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Characterization and Significance of Calnexin Phosphorylation in Mammals and *Schizosaccharomyces pombe*

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Table of Contents

Abstract

Alteration in protein folding and its subsequent trafficking has been increasingly recognized as the molecular basis of numerous diseases. Cells have acquired a quality control mechanism to ensure proper folding and assembly of proteins. Misfolded and unassembled proteins are recognized by the quality control machinery at the ER and are retained and subsequently targeted for degradation. Calnexin has been identified as one of the major constituents of the ER quality control machinery. Calnexin is a type I phosphoprotein of the ER membrane. The luminal domain of calnexin displays a lectin-like molecular chaperone activity that interacts transiently with nascent glycoproteins and promotes their efficient and productive folding. The cytosolic domain of calnexin is phosphorylated. A prolonged association of a secretion impaired α_1-antitrypsin mutant with phosphorylated calnexin has been observed. Phosphorylation of calnexin was also suggested to regulate the rate of protein transport out of the ER. Furthermore, a prolonged association of newly synthesized MHC class I heavy chains with calnexin was found in a B lymphoblastoid cell line transfected with HLA-B701 after incubation with the phosphatase inhibitor, cantharidin or okadaic acid. Hence, phosphorylation on the cytosolic domain of calnexin may provide a potential to communicate and regulate its intralumenal lectin-binding domain.

In this study, we have identified the *in vivo* phosphorylation sites of calnexin in both cultured mammalian cells and the fission yeast, *Schizosaccharomyces pombe*. By mass spectral analyses, mammalian calnexin is *in vivo* phosphorylated on three invariant serine residues, two phosphorylation sites are within CK2 recognition motifs and one is within a protein kinase C and/or proline-directed kinase (PDK) phosphorylation motif. By site-directed mutagenesis study, *S. pombe* calnexin is *in vivo* serine phosphorylated within a PDK phosphorylation motif. We have also identified ERK-1 as a candidate kinase for mammalian calnexin phosphorylation. Furthermore, we have examined the significance of mammalian calnexin phosphorylation *in vitro* employing isolated canine pancreatic microsomes. We showed that calnexin interacts with ribosomes and the interaction is regulated by the calnexin phosphorylation. ERK-1 phosphorylated calnexin was shown to

exert an enhanced interaction between calnexin and ribosomes. Hence, calnexin phosphorylation may increase the concentration of calnexin in the proximity of translocon and facilitate the translocation and folding of glycoproteins.

Resumé

Le repliement anormal des protéines ainsi que les conséquences de ces altérations sur leur traffic sont la cause de nombreuses maladies. La cellule a acquis un mécanisme de contrôle de qualité des protéines afin d'assurer le repliement correct et l'assemblage adéquat des protéines. Ainsi les protéines mal repliées et mal assemblées sont reconnues par cette machinerie présente dans le reticulum endoplasmique (RE) où elles sont retenues et écventuellement ciblées pour la dégradation. La calnexine est un des constituents majeur de ce mécanisme. C'est une protéine de type I qui agit comme une chaperonne moléculaire avec des propriétés de lectine dans son domaine luminal. La calnexine interagit de manière transitoire avec les résidus oligosaccharidiques des glycoprotéines naissantes favorisant leur repliement correct. En effet, il a été démontré que la liaison à calnexine cause la rétention et facilite le repliement des glycoprotéines ainsi que leur oligomérisation prévenant, par le fait même, leur dégradation prématurée et la formation d'aggrégats. La calnexin a été identifiée comme une protéine phopshorylée. Un mutant non sécrété de l'alpha 1-antitrypsine présente une association prolongée avec la calnexine phosphorylée. La phosphorylation de la calnexine pourrait aussi réguler la vitesse de sortie des protéines hors du RE.

Dans le cadre de ce travail nous avons examiné et identifié les sites de phosphorylation de la calnexine *in vivo* à la fois dans des cellules de mammifère en culture ainsi que chez la levure *S. pombe*. Il a été démontré *in vivo* que la calnexine de mammifère est phosphorylée au niveau de trois résidus sérine conservés. Deux des sites de phosphorylation se trouvent au niveau du motif de reconnaissance par la caséine kinase 2 (CK2) alors que le troisième est au niveau d'un motif pour la protéine kinase C et/ou au niveau d'un motif comportant un résidu proline contre lequel la kinase est dirigée (PDK). En ce qui concerne la calnexine chez *S. pombe*, celle-ci est potentiellement phosphorylée sur une sérine dans un motif PDK. Nous avons identifié ERK-1 comme une kinase potentiellement capable de phosphoryler la calnexine *in vitro* aussi bien qu'in vivo. Nous avons pu aussi montrer que les phosphorylations de la calnexine régulent son interaction avec les ribosomes et ce dans des systèmes mammifères ou levure. Ainsi nous

pouvons postuler que la phosphorylation de la calnexine par une kinase telle que ERK-1 favoriserait son recrutement à proximité du canal de translocation et de ce fait permettrait un couplage efficace entre la translocation et le repliement des chaines naissantes.

Contributions of others

I present here my original contribution to the study of calnexin phosphorylation in both mammalian cells and *Schizosaccharomyces pombe*. The work present here is largely of my own with the following exceptions:

1) Mr. Victor Dumas of the Polypeptide Hormone laboratory, Department of Medicine, McGill University, carried out the insulin-binding assay with my participation (Chapter 1, Table 4).
2) Dr. Malcolm Ward, Ms. Satty Bains, Dr. Roberto Solari and Dr. Walter Blackstock carried out the mass spectrometry analyses of calnexin immunoprecipitates (Chapter 2, Figures 18 to 22) at the mass spectrometry unit at Glaxo Wellcome Stevenage, UK.
3) The mass spectral data were analyzed with Dr. Alex Bell's help.
4) Dr. Eric Chevet performed the ERK-1 study and the ribosomal pull down assays (Chapter 4, Figures 32 to 34).
5) Sequencing reactions confirming the point mutation of *S. pombe* calnexin mutants (Chapter 5) were carried out by Mr. Daniel Dignard at BRI-NRCC.

Part of Chapter II has been published as a paper by H. N. Wong, M. A. Ward, A. W. Bell, E. Chevet, S. Bains, W. P. Blackstock, R. Solari, D. Y. Thomas, and J. J. M. Bergeron in the *Journal of Biological Chemistry*, **273**: 17227-17235 in 1998. It is used with the permission of the American Society for Biochemistry and Molecular Biology. Part of Chapter IV has been published by E. Chevet, H. N. Wong, D. Gerber, C. Cochet, A. Fazel, P. H. Cameron, J. N. Gushue, D. Y. Thomas, and J. J. M. Bergeron in the EMBO Journal, **18**(13): 3655-3666 in 1999. It is used with the permission of Oxford University Press. Manuscript for data of Chapter VI is in preparation.

Dr. Bergeron supervised the work presented in this thesis and provided editorial assistance for the entire thesis.

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Table of Figures

List of Tables

Abbreviations

ALLN	N-acetyl-leucinyl-leucinyl-norleucinal
amu	atomic mass unit
apoB	apolipoprotein B
ATP	adenosine triphosphate
BiP	immunoglobulin binding protein
BSA	bovine serum albumin
CaBP	calcium binding protein
CFTR	cystic fibrosis transmembrane conductance regulator
CHAPS	3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propane sulfonate
CK2	casein kinase II
CMG	calmegin/calnexin-t
CNL	constant neutral loss
CNX	calnexin
CRT	calreticulin
CST	castanospermine
DNJ	deoxynojirimycin
DMJ	deoxymannojirimycin
DNA	deoxyribonucleic acid
DP	docking protein (or SR)
DTT	dithiothreitol
E. coli	Escherichia coli
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(β-aminoethyl ether)N,N,N',N'-tetraacetic acid
EMM	Edinburgh minimal media
EndoH	endoglycosidase H
EOR	ER overload response
ER	endoplasmic reticulum
ERAD	ER associated degradation
ERGIC	endoplasmic reticulum-Golgi-intermediate compartment
ERK	extracellular regulated kinase
G or Glc	glucose
GlcNAc	glucosamine
gp	glycoprotein
GRP	glucose regulated protein
GST	glutathione S transferase
GTP	guanosine triphosphate
HA	Influenza hemagglutinin A glycoprotein
HBS	HEPES buffered saline solution
HEPES	N-(2-Hydroxyethyl)-1-piperazine N'-2-ethane sulfonic acid
HepG2	human hepatoma carcinoma cell line
HSP	heat shock protein
InP_3	inositol-(1,4,5) triphosphate

Ire1p/Ern1p	inositol regulated protein/ ER to nucleus signaling molecule
kb	kilo base
kDa	kilo dalton
IgG	immunoglobulin
IP	immunoprecipitates/immunoprecipitation
β_2m	β_2 microglobulin
MALDI-MS	matrix assisted laser desorption ionization mass spectrometry
Man or M	mannose
MDCK	Maden Darby canine kidney cell line
MHC	major histocompatibility complex
MS	mass spectrometry
MS/MS	tandem mass spectrometry analysis
MTP	microsomal triglyceride transfer protein
Nano-ESI	nano-electrospray ionization
PAA	phosphoamino acid analysis
PCR	polymerase chain reaction
PDI	protein disulfide isomerase
pgp	phospho-glycoprotein
PMSF	phenylmethlsulphonyl fluoride
PNGaseF	glycopeptidase F
RER	rough endoplasmic reticulum
RI	ribophorin I
RNaseB	ribonuclease B
S. cerevisiae	*Saccharomyces cerevisiae*
SDS-PAGE	sodium dodecylsulphate polyacrylamide gel electrophoresis
SERCA	sarco-endoplasmic reticulum calcium-ATPase
S. pombe	*Schizosaccharomyces pombe*
SPR	Ser-Pro-Arg
SR	SRP receptor (or DP)
SRM	stripped rough microsomes
SRP	signal recognition particle
SSR	signal sequence receptor (or TRAP)
TBS	tris buffered saline solution
TcR	T cell receptor
TRAP	translocon-associated protein (or SSR)
TRAM	translocating chain-associating membrane protein
ts	temperature sensitive
UGGT	UDP-glucose:glycoprotein glucosyltransferase
UPR	unfolded protein response
VSV-G	vesicular stomatitis virus G protein

Literature Review

The Endoplasmic Reticulum

In eukaryotic cells, newly synthesized proteins that are destined to function in various organelles within the secretory pathway or to be secreted are first targeted and translocated into the endoplasmic reticulum (ER). Morphologically, the ER is consisted of a convoluted network of interconnecting branching tubules and large cisternae enclosing a single internal space extending throughout the cytosol. In eukaryotic cells, the ER can be either coated with ribosomes, referred to as rough ER (RER), or ribosome-free, referred to as smooth ER. The rough ER is where active protein synthesis takes place. The smooth ER is the site for the maturation of lipoprotein particle and also the site for detoxification reactions that are catalyzed by the cytochrome p450 family members in hepatocytes. The amount of smooth ER and rough ER varies between different cell types, depending on the activities of the cells. In cells that secrete large amounts of proteins, such as the cells of the pancreas or salivary glands, there are extensive regions of RER. The SER is extensively developed in cells, for instance, specialized in the synthesis of steroid hormones in the endocrine cells of the gonad and adrenal cortex (Karp 1999).

The ER lumen, the internal space enclosed by the ER lipid bilayer membrane, provides a favorable oxidative environment for protein folding, post-translational modification and oligomerization of newly synthesized proteins that are translocated into the ER lumen. Numerous ER resident proteins including molecular chaperones and protein folding enzymes such as protein disulfide isomerase (PDI) are involved to ensure only properly folded proteins and assembled complexes are allowed to exit this compartment onto the next one within the secretory pathway (Gething and Sambrook 1992; Hwang, *et. al.* 1992).

The ER also functions in mediating the cytosolic delivery of a diverse range of molecules including unfolded or misfolded glycoproteins, glycopeptides and oligosaccharides from the ER lumen into the cytosol. This recently recognized novel function of the ER constitutes part of the ER quality control machinery (Ellgaard *et. al.* 1999).

The ER is an important signaling organelle and maintains a high concentration of calcium. The Ca^{2+} concentration in the ER lumen undergoes continuous fluctuation. The ER luminal Ca^{2+} concentration at steady state is approximately 400 μM, which is 2 to 3 orders of magnitude higher than the Ca^{2+} concentration in the cytosol. The ER lumen contains a high concentration of Ca^{2+}-binding proteins that facilitate the uptake of cytosolic Ca^{2+}. The ER Ca^{2+} is released to the cytosol by the inositol (1,4,5)-triphosphate (InP_3) and ryanodine receptors and is taken up by sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA) into the ER lumen (Pozzan *et. al.*1994; Meldolesi and Pozzan 1998). The regulated release of Ca^{2+} from the ER triggers specific cellular responses including the contraction of skeletal muscle cells, cell mobility, protein secretion, proliferation, and gene expression (Meldolesi and Pozzan 1998; Michalak, *et. al.* 1998; Michalak *et. al.* 1999).

The ER is sensitive to cellular changes such as the accumulation of unfolded proteins within the ER lumen. The ER is capable of sending a stress signal to the nucleus resulting in either adaptation or apoptotic induction (Kaufman 1999; Pahl 1999). For instance, perturbation of novel ER function by disturbance of ER Ca^{2+} storage or overexpression of unfolded proteins would result in the induction of the expression of a number of ER resident molecular chaperones such as BiP and PDI.

Protein synthesis and modification

Both transmembrane and soluble proteins that are destined either for secretion or to function in other organelles within the secretory pathway are targeted to the ER. During translation, an amino-terminal hydrophobic ER-targeting signal sequence, extruded from polyribosomes, is recognized by signal recognition particle (SRP) (Corsi and Schekman 1996). A pause in protein synthesis is induced by the interaction of the SRP to the signal sequence. The complex of SRP, signal sequence, and ribosomes are targeted to the translocation complex that includes the SRP receptor and a series of proteins on the cytosolic surface of the rough ER. The SRP receptor (SR or docking protein) is a heterodimeric complex consisting of a membrane associated 69 kDa α subunit (SRα or

DPα) and a 30 kDa integral membrane β subunit (SRβ). Upon binding to the SR, the SRP is released and the signal sequence interacts with the translocation machinery in a GTP-dependent manner. Protein synthesis is resumed and uni-directional translocation into the ER lumen via the translocon channel is initiated. In the budding yeast *Sacharomyces cerevisiae*, the translocon is consisting of proteins termed Sec61p, Sss1p and Sbh1p and the mammalian equivalents are termed sec61α, β and γ (Corsi and Schekman 1996). In the *S. cerevisiae*, post-translational translocation also occurs in an SRP-independent manner. The nascent polypeptides maintain their translocation competent status by interacting with cytosolic Ssa1p, a member of the HSP70 (heat shock protein) family. During post-translational translocation, additional factors are required in addition to Sec61p complex in *S. cerevisiae*. A Sec71p-Sec72p complex acting as a receptor for the nascent polypeptide at the cytosolic surface and a Sec62p-Sec63p complex assisting the Sec61p complex are required. Newly synthesized polypeptides are modified and acquire their mature tertiary or quaternary for oligomeric complex conformations in the ER lumen.

Co- and post-translational modifications of proteins provide an additional level of regulation to the activities of the proteins and increase their capacity to participate in regulatory events. The rate of transport differs among various secretory and plasma membrane proteins and the differences reside mainly in their transport from the ER to the Golgi cisternae (Lodish 1988). This may correlate with the time required for the protein to fold properly within the ER lumen. Once proteins are properly folded, they are permitted to exit the ER and subjected to other modifications as they travel through the ERGIC (ER-to-Golgi intermediate compartment) and the Golgi complex. From the trans Golgi network, proteins are subsequently transported to lysosomes (or vacuoles), storage vesicles or the plasma membrane (Palade 1975).

Upon translocation into the ER, the ER-targeting signal sequence is cleaved by signal peptidase. Secreted and membrane proteins are often modified with complex glycan structures initiated during or shortly after protein translocation across the ER membrane. The common types of glycan modifications are N-linked glycosylation of

Asn residue with a consensus NXS/T[1] sequon (X is any amino acid except proline) and glycosylphosphatidyl inositol modification of the α-carboxyl group of the carboxyl terminal amino acid of some secretory proteins in the ER lumen. Another common type of glycan modification is O-linked glycosylation of Ser or Thr residue by the addition of N-acetylglucosamine residues in the ERGIC (Kornfeld and Kornfeld 1985; Abeijon and Hirschberg 1992). Other protein modifications occurring in the ER lumen are the formation of disulfide bonds (Gilbert 1997; Ferrari and Soling 1999) and the assembly of lipoprotein particles (Wetterau *et. al.* 1991).

N-linked glycosylation pathway

In eukaryotes, the biosynthesis of N-glycosylation is initiated with the dolichol pathway in the ER. The precursor oligosaccharide dolichol linked $G_3M_9GlcNAc_2$ is assembled through a stepwise addition of monosaccharides in a series of reactions catalyzed by glycosyltransferases occurring on both sides of the ER membrane. The precursor dolichol linked oligosaccharide is co-translocationally transferred from the dolicol carrier to an Asn residue within a consensus NXS/T sequon of the polypeptides by oligosaccharyltransferase within the ER lumen. The oligosaccharides are processed by a series of trimming reactions and modifications within the secretory pathway (Kornfeld and Kornfeld 1985; Abeijon and Hirschberg 1992).

Within the ER lumen, the outermost glucose residue is subsequently removed by the action of α 1,2-glucosidase I (GI) and the remaining two glucose residues are removed by α 1,3-glucosidase II (GII) (Fig. 1A) (Herscovics 1999). Castanospermine, a plant alkaloid from *Castanospermum australe* (Saul *et. al.*1984) and deoxynojirimycin, a glucose analogue (Hettkamp *et. al.*1982) could inhibit the activity of GI and GII. Monoglucosylated glycoprotein can be re-generated by the action of UDP-glucose:glycoprotein glucosyltransferase (UGGT) (Parodi *et. al.* 1983). Following the removal of glucose, a single α 1,2-mannose residue is cleaved off, leaving a structure of $M_8GlcNAc_2$ (Byrd *et. al.*1982). In *S. cerevisiae*, the removal of mannose is restricted to a

[1] single amino acid abbreviation is used.

single mannose from the central arm of the oligosaccharide, forming a B isomer (Fig. 1A). The gene encoding the α1,2-mannosidase, *MNS1*, has been cloned and purified from *S. cerevisiae* (Camirand *et. al.* 1991). The mammalian α-mannosidase responsible for forming the B isomer oligosaccharide has recently been cloned (Gonzalez *et. al.* 1999; Tremblay and Herscovics 1999). It is a type II membrane protein in the ER with a predicted molecular weight of 79.5 kDa. It requires calcium for its enzymatic activity and is subjected to inhibition exerted by both deoxymannojirimycin (DMJ) and kifunensine (Kif) (Gonzalez *et. al.* 1999; Tremblay and Herscovics 1999). In higher eukaryotes, the removal of α1,2-mannose has been observed from nonglucosylated branch, generating a C isomer, by ER mannosidase II (Fig. 1A). The endo-α-mannosidase will cleave $G_{1-3}M_9GlcNAc_2$ and generate isomer A (Herscovics 1999). Up to four mannose residues are removed by α-mannosidase activities located within the ER and the cis-Golgi compartment in mammalian cells (Kornfeld and Kornfeld 1985). The ER mannosidase I equivalent has not been detected in the fission yeast *Schizosaccharomyces pombe* (Gemmill and Trimble 1999).

Subsequent processing of the oligosaccharide is quite different between *S. cerevisiae* and higher eukaryotes. The oligosaccharide chains of mature yeast N-linked glycoproteins consist of either a mannose core of 9 to 13 mannose residues or a branched mannan outer chain contains up to 200 monosaccharide structures. In higher eukaryotes, the maturation of glycan can proceed via different routes to generate distinct structures such as hybrid and complex oligosaccharides (Kornfeld and Kornfeld 1985; Gemmill and Trimble 1999). Lysosomal soluble glycoproteins are processed at this point to generate a mannose-6-phosphate (M6P) motif for the recognition and targeting to lysosomes by M6P receptors (Kornfeld and Kornfeld 1985).

Protein folding in the ER

In eukaryotic cells, correct protein folding and oligomerization within the ER lumen is assisted by enzymes that catalyze disulfide bond formation and molecular chaperones that inhibit hydrophobic aggregation (Gething and Sambrook 1992). The ER

maintains a high concentration of folding catalysts and molecular chaperones and provides a redox environment that favors disulfide bonds formation for protein folding (Hwang *et. al.*1992).

PDI

Protein disulfide isomerase (PDI) is an abundant folding catalyst that facilitates the correct disulfide bonds formation of newly synthesized proteins in the ER lumen. PDI is a 55 kDa Ca^{2+}-binding ER resident protein and belongs to the thioredoxin superfamily (Gilbert 1997; Ferrari and Soling 1999). PDI also participates in a number of diverse functions inside the cell. For instance, PDI is a subunit of the microsomal triglyceride transfer protein (MTP) involved in lipoprotein assembly (Wetterau *et. al.*1991). PDI exhibits chaperone activities, when it is in excess amounts, and prevents the aggregation of denatured proteins. The chaperone activity of PDI resides within its peptide binding domain (Dai and Wang 1997; Gillece *et. al.*1999). Hydrophobic interaction between PDI and peptides has been observed (Klappa *et. al.*1998). In sub-stoichiometric amount, PDI exhibits anti-chaperone activities and increases aggregate formation (Gilbert 1997). This function may facilitate the disposal of misfolded polypeptides. Furthermore, the involvement of PDI in the quality control was recently demonstrated. Pdi1p, yeast PDI homolog, was shown to interact with nascent proteins and was able to distinguish between wild type and misfolded proteins regardless of their thiol content in *S. cerevisiae* (Gillece *et. al.*1999). The expression of Pdi1p is induced in yeast in response to cellular stresses which led to protein misfolding and accumulation in the ER (Sidrauski *et. al.*1998). PDI was also found to interact with calreticulin (CRT) and this interaction may modulate their chaperone activities. Both the calcium binding capacity of CRT and the disulfide isomerase activity of PDI were compromised when they were found in a complex (Baksh *et. al.*1995).

ERp57

ERp57 (ERp60, ER60, GRP58, Q2, HIP-70 and CPT) contains two thioredoxin domains and shares significant homology to PDI (Elliott *et. al.*1997). ERp57 is a thiol oxidoreductase for the maturation of N-linked glycoproteins in concert with calnexin

(CNX) and calreticulin (CRT) (Oliver *et. al.*1996; Elliott *et. al.*1997; Oliver *et. al.*1997). The interaction between ERp57 and nascent glycoproteins, both soluble and integral membrane proteins, has been observed (Elliott *et. al.*1997; Oliver *et. al.*1997; Hughes and Cresswell 1998; Lindquist *et. al.*1998). The interaction between ERp57 and glycoproteins is glycan dependent and glucose trimming of the two outermost glucose residues is required (Van der Wal *et. al.*1998; Zapun *et. al.*1998). ERp57 does not interact directly with the modified oligosaccharide side chain (Zapun *et. al.*1998). ERp57 forms a complex with either CNX or CRT (Oliver *et. al.*1999). The disulfide isomerase activity of ERp57 on the refolding of monoglucosylated ribonuclease B (RNase B) was enhanced in the presence of either CNX or CRT *in vitro* (Zapun *et. al.*1998). Both PDI and ERp57 were found to associate with mixed disulfide bonds containing glycoproteins *in vivo* (Molinari and Helenius 1999). ERp57 has been shown to contain a cysteine protease activity towards the degradation of misfolded protein *in vivo*. The degradation activity of ERp57 can be inhibited by cysteine protease inhibitors, N-acetyl-leucinyl-leucinyl-norleucinal (ALLN) and N-acetyl-leucinyl-leucinyl-normethioninal (ALLM) (Otsu *et. al.*1995). An association of ERp57 (or ER60) with ER retained proteins such as apolipoprotein (apo) B has been observed (Adeli *et. al.*1997). ERp57 may participate in the retrograde translocation of ER retained misfolded proteins for their degradation in the cytosol by the proteasomes. The association of ERp57 with misfolded proteins may reflect a requirement for reduction and denaturation before they are shunted to the cytosol for degradation (Lindquist *et. al.*1998; Molinari and Helenius 1999). The expression of ERp57 has been shown to be induced by either glucose starvation or tunicamycin treatment that inhibits protein glycosylation (Ferrari and Soling 1999).

Other ER PDI homologues and PPI

Other ER homologs of PDI that have been identified are ERp72 (or calcium binding protein 2 or CaBP2), PDIp (PDI-like protein), P5 (CaBP1) and ERp28 in mammals and Ero1p in the budding yeast (Gilbert 1997; Ferrari and Soling 1999).

Peptidyl proline *cis-trans* isomerase (PPIs) catalyzes the isomerization of certain proline-peptidyl bonds into the cis or trans conformation on nascent polypeptides. There

are three families of PPIs and they are cyclophins that bind to cyclosporin A, FK506-binding protein (FKBPs) and parvulin family (Pliyev and Gurvits 1999).

BiP and GRP94

BiP (immunoglobulin heavy chain binding protein) is a soluble member of the HSP70 family localized in the ER lumen. BiP was originally identified as the glucose regulated protein, GRP78. Glucose starvation and cellular stress stimulated the synthesis of GRP78. BiP participates in a number of diverse functions inside the cell (Haas 1994). BiP is a peptide-dependent ATPase that interacts with exposed hydrophobic surfaces on unfolded proteins or unassembled protein subunits (Gething 1999). The molecular chaperone activity of BiP/GRP78 was first eluted by its noncovalent association with nascent immunoglobulin heavy chains in myeloma cells that do not synthesized immunoglobulin light chains (Haas and Wabl 1983). Subsequent studies showed that BiP is associated transiently with a wide variety of nascent and more permanently with misfolded or unassembled proteins (Gething and Sambrook 1992; Gething 1999). BiP contains a weak intrinsic ATPase activity that is stimulated *in vitro* by small hydrophobic peptides. ATP hydrolysis induced the release of polypeptides from BiP. BiP may be involved in the disposal of misfolded aggregates inside the ER and in the ER signaling to the nucleus (Sidrauski *et. al.*1998). Overexpression of BiP has been shown to suppress the induction of the unfolded protein response (UPR) pathway in both cultured mammalian cells and in yeast (Chapman *et. al.*1998). It has been suggested that the UPR pathway contains a sensing mechanism that detects the changes in either the concentration of free BiP or BiP found in complexes with unfolded proteins (Chapman *et. al.*1998). In addition to modulating protein folding, BiP may act as a molecular ratchet and involved in polypeptide translocation across the ER membrane (Matlack *et. al.*1999). The calcium binding capacity of BiP has also been implicated to participate in the maintenance of the ER calcium homeostasis (Lievremont *et. al.*1997).

GRP94 (endoplasmin, CaBP4, ERp99 and gp96) is a soluble ER resident protein. GRP94 expression, however, has not yet been detected in Drosophila, bacteria or yeast (Argon and Simen 1999). The expression of GRP94 is responsive to glucose deprivation

and condition that induced misfolded protein accumulation in the ER. GRP94 is involved in immunoglobulin (Melnick *et. al.*1992) and MHC I assembly (Schaiff *et. al.*1992). In addition, GRP94 has been shown to interact with thyroglobulin, apoB, collagen, protein C and bile salt-dependent lipase (Argon and Simen 1999). GRP94 is a calcium binding protein with a low affinity for either ATP or GTP. Peptide binding ability of GRP94 is not influenced by the presence of nucleotide. Unlike other ER chaperones, GRP94 is modified by both O-linked and N-linked glycosylation. The pattern of the N-linked glycosylation moiety changes, became endo H resistant, after cellular stress (Csermely *et. al.*1998; Argon and Simen 1999).

Calnexin family

Calnexin (CNX, p88 or IP90) (Degen and Williams 1991; Wada *et. al.*1991; David *et. al.*1993) along with calreticulin (CRT) (Opas *et. al.*1991) and calmegin/calnexin-t (CMG) (Ohsako *et. al.*1994; Watanabe *et. al.*1994) are the members of a recently identified molecular chaperone family. They share sequence similarity to each other. All three members are Ca^{2+}-binding proteins and reside in the ER. They contain lectin-like molecular chaperone properties that recognize newly synthesized glycoproteins. Calnexin, calmegin and calreticulin have migration mobilities on SDS-PAGE that are higher than their calculated molecular weights. The differences in migration may be contributed by their highly acidic pI values (Bergeron *et. al.*1994)(see Table 3).

Calnexin

Calnexin (CNX) is a phosphorylated integral membrane protein with a type I topology localized to the ER (Wada *et. al.*1991; David *et. al.*1993; Ou *et. al.*1995). Mammalian calnexin is a 65 kDa protein with a migration mobility of 88 to 90 kDa on SDS-PAGE (Degen and Williams 1991; Wada *et. al.*1991; David *et. al.*1993). Mammalian calnexin has a long half-life of 190 h (Vinayagamoorthy *et. al.*1993). The molecular chaperone activity of the calnexin family was first implicated by the transient association of calnexin with newly synthesized MHC I molecules, incompletely assembled forms of the T cell receptor and B cell antigen receptor (Degen and Williams

1999; Galvin *et. al.*1992; Hochstenbach 1992; David *et. al.*1993). The time of association between calnexin and a newly synthesized glycoprotein correlated with the time required for its folding and exit of the ER (David *et. al.*1993; Ou *et. al.*1993; Nauseef *et. al.*1995). A prolonged association of calnexin or CRT with misfolded proteins, either by mutation or by the incorporation of amino acid analogue, and unassembled subunits within the ER lumen have been observed (Bergeron *et. al.*1994; Trombetta *et. al.* 1998; Ellgaard *et. al.*1999). CNX or CRT along with ERp57, glucosidase II, and UGGT constitute a deglucosylation and re-glucosylation cycle or "calnexin cycle" that mediates the retention of unfolded proteins, prevents premature secretion and/or degradation and promotes productive folding of nascent glycoproteins (Chevet *et. al.*1999; Ellgaard *et. al.*1999). The constituents of the calnexin cycle and the properties of calnexin will be discussed in a greater detail in the following sections.

Calreticulin

Calreticulin is an abundant 46 kDa luminal protein of the ER. Calreticulin has also been detected in the cytoplasmic granules of the cytotoxic T cell, sperm acrosomes, tick saliva and on the cell surface (Michalak, 1999). Calreticulin is a major Ca^{2+}-binding protein of the ER lumen. Calreticulin also binds Zn^{2+} and possibly ATP. Calreticulin is retained in the ER by its carboxyl terminal (-KDEL tetrapeptide and its variables) ER retrieval signal (Opas *et. al.*1991). Calreticulin has been cloned from a variety of organisms and they are highly conserved. Calreticulin protein and the gene, however, are not found in yeast (*S. cerevisiae* and *S. pombe*) (Michalak *et. al.*1998; Michalak *et. al.*1999). Calreticulin has been implicated in the participation of several cellular functions including calcium binding and storage, Ca^{2+} signaling, cell adhesion, regulation of gene expression and autoimmunity other than the molecular chaperone activity (Michalak *et. al.*1998). Calreticulin, like calnexin, interacts transiently with newly synthesized glycoproteins (Nauseef *et. al.*1995; Peterson *et. al.*1995; Wada *et. al.*1995).

Calmegin

Calmegin or calnexin-t is a Ca^{2+}-binding protein that shares a high degree of homology to both calnexin and calreticulin (Ohsako *et. al.*1994; Watanabe *et. al.*1994)

(Tables 1-3). Cloned sequences of murine calmegin and calnexin-t differ in 19 amino acid residues and were separately cloned and sequenced by two groups of investigators (Ohsako *et. al.*1994; Watanabe *et. al.*1994). The cytsolic domains of both sequences differs at two amino acid positions ($E^{502}D$ and $V^{549}L$, from calmgein to calnexin-t, respectively) (Ohsako *et. al.*1994; Watanabe *et. al.*1994). Murine calmegin sequence (Accession number BAA0318) is used here. The cytosolic domain of calmegin is phosphorylated (Ohsako *et. al.*1998). Human calmegin homolog has also been isolated and shared an 80% identity with the mouse calmegin (Tanaka *et. al.* 1997). Calmegin is a testis-specific protein and abundantly expressed in meiotic germ cells from pachytene spermatocytes to spermatids (Watanabe *et. al.*1994). By sequence analysis of the cDNA of calmegin, it is predicted to be a type I integral membrane protein consisting of 611 amino acids. The ability of calmegin to interact with nascent polypeptides during spermatogenesis has been demonstrated (Ikawa *et. al.* 1997).

Cooperation among chaperones

Co-immunoprecipitation and cross-linking experiments demonstrating associations among various ER chaperones have suggested that the ER may function as a matrix. Incompletely folded proteins could be absorbed into this matrix in order to fold properly and to prevent premature aggregation (Kuznetsov *et. al.*1997; Tatu and Helenius 1997; Linnik and Herscovitz 1998). A balanced and coordinated chaperone function is probably the optimal condition for protein folding and assembly in the ER. For instance, BiP, GRP94, ERp72 and GRP170 have been shown to complex with thyroglobulin during its maturation *in vivo* (Kuznetsov *et. al.*1997). GRP94, calreticulin and ERp72 were co-immunoprecipitated with apoB *in vivo* (Linnik and Herscovitz 1998). Influenza hemaglutinin A (HA) was detected in a complex with BiP, calnexin and calreticulin until the homotrimer of HA is assembled (Tatu and Helenius 1997). A sequential interaction with BiP and calnexin on the folding of vesicular stomatitis virus (VSV) G ts045 glycoprotein (Hammond and Helenius 1994) or coagulation factor VIII has been observed *in vivo* (Pipe *et. al.*1998). A coordinated interaction of BiP and GRP94 on the folding of unassembled immunoglobulin chain in the ER has been observed (Melnick *et. al.*1994).

For the maturation of myeloperoxidase, a coordinated action of calreticulin and calnexin was suggested (Nauseef *et. al.*1998). BiP, calnexin, calreticulin, and tapasin have been shown to be involved in the maturation and assembly of human MHC class I molecules (York and Rock 1996).

The proper folding of transmembrane proteins containing folding domains on both sides of the ER membrane is concurrently monitored by the cytosolic molecular chaperones along with the luminal chaperones. For instance, HSP90 and HSP70 interact with the cytosolic domain of the cystic fibrosis transmembrane conductance regulator (CFTR) while its luminal domain binds to calnexin and BiP (Yang *et. al.*1993; Pind, Riordan *et. al.*1994; Loo *et. al.*1998). Similarly, the cytosolic domain of translocational paused apoB binds to HSP70 (Zhou *et. al.*1995) while its luminal domain interacts with numerous ER chaperones (Linnik and Herscovitz 1998).

ER quality control

The quality control mechanism in the early secretory pathway ensures only properly folded and assembled proteins are allow to travel further along the secretory pathway. The conformation-based ER quality control is mainly carried out by coordinated actions of molecular chaperones. Persistent misfolding or unassembled subunits are recognized. retained and eventually targeted for degradation (Brodsky and McCracken 1997; Bonifacino and Weissman 1998).

Abnormal protein trafficking due to defective protein folding has been increasingly recognized as a molecular basis for a number of human inherited diseases including cystic fibrosis and α_1-antitrypsin deficiency (Amara *et. al.*1992; Sifers *et. al.*1992; Kuznetsov and Nigam 1998).

Cystic Fibrosis

The absence of the cystic fibrosis transmembrane conductance regulator (CFTR) at the epithelial cell surface is the main contributing factor towards the manifestation of cystic fibrosis (Kopito 1999). CFTR consists of two membrane-spanning domains; each composed of 6 transmembrane segments; an extracellular region and an intracellular

domain that contains two nucleotide-binding domains and a regulatory region. CFTR molecule is glycosylated at two potential N-linked glycosylation sites. The most common mutation observed in CF patients is the deletion of a phenylalanine at position 508 of the CFTR molecule (ΔF508). ΔF508 molecules are retained and associated with calnexin and cytosolic HSP70 and HSP90 on both surfaces of the ER membrane (Yang *et. al.*1993; Pind *et. al.*1994; Loo *et. al.*1998). Intracellularly retained ΔF508 CFTR molecules are then subjected to ubiquitin-proteasome degradation via ER retrograde translocation (Ward *et. al.*1995; Kopito 1999; Xiong *et. al.*1999). Perturbation of HSP90 interaction has been shown to accelerate the intracellular degradation of nascent CFTR (Loo *et. al.*1998). ER-retained ΔF508 mutant is functional (Pasyk and Foskett 1995). Hence, interception of the normal functioning of the ER quality control machinery may represent a potential therapeutic target for the treatment of the ΔF508 CF patients.

Alpha 1-antitrypsin deficiency

Deficiency of serum α_1-antitrypsin predisposes individuals to the development of emphysema (Sifers *et. al.*1992). α_1-antitrypsin is a serine protease inhibitor (Pi) that protects elastic fibers in lung alveoli from excessive hydrolysis by neutrophil elastase. A balance between elastase and its inhibitor is crucial for the regulation of connective tissue turnover. α_1-antitrypsin is a glycoprotein of 394 amino acids with three N-linked glycosylation sites. Several mutations in human α_1-antitrypsin have been identified, which leads to either a null, or deficiency phenotype. Studies have revealed that the mutants are retained in the ER and subsequently degraded intracellularly. The PiZ variant is a full-length α_1-antitrypsin containing a Glu to Lys substitution at position 342 of mature protein. PiZ variant is partially active against elastase. Individuals homozygous for PiZ allele contain 10 to 15% of the normal level of serum α_1-antitrypsin and are predisposed to the development of emphysema. Secretion impaired PiZ variant is retained in the ER and is found in an association with calnexin (Wu *et. al.*1994). Intracellularly retained PiZ molecules are subjected to degradation involving cytosolic proteasome (Qu *et. al.*1996). The secretion of PiZ was recently shown to be enhanced by the treatment of either castanospermine, an inhibitor for both glucosidase I and II activities that prevents

calnexin binding, or deoxymannojirimycine, a mannose analogue that inhibits ER mannosidase I activity, in cultured cells (Marcus and Perlmutter 2000).

Other genetic disorders where protein misfolding has a major role towards the manifestation of the diseases include osteogenesis imperfecta, which is caused by misassemble and retention of BiP associated-collagen type I in the ER, and familial hypercholesterolemia type II where the low-density lipoprotein receptor is misfolded and ER-retained. In congenital goitrous hypoyhyroidism, defects in folding and/or assembly of thyroglobulin in the ER is the major cause (Amara *et. al.*1992; Kuznetsov and Nigam 1998). An understanding of the mechanisms of protein folding in the ER may lead to a novel therapeutic approach for misfolded protein disorders.

Calnexin as a molecular chaperone

The molecular chaperone function of calnexin, and its family members, was first proposed by its transient interaction with newly synthesized polypeptides (Degen and Williams 1991; Galvin *et. al.*1992; Hochstenbach 1992; David *et. al.*1993). The time of association between calnexin and a newly synthesized glycoprotein has been shown to correlate with the time required for its folding and exit of the ER (David *et. al.*1993; Ou *et. al.*1993; Nauseef *et. al.*1995).

The mechanism of interaction between calnexin and newly synthesized glycoproteins was first instigated by the use of tunicamycin (Ou *et. al.*1993). Tunicamycin is an analog of UDP-N-acetylglucosamine that inhibits N-linked glycosylation. Tunicamycin blocks the addition of N-acetylglucosamine to dolichol phosphate, the first step in the formation of the core oligosaccharide (Burda and Aebi 1999). The interaction between numerous nascent glycoproteins including α_1-antitrypsin, transferrin, apoB and α-fetoprotein was abrogated by tunicamycin treatment in cultured human hepatoma HepG2 cells. Calnexin does not interact with non-glycosylated albumin although its related glycosylated protein, α-fetoprotein, binds to calnexin (Ou *et. al.*1993). Subsequent studies employing glucosidase inhibitors such as castanospermine have shown that calnexin and calreticulin recognize and bind to glycoproteins containing

monoglucosylated oligosaccharides side chain (Hammond *et. al.*1994; Hebert *et. al.*1995).

Studies employing castanospermine, which prevents glucose trimming and calnexin binding to its substrates, have suggested the potential function of calnexin and mechanism of calnexin binding. Calnexin binding may act positively by facilitating glycoprotein folding and assembly or negatively by retaining misfolded glycoproteins for intracellular degradation (Chevet *et. al.*1999; Ellgaard *et. al.*1999).

In the presence of castanospermine where calnexin binding is inhibited, a reduced folding and subsequently a reduced secretion or expression was observed for a number of glycoproteins. The castanospermine affected glycoproteins include coagulation factor VIII (Pipe *et. al.*1998), tyrosinase (Toyofuku *et. al.*1999), hepatic lipase (Boedeker *et. al.*1999), VSV-G glycoprotein (Hammond and Helenius 1994), T cell receptor (TcR) alpha subunit (Kearse *et. al.*1994), insulin receptor (Bass *et. al.*1998), MHC class I heavy chain (HC) (Vassilakos *et. al.*1996; Tector *et. al.*1997; Huttinger *et. al.*1999), CD1b heavy chain (HC) (Huttinger *et. al.*1999), nicotinic acetylcholine receptor alpha subunit (Keller *et. al.*1998), and MHC class II invariant chain (Romagnoli and Germain 1995).

Inhibition of calnexin binding, however, did not alter the expression or activity of TcR β, CD3-γ and ε chains (Kearse *et. al.*1994), hemagglutinin-neuraminidase protein of Newcastle disease virus (McGinnes and Morrison 1998) or tissue specific plasminogen activator (Allen and Bulleid 1997).

Calnexin interaction has been shown to prevent premature misfolding and aggregate formation of canine gp80 (Wada *et. al.*1994), influenza hemagglutinin (Hebert *et. al.*1996), MHC class I HC (Vassilakos *et. al.*1996), and transferrin (Wada *et. al.*1997).

Dithiothreitol (DTT) appears to have an effect on calnexin binding to a subset of proteins including MHC Class I heavy chains and proteins that do not contain internal disulfide bonds (Tector and Salter 1995). Calnexin and calreticulin were also suggested to be capable of sensing the acquisition of a locally folded state (Hebert *et. al.*1995). This fuction may be contributed by ERp57 that is associated with either calnexin or

calreticulin during glycoprotein folding in the ER (Elliott *et. al.*1997; Oliver *et. al.*1997; Zapun *et. al.*1997; Hughes and Cresswell 1998; Lindquist *et. al.*1998; Zapun *et. al.*1998).

Calnexin may be involved in the intracellular degradation of a number of mutated proteins. A prolonged association of calnexin with misfolded mutant proteins including mutated MHC class I HC. (Zhang *et. al.*1995), α-1 antitrypsin PiZ and null Hong Kong variants (Le *et. al.*1994; Wu *et. al.*1994), CFTR ΔF508 (Pind *et. al.*1994), P-glycoprotein mutant (Loo and Clarke 1994), VSV-G ts045 (Hammond and Helenius 1994) or mutant myeoloperoxidase (Nauseef *et. al.*1998) has been detected.

Calnexin is phosphorylated *in vivo* (Dakour *et. al.*1993; Capps and Zuniga 1994; Hawn and Strand 1994; Le *et. al.*1994; Schue *et. al.*1994; Wu *et. al.*1994). Phosphorylated calnexin has been shown to associate with the null Hong Kong variant of α_1-antitrypsin, coinciding with its intracellular retention within the lumen of the ER (Le *et. al.*1994). Phosphorylated calnexin was also found in an association with newly synthesized MHC class I allotypes with a slow rate of exit out of the ER (Capps and Zuniga 1994). A prolonged association of newly synthesized MHC class I heavy chains with calnexin was found in a B lymphoblastoid cell line transfected with HLA-B701 after incubation with either cantharidin or okadaic acid, both are potent inhibitors for protein phosphatase 1 and 2A (Tector *et. al.*1994). An increase in the level of calnexin phosphorylation with oleic acid treatment has been observed *in vivo* in cultured McCoy cells (Schue *et. al.*1994). Hence, phosphorylation of calnexin may thereby participate in its lectin-like molecular chaperone activity.

Calnexin association with assembling protein complex

Calnexin binding has been suggested to facilitate protein assembling and to retain incomplete complexes in the ER. Calnexin in association with the subunits of hetero-oligomeric complexes including T cell receptor (Hochstenbach *et. al.*1992), monomeric immunoglobulin (Galvin *et. al.*1992), MHC class I and II (Anderson and Cresswell 1994), acetylcholine receptor (Gelman *et. al.*1995; Keller *et. al.*1996), or integrin (Lenter and Vestweber 1994; Rigot *et. al.*1999) has been detected.

T cell receptor (TcR) consists of a disulfide linked αβ heterodimer that is non-covalently associated with CD3 complex (αβδεγεζζ). Assembly of TcR complex proceeded in an orderly manner in the ER. Noncovalently associated pairs of δε and γε proteins are formed and the pairs are joined by α and β subunits to form αδε and βγε complexes. Disulfide bond formation occurs between α and β subunits and yields an incomplete αβδεγε complex. A complete αβδεγεζζ TcR complex is formed by the final addition of a disulfide-linked ζζ homodimer (Kearse *et. al.*1995). Calnexin interaction with all TcR subunits except ζ subunit has been observed (Hochstenbach *et. al.*1992; Kearse *et. al.*1994). The presence of calnexin was also detected in partially assembled TcR complexes (Hochstenbach *et. al.*1992). Castanospermine treatment has been shown to prevent calnexin binding to TcR α subunit and increased its degradation. This effect, however, was not observed with β subunit (Kearse *et. al.*1994). Recently, δ subunit was detected to exist in both monomeric and disulfide linked oligomeric forms in both $CD4^-CD8^-$ and splenic T lymphocytes. Calnexin was found to associate with both monomeric and oligomeric δ subunits (Kearse 1998). In cells that lack the expression of α subunit, a prolonged association between the β subunit of TcR complex and calnexin has been detected (David *et. al.*1993). When carboxyl terminal truncated human calnexin was co-expressed with ε subunit, a non-glycoprotein, it was found to be mislocalized with the truncated calnexin *in vivo* (Rajagopalan *et. al.*1994).

MHC I is a trimeric complex composed of a polymorphic heavy chain (HC), an invariant β_2-microglobulin (β_2m), and an antigenic peptide. The assembly of MHC class I molecules takes place in the ER and differs between human and murine species (York and Rock 1996). In the assembly of human MHC class I molecules, calnexin is immediately associated with newly synthesized MHC class I heavy chain. The heavy chain forms a non-covalent heterodimer with β_2m and calnexin is not detected in this complex (Nossner and Parham 1995). Calreticulin and ERp57 are then joined the HC-β_2m heterodimer and followed by tapasin and the TAP (transporter associated with antigen processing). Tapasin is a soluble ER resident protein that bridges the partial MHC class I complex to TAP. TAP is a heterodimer transmembrane complex that

delivers the antigenic peptides generated by cytosolic proteasomes to the ER lumen. Peptide loaded class I HC-β_2m complex is subsequently released from the ER and transported to the plasma membrane (York and Rock 1996). In the assembly of murine MHC class I molecule, calnexin has been found in a complex with either the heavy chains, the HC-β_2m heterodimer or TAP (Degen *et. al.*1992; Nossner and Parham 1995; Suh *et. al.*1996). The observed difference of the association between calnexin with either the murine or human heavy chains may rely on their glycosylation pattern (Nossner and Parham 1995; Zhang and Salter 1998). Human MHC class I heavy chain contains one glycosylation site while its murine counterparts contain two or three sites (Barbosa *et. al.*1987). When murine class I heavy chain is expressed in cultured human cells, it exhibits the murine pattern of interaction with human calnexin and human β_2m (Nossner and Parham 1995). Acquiring an additional glycosylation site by mutagenesis, human MHC class I heavy chain was found in an association with calnexin before and after β_2m association and a murine assembly pattern was observed (Zhang and Salter 1998).

In cells lacking certain components of the assembly process, such as in β_2m-negative cells, calnexin was found in a stable complex with MHC class I molecules (Nossner and Parham 1995). In calnexin-deficient *Drosophila Schneider* cells, co-expressed calnexin retains both the free murine heavy chains and peptide-deficient HC-β_2m heterodimer complex in the ER (Jackson *et. al.*1994). Calnexin binding has been shown to prevent intracellular degradation of free murine heavy chains (Jackson *et. al.*1994) and to enhance their assembly with β_2m *in vivo* (Vassilakos *et. al.*1996). Castanospermine treatment has been shown to impair the folding of MHC class I heavy chains and led to a decrease in the assembly of heavy chains with β_2m (Vassilakos *et. al.*1996). Calnexin binding may not be essential for MHC class I assembly since its folding and assembly is normal in CEM-NRK cells, a human T lymphotoid cell line lacking endogenous calnexin (Scott and Dawson 1995). Calnexin function, however, may be compensated by the endogenous calreticulin in the CEM-NRK cells (Allen and Bulleid 1997).

MHC class II is a nonameric structure consisting of α, β and invariant I (Ii) chains. The complex is assembled by a progressive addition of three αβ dimers into the Ii trimer in the ER. Calnexin has been shown to interact with newly synthesized α, β and Ii polypeptide chains. Calnexin was found to associate with the assembling αβIi complex until the final αβ heterodimer is added. Dissociation of calnexin was shown to be parallel with the egression of the nonameric structure from the ER (Anderson and Cresswell 1994).

The muscle-type nicotinic acetylcholine receptor is a pentameric membrane ion channel consisting of $\alpha_2\beta\gamma\delta$ subunits. Transient interaction between calnexin and newly synthesized α, β or δ subunits has been observed. Calnexin binding to the isolated subunits has been shown to increase their folding and assembly and decreased their degradation (Gelman *et. al.*1995; Keller *et. al.*1996; Chang *et. al.*1997; Keller *et. al.*1998).

Integrins are αβ heterodimers that mediate cell adhesion to protein of the extracellular matrix or to other cells. Calnexin was found in an association with the α_6, β_1 and β_4 monomers *in vivo* (Lenter and Vestweber 1994; Rigot *et. al.*1999). Unassembled integrin β_4 subunit was found in a stable complex with calnexin in the ER (Lenter and Vestweber 1994).

Calnexin association with viral glycoproteins

Several viral glycoproteins have been shown to interact with calnexin. These include VSV-G (Hammond and Helenius 1994); influenza HA (Chen *et. al.*1995); rabies virus glycoprotein G (RV-G) (Gaudin. 1997); rotavirus enterotoxin NSP4 (Mirazimi *et. al.*1998); hepatitis C virus glycoproteins E1 and E2 (Choukhi *et. al.*1998). Newcastle disease virus hemagglutinin-neuraminidase (NDV-HN) (McGinnes and Morrison 1998); human immunodeficiency virus (HIV) gp160 (Otteken and Moss 1996), hepatitis B virus large surface antigen (Xu, 1997) and the M envelope protein (Prange *et. al.*1999); Sendai virus envelope glycoproteins F and HN (SV-HN) (Tomita *et. al.*1999); membrane glycoproteins G1 and G2 of uukuniemi virus (UV) (Veijola and Pettersson 1999); herpes

simplex virus type I glycoprotein B, C and D (Yamashita *et. al.* 1996) and human cytomegalovirus glycoprotein B (Yamashita *et. al.*1996; Zheng *et. al.*1996).

Interaction of BiP with VSV-G (Hammond and Helenius 1994), HA (Chen, Helenius *et. al.*1995), RV-G (Gaudin 1997), UV-G1 and G2 (Persson and Pettersson 1991), or G2 SV-HN (Tomita *et. al.*1999) has been detected. Calreticulin was also found to interact with SV-HN (Tomita *et. al.*1999), UV-G1 and G2 (Veijola and Pettersson 1999), or HIV gp160 (Otteken and Moss 1996) during their maturation. For rotavirus and NDV, calnexin binding is not critical for the assembly of infection virus (McGinnes and Morrison 1998; Mirazimi *et. al.*1998). For rabies virus and hepatitis B, calnexin interaction was shown to be crucial for the assembly of virion (Gaudin 1997; Prange *et. al.*1999). Mutated herpes simplex virus type 1 glycoprotein B has been shown to be retained intracellularly in a complex with calnexin, calreticulin and BiP (Laquerre *et. al.*1998).

ATP has been shown to be requried for the productive interaction between calnexin and gp80 in cultured Mardin Darby canine kidney cells (Wada *et. al.*1994). Depletion of ATP has been shown to lead to a premature dissociation of p70, the precursor of gp80, from calnexin and the formation of p70 aggregates (Wada *et. al.*1994). A prolonged association between p70 and calnexin has also been observed in the presence of DTT which perturbs the redox condition of the cells (Wada *et. al.*1994).

Association with non-glycosylated proteins

Calnexin association with un- or non-glycosylated proteins has been detected *in vivo* which led to the hypothesis that calnexin may also recognize protein motifs. For instance, the removal of the N-linked glycosylation site by mutagenesis in both murine MHC class I heavy chain and P-glycoprotein did not affect their association with calnexin (Loo and Clarke 1994; Carreno *et. al.*1995). Tunicamycin or endoH treatment did not abrogate the interaction between calnexin and the β subunit of MHC II molecule (Arunachalam and Cresswell 1995). A complex of calnexin with non-glycosylated CD3ε aggreates (Huppa and Ploegh 1997), non-glycosylated VSV G aggregates (Cannon *et.*

*al.*1996) or DTT-treated non-glycosylated thyroglobulin (Kim and Arvan 1995) has also been detected.

Calnexin vs. calreticulin binding

Calnexin and calreticulin appear to share some differences in substrate binding specificity. They bind to partially distinct populations of substrates in cells (Nauseef *et. al.*1995; Peterson *et. al.*1995; Wada *et. al.*1995; Hebert *et. al.*1996). For instance, α_1-antitrypsin does not interact with soluble calreticulin unless it was membrane anchored with the cytosolic and transmembrane domain of calnexin to the ER membrane (Wada *et. al.*1995). Additional factors other than topological differences between CNX and CRT are likely to contribute to the following preferred interactions. CNX binds to all folding intermediates of HA while CRT associates preferentially with the earliest oxidative form of HA (Hebert *et. al.*1996). Calreticulin interacts with immature apopro-myeloperoxidase (mpo) while calnexin interacts with both apopro-mpo and enzymatically active heme-containing pro-mpo (Nauseef *et. al.*1998). Factor VIII and V are homologous coagulation cofactors that share a similar domain organization. CNX binds to Factor V only whereas CRT interacts with both Factor VIII and V (Pipe *et. al.*1998). Acetylcholine receptor α subunit is associated with calnexin but not with calreticulin (Keller *et. al.*1998).

ER associated degradation

Misfolded polypeptides and unassembled subunits are retained in the ER and are subsequently degraded in a non-lysosomal pathway in a pre-Golgi compartment and this process is often referred to as ER-associated degradation or ERAD (Brodsky and McCracken 1997; Bonifacino and Weissman 1998).

The involvement of ubiquitin-proteasome pathway in the ERAD was first demonstrated from the study of CFTR molecules (Jensen *et. al.*1995; Ward *et. al.*1995). The degradation of both normal and mutated (ΔF508) CFTR molecules was inhibited with proteasome inhibitors including ALLN, MG132 or lactacystin. An accumulation of ubiquitinated CFTR molecules was observed in the presence of the proteasome inhibitors. Co-expression of a dominant-negative ubiquitin mutant ($K^{48}R$), which prevents the

formation of polyubiquitin chains, also blocks CFTR degradation (Ward *et. al.*1995). Since then, many proteins including apoB (Fisher *et. al.*1997), TcR α subunit (Yu *et. al.*1997), and carboxypeptidase Y (CPY*) mutant (Hiller *et. al.*1996) (to just name a few) have been shown to be ubiquitinated and degraded by a process that is sensitive to proteasome inhibitors.

Proteasomes are localized mainly to the cytosol and a population of proteasomes is associated with the ER membrane in cultured mammalian cells (Rivett 1998; Sakata *et. al.*1999). In the fission yeast *S. pombe*, proteasome has also been detected in the nuclear envelope-ER network and throughout the nucleus by immunofluorescence and subcellular fractionation (Enenkel *et. al.*1998). Two ubiquitin-conjugating (ubc) enzymes that are essential for ubiquitin-proteasomal degradation, Ubc6p and Ubc7p, were localized to the ER and were found to participate in the ERAD in the budding yeast (Sommer and Jentsch 1993; Biederer *et. al.*1996; Hiller *et. al.*1996). Ubc6p is an integral membrane protein of the ER. Ubc7p is recruited to the ER membrane through the interaction with an integral membrane protein, Cue1p, of the ER membrane (Sommer and Jentsch 1993; Biederer *et. al.*1996; Hiller *et. al.*1996). Their murine homologs were recently cloned and found to reside at the similar location (Tiwari and Weissman 1998).

The dislocation of ER resident proteins for cytosolic ubiquitin-proteasome degradation via the Sec61 translocon was first suggested by studies of Wiertz *et. al.* (Wiertz *et. al.*1996; Wiertz *et. al.*1996). They have observed that in human cytomegalovirus (HCMV) infected cell, newly synthesized MHC class I heavy chain (HC) was degraded rapidly after translocation into the ER. They showed that HMCV expressed US2 and US11 viral proteins stimulated the dislocation of nascent MHC I heavy chain into the cytosolic department for degradation. The deglycosylated MHC class I heavy chain breakdown intermediates were found in an association with either the Sec61 complex or the proteasome. Furthermore, DTT-treated and glycosylated MHC class I molecule was also found in an association with the Sec61 complex (Wiertz *et. al.*1996). Ubiquitinated MHC I heavy chains have been observed to associate with the membrane fraction by subcellular fractionation (Shamu *et. al.*1999). The association of

Sec61β with either ubiquitinated CFTR or ubiquitinated apoB has been detected in the presence of proteasome inhibitors (Bebok *et. al.*1998; Chen *et. al.*1998). Ubiquitinated apoB was also found in an association with calnexin (Liao *et. al.*1998). It has been suggested that retrograde translocation is a normal process for ERAD.

Studies with *S. cerevisiae* mutants have further supported the retrograde transport of misfolded proteins via the functional translocon (Pilon *et. al.*1997; Plemper *et. al.*1997). Additional components involved in ERAD have been identified in yeast. Kar2p, a yeast homolog of BiP has been shown to be involved in the retrograde translocation of misfolded protein to the cytosol. Functional Kar2p and translocon are required for ERAD because an accumulation of mutated CPY* was observed in either the *kar2-113* mutants or the *sec61* mutants (Plemper *et. al.*1997). It has been postulated that the direction of translocation, either in or out of the ER, is directed by the composition of the translocon and/or modulated by other cellular factors. Subsequent studies have shown that protein export and import of the ER are mechanistically distinct (Brodsky *et. al.*1999). The study has shown that both import proficient *kar2* mutant and import deficient *kar2* mutant were detective for ERAD (Brodsky *et. al.*1999). Furthermore, the authors have also shown that the cytosolic HSP70 Ssa1p is not involved in directing retrograde translocation of misfolded proteins in ERAD (Brodsky *et. al.*1999). Additional components in ERAD including Der1p, Hrd1p/Der3p and Hrd3p have been identified in the budding yeast (Knop *et. al.*1996; Plemper *et. al.*1999). Der1p is predicted to be a hydrophobic protein of the ER membrane. The loss of Der1p has been shown to correlate with a defect in the ER degradation process (Knop *et. al.*1996). Der3p/Hrd3p is a type II membrane glycoprotein of the ER with a hydrophilic luminal domain (Plemper *et. al.*1999). The interaction among Der3p, Hrd3p, and Sec61p were recently demonstrated by genetics analyses (Plemper *et. al.*1999). They are the potential candidates for modulating the direction of protein trafficking through the Sec61 channel.

A potential involvement of calnexin in ERAD is controversial (Knop *et. al.*1996; McCracken and Brodsky 1996). In an *in vitro* reconstituted ERAD assay, it has been shown that cytosol, ATP and calnexin were required for the degradation of

unglycosylated pro-alpha factor (pαF). Microsomes prepared from a yeast strain containing a disrupted calnexin gene that were unable to degrade pαF (McCracken and Brodsky 1996). Another group of investigators, however, showed that the disruption of *cne1*$^{+}$ displayed no effect on the degradation of carboxypeptidase Y *in vivo* in *S. cerevisiae* (Knop *et. al.*1996).

In mammalian cells, the inhibition of calnexin binding has led to an increase in the degradation of a number of glycoproteins including apoB (Chen *et. al.*1998), coagulation factor VIII (Pipe *et. al.*1998), truncated ribophorin I_{332} (RI_{332}) (de Virgilio *et. al.*1999), alpha subunit of the nicotinic acetylcholine receptor (Chang *et. al.*1997)(Keller 1998), TcR α subunit (Kearse *et. al.*1994), asialoglycoprotein receptor polypeptides H2a (Ayalon-Soffer *et. al.*1999), CD1b HC and MHC class I HC (Huttinger *et. al.*1999). In addition, mannose trimming appears to be a directing signal for the degradation of a number of glycoproteins in both the budding yeast and mammalian cells (Jakob *et. al.*1998; Ayalon-Soffer *et. al.*1999; Huttinger *et. al.*1999; Liu *et. al.*1999; Chung *et. al.*2000).

Calnexin cycle

The core components of calnexin cycle are calnexin, glucosidase II , UGGT and Erp57 (Zapun *et. al.*1997; Zapun *et. al.*1998).

Calnexin and calreticulin lectin binding

Calnexin and calreticulin are molecular chaperones for glycoproteins. Their preference for N-linked glycoproteins was first investigated employing tunicamycin, a glycosylation inhibitor that prevents the formation of dolichol-linked $G_1M_9GlcNAc_2$ (Ou *et. al.*1993). Further evidence for the lectin binding activity of CNX came from *in vitro* studies. It has been shown that a purified lumenal domain of CNX (CNX ΔTMC) was able to bind to $G_1M_9GlcNAc_2$ oligosaccharide *in vitro*. CNX ΔTMC displayed a lesser affinity for $G_1M_{5-7}GlcNAc_2$ oligosaccharide *in vitro* (Ware *et. al.*1995; Vassilakos *et. al.*1998). CRT exhibits a higher affinity toward $G_1M_9GlcNAc_2$ oligosaccharide, whether it is peptide linked or not, than $G_1M_{5-8}GlcNAc_2$ *in vitro*. α1-6 branched mannose appears

to be important for CRT binding to monoglucosylated oligosaccharides (Spiro *et. al.*1996) (Fig. 1A). Monoglucosylated oligosaccharides on RNase B was sufficient to elicit the interaction with either the luminal domain of CNX (CNX ΔTMC) or CRT *in vitro* (Rodan *et. al.*1996; Zapun *et. al.*1997). An *in vivo* interaction between calnexin and $G_1M_5GlcNAc_2$ modified TcR beta subunit or influenza hemaglutinin molecule was recently demonstrated in cultured mutant cells that produce truncated G_1- or $G_3M_5GlcNAc_2$ oligosaccharides (Van Leeuwen and Kearse 1997; Ermonval *et. al.* 2000). In an *in vitro* reconstitution assay, it was recently shown that monoglucosylated glycan is essential and sufficient for calreticulin to bind to it substrates including HA and VSV G proteins (Peterson and Helenius 1999). It has been speculated that calmegin would share a similar preference for mono-glucosylated glycoproteins based on the high sequence homology to calnexin and calreticulin (Fig. 2). Two models have been proposed for the molecular mechanism for the interaction of calnexin or calreticulin with newly synthesized glycoproteins. The two proposed models are "lectin-only" and "dual binding" models (Rodan *et. al.*1996; Zapun *et. al.*1997; Ihara -Doyle *et. al.*1999; Saito *et. al.*1999).

In the "lectin-only" model (Fig. 3A), calnexin acts purely as a lectin that interacts with the monoglucosylated oligosaccharide moiety to retain incompletely folded glycoproteins and to prevent aggregation. The oligosaccharide-binding site has been identified to be within the proline-rich region of calnexin that consititutes its conserved repeats 1 and 2 (Fig. 2). Ca^{2+} cation is essential to the lectin binding of both calnexin and calreticulin (Le *et. al.*1994; Loo and Clarke 1994; Vassilakos *et. al.*1998). The folding status of the glycoprotein is monitored by UDP-glucose:glycoprotein glucosyltransferase (UGGT). UGGT only glucosylates non-native glycoproteins (Sousa and Parodi 1995). When a glycoprotein reaches its native conformation, it is no longer a substrate for UGGT and the native glycoprotein is released from calnexin/calreticulin cycle (Fig. 1A). The lectin-only model is supported by studies that employed the luminal domain of calnexin (CNX ΔTMC). It has been shown that CNX ΔTMC interacts with monoglucosylated RNase B regardless of its conformation, either native or non-native (Rodan *et. al.*1996; Zapun *et. al.*1997). Oligosaccharides resulting from digestion with

either endoH or PNGaseF digestion of monoglucosylated RNase B were found to co-elute with CNX ΔTMC (Zapun *et. al.*1997). Furthermore, the binding of CNX ΔTMC to monoglucosylated RNase B prevented its deglucosylation by GII and deglycosylation by PNGase F, which suggested that the recognition motifs for these glycosidases are hindered by calnexin binding (Zapun *et. al.*1997). Calnexin interaction with the human erythrocyte anion exchange 1 or Band 3 protein is glycan dependent. The location of the glycan however was not crucial (Popov and Reithmeier 1999). It has been suggested that the retention provided by calnexin would allow a residual time that is sufficient for the nascent glycoproteins to fold properly by interacting with other chaperones within the ER (Toyofuku *et. al.*1999). In this model, calnexin may act as an anchorage for the recruitment of other chaperones and folding enzymes to the unfolded proteins. Calnexin has been demonstrated to interact with ERp57, an ER luminal thiol oxidoreductase (Oliver *et. al.*1997; Oliver *et. al.*1999). Calnexin has been shown to enhance the activity of ERp57 on the folding of the calnexin-retained RNase B (Zapun *et. al.*1998). The lectin-only binding was also supported by the observation that the monoglucosylated glycan was essential and sufficient for calreticulin to bind to its substrates including HA and VSV G proteins (Peterson and Helenius 1999).

In the "dual binding" model, calnexin is both a lectin and a molecular chaperone (Fig. 3B). Calnexin was suggested to contain a polypeptide-binding site that interacts with the peptide segment of incompletely folded glycoproteins. The binding of monoglucosylated glycan was suggested to initiate the peptide-peptide interaction between calnexin and the newly synthesized glycoproteins (Arunachalam and Cresswell 1995; Ware *et. al.*1995). The release of glycoprotein from calnexin requires the concomitance of protein maturation and oligosaccharide modification. The domains responsible for the interaction between calnexin and the murine MHC class I heavy chain molecule have been suggested to be in the vicinity of their transmembrane regions (Margolese *et. al.*1993; Carreno *et. al.*1995). Unglycosylated proteins including MHC class I (Carreno *et. al.*1995; Zhang *et. al.*1995), unglycosylated thyroglobulin (Kim and Arvan 1995) and non-glycosylated VSV-G glycoprotein (Cannon *et. al.*1996) have been

shown to interact with calnexin *in vivo*. The removal of oligosaccharide was incapable of abrogating the association between calnexin and its substrates *in vitro* (Arunachalam and Cresswell 1995; Ware *et. al.*1995; Zhang *et. al.*1995; van Leeuwen and Kearse 1996; Bennett *et. al.*1998). In an *in vitro* study, the luminal domain of calnexin was shown to interact with non-glycosylated mitochondrial proteins including malate dehydrogenase and citrate synthase and prevented their heat-induced aggregate formation (Ihara *et. al.*1999). The molecular function of calnexin was shown to be enhanced by the inclusion of ATP *in vitro* (Ihara *et. al.*1999). Co-expression of calnexin has been shown to increase the expression of unglycosylated serotonin transporter mutant in a baculovirus system (Tate *et. al.*1999). Furthermore, calnexin was recently identified as one of the peptide binding protiens that associate with rat TAP-translocated peptides both *in vivo* and *in vitro* (Spee *et. al.*1999).

The dual-binding model is also supported by the study with calreticulin (Saito *et. al.*1999). Using purified components, calreticulin was shown to suppress the aggregation of either monoglucosylated glycoprotein or non-glycoproteins (Saito *et. al.*1999). The molecular chaperone function of calreticulin was enhanced in the presence of Mg-ATP and/or Zn^{2+} (Saito *et. al.*1999). In another study, purified calreticulin was shown to bind to denatured proteins in a time- and pH-dependent manner *in vitro* and the interaction was glycan independent (Svaerke and Houen 1998).

In either model, glucose trimming is important for CNX and CRT binding to and dissociation from glycoproteins (Hammond *et. al.*1994; Hebert *et. al.*1995). Pre-incubation with castanospermine, an inhibitor of glucosidase I and II, was shown to abrogate the association between calnexin and it substrates including viral hemaglutinin A and VSV G ts045 glycoproteins. The interaction was not affected by deoxymannojirimycin, an inhibitor of ER mannosidases (Hammond *et. al.*1994). The association between calnexin and *in vitro* translated HA was prevented by the removal of glucosidase II from the microsomes (employing a wash out method using pH 9.0 buffer followed by re-sealing of the microsomes). Castanospermine treatment also prevented the release of calnexin bound glycoproteins from calnexin (Hebert *et. al.*1995).

Monoglucosylated oligosaccharide intermediates can be generated by either successive de-glucosylation by glucosidase I and II or re-glucosylation by UGGT (Helenius 1994; van Leeuwen and Kearse 1996). The cycle of de-glucosylation and re-glucosylation allow multiple rounds of binding to either calnexin or calreticulin during the maturation of glycoproteins. This cycle will be referred to as "calnexin cycle" here (Chevet *et. al.*1999; Ellgaard *et. al.*1999). Multiple rounds of binding and dissociating from calnexin/calreticulin are important for the maturation of some glycoprotein while it plays a minor role for others. Repeated cycles of association and dissociation from calnexin and calreticulin have been shown to enhance the folding of transferrin and to reduce its disulfide-linked aggregates formation (Wada *et. al.*1997). It was suggested that repeated binding to calnexin/calreticulin would enhance the correct folding and trimerization of HA (Hebert *et. al.*1996). Evidences also showed the modification of most TcR subunits by UGGT persisted until the assembly of disulfide linked $\alpha\beta\delta\varepsilon\gamma\varepsilon$ incomplete complex was formed (Gardner and Kearse 1999).

Glucosidase I and II (GI and GII)

The removal of the two outermost glucose residues from the en-bloc oligosaccharide occurs immediately after its transfer onto the peptide backbone (Fig. 1A). Glucosidase I is a type II integral membrane protein of the ER (Herscovics 1999). In the calnexin cycle, glucosidase II (GII) performs two functions. By removing the second glucose from protein linked $G_2M_9GlcNAc_2$ oligosaccharide, GII allows the binding of glycoproteins to calnexin/calreticulin. GII also prevents the binding of glycoprotein to calnexin/calreticulin by removing the innermost glucose residue (Zapun *et. al.*1997)(Fig. 1A). GII is a soluble heterodimeric complex consisting of α and β subunits (Trombetta *et. al.*1996). By gene disruption and over-expression experiments, it was demonstrated that the yeasst GII α subunit contains the catalytic function. The *S. cerevisiae* GII β subunit contains a carboxyl terminal ER (-HDEL tetrapeptide) retention signal (Trombetta *et. al.*1996; Arendt and Ostergaard 1997). Alternative splicing of transcripts encoding both α and β subunits of GII has been observed in murine T lymphocytes. It was proposed that distinct isoforms may perform specialized functions (Arendt *et. al.*1999). GII displays a

preferential deglucosylation towards $G_1M_9GlcNAc_2$ over $G_1M_{7-8}GlcNAc_2$ oligosaccharides (Grinna and Robbins 1980). An *in vitro* study has shown that the deglucosylation activity of $G_1M_8GlcNAc_2$ and $G_1M_7GlcNAc_2$ were 21% and 9% of that observed for $G_1M_9GlcNAc_2$ oligosaccharide, respectively (Grinna and Robbins 1980). This preferential substrate specificity may be important for ER associated protein degradation (see below).

UDP-glucose:glycoprotein glucosyltransferase (UGGT)

UGGT is a glycoprotein folding sensor of the calnexin cycle. Rat liver UGGT was purified to homogeneity as a soluble ER resident protein containing an ER resident signal at its carboxyl terminus. UGGT was suggested to exist as a homodimer *in vivo* (Trombetta *et. al.* 1992). Both rat and *S. pombe* UGGT contain high mannose oligosaccharide (Trombetta *et. al.* 1992; Fernandez *et. al.*1994). Two human isoforms have been detected (Arnold *et. al.*1999). The enzymatic activity of one of the two human UGGT isoforms and Drosophila UGGT is stimulated with either Ca^{2+} or Mn^{2+} cation (Parker *et. al.*1995; Arnold *et. al.*1999) whereas *S. pombe* and rat UGGT requires Ca^{2+} ion for their activity (Fernandez *et. al.*1994; Trombetta. *et. al.* 1992).

In a cell free system, purified UGGT or microsomal preparation glucosylates denatured or non-native glycoproteins using UDP-Glc as the sugar donor (Sousa, *et. al.*1992; Fernandez *et. al.*1994; Parker *et. al.*1995; Trombetta, *et. al.* 1989). Rat liver UGGT recognizes both hydrophobic amino acids and the innermost GlcNAc residue of the glycan chain that are covalently linked in denatured glycoprotein (Sousa *et. al.*1992; Sousa and Parodi 1995). The catalytic domain of UGGT resides at its carboxyl terminus. Point mutation of two conserved residues, Tyr^{1312} & Asp^{1352}, in *S. pombe* UGGT has abolished its glucose transferase activity (Fanchiotti *et. al.*1998). Deletion of six amino acid residues within the conserved carboxyl terminus domain in one of the human UGGT isoforms also abolished its enzymatic activity (Arnold *et. al.*1999). The rate of *in vitro* glucosylation by UGGT is affected by the oligosaccharide structure. Oligosaccharide structure composed of $M_7GlcNAc_2$ and $M_8GlcNAc_2$ structures are glucosylated by UGGT at 15% and 50%, respectively, at the rate for $M_9GlcNAc_2$ (Sousa *et. al.*1992).

In vivo studies with *S. pombe* have revealed that misfolded conformation of the glycoprotein was not sufficient for glucosylation by UGGT (Fernandez *et. al.*1998). The additional required elements remain to be elucidated. The expression of *S. pombe* UGGT is induced by conditions that promote accumulation of misfolded proteins within the ER lumen such as heat shock and in the presence of either tunicamycin, calcium ionophore, 2-mercaptoethanol or 2-deoxyglucose. *S. pombe* UGGT is not essential for cell viability (Fernandez *et. al.*1998) whereas it became essential under cellular stress conditions (Fanchiotti *et. al.*1998).

Glucosylation of protein-linked oligosaccharide activity was not detected in S. cerevisiae UGGT homolog, Kre5p (Fernandez *et. al.*1994; Jakob *et. al.*1998).

A number of glycoproteins including TcR α, β, δ and γ subunits and transferrin have been shown to undergo reglucosylation by UGGT *in vivo* (Van Leeuwen and Kearse 1997; Wada *et. al.*1997).

ER α-mannosidase I

Trimming of mannose residues by ER mannosidase I has been proposed as a mechanism for the retention of misfolded nascent glycoproteins and the targeting of their degradation. Mutated S. cerevisiae carboxypeptidase Y (CPY*) was shown to be degraded by the ubiquitin-proteasome pathway. The degradation was glycan dependent since nonglycosylated CPY was relatively stable *in vivo* (Knop *et. al.*1996). It was subsequently shown that the degradation of misfolded CPY* in yeast correlated with the generation of $M_8GlcNAc_2$ isomer B (Fig. 1A) by ER α-mannosidase (Jakob *et. al.*1998). The presence of a mannose lectin receptor recognizing $M_8GlcNAc_2$ oligosaccharide was postulated for directing the degradation of misfolded proteins in *S. cerevisiae* (Jakob *et. al.*1998).

Supporting evidence has also implicated the requirement of mannose trimming in the degradation of numerous misfolded glycoproteins in mammalian cells (Liu *et. al.*1997; Yang *et. al.*1998; Ayalon-Soffer *et. al.*1999; Chung *et. al.*2000; Marcus and Perlmutter 2000). Intracellular degradation of a number of glycoproteins including the TcR subunit CD3-δ, a human asialoglycoprotein receptor polypeptide H2a, and the

human CD1b heavy chain was blocked in the presence of deoxymannojirimycin (DMJ), a mannose analogue (Yang *et. al.*1998; Ayalon-Soffer *et. al.*1999; Huttinger *et. al.*1999). Both DMJ and kifunensine treatments blocked the intracellular degradation of a secretion incompetent null Hong Kong variant and a secretion impaired PiZ variant of α_1-antitrypsin (Liu *et. al.*1997; Marcus and Perlmutter 2000). Initiation of mannose trimming was shown to be a prerequisite for the degradation of two mutant alpha (2)-plasmin inhibitors (Chung *et. al.*2000).

The elegant studies carried by Liu *et. al.* have illustrated the possible sequence of events of oligosaccharide processing that coupled the degradation of misfolded glycoprotein using the secretion incompetent null Hong Kong variant of α_1-antitrypsin (Liu *et. al.*1999). This is depicted in Fig. 1B. Partial deglucosylation by glucosidase I and II generates a protein-linked $G_1M_9GlcNAc_2$ intermediate which promotes its association with calnexin (Fig. 1A). The association of glycoprotein with calnexin is prevented by the removal of the innermost glucose by GII. If the protein is folded properly, the protein-linked $M_9GlcNAc_2$ intermediate will be directed for secretion (Fig. 1B, pathway 1). If the glycoprotein is not folded correctly, it will be recognized and re-glucosylated by the UGGT. The $G_1M_9GlcNAc_2$ glycoprotein will re-bind to calnexin (Fig. 1B, pathway 2). Recycling of de- and re-glucosylation will retain the misfolded glycoproteins by their binding to calnexin and will allow a chance for the glycoprotein to fold correctly within the ER. However, persistent misfolded glycoprotein will be recognized, presumably, and a mannose from the middle branch of the oligosaccharide will be removed by the ER mannosidase I generating a B isomer (Fig. 1B, pathway 3). The unfolded intermediate can be glucosylated by UGGT generating a $G_1M_8GlcNAc_2$ intermediate and allow it to re-bind to calnexin. De-glucosylation of $G_1M_8GlcNAc_2$ oligosacchariede is only 21% of the efficiency for $G_1M_9GlcNAc_2$ by GII *in vitro* (Grinna and Robbins 1980)(Fig. 1A, inset). The substrate preference of GII would favor an interaction between misfolded monoglucosylated glycoprotein and calnexin. Calnexin binding to misfolded glycoprotein containing $G_1M_8GlcNAc_2$ oligosaccharide can presumably retain it and direct it towards the ER associated degradation machinery. In this manner, it has been proposed that ER

mannosidase I may act as a clock regulating the disposal of glycoproteins that are mutant or misfolded. A recently cloned mammalian ER α-mannosidase I that is specific for generating isomer B $M_8GlcNAc_2$ intermediate (Tremblay and Herscovics 1999) (Gonzalez *et. al.*1999) will provide a more direct testing of the role of mannose trimming in targeting misfolded glycoprotein for intracellular degradation.

ERGIC 53

ERGIC 53 or MR60 is a 53 kDa non-glycosylated type I membrane protein originally identified as a marker for the ER to Golgi intermediate compartment (ERGIC) (Schweizer *et. al.*1988). ERGIC 53 circulates between ER and cis-Golgi compartment. ERGIC 53 contains a dilysine motif, as its ER retrieval signal (Schindler *et. al.*1993), and two phenylalanine residues, as its ER-exit determinant, at its cytosolic domain (Kappeler *et. al.*1997). ERGIC 53 may function as a molecular chaperone facilitating the transport of a subset of glycoproteins from the ER to the ERGIC. Mistargeting of ERGIC 53 has been shown to impair the secretion of procathepsin C (Vollenweider *et. al.*1998) and coagulation factors V and VIII (Nichols *et. al.*1998; Moussalli *et. al.*1999). ERGIC 53 has recently shown to be cross-linked to a glycorptein related to a human cysteine proteinase cathepsin Z (CatZr) in a glycan dependent manner (Appenzeller *et. al.*1999). Folded and unglucosylated CatZr was shown to be the best substrate for ERGIC 53 binding (Appenzeller *et. al.*1999).

ERGIC 53 is a lectin specific for high mannose-type glycan and the interaction is calcium dependent (Pimpaneau *et. al.*1991). Mannose trimming is not required for ERGIC 53 binding, however, inhibition of glucose trimming interfered with ERGIC 53 binding (Appenzeller *et. al.*1999). Protein linked $M_9GlcNAc_2$ oligosaccharide structure appears to be the preferred substrate for ERGIC 53 binding (Moussalli *et. al.*1999). It was also demonstrated that monomeric ERGIC53 could bind to endogenous cargo efficiently and dimerization of ERGIC 53 was the minimal requirement for their exit of the ER (Appenzeller *et. al.*1999). It was suggested that ERGIC 53 may act in concert with the calnexin cycle on the maturation of glycoprotein folding and assembly. The binding of

high mannose containing folded glycoproteins to ERGIC 53 may remove folded glycoproteins from the calnexin cycle and transport them out of the ER (Appenzeller *et. al.*1999).

ER signaling

Unfolded Protein Response (UPR)

Accumulation of unfolded proteins within the ER lumen has been shown to result in the up-regulation of a set of ER resident protein expression and the suppression of protein synthesis. The unfolded protein response (UPR) is conserved from yeast to mammalian cells (Sidrauski *et. al.*1998; Kaufman 1999). This complex signaling pathway has been well characterized in *S. cerevisiae* (Cox *et. al.*1993; Mori *et. al.*1993; Sidrauski *et. al.*1998; Kaufman 1999). The four constituents required for the yeast UPR are Ire1p/Ern1p (inositol requiring or ER to nucleus), Hac1p, Rlg1p and Ptc2p (Cox and Walter 1996; Sidrauski *et. al.*1996; Welihinda *et. al.*1998). Ire1p is an ER type I transmembrane protein and acts as a sensor of ER stress (Sidrauski *et. al.*1998; Kaufman 1999). Upon ER stress, Ire1p oligomerizes and both kinase and endoribonuclease activities within the carboxy-terminus of Ire1p are activated to initiate the UPR signaling cascade (Shamu and Walter 1996; Welihinda and Kaufman 1996). Ire1p is negatively regulated by a protein Ser/Thr phosphatase, Ptc2p (Welihinda *et. al.*1998). Hac1p is a transcription factor that binds to the UPR element in the promoter region of UPR responsive genes (Cox and Walter 1996). The binding of Hac1p to the UPR element is regulated by an unconventional mRNA splicing reaction initiated by the endoribonuclease activity of Ire1p. The endoribonuclease activity of Ire1p cleaves *HAC1* mRNA at the 5' and 3' splice site junctions to remove an intron within the 3' end of *HAC1* mRNA (Sidrauski and Walter 1997; Kawahara *et. al.*1998). The cleaved *HAC1* exons are subsequently ligated by the tRNA ligase, Rlg1p. Whereas unspliced *HAC1* mRNA is not translated, spliced *HAC1* mRNA is efficiently translated (Sidrauski *et. al.*1996; Kawahara *et. al.*1997).

The mammalian UPR shares some similarities to the yeast UPR but appears to be more complex and sophisticated. Two mammalian homologs of yeast Ire1p were recently isolated from human (h) and murine (m) genomes, *IRE1α/ERN1* (Tirasophon *et. al.*1998) and *IRE1β/ERN2* (Wang *et. al.*1998), respectively. Over-expression of *hIRE1α* or *mIRE1β* constitutively activated the UPR (Tirasophon *et. al.*1998) whereas over-expression of *mIRE1β* also induced apoptosis in the transfected cells (Wang *et. al.*1998). This suggests that the *IRE1* family may be involved in the activation of UPR pathway. Upon ER stress, both mammalian Ire1αp and Ire1βp were able to cleave the transfected *HAC1* mRNA at both splice junctions *in vivo* (Niwa *et. al.*1999). The mammalian equivalent to the yeast UPR element, has been identified and is called the ER stress response element (ERSE) (Yoshida *et. al.*1998). A mammalian mRNA substrate for hIre1αp is presently unknown although a potential transcription factor, ATF6, appears to be involved in mammalian UPR (Yoshida *et. al.*1998). ATF6 is post-translationally processed from a 90 kDa ER-localized protein to a 50 kDa nuclear-localized protein upon ER stress (Yoshida *et. al.*1998; Haze *et. al.*1999). The ligand for Ire1p has not yet been identified. It has been suggested that the amount of free BiP may be crucial for the activation of UPR. Overexpression of BiP in cultured mammalian cells and in yeast has been shown to suppress the induction of the UPR pathway. It was suggested that the UPR mechanism contain a sensing mechanism that could detect the amounts of free BiP or the amount of BiP in complex with unfolded proteins (Chapman *et. al.*1998).

Ire1p was found to associate with presenilin-1 by co-immunoprecipitation (Katayama *et. al.*1999). Mutation of presenilin-1 is associated with a subset of early-onset familiar Alzheimer's disease (Sherrington *et. al.*1995). Presenilin-1 contains either the γ-secretase activity or is involved in the activation of γ-secretase activity (Wolfe *et. al.*1999). γ-secretase cleaves the amyloid precursor proteins and generates Aβ42 which is a major constituent of the amyloid plaques present in Alzheimer's disease patients (Selkoe 1999). It was demonstrated that upon ER stress, the cytosolic domains of both mammalian Ire1αp and Ire1βp containing the activities of both kinase and endoribonuclease are cleaved and translocated into the nucleus (Niwa *et. al.*1999). The

cleavage of mammalian Ire1αp and Ire1βp and their subsequent nucleus translocation and UPR induction are dependent on the presenilin-1 activity (Niwa *et. al.*1999).

Upon UPR activation, protein synthesis is simultaneously halted to alleviate the stress within the ER lumen (Brostrom *et. al.*1996). Prompt phosphorylation of eIF-2α by PERK (PKR-like ER kinase)/PEK (pancreatic eIF-2α kinase) which impedes the formation of functional 40S translation-initiation complex and subsequently leads to a global suppression of protein synthesis (Shi *et. al.*1998; Harding *et. al.*1999). PKR (dsRNA-dependent protein kinase) is another kinase that is capable of phosphorylating eIF-2α (Srivastava *et. al.*1998). PERK is an ER stress sensor with a type I transmembrane topology. The intralumenal domain of PERK shares sequence similarity with the lumenal domain of the mammalian Ire1p. The cytosolic domain of PERK contains sequence similarity to PKR (Shi *et. al.*1998; Harding *et. al.*1999). The lumenal domain of PERK thereby is likely to sense the ER stress in a similar fashion as Ire1p and consequently phosphorylates the eIF-2α to suppress protein translation to alleviate the stress.

ER overload response (EOR)

EOR also occurs when excessive cargo proteins accumulated within the ER lumen through ,for instance, over-expression of protein subunits that cannot assemble for export (Kaufman 1999; Pahl 1999). Morphological alteration of ER, dilation and distension due to the excessive cargo proteins, is likely one major stimulating factor. Over-expression of a bacterial choloramphenicol acetyltransferase in the cytosolic or a firefly luciferase in the peroxisome did not induce EOR pathway. In response to ER overload, ER sends a signal to activate the transcription factor nuclear factor kB (NFκB) to induce the transcription of proinflammatory and immune response. NFκB is kept inactive in an association with Iκb in the cytoplasm. Phosphorylation of Iκb and its subsequent degradation by proteasomes activates NFκB. Induction of NFκB activation and its nuclear translocation via the EOR pathway may represent a physiological anti-viral host response. Upon viral infection, a large amount of viral proteins are processed in the ER. Activation of NFκB may launch a quick anti-viral response since several NFκB target genes encode anti-viral defense

proteins including cytokines, interferons and proteins involved in viral peptide presentation to T cells. Calcium chelators and anti-oxidants can block the activation of NFκB suggesting of possible second messengers such as Ca^{2+} ion and reactive oxygen intermediates. Certain reagents that activated the UPR pathway including tunicamycin and 2-deoxyglucose treatment also induced the EOR pathway. Heavy metal, dithiothreitol and castanospermine, however, specifically activate the EOR pathway and not the UPR pathway. At least two signal transducing molecules (Ire1p and PERK) for UPR has been identified whereas the same for EOR has not yet been identified. The identification of the ligand and the transducer will aid in dissecting the EOR from the UPR pathway (Kaufman 1999; Pahl 1999).

Calcium homeostasis

The ER is a major intracellular calcium storage site. A high ER Ca^{2-} concentration is maintained by Ca^{2+}-ATPase and sequestered by abundant calcium binding proteins including calreticulin, BiP, PDI family members, ERp72, GRP94, calsequestrin and calnexin in the ER lumen (Meldolesi and Pozzan 1998; Michalak *et. al.*1998; Michalak *et. al.*1999). Calcium functions as a universal second messenger and participates in numerous signaling pathways. For instance, inositol (1,4,5)-triphosphate (InP_3)-mediated release of calcium is required for the activation of calcineurin to dephosphorylate NF-AT3. Dephosphorylated transcription factor NF-AT3 is subsequently translocated into the nucleus for the transcription of genes related to cardiac developement (Molkentin *et. al.*1998). Furthermore, depletion of ER Ca^{2+} followed by Ca^{2+} ionophore treatment has also been shown to perturb protein folding and processing within the ER (Wileman *et. al.*1991).

Calcium is released by ryanodine receptors and InP_3 receptors and is taken up by sarco-endoplasmic reticulum Ca2+-ATPase (SERCA) into the ER lumen (Pozzan *et. al.*1994; Meldolesi and Pozzan 1998). In an elegant study conducted by John *et. al.* (John *et. al.*1998) employing Xenopus oocytes, they showed that the glycan-dependent interaction between calreticulin and SERCA2b modulates its calcium uptake property. SERCA2a and SERCA2b are alternative spliced forms with different Ca^{2+} sensitivity.

ATP hydrolysis and rates of calcium transport (Pozzan *et. al.*1994; Meldolesi and Pozzan 1998). SERCA2a is expressed in cardiac muscle and has a function similar to SERCA1. SERCA2b has the highest calcium sensitivity among all isoforms and is expressed in all non-muscle cells. SERCA2b differs from SERCA2a by having an additional 46 amino acids and glycosylated at Asn^{1036} at the carboxy-terminus. Upon InP_3 injection into Xenopus oocytes system, calcium is released via the InP_3 receptors. Over-expression of SERCA2b was shown to induce a high frequency and short Ca^{2+} wave upon the InP_3 injection. Co-expression of both calreticulin and SERCA2b, a sustained release of calcium without repetitive Ca^{2+} oscillation was observed with the InP_3 stimulation. Calreticulin binding to SERCA2b and its modulation is glycan dependent (John *et. al.*1998). A similar, but a less inhibitory, effect exerted by either calnexin or calmegin was also observed. Interestingly, the inhibitory effect on SERCA2b was not observed with either calnexin or calmegin mutant lacking the potential PKC phosphorylation site at their most carboxy termini (Fig. 23). It is possible that the phosphorylation of calnexin and calmegin may regulate their luminal oriented lectin- and/or calcium- binding activity or vice versa. Calnexin and calmegin was also postulated to participate in an inter-orgnaellar signaling pathway between the ER and the cytosol or other organelles (Roderick *et. al.*1998).

Calnexin phosphorylation and intracellular location

Calnexin was originally identified and purified as a constituent of a complex of four co-isolated integral membrane proteins from the canine pancreatic microsomes (Wada *et. al.*1991). The three calnexin co-purified proteins were pgp35, also known as signal sequence receptor α (SSRα), but later renamed as translocon associated protein α subunit (TRAPα) (Prehn *et. al.*1990; Hartmann *et. al.*1993); gp25H (or TRAPβ) (Gorlich *et. al.*1990; Hartmann *et. al.*1993) and gp25L, the founding member of the Emp24 family that has been implicated in protein transport (Wada *et. al.*1991; Dominguez *et. al.* 1998). The association of calnexin and TPAPα (pgp35 or SSRα) was also detected in HepG2

cells (Galvin *et. al.*1992). Turnover studies indicated that both calnexin and pgp35 are stable and they remain associated for at least 24 hours (Galvin *et. al.*1992).

Calnexin and TPAPα (pgp35 or SSRα) are exclusively phosphorylated on their cytosolically oriented serine residues *in vitro* (Wada *et. al.*1991). Casein kinase II (CK2) was subsequently purified and identified as an ER-associated kinase that is responsible for the *in vitro* phosphorylation of both calnexin and TPAPα (pgp35 or SSRα) in isolated microsomes (Ou *et. al.*1992).

By immunofluorenscence and immunogold electron microscopy, calnexin was detected at both the nuclear envelope and the rough ER in intact cultured cells (Hochstenbach *et. al.*1992). Calnexin was also observed in the nuclear envelope in addition to the ER in neuronal cell by immunohistochemical detection (Krijnse-Locker *et. al.*1995). By subcellular fractionation, calnexin was detected in nuclear fraction using both Huh7 human hepatoma cells and rat hepatic nuclear fraction (Galvin *et. al.*1992; Gilchrist and Pierce 1993). On this note, calmegin/calnexin-t was found to localize in both the ER membrane and the nuclear envelope of spermatogenic cells by immunocytochemistry (Yoshinaga *et. al.*1999).

Modification and Oligomeric formation of calnexin and calreticulin

The interaction between monomeric CNX and its monomeric substrates was observed *in vivo* by pulse-chase experiment (Ou *et. al.*1993; Le *et. al.*1994; Tatu and Helenius 1997). Oligomerization of the luminal domain of calnexin as dimer, tetramer and pentamer has been observed *in vitro* (Ou *et. al.*1995; Vassilakos *et. al.*1998; Zapun *et. al.*1998; Ihara *et. al.*1999). The luminal domain of calnexin, when transfected into COS cells, forms hetero-oligomer with the full-length or endogenous CNX (Ho *et. al.*1999). It remains to be determined whether the *in vivo* oligomerization of calnexin occurs directly or indirectly via calnexin substrate with multivalent binding sites for calnexin. Heptameric formation of calreticulin *in vitro* was also observed by size exclusion chromatography (Saito *et. al.*1999). It is unclear whether homo-oligomeric

formation of either calnexin or calreticulin contributes to the lectin-like molecular chaperone function and calcium binding ability of both CNX and CRT.

The oligomeric formation of calnexin may be influenced by its cytosolic phosphorylation modification. In the ER lumen, oligomeric BiP is phosphorylated whereas monomeric BiP is not modified by phosphorylation (Freiden *et. al.*1992). The oligomeric formation of mammalian small heat shock proteins and their subsequent action is regulated by phosphorylation (Rogalla *et. al.*1999). Calreticulin phosphorylation is observed in isolated rat hepatocytes. Phosphorylation of calreticulin was increased when the cells were treated with phorbol esters or with adrenaline plus propranolol. Calreticulin phosphorylation could be blocked in the presence of the PKC-specific inhibitor Ro 31-8220 (Rendon-Huerta *et. al.*1999). Furthermore, calreticulin was shown to interact with a number of PKC isoforms *in vivo* by co-immunoprecipitation (PKC α, β, θ, ζ, and μ) (Rendon-Huerta *et. al.*1999). Calreticulin also undergoes a prompt glycosylation in response to heat shock treatment (Jethmalani *et. al.*1994) and amino acid deprivation (Heal and McGivan 1998). This modification may also modulate the chaperone function and/or oligomeric state of calreticulin.

The interaction between ERp57 and CNX or CRT may also be modified by phosphorylation. Mass spectral analyses of TAP-associated ERp57 by MALDI-MS revealed its potential modification by tyrosine and/or serine phosphorylation (Morrice and Powis 1998).

Calnexin superfamily

The calnexin gene has been identified in a variety of eukaryotes including mammals, nematodes, yeasts and plants (Table 1 to 3). Calmegin, a testis-specific calnexin analogue, has been cloned from both mouse and human (Table 3). All calnexins and calmegins display a type I integral membrane topology and contain a stretch of charged amino acids at their carboxyl termini, except *S. cerevisiae* Cne1p (Table 2).

Mammalian calnexins have been cloned from *Homo sapien* (human) (David *et. al.*1993), *Canine familiaris* (dog) (Wada *et. al.*1991), *Rattus norvegicus* (rat), *Mus*

musculus (mouse) (Tjoelker *et. al.*1994) and they are highly conserved to each other. They share an overall of 93-98.6% amino acids identical to each other (Table 1). The cytosolic domains of mammalian calnexins are conserved, 84.3-97.7% identical to each other (Table 2). The length of their cytosolic domains ranged from 87 to 89 amino acids (Table 3).

Calnexin homologs have also been cloned from *Rana rugosa* (frog) (Yamamoto and Nakamura 1996), *Drosophila melanogaster* (fly) (Christodoulou *et. al.*1997), *Caenorhabditis elegans* (a nematode worm) (Wilson *et. al.*1994), *Schistosoma manosoni* (Hawn *et. al.*1993) and *Schistosoma japonicum* (trematode parasitic flukes) (Hooker and Brindley 1999). The overall amino acid identity from each other ranged from 40 to 85% (Table 1). Their cytosolic domains ranged from 95 to 100 amino acids in length (Table 3).

In plants, calnexin homologs have been cloned from *Arabidopsis thaliana* (thale cress) (Huang *et. al.*1993), *Pisum sativum* (pea) (Ehtesham *et. al.*1999), *Glycine max* (soybean) (Ehtesham *et. al.*1999), and *Helianthus tuberosus* (Jerusalem artichoke) (Hasenfratz *et. al.*1997)(Table 1 and 2). They have a short cytosolic tail ranging from 44 to 55 amino acids in length (Table 3).

Two calnexin homologs have been cloned from yeasts, *Schizosaccharomyces pombe* (Jannatipour and Rokeach 1995; Parlati *et. al.*1995) and *Saccharomyces cerevisiae* (de Virgilio *et. al.*1993; Parlati *et. al.*1995). *S. pombe* calnexin, Cnx1p, exhibits a greater identity to calnexin of mammalian origin than to *S. cerevisiae* (37.6% vs. 25.6%, Table 2). *S. pombe* Cnx1p is 30.1% identical to *S. cerevisiae* calnexin, Cne1p. A greater divergence between the yeast calnexin homologs resides at their cytosolic domains. *S. pombe* Cnx1p contains a short cytosolic domain of 48 amino acid in length whereas *S. cerevisiae* Cne1p contains one cytosolically orientated amino acid, Thr^{483}.

Calnexin domains

There are several amino acid motifs shared by calnexin and its homologs. The similarities among them are summarized here.

Luminal domain

Calnexin, calmegin and calreticulin contain two sets of repeating structures, both of which are rich in proline and tryptophan. All calnexins and calmegin four copies of both repeating sequence 1 (PXXIXDPDEAXKPEDWDE) and 2 (GXWXXPXIXNPX YX) whereas three copies of each is present in calreticulin (Fig. 2) (Michalak *et. al.*1999). In *Trypanosoma cruzi* calreticulin, one copy of repeat 2 is detected which is similar to calreticulin of another trypanosomatid protozoan, *Leishmania donovani* (Labriola *et. al.*1999) (Fig. 2). A conserved structure and function of the domain containing these two repeats is speculated. The first set of repeats is a high affinity and low capacity calcium-binding site (Michalak, *et. al.*1999; Tjoelker *et. al.*1994). The second set of repeating sequence does not bind calcium (Tjoelker *et. al.*1994). The domain containing both sets of repeating sequences was shown to constitute the oligosaccharide binding domain for both calnexin and calreticulin (Vassilakos *et. al.*1998).

Although the luminal domain of calnexin is highly conserved among calnexins from different species, full-length canine calnexin was unable to complement the function of *S. pombe* Cnx1p *in vivo* (Parlati *et. al.*1995). Canine and *S. pombe* calnexins were unable to substitute the activity of *S. cerevisiae* Cne1p in β1,6-glucan biosynthesis (Shahinian *et. al.*1998).

Mammalian calnexins are not glycosylated (Degen and Williams 1991; Wada *et. al.*1991; David *et. al.*1993) (Table 3). Potential glycosylation sites for *C. elegans, D. melanogaster, A. thaliana, P. sativum and G. max* calnexin and human calmegin are deduced from consensus N-linked glycosylation sequeon (NXS/T) (Kornfeld and Kornfeld 1985). *S. pombe* and *S. cerevisiae* calnexins, however, are glycosylated *in vivo* (Parlati *et. al.*1995; Parlati *et. al.*1995).

Transmembrane domain

For both murine MHC I heavy chain and TcR alpha subunit, systemic deletion analyses have suggested that their primary site of interaction to calnexin is in the vicinity of their transmembrane domains of calnexin, MHC I heavy chains and TcR α subunit (Margolese *et. al.*1993; Carreno *et. al.*1995). A soluble calnexin molecule corresponding

to the luminal domain of calnexin fails to associate with human MHC I heavy chain, Ltk, and monomeric immunoglobulin μ chain in transfected COS cells. The interaction was restored when the luminal domain of calnexin was anchored to the membrane with a heterologous transmembrane and cytosolic domain (Ho *et. al.*1999). The transmembrane interaction between calnexin and membrane proteins may represent a non-specific hydrophobic interaction within the lipid bilayer. Calreticulin does not bind to α_1-antitrypsin *in vivo* but the membrane anchored calreticulin does (Wada *et. al.*1995). It appears that the transmembrane domain of calnexin may situate it at the membrane level in the vincinity of the translocon and allows calnexin to interact with nascent glycoproteins during or shortly after they enterance into the ER lumen.

Cytosolic domain

The cytosolic domains of calnexin are hydrophilic and contain putative ER retention motif and Ca^{2+}-binding domains (Tjoelker *et. al.*1994). The cytosolic domains of mammalian calnexin and calmegin can be divided into four subdomains: a juxtamembrane basic, an acidic, a phosphorylation/signaling and an ER retrieval domain (Fig. 23).

ER type I membrane proteins contain two lysine residues at -3 and -4 (-KKXX) or -3 and -5 (-KXKXX) at their cytosolic domains that confer their ER localization (Jackson, Nilsson *et. al.*1990). The di-lysine (-KK) motif has been observed to function as an ER retrieval signals. The di-lysine motif has been shown to interact with COP1 coatomer that is involved in retrograde transport from the retrieval of ER resident proteins from the Golgi complex (Cosson and Letourneur 1994). The di-KK motif of emp24 and ERGIC53 have been shown to interact with COP1 protein and shown to be responsible for their ER retention or ER retrieval (Kappeler *et. al.*1997; Dominguez *et. al.*1998).

Sequence composed of Arg-Lys-Arg (RKR) in the cytosolic domain of ATP-sensitive K+ channel has been suggested to serve as an ER retention or retrieval signal. Oligomerization of channels was suggested to mask the RKR sequence and thus allowed the transport of the channel complex out of the ER to the cell surface. Incompletely

assembled channels were transported to the cell surface when the RKR sequence was mutated (Zerangue *et. al.*1999). Related RXR sequences are also present in the cytosolic domains of CFTR. Mutated CFTR molecules missing the RXR sequences were expressed on the cell surface (Chang *et. al.*1999).

The ER localization signal for mammalian calnexin was assigned to an -RKPRRE motif at its most carboxyl terminus (Rajagopalan *et. al.*1994). A variation of this sequence is observed in all calnexin and calmegins except *S. pombe* and *S. cerevisiae* (Table 3). *S. pombe* Cnx1p contains a -TAKNED motif at its most carboxyl terminus and *S. cerevisiae* Cne1p contains one cytosolic amino acid residue, Thr^{483}. Cne1p has been shown to be ER-localized by subcellular fractionation, epifluorescent and immunofluorescence microscopy. It has been suggested that Cne1p may be localized to the ER by association with an unknown resident membrane or luminal protein (Parlati *et. al.*1995).

Comparison of the cytosolic domains of known calnexin sequences, tabulated in Table 3, a variation of a di-basic amino acid motif (-RR, -RK, or –KR) at the position of –2 and –3 or –3 and –4 from the carboxyl termini of all calnexins except yeast calnexin is observed. The di-basic amino acid motif of calnexin may exert a similar ER retrieval function as the di-KK motif mentioned above. A conserved proline residue at position –4 or –5 of calnexin from mammals, frog, and plants may provide an additional recognition motif for the ER retrieval mechanism. The structural bend induced by the cyclic ring of the proline residue in front of the di-basic amino acid motif may protrude the di-basic motif away from the backbone and enhance the recognition by the ER retrieval mechanism. It remains to be determined whether the ER retrieval domain of calnexin interacts with ER retrieval machinery such as COP1 proteins.

The cytosolic domain of mammalian calnexin contains calcium binding domain that binds Ca^{2+} with a moderate affinity (Tjoelker *et. al.*1994).

Phosphorylation of calnexin may provide a means to regulate calnexin function and its location. Mammalian calnexins is phosphorylated *in vivo* (Dakour *et. al.*1993; Capps and Zuniga 1994; Le *et. al.*1994). *S. mansoni* calnexin is *in vivo* phosphorylated on

both serine and threonine residues (Hawn and Strand 1994). *In vitro* translated *P. sativum* calnexin can be immunoprecipitated by anti-phosphoserine antibodies. Pea calnexin can be *in vitro* phosphorylated by microsomal associated endogenous kinases (Ehtesham *et. al.*1999). *In vitro* phosphorylation of isolated intact canine microsomes followed by a limited protease digestion demonstrated that canine calnexin is phosphorylated on its cytosolically oriented serine residues (Wada *et. al.*1991). Casein kinase II (CK2) was subsequently purified as a microsomal associated kinase and shown to phosphorylate calnexin *in vitro* (Ou *et. al.*1992; Cala *et. al.*1993). Furthermore, calmegin/calnexin-t is *in vivo* phosphorylated within its carboxy-terminus domain and can be *in vitro* dephosphorylated by acid phosphatase treatment (Ohsako *et. al.* 1998). Calnexin-t/calmegin is constitutively phosphorylated during spermatogenesis and the level of protein synthesis correlates with the level of calnexin-t phosphorylation (Ohsako *et. al.* 1998).

Calnexin family members

Calnexin is absent in a NKR cell line. NKR cell line was originally derived from a human T lymphotoid cell line, CEM, which displayed resistance to the cytotoxicity of natural killer cells (Scott and Dawson 1995). The endogenous CRT may compensate for the loss of CNX in the CEM-NRK (Allen and Bulleid 1997). Calnexin is also absent in *Drosophila Schneider* cells (Jackson *et. al.*1994). A calnexin-like protein has not yet been identified in *Trypanosoma cruzi*, however, a calreticulin activity has recently been isolated (Labriola *et. al.*1999). In *S. pombe* and *S. cerevisiae*, the essentiality of calnexin was examined. Calnexin is not essential in *S. cerevisiae* whereas it is essential for the survival of *S. pombe* (Jannatipour and Rokeach 1995; Parlati *et. al.*1995; Parlati *et. al.*1995). The cytosolic domain, however, was dispensable for viability (Parlati *et. al.*1995). Interestingly, *S. pombe* was recently showed to be viable with an amino-terminal truncated Cnx1p consisting of the cytosolic domain, the transmembrane domain and a juxtamembrane stretch of 52 amino acid of the luminal domain (Elagoz *et.*

*al.*1999). The stretch of 52 amino acids of *S. pombe* calnexin constitutes its BiP-binding domain (Elagoz *et. al.*1999). *S. pombe* and *S. cerevisiae* do not express calreticulin.

The physiological function of calmegin was analyzed by a transgenic mouse study (Ikawa *et. al.* 1997). Homozygous-null male mice are infertile although their spermatogenesis is morphologically normal and mating is normal. *In vitro* fertilization assay demonstrated that the adhesion ability of the sperms from homozygous-null males towards the zona pellucida of the egg is severely compromised. This observation has led to the suggestion that calmegin may function as a chaperone for one or more sperm surface proteins that are necessary to mediate the interactions between the sperm and the egg during fertilization (Ikawa *et. al.* 1997)

Calreticulin is essential for survival. Homozygous calreticulin knockout mice were embryonically lethal. A number of embryos were dead as early as 12.5 d post coitus. Histological analyses revealed that $crt^{-/-}$ embryos failed to absorb the umbilical hernia and are defective in cardiac development. Green fluorenscence protein reporter gene under the control of the calretiulin promoter was expressed in transgenic mice to examine the expression of calreticulin during early embryogenesis. The expression of calreticulin was detected in the cardiovascular system and peaked in 9.5 d embryos. A high level of calreticulin expression was maintained until day 18 and a decreased activity was observed afterward. Immunohistological analyses revealed that calreticulin protein expression was high in myocytes during early stages of embryonic development, highest in the 13.5 d embryonic heart and absent in the mature heart. Subsequent analyses revealed that defective cardiac development in $crt^{-/-}$ embryoes is due to a reduced calcium release from the ER through the In_3P receptor resulting in an impaired activation and nuclear translocation of NF-AT3 transcription factor, which is important for cardiac development (Mesaeli *et. al.*1999).

Hypothesis:

Calnexin is a type I phosphoprotein with a lectin-like molecular chaperone activity. Prolonged association of newly synthesized MHC class I heavy chains with calnexin was found in a B lymphoblastoid cell line transfected with HLA-B701 after incubation with the phosphatase inhibitor cantharidin or okadaic acid (Tector *et. al.*1994). Phosphorylated calnexin was shown to associate with the null Hong Kong mutant of α_1-antitrypsin, coinciding with its intracellular retention within the lumen of the ER (Le *et. al.*1994). Phosphorylated calnexin was also found in an association with newly synthesized MHC class I allotypes, which egressed from the ER at slow rates. Those allotypes that transported to the Golgi apparatus at faster rates were preferentially associated with nonphosphorylated calnexin (Capps and Zuniga 1994). Phosphorylation of calnexin may provide a potential for transmembrane communication and regulation of its luminally oriented lectin chaperone activity or vice versa.

The approaches taken in this study to address the hypothesis are:

1) to test a model system where the level of calnexin phosphorylation may be modulated by external stimuli;
2) to identify the *in vivo* phosphorylation sites of mammalian calnexin;
3) to examine phosphorylation-dependent trafficking of calnexin;
4) to elucidate the significance of calnexin phosphorylation *in vitro*;
5) to study the phosphorylation and significance of calnexin employing yeast genetics.

Here, we have identified the *in vivo* sites of calnexin phosphorylation in both cultured mammalian cells and *Schizosaccharomyces pombe*. Mammalian calnexin is phosphorylated on three invariant serine residues. One of the conserved serine phosphorylation motifs of mammalian calnexin is utilized by *S. pombe*. The conserved site of calnexin phosphorylation site is suggestive of a conserved and important function in both mammals and *S. pombe*. Furthermore, we showed that the phosphorylation of mammalian calnexin affects its association with ribosomes *in vitro*.

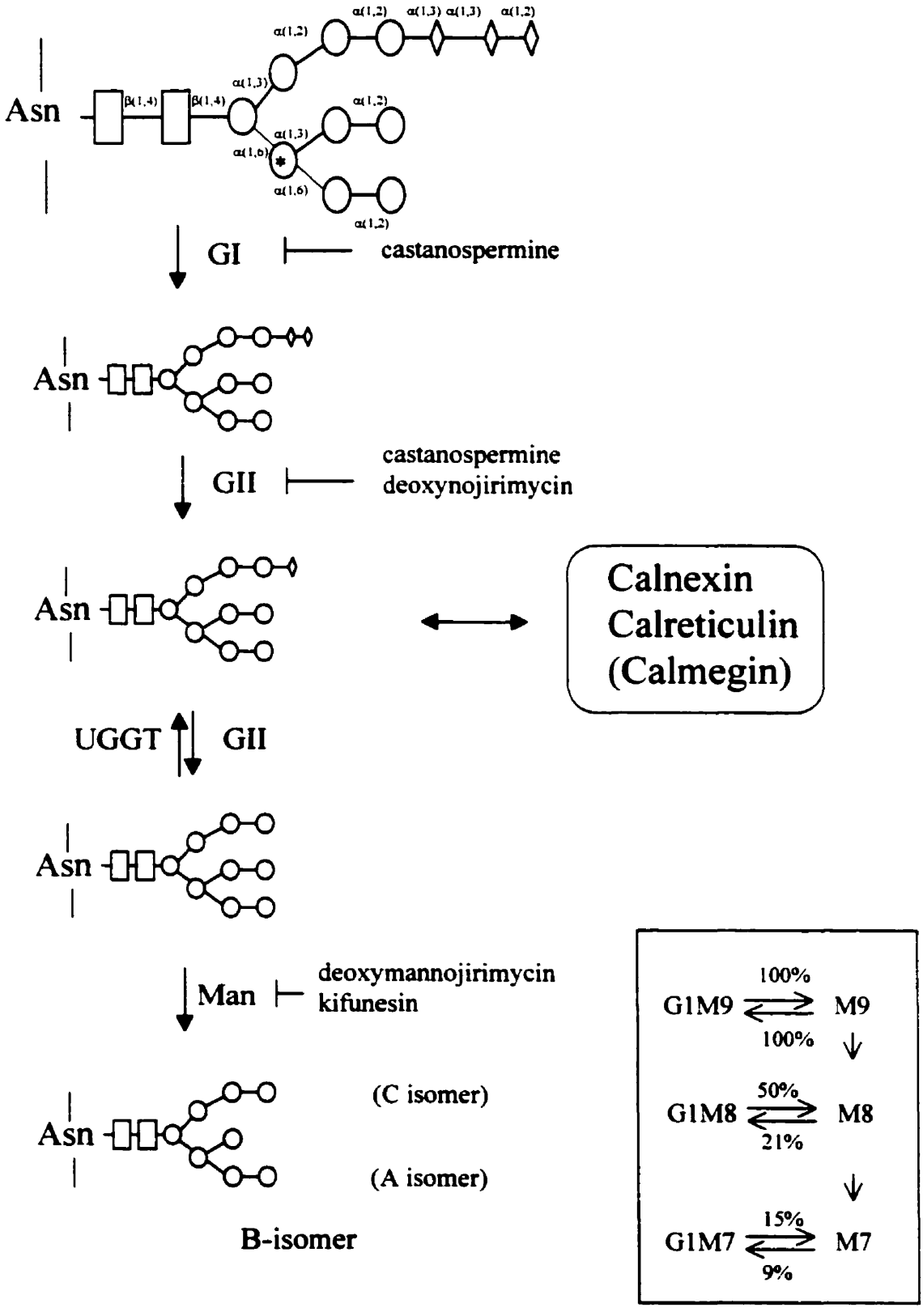
Figure 1: (A) Interaction between calnexin/calreticulin and N-linked oligosaccharide intermediates in the ER and (B) the proposed pathway for ER quality control.

Fig. 1A: Interaction between calnexin/calreticulin and N-linked oligosaccharide intermediates in the ER

Immediately following the "en-bloc" transfer of a dolichol-linked oligosaccharide onto an Asn residue on nascent glycoproteins in the ER lumen, two outermost glucoses are removed by the successive action of ER glucosidase I (*GI*) and II (*GII*). Mono-glucosylated nascent proteins can bind to calnexin and calreticulin (and calmegin). Removal of the innermost glucose by GII stimulates protein dissociation from CNX/CRT (and CMG). Protein folding takes place while being bound to CNX/CRT (and CMG). If the glycoprotein is folded correctly, it leaves the cycle. If the glycoprotein is not correctly folded, the terminal glucose could be added by the UDP-glucose:glycoprotein glucosyltransferase (*UGGT*) and allows glycoprotein to undergo cycles of binding to and release from CNX/CRT (and CMG). During glycoprotein folding, CNX/CRT recruit ERp57 to assist in disulfide bond formation (not shown here). In yeast, one mannose in the middle branch will be trimmed and generated a B isomer ($M_8GlcNAc_2$). In mammalian ER, a single mannose residue can be trimmed by ER mannosidase II and generate C isomer. Endo-mannosidase will generate isomer A from $G_{1\text{-}3}M_9GlcNAc_2$ intermediate. Glucosidase inhibitors (castanospermine and deoxynojirimycin) and mannosidase inhibitor (deoxymannojirimycin) are indicated. An asterisk indicates α1-6 branched mannose that is important for calnexin/calreticulin binding. *Rectangle*, GlcNAc; *circle*, mannose and *diamond*, glucose. Inset: The relative efficiency of glucosylation by UGGT (Sousa *et. al.*1992) and deglucosylation by GII on oligosaccharide intermediates (Grinna and Robbins 1980). Pathway is adapted from Liu *et. al.* (Liu *et. al.*1999).

Fig. 1B: Proposed pathway for ER quality control.

In pathway 1, if glycoprotein is folded properly, the protein-linked $M_9GlcNAc_2$ intermediate is targeted for secretion. In pathway 2, if the glycoprotein is not folded correctly, it will be recognized and re-glucosylated by the UGGT. The $M_9GlcNAc_2$ glycoprotein would re-bind to calnexin for a chance to fold correctly. In pathway 3, persistent misfolded glycoprotein will be recognized, presumably, by the ER mannosidase I and generate a B isomer, $M_8GlcNAc_2$ intermediate. The unfolded intermediates can be glucosylated by UGGT generating a $G_1M_8GlcNAc_2$ intermediate and allow it to re-bind to calnexin. De-glucosylation of $G_1M_8GlcNAc_2$ is less efficient, only 21% of G1M9 by glucosidase II *in vitro* (Grinna and Robbins 1980) (Fig. 1A, inset). Calnexin bound $G_1M_8GlcNAc_2$ misfolded glycoprotein could be directed for ER associated degradation machinery. Adapted from from Liu *et. al.* (Liu *et. al.*1999).


Asn
α(1,2)
α(1,3)
α(1,3)
α(1,2)
α(1,2)
α(1,3)
β(1,4)
β(1,4)
α(1,2)
α(1,3)
α(1,6)
α(1,6)
α(1,2)
GI
castanospermine
Asn
GII
castanospermine
deoxynojirimycin
Asn
Calnexin
Calreticulin
(Calmegin)
UGGT
GII
Asn
Man
deoxymannojirimycin
kifunesin
Asn
(C isomer)
(A isomer)
B-isomer
100%
G1M9
M9
100%
50%
G1M8
M8
21%
15%
G1M7
M7
9%

1B

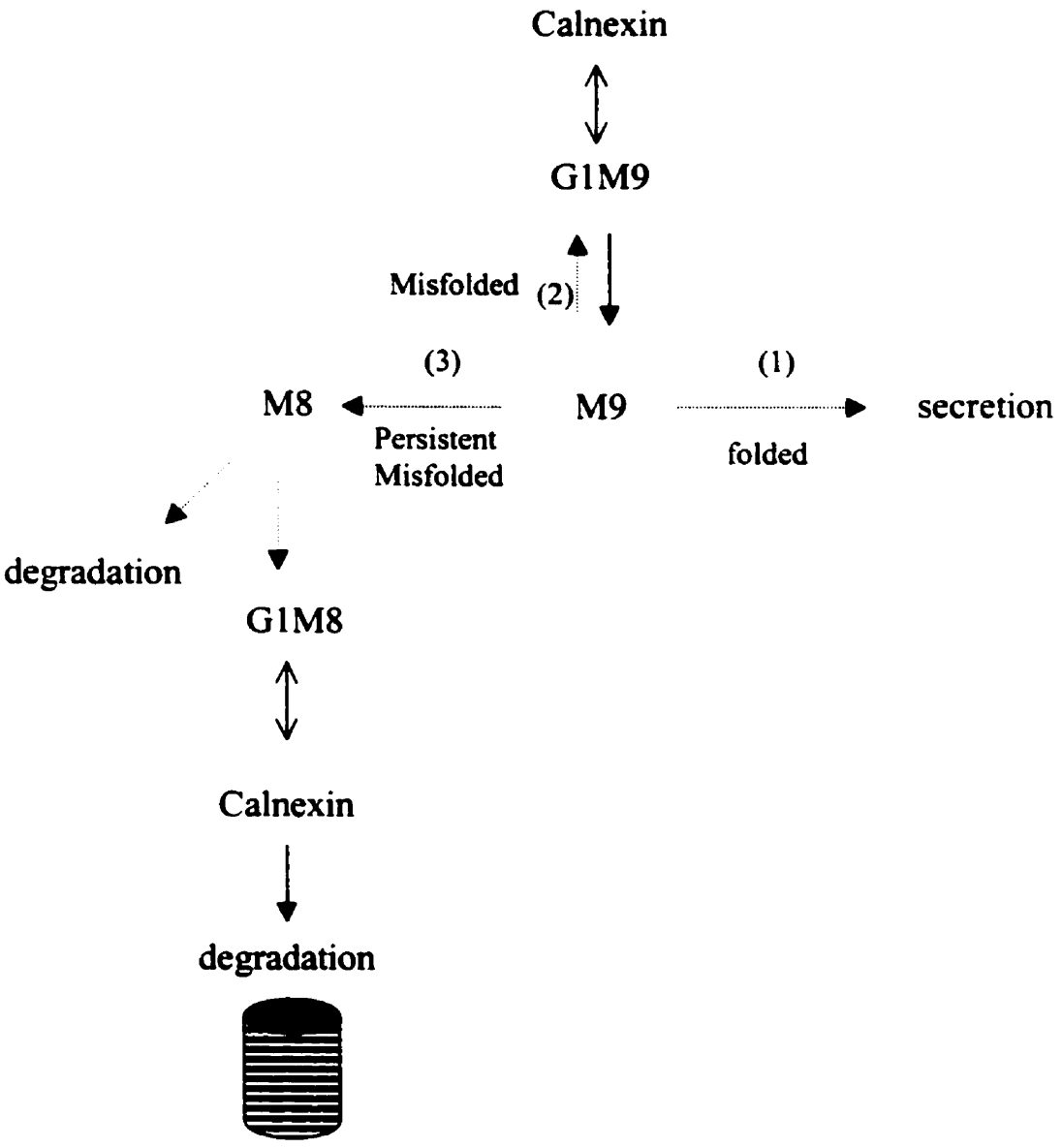

Calnexin
G1M9
Misfolded
(2)
(3)
(1)
M8
M9
secretion
Persistent
Misfolded
folded
degradation
G1M8
Calnexin
degradation

Figure 2: Comparison of the conserved regions in calnexin, calmegin and calreticulin homologues.

A linear representation of calnexin (CNX), calreticulin (CRT) and calmegin (CMG) is shown. Signal sequence, transmembrane domain, and the stretches of acidic and basic amino acids are indicated. The number of conserved repeats 1 (PXXDXDPDEAXKPEDWDE) and 2 (GXWXXPXDXNPXYX) are designated. The amino acid numbers refer to the mature calnexin (CNX), calreticulin (CRT) and calmegin (CMG). Sequence for calnexin and calreticulin are human (GeneBank accession number P27824, A37047), *Caenorhabditis elegans* (P34652, P27798), *Schistosoma mansoni* (A46637, A48573), and *Arabidopsis thaliana* (P29402, AAC49697, respectively). Human CMG (O14967), *Schizosaccharomyces pombe* Cnx1p (P36581), *Saccharomyces cerevisiae* Cne1p (P27825) and *Trypanosoma cruzi* (AAD22175). N-linked glycosylation sites are indicated, *Y*, and potential sites are in parenthesis. BiP binding domain of Cnx1p is also indicated. For CRT, the carboxyl terminal ER retention sequence is noted.

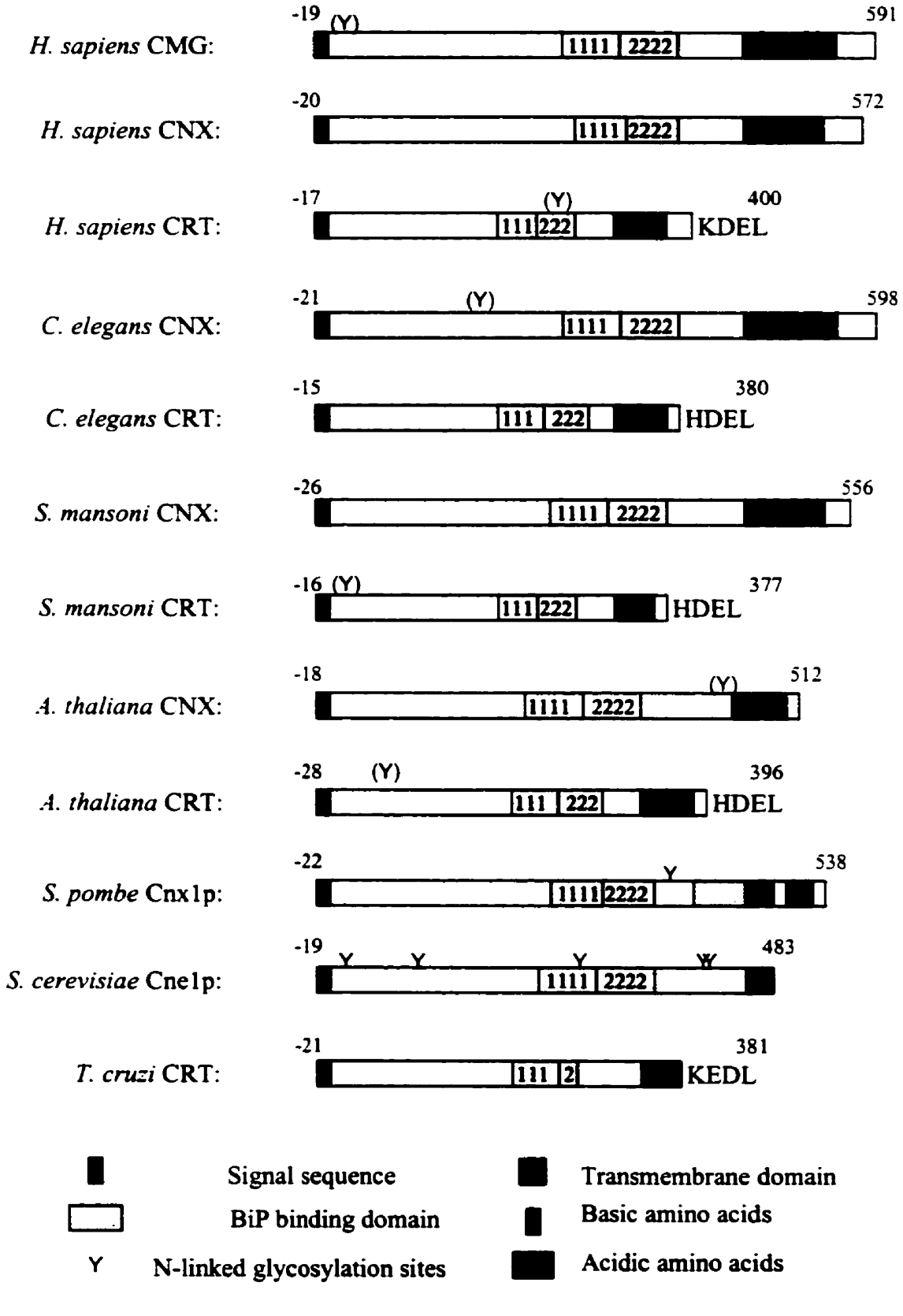
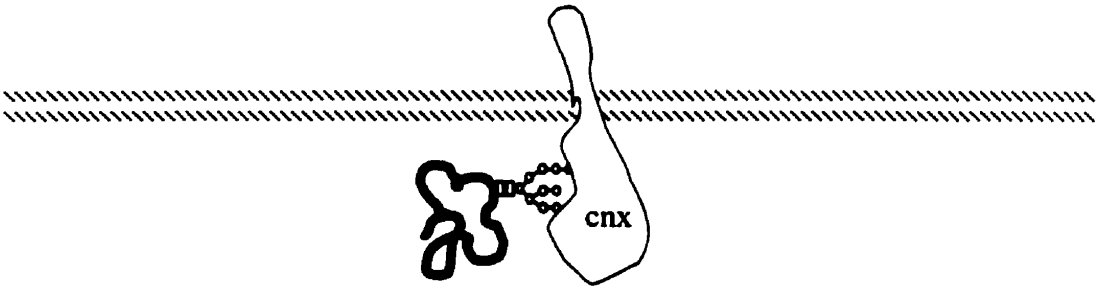

-19
(Y)
591
H. sapiens CMG:
1111
2222
-20
572
H. sapiens CNX:
-17
(Y)
400
H. sapiens CRT:
111
222
KDEL
-21
(Y)
598
C. elegans CNX:
-15
380
C. elegans CRT:
HDEL
-26
556
S. mansoni CNX:
-16
(Y)
377
S. mansoni CRT:
-18
512
A. thaliana CNX:
-28
(Y)
396
A. thaliana CRT:
-22
538
S. pombe Cnx1p:
-19
483
S. cerevisiae Cne1p:
-21
381
T. cruzi CRT:
KEDL
Signal sequence
Transmembrane domain
BiP binding domain
Basic amino acids
N-linked glycosylation sites
Acidic amino acids

Figure 3: Two proposed models of calnexin binding to glycoproteins.

A. In the lectin-only model, calnexin acts purely as a lectin. CNX interacts with glycoproteins via their monoglucosylated oligosaccharide side chain.
B. In the dual binding model, CNX is both a lectin and a molecular chaperone. Their monoglucosylated oligosaccharide side chain mediates the initial interaction between CNX and glycoproteins. The lectin binding subsequently allows peptide-peptide interaction between CNX and nascent glycoproteins to occur. ERp57 is not shown here.

A



B

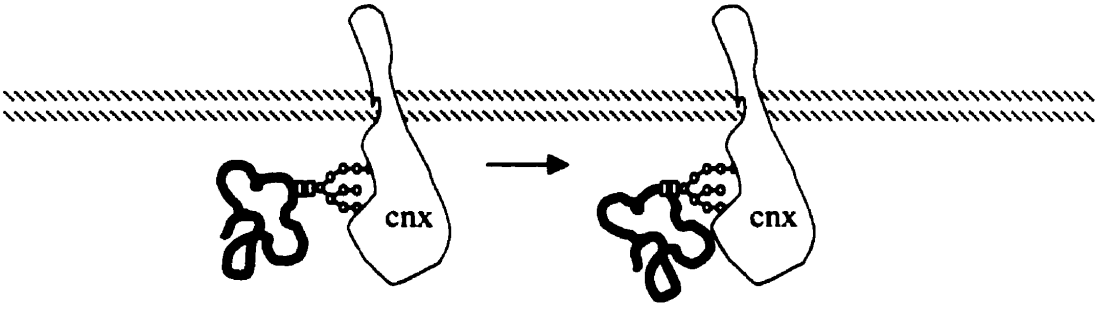


Table 1: Percentage of identity among mature calnexins after signal sequence cleavage from various species.

	Human	Canine	Rat	Mouse	Frog	Fly	Worm	*S. mansoni*	*S. japonicum*	*S. pombe*	*S. cerevisiae*	thale cress	pea	soybean	artichoke
H. sapien[1]	100	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C. familiaris	95.1	100	-	-	-	-	-	-	-	-	-	-	-	-	-
R.norvegicus	94.1	94.8	100	-	-	-	-	-	-	-	-	-	-	-	-
M. musculus	93.0	93.9	98.6	100	-	-	-	-	-	-	-	-	-	-	-
R. rugosa	76.1	75.9	76.0	75.2	100										
D.melanogaster	49.8	49.5	49.5	50.1	47.3	100	-	-	-	-	-	-	-	-	-
C. elegans	42.3	41.5	41.5	41.7	40.5	40.2	100								
S. mansoni	55.2	50.1	50.3	50.4	49.1	45.3	40.5	100	-	-	-	-	-	-	-
S. japonicum	54.4	49.4	50.3	50.4	47.8	44.5	41.2	85.4	100						
S. pombe	37.6	37.8	38.0	38.3	37.9	35.0	33.7	38.1	36.3	100	-	-	-	-	-
S. cerevisiae	25.6	25.2	25.6	25.0	24.6	26.4	23.4	24.3	26.8	30.1	100				
A. thaliana	39.2	39.6	39.3	39.4	38.0	37.2	35.1	38.7	37.9	38.9	38.9	100	-	-	-
P. sativum	39.5	37.9	38.0	37.8	38.1	35.1	35.0	37.4	37.2	37.3	37.3	72.9	100	-	-
G. max	39.9	39.8	40.5	40.0	40.1	36.7	35.1	39.5	39.0	37.9	37.9	76.3	81.4	100	-
H. tuberosus	38.9	38.0	38.5	39.2	38.8	36.2	34.2	38.9	38.1	36.5	36.5	69.9	68.1	72.3	100

[1] Percent identities were obtained by tools available at http:// dot.imgen.bcm.tmc.edu:9331/seq-search/alignment.html. Mature calnexins refers calnexins after signal sequence removal.

Table 2: Percentage of identity among the cytosolic domains of calnexin from various species[1].

	Human	Canine	Rat	Mouse	Frog	Fly	Worm	*S. mansoni*	*S. japonicum*	thale cress	pea	soybean	artichoke	*S. pombe*
H. sapien[2]	100	-	-	-	-	-	-	-	-	-	-	-	-	-
C. familiaris	94.4	100	-	-	-	-	-	-	-	-	-	-	-	-
R.norvegicus	87.6	86.5	100	-	-	-	-	-	-	-	-	-	-	-
M. musculus	85.4	84.3	97.7	100	-	-	-	-	-	-	-	-	-	-
R. rugosa	49.5	49.5	48.5	47.6	100									
D.melanogaster	33.7	32.7	32.7	34.0	27.9	100	-	-	-	-	-	-	-	-
C. elegans	30.3	28.1	24.4	24.4	26.1	25.0	100							
S. mansoni	28.2	26.5	28.4	28.4	28.4	27.5	25.8	100	-	-	-	-	-	-
S. japonicum	26.5	25.5	28.4	28.4	28.4	27.5	26.6	75.0	100					
A. thaliana	23.3	27.8	22.7	23.9	21.2	22.9	14.3	17.8	16.8	100	-	-	-	
P. sativum	24.7	23.6	23.0	24.1	23.1	20.0	16.0	18.0	19.0	52.9	100	-	-	
G. max	24.4	27.8	27.3	26.1	24.0	21.1	14.3	18.0	18.0	39.6	49.1	100	-	
H. tuberosus	24.4	24.4	25.0	25.0	23.1	16.8	14.3	19.0	16.0	37.3	39.2	43.1	100	
S. pombe	15.7	16.9	17.2	17.2	16.5	14.7	14.3	16.0	15.0	20.8	23.1	24.5	22.2	100

[1] *S. cerevisiae* calnexin is not included since it contains only one cytosolically oriented amino acid (also see Table 3).

[2] Percent identities were obtained by tools available at http:// dot.imgen.bcm.tmc.edu:9331/seq-search/alignment.html. Also see Table 3 for the cytosolic domain of calnexins.

Table 3: Selected features for calnexins and calmegins.

The "Mature" indicates the number of amino acids after signal sequence cleavage. The "cytoplasmic domain" refers to the number of amino acids in the cytosolic tail. The 10 amino acids at the most carboxyl terminal domains of each (except *S. cerevisiae*) are listed under the "C-terminal sequence". The position of the asparagine residue is indicated and the potential sites are in parenthesis.

	Mature (AA)	Cytoplasmic domain (AA)	C-terminal sequence	N-linked site	Cal.[d] MW	Cal[d] pI	Accession
Calnexin							
H. sapiens	572	89	SPRNRKPRRE	--	65395.5	4.47	P27824
C. familiaris	573	89	SPRNRKPRRE	--	65401.5	4.47	P24643
R. norvegicus	571	87	SPRNRKPRRE	--	65129.1	4.48	P35565
M. musculus	571	87	SPRNRKPRRE	--	65165.3	4.50	P35564
R. rugosa	602	103	SPRNRKPRRD	--	68510.1	4.54	BAA11426
D. melanogaster	581	95	KTRKRQARKE	(194)	65503.8	4.68	CAA67846
C. elegans	598[a,b]	119[a,b]	KRRTQARRGD	(182)[c]	66593.4	4.45	P34652
S. mansoni	556	100	SIRKRRSRKE	--	62916.5	5.1	A46637
S. japonicum	562	100	STRKRRSRKE	--	63697.2	5.01	AAC33833
S. pombe	538	48	TESPTAKNED	396	61064.1	4.36	P36581
S. cerevisiae	483	1	T	25,104,296,416,425	54685.1	5.06	P27825
A. thaliana	512	44	PRKRQPRRDN	(446)	58335.5	4.77	P29402
P. sativum	525[a]	51[b]	PPRRRPKRDN	(114,452)[c]	59754.6	4.83	CAA76741
G. max	521	50	AARRRPRRET	(448)	59444.2	4.77	Q39817
H. tuberosus	504	50	APRRRPRRDT	--	57882.2	4.86	CAA84491
Calmegin							
H. sapiens	591	118	SVRKRRVRKD	(22)	67831.9	4.56	O14967
M. musculus[e]	592	119	SLRKRRVRKD	--	67368.5	4.54	P52194

a, signal sequence prediction using tools available at : http://www.cbs.dtu.dk/services/SignalP/#submission
b, prediction of protein sorting signals and cytoplasmic domain using tools available at: http://psort.nibb.ac.jp:8800/
c, Asn-linked glycosylation sites, potential sites in parentheses are predicted using tools available at http://www.expasy.ch/tools/scnpsite.html
d, Calculation based on mature protein after removal of signal sequence, glycan was not considered (either from published or predicted signal sequence) using tools available at: http://www.expasy.ch/tools/protparam.html e, minor differences between mouse calmegin and mouse calnexin-t sequences and mouse calmegin is used here for simplicity.

Materials and Methods

Antibodies

Three rabbit polyclonal antibodies termed anti-C1, anti-C3 and anti-C4 calnexin antibodies were used in this study. They were raised against the synthetic peptides corresponding to amino acids 30-48, 487-505 and 555-573 of mature canine calnexin, respectively (Ou *et. al.*1993; Le *et. al.*1994; Ou *et. al.*1995). It is worthy to mention that the synthetic peptides used for antibodies generation were conjugated to different carriers prior immunization. C1 and C3 synthetic peptides were conjugated to BSA whereas C4 synthetic peptides were conjugated to keyhole limpet hemocyanin prior immunization. Thus, BSA at 1 mg/ml was added to both anti-C1 and C3 antisera when they were used in this study. Polyclonal sheep anti-human ApoB antibodies were purchased from Boehringer Mannheim (Montreal, PQ). Dr. Walter E. Mushynski (McGill University, PQ) kindly provided the monoclonal anti-NFL antibodies. Polyclonal rabbit antisera raised against the *E. coli* expressed GST fusion protein of the luminal domain of precursor *Schizosaccharomyces pombe* calnexin (amino acids 23-492) were provided by Dr. David Y. Thomas (NRC BRI, Montreal, Quebec) (Parlati *et. al.*1995). *S. pombe* Cnx1p antibodies were affinity purified using the GST-Cnx1p (23-492) as described (Parlati *et. al.*1995).

Materials

^{32}P-orthophosphoric acid (S.A. of 285 Ci/mg), [γ-^{32}P]-ATP (S.A. of 3000 Ci/mmol) and [γ-^{32}P] GTP (S.A. of 25-42 Ci/mmol) were purchased from NEN-Mandel Scientific (Toronto, Ontario). Tran^{35}S-label (S.A. of 1048-1504 Ci/mmol) and ^{3}H-UDP galactose (S.A. of 50 Ci/mmol) were from ICN Canada Ltd. (Montreal, Quebec) and NEN-Mandel Scientific respectively. Protein A-Sepharose beads and glutathione sepharose beads were from Pharmacia LKB Biotechnology Inc. (Montreal, PQ). Pyridine, isobutyric acid, TPCK treated trypsin, phenyl methyl sulfonyl fluoride (PMSF), leupeptin, N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES), staurosporine, alkaline phosphatase (P-2276) were from Sigma (Ontario, Canada). 3-[(3-

cholamidopropyl)-dimethyl-amminio]-1-propanesulfonate (CHAPS), recombinant PKC, recombinant CK2, and sequencing graded bovine trypsin were from Boehringer Mannheim (Montreal, Quebec, Canada). Olomoucine, 5,6-Dicholoro-1-β-D-ribofuranosylbenzimidazole (DRB), iso-olomoucine, roscovitine and heparin were purchased from Calbiochem (San Diego, Calf.) Thin layer microcrystalline cellulose plates (0.1 mm thickness) were purchased from BDH (Toronto, Ont.). Kodak XAR-5 OMAT x-ray film from Picker International Canada Inc. (Montreal, PQ). Reagents for protein determination, SDS-PAGE, HRP-conjugated goat anti-rabbit IgG, and protein A-HRP were obtained from BioRad Laboratories (Mississauga, Ont.). Pre-stained broad range protein marker was from New England BioLabs (Beverly, MA). Nitrocellulose (BA85) and PVDF membranes (pore size 0.45 μm) were obtained from Xymotech (Mt. Royal, PQ). The ECL Detection kit was purchased from Amersham (Oakville, Ontario). Restriction enzymes and recombinant Taq polymerase were from Pharmacia Biotech Inc. (Montreal, PQ). The GeneClean II Kit for DNA fragment purification was from Bio101 Inc. (La Jolla, CA). The primers were purchased from both Hukabel Scientific Ltd (Montreal, PQ) and Gibco Life Technologies (Burlington, Ont.) respectively.

Unless otherwise stated, all other reagents and chemicals were of reagent graded and obtained from Sigma Chemical Company (St-Louis, MO), Anachemia Canada Inc. (Lachine, PQ), Gibco BRL (Burlington, PQ), Fisher Scientific (Montreal, PQ), Pharmacia Biotech Inc. (Montreal, PQ) and Boehringer Mannheim (Montreal, PQ).

Cell lines and canine pancreata

Madin Darby canine kidney cells were gift from Dr. Paul Walton (University of Western Ontario, London, Ont.). HepG2, MDCK and FR3T3 cells were cultured in Dubecco's modified Eagle medium (DMEM) supplemented with final concentration of 50 mM HEPES, pH 7.5, 10% fetal bovine serum (FBS) (Hyclone, Logan, UT), 1% penicillin and 1% streptomycin (Life Technologies, Burlington, Ont.). They were maintained at 37°C incubator at 5% CO2 containing atmosphere. Cells were passaged weekly and were used approximately 70 to 90% confluence after plating. A rat hepatoma

cell line transfected with expression plasmids of human insulin receptor, HTC-IR cell, was maintained and provided by Mr. Victor Dumas (Polypeptide laboratory, McGill University, PQ) (Band *et. al.*1997).

The laboratories of Drs. Bruce Jamieson and Mark Levy (McGill University, PQ) kindly provided canine pancreata.

Schizosaccharomyces pombe strains and medium

Schizosaccharomyces pombe strain Q358 (h^- ade6-M210 leu1-32 ura4-D18) was used as a wild-type control in experiments done in this study. *S. pombe* Δ*cnx1* pCNX560, *S. pombe* Δ*cnx1* pCNX524, *S. pombe* Δ*cnx1* pCNX484 and *S. pombe* Δ*cnx1* pCNX474 strains are no longer harboring the genomic copy of $cnx1^+$ but containing a plasmid expressing different carboxyl truncated $cnx1^+$ (derived from *LEU2* based vector pREP1 expression plasmid) were generously provided by Dr. David Y. Thomas (NRC BRI, Montreal, Quebec) (Parlati *et. al.*1995). The sequencing number for precursor Cnx1p is used in this study. All strains were grown at 30°C in Edinburg Minimal Medium (EMM) supplemented with required nutrients (Moreno *et. al.*1990). EMM, phosphate free EMM (EMMP), other medium and required nutrients were prepared as described (Moreno *et. al.*1990). Nonsulfate medium (NSM) is the same as EMM except that all sulfate salts in both salt and mineral stock solution were replaced with chloride salt instead (Jannatipour *et. al.*1998).

GST-fusion proteins

The *E. coli* strains carrying different recombinant GST fusion protein constructs of various length of the cytosolic domain of *S. pombe* calnexin were provided by Mr. Marc Pelletier and Dr. David Y. Thomas (NRC, BRI) (Fig. 45). Briefly, the GST-Cnx1p constructs were made by inserting PCR fragments encoding either amino acids 511-560 (GST-FL), AA 511-525 (GST-A), AA 526-542 (GST-B), and AA 543-560 (GST-C) into pGEX-2T plasmid. The E. coli strain DH5α was used for plasmid amplification and

isolation. Bacterial expressed fusion constructs were isolated by affinity chromatography using glutathione-sepharose per manufacturer's instruction (Amersham Pharmacia).

Methods

Subcellular fractionation of canine pancreata

Translocation competent dog pancreatic rough microsomes were prepared and ribosomes were EDTA stripped as previously described (Paiement and Bergeron 1983; Walter and Blobel 1983). The concentration of protein and nucleotides in the samples were determined by spectrophotometric measurement of the absorbance at wavelength of 280 and 260 nm respectively. Microsomes were stored (10-12 mg/ml as determined by Bradford method (Bradford 1976) using bovine serum albumin as a standard) in aliquots frozen at -70 °C in 20 mM HEPES-NaOH, pH 7.4, 50% glycerol and 1 mM DTT.

Metabolic radiolabeling and immunoprecipitation of ApoB

ApoB100 were *in vivo* radiolabeled and immunoprecipitated as described (Yao *et. al.*1992; McLeod *et. al.*1994). Briefly, HepG2 cells plated in 60-mm dishes were pre-incubated, labeled and chased at 37°C in medium containing either 1.5% BSA (essentially fatty acid free) (control) or a final concentration of 0.4 mM oleate/1.5% BSA (0.4 mM oleate). 10 mM oleate/BSA (Molar ratio of 6.9:1) was prepared as described (Dixon, Furukawa *et. al.*1991). The cells were washed twice with methionine-free DMEM, pre-incubated in 2 ml of the same medium with 1.5%BSA or 0.4 mM oleate for 1 h and pulse-labeled with Tran^{35}S-lable (150 µCi/dish) in 1.5 ml for 30 min. At the end of the pulse, the labeling medium was removed and the cells were incubated with 2 ml of DMEM supplemented with 200 µM methionine for 0, 1/2, 1, 2, and 3 h at 37°C. At the end of each chase, the medium was collected and the cells were lysed with 0.5 ml of 1% SDS-RIPA buffer [1% SDS (w/v), 1% TritonX-100 (v/v), 1% deoxycholic acid (w/v), 1 mM EDTA, 1 mM dithiothreitol, 150 mM NaCl, 0.015% PMSF (w/v), 50 mM Tris-HCl, pH 8.0]. Both cell lysate and medium samples were heated for 15 min at 75°C and adjusted to 0.1% SDS. The samples were pre-cleared with protein A sepharose for 2 h at room temperature (RT) and followed by an overnight incubation at RT with either anti-

human apoB or anti-C4 antibodies. The immunocomplexes were collected by a 2-h rotation with protein A sepharose beads at RT and washed with 0.1% SDS-RIPA buffer. For single immunoprecipitation, the samples were solubilized with Laemmli sample preparation buffer (Laemmli 1970). For sequential immunoprecipitation, anti-C4 immunocomplexes were resuspended in 100 µl of 1%SDS-RIPA, heated at 90°C for 5 min, allowed it to cool to RT and adjusted to 0.05% SDS. Quick spin and divided the supernatant to 2 vials and subjected to overnight immunoprecipitation with either anti-C4 or anti-apoB antibodies. The immunocomplexes were collected, washed, and eluted as above. The samples were heated for 5 min, resolved on 5% SDS-PAGE, Coomassie stained, dried and autoradiography as below.

Insulin binding assay

Insulin binding assays were carried out as previously described (Burgess *et. al.*1992; Band *et. al.*1997). Briefly, nearly confluent HepG2 and HTC-IR cells were lysed in 1.5% Triton X-100 solubilization buffer with various protease and phosphatase inhibitors (Band *et. al.*1997). Insulin receptors purified from cell extract by wheat germ agglutinin lectin in serial dilutions were incubated with ^{125}I-insulin (S. A. of 130-200 µCi/µg) in triplicate as described (Burgess *et. al.*1992). Nonspecific binding was determined in the presence of a 10-fold excess of non-radiolabeled insulin and was subtracted from total binding quantified by scintillation detection. The percentage of specific binding in each cell line was corrected to the same amount of protein present in each wheat germ agglutinin purified proteins.

In vivo metabolic radiolabeling of calnexin from cultured cells

Cells were metabolically radiolabeled as previously described (Ou *et. al.*1993). Briefly, cells at approximately 80 to 90% confluence were washed in methionine-free DMEM supplemented with 10% dialyzed FBS and incubated for 1 h in the same media. Cells were radiolabeled with 100 µCi/ml of Tran^{35}S-label for the indicated time. The radiolabeled was removed at the end of the labeling period. For pulse and chase experiments, cells were washed briefly with chase medium (complete DMEM with 10% FBS plus 2 mM methionine) after the removal of labeled medium. At the end of the

pulse or chase period, the cells were lysed as described with the following modification (Ou *et. al.* 1992). Briefly, cells were washed twice with ice cold phosphate buffered saline (20mM $NaPO_4$, pH7.5, 150mM NaCl) (PBS) and once with ice cold HEPES buffered saline (50mM HEPES, pH7.6, 200mM NaCl) (HBS) before harvesting. Cells were then lysed in 2% CHAPS lysis buffer [2% (w/v) CHAPS, 50mM HEPES, pH7.6, 200mM NaCl, 1mM PMSF, 10 µg/ml each of leupeptin and aprotinin, 10mM NaF, 10mM NaPPi, 0.4mM $NaVO_4$ and 5mM $NaMoO_4$] for 30 min on ice. For lysis with 2% DOC lysis buffer, sodium deoxycholic acid was used instead of CHAPS.

In vivo [^{32}P]O_4 labeling of calnexin from cultured cells

Cells were radiolabeled as described previously (Capps and Zuniga 1994; Le *et. al.*1994). Briefly, cells at 70 to 85% confluence were washed in phosphate-free DMEM medium supplemented with 1% dialyzed FBS. Cells were incubated in the same medium for 1 h at 37°C. Cells were then pulse-labeled by addition of indicated amount of carrier free [^{32}P]-orthophosphate for the indicated time. At the end of labeling, the cells were lysed as described above.

Immunoprecipitation of calnexin.

The same procedures were used to immunoprecipitate calnexin and its associated proteins from radiolabeled and non-radiolabeled cellular extracts as previously described (Ou *et. al.*1993). Briefly, lysates were centrifuged at 100.000 rpm at 4°C for 30 min using Beckman rotor TLA 100.2, and pre-cleared with an aliquot of protein A sepharose CL-4B for 30 min at 4°C with rotation. The protein concentration of the pre-cleared lysates was determined by Bradford method (Bradford 1976). For immunoprecipitation from labeled medium, equal amount of medium was used for each immunoprecipitation. An equal amount of protein was used for each immunoprecipitation. The pre-cleared lysates were incubated with an aliquot of the indicated antiserum for either 2 h or overnight at 4 °C. For immunoprecipitation with anit-C3 antibodies, BSA was added to the immunoprecipitation buffer to neutralize possible cross-reacting anti-albumin antibodies. The immunocomplexes were isolated with protein A-Sepharose beads at 4°C with rotation, washed extensively with 1%CHAPS (w/v) in HBS and with HBS as the final

wash. Immunoprecipitated proteins were eluted from the beads by the addition of Laemmli sample preparation buffer (Laemmli 1970). Proteins were recovered by heating at 65 °C for 10 min.

Alkaline phosphatase treatment

Duplicate immunoprecipitates were obtained as above. The beads were washed extensively with 1%CHAPS (w/v) in HBS followed by washing with alkaline phosphatase buffer (10 mM Tris, pH 9.5 and 1 mM $MgCl_2$) and then suspended in 15 µL of the same buffer. To one set of immunoprecipitates, 2 µL or 45 DEA unit of alkaline phosphatase was added to the immunoprecipitates followed by a 30-min incubation at 37 °C. The duplicate set of immunoprecipitates was mock treated by the same incubation in buffer alone. For ^{35}S-labeled immunoprecipitates, the samples were quickly spun and the supernatants were transferred to new tubes containing equal volume of 2X Laemmli sample buffer by Hamilton syringe. The samples associated with the beads were recovered by the addition of Laemmli sample buffer. For ^{32}P immunoprecipitates, the reaction was stopped by the addition of 2X Laemmli sample buffer. The samples were heated for 10 min at 65°C prior separation by SDS-PAGE.

SDS-PAGE and radioautography

For Tans^{35}S-labeled samples, proteins were separated by SDS-polyacrylamide gels followed by coomassie stained (0.1% Coomassie blue R in 50% methanol and 10% glacial acetic acid) and destained in destain solution (30% methanol and 7% glacial acetic acid). The polyacrylamide gels were processed for fluorography with EN^{3}Hance (NEN-Mandel, Boston, MA) according to the manufacturer's instructions and dried under vacuum. For ^{32}P-labeled samples, proteins were separated by SDS-PAGE followed by coomassie stained and destained as above. The gels were dried for 2 h at 80°C under vacuum or transferred onto either nitrocellulose membrane(s) or PVDF membrane for indicative time in 192 mM glycine, 25 mM Tris base buffer at 12V at 4°C (Wada *et. al.*1991). The radiolabeled bands were visualized by radioautography either at room temperature or at –80°C with or without an enhancing screen. The non-radiolabeled calnexin was visualized by coomassie blue R250 stain.

Immunoblotting

Samples were separated by SDS-PAGE (Laemmli 1970) and transferred onto nitrocellulose membranes as above. The blots were incubated in 5% non-fat skimmed milk (NSM) (w/v) in TNT buffer (10 mM Tris, pH 7.5, 150 mM NaCl and 0.05% Tween20) for 2 h at room temperature. The blots were probed with various antibodies as indicated in 5% NSM (w/v) in TNT for 2 h at room temperature with gentle rotation followed by successive washes in 0.5% NSM (w/v) in TNT buffer. Secondary antibodies conjugated to alkaline phosphatase and protein A-HRP (1:10,000) in 0.5% NSM (w/v) in TNT buffer for 45 min at room temperature were utilized to visualize the detection by the primary antibodies on the blots. The blots were washed with 5 changes of TNT buffer gentle. The chemiluminescence reaction was done as described by the manufacturer and visualized by exposure to Kodak X-OMAT X-ray film. Colorimetric visualization using alkaline phosphatase secondary antibodies was performed as described (Smith and Fisher 1984). ^{125}I-goat anti-rabbit secondary antibodies (Fig. 26) were also used at 10,000 cpm or 2 μCi/mL according to the assay date for visualization by radioautography at -70°C with an enhancing screen.

In vitro phosphorylation of calnexin from cultured cells

In vitro phosphorylation of canine pancreatic membrane was carried out as described (Wada *et. al.*1991; Rindress *et. al.*1993). Briefly, microsomal membranes at a final concentration of 1 mg/mL were incubated with 8-8.5 μM of either [γ- ^{32}P] GTP or [γ- ^{32}P] ATP in the reaction buffer containing 2.5 mM $MnCl_2$, 7.5mM $MgCl_2$, 40 mM KCl and 40 mM Tris-HCl, pH7.4. Phosphorylations were performed for 30 min on ice. For *in vitro* inhibition studies, various inhibitors were included at the concentration indicated during the phosphorylation reaction. The reaction was terminated by the addition of a concentrated stop solution to yield a final concentration of 1 mM GTP or ATP, 2 mM Na_3VO_4, 2 mM NaF, 20 mM β-glycerolphosphate in 12.5 mM Tris-HCl, pH7.4. An aliquot, usually 10 μg, of the phosphorylated samples was separated and analyzed by SDS-PAGE (Laemmli 1970). The remainder of sample was lysed by the addition of an equal volume of 4%CHAPS lysis buffer in HBS [4% CHAPS (w/v) 2 mM

PMSF, 20 µg/ml each of leupeptin and aprotinin, 20 mM NaF, 20 mM NaPPi, 0.8 mM $NaVO_4$ and 10 mM $NaMoO_4$] and left for 30 min on ice. Lysates were microfuged for 5 min and pre-cleared with an aliquot of protein A-sepharose beads. Either Anti-C3 or anti-C4 antibodies were added to the pre-cleared lysates and phosphorylated calnexins were isolated by the immunoprecipitation and separated by SDS-PAGE procedures as described before.

Phosphoamino acid analysis

Phosphoamino acid analysis was performed as described (Boyle *et. al.*1991). Briefly, after either *in vivo* or *in vitro* phosphorylation reactions, immunoprecipitated phosphorylated calnexins were separated by SDS-PAGE and electroblotted onto a PVDF membrane, the radioactive bands were detected by autoradiography and the corresponding blots were excised. Alternatively, the radioactive bands detected by autoradiography were extracted from the polyacrylamide gel as described (Boyle *et. al.*1991). The membrane containing the phosphorylated calnexin was washed extensively with distilled water and was hydrolyzed in 6N HCl at 110°C for 60 min. The hydrolysate was lyophilized and dissolved in pH1.9 buffer [88% formic acid, glacial acetic acid, H_2O: 1.3.5.40.5 (v/v/v)] and separated electrophoretically in two dimensions on cellulose thin layer plates in the presence of phosphoamino acid standards; phospho-serine, phospho-threonine and phospho-tyrosine. First electrophoresis was carried out in pH1.9 buffer for 20 min at 1.3kV using a Hunter thin layer electrophoresis system (C.B.S. Scientific, Del Mar, CA). The second dimensional electrophoresis was carried out in pH3.5 buffer [pyridine, glacial acetic acid, H_2O: 1.10.200 (v/v/v)] at 1.5kV for 20 min. The standards were visualized by 0.25% (w/v) ninhydrin staining followed by a brief incubation at 65°C. The radiolabeled amino acids were detected either by radioautography with an enhancing screen or by a Fuji PhosphoImager screen and visualized using a BAS 2000 Fuji Bio-Imaging Analyzer (Fuji BioMedical Systems Inc., Bethesda, MD). Cerenkov counting monitored the recovery from each step was done.

Phosphopeptide mapping analysis

Phosphopeptide mapping was carried out according to the methods described (Boyle *et. al.*1991; van der Geer *et. al.*1993). Briefly, after either *in vivo* or *in vitro* phosphorylation reactions, phosphorylated calnexins were transferred onto nitrocellulose membrane after separation by SDS-PAGE. Radiolabeled calnexin was detected by radioautography, the corresponding region of the nitrocellulose was excised, and blocked in 0.5% PVP360 in 100 mM acetic acid for 30 min at 37°C. It was then washed extensively with distilled water and twice with the 50 mM NH_4HCO_3, pH 7.8. The membrane-bound calnexin was subjected to TPCK-trypsin digestion in 50 mM NH_4HCO_3, pH7.8. The tryptic peptides were lyophilized and subjected to oxidation by freshly prepared performic acid [99% formic acid, 30% H_2O_2: 9.1 (v/v)] for 1 h on ice. The tryptic peptides were lysophilized and washed extensively. The dried tryptic phosphopeptides were dissolved in pH1.9 buffer as above. The peptides were separated first by electrophoresis in pH1.9 buffer for 30 min at 1 kV employing a Hunter thin-layer electrophoresis system and followed by overnight ascending chromatography in isobutyric chromatography buffer [isobutyric acid, n-butanol, pyridine, glacial acetic acid, H_2O: 625.19.48.29.279 (v/v/v/v/v)]. For Figs. 27 and 30A, phospho-chromatography buffer [n-butanol, pyridine, glacial acetic acid, H_2O: 375.250.75.300 (v/v/v/v/v)] was used. Marker dye composed of 5 mg/ml of ε-dinitrophenyl-lysine and 1 mg/ml of xylenen cyanol FF blue were spotted onto the TLC plate for both electrophoresis and chromatography to monitor the migration mobility. Phosphopeptide maps were visualized either by radioautography with enhancing screens or with a Fuji PhosphoImager screen and visualized using a BAS 2000 Fuji Bio-Imaging Analyzer (Fuji BioMedical Systems Inc., Bethesda, MD) with the gradation, sensitivity, and resolution set as described by the manufacturer. Cerenkov counting monitored the efficiency of recovery from each step.

Tricine-SDS-PAGE analysis

Tricine-SDS polyacrylamide gel was prepared as described (Schagger and von Jagow 1987). Lyophilized ^{32}P-labeled trypsinized samples after oxidation as above were resuspended in 1X Laemmli sample buffer. The polyacrylamide gel was initially

electrophoresized for 1.5 h at 30 V and followed by 90 V for 20 h. The gel was Coomassie stained and destained in destain solution containing 3% glycerol. The gel was dried under vacuum for 3 h at 60 oC followed by visualization by radioautography with an enhancing screen at –80 oC.

In-gel digestion and nanoelectrospray ionization (nanoES) mass spectrometry (MS)

Calnexin antibodies immunoprecipitated samples from non-radiolabeled HepG2 and MDCK cell lysates were separated by SDS-PAGE and stained with 0.2% (w/v) Coomassie Brilliant Blue R250 in 50% (v/v) methanol and 2% (v/v) acetic acid and destained in 50% (v/v) methanol and 2% (v/v) acetic acid. The excised coomassie stained band bound on the polyacrylamide gel was reduced and alkylated with 15 mM DTT and 1.3 mM iodoacetamide and followed by trypsin digestion as described (Wilm *et. al.*1996). Tryptic peptides were extracted twice with 100 mM NH_4HCO_3 and acetonitrile followed by two extractions with 5% formic acid and 50% methanol before analysis by Delayed Extraction Matrix Assisted Laser Desorption Ionization (DE-MALDI) and nanoES-MS. DE-MALDI time of flight (TOF) spectra were acquired for each digest and the peptide masses were used to confirm that the coomassie blue stained band was in fact calnexin. NanoES-MS experiments were performed as described (Betts *et. al.*1997). Briefly, each sample was desalted via a POROS R2 capillary column (PerSeptive Biosystems, Farmingham, MA) and eluted in 10 µl of spraying solution [50% (v/v) methanol, 5% (v/v) ammonia in water for negative ion mode detection or 50% (v/v) methanol, 1% (v/v) formic acid in water for positive ion mode detection]. 1 µl of eluate was inserted into the needle made with a micropipette puller (Sutter Instrument Co., Novato, Ca) from borosilicate glass capillary (Clark Electromedical Instruments, Pangbourne, Reading, UK). Electrospray mass spectra were acquired on a Sciex API III triple quadrupole machine (Perkin-Elmer Sciex, Ontario, Canada) equipped with a nanoES ion source developed by Wilm and Mann (Wilm and Mann 1994; Wilm and Mann 1996). The instrument was operated in negative ion mode to generate a phosphate specific fragment, PO_3^- at m/z of 79 (Carr *et. al.*1996). The instrument was set to record only those peptides give rise to this fragment. This was termed a Precursor of m/z

negative 79 scan. Similarly, the instrument was also operated in positive ion mode to scan peptides yielding a neutral constant loss at m/z 49, loss of H_3PO_4 with a molecular mass of 98, for doubly charged molecular ions of phosphate containing peptides. Spectra interpretation was performed using BioMultiView (Sciex) software.

Embryonic dorsal root ganglia experiment

Embryonic dorsal root ganglia (DRG) were isolated and cultured as previously described (Giasson and Mushynski 1997). Briefly, embryos were removed from a Sprague-Dawley 14-d pregnant rat that was previously anesthetized with ether and sacrificed by cervical dislocation. Embryos were placed in defined medium N1 containing 30 µg/ml bovine apotransferrin, 0.9% BSA 6 ng/ml of 2.5 S nerve growth factor (NGF) and antibiotics. Embryonic sacs were removed and embryos were transferred to a new dish containing fresh medium. Under microscope, the entire spinal cord of each embryo was removed and pulled apart gently to expose the dorsal root ganglia. DRGs were detached gently and plated in a small volume of medium at the center of a 35-mm culture dish. The cells were allowed to attach for 30 min before flooding with defined medium.

The localization of neuronal perikarya in a small centrally located region allowed manual separation of cell bodies from the neurites (axons and dendrites). Neurites extended radially from the center of the dish to form a halo surrounding the cell body mass. For cultures treated with antimitotic agents, the cells were cycled among 10^{-5} M 5-fluoro-2'-deoxyuridine, 10^{-6} M cytosine β-D-arabino-furanoside and 5×10^{-6} M 5-fluoro-2'-deoxyuridine, 5×10^{-7} M cytosine β-D-arabino-furanoside every 4 d for 16 d, starting 24 hr after plating. For fibroblast-only cultures, the DRG cultures were maintained as above without the addition of NGF.

Cultures were pulsed with 1 mCi of ^{32}P-orthophosphate per ml of medium for 1.5 h, dissected as described (Giasson and Mushynski 1997) and lysed with modification. Briefly, at the end of the pulse, cells were washed briefly with HBS. Cell bodies were manually separated using a cut-off 1-ml pipetteman tip attached to a pipetteman (Fig. 24) and transferred gently to a microfuge tube containing 1% CHAPS lysis buffer [1% (w/v) CHAPS, 50mM HEPES, pH7.6, 200mM NaCl, 1mM PMSF, 10 µg/ml each of leupeptin

and aprotinin, 10mM NaF, 10mM NaPPi, 0.4mM $NaVO_4$ and 5mM $NaMoO_4$]. Neurites were collected subsequently by gentle swirling with a 1-mL pipetteman tip attached to a pipetteman and transferred to a microfuge tube containing 1% CHAPS lysis buffer. On ice for 30 min and pellets were not dissolved. A final concentration of 1% SDS was added and boiled for 5 min. Cell debris was removed by ultracentrifugation at maximum speed for 10 min. Protein concentration of each supernatant was determined. Equal amount of protein from each fraction was used for immunoprecipitation and a final concentration of 0.1% SDS was adjusted with HBS containing the same concentration of protease inhibitors and phosphatase inhibitors in lysis buffer. Immunoprecipitation with either anti-NFL or anti-C3 CNX antibodies. SDS-PAGE, electroblotting and radioautography were done as described above.

Ribosome associated proteins.

The association of membrane proteins with ribosomes was done as described (Gorlich *et. al.*1992; Kalies *et. al.*1994; Chevet *et. al.*1999). Briefly, canine pancreatic microsomes were solubilized in lysis buffer [50 mM Tris-HCl, pH7.6, 400 mM Kacetate, 10 mM MgAcetate, 15% glycerol, 5 mM b-mercaptoethanol and protease inhibitors] containing either 1.5% digitonin, or 1% CHAPS or 1% TX100 on ice for 30 min. The ribosomal complexes were centrifuged through a 100 ml cushion of 1.5M sucrose in lysis buffer containing 0.1% of the corresponding detergent. Proteins in the ribosomal pellets were analyzed by immunoblotting after SDS-PAGE separation. Ribosomes in the sedimented pellet were verified by immunoblotting with chicken antibodies against the ribosomal proteins L3/L4 (Murphy *et. al.*1997).

Heat inactivated EsRM (Ou *et. al.*1992) were incubated with either 0.5mg of CK2, or activated ERK-1 (isolated from 2 mg of rat liver cytosol from EGF treated rat as described by DiGuglielmo *et. al.* (Di Guglielmo *et. al.*1994) or both for 30 in at 30°C in the presence of 2 μCi [γ-^{32}P]-ATP. The reaction was placed on ice and incubated with 50 mg of competent isolated ribosomes (Chevet *et. al.*1999) for 30 min. Microsomes were solubilized in 1% CHAPS lysis buffer and ribosomal associated proteins were purified as described above. Proteins in the pellet were solubilzed in lysis buffer containing 1% SDS

and heated for 10 min at 65 oC and then diluted 10 fold in the same buffer without SDS but 1% TX100. Calnexin was immunoprecipitated by anti-C3 calnexin antibodies, separated by SDS-PAGE, transferred onto a nitrocellulose membrane, and immunoblotted with anti-C1 calnexin antibodies. Proteins in the ribosomal supernatant were separated by SDS-PAGE and immunoblotted with anti-C1 antibodies.

In-gel kinase assay.

In-gel kinase assay was done as described (Chevet *et. al.*1999). Briefly, Following immunoprecipitation with either anti-C3 or C4 calnexin antibodies, proteins were separated by 10% SDS-PAGE in a gel containing 0.5 mg/ml of myelin basic protein (MBP). The gel was washed twice with 20% isopropanol in 30 mM Tris-HCl pH 7.5, and twice with 2 mM DTT in 30 mM Tris-HCl pH 7.5, 30 min per wash. The gel was then incubated in 30 mM Tris-HCl pH containing 2 mM DTT and 6 M urea for 45 min. The proteins were renatured by three washes of 45 min in renaturation buffer [30 mM Tris-HCl pH 7.5, 2 mM DTT and 0.05% Tween 20] containing 3, 1.5 and 0.75 M urea. The gel was incubated in renaturation buffer for 2 h followed by a 30 min incubation in kinase buffer [30 mM Tris-HCl pH 7.5, 2 mM DTT, 10 mM $MgCl_2$, 10 mM $MnCl_2$]. The phosphorylation reaction was initiated by the incubation of the gel in the kinase buffer containing 10 µCi/ml of [γ-^{32}P]-ATP and 0.1 mM ATP for 45 min at room temperature. At the end of phosphorylation, the gel was extensively washed and fixed in 5% (v/v) trichloroacetic acid and 1% (w/v) NaPPi.

Exogenous O-linked glycosylation assay

The incorporation of UDP-[^{3}H]-galactose by exogenous galactosyl transferase onto isolated canine pancreatic microsomes was carried with method described previously (Paiement *et. al.*1982). Briefly, the galactosyl transferase, obtained from Sigma, was autogalactosylated using non-radiolabeled galactose accordingly prior reaction (Whiteheart *et. al.*1989; Roquemore *et. al.*1994). The isolated canine pancreatic microsomes were either intact or sonicated for 10 sec prior galactosylation reaction. At the end of reaction, microsomes were lysed by 1%TX100 and calnexin was

immunoprecipitated as described before. Samples were separated by SDS-PAGE, coomassie blue stained and destained as described above. Signals were amplified with EN^{3}Hance (NEN-Mandel, Boston, MA) and visualized by radioautography at -80°C. Ovalbumin was included as a control.

In vivo phosphorylation of S. pombe

S. pombe were radiolabeled as previously described with modification (Gould *et. al.*1991; Park *et. al.*1995). Briefly, *S. pombe* cultured in EMM with essential nutrients were grown to a logarithm stage and resuspended to $2x10^8$ cells/ml in 1 mL volume. The cells were maintained in phosphate free medium (EMMP) supplemented with essential nutrients for 7 h. The growth of the cells were not hindered as monitored by their OD reading at the end of pre-labeling period (data not shown). Cells were subsequently radiolabeled with 0.5 or 1 mCi/ml of ^{32}P-orthophosphate for 4h. At the end of the pulse, a final concentration of 0.1% NaN_3 was added to the cells and cells were collected by centrifugation and proceeded to lysis and immunoprecipitation (see below).

In vivo metabolic radiolabeling of S. pombe calnexin

S. pombe were metabolically radiolabeled as described (Jannatipour *et. al.*1998). Briefly, *S. pombe* were grown in sulfate-free EMM medium (EMS) with supplement required nutrients and 100 µg/ml NH_4SO_3 to logarithm state. The cells were washed in EMS medium, resuspended to $2x10^8$ cells/ml and into 1mL aliquot. The cells were then pulsed by the addition of 0.2 mCi/mL of Tran^{35}S-Methionine and grown for indicative time at 30°C shaking waterbath. At the end of the pulse, a final concentration of 25 µg/ml of methionine and cysteine was added to the cells and further incubated for another 10 min at 30°C. A final concentration of 0.1 % (w/v) sodium azide was added to the cells and placed on ice. *S. pombe* were washed in 1xPBS containing 0.02% NaN_3.

Immunoprecipitation of S. pombe calnexin

S. pombe Cnx1p was immunoprecipitated as described (Jannatipour *et. al.*1998). Briefly, washed *S. pombe* were resuspended into 150 µl of RIPA lysis buffer [50 mM HEPES, pH 7.0, 50 mM NaCl, 10 mM iodoacetamide, 1% NP-40, 0.5% DOC, 0.1%

SDS, 1 mM PMSF, 10 mg/ml of each protease inhibitors (leupeptin, chymostatin, bestatin and pepstatin), 10mM NaF, 10mM NaPPi, 0.4mM $NaVO_4$ and 5mM $NaMoO_4$]. A volume of 300 µL of acid washed glass beads was added to cell suspension. The cells were lyzed by mechanical vortexing, 10 times of 30 sec vortexing with 30 sec on ice in between the vortexes. The lysates were transferred to a new tube and microfuged for 10 min at 4°C. *S. pombe* lysates were pre-cleared and immunoprecipitated with affinity purified anti-Cnx1p antibodies. *S. pombe* Cnx1p were separated by SDS-PAGE, silver stained (Just *et. al.*1995) and analyzed by radioautography. For Fig. 40, Spheroplasts were prepared (Moreno *et. al.*1990) and were lysed with 1%TX100/0.1%SDS lysis buffer [150 mM NaCl, 10 mM DTT, 1 mM PMSF, 10 mg/ml of each protease inhibitors (leupeptin, aprotinin and pepstatin) as described (Parlati *et. al.*1995). Cnx1p was subsequently immunoprecipitated as described above.

Polymerase chain reaction.

Polymerase chain reaction (PCR) using the designated PCR primers (see below) performed site-directed mutagenesis experiments. Genomic DNA isolated from *S. pombe* Q358 strain was the template. Amplification reactions were performed as previously described using a Perkin-Elmer Cetus thermocycler with Taq DNA polymerase (Parlati *et. al.*1995). In oligonucleotide sequences (see below), the Hind III restriction site is underlined, the BamHI site is double underlined, the corresponding nucleotide sequence for the stop codon and mutagenized amino acid residue is in italicized and boldface characters respectively. Nucleotide numbers (nt) and sequence numbers correspond to the position on *cnx1*$^+$ (GeneBank #M98799) and precursor Cnx1p, respectively (Parlati *et. al.*1995). Oligonucleotides used for the construction of each mutant (see Fig. 39A) also contained restriction enzyme sites. For S546A mutant, the primers used were CUP-1, 5'-ctccattattctttcgattaagcttgctgatccatctcatcg-3' (nt -109 to -78) and S546A, 5'-attttagca ggatcc*tta*gtcttcattcttcgcagttggtgattcagtttcgggagcgta**agc**aacgtctat-3' (nt 1627-1708). For S553A mutant, the primers used were CUP-1, and S553A, 5'-attttagcaggatcc *tta*gtcttcattcttcgcagttgg**tgc**ttcagtttcggg -3' (nt 1645-1708). For AA mutant, the primers used were CUP-1 and AA, 5'-attttagcaggatcc*tta*gtcttcattcttcgcagttgg**tgc**ttcagtttcggg

agcgtaagcaacgtctat-3' (nt 1627-1708). For S546E mutant, the primers used were CUP-1 and S546E, 5'-attttagcaggatcc*tta*gtcttcattcttcgcagttggtgattcagtttcgggagcgtactcaacgtctat-3' (nt 1627-1708). For S553E mutant, the primers used were CUP-1 and S553E, 5'-attttagcaggatcc*tta*gtcttcattcttcgcagttggctcttcagtttcggg -3' (nt 1645-1708). For EE mutant, the primers used were CUP-1 and EE, 5'-attttagcaggatcc*tta*gtcttcattcttcgcagttggctcttca gtttcgggagcgtactcaacgtctat-3' (nt 1627-1708). The PCR products were ethanol precipitated and an aliquot was electrophoresed on 0.7% agarose and stained with ethidium bromide. The PCR products were subsequently digested with Hind III and Bam HI and a band of approximately 1.9 kb was obtained.

Plasmids

Dr. Miho Shida (NRC-BRI, Montreal, Quebec) provided pMS vector constructed from pSP72 plasmid, see Fig. 42. The PCR product of *cnx1*$^{-}$ containing the specific mutation from above and the pMS vector were digested with HindIII and BamHI. The products were separated in a 0.7% agarose gel and the bands corresponding to the PCR fragments (~1.9 kb) and linearalized vector (~ 7.6 kb) were excised and extracted from agarose using GeneClean method with manufacturer's instruction (Bio101, La Jolla, CA). The HindIII-BamHI fragments were allowed to ligate in the presence of T4 DNA ligase. Plamids were amplified in *E. coli* strain MC1061, grown on 2YT plates supplemented with ampicillin and plasmids were isolated (Maniatis, Fritsch *et. al.*1982). The mutation was confirmed by sequencing the plasmids using primers TM-1 5'-ggattgcaattgttgcc-3' (nt 1466-1482) and ura4 5'-cagagatgccgacgaagc-3'. Sequencing reaction were performed using a ABI Prism Dye Terminator (Perkin Elmer, CA). The resulting plasmids containing the correct constructs were amplified and used for transformation.

Transformation

S. pombe strain Q358 was used for transformation by Lithium acetate method as described (Moreno *et. al.*1990). Briefly, strain Q358 was cultured overnight at 30°C in EMM medium with essential nutrients to an OD600 of 1 to 2. Cells were resuspended in 0.1M lithium acetate at $1x10^7$ cells/ml and kept at 30°C for 1 h. The constructed plasmids were added to the cells and mixed. 3 vol of pre-warmed 40% PEG4000 in 0.1M

Lithium acetate was added to this cell suspension. After a 1-h incubation at 30°C, cells were heat shocked for 15 min at 42°C. The supernatant was removed after a quick spin and the cells were resuspended in YE medium, grown in 30°C shaker for 1 h, and plated onto EMM plates supplemented with required nutrients. Ura4+ transformants were selected and genomic DNA was prepared from these transformants. Homologous recombination was confirmed by PCR using primers CUP-3 5' gatacctgacagttg-3' (nt -497 to -483) and ura4. The mutation in the selected transformants was confirmed by sequencing reaction from both ends with primers TM-1 and ura4.

GST pull down assay

Metabolic Tran^{35}S radiolabeled lysate prepared as above was pre-cleared with an aliquot of glutathione sepharose beads for 1 h at 4°C with rotation. Approximately 300 µg of lysate was incubated with an aliquot of glutathione sepharose beads containing the different GST fusion constructs (GST, GST-FL, GST-A, GST-B and GST-C) overnight with rotation at 4°C. The beads were washed extensively three times with lysis buffer and once with HBS. The samples were eluded with Laemmli sample buffer and separated by SDS-PAGE as above. The gel was Coomassie blue stained, destained was treated with Amplify solution prior drying. The GST fusion interacting proteins were visualized by radioautography with an enhancing screen at –80°C.

Chapter I

ApoB100 interaction with calnexin

Introduction

Transport of endogenous triacylglycerol and cholesterol in the blood stream from the liver to other tissues are in the form of low density lipoprotein (LDL) particles and their precursors are very low density lipoprotein (VLDL) particles. Conversion of VLDL particles to LDL particles is mediated by the action of lipoprotein lipase on the epithelial surface of capillaries in the plasma (Eckel 1989). Elevated concentration of plasma LDL has been implicated with the risk for the development of hyperlipidemia and atherosclerosis (Kissebah *et. al.*1981; Kissebah *et. al.*1984; Grundy *et. al.*1985; Howard 1999). The homeostasis of plasma LDL is maintained by both the LDL receptor activity and by the rate of VLDL secretion and its conversion to LDL (Brown and Goldstein 1986; Ginsberg 1987). One possibility of lowering the concentration of plasma LDL particles is by limiting the amount of secreted VLDL particles. VLDL particles are triacylglycerol rich and contain numerous apolipoproteins (Zannis *et. al.*1993). ApoB-100 is the major structural component responsible for the assembly of VLDL particles and their transport (Homanics *et. al.*1993). ApoB is also involved in the clearance of LDL from plasma via interaction with LDL receptors (Brown and Goldstein 1986; Pease *et. al.*1990). Hence, understanding of the regulation on the synthesis of apoB is of considerable importance.

Human apoB100 is a glycoprotein of 4536 amino acids, including an amino terminal 27 amino acid signal peptide that is cleaved in the lumen of the ER (Knott *et. al.*1986; Yang *et. al.*1986; Boerwinkle and Chan 1989; Yang *et. al.*1989). ApoB100 has a relative molecular mass of 550 kDa by SDS-PAGE and approximately 10% of which is contributed to oligosaccharide moieties (Swaminathan and Aladjem 1976; Yang *et. al.*1989). Human apoB100 contains nineteen potential N-glycosylation sites and sixteen of which are glycosylated. Five of the sixteen N-glycosylation sites are situated within the amino half of human apoB100 (Yang *et. al.*1989). Twelve of the twenty-five cysteine residues that are involved in the formation of intra-molecular disulfide bonds is clustered within the amino-terminal 500 amino acids of the apoB molecule (Yang *et. al.*1990).

ApoB is an atypical secretory glycoprotein and is insoluble even in 4.2 M urea. The primary sequence of apoB lacks classical transmembrane regions (Leiper *et. al.*1996). Two models of apoB translocation across the ER membrane have been suggested. In one proposed model, apoB is co-translationally translocated across the translocon and inserted into the inner leaflet of the ER membrane (Pease *et. al.*1991; Boren *et. al.*1992). In the second model, apoB contains twenty-three pause transfer sequences that induced translocational pauses at the translocon (Chuck *et. al.*1990; Chuck and Lingappa 1992; Du *et. al.*1994; Kivlen *et. al.*1997).

ApoB exists in two forms and they are apoB48 and apoB100 (Krishnaiah *et. al.*1980; Elovson *et. al.*1981; Sparks *et. al.*1983; Hardman *et. al.*1987). ApoB100 is synthesized in liver and secreted as an obligatory part of the VLDL particles (Homanics *et. al.*1993). ApoB48 is a 250 kDa polypeptide consisting of 2152 amino acids. ApoB48 consists of 48% of the mature apoB100. ApoB48 is generated from a sequence-specific mRNA editing of the apoB100 transcript (Hardman *et. al.*1987; Powell *et. al.*1987). Synthesis of apoB48 is confined to the human small intestine and secreted as an integral part of the chylomicron (Teng *et. al.*1990). In the rat, apoB48 is also synthesized in the liver and secreted as a component of VLDL particles (Sparks *et. al.*1983; Davidson *et. al.*1988). Recent studies have showed that heart muscles are capable of secreting ApoB-containing lipoprotein molecules (Boren *et. al.*1998).

The secretion of ApoB100 has been shown to be regulated mainly at the post-translational level from cultured human hepatoma HepG2 cells (Dashti *et. al.*1989; Pullinger *et. al.*1989; Moberly *et. al.*1990) and in primary rat hepatocytes (Jackson *et. al.*1988; Sparks and Sparks 1990; Bjornsson *et. al.*1992). The mRNA for apoB has been shown to be constitutively expressed and relatively stable under many conditions, whereas the secretion of apoB-containing lipoproteins was altered (Dashti *et. al.*1989; Pullinger *et. al.*1989; Moberly *et. al.*1990; Wang *et. al.*1993). In HepG2 cells, the secretion of apoB100-containing lipoproteins has been shown to be stimulated in the presence of oleate and reduced in the presence of insulin (Pullinger *et. al.*1989; Dixon *et. al.*1991; Furukawa *et. al.*1992). Altered secretion of apoB100-containing lipoproteins

with either oleate or insulin treatment was similarly observed in primary rat hepatocytes (Patsch *et. al.*1986; Sparks *et. al.*1986; Jackson *et. al.*1988; Bjornsson *et. al.*1992). A reduced apoB100 secretion has been shown to correlate with an increase in post-translational degradation that occurred at a pre-Golgi compartment (Sato *et. al.*1990; Furukawa *et. al.*1992; Adeli 1994).

The rate-limiting step for apoB secretion was found to correlate with the rate of apoB transport out of the ER (Borchardt and Davis 1987). The fate of newly synthesized apoB was determined by its translocation efficiency (Bonnardel and Davis 1995). The existence of two functionally distinct pools of apoB molecules in the ER has been demonstrated. One pool of apoB was shown to translocate across the ER. The translocated apoB was assembled and secreted as a component of lipoprotein particles. The other pool of apoB was membrane associated and not translocated across the ER membrane. Domains of membrane-associated apoB were exposed on the cytoplasmic surface of the ER (Davis *et. al.*1990; Cartwright *et. al.*1993). Proteolytic fragments of apoB were detected in both rough and smooth ER. It was suggested that membrane-associated apoB was diverted for intracellular degradation (Davis *et. al.*1989). In HepG2 cells, membrane-associated apoB100 and a fraction of the ER luminal apoB have been shown to be degraded intracellularly (Boren *et. al.*1992; Adeli *et. al.*1997).

Lipoprotein assembly requires the presence of microsomal triglyceride transfer protein (MTP), a heterodimer consisting of proline disulfide isomerase (PDI) and a 97 kDa polypeptide (Wetterau *et. al.*1991; Wetterau *et. al.*1991; Gordon *et. al.*1994; Leiper *et. al.*1994). ApoB translocation and lipoprotein assembly was blocked either in the absence of MTP lipid transferring activity (Thrift *et. al.*1992; Wetterau *et. al.*1992; Gordon *et. al.*1994; Leiper *et. al.*1994; Wang *et. al.*1996), or in the presence of the MTP inhibitor (Benoist and Grand-Perret 1997; Wetterau *et. al.*1998; Zhou *et. al.*1998), or with insufficient lipid (Gordon *et. al.*1994). Increasing the availability of cellular triglyceride for the assembly of the lipoprotein particles has been shown to enhance the translocation of apoB molecules into the ER lumen (Sakata *et. al.*1993). In the presence of supplemented oleic acid in the cultured medium, an enhanced translocation of apoB

molecules and their subsequent secretion have been observed in both the HepG2 cells and primary rat hepatocytes (Pullinger *et. al.*1989; Dixon *et. al.*1991; Furukawa *et. al.*1992; Dixon *et. al.*1991; Gibbons *et. al.*1992; Sakata *et. al.*1993).

The disposal of the majority of apoB has been suggested to occur at the level of ER membrane in the studies employing both human and rat hepatoma cell lines (Davis *et. al.*1990; Sato *et. al.*1990; Boren *et. al.*1991; Furukawa *et. al.*1992). A population of nascent apoB molecules was degraded by an ALLN-sensitive and ATP-dependent intracellular degradation mechanism on the cytosolic surface of the ER (Sakata *et. al.*1993; Adeli 1994) and hence the involvement of cysteine protease was suggested. ApoB-associated heavy density lipoprotein-like particles inside the ER lumen was also degraded (Boren *et. al.*1990; Adeli *et. al.*1997). The degradation of lipoprotein-associated apoB was also ALLN sensitive (Adeli *et. al.*1997). A second degradation site for apoB at a post-ER compartment was observed in primary rat and rabbit hepatocytes (Verkade *et. al.*1993; Wang *et. al.*1995; Cartwright and Higgins 1996; Sparks *et. al.*1996). This latter site of degradation was also demonstrated in the HepG2 cells to be ALLN-insensitive and DTT-sensitive (Wu *et. al.*1997).

ApoB has been shown to be intracellularly degraded by the ubiquitin-proteasome pathway (Yeung *et. al.*1996). An accumulation of ubiquitin conjugated apoB molecules was observed in the presence of lactacystin and ALLN (Yeung *et. al.*1996; Benoist and Grand-Perret 1997; Fisher *et. al.*1997; Zhou *et. al.*1998; Du *et. al.*1999; Sakata and Dixon 1999). Lactacystin and ALLN are inhibitors for proteasome activities (Rock *et. al.*1994; Fenteany *et. al.*1995; Craiu *et. al.*1997). Translocation-arrested apoB has been demonstrated to be ubiquitinated and targeted for proteasomal degradation (Benoist and Grand-Perret 1997; Fisher *et. al.*1997; Zhou *et. al.*1998; Du *et. al.*1999; Sakata and Dixon 1999; Sakata *et. al.*1999). Co-translational ubiquitination of apoB has been observed (Benoist and Grand-Perret 1997; Du *et. al.*1999). ER-associated proteasome molecules were detected in the perinuclear region in the digitonin-permeabilized HepG2 cells (Sakata *et. al.*1999). Furthermore, two ubiquitin-conjugating enzymes that are essential for ubiquitin-proteasomal degradation, Ubc6p and Ubc7p, were localized to the ER in

yeast (Sommer and Jentsch 1993; Biederer *et. al.*1996). Their murine homologs have recently been cloned (Tiwari and Weissman 1998). Ubiquitinated and glycosylated apoB molecules have been was found in a complex with Sec61β, a subunit of the translocon (Chen *et. al.*1998; Mitchell *et. al.*1998). Retrograde translocation of luminal localized apoB molecules for cytosolic proteasomal degradation was proposed (Liao *et. al.*1998).

The signals responsible for initiating ubiquitin conjugationof apoB and its entrance into the ubiquitin-dependent proteasome pathway are not well understood. ApoB has been shown to interact with numerous cellular proteins. The cytosolically exposed domains of apoB have been shown to interact with the cytosolic molecular chaperone HSP70 (Zhou *et. al.*1995; Fisher *et. al.*1997). MTP (Patel and Grundy 1996; Wu *et. al.*1996), calnexin (Ou *et. al.*1993; Chen *et. al.*1998; Liao *et. al.*1998), ERp57 (or ER60)(Adeli *et. al.*1997), BiP, ERp72, GRP94, and calreticulin (Linnik and Herscovitz 1998) have been shown to interact with apoB within the ER lumen. These chaperones may participate in the regulation of apoB translocation into the ER and/or the degradation of apoB by the cytosolic proteasome.

Insulin has been demonstrated to reduce the secretion of apoB with a concomitant stimulation of its intracellular degradation in primary rat hepatocytes (Sparks *et. al.*1986; Jackson *et. al.*1988; Sparks and Sparks 1990; Bjornsson *et. al.*1992). The insulin-mediated degradation of apoB in primary rat hepatocytes was partly inhibited by ALLN and was blocked by brefeldin A (Sparks *et. al.*1996). Inhibition of apoB secretion by insulin has been observed in HepG2 cells (Dashti *et. al.*1989; Pullinger *et. al.*1989). In primary rat hepatocytes, the effect of insulin on the degradation of apoB was shown to be mediated by insulin receptors (Patsch *et. al.*1986). The effect of insulin could be blocked by wortmannin and LY 294002, two inhibitors of phosphatidylinositol 3-kinase (PI3-K), suggesting of the requirement of PI3-K activation (Sparks *et. al.*1996; Phung *et. al.*1997). In response to insulin, PI3-K activity has been demonstrated to re-distribute and co-localize with apoB in both rough and smooth microsomes (Phung *et. al.*1997). Insulin may exert its effect on apoB secretion and degradation at the ER membrane via the activity of PI3-K. Calnexin is a phosphorylated lectin-like molecular chaperone that

interacts transiently with newly synthesized glycoproteins of the ER membrane (Wada *et. al.*1991; Ou *et. al.*1993). A role of calnexin phosphorylation in the regulation of the rate of glycoprotein transport was postulated (Capps and Zuniga 1994). Phosphorylated calnexin has been shown to associate with the null Hong Kong mutant of α_1-antitrypsin, coinciding with the retention of this misfolded glycoprotein within the lumen of the ER (Le *et. al.*1994). A protective role towards apoB molecules exerted by calnexin was also suggested (Chen *et. al.*1998). Manipulation of the glycosylation machinery by tunicamycin also caused a reduction in apoB100 secretion in HepG2 cells (Adeli 1994; Bonen *et. al.*1998). Therefore, calnexin appears to be situated at a strategic position to regulate the degradation of the membrane-bounded apoB in response to insulin. The level (or site) of calnexin phosphorylation may be affected by insulin. A plausible quality control effect on apoB molecules mediated by calnexin may exist. An attempt to probe the existence of a new signal transduction pathway from insulin receptors mediated by calnexin was initiated in this study employing HepG2 cells.

Here, we showed that calnexin interacts only with full-length apoB molecules in HepG2 cells. The treatment of oleic acid increased both the apoB secretion and the rate of dissociation of apoB molecules from calnexin. An attempt to examine the temporal interaction between apoB100 and calnexin in response to insulin was not pursued due to the insufficient expression of insulin receptors displayed by our cultured HepG2 cells.

Results

Oleic acid treatment increases apoB secretion.

Pulse-chase experiments were conducted to examine the synthesis and secretion of apoB100 in HepG2 cells. The cells were metabolically pulsed for 30 min and subsequently chased up to 3 h in the medium containing excess amounts of unlabeled methionine. Cells were harvested at the indicated time points and lysed as described in Materials and Methods. ApoB was immunoprecipitated, resolved by SDS-PAGE, and analyzed by radioautography. At the end of the pulse (at 0 chase time), radioautography revealed that apoB immunoprecipitates contained a band corresponding to mature human apoB100 and a number of bands with faster migration mobility corresponding to the incompletely translated nascent apoB chains (Fig. 4A, lanes 1 and 6). The nascent polypeptides of apoB100 disappeared upon a 30-min chase, coinciding with the appearance of full-length apoB100 (Fig. 4A). This is in agreement with the reported time of 14-17 min that is required for the synthesis of apoB100 (Bostrom *et. al.*1986). Radiolabeled apoB100 molecules were detected in the medium upon a 30 min of chase (Fig. 4C, lane 2), as similarly demonstrated by other investigators (Furukawa *et. al.*1992).

A stimulation of apoB secretion in responses to oleic acid has been observed in HepG2 cells (Dashti and Wolfbauer 1987; Bostrom *et. al.*1988; Dixon *et. al.*1991; Sakata *et. al.*1993). Oleic acid was reported to stimulate apoB secretion by increasing the amount of available triglyceride to nascent apoB and thus enhanced apoB translocation across the ER membrane (Wu *et. al.*1996). We also examined the effect of oleic acid treatment on apoB synthesis and secretion in our HepG2 cells. Oleic acid treatment increased both the intracellular and extracellular populations of apoB (Fig. 4, A and C).

Calnexin interacts with full-length apoB100.

Calnexin was immunoprecipitated from the lysates to examine the temporal interaction of calnexin and apoB100. Radioautography of calnexin immunoprecipitates revealed that calnexin only interacted with the mature or full-length apoB (Fig. 4B, lane 1). This was confirmed by sequential immunoprecipitation using anti- apoB antibodies (Fig. 4D, lane 1). In this procedure, calnexin immunoprecipitates consisting of calnexin

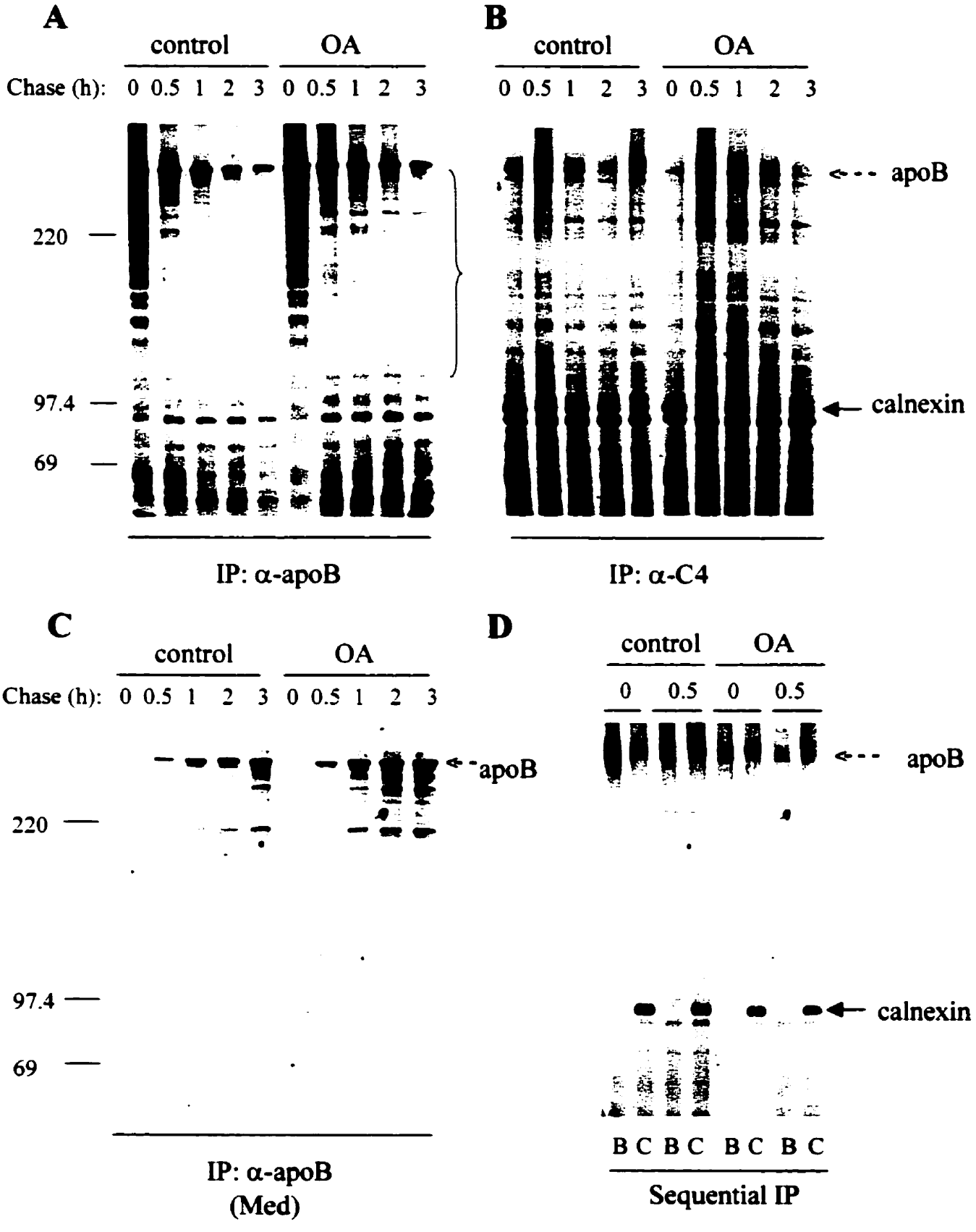
and its associated proteins were dissociated with 1% SDS. The SDS-containing supernatant was separated from the protein A-sepharose beads that were used in immunoprecipitation. The concentration of SDS in the supernatant was diluted and quenched with 1% TX100 buffer. ApoB and calnexin were subsequently immunoprecipitated using anti-apoB and anti-C4 calnexin antibodies, respectively (see Fig. 5 and Materials and Methods for description of anti-C4 calnexin antibodies). Radioautograms of the sequential immunoprecipitates of anti-apoB revealed that a smear of radiolabeledmaterials containing a band corresponding to the migration mobility of full-length apoB (Fig. 4D, lane 1). It was not confirmed here, but the smearing appearance of calnexin-associated apoB may be due to ubiquitin conjugation of apoB molecules. Calnexin has been found in a complex with ubiquitinated apoB (Liao *et. al.*1998). A longer exposure of the polyacrylamide gel was needed to detect the presence of apoB in calnexin immunoprecipitates by sequential immunoprecipitation (Fig. 4D). The association was brief since the majority of the apoB molecules were dissociated after 30 min of chase (Fig. 4D). As a control, calnexin was sequentially immunoprecipitated from lysates collected at chase time points of 0 and 30 min. Relatively similar amounts of calnexin were immunoprecipitated from both chase time points (Fig. 4D, "C" lanes). Other investigators also observed the interaction between calnexin and full-length apoB molecules(Ou *et. al.*1993; Chen *et. al.*1998; Tatu and Helenius 1999). In the presence of supplemented 0.4 mM oleic acid, apoB secretion was increased in our HepG2 cells (Fig. 4C). The amount of apoB associated with calnexin was minimal at time 0 of chase in the presence of oleic acid (Fig. 4D, compares Lane 5 to Lane 1). Oleic acid treatment has been shown to enhance apoB translocation across the ER (Sakata *et. al.*1993) and this may correlate with the observation made here. Oleic acid treatment appeared to increase the rate of dissociation of full-length apoB molecules from calnexin. Calnexin associated apoB was barely detectable at time 0 of chase with oleic acid treatment (Fig. 4B, lane 6 and 4D, lane 5).

Insufficient insulin receptors on our cultured HepG2 cells.

Variation in responses to insulin treatment by HepG2 has been observed ranging from no effect (Moberly *et. al.*1990) to a reduction of apoB secretion (Pullinger *et. al.*1989). The effect of insulin on apoB secretion is insulin receptor mediated (Patsch *et. al.*1986). The variation of the levels of insulin receptor expression by cultured cells may underline the various responses observed with insulin. Hence, we set forth to determine if our cultured HepG2 cells contain sufficient surface insulin receptors to mediate the effect of insulin on apoB secretion. Insulin receptors were partially purified from cellular extracts by wheat germ agglutinin and insulin binding assays were done as described (Burgess *et. al.*1992; Band *et. al.*1997). HTC-IR cells, rat hepatocytes over-expressing human insulin receptors, were included for comparison. Our cultured HepG2 cells displayed relatively a four-fold less insulin binding capacity than the HTC-IR cells (Table 4). Hence, the study of insulin regulation of apoB secretion via calnexin phosphorylation was not pursued further.

Figure 4: Evaluation of apoB100 synthesis in HepG2 cells.

HepG2 cells were incubated in the absence or presence of a final concentration of 0.4 mM oleic acid in methionine free medium for 1 h prior metabolic labeling. The cells were subsequently pulsed for 30 min with 0.2 mCi/ml of Trans^{35}S-labels and chased for 0 to 3 h time intervals as indicated. Oleic acid at 0.4 mM was present during both pulse and chase where it is indicated. Cells were lysed using RIPA lysis buffer as described in Materials and Methods and immunoprecipitated with either anti-apoB antibodies or anti-C4 calnexin antibodies. Panels A and B, intracellular apoB and calnexin immunoprecipitated with either anti-apoB or ant-C4 calnexin antibodies, respectively. Panel C, extracellular apoB immunoprecipitated with anti-apoB antibodies. Panel D, anti-C4 calnexin immunoprecipitates at time of 0 and 0.5 h of chase were eluted from protein A-sepharose beads and sequential immunoprecipitated with either anti-apoB antibodies (*B*) or anti-C4 calnexin antibodies (*C*). Immunoprecipitates were resolved by 5% SDS-PAGE, Coomassie blue stained, enhanced and visualized by radioautography. The exposure time for panels A, B, and C was 37 h whereas the exposure time for panel D was 11 d at -70°C. Nascent apoB polypeptides are grouped (*bracket*). A dashed arrow indicates the migration position of Coomassie blue stained human plasma apoB100. An arrow indicates the migration mobility of calnexin. The migration position of ^{14}C-labeled molecular mass markers is indicated on the left.


A
control
OA
Chase (h): 0 0.5 1 2 3 0 0.5 1 2 3
220
97.4
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IP: α-apoB
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calnexin
IP: α-C4
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Chase (h): 0 0.5 1 2 3 0 0.5 1 2 3
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apoB
calnexin
B C B C B C B C
Sequential IP

Table 4: Assessment of insulin receptors by insulin binding assay.

Cell lines	Specific binding (%)	Factor
HTC-IR	3.19%	4
HepG2	0.76%	1

Insulin binding assays were carried out as previously described (Burgess *et. al.*1992; Band *et. al.*1997). Nearly confluent HTC-IR and HepG2cells were lysed in 1.5% Triton X-100 solubilization buffer with various protease and phosphatase inhibitors (Band *et. al.*1997). Insulin receptors were partially purified by wheat germ agglutinin (WGA) chromatography and eluted with N-acetyl-D-glucosamine. An equivalent volume of WGA eluate from each cell line was assayed for ^{125}I-insulin binding. Approximately 50,000 cpm of ^{125}I-insulin was included in each assay tube. For the assay, a serial dilution of each eluate was made and each dilution was done in triplicate. The protein concentration of each WGA partially purified glycoproteins was determined by Bradford method (Bradford 1976). Nonspecific binding was determined in the presence of a 10-fold excess of non-radiolabeled insulin and was subtracted from total binding quantified by scintillation detection. The percentage of specific binding was calculated by dividing the corrected specific binding over the total amount of ^{125}I-insulin present and was corrected to the 10 µg of WGA eluate of each cell line.

Discussion

Calnexin was originally purified as a complex consisting of three other membrane proteins of the ER (Wada *et. al.*1991) and they are pgp35 or SSRα (later renamed TRAPα), gp25H or SSRβ (or TRAP β) and gp25L (Gorlich *et. al.*1990; Prehn *et. al.*1990; Wada *et. al.*1991; Hartmann *et. al.*1993). TRAPα and TRAPβ are components of the tetrameric TRAP complex (Hartmann *et. al.*1993). The association of calnexin with the tetrameric TRAP complex suggested that calnexin is likely to be situated in close proximity to the translocon if not a part of the translocon where newly synthesized ER targeted proteins are funneled into the ER lumen. The close proximity of calnexin to translocon and the lectin-like property of calnexin, one would predict that calnexin would interact with the nascent polypeptide chains of glycosylated apoB. The amino terminal half of apoB is glycosylated at five Asn residues (Yang *et. al.*1989). Our results, however, showed that calnexin does not associate with nascent apoB and rather associates with full-length apoB molecules only (Fig. 4). This observation suggested that calnexin is associated with apoB after it has been released from the translocation machinery. This is similar to the interaction between calnexin and the human erythrocyte anion exchanger 1 (Popov and Reithmeier 1999). Other investigators have shown that the interaction between calnexin and apoB is glycan-dependent because this association was prevented in the presence of either tunicamycin, an inhibitor of the oligosaccharide assembly, or castanospermine, an inhibitor of glucosidase activities (Ou *et. al.*1993; Chen *et. al.*1998; Liao *et. al.*1998; Tatu and Helenius 1999). Tunicamycin treatment has been shown to stimulate apoB degradation (Macri and Adeli 1997).

ApoB was also observed as a secreted phosphoprotein in both the primary rat hepatocytes and the HepG2 cells (Davis *et. al.*1984; Sparks *et. al.*1988; Jackson *et. al.*1990; Swift 1996). Phosphorylated apoB48 was reported to be more readily detected than phosphorylated apoB100 (Sparks *et. al.* 1988; Swift 1996). Phosphorylation of apoB has been suggested to play a role in the regulation of its secretion and degradation (Jackson *et. al.*1990; Swift 1996). We have also examined the phosphorylation status of

apoB in our HepG2 cells. However, we did not detect any phosphorylated apoB100 either intracellularly or extracellularly in our HepG2 cells up to 6 h of *in vivo* radiolabeling with ^{32}P-orthophosphate (data not shown) although the metabolic radiolabeled apoB was readily detected in both compartments (Fig. 4).

ApoB molecule is a unique secretory protein. The primary sequence of apoB lacks classical transmembrane domains (Leiper *et. al.*1996). Studies of apoB translocation have suggested two different views of translocation of apoB. One group of investigators suggested translocational pausing and transient transmembrane intermediates of apoB have been observed because of the presence of pause transfer sequences (Chuck *et. al.*1990; Chuck and Lingappa 1992). In this model, uncoordinated translation and translocation would result in the exposure of parts of nascent apoB to the cytoplasm. This model has been supported by other investigators where they observed protease sensitive domains of apoB in intact microsomes (Boren *et. al.*1992; Du *et. al.*1994) and co-translational ubiquitination of apoB (Du *et. al.*1998). Another group of investigators, however, suggested that the translocation of apoB is similar to other secretory proteins. ApoB is co-translational translocated across the translocon and inserted into the inner membrane leaflet of the ER membrane (Pease *et. al.*1991). This model has been supported by examining the glycosylation modification at the most carboxyl terminal of apoB (Huang and Shelness 1999).

The two existing pools of apoB, either membrane-associated or lipid-associated, are distinctively present in the cells (Davis *et. al.*1990; Boren *et. al.*1991). Inefficient translocation of apoB would result in its association with the ER membrane. Studies have indicated that it is the membrane-associated form that is subjected to intracellular degradation by the ubiquitin proteasome pathway (Sato *et. al.*1990; Boren *et. al.*1991; Furukawa *et. al.*1992; Yeung *et. al.*1996). Furthermore, translocation efficiency was shown to determine the fate of nascent apoB polypeptides chains (Bonnardel and Davis 1995). Oleic acid treatment exerted a protective role to apoB molecules by facilitating the translocation process (Dixon *et. al.*1991; Chen *et. al.*1998). Ubiquitinated apoB in the sec61 complex was reduced with oleate treatment (Chen, *et. al.*1998). An increase in

the association between ubiquitinated apoB to Sec61 proteins has been observed when the interaction between calnexin and apoB was inhibited by castanospermine treatment (Chen *et. al.*1998). In our studies, we showed that oleic acid supplement increased both the secretion of apoB and its rate of dissociation from calnexin (Fig. 4C and 4D). This observation may correlate with the enhanced translocation of apoB across the ER membrane and the subsequent increases in the secretion of apoB-containing lipoproteins with oleic acid treatment (Sakata *et. al.*1993) (Fig. 4C). In the absence of oleic acid supplement, we detected an association between calnexin and full-length apoB by sequential immunoprecipitation (Fig. 4D). Calnexin-associated apoB may be ubiquitinated based on their peculiar migration on SDS-PAGE. The smearing appearance of calnexin-associated apoB may be due to poly-ubiquintin modification of apoB. Ubiquitinated apoB has been found in a complex with calnexin and sec61β subunit of the translocon (Chen *et. al.*1998). Under limited triglyceride supply, membrane-associated apoB has been suggested to be diverted for degradation (Davis *et. al.*1989; Boren *et. al.*1992; Adeli *et. al.*1997). The temporal interaction between apoB and calnexin may participate in determining the fate of nascent apoB polypeptides. The anchorage provided by calnexin at the ER membrane may represent a possible regulatory mechanism that may either act positively to assist apoB folding and assembly or act negatively to direct apoB towards proteasomal degradation.

Insulin treatment has been shown to inhibit apoB secretion and stimulate apoB degradation (Sparks *et. al.*1986; Jackson, *et. al.*1988; Sparks and Sparks 1990; Bjornsson *et. al.*1992). Recent studies have indicated that PI3 kinase activity in rough ER is required for insulin-dependent inhibition of apoB secretion by primary rat hepatocytes (Sparks *et. al.*1996; Phung *et. al.*1997). The temporal interaction between calnexin and apoB at the ER membrane may present a potential regulatory checkpoint. Together with our result obtained from oleic acid treatment, one would expect a prolonged interaction of apoB with calnexin in response to insulin. Calnexin is a lectin-like phosphorylated transmembrane protein of the ER. Hence, the phosphorylation status of calnexin might provide a potential in the regulation of apoB secretion in response to insulin. Our

cultured HepG2 cells, however, do not display a sufficient amount of insulin receptors to allow us to examine this possibility and hence this study was not pursued further.

Chapter II

Characterization of *in vivo* calnexin phosphorylation

Introduction

Calnexin is a lectin-like molecular chaperone and it is one of the constituents of the quality control machinery of the endoplasmic reticulum (ER) of eukaryotic cells (Bergeron *et. al.*1994; Williams 1995; Trombetta and Helenius 1998). Calnexin is a type I transmembrane protein that is phosphorylated within its cytosolically oriented carboxy-terminus domain (Wada *et. al.*1991). Newly synthesized N-linked glycoprotein has been demonstrated to associate with calnexin transiently within the ER compartment during their maturation (Ou *et. al.*1993). The molecular interaction between the luminal domain of calnexin and the newly synthesized glycoproteins has been elucidated. Calnexin recognizes and interacts with the mono-glucosylated oligosaccharide side chain of the N-linked glycoproteins (Rodan. *et. al.*1996; Zapun *et. al.*1997). Calnexin couples the oligosaccharide modification with the productive folding of glycoproteins. Prolonged association of calnexin with misfolded proteins, either induced by amino acid analogues or caused by genetic mutations, has been observed (Ou *et. al.*1993; Loo and Clarke 1994; Le *et. al.*1994). Misfolded and ER retained proteins are subsequently subjected to retrograde translocation and degradation by the proteasomal pathway (Brodsky and McCracken 1997; Bonifacino and Weissman 1998). The phosphorylation of calnexin presents a plausible means of assuring that only properly folded glycoproteins are allowed to exit the ER compartment for secretion.

Calnexin was originally identified as a substrate of ER microsomal-associated kinases (Wada *et. al.*1991). Purification of the ER-associated kinase that phosphorylated calnexin in microsomes led to the identification of CK2 (Ou *et. al.*1992). The properties of this kinase were consistent with the conditions that originally revealed calnexin phosphorylation (Wada *et. al.*1991; Ou *et. al.*1992). Furthermore, purified CK2 has been found to phosphorylate calnexin on putative CK2 sites found within the cytosolic domain of calnexin (Ou *et. al.*1992; Cala *et. al.*1993).

Calnexin is phosphorylated *in vivo* (Dakour *et. al.*1993; Capps and Zuniga 1994; Hawn and Strand 1994; Le *et. al.*1994; Schue *et. al.*1994; Wu *et. al.*1994).

Phosphorylated calnexin has been shown to associate with the null Hong Kong mutant of α-1-antitrypsin, coinciding with retention of this misfolded glycoprotein within the lumen of the ER (Le *et. al.*1994). Phosphorylated calnexin was also found in association with newly synthesized major histocompatibility complex class I allotypes, which egressed from the ER at slow rates. Those allotypes that transported to the Golgi apparatus at more rapid rates were preferentially associated with nonphosphorylated calnexin (Capps and Zuniga 1994). Prolonged association of newly synthesized major histocompatibility complex class I heavy chains with calnexin was found in a B lymphoblastoid cell line transfected with HLA-B701 after incubation with the phosphatase inhibitor cantharidin or okadaic acid (Tector *et. al.*1994). Furthermore, when human synovial epithelial (McCoy) cells were treated with okadaic acid, the major cellular protein whose phosphorylation was shown to increase (based on two-dimensional gels followed by protein microsequencing) was calnexin (Schue *et. al.*1994). Calnexin phosphorylation also increased 3-fold when McCoy cells were treated with *Clostridium difficile* cytotoxin B (Schue *et. al.*1994), a protein that glucosylates Rho protein of the Ras superfamily (Just *et. al.*1995).

Biological significance elicited by calnexin phosphorylation can be explored by the identification of the sites of calnexin phosphorylation. Although some progress has been made on the kinases and sites of phosphorylation of calnexin after *in vitro* phosphorylation of intact microsomes (Ou *et. al.*1992; Cala *et. al.*1993), little is known of the sites of calnexin phosphorylation *in vivo*. Here we have characterized calnexin phosphorylation by both phosphoamino acid analyses and two-dimensional tryptic phosphopeptide mapping analyses. Combining mass spectral analyses of calnexin immunoprecipitates, we have identified the *in vivo* phosphorylation sites of calnexin in two mammalian cell lines, human hepatoma (HepG2) cells (human) and Madin-Darby canine kidney (MDCK) cells. Both cell lines revealed phosphorylation of the cytosolic domain of calnexin exclusively on serine residues within CK2 motifs as well as a protein kinase C (PKC) and/or proline-directed kinase (PDK) motif.

Results

Evaluation of the three different anti-calnexin antibodies.

Three different calnexin antibodies referred to as anti-C1, anti-C3 and anti-C4 raised against synthetic peptides from both the amino and carboxyl termini of mature canine calnexin were available to characterize the *in vivo* phosphorylation sites of calnexin (Fig. 5A). They were assessed to determine their abilities to immunoprecipitate phosphorylated calnexins.

The HepG2 cells were *in vivo* phosphorylated with ^{32}P-orthophosphate for 3 h and lysed with either 2% deoxycholate (DOC) or CHAPS lysis buffer followed by anti-calnexin immunoprecipitation and visualization by radioautography (Fig. 5B). CHAPS appeared to be a better detergent than DOC to immunoprecipitate ^{32}P-labeled calnexin (Fig. 5B, compare left and right panels). A protein with a migration mobility above 220 kDa interacted non-specifically to the protein A-sepharose beads used in the pre-clearing step in immunoprecipitation in CHAPS lysate (Fig. 5B, left panel, lane 1, indicates by an asterisk). Anti-C1 calnexin antibodies were able to bring down three phosphorylated species but not a band corresponding to the expected mobility of human calnexin at 90 kDa (Fig. 5B left panel, lane 2). Anti-C3 and anti-C4 calnexin antibodies were able to immunoprecipitate calnexin, however, each appeared to bring down a different set of phosphorylated species (Fig. 5B, left panel, compare lanes 3 and 4) as well as the non-specific binding protein. It is unclear whether the different phosphorylated proteins associated with either anti-C3 or anti-C4 antibodies share similar antigenic epitopes and thus for the recognition by either anti-C3 or anti-C4 antibodies. It is also possible that the proteins associated with anti-C3 and anti-C4 calnexin immunoprecipitates represented different populations of phosphorylated proteins that are associated with calnexin under non-denaturing condition. Non-denaturing condition provided by CHAPS has been employed to detect the interaction between calnexin and its associated newly synthesized N-linked glycoproteins (Ou *et. al.* 1993).

In order to assess if anti-C3 and anti-C4 calnexin antibodies bring down the same or different pools of phosphorylated calnexin, two-dimensional (2-D) tryptic

phosphopeptide mapping analyses were performed. HepG2 cells were *in vivo* radiolabeled for 6 h and calnexins were subsequently immunoprecipitated under non-denaturing conditions in duplicate. One set of calnexin immunoprecipitates (IP) was separated by SDS-PAGE and visualized directly by radioautography (Fig. 6A, left panel). The second set of IPs was separated by SDS-PAGE, electroblotted onto a layer of nitrocellulose membrane and visualized by radioautography (Fig. 6A, middle and right panels, respectively). There was still a substantial amount of radiolabeled materials left behind in the polyacrylamide gel after a 40-min transfer. The bands corresponding to the phosphorylated calnexin on the nitrocellulose membrane were subjected to trypsin digestion and analyzed by 2-D phosphopeptide mapping. The phosphorylated species were grouped into phosphopeptides group A and B (Fig. 6B). Radioautograms of their corresponding 2-D tryptic phosphopeptide maps revealed that both anti-C3 and anti-C4 IP provided a similar mapping pattern although less signals or materials are observed in group A peptides in anti-C4 IP map (Fig. 6B).

The tryptic ^{32}P-labeled peptides from both human and canine calnexins migrated within the region enclosed by the dotted lines on a 20x20cm plate (Fig. 7). Hence, for simplicity, only the region within the dotted lines is displayed here for all 2-D phosphopeptide mapping.

Mammalian calnexin is exclusively in vivo phosphorylated on serine residues.

In order to determine the *in vivo* phosphorylated amino acid residue in human calnexin, ^{32}P-labeled calnexin was immunoprecipitated with both anti-C3 and anti-C4 antibodies from HepG2 cell lysates. SDS-PAGE resolved calnexin immunoprecipitates were electroblotted onto a layer of PVDF membrane and detected by radioautography (Fig. 8A). The bands corresponding to the phosphorylated calnexin were excised and subjected to phosphoamino acid analyses. Radioautograms of the two-dimensional TLC plates for both anti-C3 and anti-C4 calnexins revealed only ^{32}P-labeled serine comigrated with the non-radiolabeled phosphoserine standard as detected by ninhydrin staining (Fig.8B left and right panels respectively). Hence, human calnexin, after 6 h of *in vivo* labeling, is exclusively phosphorylated on serine residues (Fig. 8).

Another mammalian cell line was used in this study to determine the *in vivo* phosphorylation site of calnexin. Madin Darby canine kidney, MDCK, cells were similarly *in vivo* ^{32}P-orthophosphate labeled for 6 h and lysed by 2% CHAPS lysis buffer. Calnexin was immunoprecipitated from the pre-cleared lysates and electroblotted onto 2 layers of nitrocellulose membranes for 40 min (Fig. 9A, middle and right panels, respectively). In this setup, phosphorylated calnexin was also detected on the second nitrocellulose (Fig. 9A, right panel) and there was still a significant amount of ^{32}P-labeled calnexin left behind in the polyacrylamide gel after transfer (Fig. 9A, left panel). Anti-C3 antibodies appeared to bring down more calnexin than anti-C4 antibodies but also other phosphorylated proteins at migration mobility above calnexin. The non-specific phosphorylated protein with a migration mobility of 220kDa observed in calnexin IPs from HepG2 cell lysates was also observed here (compare Figs. 9A and 5B). Membrane bound calnexin immunoprecipitates, however, displayed mainly phosphorylated calnexin by radioautography (Fig. 9A, middle and right panels).

^{32}P-labeled calnexins from the polyacrylamide gel were extracted and subjected to phosphoamino acid analyses as described in Materials and Methods. Canine calnexin was also phosphorylated only on serine residues after a 6-h pulse (Fig. 9B). By 2-D tryptic phosphopeptide mapping analyses, canine calnexin gave a similar pattern regardless of which antiserum was used to immunoprecipitate calnexin. However, less signals or materials in group A phosphorylated species on the anti-C4 IP peptide map were detected by radioautography (compare left and middle panels of Fig. 9C). This was also observed on the 2-D tryptic phosphopeptide map of anti-C4 immunoprecipitated and trypsinized HepG2 calnexin (Fig. 6B). The difference between anti-C3 and anti-C4 calnexin IPs may be from the modification of the epitope that is being recognized by the anti-C4 antiserum (see below). An equal amount of both anti-C3 and anti-C4 calnexin tryptic digests, determined by Cerenkov counting, were mixed and spotted onto the same TLC plate to confirm that both antibodies do indeed recognize the same phosphorylated form of calnexin (Fig. 9C). For the subsequent ^{32}P-labeled experiments, anti-C3 antibodies were used to immunoprecipitate calnexin since this antiserum appeared to

isolate calnexins that are evenly phosphorylated at sites that are represented by phosphorylated peptides in both groups A and B (Fig. 9C).

Calnexin has been demonstrated to associate transiently with newly synthesized N-linked glycoproteins (Ou *et. al.*1993). Therefore, it would be of interest to determine whether the calnexins, recognized by these two antisera, are interacting with the same or different subsets of proteins. HepG2 cells were metabolically labeled with Tran^{35}S-label for 10 min and 1 h, respectively. For the 10-min pulse, metabolically pulsed proteins were chased for an hour to detect the dissociation kinetics of the calnexin-associated proteins from calnexin. Calnexin was immunoprecipitated with either anti-C3 or anti-C4 antiserum under non-denaturing condition, separated by SDS-PAGE and Coomassie blue stained prior exposure to the film. Although relatively similar amounts of antibodies were used for both anti-C3 and anti-C4 antisera as determined by their Coomassie blue staining intensities (Fig. 10C), anti-C3 immunoprecipitates appeared to bring down more radiolabeled proteins than anti-C4 immunoprecipitates (Fig. 10A, compare lanes 1 to 2). A shorter exposure of the same gel was included as Fig. 10B. For HepG2 cells, both anti-C3 and anti-C4 calnexin antibodies were able to bring down a similar profile of metabolically labeled proteins (Fig. 10).

MDCK cells were similarly radiolabeled as the HepG2 cells in Fig. 10. Anti-C3 calnexin antiserum brought down an additional metabolically protein with a migration mobility at 97 kDa which was not observed in anti-C4 immunoprecipitates (Fig. 11A, lane 3 and see below). The detection of this 97 kDa protein became predominant upon 1 h of chase (it was not detected at the end of the 10-min pulse, Fig. 11A lane 1) and Coomassie staining (Fig. 11C) readily observed this protein in anti-C3 immunoprecipitates. This additional protein was not observed in calnexin IPs from HepG2 cells. This 97 kDa protein may be species-specific. It is not clear whether anti-C3 calnexin antiserum or calnexin recognizes this 97 kDa protein. Nevertheless, since both antisera recognize the same phosphorylated form of calnexin and anti-C3 antibodies provide an evenly distributed phosphorylated calnexin tryptic peptides in both groups A

and B (see 2-D tryptic phosphopeptide maps in Fig. 6 and 9), anti-C3 antiserum was utilized to characterize the *in vivo* phosphorylation sites of calnexins.

Long term labeling of canine calnexin.

MDCK cells were *in vivo* labeled for 3, 6, 12 and 24 h at different concentrations of ^{32}P-orthophosphate from 0.065 to 2 mCi per ml of medium (Fig. 12). Calnexin was isolated with anti-C3 antiserum, SDS-PAGE resolved and electroblotted onto 2 layers of nitrocellulose membranes for 40 min. Only the radioautogram of the first nitrocellulose membrane that is closest to the gel is displayed for simplicity (Fig. 12A). Calnexin was strongly phosphorylated at a high concentration of ^{32}P-orthophosphate with a long pulse time. The bands corresponding to phosphorylated calnexins labeled for 3, 6, 12, and 24 h at 2 mCi/mL were excised, trypsin digested and analyzed by both tricine gel separation and 2-D phosphopeptide mapping. Radioautograms of the tricine gel indicated that there were 2 main populations of small-phosphorylated species at different migration mobilities (Fig. 12B). For simplicity, only the radioautograms of the 2-D tryptic phosphopeptide maps of 3 h- and 24 h-labeled calnexins were shown. They gave virtually an identical pattern whether calnexin was radiolabeled for 3 h or 24 h (Fig. 12C). The phosphorylated residues were not altered, remained on serine residues, from 3 to 24 h *in vivo* radiolabeling as examined by phosphoamino acid analyses (data not shown).

Calnexin has been demonstrated to be *in vitro* phosphorylated on its cytosolic domain by microsomal associated kinase(s) in isolated microsomes (Wada *et. al.*1991). The cytosolic COOH-terminal domains of canine (MDCK) and human (HepG2) calnexins (Wada *et. al.*1991; David *et. al.*1993) share 94% identity (Fig. 13). The cytosolic domain of canine calnexin contains six serine residues, and the cytosolic domain of human calnexin contains five equivalent serines and a threonine. The five conserved cytosolic serine residues are in primary sequence motifs that are predicted to be recognized by CK2 (Pinna 1990), PKC (Kishimoto *et. al.*1985; Woodgett *et. al.*1986), PDK (Nigg 1995; Robinson and Cobb 1997), or PKA (Feramisco *et. al.*1980; Glass *et. al.*1986) (Table 5). There are four other amino acids differences between the cytosolic domains of human and canine calnexins (Fig. 13). If all the serines within the cytosolic

domain of canine or human calnexin were being utilized in the phosphorylation, one would expect four completely trypsinized ^{32}P-labeled peptides that were depicted between the arrows, trypsin digestion sites (Fig. 13).

MDCK and HepG2 cells were *in vivo* labeled for 3 h with 2 mCi/ml of ^{32}P-orthophosphate and calnexin was immunoprecipitated with anti-C3 antiserum. SDS-PAGE resolved calnexin immunoprecipitates were electroblotted as in Fig. 12. Membrane-bound calnexins from the first nitrocellulose membrane closest to the polyacrylamide gel (Fig. 14A) were analyzed by 2-D tryptic phosphopeptide mapping (Fig. 14B). The 2-D tryptic phosphopeptide map of canine calnexin was slightly different from the one observed before (compare Fig. 14B, MDCK panel to Figs. 9 and 12) although a similar pattern was still observed. An increased amount of TPCK-trypsin was used for digestion. The additional phosphorylated species seen on Fig. 12C may represent partial digestion. Tryptic phosphorylated peptide maps of both canine and human calnexins appeared similar by radioautography (Fig. 14B). An equivalent amount of canine and human tryptic digests, determined by Cerenkov count, was mixed and spotted onto the same TLC plate and visualized by radioautography (Fig. 14B, MDCK + HepG2). Group A phosphopeptides were virtually identical between canine and human calnexins. Upon comparison of the sequences between the cytosolic domains of canine and human calnexins, the region composed of the 20 amino acids at the most carboxyl terminus matches between the two sequences (^{554}AEEDEILNRS^{563}PRNRKPRRE^{572}, human numbering and see Figs. 13 and 23). This conserved region contains one potential serine phosphorylation residue (Ser^{563}) which is followed by a Pro and an Asp residue (SPR). The SPRsequence is a potential PKC or a PDK phosphorylation motif (Fig. 13 and Table 5). The same region of 20 amino acids is identical among the mammalian calnexins (Fig. 23). Ser^{563} (will be referred as "SPR" here onward) phosphorylation is likely represented by the two phosphorylated species observed by the group A peptides (Fig. 14B, MDCK+HepG2 map). The two co-migrating species of group A phosphopeptides may arrive from incomplete trypsin digestion from the region containing the SPR phosphorylation site. Alternatively, they may come from the same peptide but one is

modified such that one has a different chromatography mobility. For the group B phosphorylated species, combination of both canine and human calnexin tryptic digests provided an additive effect. Group B tryptic peptides appeared to share similar electrophoretic mobility yet different chromatographic mobility (Fig. 14B). They are likely to arrive from phosphorylation of Ser^{535} and Ser^{545} from both species (see below and Chapter 4).

Alkaline phosphatase treatment

Alkaline phosphatase was employed to determine if it could dephosphorylate calnexin. This could provide a possible tool to study the biological role of calnexin phosphorylation. ^{32}P-labeled calnexins were isolated from HepG2 and MDCK cells as done in Fig. 14. After extensive washes, one set of calnexin immunoprecipitates was subjected to alkaline phosphatase treatment and one set was mock treated. The level of calnexin phosphorylation was reduced but not abolished as detected by radioautography (Fig. 15A). By 2-D tryptic phosphopeptide mapping analyses, alkaline phosphatase appeared to affect mainly on the phosphorylation level of group B peptides, with little effect on group A peptides or "SPR" phosphorylation site (Fig. 15B).

Phosphorylation of the cytosolic domain of calnexin may provide a possible mean for transmembrane communication and regulation of the luminally oriented lectin chaperone activity of calnexin. Alkaline phosphatase was employed to determine whether this treatment would affect the calnexin's lectin activity. MDCK cells were metabolically radiolabeled and calnexin was immunoprecipitated under non-denaturing conditions as in Fig. 11. Calnexin immunoprecipitates were subjected to either mock or alkaline phosphatase treatment. Supernatant and beads of each immunoprecipitate were separately resolved by SDS-PAGE. The 30°C treatment did not abrogate the interaction between the newly synthesized glycoproteins and calnexin (compare Fig 16, lanes 1 and 2) and neither did alkaline phosphatase treatment (Fig. 16). Alkaline phosphatase treated calnexin, however, is still phosphorylated, at least at the SPR site (Fig. 15B). Hence, the possible regulatory role of phosphorylation towards the lectin activity of calnexin could not be excluded completely since calnexin was only partially dephosphorylated by

alkaline phosphatase. This, however, was not pursued since the phosphatase(s) that acts on the SPR phosphorylation site is unknown.

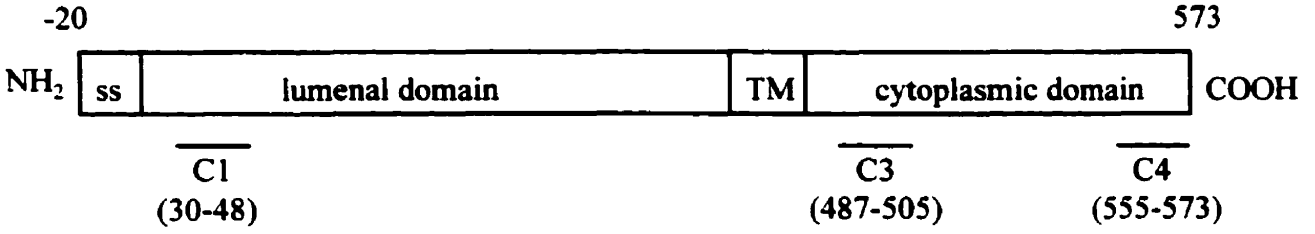
Attempts were made to identify the phosphorylated species on the tryptic peptide maps however they were not successful. Alternatively, we proceeded to mass spectrometry analyses to identify the *in vivo* phosphorylation sites of both canine and human calnexins.

Figure 5: Evaluation of anti-calnexin antisera and different detergent using HepG2 cells.

A. A schematic diagram of the canine calnexin (CNX). *ss*, signal sequence; luminal domain; *TM*, transmembrane domain; and cytoplasmic domains are indicated. Three different antibodies raised against synthetic peptides of amino acids (AA) 30-48 (*C1*), AA 487-505 (*C3*) and AA 555-573 (*C4*) of mature canine CNX were used in this study.

B. HepG2 cells were *in vivo* labeled with ^{32}P-orthophosphate at 125 μCi/ml for 3h and were lysed with 2% CHAPS lysis buffer (*2% CHAPS*) (see Materials and Methods), left panel; or 2% deoxycholic acid lysis buffer (*2% DOC*), right panel. Each cell lysate was pre-cleared with an aliquot of protein A-sepharose bead (*PC*) and followed by immunoprecipitation (IP) with 4 μl of either anti-C1 (*C1*), anti-C3 (*C3*), or anti-C4 (*C4*) CNX antisera. The pre-clearing protein A-sepharose beads and protein A-sepharose beads containing the immunocomplexes were washed extensively, separated by 6% SDS-PAGE and visualized by radioautography with intensifying screens for 18 h at –70°C. The mobility of molecular standards in kDa is indicated on the left. An *a*sterisk indicates the non-specific binding protein that is also found in pre-clearing protein A-sepharose beads.

A


-20
573
NH2
ss
lumenal domain
TM
cytoplasmic domain
COOH
C1
(30-48)
C3
(487-505)
C4
(555-573)

B

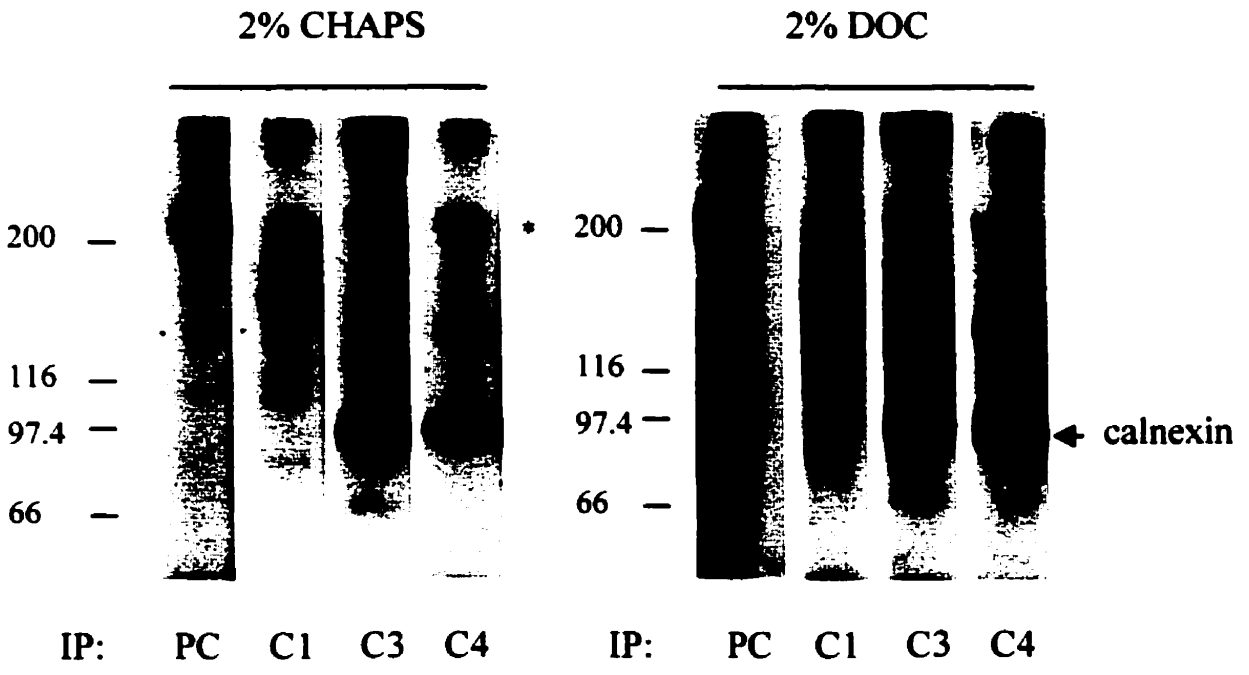
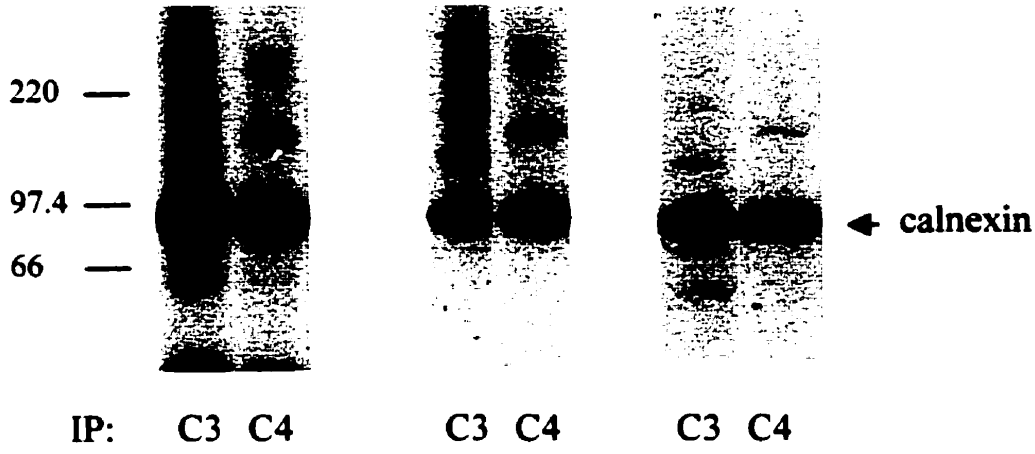

2% CHAPS
2% DOC
200
116
97.4
66
calnexin
IP: PC C1 C3 C4
IP: PC C1 C3 C4

Figure 6: Two-dimensional tryptic phosphopeptide mapping of *in vivo* phosphorylated calnexin from HepG2 cells.

A. HepG2 cells were *in vivo* labeled with ^{32}P-orthophosphate at 125 µCi/ml for 6 h, lysed with 2% CHAPS lysis buffer (see Materials and Methods), 5 µl of either anti-C3 (*C3*) or anti-C4 (*C4*) CNX antisera was added to pre-cleared lysates to immunoprecipitate CNX in duplicate. One set of immunoprecipitates (IP) was separated by SDS-PAGE (left panel). The second set of SDS-PAGE separated IP was electroblotted onto a nitrocellulose membrane (right panel) for 40 min and the polyacrylamide gel after electroblotting was saved (middle panel). Both polyacrylamide gels (left and middle panels) were dried under vacuum and visualized by radioautography for 21 h at –70oC with intensifying screens. Membrane bound and phosphorylated proteins were visualized by radioautography after a 15-h exposure (right panel). The mobility of ^{14}C-molecular standards in kDa is indicated on the left.

B. 2-D tryptic phosphopeptide mapping of ^{32}P-labeled CNX digested from the nitrocellulose membrane (Fig. 6A, right panel) were done as described in Materials and Methods. *Arrows* indicate the direction of electrophoresis towards anode and ascending chromatography. Positively charged tryptic peptides migrated toward cathode. Phosphopeptide mapping were visualized by a Fuji PhosphoImager screen. Phosphorylated species are grouped into phosphopeptides group A (*A*) and group B (*B*). *o*, origin.

A


220
97.4
66
calnexin
IP: C3 C4 C3 C4 C3 C4

B

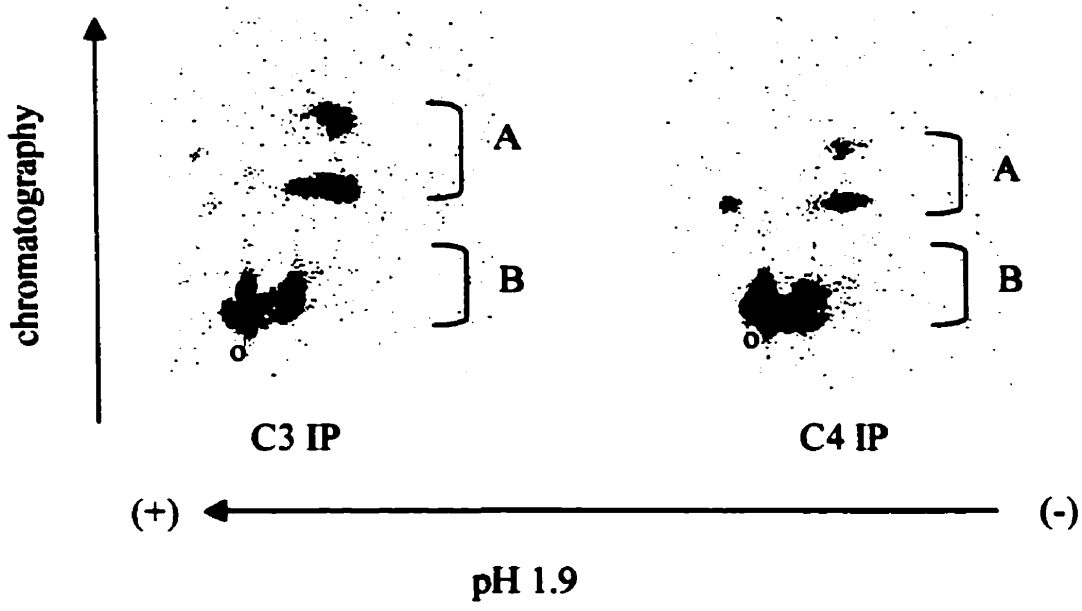

chromatography
A
B
A
B
C3 IP
C4 IP
(+)
(-)
pH 1.9

Figure 7: Analysis of the 2-D tryptic phosphopeptide mapping.

An outline of a typical 2-D phosphopeptide mapping of CNX on a 20 by 20cm TLC plate is shown. Prior first separation by electrophoresis, tryptic sample was spotted on the region marked by a *cross* with origin written next to it at the lower left-hand corner. Marker dye was spotted directly above the sample at the top of the plate, marked by a *cross*, to provide visual aid at the end of electrophoresis. The marker dye is composed of ε-dinitrophenyl-lysine yellow (*Y*) and xylene cyanol FF blue (*B*). Marker dye was also spotted at the left-hand side of the plate, indicated by a *circled cross*, prior second separation by ascending chromatography. *Dotted circles* indicate the positions of the marker dyes at the end of electrophoresis and chromatography of a typical map in isobutyric chromatography buffer. For Fig. 26C and 29A, phosphopeptide chromatography buffer was used. The direction of electrophoresis and chromatography are indicated. Unless indicated, chromatography was usually carried out until the buffer migration front reaches approximately 1 cm before the edge of the plate as indicated by an *arrowhead*. Radioactive and colored marker was placed at the edge of the plate (left-handed corner) for alignment between the film and the plate after radioautography. The area enclosed by the *dotted box* is shown in this study since all phosphorylated tryptic peptides migrated within this region

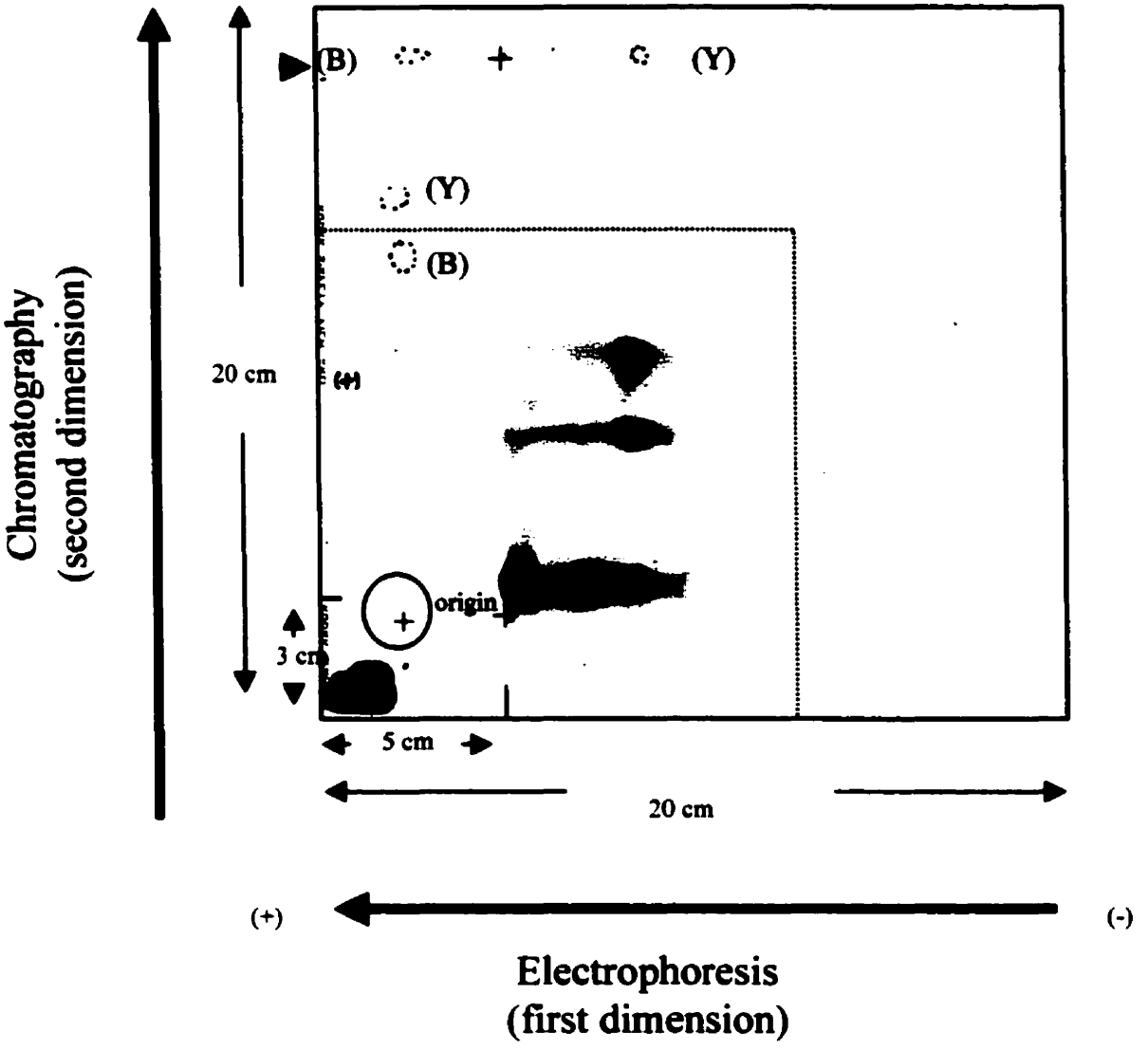
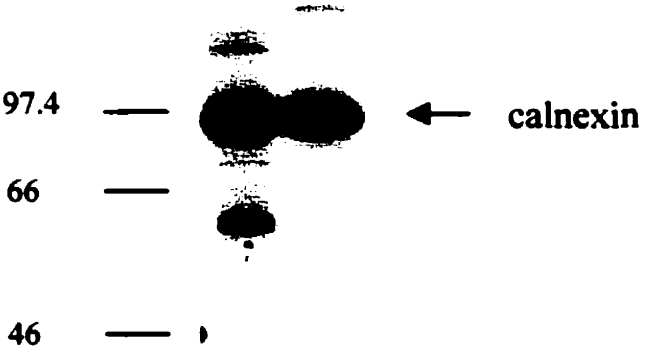

(B)
(Y)
(Y)
(B)
20 cm
origin
3 cm
5 cm
20 cm
Chromatography
(second dimension)
(+)
(-)
Electrophoresis
(first dimension)

Figure 8: HepG2 calnexin is *in vivo* phosphorylated on serine residues.

A. HepG2 cells were *in vivo* labeled as in Fig. 6 for 3 h at 125 μCi/ml of ^{32}P-orthophosphate. The anti-C3 (*C3*) and annti-C4 (*C4*) CNX immunoprecipitates from 2% CHAPS lysates were resolved by SDS-PAGE, electroblotted onto a PVDF membrane for 45 min and visualized by radioautography for 35 h at -70oC with intensifying screens. The mobility of ^{14}C-molecular standards in kDa is indicated on the left.

B. Phosphoamino acid analyses of ^{32}P-labeled CNX extracted from PVDF membrane (panel A) was done as described in Materials and Methods. Radioautogram of a 12-d exposure at -70oC intensifying screens is shown. *Arrows* indicate the direction of electrophoresis towards anode. Standard phosphoamino acids; phospho-threonine (*pT*), phospho-tyrosine (*pY*), and phospho-serine (*pS*); were visualized by ninhydrin staining. *Pi*, free phosphate; *partial*, incomplete peptide hydrolysis; and *o*, origin.

A


97.4
66
46
calnexin
IP: C3 C4

B

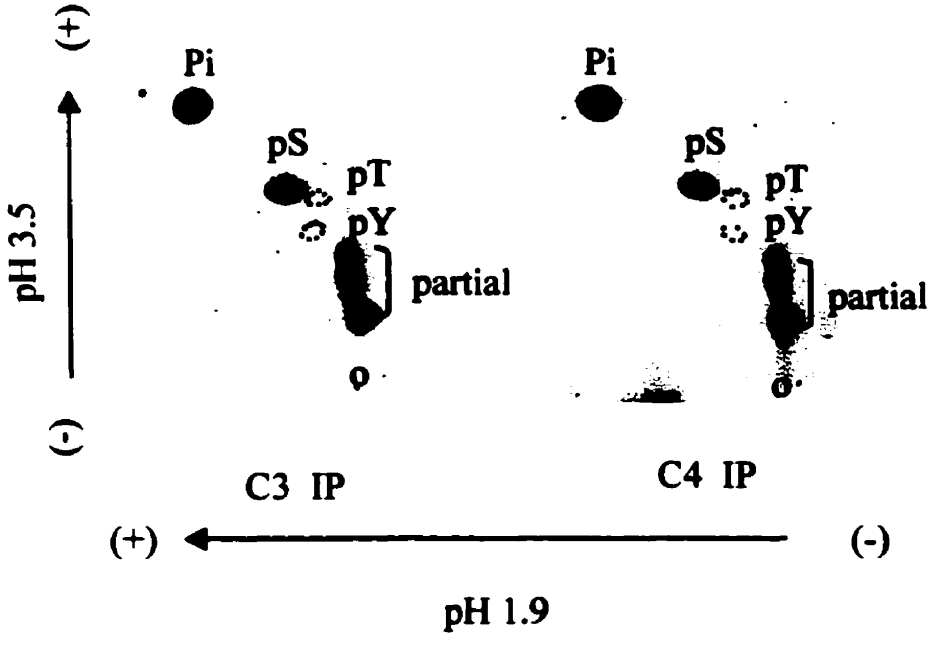

(+)
pH 3.5
(-)
Pi
pS
pT
pY
partial
Pi
pS
pT
pY
partial
C3 IP
C4 IP
(+)
(-)
pH 1.9

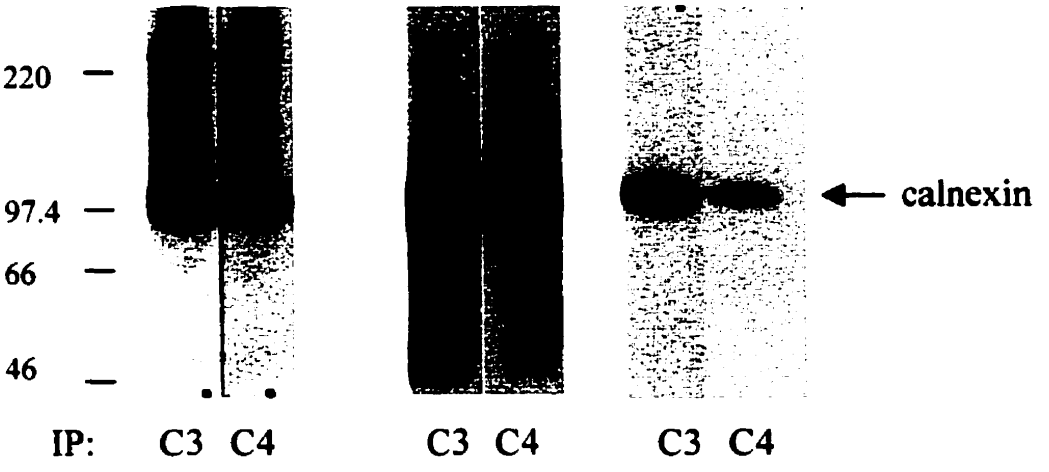
Figure 9: *In vivo* phosphorylation of canine calnexin.

A. MDCK cells were *in vivo* labeled with ^{32}P-orthophosphate at 125 μCi/ml for 6 h, lysed with 2% CHAPS lysis buffer and immunoprecipitated with either anti-C3 or anti-C4 CNX antisera. The anti-C3 (*C3*) and anti-C4 (*C4*) calnexin immunoprecipitates were resolved by SDS-PAGE and electroblotted onto 2 layers of nitrocellulose membrane for 40 min. The polyacrylamide gel after electroblotting was saved and dried (left panel). The gel (left panel), first nitrocellulose membrane that is next to the gel (middle panel) and second nitrocellulose membrane (right panel) were visualized by radioautography with intensifying screens at –70°C. The exposure times were 12 h for left panel and 21 h for middle and right panels respectively. The mobility of ^{14}C-molecular standards in kDa is indicated on the left.

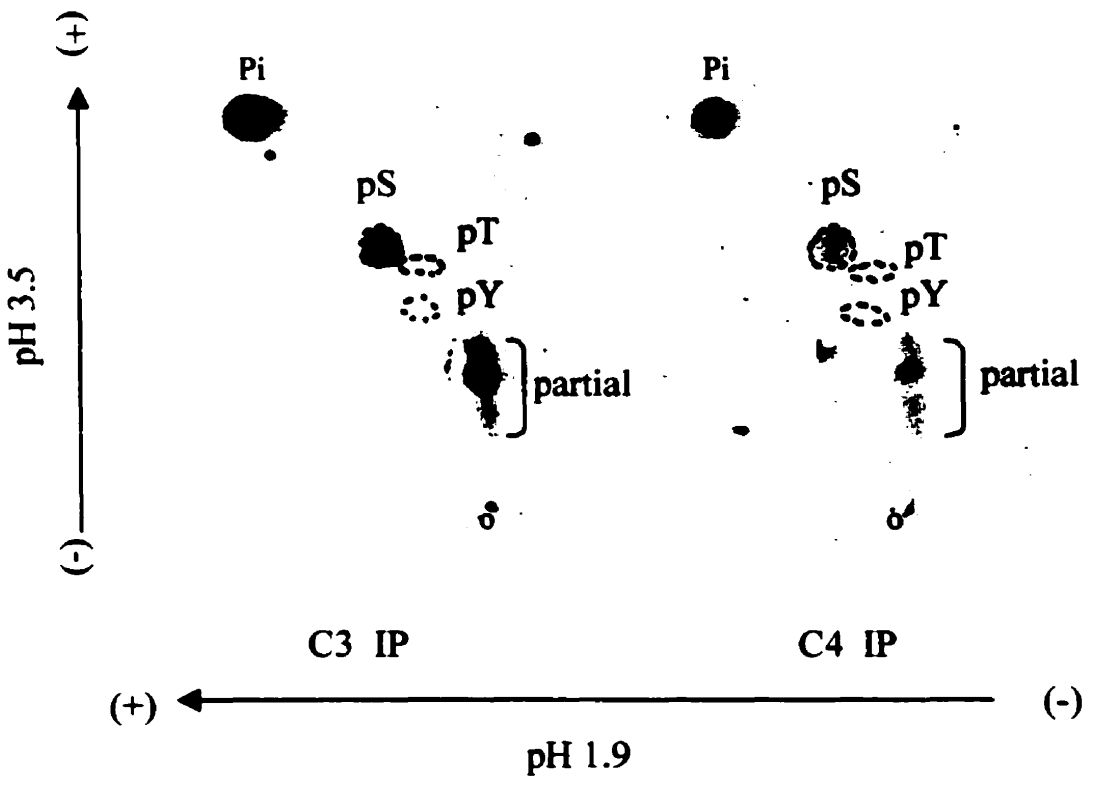
B. Phosphoamino acid analysis of ^{32}P-labeled CNX extracted from the SDS-PAGE (A, left panel) was processed as described in Materials and Methods and visualized by radioautography for 16 d at -70°C with intensifying screens. *Arrows* indicate the direction of electrophoresis. Standard phosphoamino acids; phospho-threonine (*pT*), phospho-tyrosine (*pY*), and phospho-serine (*pS*); were visualized by ninhydrin staining. *Pi*, free phosphate; *partial*, incomplete peptide hydrolysis; and *o*, origin.

C. 2-D tryptic phosphopeptide mapping of ^{32}P-labeled CNX from the first nitrocellulose membrane (A, middle panel) were done as described in Materials and Methods. *Arrows* indicate the direction of electrophoresis and ascending chromatography. Radioautogram after a 16-d exposure at –70°C are shown. Top panel, anti-C3 IP; middle panel, anti-C4 IP and bottom panel, an equal amount of anti-C3 and anti-C4 tryptic digests, determined by Cerenkov counting, were mixed and spotted on the same TLC plate. Phosphorylated species are grouped into group A (*A*) and group B (*B*). *o*, origin.

A


220
97.4
66
46
calnexin
IP: C3 C4 C3 C4 C3 C4

B


(+)
pH 3.5
(-)
Pi
pS
pT
pY
partial
Pi
pS
pT
pY
partial
C3 IP
C4 IP
(+)
(-)
pH 1.9

C

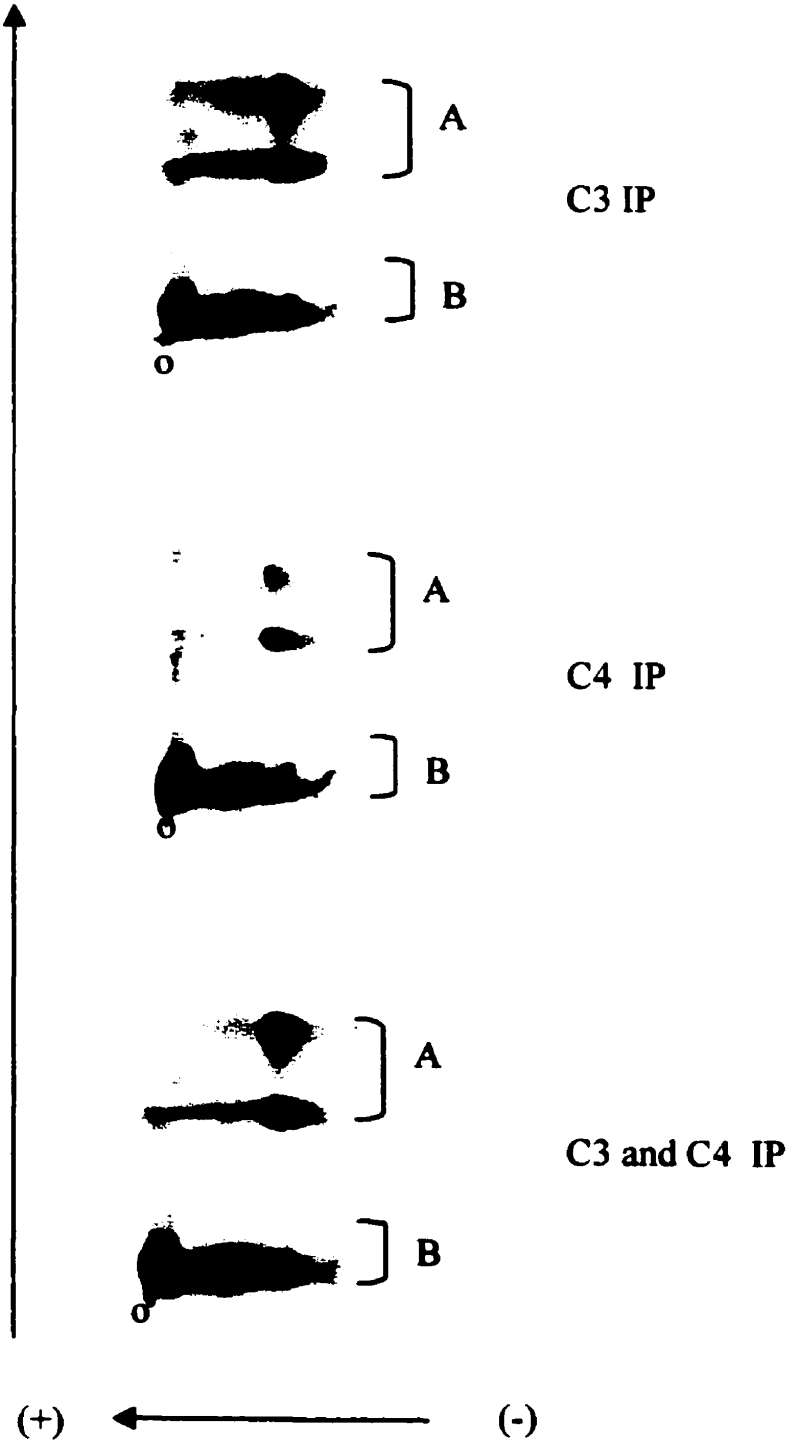


Figure 10: Comparison of calnexin associated proteins with different antisera in HepG2 cells.

HepG2 cells were metabolically pulsed with 0.2 mCi/ml of Tran^{35}S-label for 10 min with 0 and 60 min chase or pulsed for 1 h. At the end of the pulse and chase, cells were lysed with 2% CHAPS lysis buffer. Metabolically labeled lysates were pre-cleared with an aliquot of protein A-sepharose beads and only the one from 1-h chase is shown (*PC*). CNX and its associated proteins were immunoprecipitated with either anti-C3 (*3*) or anti-C4 (*4*) CNX antibodies from pre-cleared lysates. The immunoprecipitates were resolved by 8% SDS-PAGE, Coomassie blue stained, enhanced and visualized by radioautography for 20 h (panel A) and 8 h (panel B) at –70°C. Panel C, the corresponding Coomassie blue stained gel is shown. *Arrowheads* indicate CNX; IgG, immunoglobulin heavy chain and exogenously added BSA, bovine serum albumin. An a*sterisk* indicates non-specific binding protein. The mobility of ^{14}C-molecular standards in kDa is indicated on the left.

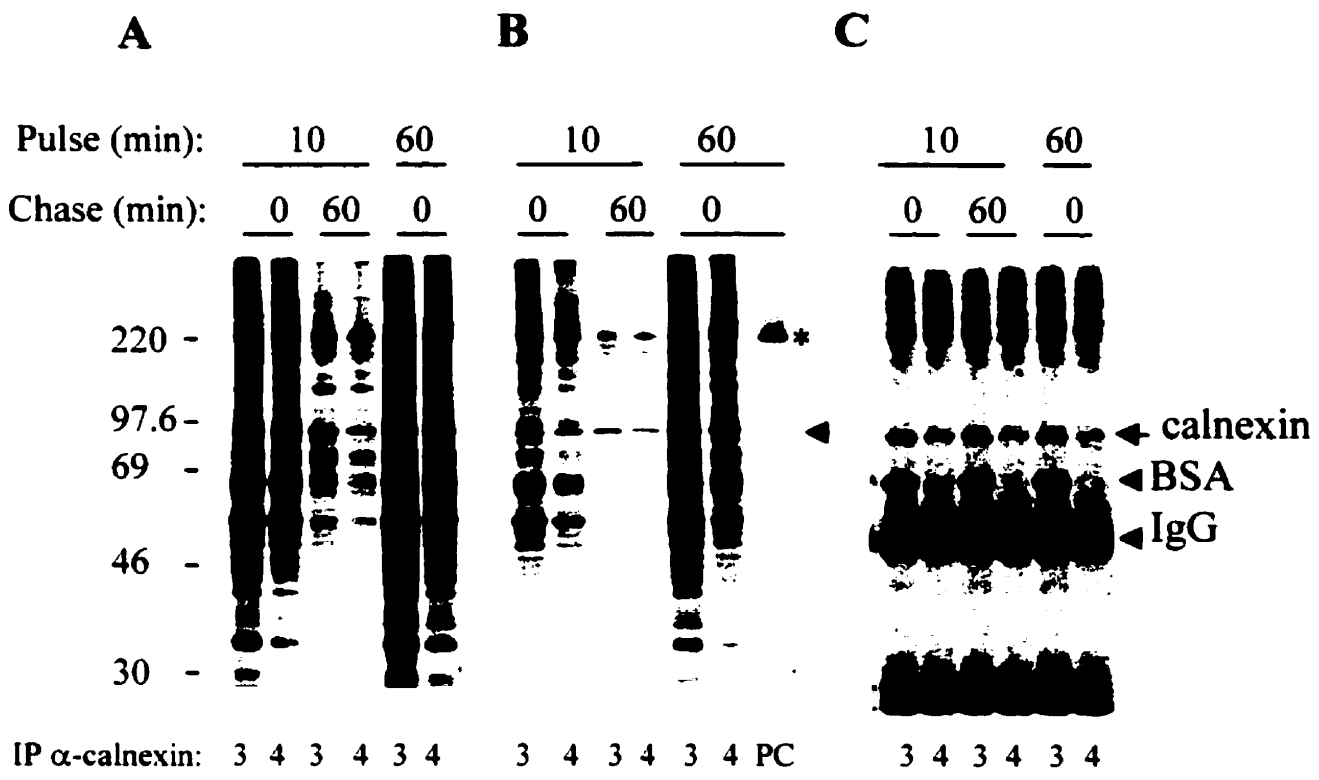

A
B
C
Pulse (min): 10 60 10 60 10 60
Chase (min): 0 60 0 0 60 0 0 60 0
220 -
97.6 -
69 -
46 -
30 -
*
calnexin
BSA
IgG
IP α-calnexin: 3 4 3 4 3 4 3 4 3 4 3 4 PC 3 4 3 4 3 4

Figure 11: Comparison of calnexin associated proteins with different antisera in MDCK cells.

MDCK cells were metabolically pulsed with 0.2 mCi/ml of Tran^{35}S-label for 10 min with 0 and 60 min chase or pulsed for 1 h as in Fig. 10. At the end of the pulse and chase, cells were lysed with 2% CHAPS lysis buffer. Metabolically labeled lysates were pre-cleared with an aliquot of protein A sepharose and only the one from 1-h chase is shown (*PC*). CNX and its associated proteins were immunoprecipitated with either anti-C3 (*3*) or anti-C4 (*4*) CNX antibodies from pre-cleared lysates. The immunoprecipitates were resolved by 8% SDS-PAGE, Coomassie blue stained, enhanced and visualized by radioautography for 20 h (panel A) and 8 h (panel B) at -70°C. Panel C, the corresponding Coomassie blue stained gel is shown. *Arrowheads* indicate CNX: IgG, immunoglobulin heavy chain and exogenously added BSA, bovine serum albumin. A d*ashed arrow* indicates the additional Coomassie and metabolic labeled protein observed in anti-C3 calnexin immunoprecipitates. An a*sterisk* indicates non-specific binding protein. The mobility of ^{14}C-molecular standards in kDa is indicated on the left.

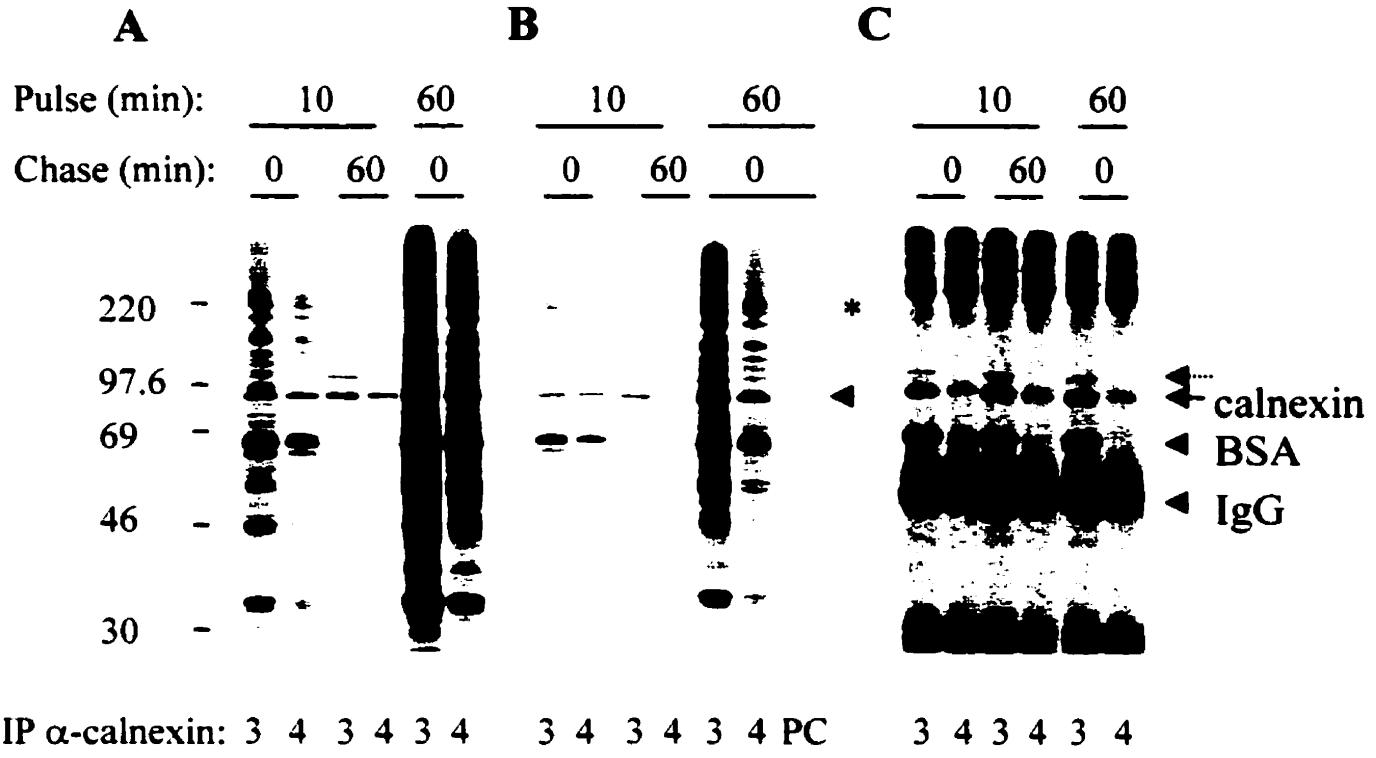
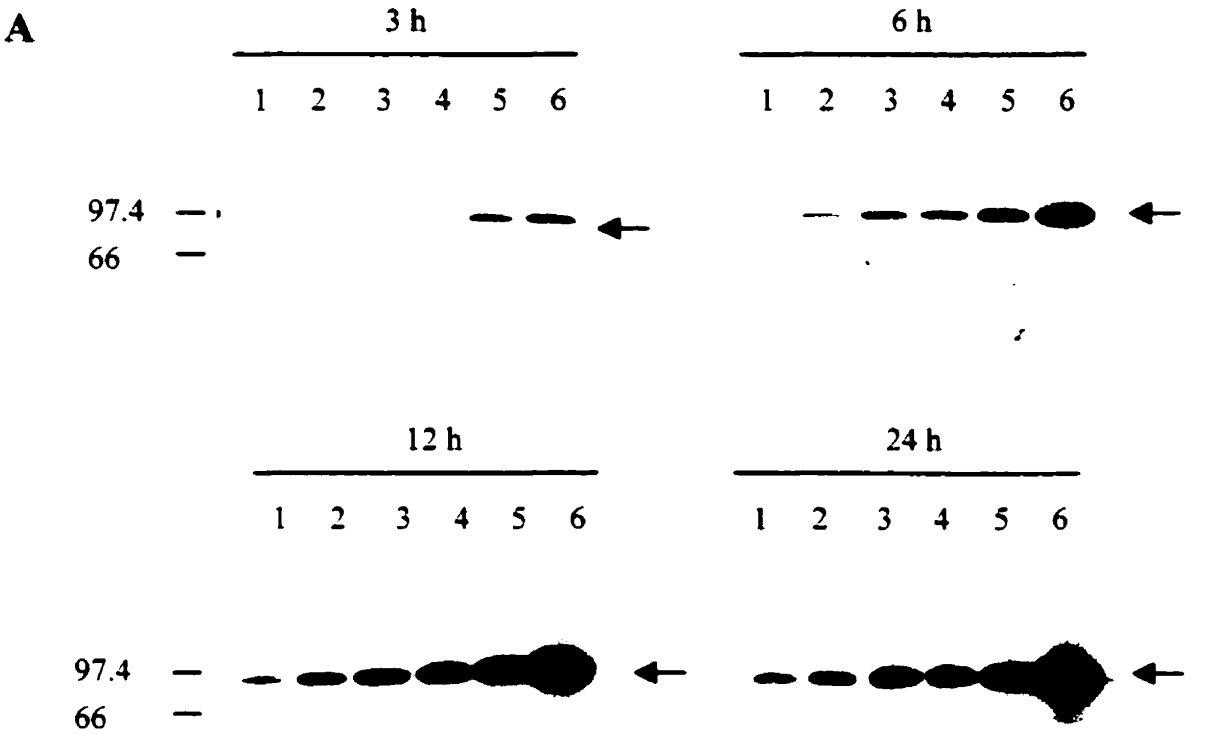

A
B
C
Pulse (min): 10 60 10 60 10 60
Chase (min): 0 60 0 0 60 0 0 60 0
220
97.6
69
46
30
*
calnexin
BSA
IgG
IP α-calnexin: 3 4 3 4 3 4 3 4 3 4 3 4 PC 3 4 3 4 3 4

Figure 12: Long term ^{32}P-orthophosphate labeling of MDCK calnexin.

A. MDCK cells were pulsed with either 0.065 (lane 1), 0.125 (lane 2), 0.25 (lane 3), 0.5 (lane 4), 1 (lane 5) or 2 (lane 6) mCi/ml of ^{32}P-orthophosphate for 3 h, 6 h, 12 h, or 24 h as indicated. Cells were lysed with 2% CHAPS lysis buffer and CNX was immunoprecipitated from 200 µg of pre-cleared lysate with 2 µl of anti-C3 antisera. The immunoprecipitates were resolved by 8% SDS-PAGE and electroblotted onto 2 layers of nitrocellulose membranes for 80 min and visualized by radioautography for 1 h at -70°C with intensifying screens. For simplicity, only the nitrocellulose membrane next to the polyacrylamide gel of each set is shown here. An *arrow* indicates immunoprecipitated CNX. The mobility of ^{14}C-molecular standards in kDa is indicated on the left.

B. Membrane-bound CNX pulsed for 3, 6, 12 and 24 h, were subjected to trypsin digestion. An aliquot of each tryptic digest was resolved by a tricine SDS-PAGE (see Materials and Methods) and visualized by radioautography with intensifying screens after a 26-d exposure at -70°C. The mobility of polypeptide standards in kDa is indicated on the left. *Arrows* indicate two main phosphorylated tryptic peptides.

C. Tryptic digests of ^{32}P-labeled calnexins that were pulsed for 3 and 24 h were analyzed by 2-D phosphopeptide mapping (see Materials and Methods) and visualized by radioautography for 3 d at –70°C with intensifying screens. *Arrows* indicate the direction of electrophoresis and ascending chromatography. *o*, origin.


A
3 h
6 h
1 2 3 4 5 6
1 2 3 4 5 6
97.4
66
12 h
24 h
1 2 3 4 5 6
1 2 3 4 5 6
97.4
66

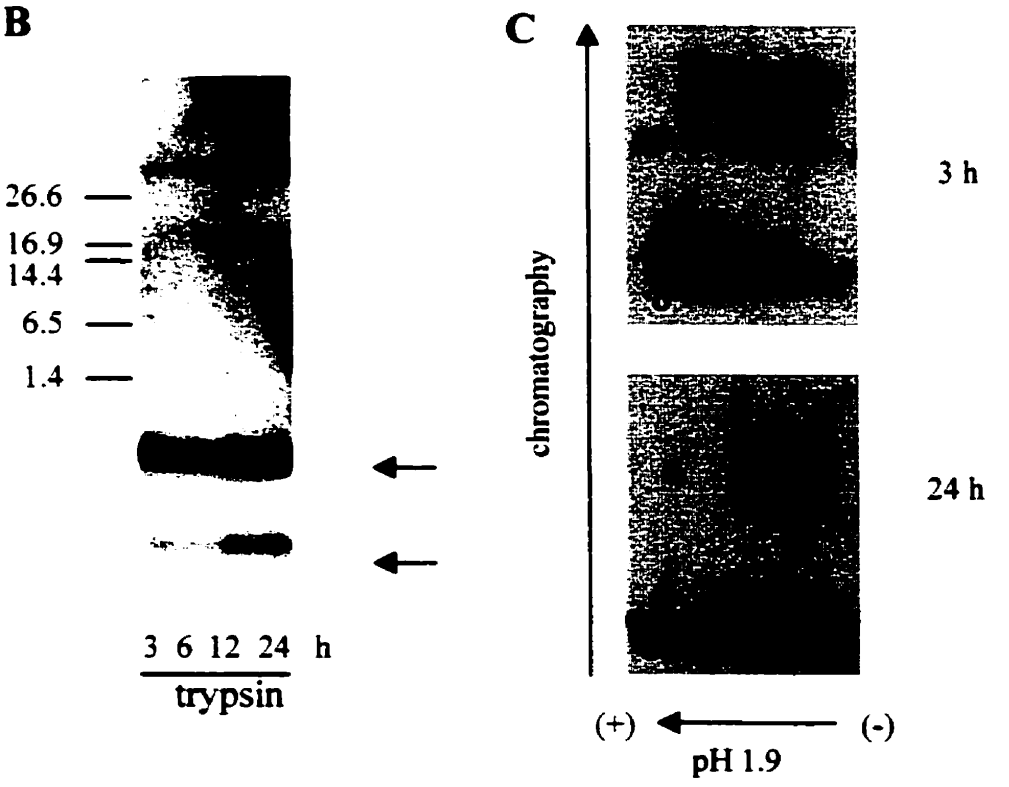

B
26.6
16.9
14.4
6.5
1.4
3 6 12 24 h
trypsin
C
chromatography
3 h
24 h
(+)
(-)
pH 1.9

Figure 13: Sequence alignment of the cytosolic domains of canine and human calnexins and summary of cytosolic tryptic fragments characterized by MS.

Alignments of the cytosolic domains of mature human, *Hu*, (amino acids 482-572) and canine, *Ca*, (amino acids 483-573) calnexin (*cnx*). *CC* (italic type) are amino acid residues predicted to be the last two residues of the transmembrane domain. Serine residues are in *boldface* type; five amino acid differences between human and canine CNX i.e. Ser^{490}, Pro^{492}, Val^{493}, Ala^{544}, and Asp^{549} are shown for canine calnexin (*Ca cnx*), and identical amino acids are indicated by *dashes*. *Arrows* indicate the predicted sites of trypsin cleavage. Trypsin will not cleave K-P or R-P bonds and may generate partial digestion products, where Arg and Lys residues are arranged in tandem (Boyle, van der Geer *et. al.*1991). Bottom, peptide fragments detected by DE-MALDI-ToF MS (Fig. 18 and Table 6) or Q1 nano-ESI MS are indicated by *thin solid lines*, and phosphorylated fragments identified by precursors m/z 79 or CNL scans (Figs. 20-22) are indicated by *thick solid lines*. *Hu* and *Ca* are the tryptic fragments detected from HepG2 and MDCK CNX, respectively.

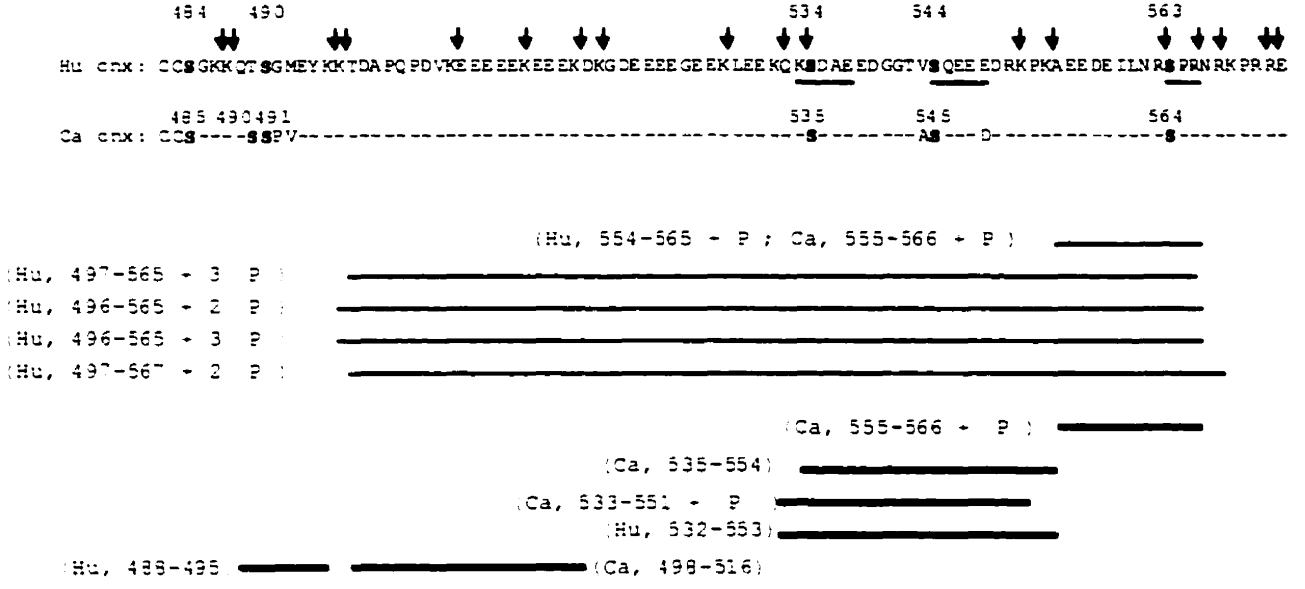

484 490 534 544 563
Hu cnx: CCSGKKQTSGMEYKKTDAPQPDVKEEEEKEEEKDKGDEEEEGEEKLEEKQKSDAEEDGGTVSQEEEDRKPKAEEDEILNRSPRNRKPRRE
485 490491 535 545 564
Ca cnx: CCS----SSPV----------S----AS---D----------S----------
(Hu, 554-565 + P ; Ca, 555-566 + P)
(Hu, 497-565 + 3 P)
(Hu, 496-565 + 2 P)
(Hu, 496-565 + 3 P)
(Hu, 497-567 + 2 P)
(Ca, 555-566 + P)
(Ca, 535-554)
(Ca, 533-551 + P)
(Hu, 532-553)
(Hu, 488-495
(Ca, 498-516)

Table 5: Potential serine phosphorylation site(s) of the cytosolic domains of canine and human calnexins

Parenthesis indicates motif residues that are not conserved between canine and human calnexins.

Canine calnexin[1]	Ser^{485}	Ser^{490}	Ser^{491}	Ser^{535}	Ser^{545}	Ser^{564}
CK2	-	-	+	+	+	-
PKC	+	-	-	-	-	+
PDK	-	-	(+)	-	-	+
PKA	-	(+)	-	-	-	-

Human calnexin	Ser^{484}	Ser^{490}	Ser^{534}	Ser^{544}	Ser^{563}
CK2	-	+	+	+	-
PKC	+	-	-	-	+
PDK	-	-	-	-	+

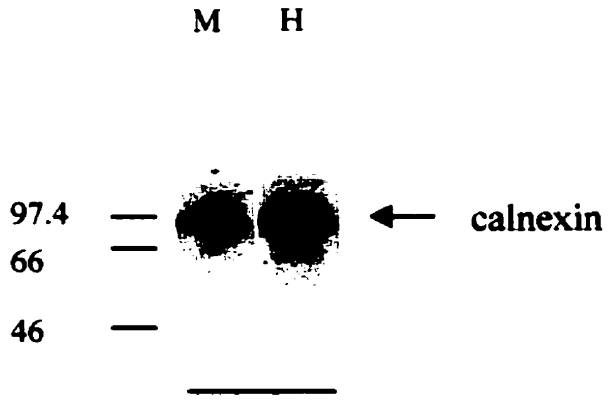
[1] CK2, predicted casein kinase II site; PKC, predicted protein kinase C site; PDK, predicted proline-directed kinase site; PKA, predicted protein kinase A site.

Figure 14: Comparison of *in vivo* ^{32}P-phosphorylated canine and human calnexins.

A. MDCK (*M*) and HepG2 (*H*) cells were pulsed for 3 h with 2 mCi/ml of ^{32}P-orthophosphate and lysed with 2% CHAPS lysis buffer. Calnexin was immunoprecipitated with anti-C3 calnexin antisera from pre-cleared lysates as in Fig. 12. SDS-PAGE resolved calnexin immunoprecipitates were transferred onto two layers of nitrocellulose membrane for 80 min as in Fig. 12. Nitrocellulose membrane next to the polyacrylamide gel was visualized by radioautography for 2 h at room temperature with intensifying screens. The mobility of ^{14}C-molecular standards in kDa is indicated on the left

B. 2-D tryptic phosphopeptide mapping of ^{32}P-labeled CNX from the nitrocellulose membrane was done as described in Fig. 9 and visualized by radioautography after a 4.6-d exposure at -70°C with intensifying screens. *MDCK*, canine CNX; *HepG2*, human CNX; and *MDCK+HepG2*, an equal amount of canine and human tryptic digests determined by Cerenkov counting was mixed and spotted on the same TLC plate. Phosphorylated species are grouped into group A (*A*) and group B (*B*) as in Fig. 6. *Arrows* indicate the direction of electrophoresis and ascending chromatography. *o*, origin.

A


M
H
97.4
66
46
calnexin
IP: α-C3

B

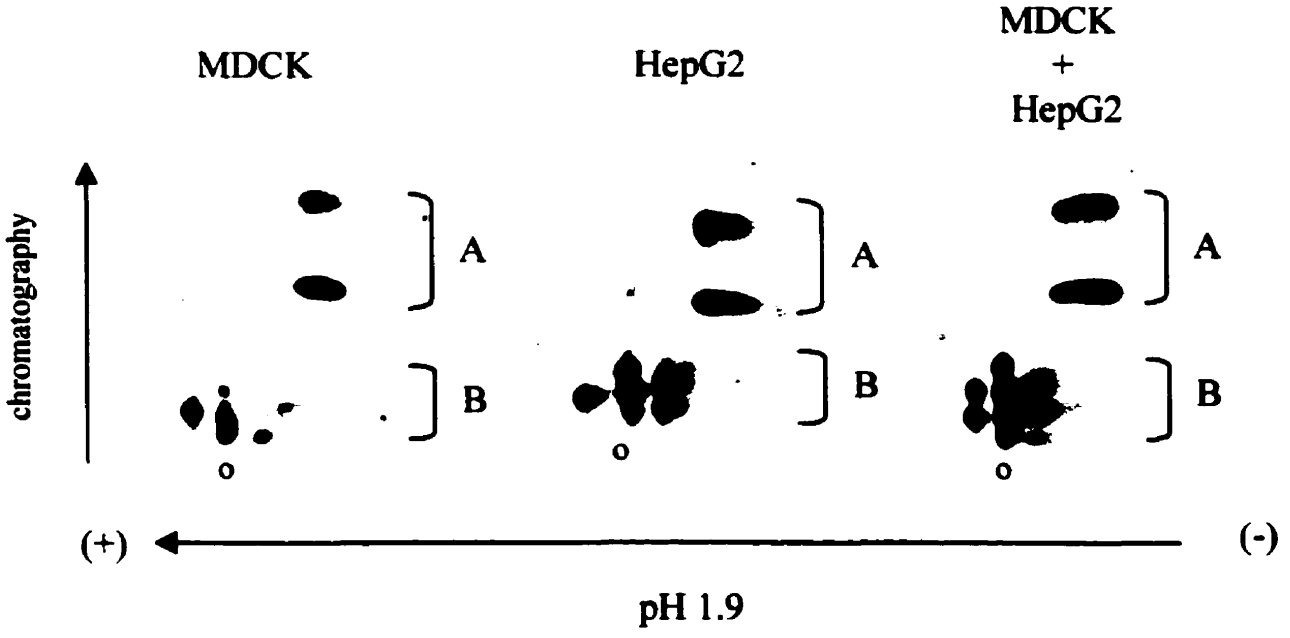

MDCK
HepG2
MDCK
+
HepG2
chromatography
A
B
o
(+)
(-)
pH 1.9

Figure 15: Alkaline phosphatase treatment of *in vivo* phosphorylated calnexins.

A. *In vivo* phosphorylated calnexins were immunoprecipitated with anti-C3 calnexin antisera from both MDCK and HepG2 cells in duplicates as in Fig. 14. Calnexin immunoprecipitates were washed extensively with alkaline phosphatase buffer and resuspended in the same buffer. Immunoprecipitates were either alkaline phosphatase or mock treated and incubated at 30°C for 30 min. An equal volume of 2X Laemmeli samples buffer was added to each immunoprecipitate at the end of the incubation. The immunoprecipitates were resolved and electroblotted as in Fig. 14. The nitrocellulose membrane next to the polyacrylamide gel was visualized by radioautography for 2 h at room temperature. The mobility of the ^{14}C-molecular standards in kDa is indicated on the left.

B. 2-D tryptic phosphopeptide mapping of the isolated HepG2 or MDCK calnexins after either mock (*-AP*) or alkaline phosphatase (*+AP*) treatment was done as described in Fig. 9. A Fuji PhosphoImager screen visualized the maps. Phosphorylated species are grouped into group A (*A*) and group B (*B*). *Arrow* indicates the directions of electrophoresis and ascending chromatography. *o*, origin.

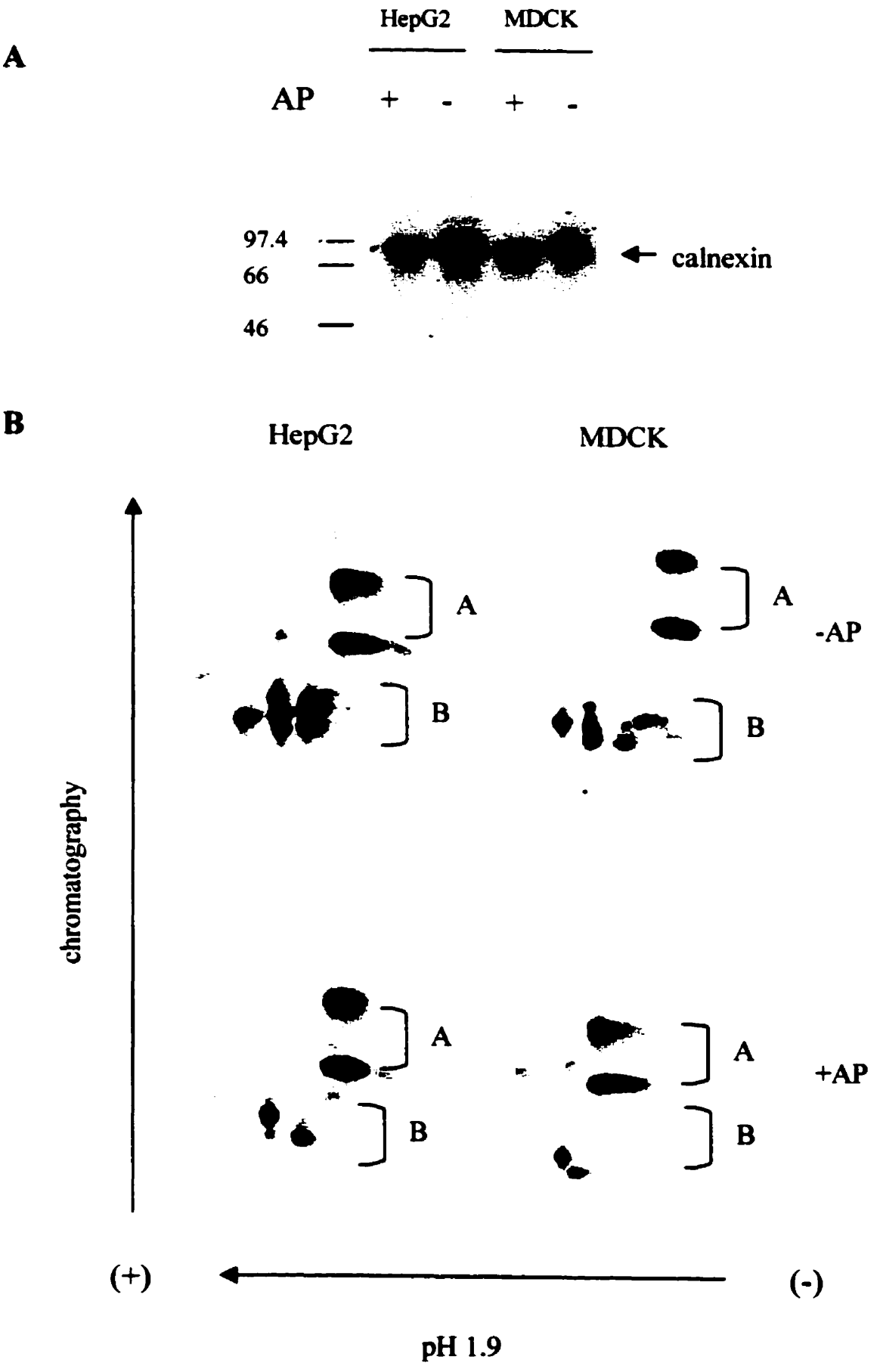
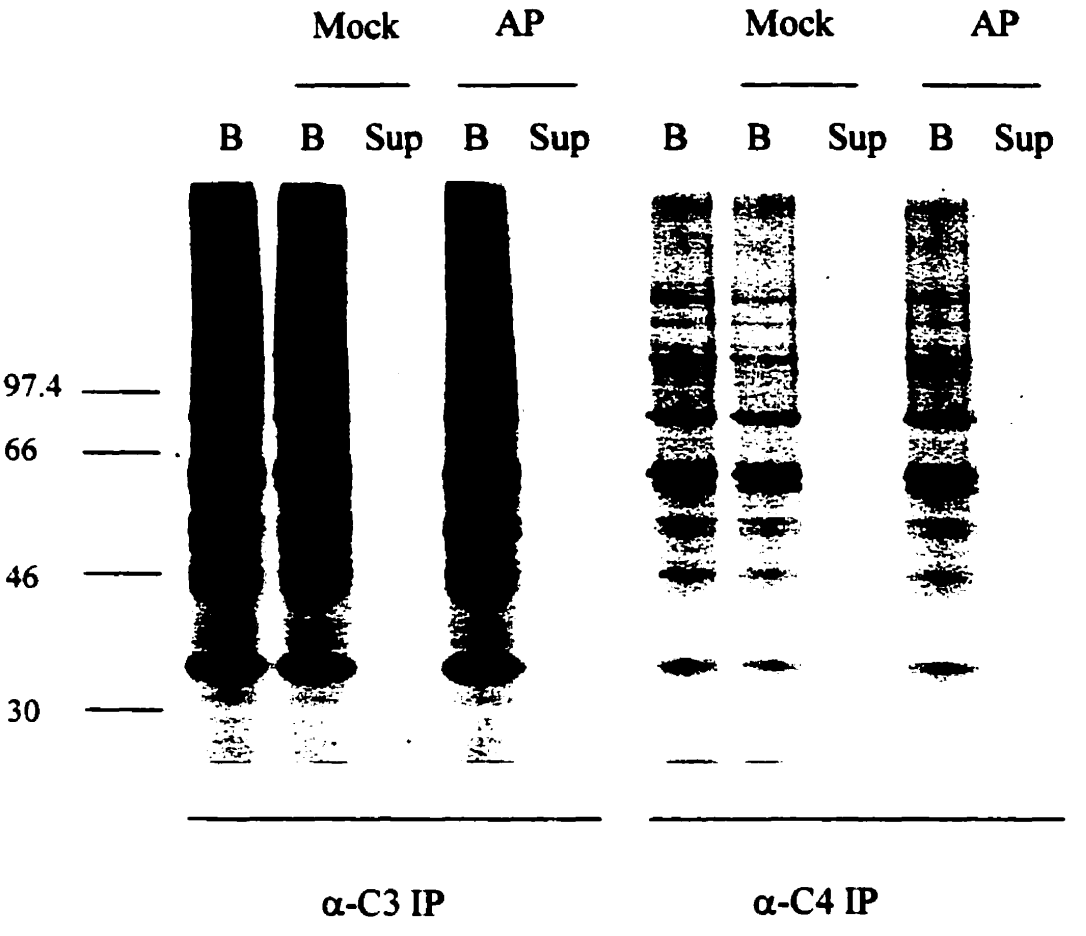

A
HepG2
MDCK
AP
+
-
+
-
97.4
66
46
calnexin
B
HepG2
MDCK
A
B
-AP
+AP
chromatography
(+)
(-)
pH 1.9

Figure 16: Evaluation of the effect of alkaline phosphatase treatment on calnexin associated proteins.

MDCK cells were metabolically pulsed with 0.2 mCi/ml of Tran^{35}S-label for 1 h and lysed with 2% CHAPS lysis buffer. Calnexin was immunoprecipitated with either anti-C3 or anti-C4 calnexin antibodies from pre-cleared lysates in triplicates. After extensive washes, one set of immunoprecipitate (IP) (lane 1) was resuspended in 1X Laemmeli samples buffer. The other two sets of calnexin IP were subjected to either alkaline phosphatase or mock treated as described in Fig. 15A. At the end of the incubation, each supernatant (*Sup*) was transferred using a Hamilton syringe to a new tube containing an equal volume of 2X Laemmeli samples buffer. The remaining beads (*B*) were washed extensively and resuspended in 1X Laemmeli samples buffer. The samples were resolved by 8% SDS-PAGE, enhanced, and visualized by radioautography at -70°C for 12.5 h. The mobility of the ^{14}C-molecular standards in kDa is indicated on the left.


Mock
AP
Mock
AP
B
B
Sup
B
Sup
B
B
Sup
B
Sup
97.4
66
46
30
α-C3 IP
α-C4 IP

Analyses of tryptic digests of calnexin by DE-MALDI-ToF MS and Nano-ESI MS.

Calnexin was immunoprecipitated from non-radiolabeled cell lysates from both cultured MDCK and HepG2 cells. Calnexin immunoprecipitates were resolved by SDS-PAGE and visualized by Coomassie blue staining (Fig. 17). Anti-C3 and anti-C4 calnexin immunoprecipitates consist mainly of three major Coomassie stained materials: immunoglobulin heavy chain (50 kDa), BSA (66 kDa) and calnexin (90 kDa) (Fig. 17). Anti-C3 calnexin immunoprecipitates obtained from MDCK lysate however contains an additional minor Coomassie blue stained material with a slower migration mobility than calnexin with an apparent molecular weight of 97 kDa. This 97kDa protein is likely to be the same protein observed in anti-C3 calnexin IP from MDCK cells in Fig. 11. The identity of this 97 kDa material was anlayzed by mass spectrometry. By its sequence tag information, this 97 kDa material is canine C-1 tetrahydrofolate synthase (Swiss Prot ID P11586 for human variety) (data not shown). Sequence comparison between the C-1 tetrahydrofolate synthase and the synthetic peptide of calnexin used to raise anti-C3 antibodies (AA 487-505 of mature canine calnexin) revealed a number of regions, constitutes of four to six amino acids, that are identical to each other (data not shown). The common regions between C-1 tetrahydrofolate synthase and calnexin may render the immunoreactivity by anti-C3 calnexin antibodies. The Coomassie blue stained material at 90 kDa, which was common to both anti-C3 and anti-C4 calnexin immunoprecipitates, was excised from the gel and subjected to in-gel trypsin digestion. The tryptic digests were characterized by mass spectral analyses.

DE-MALDI-ToF mass spectra for the tryptic peptides of calnexin from MDCK and HepG2 cells were collected to confirm the identity of the bulk of the Coomassie blue stained material at 90 kDa was indeed calnexin (Fig. 18 A and B, respectively). Peptide masses not observed in the mock in-gel trypsin digest (data not shown) were used to compare and confirm the identity of MDCK and HepG2 calnexins. The identities of the numbered peaks are tabulated (Table 6). The total coverage for MDCK calnexin was 165 out of 573 (28.8%) amino acid residues. The coverage from HepG2 calnexin was 171 out

of 572 (29.9%) amino acids. With respect to the cytosolic domain of calnexin, however, the degree of coverage was 58.4 and 33.7% from MDCK and HepG2 cells, respectively. Coverage of the cytosolic domain was increased by nano-ESI MS and was greater than 90% by combining data generated by both mass spectrometric techniques (see below and Fig. 13). Two phosphopeptides, peak 1 and peak 7, were detected by DE-MALDI-ToF (Fig. 18A) and summarized in Table 6. Poor coverage of the luminal domain of calnexin was consistent with the generation of a protease resistant core in the presence of Ca^{2+} (Ou *et. al.*1995). Ca^{2+} was present during the in-gel trypsin digestion of calnexin to prevent autodigestion of trypsin. Calnexin identity was further confirmed by tandem MS-MS sequence specific sequence tags information (Fig. 19).

The Q1 positive ion MS spectrum between *m/z* 800 and 1150 of HepG2 calnexin tryptic digests displays four major ions (P1-P4) that are tentatively assigned as tryptic fragments of calnexin by comparison with mock in gel trypsin digest (Figs. 19, A and B). By comparison of the observed masses and the cDNA predicted calculated average masses (David *et. al.*1993), the P1 (*m/z* 817) ion correspond to the doubly positvely charged states of the tryptic fragment ^{91}ESKLPGDKGLVLMSR^{105}. The calculated average mass for this tryptic fragment is 1631.0 atomic mass units (amu) which corresponds well to its observed mass of 1632 amu (817x2-2). The P2 ion (*m/z* 886.3) correspond to the doubly positively charged states of the tryptic fragment ^{42}APVPTGEVYFADSFDR^{57} (calculated average mass, 1771.9 amu) of human calnexin. The P3 (*m/z* 903) and P4 (*m/z* 910) ions correspond to the triply positively charged states of the tryptic fragments ^{151}TPELNLDQFHDKTPYTIMFGPDK^{173} (calculated average mass, 2709.1 amu) and ^{191}TGIYEEKHAKRPDADLKTYFTDK^{213} (calculated average mass, 2728.0 amu), respectively, of human calnexin. The tentative assignment of the tryptic peptide ion P2 (*m/z* 886.3) was confirmed by generation of sequence-specific sequence tags by MS/MS (tandem mass spectrometry). Fragmentation of the P2 peptide ion by collision-induced dissociation (CID) tandem MS in positive ion mode resulted in product ion spectra (Fig. 19, inset). Prominent product peptide ions with m/z values of 1120.6, 1306.4, 1407.6, and 1504.8 correspond to the y-ion series of singly positively

charged ions: y_9 (calculated *m/z* 1120.2), y_{11} (calculated *m/z* 1306.4), y_{12} (calculated *m/z* 1407.5), y_{13} (calculated *m/z* 1504.6) for CID of the calnexin tryptic peptide ^{42}APVPTGEVYFADSFDR^{57} (using ProteinProspector search tools at http://prospector.ucsf.edu). The mass differences between product ions provide sequence information for the precursor ion. For instance, the difference between y_{13} and y_{12} ions is 97.1, which corresponds to the mass of a proline residue. This identification of the P2 peptide ion is unambiguous. Search parameter wiith a mass tolerance of 0.5 Da for both parent and fragmented ions were used with search engine provided by ProteinProspector tools. The partial sequence ... (Val^{44})-Pro-Thr-(Gly-Glu)-(Val^{49}) ... (weak assignments are shown in parentheses) can be assigned from the MS/MS spectrum, taking into account potential weak signals for y_8 (calculated *m/z* 1021.1), y_{10} (calculated *m/z* 1249.3) and y_{14} (calculated *m/z* 1603.7) (Fig. 19, inset).

Identification of in vivo phosphorylation sites by Nano-ESI MS.

Only two phosphopeptides were encountered by DE-MALDI-ToF MS. Hence, we proceeded to characterize in greater detail the phosphorylated peptides from the total tryptic digests of non-radiolabeled canine and human calnexins employing two selective scans for identification of phosphorylated fragments. The two selective scans are precursors m/z 79 in negative ion mode (Carr *et. al.*1996; Wilm *et. al.*1996) and constant neutral loss (CNL) of H_3PO_4 (m/z 49 for doubly charged tryptic peptides) in positive ion mode (Covey *et. al.*1991). Precursor m/z 79 scans reveal precursor ions that fragment to produce a characteristic product ion of m/z 79 that corresponds to the phosphate anion, PO_3^-. CNL scans reveal the masses of precursor ions that lose the phosphate group, H_3PO_4 with a molecular mass of 98, as a neutral fragment. A loss of m/z 98 would be observed for singly charged precursor ions, $[M-H_3PO_4 + 1H]^{1+}$ and a loss of m/z 49 would be observed from doubly charged precursor ions, $[M-H_3PO_4 + 2H]^{2+}$.

Analyses of the tryptic digests of MDCK calnexin in positive ion by CNL m/z 49 scans detected a $[M+2H]^{2+}$ peptide ion with a m/z value of 755.2 (Fig. 20A). An equivalent doubly charged peptide ion was similarly observed for trypsinized HepG2 calnexin (Fig. 20B). This corresponds to doubly charged states of the phosphorylated

tryptic fragment of ^{555}AEEDEILNRpSPR^{566} (canine numbering with a calculated average mass of 1509.5 amu, pS represents phosphoserine). The presence of this phosphorylated tryptic peptide was confirmed in negative ion mode by Precursors m/z 79 scans. Precursors m/z 79 scans of the tryptic peptides from MDCK calnexin were dominated by three major phosphorylated peptide ions (Fig. 21A), two of the three m/z values (753.4 and 502.2) correspond to the doubly and triply charged states of the phosphorylated tryptic fragment ^{555}AEEDEILNRpSPR^{566} (canine numbering). The ion at m/z 405.0 is a commonly observed nonspecific background peak.

Mass spectral analyses of the tryptic peptides from HepG2 calnexin by Precursors of m/z 79 scan revealed a clustered series of multiply charged ions (Fig. 21B). Deconvolution of this clustered series of ions identified several large phosphorylated partial tryptic fragments that were derived from the cytosolic domain of HepG2 calnexin (see below). The phosphorylated tryptic fragment detected by CNL 49 scans was not observed strongly (m/z, 755.2, Fig. 20B) by Precursors m/z 79 scans of trypsinized HepG2 calnexin as it was observed for trypsinized MDCK calnexin (Fig. 21 compare A and B).

The clustered series of multiply charged ions recorded by Precursors m/z 79 scans of trypsinized HepG2 calnexin was subjected to deconvolution employing the Hypermass Reconstruction software (Covey *et. al.*1988). The reconstruct algorithm was initially carried out over the range of 2000-10,000 Da and then focused in on the range of interest. No discernible species could be identified at other masses. The Reconstructed profile (Fig. 22) indicated that the clustered series of multiply charged ions was derived from partial digestion of the same phosphorylated region of the cytosolic domain of HepG2 calnexin. Partial digestion of calnexin was also observed by 2-D tryptic phosphopeptide mapping analyses. We have observed a decrease in the number of phosphorylated species migrated in phosphopeptides group B when an increased amount of TPCK-treated trypsin was used (data not shown). As noted above, the inclusion of Ca^{2+} to prevent autodigestion of trypsin that may have increased the proteolytic resistance of the cytosolic domain of calnexin as this domain also binds Ca^{2+} (Tjoelker, Seyfried *et. al.*1994). The

partial tryptic fragments (Fig. 22) correspond to the region (K^{496})T^{497}DAPQPDVKEEEEEKEEEKDKGDEEEEGEEKLEEKQKSDAEEDGGTVSQ EEEDRKPKAEEDEINRSPR^{565}(NR^{567}) of HepG2 calnexin (boldface type represents potential sites of phosphorylation), as indicated by the comparison between the observed masses and calculated monoisotopic and average masses (tabulated, Fig. 22). These phosphorylated partial trypsin digested fragments contained either two or three phosphate groups and included three of the five cytosolic serine residues, S^{534} , S^{544} and S^{563}, of human calnexin. Thus, these three serine residues represent the *in vivo* phosphorylation sites of calnexin. Close inspection of the precursors m/z 79 scans for phosphorylated trypsinized MDCK calnexin revealed a similar clustered series of multiply charged ions (Figs. 21). Attempts to deconvolute this clustered series of ions were unsuccessful due to the presence of dominant ions (m/z; 405.0, 502.2 and 753.4). Differences in sensitivity to trypsinization are unclear but these data are consistent with MDCK calnexin being phosphorylated on the equivalent three serine residues, i.e. S^{534} , S^{544} and S^{563} from both the mass spectral analyses (Figs. 20-22) and 2-D-tryptic phosphopeptide maps (Fig. 14) (summarized in Figs. 13 and 23).

Figure 17: Coomassie blue stained calnexin immunoprecipitates from HepG2 and MDCK cells.

Calnexin was immunoprecipitated with either anti-C3 (*C3*) and C4 (*C4*) calnexin antibodies from non-radiolabeled cultured HepG2 and MDCK cell lysates as described in Materials and Methods. Calnexin immunoprecipitates (IP) were resolved by SDS-PAGE and visualized by Coomassie blue stains. The Coomassie blue stained band corresponding to the expected mobility of calnexin at 90 kDa was excised and in-gel trypsin digested. The tryptic digests were proceeded for mass spectrometry analyses. Arrowheads indicate exogenously added BSA and immunoglobulin heavy chain (IgG). An additional Coomassie blue stained protein is observed in anti-C3 calnexin IP from MDCK cell is indicated by a *dashed arrow*. The mobility of the broad-range molecular weight standards in kDa is indicated on the left.

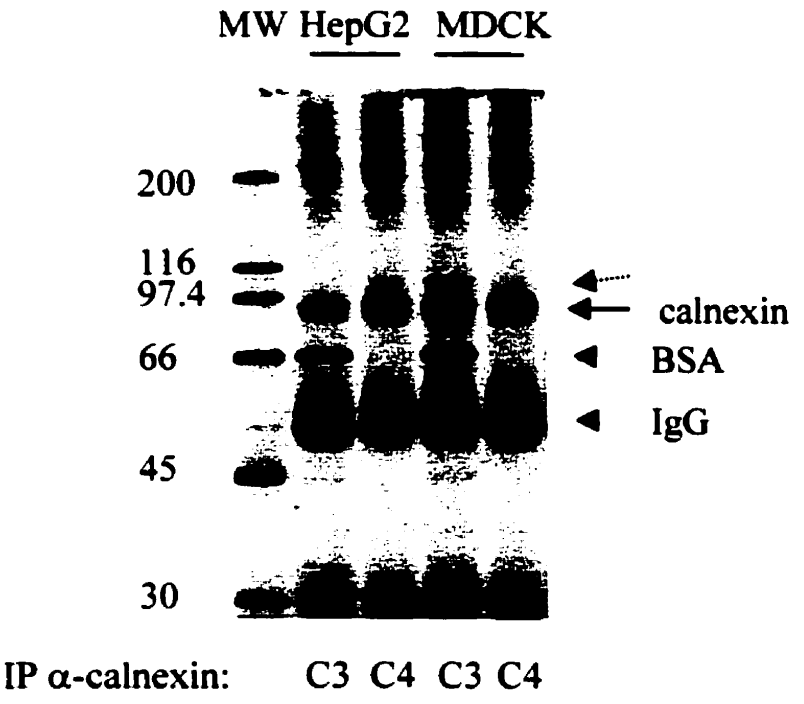
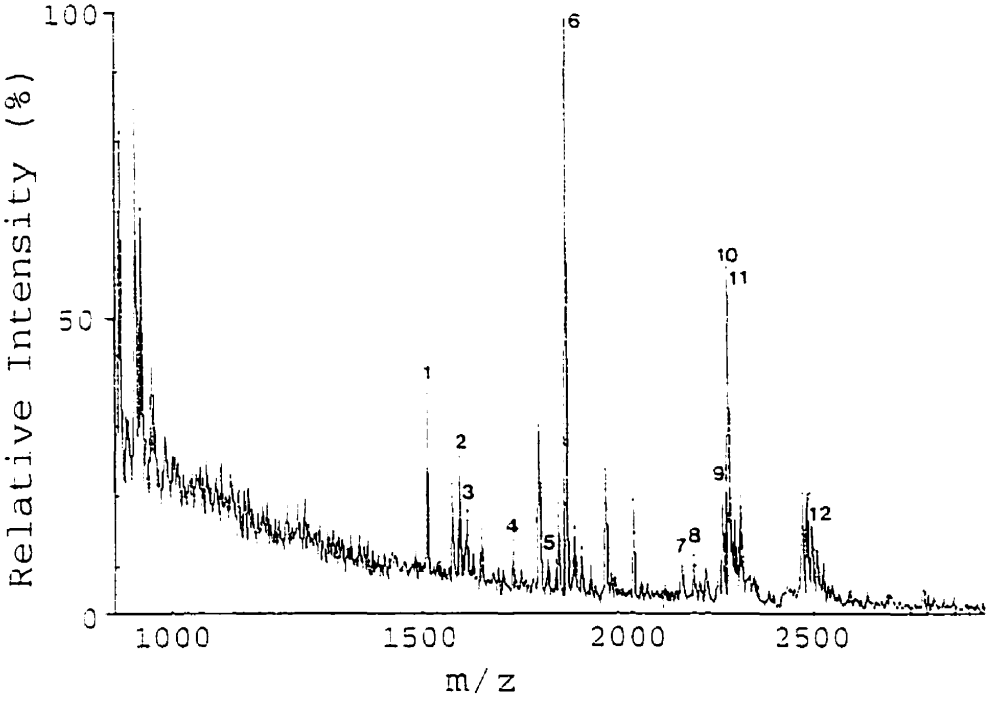

MW
HepG2
MDCK
200
116
97.4
66
45
30
calnexin
BSA
IgG
IP α-calnexin:
C3
C4
C3
C4

Figure 18: DE-MALDI-ToF mass analyses of tryptic fragments of calnexins.

Panels A and B, positive ion mode DE-MALDI-ToF mass spectra of tryptic digests of non-radiolabeled and Coomassie blue stained calnexin from MDCK and HepG2 cells, respectively. The identities of the numbered peaks are shown in Table 6. The x axis indicates the mass-to-charge (*m/z*) values and the y axis indicates the relative ion intensity.


100
50
0
Relative Intensity (%)
1000
1500
2000
2500
m/z
1
2
3
4
5
6
7
8
9
10
11
12

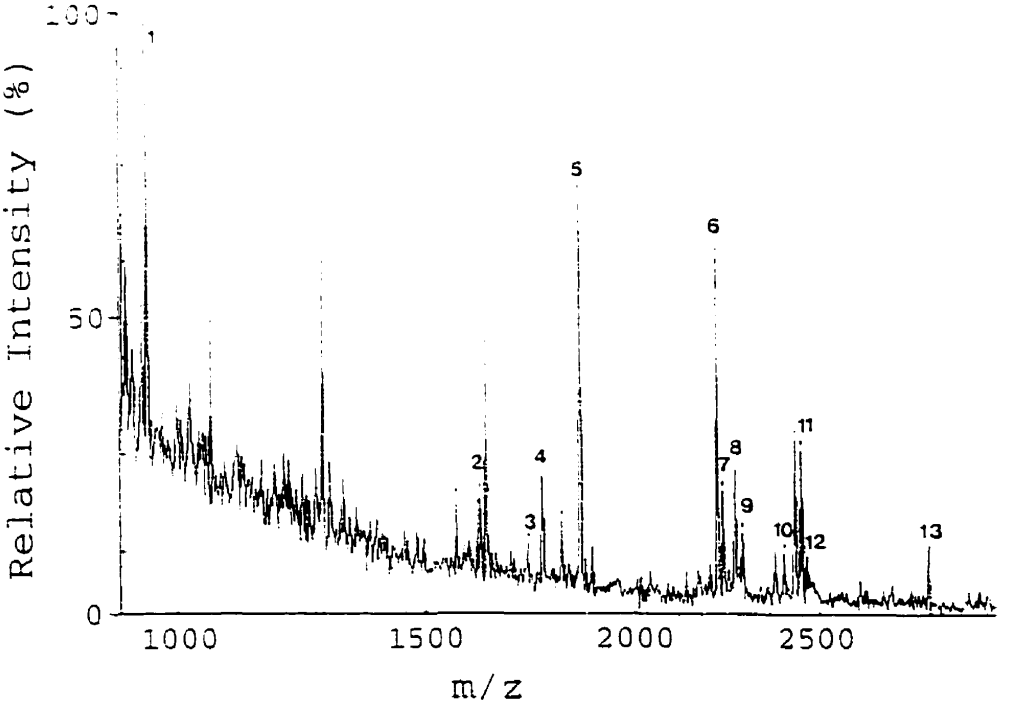

100
50
0
Relative Intensity (%)
1000
1500
2000
2500
m/z
1
2
3
4
5
6
7
8
9
10
11
12
13

Table 6: Summary of DE-MALDI-ToF mass spectral data (Fig. 18, A and B)

The observed masses and calculated monoisotopic (*Mi*) and average (*Avg*) masses for specific monoprotonated tryptic fragments ($[M+H]^+$) from MDCK and HepG2 calnexins are shown.

Peak #	Observed mass	Calculated Mi mass	Calculated Avg. mass	Residue	Modification[1]
MDCK (Fig. 18A)					
1	1508.76	1508.67	1509.5	555 - 566	1 PO_4
2	1584.85	1584.76	1585.8	175 - 186	
3	1602.86	1602.79	1603.8	427 - 440	
4	1707.75	1707.83	1708.9	383 - 396	
5	1811.76	1811.87	1812.9	69 - 84	
6	1835.90	1835.92	1837.1	382 - 396	
7	2130.90	2129.78	2130.9	533 - 551	Pyro Q,1 PO_4
8	2163.98	2163.93	2165.2	535 - 554	
9	2240.14	2240.14	2241.6	364 - 381	
10	2254.86	2255.01	2256.4	256 - 273	
11	2259.10	2259.01	2260.3	498 - 516	
12	2479.04	2479.10	2480.8	341 - 361	
HepG2 (Fig. 18B)					
1	943.42	943.42	944.1	488 - 495	
2	1616.84	1616.81	1617.8	426 - 439	
3	1735.87	1735.83	1736.9	382 - 395	
4	1770.87	1770.83	1771.9	42 - 57	
5	1863.93	1863.93	1865.1	381 - 395	
6	2213.09	2213.13	2214.6	363 - 380	
7	2229.12	2229.13	2330.6	363 - 380	Met-ox
8	2262.14	2262.11	2263.5	38 - 57	
9	2283.05	2283.05	2284.4	255 - 272	
10	2398.31	2398.25	2399.8	361 - 380	
11	2447.24	2447.12	2448.8	340 - 360	
12	2462.14	2462.13	2463.5	532 - 553	
13	2813.14	2813.42	2815.2	42 - 67	

[1] PO_4, phosphate group: Met-ox, methionine sulfoxide; pyro-Q, pyroglutamic acid.

Figure 19: Nano-ESI MS and MS/MS of tryptic fragments of HepG2 calnexin.

Panels A and B, positive ion Q1 scans (800-1150 m/z) of calnexin and mock in-gel tryptic digests, respectively. The identities of the labeled peaks are as follows: P1 (m/z 817), the +2 charged tryptic fragment of ^{91}ESKLPGDKGLVLMSR^{105} (calculated average mass, 1631.0 atomic mass units (amu)); P2 (m/z 886.3), the +2 charged tryptic fragment of ^{42}APVPTGEVYFADSFDR^{57} (calculated average mass, 1771.9 amu); P3 (m/z 903), the +3 charged tryptic fragment of ^{151}TPELNLDQFHDKTPYTIMFGPDK^{173} (calculated average mass, 2709.1 amu); and P4 (m/z 910), the +3 charged tryptic fragment of ^{191}TGIYEEKHAKRPDADLKTYFTDK^{213} (calculated average mass, 2728.0 amu) of HepG2 calnexin. Inset, a positive ion MS/MS profile of m/z 886.3 (P2), identifying the y ion series corresponding to ... (V)PT(GE)(V) ... (weak assignments identified by parentheses; see "Results"). The x axis indicates the mass-to-charge (*m/z*) values and the y axis indicates the relative ion intensity.

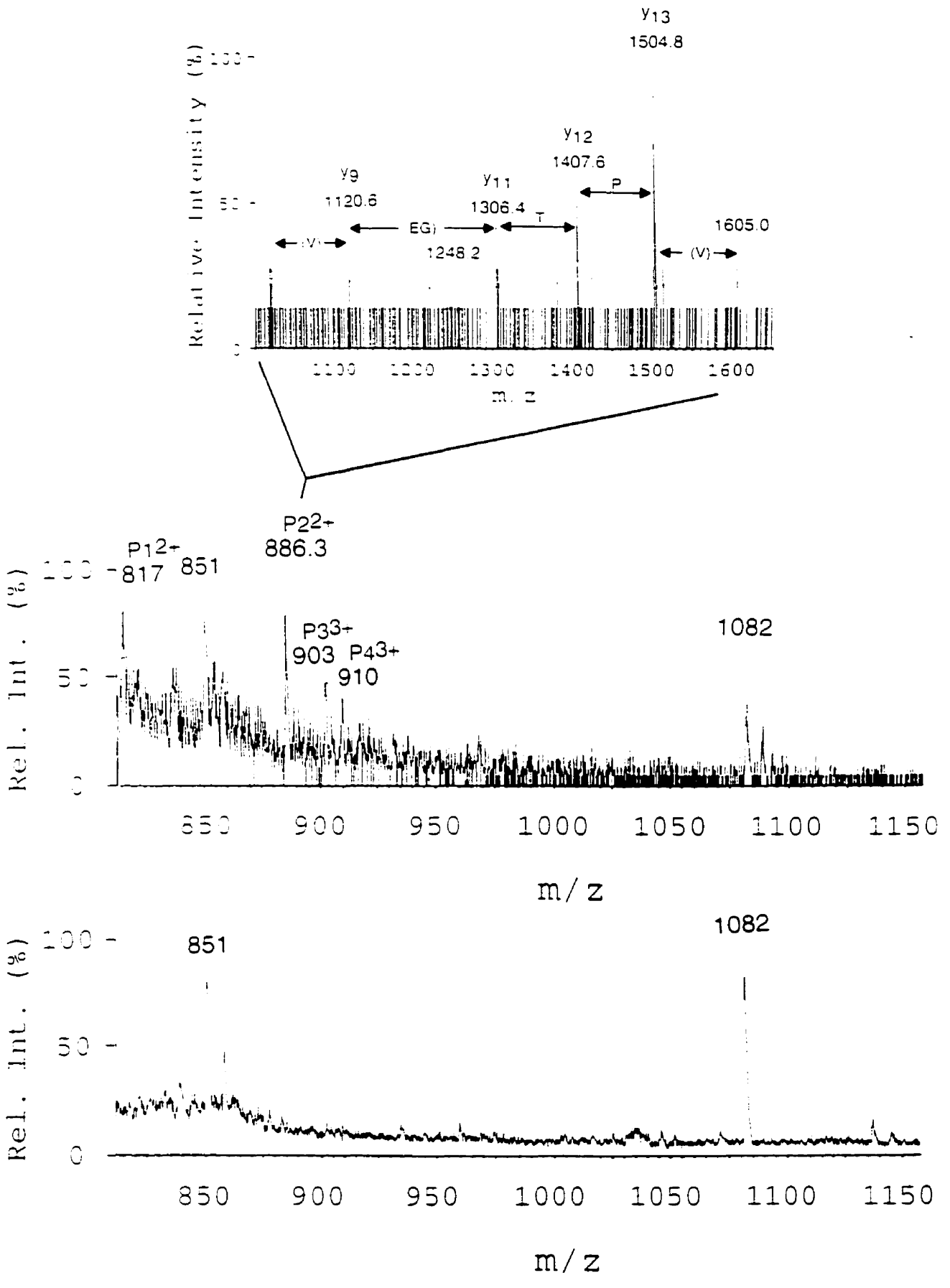

y13
1504.8
y12
1407.6
y9
1120.6
y11
1306.4
P
T
EG)
1248.2
1605.0
(V)
m/z
P22+
886.3
P12+
817
851
P33+
903
P43+
910
1082
Rel. Int. (%)
m/z
851
1082
Rel. Int. (%)
m/z

Figure 20: Constant neutral loss mass analyses of phosphorylated calnexin tryptic fragments.

Panels A and B, constant neutral loss (CNL) scans of the tryptic peptides of MDCK and HepG2 calnexin, respectively. Precursor ions that fragment to lose a neutral fragment equivalent to m/z 49 (H_3PO_4) were detected for doubly charged peptide ions. The peptide ions, $[M+2H]^{2+}$, with the m/z values of 754.6 or 755.2, correspond to the +2 charged phosphorylated tryptic peptide, AEEDEILNRpSPR (calculated average mass, 1509.5 amu), of MDCK (AA 555-566) or HepG2 (AA 554-565) CNX. The x-axis indicates the mass-to-charge (*m/z*) values and the y-axis indicates the relative ion intensity.

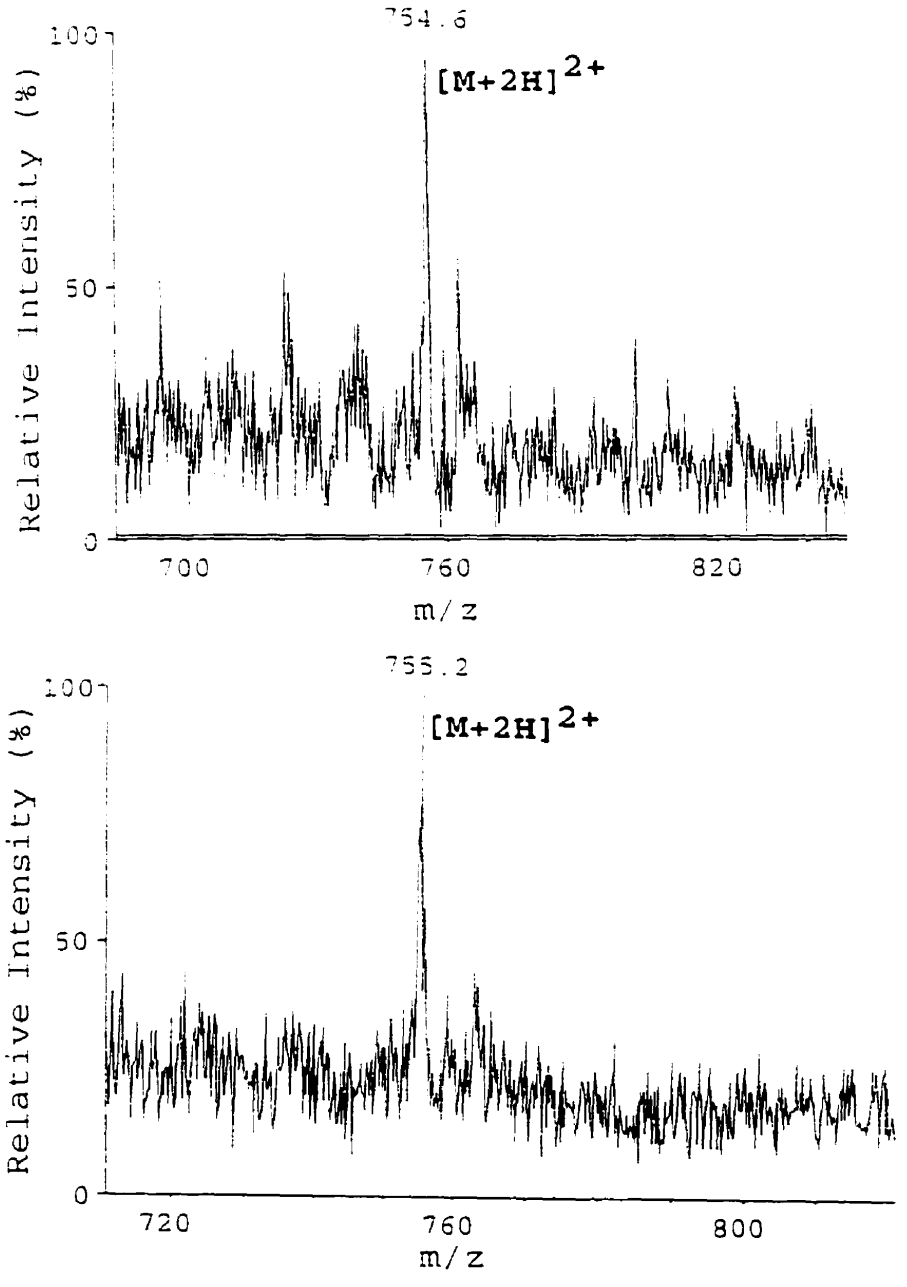
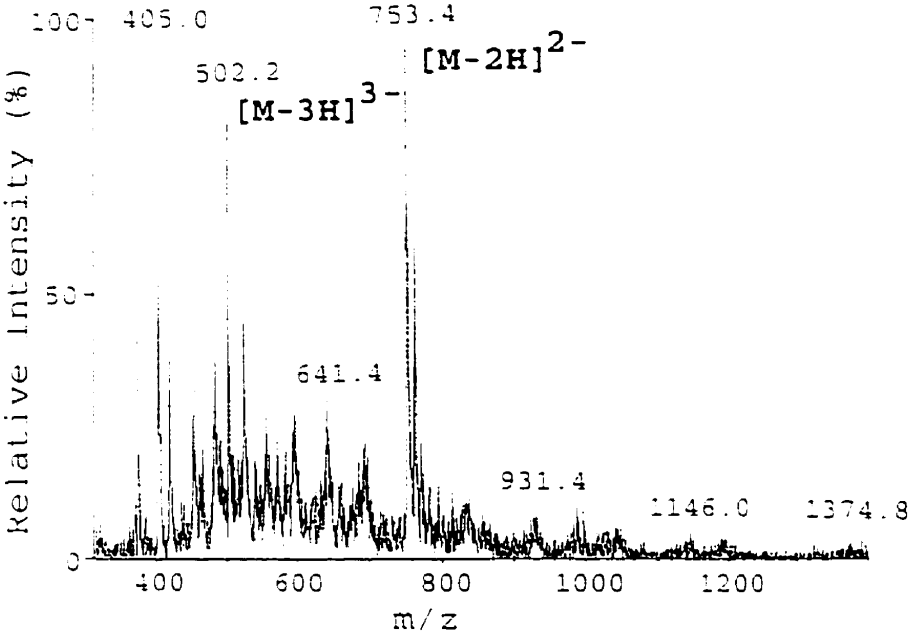

754.6
100
50
0
[M+2H]2+
Relative Intensity (%)
700
760
820
m/z
755.2
100
50
0
[M+2H]2+
Relative Intensity (%)
720
760
800
m/z

Figure 21: Precursors m/z 79 mass analyses of phosphorylated tryptic fragments of calnexin.

Panels A and B are precursors m/z 79 negative ion scans of the tryptic peptides of calnexin from MDCK and HepG2 cells, respectively. Precursor ions that fragment to produce an ion at m/z 79 (PO_3^-) are detected. The -3 ($[M-3H]^{3-}$; 502.2 amu) and -2 ($[M-2H]^{2-}$; 753.4 amu) charged states of the phosphorylated fragment ^{555}AEEDEILNRpSPR^{566} (calculated average mass, 1509.5 amu) (panel A) and the multiply charged states of the clustered ion series (panel B) are indicated. The x-axis indicates the mass-to-charge (*m/z*) values and the y-axis indicates the relative ion intensity.


100
405.0
753.4
[M-2H]2-
502.2
[M-3H]3-
50
641.4
931.4
1146.0
1374.8
0
400
600
800
1000
1200
m/z
Relative Intensity (%)

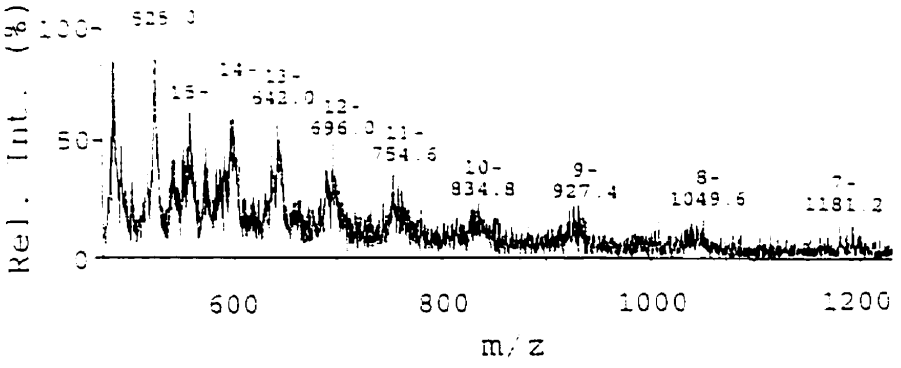

100
525.0
14-
13-
15-
642.0
12-
696.0
11-
754.6
10-
934.8
9-
927.4
8-
1049.6
7-
1181.2
50
0
600
800
1000
1200
m/z
Rel. Int. (%)

Figure 22: Reconstruct profile of Precursors m/z 79 scans of HepG2 calnexin.

Hypermass reconstruction software was employed (Covey, Bonner *et. al.*1988) to deconvolute the clustered series of multiply charged ions of HepG2 calnexin (Fig. 21B). The identities of the numbered fragments are tabulated. Na^+ and K^+ adducts are indicated. *mi*, monoisotopic; *av*, average. The x-axis indicates the mass values (atomic mass units (*amu*)) and the y-axis indicates the relative ion intensity.

	Obs. Mass	Cal. Mi Mass	Cal. Av.Mass
Peak 1: 497-565 + 3 HPO_3	8224.0	8225.6	8230.3
Peak 2: 496-565 + 2 HPO_3	8278.0	8273.7	8278.4
Peak 3: 496-565 + 3 HPO_3	8356.0	8353.7	8358.4
Peak 4: 497-567 + 2 HPO_3	8415.0	8415.8	8420.5

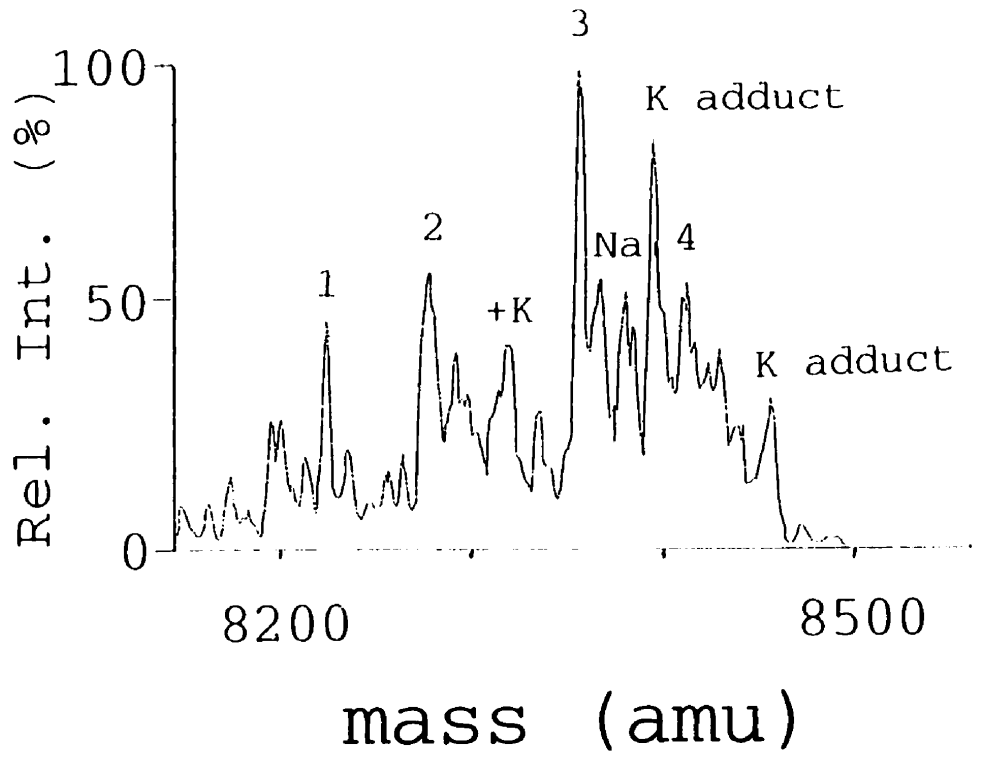
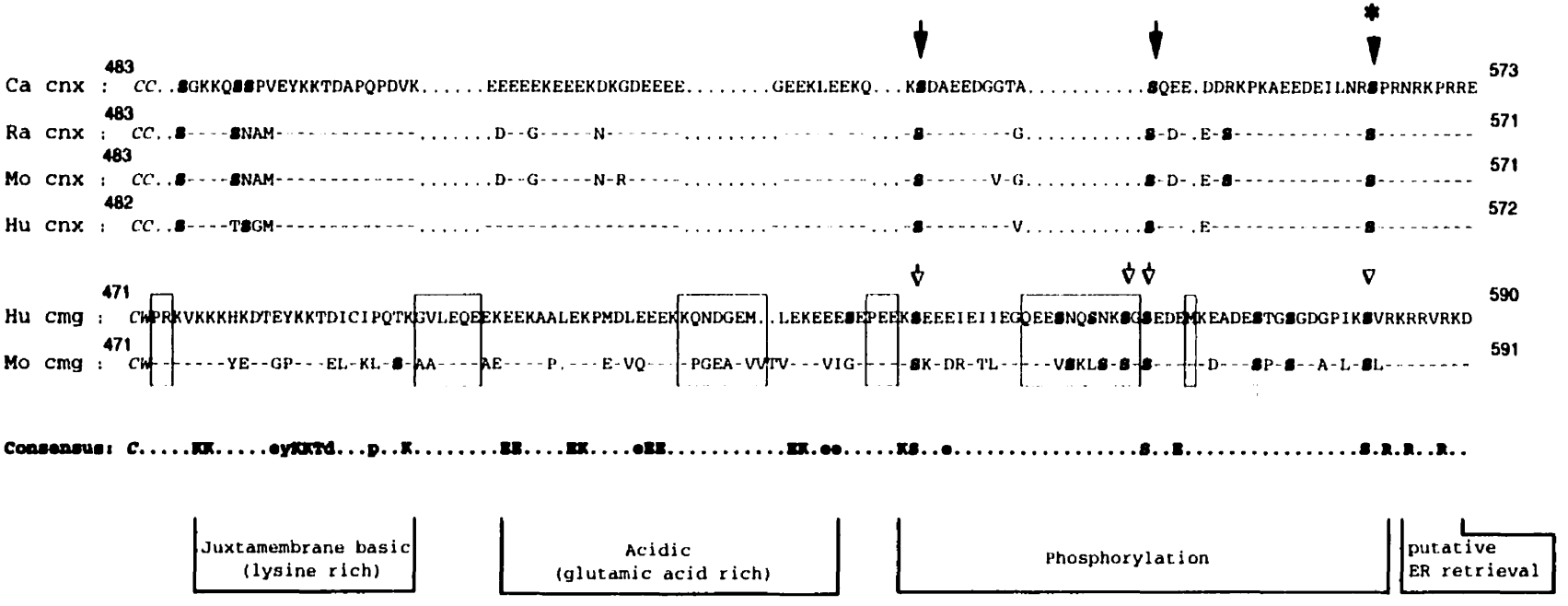


Figure 23: Sequence alignment of the cytosolic domains of known mammalian calnexins and calmegins.

Predicted cytosolic domains of canine (*Ca*) (Wada *et. al.*1991)), rat (*Ra*) (Tjoelker *et. al.*1994), mouse (*Mo*) (Tjoelker *et. al.*1994), and human (*Hu*) (David *et. al.*1993) calnexins (*cnx*) and human and mouse calmegins (*cmg*) (Watanabe *et. al.*1994) are shown. *CC* and *CW* (italic type), amino acid residues predicted to be the last two residues of the transmembrane domains of calnexin and calmegin, respectively. CNX identified (*closed symbols*) and CMG putative (*open symbols*) phosphorylation sites are shown. Potential kinases are as follows: CK2 (*arrows*), PKC (*arrowheads*), and PDK (*asterisks*). Residue numbers refer to the mature CNX or CMG. Serine residues are in *boldface* type; amino acid differences among the calnexins and among the calmegins are indicated; and identities are indicated by *dashes*. Gaps introduced to optimize alignments are shown as *dots*. Calnexin alignment identifies two deletions of one amino acid at position 507 and 524 of rodent CNX; similarly, CMG alignment identifies two deletions of one (*Mo*, 510) or two (*Hu*, 528) amino acids. CNX/CMG alignment identifies six deletions/insertions of 2, 6, 8, 3, and 11 residues and 1 residue (*enclosed boxes*, CMG). Consensus sequence is as follows: invariant (*boldface uppercase type*) and five of six identities (*bold lowercase type*). *Boldface dots* indicate differences. The four subdomains (i.e. the juxtamembrane basic, acidic, phosphorylation, and the predicted ER retrieval domains (Rajagopalan *et. al.*1994) are indicated


Ca cnx
Ra cnx
Mo cnx
Hu cnx
Hu cmg
Mo cmg
Consensus:
Juxtamembrane basic (lysine rich)
Acidic (glutamic acid rich)
Phosphorylation
putative ER retrieval

Discussion

A recently uncovered family of resident ER proteins has revealed properties of novel lectin-like molecular chaperones. These recognize *N*-linked glycoproteins and couple *N*-linked oligosaccharide modification with productive glycoprotein folding (Ou *et. al.*1993; Bergeron *et. al.*1994; Hammond *et. al.*1994; Hebert *et. al.*1995; Nauseef, *et. al.*1995; Peterson *et. al.*1995; Ware *et. al.*1995; Rodan *et. al.*1996; Helenius *et. al.*1997; Ikawa *et. al.*1997; Zapun *et. al.*1997; Zapun *et. al.*1998). The family is composed of calnexin, a type I transmembrane protein of the ER (Wada *et. al.*1991; Ou, Bergeron *et. al.*1995); calreticulin, a KDEL-terminated soluble ER-resident protein (Opas *et. al.*1991); and calmegin, a testis-specific ER transmembrane protein with sequence conservation at the predicted luminal domain (Watanabe *et. al.*1994).

Major differences among the three proteins are found at their COOH termini. Mammalian calnexins reveal 89 cytosolically oriented residues (Wada *et. al.*1991; David *et. al.*1993; Tjoelker *et. al.*1994; Ou *et. al.*1995), that were here shown to be phosphorylated at three of the four invariant serine residues. The calmegin deduced protein sequences predict 119 amino acids cytosolically oriented (Watanabe *et. al.*1994; Tanaka *et. al.*1997) with six conserved (human and mouse) potential serine phosphorylation sites (Fig. 23). The calmegin conserved potential serine phosphorylation sites are, as with the observed sites of serine phosphorylation in calnexin, also in the COOH-terminal half of the respective cytosolic domain. Five of the six potential serine phosphorylation sites of calmegin are within motifs similar to those observed for calnexin; three are in CK2 motifs, one is in a PKC motif, and another (mouse sequence only) is in a PDK motif (Fig. 23). Calnexin-t/calmegin is *in vivo* phosphorylated within its carboxy-terminus domain (Ohsako *et. al.* 1998). Thus, calmegin is predicted to be phosphorylated on equivalent serines to those in calnexin (Fig. 23). The alignment of the cytosolic domains of calnexins and calmegins identifies three major (and three minor) loops (*boxed* in Fig. 23) that are unique to the calmegins. Furthermore, this alignment reveals that the cytosolic domains of both calnexin and calmegin can be divided into four

subdomains: a juxtamembrane basic, lysine-rich subdomain; a central acidic, glutamic acid-rich subdomain; a phosphorylation signaling subdomain; and a putative COOH-terminal ER retrieval subdomain (Rajagopalan *et. al.*1994) (Fig. 23). Calreticulin is a luminal ER-resident protein that has no cytosolically oriented sequences but rather a COOH-terminal KDEL ER retrieval signal (Sonnichsen *et. al.*1994).

For yeast, greater evolutionary divergence has occurred at the cytosolic domain of calnexin as opposed to the intraluminal domain. The 48-amino acid cytosolic domain of the calnexin homolog Cnx1 in the fission yeast, *Schizosaccharomyces pombe*, is phosphorylated *in vivo* (see Chapter 5). The *S. pombe* calnexin gene is essential for viability, but the cytosolic domain is dispensable for that essential feature (Parlati *et. al.*1995). The *Saccharomyces cerevisiae* calnexin homolog Cne1p reveals only one potential cytosolic amino acid (Thr^{482}), and this calnexin gene, *CNE1*, is nonessential for *S. cerevisiae* viability (Parlati *et. al.*1995). The riddle of the evolution of a cytosolic domain for *S. pombe* calnexin coincident with essential function(s) for viability for which the cytosolic domain appears to be dispensable may be ultimately resolved by an analysis of the signaling cascades that phosphorylate calnexin in mammalian species and in *S. pombe*.

The cytosolic domains of all known mammalian calnexins display an 83% identity with four invariant serines (Fig. 23). As a consequence of this high degree of sequence identity, we set out to identify the sequences constitutively phosphorylated in two cell lines, *i.e.* human HepG2 and canine MDCK cells. By phosphoamino acid analyses, both calnexins were exclusively *in vivo* phosphorylated on serine residues. The phosphorylating residue was the same up to 24 h of radiolabeling (data not shown). By 2-D tryptic phosphopeptide mapping, both HepG2 and MDCK calnexins gave a similar tryptic peptide pattern (Fig. 14).

The first cytosolic serine residue, Ser^{484} and Ser^{485} of HepG2 and MDCK calnexins, respectively, is a juxtamembrane serine residue that is invariant among mammalian calnexins (Fig. 23) and corresponds to a potential PKC phosphorylation site. A tryptic peptide containing the transmembrane domain and thus the juxtamembrane

serine residue was not detected by MS analyses. Low recovery of this tryptic fragment may be due to poor extractability (Schaller *et. al.*1997) of such a peptide and/or due to partial proteolysis generating very large hydrophobic poorly extractable fragments probably linked to the protease-resistant Ca^{2+} luminal core of calnexin (Ou *et. al.*1995). The next COOH-terminal serine residue was conserved in HepG2 (Ser^{490}, a potential CK2 phosphorylation site) and MDCK (Ser^{491}, a potential CK2/PDK phosphorylation site) calnexins but not in rodent calnexins (Fig. 23). This site was detected only as a nonphosphorylated fragment by MS analyses of the HepG2 tryptic digests (Fig. 18 and Table 4).

As summarized in Fig. 22, only Ser^{534}, Ser^{544}, and Ser^{563} (human numbering, invariant in mammalian calnexins) were *in vivo* phosphorylated as detected by two selective nano-ESI MS techniques for detection of phosphorylated peptides. Two of these three invariant serine phosphorylation sites, Ser^{534} and Ser^{544}, are within well recognized CK2 motifs (Pinna 1990). This coincides with earlier observations that calnexin in microsomes was *in vitro* phosphorylated by CK2 (Ou *et. al.*1992; Cala *et. al.*1993) and that CK2 was purified as an ER membrane-associated kinase (Ou *et. al.*1992). The identification of a third site of calnexin phosphorylation (Ser^{563} in HepG2 calnexin; Ser^{564} in MDCK calnexin) was not predicted from previous *in vitro* studies (Wada *et. al.*1991) (Ou *et. al.*1992; Cala *et. al.*1993). This site, invariant in mammalian calnexins, is within a motif potentially recognized by either PKC or PDK (Kishimoto *et. al.*1985; Woodgett *et. al.*1986; Nigg 1995; Robinson and Cobb 1997).

We have presented evidences for diphosphorylated and triphosphorylated (Figs. 22) calnexins but no conclusive data for a monophosphorylated form, *i.e.* with only one of the two CK2 sites or only the $S^{563}PR$ site being phosphorylated, since no singly phosphorylated fragments encompassing the three sites were identified. Nonphosphorylated peptides encompassing the observed two CK2 sites of serine phosphorylation were identified by DE-MALDI-ToF MS analyses: ^{532}QKSDAEEDGGTVSQEEEDRKPK^{553} of HepG2 calnexin and ^{535}SDAEEDGGTAS QEEDDRKPK^{554} of MDCK calnexin (Figs. 13 and 18 and Table 6). The strongest

evidence for only one of the two CK2 phosphorylation sites that being phosphorylated was observed from MDCK trypsinized calnexin (Fig. 18A and Table 4). The peptide ion, *m/z* 2130.9 (Fig. 18A and Table 4) corresponds to the partial tryptic fragment of ^{533}pyro-QKSDAEEDGGTASQEEDDR^{551}, containing one phosphate group and cyclization of the NH2-terminal glutamine (calculated average mass, 2130.9 amu) (Allen 1989). The cyclization of the NH_2-terminal glutamine of this tryptic fragment was suggested also by Cala and co-workers (Cala *et. al.*1993). For both calnexins, the two phosphorylated CK2 sites (Ser^{534} and Ser^{544} of HepG2 calnexin) are contained within the same tryptic fragment, and thus by our strategies, these two phosphorylation sites could not be characterized individually. Evidence for a nonphosphorylated state of the most COOH-terminal serine residue was not obtained. This may be a consequence of complete proteolytic digestion of this nonphosphorylated form and the subsequent loss of the corresponding tryptic tripeptide, (R)S^{563}PR, during desalting/washing steps prior to MS analyses. However, the S^{563}PR site in the diphosphorylated large partial tryptic fragment (Fig. 22) that contains three potential sites of phosphorylation may correspond to a nonphosphorylated Ser^{563} site. On this basis, there are six potentially different (three mono- and three diphosphorylated) partially phosphorylated states of calnexin that probably represent a regulatory mechanism for calnexin action.

The phosphorylation results presented in this paper extend the previous finding by Capps and Zuniga (Capps and Zuniga 1994) and Le *et. al.* (Le *et. al.*1994) that a significant proportion of calnexin was phosphorylated *in vivo*. The observation of phosphorylated calnexin in association with a subset of incompletely folded MHC class I allotypes (Capps and Zuniga 1994) or of the misfolded null Hong Kong mutant of α_1-antitrypsin (Le *et. al.*1994) is suggestive of a coincident and perhaps a regulatory role with the action of the luminal domain of calnexin in glycoprotein folding and quality control. Conservation of the three serine targets of protein kinases as elucidated here predicts that this conservation and their phosphorylation are under strict control. Elucidation of the signaling cascades that trigger calnexin phosphorylation at the PKC/PDK site as well as the CK2 sites may lead to new insights in the regulation of

cargo folding and transport from the ER. These studies may also lead ultimately to a rationale for the evolution of three distinct genes in mammals that encode this family of molecular chaperones.

Chapter III

Calnexin phosphorylation in embryonic dorsal root ganglia

Introduction

Neurons are polarized cells with morphologically and functionally distinct cell body or perikaryon and neurite (axonal and dendritic) domains. By immunofluorescence detection, markers for rough ER, (TRAPα) the ERGIC (p58, KDEL receptor, and rab1) and the Golgi complex compartments (TGN38 and mannosidase II) were predominantly localized to the cell body in both embryonic dorsal root ganglia (DRG) and hippocampal neurons (Krijnse-Locker *et. al.*1995; Cook *et. al.*1996; Weclewicz *et. al.*1998). Several ER resident proteins were also found into the processes beyond the perikaryon compartment. In cultured embryonic DRG, calnexin and calreticulin were detected in the axons beside the cell bodies whereas BiP was confined to the perikarya (Weclewicz *et. al.*1998). In cultured embryonic hippocampal neurons, the expression of calnexin, calreticulin and protein disulfide isomerase (PDI) was observed in the neurite domain beside the perikarya (Krijnse-Locker *et. al.*1995; Weclewicz *et. al.*1998). The presence of BiP was detected in the dendritic spines of cultured rat purkinje cells and hippocampal pyramidal neuron (Villa *et. al.* 1992; Rubio and Wenthod 1999). It appears that the neuronal ER has a heterogeneous composition and is continuous throughout the neuron.

The presence of ER, polyribosomes, mRNA transcripts and glycosylation machinery in the neurite fraction has supported the observation of local protein synthesis (Torre and Steward 1992; Torre and Steward 1996; Koenig and Giuditta 1999; Schuman 1999). The continuous ER membrane system extended from cell body to neurite was also suggested to represent a major channel for the transport of membrane proteins from the cell body to their functional location where assembly of subunits could occur (Kiss 1977; Rubio and Wenthod 1999; Rubio and Wenthold 1999). ER resident proteins such as BiP, calnexin, calreticulin, and PDI could assist the maturation and/or assembly of subunits at their functional location. Calnexin phosphorylation at its cytosolic domain may regulate its trafficking in conjunction with calnexin-associated proteins along the continuous ER membrane system within the neuron.

A chaperone function of CNX for neurotransmitter receptor was recently suggested. In a baculovirus expression system, co-expression of calnexin increased the expression of functional serotonin transporter (SERT) by three folds. The *in vivo* interaction between calnexin and SERT remains to be explored (Tate *et. al.*1999). In cultured hippocampal neuron, glycosylated α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptor subunits were shown to interact with BiP and calnexin (Rubio and Wenthod 1999). BiP and calnexin are likely to interact with AMPA receptor subunits in the cell body where they are synthesized and both BiP and calnexin are abundantly expressed. By immunofluorescence labeling, the investigators detected the co-localization of AMPA receptor subunits, BiP, and calnexin in both the dendrites and the cell body of cultured hippocampal pyramidal neurons. The investigators suggested that BiP and calnexin may participate in the maturation and targeting of AMPA receptor subunits to appropriate synaptic location within the neuron (Rubio and Wenthod 1999).

Another interesting feature of neuronal cultures is the topological regulation of neurofilament (NF) phosphorylation. Neurofilaments (NFs) are components of the cytoskeleton structures of neurons and are composed of three subunits. The phosphorylation levels of NF subunits correlate with their assembly and are important for the maintenance of axonal calibre *in vivo*. Both ends of NF subunit are phosphorylated but subjected to different forms of regulation. Phosphate incorporation to the amino terminal of NF-L (low molecular mass NF) subunits prevents polymerization in the perikarya. As the NF subunits are being assembled in the axonal processes, the location of the phosphate incorporation is altered. They are predominantly phosphorylated at their carboxy termini, especially for NF-M and NF-H (medium and high molecular mass NF) in the axonal processes. It appears that there is a subset of protein kinases and phosphatases in the axonal compartment that is different from the one in cell body (Nixon 1993; Pant, 1995; Julien and Mushynski 1998).

Here, we have attempted to investigate the role of calnexin phosphorylation in its trafficking along the secretory pathway employing polarized cultured dorsal root ganglia

(DRG). Calnexin phosphorylation was examined in both cell body and neurite enriched fractions of cultured DRG. The level of calnexin phosphorylation is relatively the same in both fractions.

Results

Calnexin is phosphorylated in cultured embryonic dorsal roots ganglia.

In an attempt to investigate whether phosphorylation of calnexin may play a role in its trafficking along the secretory pathway, cultured embryonic dorsal root ganglia (DRG) were employed. A schematic diagram of manual separation of cultured DRG into cell body and neurite (axons and dendrites) enriched fractions is depicted in Fig. 24. Compartments of the secretory pathway were grossly separated into two fractions.

Fibroblasts such as Schwann cells are normally found in the cultured DRG. The inclusion of antimitotic agents in the cultured medium prevented the proliferation of fibroblasts. However, a population of quiescent fibroblasts that is resistant to antimitotic treatment is found in cultured DRG (Giasson and Mushynski 1997). A dish of cultured DRG treated with antimitotic agents in the absence of nerve growth factor (NGF), which prevented the neuronal proliferation, was obtained in order to compensate for the contamination by the quiescent fibroblasts (Giasson and Mushynski 1997). The NGF minus and antimitotic treated DRG is referred to as fibroblast sample here. An equal amount of each: total DRG, cell body enriched fraction, neurite enriched fraction and fibroblast sample; was loaded and the amount of calnexin in each fraction was determined by immunoblotting with anti-C4 CNX antibodies followed by secondary ^{125}I-conjugated secondary antibodies (Fig. 25A). Radioautograms revealed the presence of calnexin in both the cell body and neurite enriched fractions of cultured DRG as well as in quiescent fibroblast extracts (Fig. 25A). The level of calnexin expression in fibroblast protein, however, was much less in comparison to other fractions (Fig. 25A, Lane 5). The detection of calnexin in both cell body and neurite enriched fractions is in agreement with the earlier observation by immunogold labeling (Krijnse-Locker *et. al.*1995; Cook, *et. al.*1996).

Cultured DRG and fibroblasts were *in vivo* radiolabeled with ^{32}P-orthophosphate for 1.5 h and manually separated. Each fraction was immunoprecipitated with either anti-NF-L or anti-C4 calnexin antibodies. SDS-PAGE resolved immunoprecipitates were transferred onto a nitrocellulose membrane and visualized by radioautography (Fig. 25B).

Radioautograms revealed a comparative level of NF-L phosphorylation in both the cell body and neurite enriched fractions (Fig. 25B, Lanes 1 and 2). No phosphorylated NF-L molecule was detected in fibroblast sample prepared from cultured DRG without NGF treatment (Fig. 25B, Lane 3). This observation correlates with the lack of NF-L protein in fibroblast sample (Giasson and Mushynski 1997). Radioautograms also revealed the presence of ^{32}P-labeled calnexin in all fractions examined (Fig. 25B). A similar level of ^{32}P-labeled calnexin was observed in both the cell body and neurite enriched fractions (Fig.25B, lanes 4 and 5). Calnexin immunoprecipitated from fibroblast sample, however, displayed a greater level of ^{32}P-orthophosphate incorporation than calnexin in either the cell body or neurite enriched fractions, albeit with much less calnexin detected (Compare Figs. 25B and 25A). It appears that the majority of calnexin in cultured DRG may not be phosphorylated *in vivo*.

Figure 24: A schematic diagram of manual dissection of cultured embryonic dorsal root ganglia.

Embryonic dorsal root ganglia (DRG) were plated in the center of the culture dishes and allow radial growth of neurite extensions as described (Materials and Methods). A visible halo center containing the cell body was manually dissected with a trimmed 1-ml pipetteman tip attached to a P-1000 pipetteman. The mass containing the cell body was gently removed and transferred to a microfuge tube containing some volume of lysis buffer. The remaining neurites in the dish were swirled up with a 1-ml pipetteman tip attached to a P-1000 pipetteman and transferred in a microfuge tube containing some volume of lysis buffer.

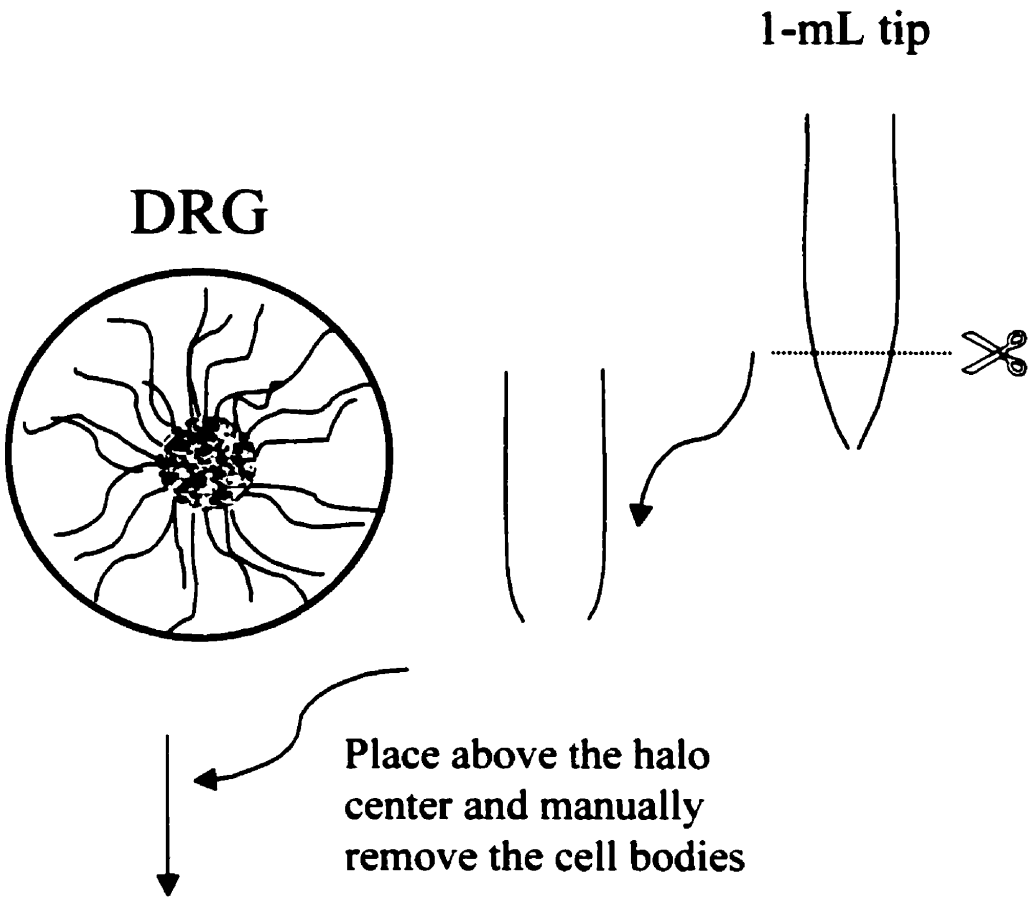
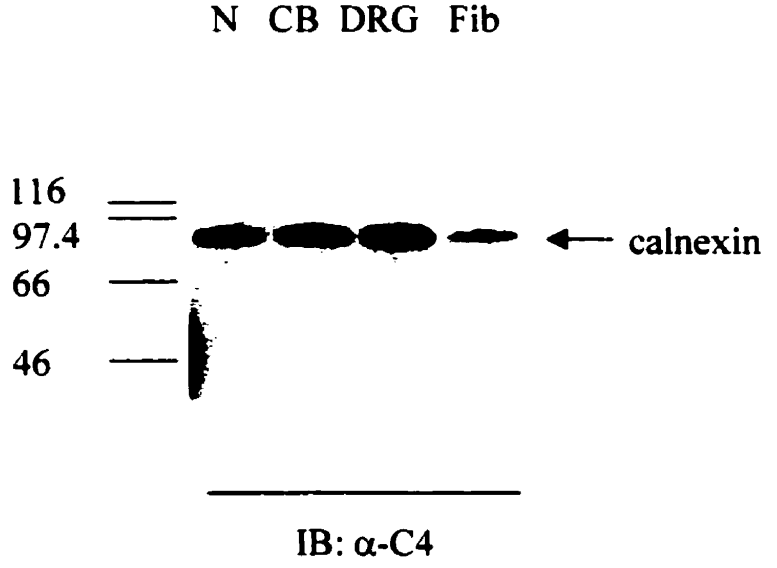

1-mL tip
DRG
Place above the halo
center and manually
remove the cell bodies

Figure 25: *In vivo* phosphorylation of calnexin from embryonic dorsal root ganglia.

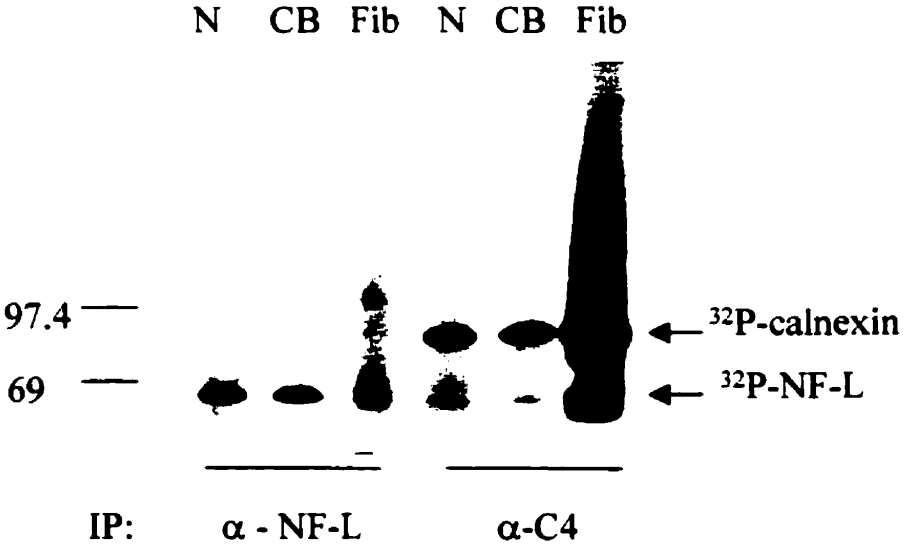
A. Western blot of calnexin in manually dissected cell body (*CB*) and neurites (*N*) enriched fractions from cultured dorsal root ganglia (*DRG*) from 16-d rat embryos as in Fig. 24, whole DRG and fibroblasts (*Fib*), prepared from DRG without NGF treatment (see Materials and Methods). Approximately 7 µg of proteins was loaded in each lane. The blot was probed with anti-C4 CNX antibodies and visualized using ^{125}I-GAR at -70°C for 11 d with intensifying screens.

B. Cultured DRG and fibroblasts (*Fib*) were *in vivo* radiolabeled for 1.5 h with 1 mCi/ml of ^{32}P-orthophosphate. Calnexin and low molecular mass neurofilament protein (NF-L) were immunoprecipitated from fibroblasts (*Fib*) and dissected neurites (*N*) and cell bodies (*CB*) enriched fractions with anti-NF-L (*α -NF-L*) and anti-C4 CNX (*α-C4*) antibodies. SDS-PAGE resolved immunoprecipitates were electroblotted onto a nitrocellulose membrane for 80 min. The blot was visualized at -70°C with intensifying screens for 36 h.

A


N CB DRG Fib
116
97.4
66
46
calnexin
IB: α-C4

B


N CB Fib N CB Fib
97.4
69
32P-calnexin
32P-NF-L
IP: α - NF-L α-C4

Discussion

Manipulation of the cytosolic domain of calnexin has shed an insight into the possibility of calnexin trafficking along the secretory pathway. Mammalian calnexin devoid of the entire cytosolic domain has been detected along the secretory pathway as well as the plasma membrane and lysosomes (Rajagopalan *et. al.*1994). The ER retention motif for calnexin was subsequently mapped to the six amino acids (-RKPRRE) at the most extreme carboxyl terminus of calnexin (Rajagopalan *et. al.*1994).

Phosphorylation of calnexin may participate in regulating its trafficking along the secretory pathway. We have attempted to address this possibility employing cultured embryonic DRG by *in vivo* radiolabeling and immunoprecipitation. From the radioautogram, calnexin appeared be *in vivo* phosphorylated throughout the cultured DRG (Fig. 25B). The level of calnexin phosphorylation in fibroblast proteins, however, was much greater than both cell body and neurite enriched fractions combined. It is not certain whether the calnexin phosphorylation observed in DRG came from calnexin present in the DRG or from the quiescent fibroblasts.

Immunohistochemical studies have revealed that the carboxyl termini of both medium and high molecular mass neurofilament (NF-M and NF-H) are highly phosphorylated in the axonal processes (Cohen *et. al.*1987). Most of the carboxyl phosphorylation sites of NF-M and NF-H are on a serine residue found within either a KSPXX or KSPXK/E sequence. Several proline-directed kinases (PDK) have been shown to phosphorylate NF-H and NF-M *in vitro*. These include cyclin dependent kinase 5, stress-activated protein kinase γ, ERK and glycogen synthase kinase 3 (Nixon 1993; Pant and Veeranna 1995; Julien and Mushynski 1998). The involved PDK in DRG for NF phosphorylation may not be involved in calnexin phosphorylation at its SPR phosphorylation site (Fig 23). This conclusion was drawn based on the low level of calnexin phosphorylation observed in cultured DRG in comparison to calnexin immunoprecipitated from the fibroblast extracts (Fig. 25B). Alternatively, the phosphatase(s) in the cell body and neurite enriched samples may dephosphorylate

calnexin. The level of calnexin phosphorylation in fibroblast protein is much higher than the one observed in either the cell body or neurite enriched fraction (Fig. 25B). The phosphatase(s) from the neuronal samples may act on phosphorylated calnexin during lysis and immunoprecipitation incubation despite the presence of phosphatase inhibitors in the immunoprecipitation buffer (see Materials and Methods). A protein phosphatase-2A-like activity has been reported to dephosphorylate KSP repeats in NF-H (Veeranna *et. al.*1995). An increase in calnexin phosphorylation was observed *in vivo* with okadaic acid, an inhibitor to protein phosphatase 1A and 2A (Schue *et. al.*1994). Dephosphorylation of calnexin by phosphatase(s) may provide an alternative explanation for the low level of ^{32}P-orthophosphate incorporation observed in neuronal calnexin.

The site or extend of calnexin phosphorylation, however, was not investigated since no distinct difference on calnexin phosphorylation was observed between the cell body and neurite enriched fractions. Mammalian calnexin is *in vivo* phosphorylated on three serine residues, two are within CK2 phosphorylation motifs and one is within a PKC/PDK phosphorylation motif (Wong *et. al.*1998) (and Chapter 2). Calnexin could potentially exist in eight different phosphorylation states (non-, mono-, di- and tri-phosphorylated species). It is still probable that calnexin is phosphorylated at different serine residues in different compartments along the secretory pathway. Hence, the possible role of calnexin phosphorylation in directing its trafficking along the secretory pathway can not be excluded completely with the study performed here.

Chapter IV

In vitro characterization and significance of calnexin phosphorylation

Introduction

Calnexin is a type I transmembrane phosphoprotein of the ER that exhibits lectin-like molecular chaperone functions. Calnexin is a constituent of the deglucosylation and re-glucosylation cycle or "calnexin cycle" of the ER quality control mechanism (Bergeron *et. al.*1994; Williams, 1995; Trombetta *et. al.* 1998). The luminal domain of calnexin (along with calreticulin and calmegin) interacts transiently with the newly synthesized N-linked glycoproteins within the ER lumen (Ou *et. al.*1993; Peterson, Ora *et. al.*1995; Ikawa *et. al.*1997). Calnexin and calreticulin specifically recognize and interact with glycoproteins that have monoglucosylated intermediates of the N-linked oligosaccharide (Hebert *et. al.*1995; Rodan *et. al.*1996; Zapun *et. al.*1997). Calnexin (or calreticulin) in complex with ERp57, a thiol oxidoreductase, mediates the retention and promotes the proper folding of disulfide bridges containing glycoproteins (Oliver *et. al.*1997; Zapun *et. al.*1998; Oliver *et. al.*1999). In the calnexin/calreticulin cycle, glycoproteins remodeling are effected by the participation of other ER luminal resident proteins, α-Glucosidase II (GII) and UDP-glucose:glycoprotein glucosyltransferase (UGGT). The removal of the innermost glucose by GII prevents the interaction between N-linked glycoprotein with calnexin (Zapun *et. al.*1997; Trombetta and Helenius 1998). UGGT is a glycoprotein folding sensor. UGGT recognizes glycoproteins in non-native conformations and only adds a glucose residue to the oligosaccharide side chain. The glucosylation activity of UGGT allows incompletely folded glycoproteins to re-bind to calnexin (Sousa and Parodi 1995; Petrescu *et. al.*1997; Wada *et. al.*1997; Cannon and Helenius 1999). Folded glycoproteins that are no longer substrates for UGGT can exit the "calnexin cycle" of retention and move further down the secretory pathway (Parodi, *et. al.*1983; Trombetta *et. al.*1989; Trombetta *et. al.* 1998; Liu *et. al.*1999).

Calnexin was originally identified as a major integral membrane protein substrate of kinase(s) associated with the ER (Wada *et. al.*1991). Calnexin was co-purified along with three other transmembrane proteins of the ER from isolated canine pancreatic microsomes (Wada *et. al.*1991). The three calnexin co-purified proteins are pgp35, also

known as SSRα and later renamed TRAPα (Prehn *et. al.*1990; Hartmann *et. al.*1993); gp25H or SSR β (also later renamed TRAPβ) (Gorlich *et. al.*1990; Hartmann *et. al.*1993) and gp25L, the founding member of the p24 family that has been implicated in cargo transport (Dominguez *et. al.* 1998). The co-purification of calnexin with TRAP α and β suggested that calnexin is in the vicinity, if not a part, of the translocon machinery. Calnexin is situated at an advantageous position to assist the maturation of nascent glycoproteins that are either being translocated or shortly after translocation. Calnexin association with incompletely translocated polypeptides (Chen *et. al.*1995; Oliver, *et. al.*1996; de Virgilio *et. al.*1998) further supported a close association of a population of calnexin with the translocon.

The biological significance of the phosphorylation of the cytosolic domain of calnexin, however, remains elusive. Phosphorylated calnexin has been shown to associate with a secretion incompetent null Hong Kong variant of α_1-antitrypsin (Le, Steiner *et. al.*1994). Phosphorylation of calnexin has also been suggested to regulate the rate of murine MHC class I heavy chains transport out of the ER (Capps and Zuniga 1994). Okadaic acid, an inhibitor for protein phosphatase 1 and 2A, has been shown to prolong the association between calnexin and its associated proteins (Tector *et. al.*1994) and also increased the level of calnexin phosphorylation *in vivo* (Schue *et. al.*1994).

The cytosolic domain of mammalian calnexin is *in vivo* phosphorylated on three invariant serine residues, two are within CK2 phosphorylation motifs and one is within a PKC/PDK phosphorylation motif (Wong *et. al.*1998) (and Chapter 2 here). Calnexin is *in vitro* phosphorylated on isolated microsomes by microsomal associated kinases when supplemented with radiolabeled adenosine and/or guanosine triphosphates (Wada *et. al.*1991; Galvin *et. al.*1992). Casein kinase II (CK2) was subsequently purified as an ER-associated kinase responsible for the *in vitro* phosphorylation of calnexin in microsomes, coinciding with the identification of calnexin's *in vivo* phosphorylation sites (Ou *et. al.*1992; Wong *et. al.*1998). The kinase responsible for the PKC/PDK serine phosphorylation site is unknown. The elucidation of the kinase responsible for the

PKC/PDK phosphorylation site may provide an insight into the possible function of calnexin phosphorylation.

Here, we have characterized calnexin phosphorylation employing isolated canine pancreatic microsomes and known kinase inhibitors. We show that the *in vivo* phosphorylation residues of calnexin are also *in vitro* phosphorylated by ER-associated kinases. Various kinase inhibitors can inhibit the ER-associated kinases responsible for calnexin phosphorylation. We have identified that ERK-1 is a candidate kinase for calnexin phosphorylation *in vitro*. Furthermore, we have shown that calnexin interacts with ribosomes in a phosphorylation dependent manner *in vitro*. An enhanced calnexin association with ribosomes is observed with the synergic action of ERK-1 and CK2 in *vitro*.

Results

In vitro phosphorylation of calnexin using isolated canine pancreatic microsomes.

Calnexin was originally identified as a major integral membrane protein substrate of kinase(s) associated with the isolated canine pancreatic microsomes using either GTP or ATP as phosphate donor (Wada *et. al.*1991; Galvin *et. al.*1992). We have shown that calnexin is *in vivo* phosphorylated on its three cytosolically oriented serine residues (Wada *et. al.*1991; Wong *et. al.*1998) (Fig. 13), two of the three serines are within well-recognized CK2 phosphorylation motifs. This coincides with earlier observations that calnexin in isolated microsomes was *in vitro* phosphorylated by CK2 (Ou *et. al.*1992; Cala *et. al.*1993) and that CK2 was purified as an ER-associated kinase (Ou *et. al.*1992). Hence, it would be of interest to determine whether the kinase(s) responsible for the third serine phosphorylation residue that is within a potential PKC/PDK phosphorylation motif or the SPR phosphorylation site of calnexin is (are) also associated with the ER.

Canine pancreatic rough microsomes were isolated and EDTA stripped as described previously (Walter and Blobel 1983; Wada *et. al.*1991). EDTA stripped rough microsomes (EsRM) were *in vitro* phosphorylated with either [γ-^{32}P] ATP or [γ-^{32}P] GTP as previously described (Wada *et. al.*1991). Electrophoresis and radioautography (Fig. 26A) analyzed an aliquot of each phosphorylation product. Similar to previously reported, numerous proteins were phosphorylated by ER-associated kinases with [γ-^{32}P] ATP and four predominantly proteins were *in vitro* phosphorylated with [γ-^{32}P] GTP (Fig. 26A). The four [γ-^{32}P] GTP-phosphorylated proteins were calnexin, pp56, pgp35 (SSRα or TRAPα) and pp15 as previously reported (Wada *et. al.*1991). Calnexin was immunoprecipitated from both reaction mixtures and a comparable level of phosphorylation was observed (Fig. 26B). Two-dimensional (2-D) tryptic phosphopeptide mapping analyses of either [γ-^{32}P] ATP- or [γ-^{32}P] GTP-phosphorylated calnexin revealed similar 2-D tryptic phosphopeptide maps. These maps were comparable to the 2-D tryptic map of *in vivo* phosphorylated canine calnexin (Fig.26C). In addition, *in vitro* ATP-phosphorylated canine calnexin from isolated microsomes co-migrated with *in vivo* phosphorylated calnexin from MDCK cells on the same TLC plate by 2-D tryptic peptide

mapping analyses (data not shown). Taken together, *in vivo* phosphorylation residues of calnexin are also phosphorylated *in vitro* by ER-associated kinases. Phosphorylated species of phosphopeptide group A were speculated to correspond to $S^{564}PR$ phosphorylation (canine numbering, Fig. 26C, also see Figs. 13 and 14). This site could be *in vitro* phosphorylated with either [γ-^{32}P] ATP or [γ-^{32}P] GTP as the phosphate donor. The kinase(s) responsible for the phosphorylation of $S^{564}PR$ site (will be referred to as "SPR" kinase from here onward) is also ER membrane-associated. Isolated microsomes appear to be an attractive model to study calnexin phosphorylation and its biological function.

Ionic requirement for the "SPR" kinase on isolated microsomes.

In the absence of either Mg^{2+} or Mn^{2+} cation, no incorporation of ^{32}P-phosphate into calnexin was detected although the presence of calnexin was readily visible by Coomassie blue stain (Lane 2 of Fig. 27 panels A and B). The kinases, CK2 and "SPR" kinase(s), responsible for calnexin phosphorylation on all three serine phosphorylation sites could utilize either Mg^{2+} or Mn^{2+} cation as revealed by their 2-D tryptic phosphopeptide mapping pattern (Fig. 27C).

The identity of the "SPR" kinase would allow us to decipher the possible regulation of calnexin phosphorylation and its function. Kinase purification from isolated intact pancreatic microsome has been extensively carried out and only CK2 was subsequently identified (Ou *et. al.* 1992). Alternatively, a different approach employing kinase inhibitors ad recombinant kinases on heat inactivated microsomes was taken to elucidate the candidate kinase responsible for calnexin phosphorylation on its $S^{564}PR$ motif.

CK2 inhibitors studies.

Various inhibitors were utilized to dissect and characterize the ER-associated kinases that are responsible for calnexin phosphorylation.

EsRM were *in vitro* phosphorylated with either [γ-^{32}P] ATP or [γ-^{32}P] GTP in the presence of various compounds that were known to modify CK2 activity and the effect on calnexin phosphorylation was examined by immunoprecipitation and radioautography. In

the presence of exogenous recombinant CK2, the level of calnexin phosphorylation was increased (Fig. 28, lane 2). In the presence of heparin, an inhibitor on CK2 activity (Hathaway *et. al.*1980), the level of calnexin phosphorylation was significantly reduced but not completely inhibited (up to 1.5 mg/mL of heparin, data not shown) (Fig. 29, Lane 3). The residual level of calnexin phosphorylation was probably due to the phosphorylation at its SPR motif. This further supports the presence of the "SPR" kinase on the microsomes. Histone H1 was included as a competitive substrate for a set of proline-directed kinases such as cyclin dependent kinases. It did not exert any effect on the level of calnexin phosphorylation (Fig. 28, Lane 5). Similar effects of these compounds on calnexin phosphorylation were observed in the presence of either [γ-^{32}P] ATP or [γ-^{32}P] GTP (Fig. 28, panels A and B, respectively).

2-D tryptic phosphopeptide mapping analyses of [γ-^{32}P] GTP-phosphorylated calnexin in the presence of CK2, heparin and histone H1 provided some identity to the phosphorylated tryptic species on the map (Fig. 29). In the presence of exogenous CK2, the increased level of calnexin phosphorylation (Fig. 28B, Lane 2) was reflected on group B phosphopeptides (Fig. 29A, CK2 panel). The phosphorylation level of group B peptides was greatly reduced, compared to group A phosphopeptides on the same map, when calnexin was *in vitro* phosphorylated in the presence of heparin (Fig. 29A, heparin panel). No effect on either group A or B phosphopeptides was observed when histone H1 was used (Fig. 29A, histone panel).

In a separate experiment, the effect of heparin was confirmed by 2-D tryptic phosphopeptide mapping analyses of [γ-^{32}P] ATP-phosphorylated calnexin. The phosphorylation level of group B phosphopeptides was greatly reduced, compared to group A phosphopeptides, when heparin was included in the reaction (Fig. 29B, heparin panel). The majority of group B phosphopeptides is most likely consisting of peptides containing the CK2 phosphorylation motifs (Ser^{534} and Ser^{544}). This was confirmed with another CK2 specific inhibitor, DRB (Zandomeni *et. al.*1986), as revealed by the corresponding 2-D tryptic phosphopeptide map (Fig. 29B, DRB panel).

PKC/PDK inhibitors studies.

Various inhibitors for PKCs and proline-directed kinases were included in the phosphorylation reaction to examine their respective effects on the level of calnexin phosphorylation using [γ-^{32}P] GTP (Fig. 30).

In the presence of 7 μM staurosporine, an inhibitor for a broad spectrum of kinases (Couldwell, Hinton *et. al.*1994; Nishimura and Simpson 1994), *in vitro* phosphorylation level of calnexin was significantly decreased (Fig. 30A, Lane 2). In the presence of 0.5 mM calphostin C, an inhibitor that competes for the binding site of diacylglycerol and phorbol esters of PKCα, β, γ, δ, and ε isoforms (Kobayashi *et. al.*1989), the level of calnexin phosphorylation was not affected (Fig. 30A, Lane 3). Similarly, no effect was observed when peptide 19-36, a pseudo-substrate peptide for PKC, was included in the reaction (Fig. 30A, Lane 4). This coincides with the earlier observation that the *in vivo* phosphorylation level of calnexin was not affected by phorbol esters (Capps and Zuniga 1994). The level of calnexin phosphorylation was reduced in the presence of 70 μM DRB, a CK2 inhibitor (Fig. 30A, Lane 5). Interestingly, calnexin phosphorylation level was affected in the presence of olomoucine, an inhibitor for proline directed kinases such as p34^{cdc2}/cylcin A kinase and p44^{MAPK}/ERK-1 (Vesely, *et. al.*1994). Calnexin phosphorylation, however, was not completely abolished when olomoucine was added simultaneously with either staurosporine or DRB in the reaction (Fig. 30A, Lanes 7 and 9).

We also analyzed an aliquot of each reaction prior to immunoprecipitation by SDS-PAGE and radioautography (Fig. 30B). Interestingly, phosphorylation of TRAPα or pgp35 was subjected to a similar level of inhibition as calnexin with the compounds tested here (Fig. 30B, indicated by arrowhead and solid arrow respectively). The cytosolic domain of TRAPα also contains one potential CK2 phosphorylation motif and one SPR tripeptide motif (Fig. 36). CK2 has been previously shown to phosphorylate TRAPα (pgp35 or SSRβ). The spatial difference between CK2 and SPR phosphorylation motifs is similar between TRAPα and calnexin (Wada *et. al.*1991)(Fig. 36). Taken together, phosphorylation of calnexin and TRAPα may be regulated by the same

mechanism. Phosphorylation of p56 is mainly affected by staurosporine and DRB (Fig. 30B, indicated by dotted arrow). This observation suggests that the regulatory mechanism for p56 phosphorylation is different from the one for calnexin and TRAPα (Fig. 30B).

2-D tryptic phosphopeptide mapping analyses were carried out to examine the effects of staurosporine and olomoucine on [γ-^{32}P] ATP-phosphorylated calnexin (Fig. 30A). Staurosporine at 50 μM was mainly affecting the group A phosphopeptides and having little effect on the group B phosphopeptides (Fig. 31, staurosporine panel). Group A phosphopeptides likely corresponded to SPR phosphorylation whereas group B phosphopeptides likely corresponded to CK2 phosphorylation as mentioned earlier (Fig. 31). This was confirmed by comparing the effect of heparin on calnexin's tryptic phosphorylated peptide mapping pattern (Fig. 31, heparin panel). On the other hand, olomoucine at 50 μM was mainly affecting the phosphorylation level of group A phosphopeptides (Fig. 31, olomoucine panel). The effect was specific since iso-olomoucine (Vesely, Havlicek *et. al.*1994), a structural isomer of olomoucine, did not exert any effect on group A phosphopeptides (Fig. 31, iso-olomoucine panel). Roscovitine (Meijer, Borgne *et. al.*1997), a potent inhibitor of cyclin-dependent kinases and MAPK, at 50 μM exerted a similar effect as staurosporine and olomoucine (Fig. 31, roscovitine panel).

ERK-1 as a candidate kinase for calnexin phosphorylation.

Immunoprecipitation and in-gel kinase assay were also performed to determine whether the kinase(s) that are responsible for calnexin phosphorylation may associate with it. Calnexin was immunoprecipitated from either proliferating or heat shocked Rat-2 fibroblasts with either anti-C3 or anti-C4 calnexin antibodies. The kinase activities associated with calnexin immunoprecipitates were assayed by in-gel kinase assay with myelin basic protein (MBP) as substrates embedded in the polyacrylamide gel. Anti-C3 calnexin immunoprecipitates displayed a weak MBP kinase activity with a migration mobility of 42 kDa whereas anti-C4 calnexin immunoprecipitates contained an additional strong kinase activity with an apparent molecular mass of 44 kDa (Fig. 32A). The co-

precipitated kinases activities were not changed when cells were heat shocked for 15 min at 51°C (Fig. 32A)

The sensitivity of SPR kinase(s) to staurosporine, olomoucine and roscovitine and a co-immunoprecipitated p44 protein kinase to calnexin has foreseen the possible involvement of $p44^{MAPK}$/ERK-1 on calnexin phosphorylation. We next evaluate whether ERK-1 is capable of phosphorylating calnexin *in vitro* using heat-treated EsRM.

Heat inactivated microsomes were *in vitro* phosphorylated with recombinant GST-ERK-1 to determine whether this kinase could phosphorylate the SPR motif on calnexin with [γ-^{32}P] ATP. A brief heat treatment has been shown to abolish the kinase activities associated with the microsomes for calnexin phosphorylation (Ou, Thomas *et. al.*1992). Calnexin was immunoprecipitated, resolved by SDS-PAGE, electroblotted onto nitrocellulose membranes and analyzed by radioautography (Fig.32B, top panel, Lane 1). The presence of calnexin was detected by immunoblotting and chemiluminescence detection (Fig. 32B, bottom panel). In heat-treated microsomes, calnexin was not phosphorylated although the presence of calnexin was vividly detected by immunoblotting (Fig. 32A and B, Lane 1). In the presence of either GST-MEK-1 or GST-ERK-1, no incorporation of ^{32}P-phosphate into calnexin was observed (Fig. 32B, top panel, Lanes 2 and 3). Calnexin was ^{32}P-labeled in the presence of GST-ERK-1 activated by GST-MEK-1 (Fig. 32B, top panel, Lane 4). MEK-1 is one of the upstream activators of ERK-1 and the incubation with MEK-1 would activate ERK-1 (Robinson and Cobb 1997). By 2-D tryptic phosphopeptide mapping analysis, ERK-1 was mainly affecting the level of ^{32}P-phosphate incorporation into group A phosphopeptides that corresponded to SPR phosphorylation (data not shown). The presence of ERK-1 on the microsomes has been reported (Chevet *et. al.*1999).

Calnexin association with ribosomes.

Calnexin was originally co-purified with three other membrane proteins from isolated microsomes and two of which were TRAPα (pgp35 or SSRα) and TRAPβ (gp 25H or SSRβ) (Gorlich *et. al.*1990; Prehn *et. al.*1990; Wada *et. al.*1991; Hartmann, *et. al.*1993). TRAPα and β have been shown to interact with ribosomes (Gorlich, Prehn *et.*

*al.*1992). In order to determine whether calnexin is similarly associated with ribosomes, a ribosome pull down assay was performed as described (Gorlich *et. al.*1992; Kalies, *et. al.*1994).

Canine pancreatic rough microsomes (RM) were solubilized with different detergents and ribosome associated proteins were purified through a 1.5 M sucrose cushion (Gorlich *et. al.*1992; Kalies *et. al.*1994). The ribosome-associated constituents were resolved by SDS-PAGE and analyzed by immunoblotting (Fig. 33A). Sec61α, the translocon subunit, previously shown to associate with ribosomes (Gorlich *et. al.*1992) was found in the ribosomal pellet with either digitonins, CHAPS, or TX100 was used (Fig. 33A). Similarly, calnexin and TRAPα were found to associate with ribosomes (Fig. 33A).

The interaction between ribosomes and calnexin was further confirmed by a protease protection assay (Fig. 33B). In the presence of trypsin, the cytosolic domain of calnexin in EDTA stripped microsomes was digested as determined by the faster mobility of trypsinized calnexin by SDS-PAGE (Fig. 33B, top panel). Anti-C1 calnexin antibodies, recognizing the amino-terminal domain of calnexin (Fig 5A), were used to detect both the full-length and trypsinized calnexin by immunoblotting. On rough microsomes, trypsin digestion of calnexin was tethered (Fig. 33B, middle panel). The protection conferred by ribosomes was confirmed by pre-incubation of purified ribosomes with EsRM membranes before trypsin digestion (Fig. 33B, bottom panel).

Phosphorylation regulates calnexin association with ribosomes.

In order to determine whether the interaction between ribosomes and calnexin could be regulated by calnexin phosphorylation, experiments with EsRM and heat-inactivated EsRM were performed. EsRM were *in vitro* phosphorylated, incubated with purified ribosomes and subjected to ribosomal pull down assay. Calnexin was then immunoprecipitated with anti-C3 antibodies from the ribosomal pellets and visualized by immunoblotting and revealed by enhanced chemiluminescence reagents. *In vitro* phosphorylated calnexin was associated with ribosomes (Fig. 34, Lane 1).

Calnexin is phosphorylated potentially by two kinases and on three serine residues (Chapter II and IV here). The contribution of each phosphorylation motif was assessed by specific phosphorylation by exogenous kinases of heat-inactivated EsRM.

The amount of ribosomal associated calnexin was greatly reduced after heat treatment of EsRM and incubation with ribosomes (Fig. 34, Lane 2). The residual amount of ribosomal associated calnexin was likely to represent the already endogenous phosphorylated population of calnexin prior heat treatment. Similarly, heat inactivated EsRM were *in vitro* phosphorylated in the presence of recombinant CK2, activated ERK-1 or both. The association of calnexin with ribosomes was evaluated in each condition. Virtually no calnexin was associated with ribosomes when calnexin was phosphorylated by CK2 (Fig. 34, compare Lane 3 to Lane 2). It appears that CK2 phosphorylated calnexin may exert a negative regulatory effect on ribosomal association. When calnexin was *in vitro* phosphorylated by activated ERK-1 (immunoprecipitated from EGF treated rat liver cytosol) (Di Guglielmo *et. al.*1994) the amount of calnexin associated with ribosomes was increased significantly (Fig. 34, compare Lane 4 to Lane 2). This was confirmed with *in vitro* phosphorylation of heat-inactivated EsRM with activated ERK-1 in the presence of synthetic peptides that corresponds to the AA 555-573 of mature canine calnexin, which contains the SPR phosphorylation motif. In the presence of the synthetic peptides and activated ERK-1, the level of association between calnexin and ribosomes was returned to the basal level observed (Fig. 34, compare Lane 5 to Lane 2).

Figure 26: *In vitro* phosphorylation of calnexin from isolated canine pancreatic microsomes.

A. *In vitro* phosphorylation of EsRM with either [γ-^{32}P] ATP (*A*) or [γ -^{32}P] GTP (*G*) by endogenous microsomal associated kinases on ice for 30 min was performed as described (Wada *et. al.* 19991). An aliquot of each reaction was resolved by 12% SDS-PAGE and visualized by radioautography for 12 h at -70°C with intensifying screens. *Asterisks* indicate the three other major proteins (pp15, pgp35, pp56) as well as calnexin (*arrow*) that were phosphorylated with [γ-^{32}P] GTP reported previously (Wada *et. al.* 19991).

B. Calnexin was immunoprecipitated with anti-C3 calnexin antibodies and resolved by 6% SDS-PAGE. Calnexin immunoprecipitates were visualized by radioautography for 12 h at -70°C with intensifying screens. The mobility of ^{14}C-molecular standards in kDa is indicated on the left.

C. Calnexin immunoprecipitates from panel B were subjected to in-gel trypsin digestion and analyzed by 2-D phosphopeptide mapping as in Fig. 6 followed by detection with a Fuji PhosphoImager screen. Phosphorylated species are grouped into group A (*A*) and B (*B*). 2-D phosphopeptide mapping of *in vivo* phosphorylated canine (*MDCK*) calnexin prepared as in Fig. 9 is included for comparison. Phospho-chromatography buffer was used in the ascending chromatography in the second dimension. The migration front was stopped approximately 1 cm before the edge of the TLC plate for *in vivo* phosphorylated canine calnexin (MDCK panel) whereas the migration front for *in vitro* phosphorylated calnexin had ran off (ATP and GTP panels). *Arrows* indicate the direction of electrophoresis towards anodein pH 1.9 buffer and ascending chromatography. *o*, origin.

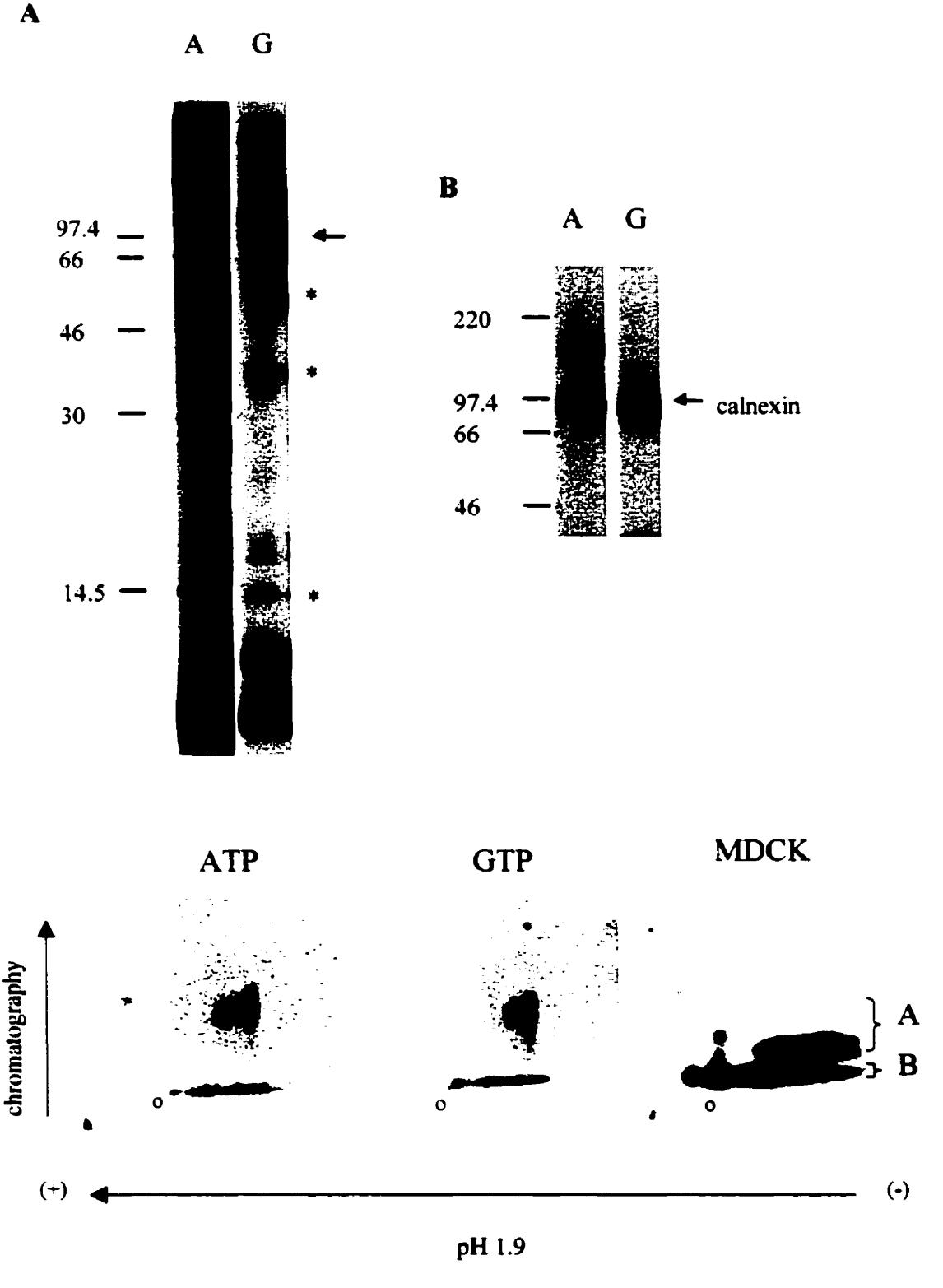

A
A G
97.4
66
46
30
14.5
B
A G
220
97.4
66
46
calnexin
C
ATP
GTP
MDCK
chromatography
A
B
(+)
(-)
pH 1.9

Figure 27: Evaluation of cation requirement for the microsomal associated kinases.

Panels A and B. EsRM, were *in vitro* phosphorylated with [γ-^{32}P] ATP on ice for 30 min either in the presence (Lanes 1, 3- 6, 8-11) or the absence (Lane 2) of $MnCl_2$ and/or $MgCl_2$ with the indicated concentration. For lane 1, 20 mM $MgCl_2$ and 2.5 mM $MnCl_2$ were present during the reaction. Calnexin was immunoprecipitated with anti-C3 calnexin antibodies, resolved by 8% SDS-PAGE, and Coomassie blue stained. Radioautogram after a 10-h exposure at -70°C and the corresponding Coomassie blue stained gel are shown as panels A and B, respectively. Lane 7 contains an aliquot of the broad-range molecular weight standards from BioRad. *Arrows* indicate calnexin and heavy chain (IgG). The mobility of the molecular standards in kDa is indicated on the left.
Panel C. Phosphorylated calnexin from lanes 1 (Mg^{2+}+Mn^{2+}), 6 (Mg^{2+}), and 11 (Mn^{2+}) were in-gel trypsin digested and analyzed by 2-D tryptic phosphopeptide mapping (see Materials and Methods) followed by detection using a Fuji PhosphoImager screen. *Arrows* indicate the direction of electrophoresis towards anode in pH 1.9 buffer and ascending chromatography in isobutyric chromatography buffer. *o*, origin.

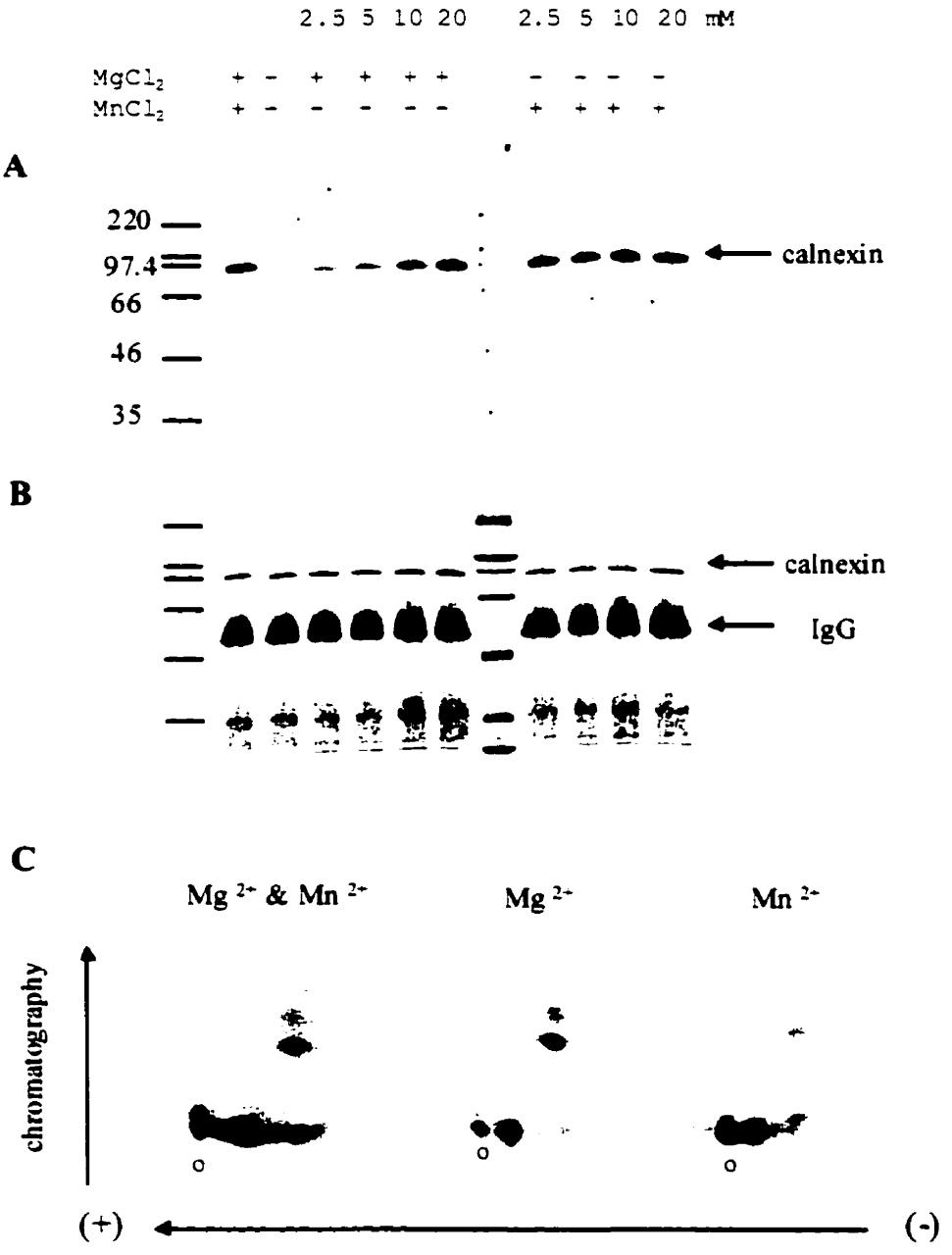

2.5 5 10 20 2.5 5 10 20 mM
MgCl2 + - + + + + - - - -
MnCl2 + - - - - - + + + +
A
220
97.4
66
46
35
calnexin
B
calnexin
IgG
C
Mg 2+ & Mn 2+
Mg 2+
Mn 2+
chromatography
o
o
o
(+)
(-)
pH 1.9

Figure 28: Evaluation of various CK2 inhibitors on microsomal associated kinases.

EsRM were *in vitro* phosphorylated with [γ-^{32}P] GTP (panel A) or [γ -^{32}P] ATP (panel B) and lysed with 2% CHAPS lysis buffer. Calnexin was immunoprecipitated with anti-C3 calnexin. The reaction was done on ice for 30 min either in the absence (lane 1) or in the presence of 2 µg/mL of recombinant CK2 (lanes 2, 4, and 6), 0.5 mg/ml heparin (lanes 3, 4, and 7), or 125 µg/ml of histone H1 (lanes 5 to 7). SDS-PAGE resolved calnexin immunoprecipitates were visualized by radioautography for 12 h at -70°C with intensifying screens. An *arrow* indicates the band corresponding to calnexin. The mobility of the ^{14}C-molecular standards in kDa is indicated on the left.

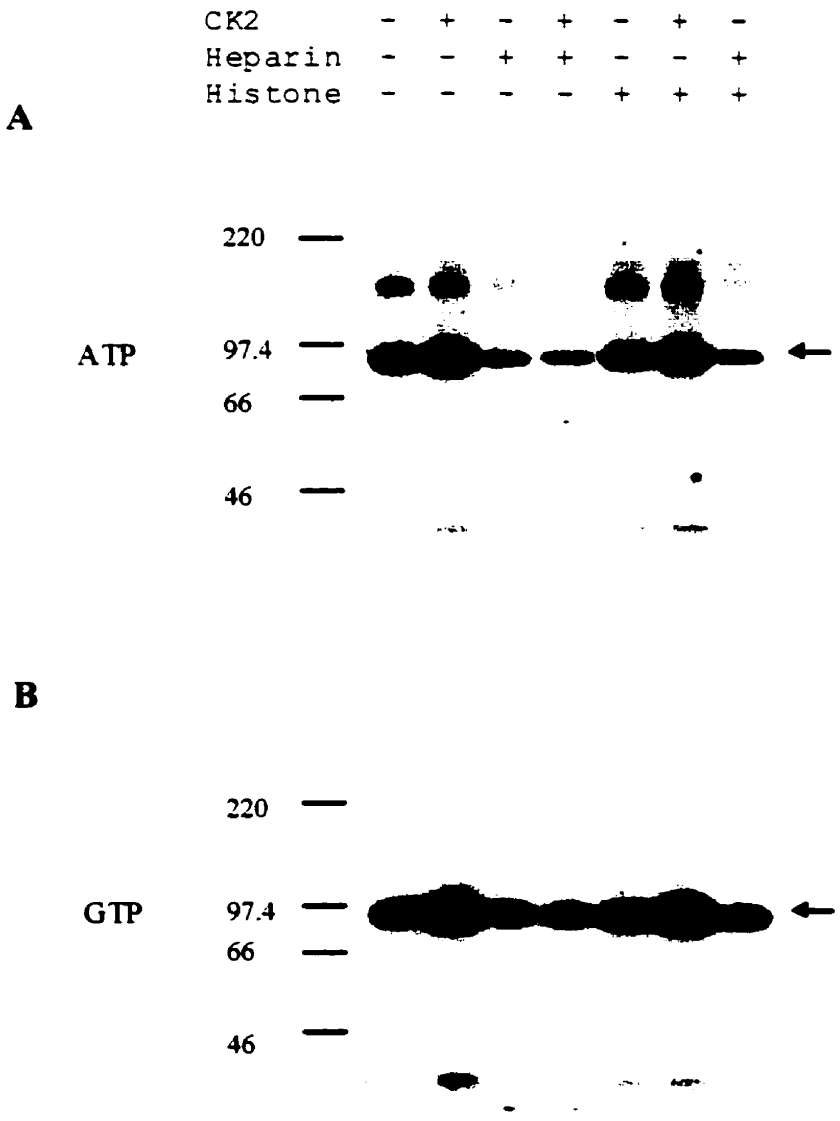
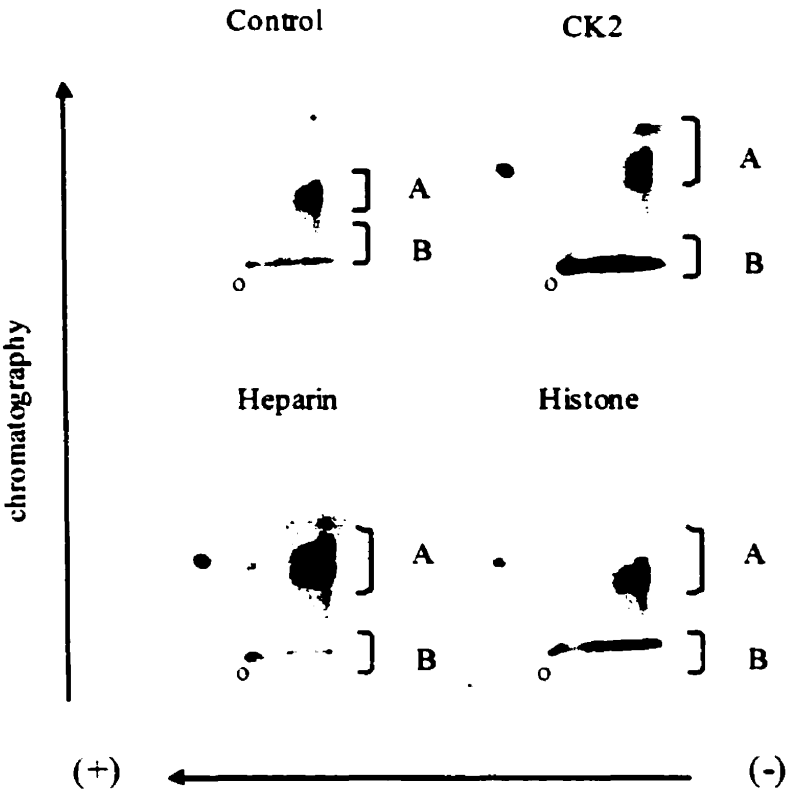

CK2 - + - + - + -
Heparin - - + + - - +
Histone - - - - + + +
A
220
ATP
97.4
66
46
B
220
GTP
97.4
66
46

Figure 29: Evaluation of CK2 phosphorylation sites within the cytosolic domain of calnexin.

A. *In vitro* phosphorylated canine calnexin with [γ -^{32}P] GTP from lanes 1 (*Control*), 2 (*CK2*), 3 (*Heparin*) and 5 (*Histone*) of Fig. 29B were in-gel trypsin digested and analyzed by 2-D tryptic phosphopeptide mapping in phospho-chromatography buffer as described in Materials and Methods.

B. In a separate experiment, [γ-^{32}P] ATP phosphorylated calnexin in the absence (*Control*) or presence of 0.5 mg/ml of heparin or 70 μM DRB were immunoprecipitated with anti-C3 calnexin antibodies. Calnexin immunoprecipitates were resolved by SDS-PAGE, in-gel trypsin digestion and analyzed by 2-D tryptic phosphopeptide mapping as in Panel A. The 2-D tryptic phosphopeptide maps were visualized by a Fuji PhosphoImager screen. Phosphorylated species are grouped into either groups A (*A*) or B (*B*) as in Fig. 6. *Arrows* indicate the direction of electrophoresis towards anodein pH 1.9 buffer and ascending chromatography in isobutyric chromatography buffer. *o*, origin.

A


Control
CK2
A
B
A
B
chromatography
Heparin
Histone
A
B
A
B
(+)
(-)
pH 1.9

B

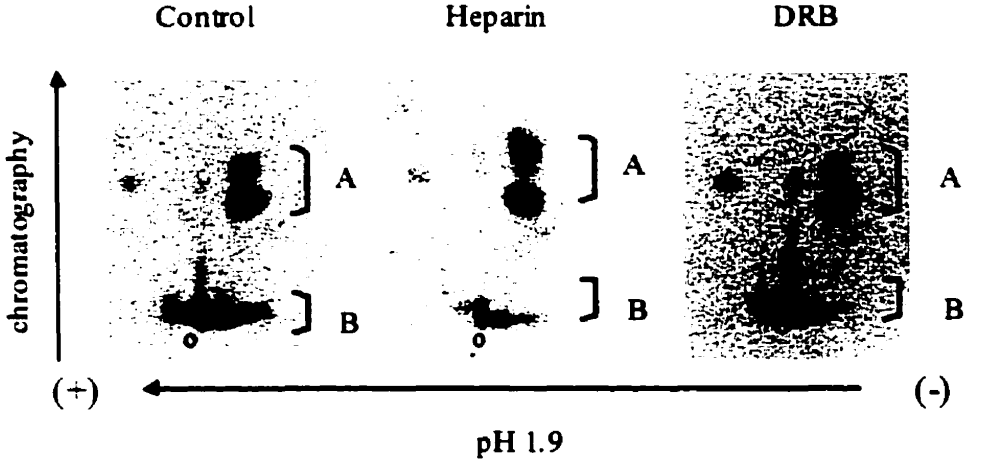

Control
Heparin
DRB
chromatography
A
B
A
B
A
B
(+)
(-)
pH 1.9

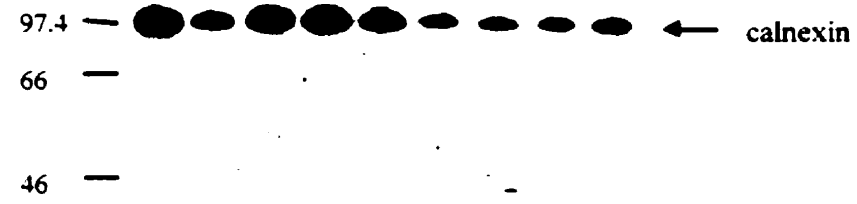
Figure 30: Evaluation of various inhibitors on microsomal associated kinases.

EsRM were *in vitro* phosphorylated with [γ-^{32}P] GTP on ice for 30 min. The reactions were done either in the absence (lane 1) or in the presence of 7 μM staurosporine (lanes 2, 7 and 8), 0.5 mM calphostin C (lane 3), PKC pseudopeptide substrate 19-36 (lane 4), 70 μM DRB (lanes 5, 8 and 9), or 70 μM olomoucine (lanes 6 and 7). At the end of the reaction, microsomes were lysed with 2% CHAPS lysis buffer.

A. Calnexin was immunoprecipitated with anti-C3 calnexin antibodies, resolved by SDS-PAGE and visualized by radioautography for 9 h at -70°C with intensifying screens.
B. Prior to immunoprecipitation of calnexin, 10 μg of the total microsomal extract was aliquoted from each reaction and resolved by 8% SDS-PAGE. Radioautograms after a 15-h exposure at -70°C with intensifying screens. TRAPα (SSRα or pgp35) and pp55 are indicated by an *arrowhead* and *dashed arrow*, respectively. The mobility of the ^{14}C-molecular standards in kDa is indicated on the left.

A

Staurosporine	-	+	-	-	-	-	+	+	-
Calphostin C	-	-	+	-	-	-	-	-	-
peptide 19-36	-	-	-	+	-	-	-	-	-
DRB	-	-	-	-	+	-	-	+	+
olomoucine	-	-	-	-	-	+	+	-	+



B

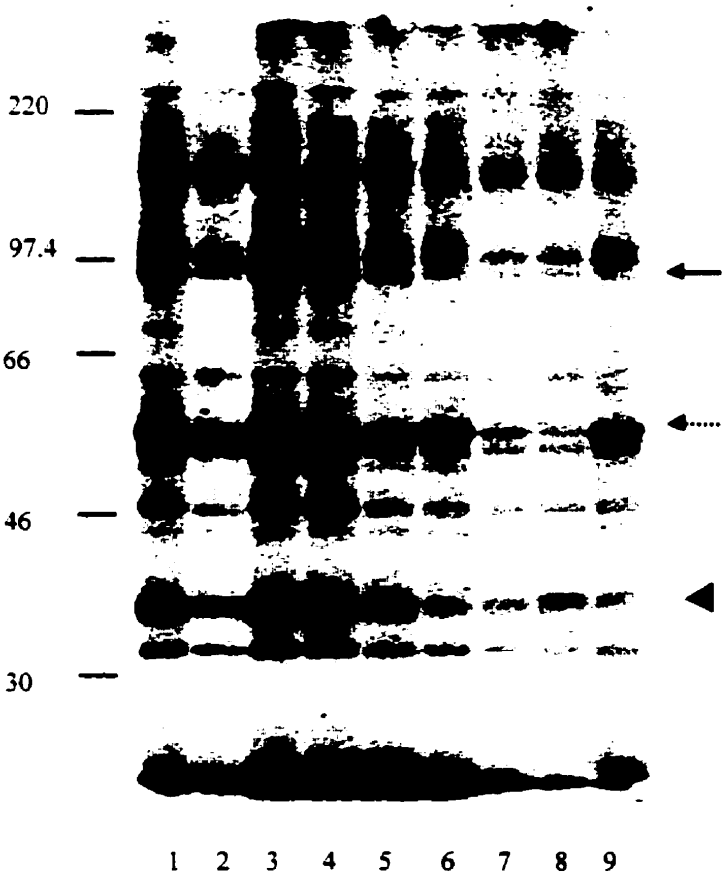


Figure 31: Evaluation of "SPR" phosphorylation site of calnexin.

EsRM were *in vitro* phosphorylated with [γ-^{32}P] ATP on ice for 30 min. The reactions were carried out either in the absence (*CTR*) or in the presence of 50 μM staurosporine, 0.5 mg/ml heparin, 10 μM roscovitine, 10 μM olomoucine or 10 μM iso-olomoucine. At the end of the reaction, microsomes were lysed with 2% CHAPS lysis buffer. Calnexin was immunoprecipitated with anti-C3 calnexin antibodies and resolved by SDS-PAGE. Phosphorylated calnexin immunoprecipitates were in-gel trypsin digested, analyzed by 2-D phosphopeptide mapping and visualized by a Fuji PhosphoImager screen. Phosphorylated peptides are grouped into groups A (*A*) and B (*B*) as before. *Arrows* indicate the direction of electrophoresis towards anode in pH 1.9 buffer and ascending chromatography in isobutyric chromatography buffer. *o*, origin.

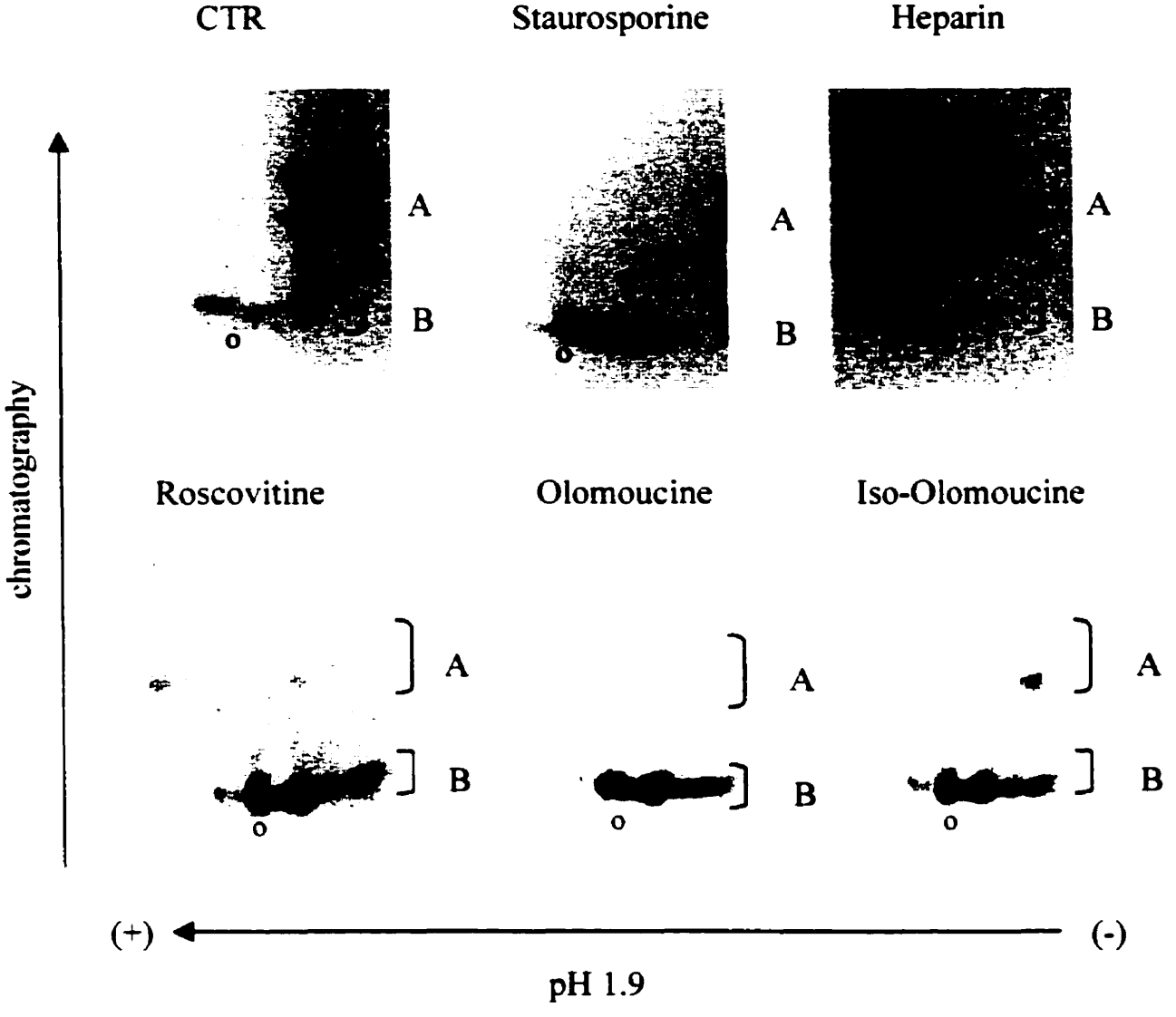
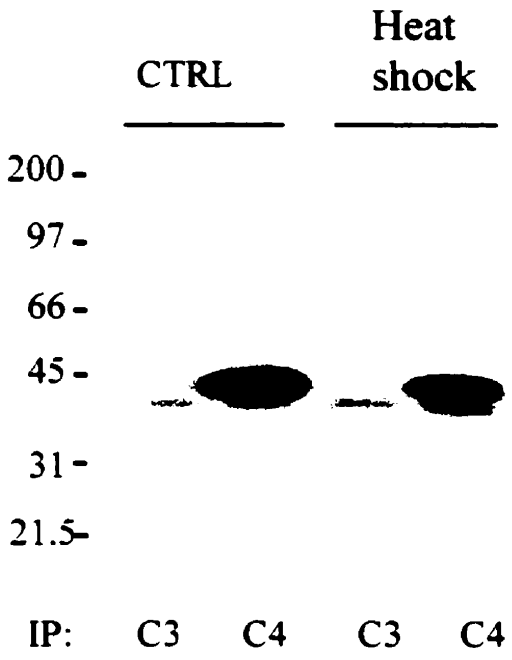

CTR
Staurosporine
Heparin
A
B
o
A
B
o
A
B
chromatography
Roscovitine
Olomoucine
Iso-Olomoucine
A
B
o
A
B
o
A
B
o
(+)
(-)
pH 1.9

Figure 32: ERK-1 is a potential PDK kinase for mammalian calnexin.

A. Rat-2 fibroblasts were heat shocked cells (15 min at 51°C) or not (CTRL) and lysed with 1% CHAPS lysis buffer for 30 min at 4°C. Calnexin was then immunoprecipitated using either anti-C3 (*C3*) or anti-C4 (*C4*) calnexin antibodies. Calnexin immunoprecipitates were resolved by 10% SDS-PAGE containing 0.5 mg/ml MBP. An in-gel kinase assay was then carried out as described in the Materials and Methods. After Coomassie blue staining and drying, the gel was radioautographed for 15 h at -80°C with intensifying screens. The mobility of the molecular standards in kDa is indicated on the left.

B. EsRM were heat-inactivated at 65°C for 10 min as previously described (Ou, Thomas *et. al.*1992). The microsomes were incubated either in the absence (lane 1) or in the presence of 0.5 µg of GST-ERK-1 (lane 2), 0.5 µg of GST-MEK-1 (lane 3), or both kinases together (0.5 µg of each) (lane 4) with 2 µCi of [γ-^{32}P] ATP for 30 min at 30°C. Microsomes were then solubilized with 1% CHAPS lysis buffer and calnexin was immunoprecipitated with anti-C4 CNX antibodies. Calnexin immunoprecipitates were resolved by 10% SDS-PAGE, transferred onto a nitrocellulose membrane and exposed to X-OMAT AR film for 6 h at -80°C with intensifying screens (upper panel) or immunoblotted using anti-C4 calnexin antibodies, and visualized using enhanced chemiluminescence (bottom panel).

A


CTRL
Heat shock
200
97
66
45
31
21.5
IP: C3 C4 C3 C4

B

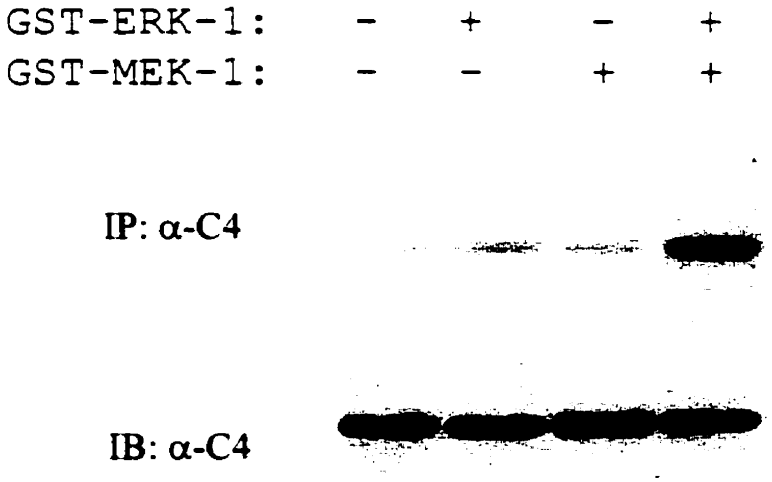
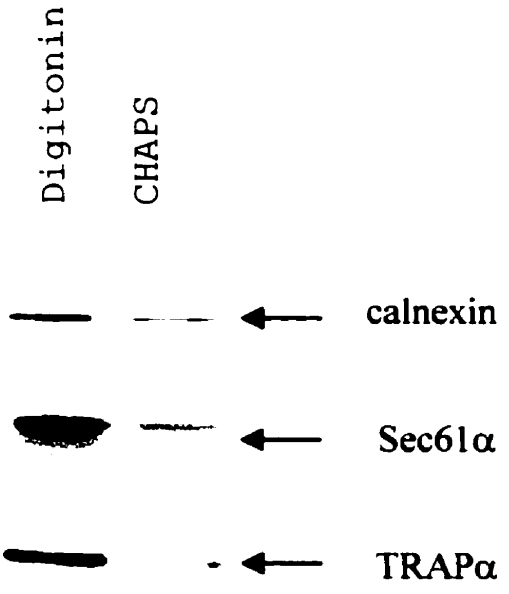

GST-ERK-1: - + - +
GST-MEK-1: - - + +
IP: α-C4
IB: α-C4

Figure 33: Mammalian calnexin is associated with ribosomal proteins.

A. Isolated canine pancreatic rough microsome were solubilized with either 1% Triton X-100 (*TX100*), 1.5% digitonin (*Digitonin*) or 1% CHAPS (*CHAPS*). Ribosome-associated protein fractions were purified through a 1.5 M sucrose cushion, the pellet was resuspended in SDS-PAGE Laemmli sample buffer, resolved by SDS-PAGE, transferred onto a nitrocellulose membrane and immunoblotted with anti-C1 calnexin, anti-Sec61, or anti-TRAPα antibodies, followed by [^{125}I] goat anti-rabbit IgG detection and visualized by radioautography -80°C.

B. Top panel, isolated canine pancreatic rough microsomes (*RM*); middle panel, EDTA-stripped rough microsomes (*EsRM*) and bottom panel, EsRM pre-incubated with competent ribosomes (*EsRM+Rib*) were incubated on ice for 15 or 30 min with trypsin (10 μg/ml). The reaction was stopped by the addition of 1X Laemmli sample buffer. Proteins were resolved by SDS-PAGE, and transferred onto a nitrocellulose membrane. The blot was probed with anti-C1 CNX antibodies, followed by [^{125}I] goat anti-rabbit IgG and visualized by radioautography at -80°C. Full length and cytoplasmic terminally truncated (Δ) calnexins are indicated.

A


TX100
Digitonin
CHAPS
calnexin
Sec61α
TRAPα

B

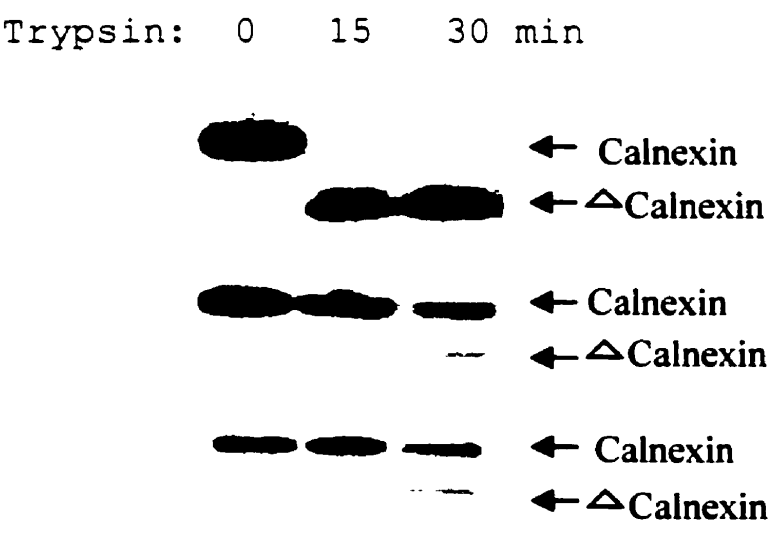

Trypsin: 0 15 30 min
Calnexin
△Calnexin
Calnexin
△Calnexin
Calnexin
△Calnexin

Figure 34: Phosphorylation regulates calnexin binding to ribosomes *in vitro*.

Association of calnexin with ribosomes *in vitro* was carried out as described in the Materials and Methods. EsRM (lane 1) or heat-inactivated EsRM (lanes 2 to 6) were phosphorylated with either CK2 (lane 3), or ERK-1 + CK2 (lane 4), or ERK-1 (lane 5) or ERK-1 + CK2 in the presence of 10 µg of C4 peptide (AA 555-573 of mature canine calnexin) (lane 6). After the reaction, samples were separated into ribosomal pellet and supernatant by centrifugation through a 1.5 M sucrose cushion. The pellets and supernatants were denatured and immunoprecipitated with anti-C4 calnexin antibodies. Calnexin immunoprecipitates were resolved by 8% SDS-PAGE, transferred onto PVDF membranes, immunoblotted with anti-C1 calnexin antibodies and visualized by enhanced chemiluminescence.

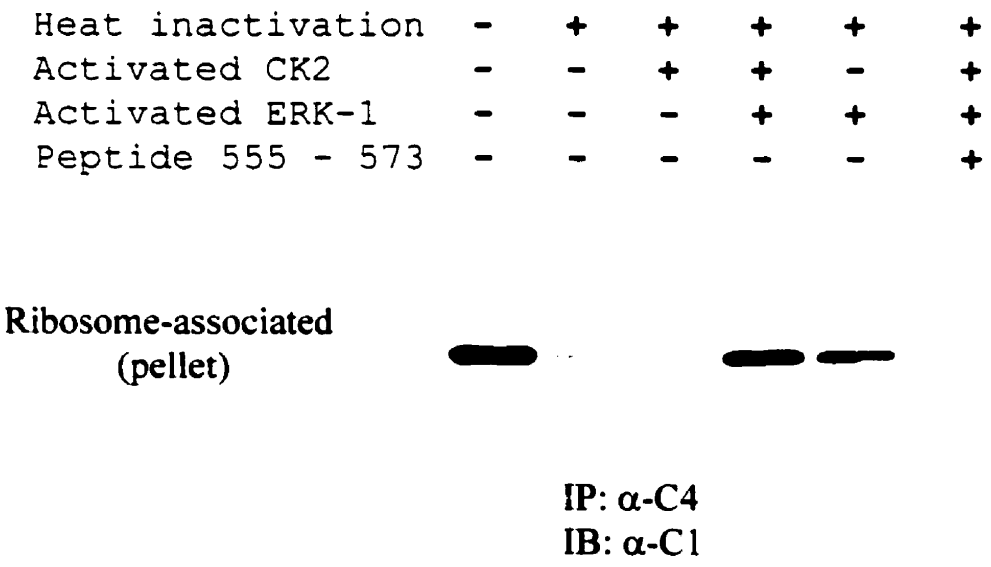
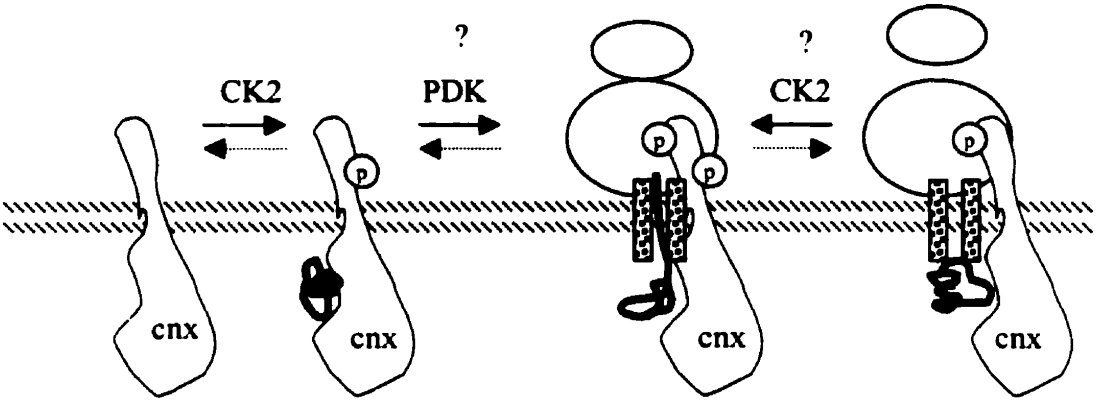

Heat inactivation - + + + + +
Activated CK2 - - + + - +
Activated ERK-1 - - - + + +
Peptide 555 - 573 - - - - - +
Ribosome-associated
(pellet)
IP: α-C4
IB: α-C1

Figure 35: Calnexin association with ribosomes and regulation by calnexin phosphorylation

Calnexin is *in vivo* phosphorylated at three invariant serine residues (Wong, Ward *et. al.*1998) (and Fig 13). Calnexin could potentially exist in triply-, di-, mono- or non-phosphorylated state. From the observation made with the ribosomal pull down assay (Fig. 35), calnexin phosphorylated by PDK such as ERK-1 was shown to enhance the interaction between calnexin and ribosomes. This interaction was strengthened by the synergic action of CK2. Phosphorylation by CK2, however, appeared to prevent or abolish the interaction between calnexin and ribosomes. This may represent a possible regulation of calnexin binding to ribosomes and place calnexin to the proximity of the translocon to anticipate enhanced glycoprotein synthesis. Phosphorylation and dephosphorylation thereby tightly regulate calnexin binding to ribosomes. Hypothetically, phosphorylation of calnexin by PDK such as ERK-1 may increase calnexin concentration in proximity to the translocon to couple it to enhanced glycoprotein synthesis. The release of calnexin from ribosomes is presumably achieved by a phosphatase acting on the SPR phosphorylation motif on triply phosphorylated calnexin. The phosphatases for calnexin dephosphorylation have not yet been identified although okadaic acid has been shown to increase calnexin phosphorylation *in vivo* (Schue, Green *et. al.*1994). The order of phosphate incorporation into calnexin is presently unknown and it may contribute to the regulation of calnexin phosphorylation and its binding to ribosomes. The phosphorylation states of calnexin may also be potentially regulated by the folding status of its associated substrates (indicated by thick lines).



CK2 (Ser^{535}):	-	+/-	+	+/-
CK2 (Ser^{545}):	-	+/-	+	+/-
PDK (Ser^{564}):	-	-	+	+
no. of Ⓟ :	0	1 or 2	3	1 or 2

translocon

Ⓟ phosphate

dephosphorylation

Discussion

In this study, we have examined calnexin phosphorylation and its potential biological significance employing isolated canine pancreatic microsomes. We have employed various kinases and kinase inhibitors to dissect the phosphorylated peptides observed on the 2-D tryptic phosphopeptide maps. *In vitro* phosphorylated calnexin with either [γ-^{32}P] ATP or [γ-^{32}P] GTP by ER-associated kinases displayed a similar 2-D tryptic phosphopeptide mapping pattern as *in vivo* phosphorylated calnexin (Fig. 26C). Employing various kinases and kinases inhibitors, we were able to provide a general assignment to the two designated groups of peptides, phosphopeptides groups A and B (Figs. 26 to 31). The identity of each phosphopeptides on the 2-D tryptic peptide maps, however, remains unknown. Phosphopeptides group A is likely to correspond to serine phosphorylation at the SPR phosphorylation motif of calnexin. Phosphorylation of two serines within the two CK2 phosphorylation motifs of calnexin are likely to provide the signal observed in phosphopeptides group B on the map (Fig. 26C). In addition, partial digestion of calnexin resulting in phosphopeptides containing all three phosphorylation sites may also be present on the 2-D tryptic phosphopeptide maps. The kinases responsible for all three phosphorylation sites are ER-associated. One of the kinases responsible for calnexin phosphorylation is CK2 (Ou *et. al.*1992; Wong *et. al.*1998). CK2 was previously purified and shown to be capable of phosphorylating calnexin in isolated microsomes *in vitro* (Ou *et. al.*1992; Cala *et. al.*1993). The kinase(s) responsible for the serine phosphorylation within the SPR tripeptide, a potential PKC and/or PDK phosphorylation motif, of calnexin is also ER-associated. The involvement of PKC in SPR phosphorylation of calnexin appears unlikely because (1) phorbol esters were unable to increase the level of calnexin phosphorylation *in vivo* (Capps and Zuniga 1994) and (2) the inability of calphostin-C to inhibit calnexin phosphorylation *in vitro* here (Fig. 30A, Lane 3). Here, we have identified ERK-1 as a candidate kinase for calnexin phosphorylation based on these characteristics: (1) the sensitivity towards olomoucine and roscovitine (Fig. 30 and 31), (2) the molecular mass of calnexin associated kinase as

obtained by in-gel kinase assay (Fig. 32A), and (3) the ability of activated recombinant ERK-1 to phosphorylate calnexin *in vitro* (Fig. 32B).

We further showed that calnexin is associated with ribosomes (Fig. 33) and this interaction is regulated by calnexin phosphorylation (Fig. 34). Synergic phosphorylation of calnexin by both CK2 and ERK-1 enhanced the interaction between calnexin and ribosomes (Fig. 34). CK2 phosphorylation alone, however, appears to exert a negative effect on calnexin association to ribosomes (Fig. 34, Lane 3). The negative effect by CK2 phosphorylation, however, was outweighed by ERK-1 activity (Fig. 34, Lane 4). This observation implicates a possible involvement of a phosphatase(s) acting on phosphorylated SPR motif, which may allow the dissociation of calnexin from ribosome by CK2 phosphorylation. The order of phosphate incorporation into calnexin, which is unknown, may be relevant to its function and its regulation. Calnexin could potentially exist in non-, mono-, di- and triphosphorylated states (see discussion of Chapter II). Taken together, all these data indicates that calnexin phosphorylation appears to be tightly regulated by the activities of protein kinases and phosphatases (Fig. 35).

Conditions that enhance calnexin abundance near the translocon (i.e. over-expression of calnexin by transient transfection) has been shown to increase the productive folding of co-transfected subunits of the nicotinic acetylcholine receptor (Chang *et. al.*1997) or tyrosinase (Toyofuku *et. al.*1999). The synergic action of CK2 and ERK-1 which enhances the association of calnexin with ribosomes provides a regulatory mechanism whereby a stimulation of protein synthesis via the MAPK pathway (Fukunaga and Hunter 1997) would be rapidly accompanied by an increase in glycoprotein folding capacity near the translocon.

Prolonged interaction of calnexin with misfolded proteins, either caused by mutation or incorporation of amino acid analogs, is suggestive of the involvement of calnexin in protein quality control in the ER (Ou *et. al.*1993; Le *et. al.*1994). Misfolded proteins and unassembled subunits are retained in the ER lumen and eventually degraded and this is generally referred to as ER associated degradation (ERAD). Retrograde translocation of misfolded proteins or unassembled subunits through the translocon has

been suggested to transport the misfolded proteins from the ER lumen to the cytosol for degradation via proteasomes (Brodsky and McCracken 1997; Bonifacino and Weissman 1998). The involvement of calnexin in ERAD was implicated by an *in vitro* reconstituted assay where the investigators have shown that calnexin was essential for the degradation of unglycosylated pro-alpha factor (McCracken and Brodsky 1996). However, another group of investigators showed that gene disruption of *S. cerevisiae* calnexin does not exhibit any effect on the degradation of a carboxypeptidase Y mutant *in vivo* (Knop *et. al.*1996).

Calnexin has been found in a complex with ubiquitinated apolipoprotein B and sec61β (Liao *et. al.*1998). Calnexin was also found in a complex with PiZ variant of α_1-antitrypsin prior to its degradation by cytosolic proteasomes (Qu *et. al.*1996). These observations support an involvement of calnexin in ERAD. Taken together with the lectin-like molecular chaperone function, association with ubiquitinated substrates, membrane anchorage, the close vicinity to translocon and phosphorylation-dependent association and dissociation with ribosomes of calnexin, it is probable to envisage an involvement of calnexin in protein retro-translocation from the ER lumen to the cytosol. The phosphorylation of calnexin may play an important role in regulating the biological function of calnexin either in assisting the translocation of nascent proteins into the ER lumen or in the retrograde translocation of misfolded proteins out of the ER for protein degradation in the cytosol (Fig. 35).

TRAPα is a constituent of a tetrameric complex of the ER (Hartmann *et. al.*1993). The function of the TRAP complex during protein translocation remains unclear. The proximity of the TRAP complex to preproteins and tranlsocon has suggested that it may participate in the insertion of membrane proteins into the lipid bilayer or retention of ER membrane proteins (Gorlich *et. al.*1990; Hartmann *et. al.*1993). Reconstituted proteoliposomes devoid of the TRAP complex by immunodepletion showed unimpaired translocation activity (Migliaccio *et. al.*1992). Interestingly, phosphorylation of TRAPα or pgp35 was subjected to similar inhibitory effects by the inhibitors used here as calnexin (Fig. 30B, indicated by arrowhead and solid arrow respectively). TRAPα has

been demonstrated to be phosphorylated on its cytosolically oriented serine residue(s) both *in vivo* and *in vitro* (Prehn *et. al.*1990; Wada *et. al.*1991). The cytosolic domain of canine TRAPα also contains one potential CK2 phosphorylation site and a phosphorylation motif composed of a SPR tripeptide sequence (Prehn *et. al.*1990) (Fig. 38). CK2 has been shown to be capable of phosphorylating both TRAPα and calnexin *in vitro* in isolated microsomes (Ou *et. al.*1992). The spatial separation between the CK2 and SPR phosphorylation sites of TRAPα is similar to the one observed within the cytosolic domain of calnexin (Fig. 36).

Figure 36: Sequence alignment and potential serine phosphorylation sites of the cytosolic domains of canine calnexin and TRAPα.

Alignments of the cytosolic domains of canine mature calnexin, CNX (amino acids 541-573) and precursor TRAPα (amino acids 244-286) (Accession P16967). Potential CK2 phosphorylation motifs are underlined and potential PKC/PDK phosphorylation motifs are double underlined.

CNX: ^{541}GGTASQEEDDRKPKAEEDEILNRSPRNRKPRRE^{573}

TRAPα: ^{244}GTSSQNDVDMSWIPQETLNQINKASPRRLPRKRAQKRSVGSDE^{286}

The serine residue within the SPR phosphorylation motif of TRAPα may also be utilized. Taken together with the co-purification of TRAPα with calnexin (Wada *et. al.*1991) and their common potential phosphorylation motifs (Fig. 36), it is conceivable that both calnexin and TRAPα phosphorylation could be regulated by the same mechanisms for their coordination of action in protein synthesis, translocation and modification.

Chapter V

Mammalain calnexin is not O-linked modified

Introduction

Numerous nuclear, cytosplasmic and plasma membrane proteins undergo a post-translational modification by the addition of a single N-acetylglucosamine (GlcNAc) monosaccharide to the hydroxyl group of serine and threonine residues (Hart 1997; Comer and Hart 1999). Elongation of the O-linked GlcNAc monosaccharide modification has not been observed (Hart 1997; Comer and Hart 1999). The enzymes responsible for the addition, UDP-GlcNAc:polypeptide N-acetylglucosaminyl-transferase (Kreppel *et. al.*1997), and the removal, O-GlcNAc-specific β-D-N-acetylglucosaminidase, of the sugar have been identified and are localized to the cytoplasm and nucleus (Dong and Hart 1994).

The exact function of the O-linked GlcNAc modification remains to be established. Many lines of evidence, however, have suggested that O-linked GlcNAc modification is dynamic and responsive to cellular signals (Hart 1997; Comer and Hart 1999). A transient and rapid change in the level of O-GlcNAc on numerous nuclear and cytoplasmic glycoproteins has been observed in activated T lymphocytes (Kearse and Hart 1991). O-linked GlcNAc modified proteins identified so far are also phosphoproteins (Hart 1997; Comer and Hart 1999). The function of O-linked GlcNAc has been suggested to be analogous to phosphorylation and dephosphorylation and may modulate the function of numerous phosphoproteins (Hart 1997; Comer and Hart 1999). One of the major O-GlcNAc modification sites for c-Myc oncoprotein, a nuclear transcription factor, is Thr^{58} which is also an *in vivo* phosphorylation site within the transactivation domain and is a mutational hot spot in numerous human lymphomas (Chou *et. al.*1995). The major sites for O-linked GlcNAc modification for transcription factor SV40 large T-antigen has been mapped to Ser^{111} and Ser^{112} which are also phosphorylated (Medina *et. al.*1998). The competition between O-GlcNAc modification and phosphorylation has been suggested to be involved in modulating the functions of both c-Myc and SV40 large T-antigen (Hart 1997; Comer and Hart 1999). The O-linked GlcNAc modified phosphoproteins also form reversible multimeric complexes with other

polypeptides via associations that are regulated by phosphorylation. It has been suggested that O-GlcNAc modification may modulate the interaction of O-lined GlcNAc modified phosphoproteins with each other or other cellular proteins (Hart 1997; Comer and Hart 1999). In the case of transcription factors Sp1, O-linked GlcNAc modified Sp1 was found to be 3-5 fold more efficient in activating transcription than unglycosylated recombinant transcription factor Sp1 produced in E. coli (Jackson and Tjian 1988). It has also been observed that underglycoylated Sp1 was subjected to a rapid proteasome degradation (Han and Kudlow 1997). Furthermore, the presence of the O-GlcNAc modification inhibited both Sp1 dimerization and interaction of the Sp1 transcription domain with TATA binding protein associated factor 110 in vitro (Roos *et. al.*1997). The three subunits of neurofilaments (NF-L, NF-M and NF-H) that are essential for axonal caliber are also modified by O-GlcNAc (Dong, Xu *et. al.*1993). The site of O-linked GlcNAc modification was identified to the head domains of the three neurofilament subunits. Furthermore, O-GlcNAc modification was detected to occur at multiple sites within the KSP repeat motif in the tail domain of NF-H. KSP repeat motif of neurofilaments is highly phosphorylated in the axonal processes (Cohen *et. al.*1987). It was suggested that O-GlcNAc modification may play a role in neurofilament assembly and network formation for neuronal cells (Dong *et. al.*1993; Dong *et. al.*1996). Post-translational modification by both O-GlcNAc and phosphate moieties on the same protein could potentially increase the level of control on a given protein and its function.

O-linked GlcNAc modification may play a role in the regulation of protein translation and protein degradation. A 67 kDa eIF-2 associated protein (p67) has been identified (Datta *et. al.*1989). It was demonstrated that O-linked GlcNAc modified p67 interacted with eIF-2a and prevented it from phosphorylation (Ray *et. al.*1992; Datta *et. al.*1999). Phosphorylation of eIF-2a has been shown to inhibit protein synthesis. Under conditions of serum starvation or heme depletion, p67 was found to be deglycosylated and resulted in its degradation (Ray *et. al.*1992).

Numerous integral membrane proteins of the ER and Golgi apparatus were found to be modified by O-GlcNAc moieties on their cytosolic domains (Abeijon and

Hirschberg 1985; Capasso *et. al.*1988). GRP94, an ER resident molecular chaperone, has been reported to be modified by O-linked glycosylation and this could potentially regulate its molecular chaperone function (Csermely *et. al.*1998). Calnexin is a phosphoprotein with a lectin-like molecular chaperone function (Chevet *et. al.*1999; Ellgaard *et. al.*1999). Calnexin is phosphorylated at its cytosolic domain at three serine residues that could be potentially phosphorylated by CK2, PKC and/or PDK (Wong *et. al.*1998) (and chapter II). Calnexin has been shown to interact with ribosomes and this interaction is regulated by calnexin phosphorylation (Chevet *et. al.*1999) (and chapter IV). We proceeded to examine the possible regulation exerted by O-linked GlcNAc modification on calnexin phosphorylation by galactose labeling assay (Paiement, *et. al.*1982; Roquemore *et. al.*1994). Here, we show that calnexin is not O-GlcNAc modified.

Results

Calnexin is not O-linked GlcNAc modified.

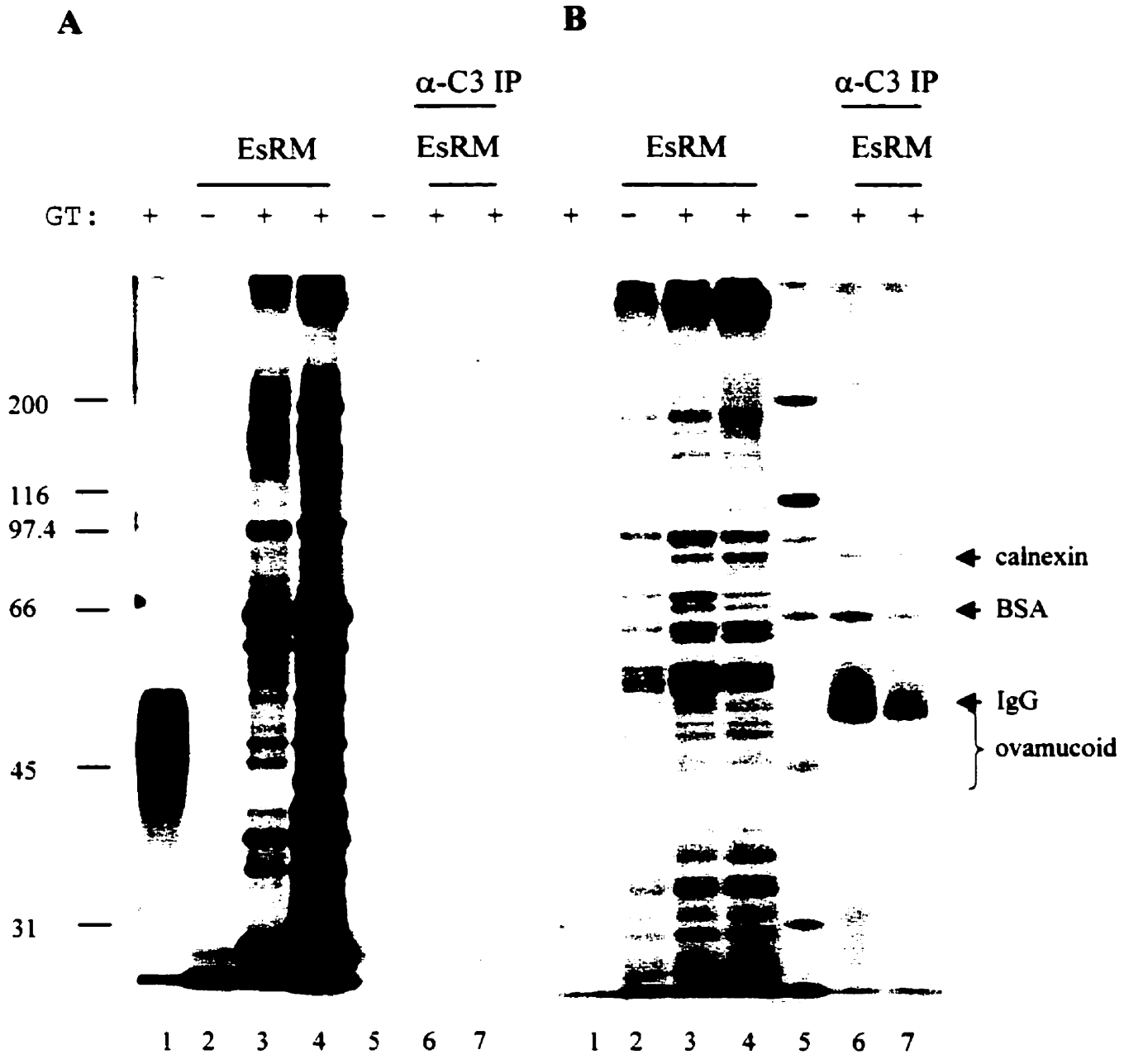
Several integral membrane proteins of the ER and Golgi apparatus were found to be modified by O-GlcNAc moieties on their cytosolic domains (Abeijon and Hirschberg 1985; Capasso *et. al.*1988). Given the dynamic nature of O-GlcNAc modification, we proceed to examine whether this modification could modulate the function of calnexin phosphorylation and its interaction with ribosomes. We proceeded to examine the possible regulation by O-GlcNAc modification of calnexin phosphorylation employing isolated intact canine pancreatic microsomes by a galactose labeling assay (Paiement, *et. al.*1982; Roquemore *et. al.*1994).

Intact isolated canine pancreatic EDTA stripped rough microsomes (EsRM) were prepared as previously described (Walter and Blobel 1983; Wada *et. al.*1991). Incubation of isolated intact canine pancreatic EsRM with radiolabeled UDP-[^{3}H] Gal did not yield any radiolabeled proteins by radioautography (Fig. 37 Lane 2). This demonstrated that there is no endogenous galactosyl transferase activity associated with the cytosolic surface of the microsomes. Ovamoucoid, a known galactosyl transferase substrate (Paiement *et. al.*1982), was used to confirm the activity of the autogalactosylated galactosyl transferase (Fig. 37A. Lane 1). The amount of ovamoucoid used was not readily visible by Coomassie blue staining (Fig. 37B. Lane 1) however it had a good incorporation of UDP- [^{3}H]-galactose as visualized by radioautography (Fig. 37A. Lane 1). Isolated intact canine pancreatic microsomes were incubated with autogalactosylated galactosyl transferase and radiolabled sugar (Fig. 37 A and B, Lane 3). There are a number of good substrates with receptor sites for ^{3}H-galactose on isolated intact canine pancreatic EsRM which is compatible to the ones observed previously on rat liver microsomes (Abeijon and Hirschberg 1985; Capasso *et. al.*1988). The number of galactose acceptor proteins increased when membranes were lightly sonicated prior to the incubation with galactosyl transferase and radiolabeled sugar. Radioautogram revealed a radiolabeled protein with an apparent molecular weigh at 90 kDa by SDS-PAGE with

either intact or sonicated microsomes (Fig. 37A. Lanes 3 and 4). In order to confirm whether the 90 kDa-galactosylated band is calnexin, calnexin was immunoprecipitated from both GT-galactosylated intact and sonicated EsRM membranes with anti-C3 calnexin antibodies. Immunoprecipitated calnexin were readily visible by Coomassie staining (Fig.37B. Lanes 6 and 7) and its corresponding radioautogram revealed that calnexin is not galactosylated (Fig. 37A. Lanes 6 and 7).

Figure 37: Evaluation of possible regulation of calnexin phosphorylation by O-linked glycosylation.

Galactosyl transferase (GT) were autogalactosylated and the reactions were done as previously described (Paiement *et. al.*1982; Roquemore *et. al.*1994) (also see Materials and Methods). Lane1, 100 µg of ovamoucoid with GT; lane 2, EsRM incubated without GT; lane 3 and 4 were GT incubated with either EsRM or briefly sonicated EsRM prior reaction. 2 µCi of UDP-[^{3}H]Gal was present in all tubes. At the end of the incubation, approximately 100 µg of each reaction was separated by SDS-PAGE. For reactions of lanes 3 and 4, calnexin was immunoprecipitated with anti-C3 calnexin antibodies and separated by SDS-PAGE in lanes 6 and 7, respectively. Lane 5, broad-rang molecular weighs standards and the mobility is indicated on the left. The polyacrylamide gel was Coomassie blue stained, enhanced and exposed to film for 57 d at -70°C. Radioautograms (panel A) and the corresponding Coomassie blue stained (panel B) are shown. *Arrows* indicate calnexin, exogeneously added BSA, and immunoglobulin heavy chain (*IgG*). *Bracket* indicates ovamoucoid.


A
B
α-C3 IP
α-C3 IP
EsRM
EsRM
EsRM
EsRM
GT: + - + + - + + + - + + - + +
200
116
97.4
66
45
31
calnexin
BSA
IgG
ovamucoid
1 2 3 4 5 6 7
1 2 3 4 5 6 7

Discussion

Calnexin is a phosphoprotein with a lectin-like molecular chaperone function of the ER membrane (Wada *et. al.*1991; Ou *et. al.*1993). Calnexin interacts transiently with numerous nascent glycoproteins. Calnexin together with ERp57, an ER thiol oxidoreductase, mediate the retention and promote proper folding of nascent glycoproteins (Chevet *et. al.*1999; Ellgaard *et. al.*1999).

Calnexin has been shown to interact with ribosomes and the interaction is regulated by calnexin phosphorylation (Chevet *et. al.*1999). Calnexin is phosphorylated at its cytosolic domains at three serine residues that could be recognized by protein kinases CK2, PKC and/or PDK (Wong *et. al.*1998). The phosphatases responsible for calnexin dephosphorylation have not yet been identified. Okadaic acid, an inhibitor of serine protein phosphatase 1 and 2A (Cohen *et. al.*1990), has been shown to increase the level of calnexin phosphorylation *in vivo* (Schue *et. al.*1994). Calnexin phosphorylation is potentially regulated tightly by phosphorylation by kinases and dephosphorylation by phosphatases.

The cytosolic domains of numerous integral membrane proteins of the ER and Golgi apparatus were found to be modified by O-linked GlcNAc addition (Abeijon and Hirschberg 1985; Capasso *et. al.*1988). The exact function of O-GlcNAc modification remains to be established. Modification by O-linked GlcNAc however has been suggested to be reciprocal to phosphorylation of some proteins and may play a role in modulating the function of numerous phosphoproteins and their interactions with other cellular proteins (Hart 1997; Comer and Hart 1999). O-linked GlcNAc modification has been suggested to modulate the molecular chaperone function of GRP94 (Csermely, Schnaider *et. al.*1998). Elucidation of the possible regulation of calnexin by O-linked GlcNAc modification may provide an insight into the regulatory mechanism of calnexin phosphorylation. We showed here that calnexin is not radiolabeled in either intact or sonicated isolated canine pancreatic microsomes with radioalebeled galactose (Fig. 37). Hence, calnexin is not modified by O-linked GlcNAc modification.

Chapter VI

Schizosaccharomyces pombe calnexin phosphorylation

Introduction

Membrane-anchored calnexin and calmegin/calnexin-t, a testis-specific calnexin homologue, and the soluble calreticulin are authentic lectin-like molecular chaperones of the ER in mammalian cells (Ou *et. al.*1993; Peterson *et. al.*1995; Ikawa *et. al.*1997). Calnexin and calreticulin recognize monoglucosylated high mannose-type oligosaccharide side chains present on nascent glycoproteins (Rodan *et. al.*1996; Zapun, *et. al.*1997; Vassilakos *et. al.*1998). Calnexin binding to nascent glycoproteins has been shown to promote their folding and assembly. Prolonged association of calnexin with misfolded glycoproteins either by mutations or by the incorporation of amino acid analog has also been shown (Bergeron *et. al.*1994; Chevet *et. al.*1999; Ellgaard *et. al.*1999). Both calnexin and calmegin/calnexin-t are type I transmembrane proteins and are phosphorylated at their cytosolic termini (Wada *et. al.*1991; Ohsako *et. al.*1998; Wong *et. al.*1998). Furthermore, mammalian calnexin is phosphorylated on three conserved serine residues *in vivo* (Wong *et. al.*1998)(and Chapter 2). Prolonged association of phosphorylated calnexin with the null Hong Kong variant of α_1-antitrypsin has been observed *in vivo* (Le *et. al.*1994). The phosphorylation of calnexin has also been suggested to regulate the rate of protein transport out of the ER (Capps and Zuniga 1994). Calnexin phosphorylation has been demonstrated to regulate its interaction to ribosomes. It was proposed that calnexin phosphorylation may increase calnexin concentration in proximity to the translocon to couple it to enhanced glycoprotein synthesis (Chevet *et. al.*1999) (and Chapter 4).

The fission yeast *Schizosaccharomyces pombe* shares many similar properties as mammalian cells with respect to glycoprotein folding and secretion (Beaulieu *et. al.*1999). For glycoprotein processing, the dolichol-linked $G_3M_9GlcNAc_2$ oligosaccharide is transferred onto an asparagine residue of newly synthesized glycoproteins. The generation of protein-linked $M_9GlcNAc_2$ has been observed in the *S. pombe* (Ziegler *et. al.*1994). The genes encoding the two subunits of glucosidase II (GII) have recently been cloned (D'Alessio *et. al.*1999). The *S. pombe* alpha subunit of GII contains the catalytic activity, which is similar to its mammalian counterpart (Trombetta, Simons *et. al.*1996;

D'Alessio *et. al.*1999). The cDNA encoding the *S. pombe* UDP-glucose:glycoprotein glucosyltransferase (UGGT), a glycoprotein folding sensor, has been cloned and sequenced (Fernandez *et. al.*1994; Sousa and Parodi 1995). Misfolded high mannose-containing glycoproteins can be re-glucosylated by UGGT in *S. pombe*. UGGT has been shown to be essential under extreme stress conditions in *S. pombe* (Fernandez *et. al.* 1996). Furthermore, the glucose-trimmed oligosaccharide is processed in the Golgi apparatus involving the addition of mannose and galactose (Chappell *et. al.*1994; Ziegler *et. al.*1994; Huang and Snider 1995). This further highlights the conservation of glycoprotein processing between *S. pombe* and mammalian cells. The ER mannosidase I equivalent, however, has not been detected in the fission yeast *S. pombe* (Gemmill and Trimble 1999).

The fission yeast contains the core components of the calnexin cycle for the productive glycoprotein folding in the ER (Fernandez *et. al.*1994; Jannatipour and Rokeach 1995; Parlati *et. al.*1995; Beaulieu *et. al.*1999; D'Alessio *et. al.*1999). *S. pombe* has a calnexin (Cnx1p) molecule that is between 37.6% to 38.3% identical to its mammalian homologue (Table 1) and shares many similar features (Jannatipour and Rokeach 1995; Parlati *et. al.*1995). Cnx1p is a type I transmembrane protein with a short cytosolic domain consisting of 48 amino acid residues which is dispensable for viability (Jannatipour and Rokeach 1995; Parlati *et. al.*1995). The luminal portion of Cnx1p contains the conserved domains for calcium binding and ligand binding (Jannatipour and Rokeach 1995; Parlati *et. al.*1995). *S. pombe* Cnx1p can potentially dissociate and re-bind to monoglucosylated proteins by the activities of GII and UGGT, respectively (Fernandez *et. al.*1994; D'Alessio *et. al.*1999). The luminal domain of Cnx1p is essential for the viability of *S. pombe* (Jannatipour and Rokeach 1995; Parlati *et. al.*1995; Jannatipour *et. al.*1998). It has been suggested that the BiP-binding region within the 52 amino acids at the carboxyl terminal of the luminal domain of Cnx1p was responsible for the essentiality of Cnx1p (Elagoz *et. al.*1999). A glycan independent interaction between Cnx1p and a glycoprotein, acid phosphatase, was recently shown. Cnx1p was found in a complex with BiP and nascent acid phosphatase (Jannatipour *et. al.*1998). It has been suggested that the

coordinated chaperone function of BiP and Cnx1p enhances the glycoprotein folding efficiency in *S. pombe* (Fernandez *et. al.*1994; Fanchiotti *et. al.*1998; Jannatipour *et. al.*1998; D'Alessio *et. al.*1999; Elagoz *et. al.*1999).

The similarities between *S. pombe* and mammalian cells with respect to protein glycosylation and secretion and the ease of gene manipulation have made *S. pombe* an attractive model for the genetic study of calnexin in the quality control mechanism of the ER. Here, we have used this organism to study calnexin phosphorylation. We show that *S. pombe* Cnx1p is *in vivo* serine phosphorylated within a potential proline-directed kinase (PDK) phosphorylation motif by site-directed mutagenesis. Employing fusion protein constructs containing different regions of the cytosolic domain of Cnx1p, a Cnx1p-interacting protein with a relative migration mobility of 150 kDa by SDS-PAGE was observed.

Results

S. pombe Cnx1p is in vivo phosphorylated on its cytosolic domains at serine residues.

The *S. pombe* strains used in this study are listed in Fig. 38. PCR and homologous recombination were employed to generate the strains expressing Cnx1p containing either an alanine or glutamic acid substitution at either Ser546 or Ser553 position (Figs. 38A and 41). A schematic diagram of the *S. pombe* expression plasmids encoding either the full-length or carboxyl truncated Cnx1p are also shown (Fig. 38B) (Parlati *et. al.*1995). The plasmids are expressed in a *S. pombe Δcnx1* deletion strain that no longer express its endogenous Cnx1p (Parlati *et. al.*1995).

S. pombe wild type (WT) and *S. pombe Δcnx1* deletion strains expressing a plasmid copy of either the full length, pCNX560 (p560), or carboxyl truncated Cnx1p, pCNX524 (p524), (Fig. 38B) were *in vivo* ^{32}P-orthophosphate radiolabeled for 4 h. Cnx1p was immunoprecipitated with affinity purified anti-Cnx1p antibodies. Polyclonal anti-Cnx1p antibodies were raised against a bacterial expressed GST-fusion construct containing the luminal domain of precursor Cnx1p (amino acid 23-492) (Parlati *et. al.*1995). SDS-PAGE resolved Cnx1p immunoprecipitates were electroblotted onto a nitrocellulose membrane and visualized by radioautography (Fig. 40A). Upon an initial exposure of 43 h at -70°C, Cnx1p immunoprecipitates from wildtype and p560 mutants were phosphorylated (Fig. 39A, top panel). Upon a second exposure of the same blot for 6 d at -70°C, Cnx1p immunoprecipitates from both wildtype and p560 *S. pombe* were strongly phosphorylated whereas Cnx1p from p524 mutant was slightly phosphorylated (Fig. 39A, bottom panel). Cnx1p expressed by p524 mutant contains the transmembrane domain and the 12 juxtamembrane cytosolically oriented amino acid residues of Cnx1p (Fig. 38B). The ^{32}P-orthophosphate incorporation into Cnx1p immunoprecipitated from p524 *S. pombe* may not represent an authentic phosphorylation of Cnx1p. A non-specific phosphorylated band with a similar migration mobility of p524-Cnx1p by SDS-PAGE is sometimes observed to interact with protein A-sepharose beads (see Fig. 41A Lane 1).

The band corresponding to the phosphorylated Cnx1p was excised from the nitrocellulose membrane (Fig. 39A, Lane 1), trypsin digested and subjected to

phosphoamino acid analyses. Radioautogram of the two-dimensional TLC plate of hydrolyzed Cnx1p revealed only ^{32}P-labeled serine that co-migrated with the non-radiolabeled phosphoserine standard as detected by ninhydrin staining (Fig. 39B). Hence, *S. pombe* Cnx1p is *in vivo* phosphorylated on serine residue(s).

In order to dissect the phosphorylation domain of *S. pombe* Cnx1p, *S. pombe* mutants expressing either the full-length of Cnx1p (p560) or the carboxyl truncated Cnx1p (p484 and p474) (Fig. 38B) were *in vivo* ^{32}P-orthophosphate radiolabeled for 4 h and calnexin immunoprecipitated with affinity purified anti-Cnx1p antibodies. SDS-PAGE resolved Cnx1p immunoprecipitates were visualized by silver nitrate staining and radioautography (Fig. 40, A and B). Radioautograms of the polyacrylamide gel showed that full-length Cnx1p isolated from p560 is phosphorylated whereas the Cnx1p lacking both the cytosolic and transmembrane domains from p484 and p474 mutants are not phosphorylated (Fig. 40A). A comparable amount of Cnx1p was immunoprecipitated from each strain as revealed by silver nitrate staining (Fig. 40B). *S. pombe* Cnx1p is thereby *in vivo* phosphorylated within its cytosolic domain. Taken together with the result observed in Fig. 40A, the phosphorylation amino acid resiude(s) is likely to reside within amino acid position 525 to 560 of the cytosolic domain of precursor Cnx1p (Fig. 38A).

Cnx1p is in vivo phosphorylated at a proline-directed kinase motif.

There are two serine residues residing between amino acid position 525 to 560 of the precursor of Cnx1p (Fig. 38A). We proceeded to identify the *in vivo* site of phosphorylation by site-directed mutagenesis and *in vivo* ^{32}P-orthophosphate radiolabeling experiments. Ser^{546} and Ser^{553} of Cnx1p were either singly or doubly mutated to alanine or glutamic acid (Fig. 38A).

A pMS plasmid, modified from pSp72 plasmid, containing the coding sequence for *cnx1*$^+$, *ura4*$^+$ cassette, poly A tail and *nmt1* promoter was used to create site-directed mutagenized Cnx1p. PCR and subcloning obtained a 4.2-kb linear restriction fragment containing the *cnx1*$^+$ with the appropriate point mutation and the *ura4*$^+$ cassette. Flanking sequences of 0.1 kb on the 5' side and 0.5 kb on the 3' side were retained in order to direct

homologous recombination upon transformation into the haploid *S. pombe* wild type strain, Q358 (Fig. 41). Ura+ transformants were selected. To confirm that homologous recombination, PCR was performed using a sense primer that hybridizes a region outside of the 4.2-kb PCR fragment and an anti-sense ura4 primer that recognizes the *ura4*$^+$ cassette (Fig. 41). Mutation was confirmed by sequencing the genomic DNA from the Ura+ transformants with primer ura4 and TM-1 from both ends.

The synthesis of Cnx1p from Ura+ transformants was examined by metabolic radiolabeling with Tran^{35}S-labels for 4 h. Cells were lysed with RIPA lysis buffer and Cnx1p was immunoprecipitated as described (Beaulieu *et. al.*1999). SDS-PAGE resolved Cnx1p immunoprecipitates were visualized by radioautography (Fig. 42). A compatible amount of newly synthesized Cnx1p was observed in each transformant as compared to the parental strain (Fig. 42). Cnx1p immunoprecipitates also brought down two additional metabolically radiolabeled proteins with a faster migration mobility than Cnx1p by SDS-PAGE. They are also found to bind to protein A-sepharose beads in the pre-clearing step and thus considered non-specific (Fig 42, Lane 1).

No morphological differences were observed among the point mutants as compared to the wild type at both 30°C and 37°C by light microscopy. The growth rate among the point mutants was compatible to the one displayed by the wildtype *S. pombe* at either temperature. The point mutants do not display a better or worse protection to cellular stresses elicited by 10 mM DTT or 1 μg/ml of tunicamycin (data not shown).

Wildtype and point mutants *S. pombe* were *in vivo* radiolabeled with ^{32}P-orthophosphate as in Fig. 40. Cnx1p was immunoprecipitated, resolved by SDS-PAGE and visualized by silver nitrate staining and radioautography (Fig. 43A). Cnx1p containing alanine substitution of Ser^{546} was phosphorylated whereas Cnx1p with alanine substitution at Ser^{553} was not phosphorylated (Fig. 43A, Lanes 'S546A' and 'S553A'). Cnx1p containing alanine substitution at both serine residues was not phosphorylated either (Fig. 43A, Lane 'AA'). The same phosphorylation pattern was observed with glutamic acid substituted Cnx1p (Fig. 43A, Lanes 'S546E', 'S553E' and 'EE'). The lack of detection by radioautography is not due to the lack of Cnx1p as judged by the silver

staining of the same polyacrylamide gel (Fig. 43B). From this observation, Ser^{553} is a potential *in vivo* phosphorylation site for *S. pombe* Cnx1p. Interestingly, Ser^{553} is found to be within a potential PDK phosphorylation motif (Fig. 38A).

Potential cellular proteins interacting with the cytosolic domain of Cnx1p.

The cytosolic domain of Cnx1p has been demonstrated to interact with mammalian bak by the yeast two-hybrid system (Torgler *et. al.*1997). The interaction between the cytsolic domain of Cnx1p, between amino acid 525 to 560 of Cnx1p, and mammalian bak was in part responsible for the apoptosis induction (Torgler *et. al.*1997). The cytosolic domain of Cnx1p may interact with other cellular proteins in *S. pombe*. We proceeded to search for potential Cnx1p interacting proteins employing GST fusion constructs containing different domains of the cytosolic tail of Cnx1p.

The carboxyl terminal 50 amino acids of Cnx1p (AA 511 to 56) consisting of the entire cytosolic domain of Cnx1p and two amino acids of the predicted transmembrane domain was either expressed as one construct or divided into three segments and separately expressed. Bacterial expressed GST fusion constructs were purified with glutathione sepharose beads (Fig. 44A). The fusion constructs were incubated with metabolically radiolabeled *S. pombe* cell extracts, prepared as in Fig. 43. The resulting glutathione sepharose beads were washed extensively, resolved by SDS-PAGE and visualized by Coomassie blue stain and radioautography (Fig. 44B). GST was also included to detect non-specific binding proteins. A radiolabeled protein with a relative migration mobility of 150 kDa by SDS-PAGE was found to interact with both the GST-FL (amino acid 511-560) and GST-A (amino acid 511-525) fusion constructs (Fig. 44B, left panel). The 150 kDa protein was not observed with the incubation of GST, GST-B or GST-C containing sepharose beads. The region within amino acid 511 to 525 of Cnx1p is likely to be responsible for the interaction with the 150 kDa protein. The identity of the 150 kDa protein is presently not known.

Figure 38: Sequence of the cytosolic domain of *S. pombe* calnexin and mutants used.

A. The cytosolic domain of precursor *S. pombe* calnexin, Cnx1p (amino acids 511-560) is shown. The amino acid numbering of precursor Cnx1p, including the signal sequence, is used. *FA* (italic type) are amino acids predicted to be the last two residues of the transmembrane domain. Serine residues are in *boldface* type. Ser^{546} and Ser^{553} were either singly or doubly mutated to either alanine or glutamic acid by site-directed mutagenesis (also see Fig. 42).

B. A schematic diagram of the *S. pombe* expression plasmids containing either the full-length *S. pombe cnx1*$^+$ (pCNX 560) or carboxyl terminal truncated *cnx1*$^+$, pCNX 524 and pCNX 484, and pCNX 474 are shown. Plasmids pCNX 524, pCNX 484 and pCNX 474 encode Cnx1p truncated at amino acid 524, 484 and 474, respectively (Parlati *et. al.*1995). The plasmids are expressed in a strain that no longer contain the genomic copy of *cnx1*$^+$ (Parlati *et. al.*1995). Signal sequence (*SS*), the luminal, and the transmembrane (*TM*, AA 490-512) domains are indicated. The proposed BiP-binding region (AA 438-490) is also indicated. The cytosolic domain of pCNX 560 and pCNX 524 are in hatched boxes.

A

```
   WT:511FASSSPASLSTGTTEAEKEQQEKFKQETETEKIDVSYAPETESPTAKNED560
S546A:  ------------------------------------A--------------
S553A:  ------------------------------------------A--------
   AA:  ------------------------------------A------A--------
S546E:  ------------------------------------E--------------
S553E:  ------------------------------------------E--------
   EE:  ------------------------------------E------E--------
```

B

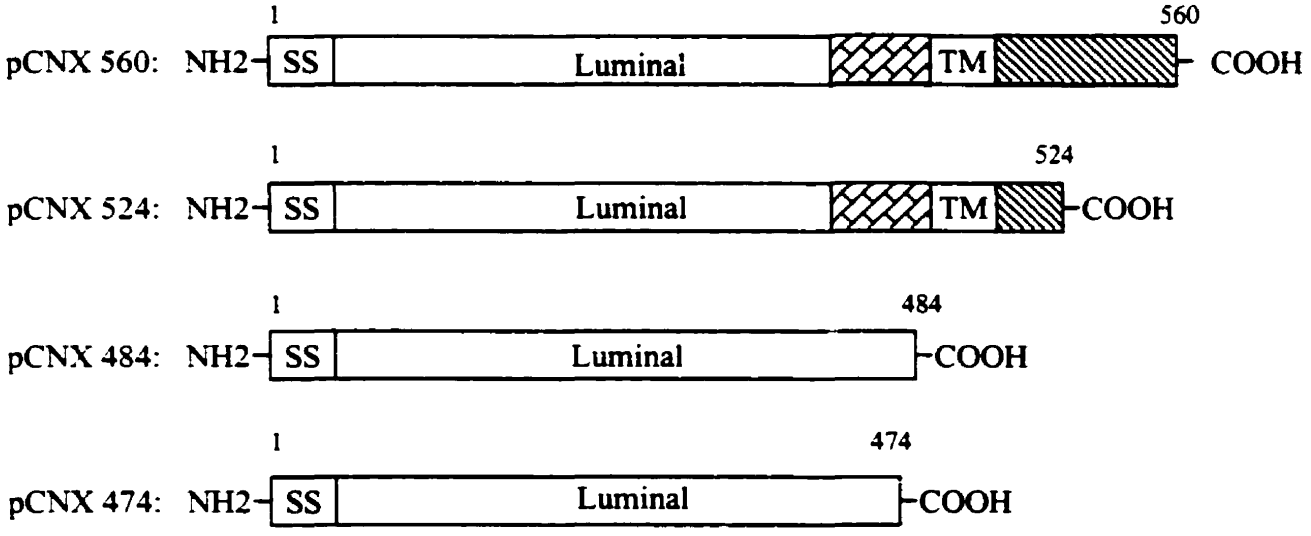


Figure 39: *S. pombe* Cnx1p is *in vivo* phosphorylated on serine residues.

A. *S. pombe* WT (strain Q358), *S. pombe* Δ*cnx1* pCNX560 (p560), and *S. pombe* Δ*cnx1* pCNX524 (p524) were *in vivo* labeled for 4 h with 0.5 mCi/ml of ^{32}P-orthophosphate. Spheroplasts were prepared and solubilized with 1%TX100/0.1%SDS lysis buffer (Parlati *et. al.*1995). Cnx1p was immunoprecipitated with affinity purified anti-Cnx1p antibodies. SDS-PAGE resolved Cnx1p immunoprecipitates were electroblotted onto nitrocellulose membranes and visualized by radioautography for 43 h and then for 6 d (top and bottom panel, respectively) at -70°C with intensifying screens. The mobility of the ^{14}C-molecular standards in kDa is indicated on the left

B. Membrane-bound Cnx1p (Lane 1) was trypsin digested and analyzed by phosphoamino acid analysis as described in Materials and Methods. *Arrows* indicate the direction of electrophoresis towards anode. Radioautograms after a 17-d exposure at -70°C with intensifying screens is shown. Standard phosphoamino acids; phospho-threonine (*pT*), phospho-tyrosine (*pY*), and phospho-serine (*pS*); were visualized by ninhydrin staining and indicated by *dashed circles*. *Pi*, free phosphate; and *o*, origin.

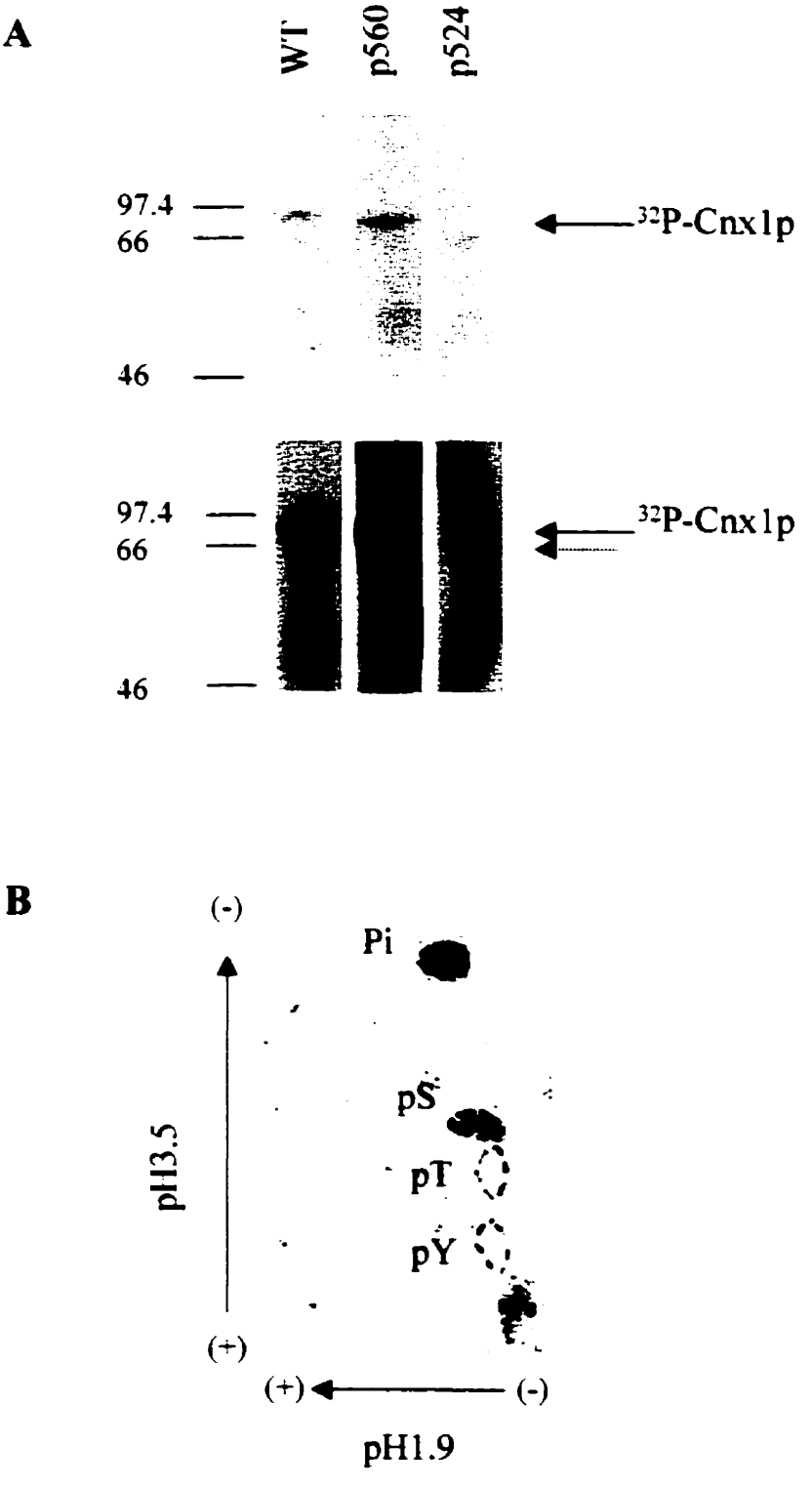

A
WT
p560
p524
97.4
66
46
32P-Cnx1p
97.4
66
46
32P-Cnx1p
B
(-)
Pi
pS
pT
pY
pH3.5
(+)
(+)
(-)
pH1.9

Figure 40: *S. pombe* Cnx1p is *in vivo* phosphorylated on its cytosolic domain.

S. pombe Δ*cnx1* pCNX560 (p560), *S. pombe* Δ*cnx1* pCNX484 (p484), and *S. pombe* Δ*cnx1* pCNX474 (p474) were *in vivo* phosphorylated for 4 h with 1 mCi/ml of ^{32}P-orthophosphate. Approximately $2x10^8$ cells were resuspended in RIPA lysis buffer and lysed with glass beads by manual vortexing as described (Jannatipour *et. al.*1998). Lysates were pre-cleared and Cnx1p was immunoprecipitated. Pre-cleared beads (*PC*) and Cnx1p immunoprecipitates were resolved by SDS-PAGE and visualized by silver staining and radioautography for 6 d at –70°C with intensifying screens. Panels A and B are the radioautogram and the corresponding region of the silver stained polyacrylamide gel, respectively. An *Asterisk* indicates a non-specific binding protein that is also found in PC beads. The mobility of the ^{14}C-molecular standards in kDa is indicated on the left.

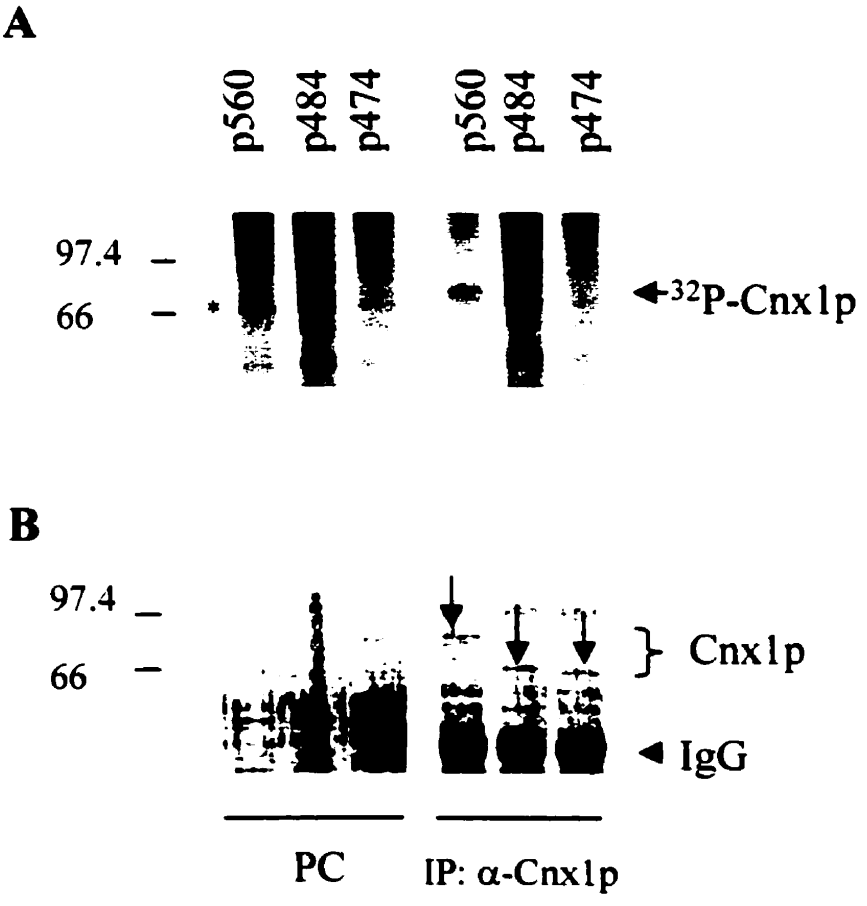

A
p560
p484
p474
p560
p484
p474
97.4
66
*
32P-Cnx1p
B
97.4
66
Cnx1p
IgG
PC
IP: α-Cnx1p

Figure 41: A schematic diagram of pMS plasmid and the strategy used to generate site-directed mutagenized Cnx1p.

A linearized pMS vector modified from pSp72 vector is shown. Restriction sites of interest are indicated. pMS vector contains the coding region of *cnx1*$^+$ (nt -360 to 1683) nmt1 promoter (*nmt*), poly A, *ura4*$^+$ cassette (*ura4*$^+$), and 3' untranslated region of *cnx1*$^+$ (*3' UTR*). PCR fragment containing the site-directed mutagenized amino acid residue, using the CUP-1 primer and mutagenesis primers. The genomic DNA isolated from the *S. pombe* haploid wild type strain (Q 358) was used as the template for the PCR (see Material and Methods). Each 1.9kb-PCR fragment was digested and ligated into pMS vector. The ligation was confirmed by the size of the vector and by sequencing using primers TM-1 and ura4. Modified pMS vector was amplified and used to transform the wildtype *S. pombe* Q358. Ura+ transformants were selected. PCR using primers CUP-3 and ura4 confirmed the homologous replacement of the endogenous *cnx1*$^+$. Sequencing from both ends using primers TM-1 and ura4 confirmed the presence of the mutagenized amino acid residues in Ura4+ transformants. Arrows at the bottom indicate primers. An *asterisk* indicates mutagenized amino acid residue.

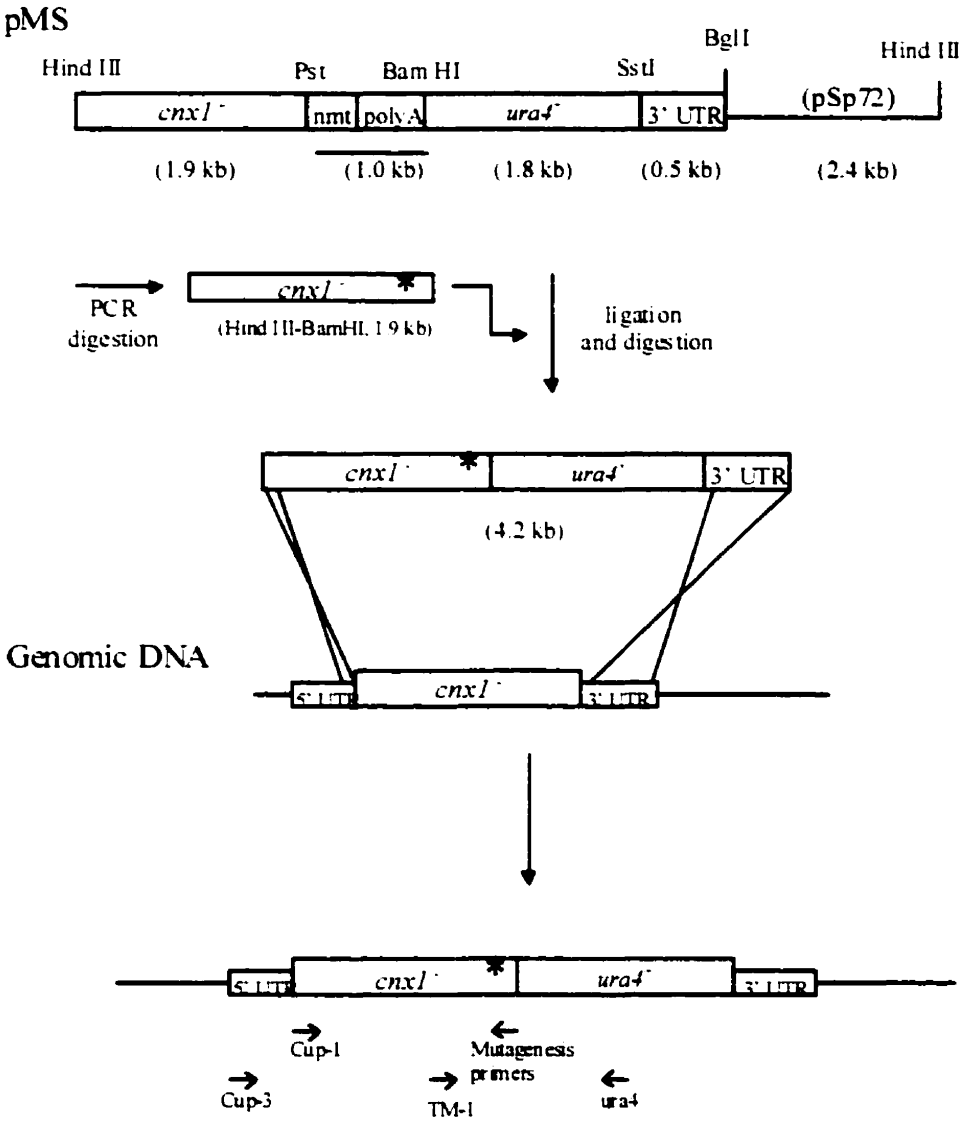

pMS
Hind III
Pst
Bam HI
SstI
BglI
Hind III
cnx1+
nmt
polyA
ura4+
3' UTR
(pSp72)
(1.9 kb)
(1.0 kb)
(1.8 kb)
(0.5 kb)
(2.4 kb)
PCR
digestion
cnx1+
(Hind III-BamHI, 1.9 kb)
ligation
and digestion
Genomic DNA
cnx1+
ura4+
3' UTR
(4.2 kb)
5' UTR
cnx1+
3' UTR
5' UTR
cnx1+
ura4+
3' UTR
Cup-1
Mutagenesis
primers
Cup-3
TM-1
ura4

Figure 42: Metabolic labeling of *S. pombe* Cnx1p point mutants.

S. pombe wildtype (*WT*) and point mutants (*S546A, S553A, AA, S546E, S553E and EE*) were pre-incubated and metabolically pulsed with 0.2 mCi/ml of Trans^{35}S-labels as described (Jannatipour *et. al.*1998). Cnx1p was immunoprecipitated as described in Fig. 41. Pre-cleared beads (*PC*) and Cnx1p immunoprecipitates were separated by SDS-PAGE, enhanced, and visualized by radioautography at -70°C for 19 h. *Asterisks* and *arrow* indicate non-specific binding proteins and Cnx1p, respectively.

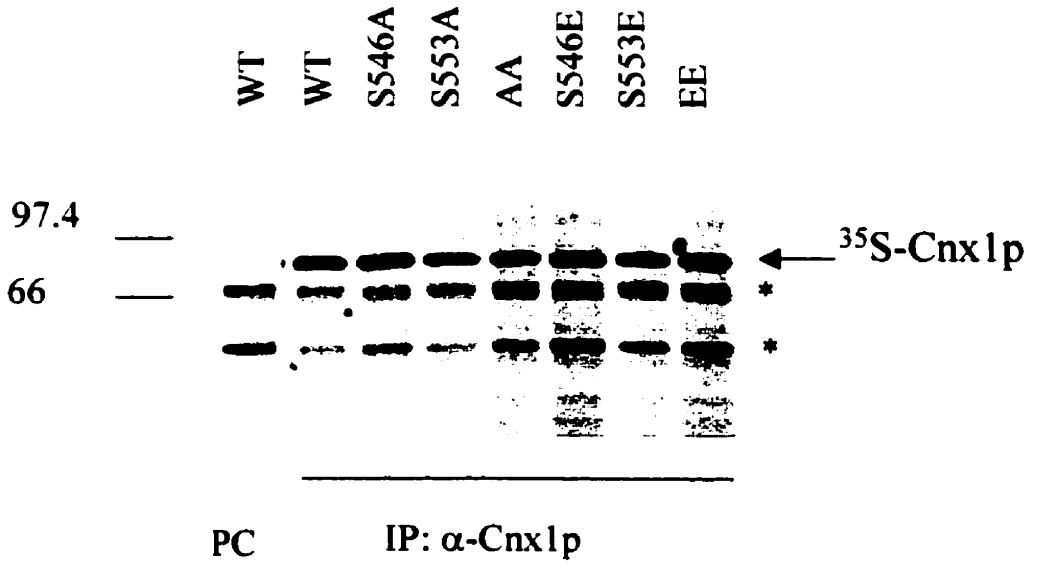
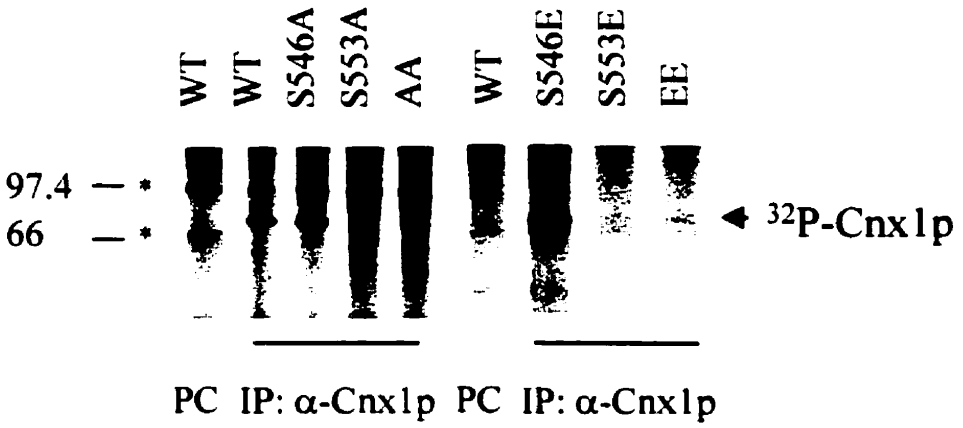

WT
WT
S546A
S553A
AA
S546E
S553E
EE
97.4
66
35S-Cnx1p
PC
IP: α-Cnx1p

Figure 43: *S. pombe* Cnx1p is *in vivo* phosphorylated within a proline-directed kinase motif.

S. pombe wildtype (*WT*) and point mutants (*S546A, S553A, AA, S546E, S553E and EE*) were *in vivo* ^{32}P-labeled and solubilized as in Fig. 41. Lysates were pre-cleared and Cnx1p was immunoprecipitated with affinity purified anti-Cnx1p antibodies. Pre-cleared beads (*PC*) and Cnx1p immunoprecipitates were resolved by SDS-PAGE and visualized by silver staining and radioautography for 7 d at -70°C with intensifying screens. The radioautogram and the corresponding region of the silver stained polyacrylamide gel, panels A and B respectively, are shown. *Asterisks* indicate non-specific binding proteins that are also found in pre-clearing beads (*PC*). The mobility of the ^{14}C-molecular standards in kDa is indicated on the left.

A


WT
WT
S546A
S553A
AA
WT
S546E
S553E
EE
97.4
66
32P-Cnx1p
PC IP: α-Cnx1p PC IP: α-Cnx1p

B

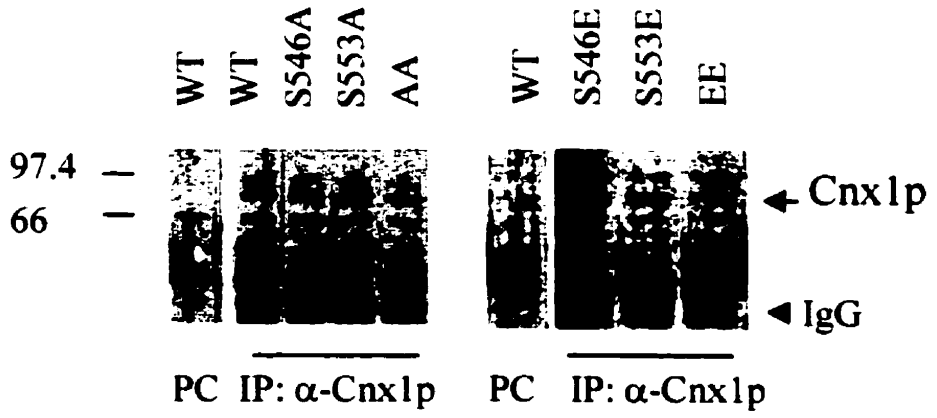
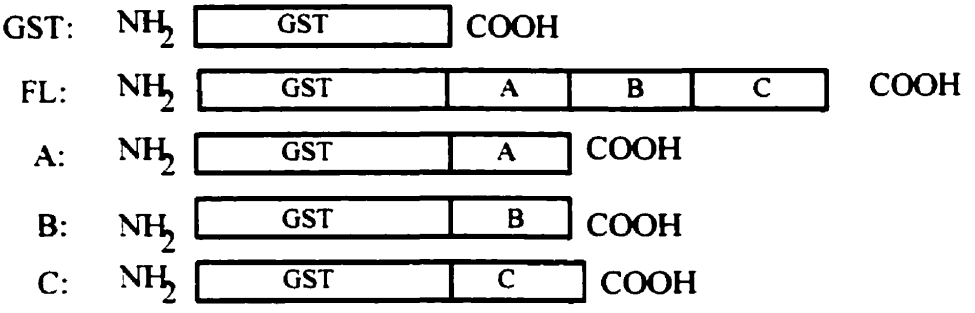

WT
WT
S546A
S553A
AA
WT
S546E
S553E
EE
97.4
66
Cnx1p
IgG
PC IP: α-Cnx1p PC IP: α-Cnx1p

Figure 44: Cnx1p-interacting proteins.

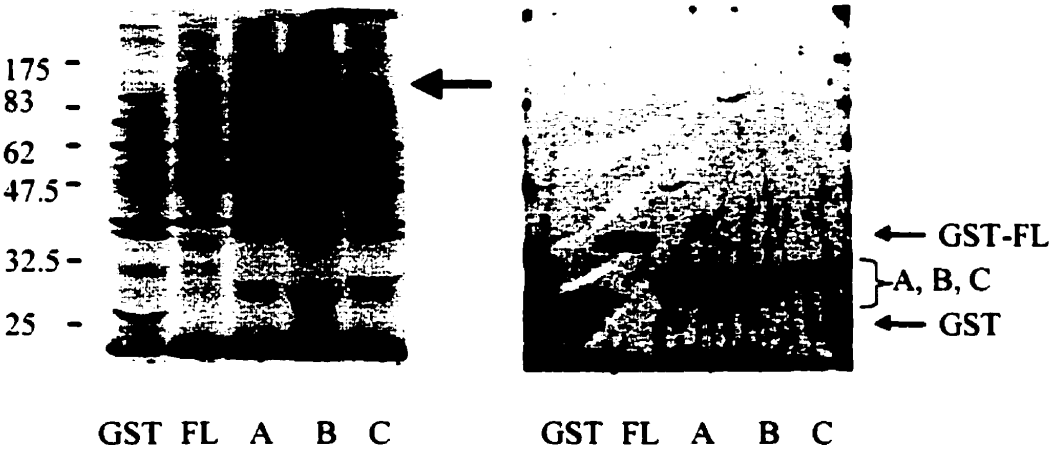
A. A schematic diagram of GST fusion proteins used in this study. The region of amino acid 511 to 560 (Fig. 39A) of *S. pombe* Cnx1p is divided into 3 domains: A (AA 511-525), B (AA 526-542), and C (AA 543-560). *GST*, GST alone; *FL*, GST fusion construct containing the entire region of AA 511 to 560 of Cnx1p.

B. Bacterially expressed GST constructs were isolated with glutathione sepharose beads (see Materials and Methods). An equal amount of Tran^{35}S-labeled *S. pombe* wild type cell extract, prepared as in Fig. 43, was incubated with each GST construct non-covalently coupled onto glutathione sepharose beads. At the end of the incubation, the complexes were washed extensively, resuspended in 1X Laemmeli sample buffer and resolved by SDS-PAGE. The polyacrylamide gel was Coomassie blue stained (right panel), enhanced, and visualized by radioautography for 5 d at -70°C (left panel). A *thick arrow* indicates a potential protein that interacts with the juxtamembrane domain of the cytosolic tail of Cnx1p. GST fusion constructs are indicated with *arrows* and *bracket* on the right. The mobility of the pre-stained molecular standards (New England BioLab) in kDa is indicated on the left.

A


GST:
NH2
GST
COOH
FL:
NH2
GST
A
B
C
COOH
A:
NH2
GST
A
COOH
B:
NH2
GST
B
COOH
C:
NH2
GST
C
COOH

B


175
83
62
47.5
32.5
25
GST-FL
A, B, C
GST
GST FL A B C
GST FL A B C

Discussion

Phosphorylation may represent an additional regulation and function of calnexin with respect to its intralumenal lectin activity. Furthermore, mammalian calnexin is phosphorylated at three invariant serine residues, two of which are within CK2 phosphorylation motifs and one is within a potential PKC/PDK recognition motif (Wong *et. al.*1998) (and Chapter 2). Here, we have examined calnexin phosphorylation in *S. pombe*.

By phosphoamino acid analyses, Cnx1p is *in vivo* phosphorylated on serine residue(s) (Fig. 39). Employing site-directed mutagenesis and *in vivo* radiolabeling, we have identified a potential *in vivo* phosphorylation site of *S. pombe* Cnx1p, Ser^{553}. Mutation of Ser^{553} to either alanine or glutamic acid abolishes the incorporation of ^{32}P-orthophosphate into Cnx1p (Fig. 43). Interestingly, Ser^{553} is found within a PDK phosphorylation motif. It appears that PDK phosphorylation of calnexin is conserved between mammals and *S. pombe* and a similar regulatory mechanism might be utilized in both organisms. PDK phosphorylation may represent a conserved function of calnexin. PDK phosphorylation of mammalian calnexin has been shown to regulate its interaction with ribosomes and ERK-1 has been identified as a candidate kinase responsible for calnexin phosphorylation at its SPR tripeptide motif (Chevet *et. al.*1999) (and Chapter 4). It has been proposed that phosphorylation of calnexin may increase the concentration of calnexin in the proximity of the translocon to couple it to enhanced glycoprotein synthesis (Chevet *et. al.*1999). Phosphorylated Cnx1p at its PDK motif is likely to interact with ribosomes and may perform a similar function as its mammalian counterpart (Chevet *et. al.*1999). Phosphorylation of Cnx1p may also increase its concentration at the translocon to facilitate and couple protein synthesis and co-translocation into the ER.

We cannot, however, exclude the possibility that Ser^{546} is also *in vivo* phosphorylated. Phosphorylation of Ser^{546} may be dependent on prior phosphate incorporation at Ser^{553}. Phosphorylation mimicking by glutamic acid at position 553 may be insufficient to allow phosphorylation at Ser^{546} to occur. This issue may be resolved by

mass spectral analyses of Cnx1p immunoprecipitates, as similarly done for mammalian calnexin in Chapter 2.

Neither abolishing the site of phosphorylation, alanine mutants, nor creating a phosphorylation mimicking, glutamic acid mutants, provide any discernible phenotype. Cells expressing either the wildtype and point mutated Cnx1p were able to grow at both 30°C and 37°C. The cells were able to elicit a similar level of response toward cellular stresses tested here. The non-discernible phenotype displayed by point mutants may correspond to the earlier observation that the cytosolic domain of Cnx1p was dispensable for viability (Parlati *et. al.*1995). The conserved function exerted by PDK phosphorylation (i.e. interaction with ribosomes as deciphered from the conserved phosphorylation motif between *S. pombe* and mammalian calnexins) may not be essential for cellular function in *S. pombe* under normal conditions. The fission yeast may adapt to a post-translational translocation in the absence of co-translational translocation pathway (Brennwald, 1994). In the budding yeast *S. cerevisiae*, impaired translocation of soluble and membrane proteins across the ER membrane was observed in SPR deficient cells (Ogg *et. al.*1992). Cells that were grown for a prolonged period in the absence of SRP or SRP receptor no longer showed pronounced protein translocation defects. Post-translocation via the SRP-independent pathway was suggested to be activated and compensated for the disruption of the co-translocation pathway in the budding yeast (Ogg *et. al.*1992).

Interestingly, a recent study revealed that an essential domain for cell viability was mapped to the carboxyl-terminal 123 amino acid residues of Cnx1p (Elagoz *et. al.*1999). This truncated Cnx1p is consisting of the cytosolic domain (48 AA), the transmembrane domain (23 AA) and the carboxy terminal 52 amino acids of the luminal domain. The essential function(s) of Cnx1p was suggested to reside within the 52 amino acid region and this region appears to share little sequence conservation to calnexins from other species (Elagoz *et. al.*1999). It was suggested that the observed lack of complementation of the luminal domain of canine calnexin to *S. pombe* Cnx1p (Parlati *et. al.*1995), despite the high similarity of the overall luminal domains among calnexins, was due to the

unique sequence of this stretch of 52 amino acids (Elagoz *et. al.*1999). BiP was found in a complex with the full-length Cnx1p and nascent acid phosphatase (Jannatipour *et. al.*1998). The binding of Cnx1p and BiP to acid phosphatase, a glycoprotein with nine potential N-linked glycosylation sites, is independent of glucose trimming and reglucosylation by UGGT (Jannatipour *et. al.*1998). BiP was also found to interact with the 123AA amino-terminal truncated Cnx1p (Elagoz *et. al.*1999). The carboxy terminal 52 amino acids of the luminal domain of Cnx1p is likely the region of Cnx1p interacts with BiP (Elagoz *et. al.*1999).

The coordinated chaperone function exerted by BiP and Cnx1p on protein folding was suggested to enhance the efficiency of yeast's protein synthesis and secretion (Jannatipour *et. al.*1998; Elagoz *et. al.*1999).

Monoglucosylated oligosaccharide formation has been shown to facilitate the efficiency of glycoprotein folding (D'Alessio *et. al.*1999). The UGGT mediated formation of monoglucosylated oligosaccharide was essential for *S. pombe* survival only under extreme cellular stresses (Fanchiotti *et. al.*1998). In *S. pombe* lacking either the activity of glucosidase II (GII) or UGGT, UPR pathway was found to be activated as assessed the induction of BiP mRNA despite the absence of exogenous stress (D'Alessio *et. al.*1999; Fernandez *et. al.*1996). It was suggested that the up-regulation of other chaperones by the UPR pathway may alleviate the stress due to the missing GII and UGGT activities and thus for the absence of a discernible phenotypes (Fanchiotti, *et. al.*1998; D'Alessio *et. al.*1999). The lack of monoglucosylated oligosaccharide generation has been shown to quicken the folding process but with a reduced productive folding (D'Alessio *et. al.*1999). It appears that the retention of monoglucosylated glycoproteins by calnexin may increase the quality and efficiency of glycoprotein folding by the interaction with other chaperones such as BiP and may enhance the survival of *S. pombe* (Fanchiotti *et. al.*1998; Jannatipour *et. al.*1998; D'Alessio *et. al.*1999; Elagoz *et. al.*1999).

Greater divergence was found in the cytosolic domains of calnexin between *S. cerevisiae* and *S. pombe*. *S. pombe* Cnx1p contains a short cytosolic domain (Jannatipour and Rokeach 1995; Parlati *et. al.*1995) whereas *S. cerevisiae* calnexin contains one

cytosolically oriented amino acid (de Virgilio *et. al.*1993; Parlati *et. al.*1995) (Table 3). The cytosolic domain of Cnx1p may exert an additional function or regulation by interacting with other cellular proteins. *S. pombe* Cnx1p has been demonstrated to interact with mammalian bak by the yeast two hybrid assay (Torgler *et. al.*1997). The mammalian bak induced apoptosis in *S. pombe* has been shown to be mediated in part through the interaction with the cytosolic domain (AA 525 to 560) of Cnx1p (Torgler *et. al.*1997). The cytosolic domain of Cnx1p may interact with other cellular proteins in *S. pombe*. We have identified a potential Cnx1p-interacting protein with a relative migration mobility of 150 kDa by SDS-PAGE using fusion protein constructs. The region responsible for the interaction was found to be within amino acid residues 510 to 525 of the cytosolic domain of Cnx1p (Fig. 44) which does not contain the phosphorylation residue, Ser^{553}. The identity of this 150 kDa protein is presently unknown. It remains to be determined whether the interaction between the 150 kDa protein and Cnx1p is phosphorylation dependent.

Here, we have identified a potential *in vivo* phosphorylation site of Cnx1p and detected a protein that interacts with the cytosolic domain of Cnx1p. Analyses of the lectin binding capacity of the phosphorylation Cnx1p mutants and their association with BiP and ribosomes may reveal the possible functions of Cnx1p phosphorylation. The identification of the 150 kDa protein may provide an insight into the possible acquired regulation or function of Cnx1p.

Discussion and Future Perspectives

Calnexin, calmegin/calnexin-t, and calreticulin are the members of a recently identified molecular chaperone family that participates in the quality control of glycoprotein synthesis (Chevet *et. al.*1999; Ellgaard *et. al.*1999; Michalak, 1999). Calnexin and calmegin are phosphorylated at their cytosolic termini (Wada *et. al.*1991; Ohsako *et. al.*1998; Wong *et. al.*1998). Prolonged association of phosphorylated calnexin with a secretion incompetent null Hong Kong variant of α_1-antitrypsin was observed, coinciding with its intracellular retention and degradation (Le *et. al.*1994). Calnexin phosphorylation was also suggested to regulate the rate of protein transport out of the ER (Capps and Zuniga 1994). Furthermore, phosphatase treatment was shown to prolong the association between calnexin and its substrate, MHC class I heavy chain (Tector *et. al.*1994). Phosphorylation of calnexin may provide a potential for intralumenal communication or vice versa. Furthermore, calnexin phosphorylation may represent a prompt mechanism in response to both external and internal stimuli. Here, we have examined calnexin phosphorylation in both cultured mammalian cells and *S. pombe*.

We have attempted to investigate the role of calnexin phosphorylation in ER associated degradation of apoB100 in human hepatoma HepG2 cells (Chapter I). The secretion of ApoB100 has been shown to be regulated mainly at the post-translational level from cultured HepG2 cells (Dashti *et. al.*1989; Pullinger *et. al.*1989; Moberly *et. al.*1990) and in primary rat hepatocytes (Jackson *et. al.*1988; Sparks and Sparks 1990; Bjornsson *et. al.*1992). Reduced apoB100 secretion has been shown to correlate with an increase in intracellular degradation that occurred at a pre-Golgi compartment (Sato, *et. al.*1990; Furukawa *et. al.*1992; Adeli 1994). ApoB100 has been shown to be intracellularly degraded by the ubiquitin-proteasome pathway (Yeung *et. al.*1996). Here, we have examined the temporal interaction between calnexin and apoB100 with supplemented oleic acid in the cultured media (Chapter I, Fig. 4). By co- and sequential immunoprecipitation, we detected that only full-length apoB100 is associated with calnexin (Fig. 4D) despite the presence of oligosaccharide moieties at the amino terminal

of apoB molecules (Yang *et. al.*1986; Yang *et. al.*1989). By the same experiment, we also observed that oleic acid treatment increased the rate of apoB dissociation from calnexin (Fig. 4D) concomitant with an increased in apoB secretion as previously reported (Fig. 4C) (Pullinger *et. al.*1989; Dixon *et. al.*1991; Furukawa *et. al.*1992). Furthermore, calnexin associated apoB molecules may be ubiquitinated by their animosity appearance by SDS-PAGE (Fig. 4D). This was not confirmed here, however, ubiquitin modified apoB molecules have been observed by other investigators (Yeung, *et. al.*1996; Benoist and Grand-Perret 1997; Fisher *et. al.*1997; Zhou *et. al.*1998; Du *et. al.*1999; Sakata and Dixon 1999). Insulin has been demonstrated to reduce the secretion of apoB with a concomitant stimulation of its intracellular degradation in primary rat hepatocytes (Sparks *et. al.*1986; Jackson *et. al.*1988; Sparks and Sparks 1990; Bjornsson *et. al.*1992). The level (or site) of calnexin phosphorylation may be affected by insulin. A plausible quality control effect on apoB molecules mediated by calnexin may exist. An attempt to probe the existence of a new signal transduction pathway from insulin receptors mediated by calnexin was initiated and later abandoned because of the insufficient insulin receptors expressed by our cultured HepG2 cells (Table 4).

Manipulation of the cytosolic domain of calnexin has shed an insight into the possibility of calnexin trafficking along the secretory pathway (Rajagopalan *et. al.*1994). The ER retention motif for calnexin was subsequently mapped to the six amino acids (-RKPRRE) at the most extreme carboxyl terminus of calnexin (Rajagopalan *et. al.*1994). Phosphorylation of calnexin may participate in regulating its trafficking along the secretory pathway. We have attempted to address this possibility employing cultured embryonic dorsal root gangalia (DRG) by *in vivo* radiolabeling and immunoprecipitation. Calnexin appeared to be *in vivo* phosphorylated throughout the cultured DRG (Chapter III, Fig. 25B). The level of calnexin phosphorylation in fibroblast proteins devoid of neuronal samples, however, was much greater than both cell body and neurite enriched fractions combined (Fig. 25). It is not certain whether the calnexin phosphorylation observed in DRG came from calnexin present in the DRG or from the quiescent fibroblasts.

Modification of Ser and Thr residues by nt attachment of O-linked N-acetylglucosamine (O-GlcNAcylation) have been observed for numerous cytosolic and nuclear phosphoproteins that also modified by phosphorylation (Hart *et. al.*1996; Hart 1997). It has been suggested that O-GlcNAc modification may have a reciprocal relationship to phosphorylation. The activity of the phosphoproteins may potentially be regulated by O-GlcNAc modification (Hart *et. al.*1996; Hart 1997). Calnexin could be regulated by O-GlcNAc modification. Using galactosyltransferase to probe for O-GlcNAc modification, we did not detect calnexin modification by O-GlcNAc in our study (Chapter V, Fig. 37).

We have characterized *in vivo* phosphorylation of mammalian calnexin by 2-D tryptic phosphopeptide mapping and mass spectral analyses employing two different cultured mammalian cell lines, HepG2 and MDCK cells (Chapter II). By 2-D phosphoamino acid analyses and 2-D tryptic phosphopeptide mapping analyses, calnexins from both HepG2 and MDCK cells were shown to be serine phosphorylated and displayed a similar mapping pattern (Figs. 6, 8, 9, 12). By co-migration on the same TLC plate, two populations of phosphopeptides could be grossly separated (Fig. 14). Comparison of the sequences of calnexin from both species, we were able to speculate one phosphorylation site at the cytosolic domain of calnexin (Fig. 13). Phosphorylation at the conserved Ser-Pro-Arg (SPR) motif would likely give rise to phosphopeptides observed in group A (Figs. 13 and 14). Phosphorylation of calnexin at its SPR motif could not be dephosphorylated by alkaline phosphastase with the condition used in this study (Fig. 15). Alkaline phosphatase, however, was able to dephosphorylate typtic phosphopeptides designated as group B on the TLC maps (Fig. 15). Furthermore, alkaline phosphatase treatment did not abrogate the interaction between calnexin and its associated substrates (Fig. 16). The significance of this observation is unclear; however, it may provide a tool to dissect and study calnexin phosphorylation. By mass spectral analyses, we were able to identify the *in vivo* phosphorylation sites of calnexin employing non-radiolabeled calnexin immunoprecipitates from both HepG2 and MDCK cells (Fig. 20-23). The identity of calnexin at the migration position of 90 kDa by SDS-PAGE was

confirmed by sequence tag information (Figs. 18 and 19 and Table 6). Mammalian calnexin is *in vivo* phosphorylated on three invariant serine residues, two are within CK2 phosphorylation motifs, and one is within a PKC/PDK phosphorylation motif (Wong *et. al.*1998)(Figs. 14, 15, 18, 20-23).

Employing isolated and intact canine pancreatic microsomes, we were able to study the significance of calnexin phosphorylation *in vitro*. 2-D phosphopeptide mapping analyses revealed that the *in vivo* phosphorylation sites of calnexin are *in vitro* phosphorylated by microsomal associated kinases (Wada *et. al.*1991) (Fig. 26C). Calnexin kinases associated with the microsomes could use either magnesium or manganese cation for the phosphorylation reaction (Fig. 27). Employing various kinases and kinase inhibitors, we were able to provide a general assignment to the phosphopeptides observed on the 2-D tryptic peptide maps (Figs. 28-31). Phosphopeptides group A is likely to correspond to serine phosphorylation at the SPR phosphorylation motif of calnexin. Phosphorylation of two serines within the two CK2 phosphorylation motifs of calnexin are likely to provide the signal observed in phosphopeptides group B on the 2-D tryptic peptide maps (Fig. 26C). We have also identified ERK-1 as a candidate kinase for calnexin phosphorylation based on these characteristics: (1) the sensitivity towards olomoucine and roscovitine (Fig. 30A and 31). (2) the molecular mass of calnexin associated kinase as obtained by in-gel kinase assay (Fig. 32A), and (3) the ability of activated recombinant ERK-1 to phosphorylate calnexin in vitro (Fig. 32B). . However, we cannot exclude the other possible PDK kinase that can phosphorylate calnexin at its SPR phosphorylation motif. The observation that the SPR site could be phosphorylated *in vitro* by microsomal associated kinases using GTP nucleotide as the phosphate donor (Fig. 26C). We have not excluded the possibility that the exchange of GTP to ATP might occur for SPR phosphorylation by microsomal associated kinases. This issue should be resolved by including the ATP glycerol kinase in the presence of [γ-^{32}P]-GTP as previously performed (Ou, Thomas *et. al.*1992) and followed by phosphopeptide mapping analyses.

Employing heat-inactivated intact microsomes, calnexin was shown to interact with ribosomes in a phosphorylation dependent manner (Figs 33 and 34). It was proposed that calnexin phosphorylation may increased calnexin concentration in the proximity to the translocon and couple it to enhanced glycoprotein synthesis (Chevet *et. al.*1999).

We have also shown that *S. pombe* Cnx1p is *in vivo* serine phosphorylated within a potential proline-directed kinase (PDK) phosphorylation motif by site-directed mutagenesis (Fig. 39, 40, and 43). Employing fusion protein constructs containing different regions of the cytosolic domain of Cnx1p, a Cnx1p-interacting protein with a relative migration mobility of 150 kDa by SDS-PAGE was also observed (Chapter VI, Fig. 44).

Mammalian calnexin is *in vivo* phosphorylated on three invariant serine residues, two are within CK2 phosphorylation motifs and one is within a PKC/PDK phosphorylation motif (Wong *et. al.*1998) (Figs. 14, 15, 18, 20-23). The identification of CK2 phosphorylation sites coincides with earlier observation that calnexin can be phosphorylated *in vitro* by CK2 (Ou *et. al.*1992; Cala *et. al.*1993). The PKC/PDK phosphorylation motif identified here is highly conserved among mammalian calnexins (Fig. 23). Furthermore, *S. pombe* calnexin is phosphorylated within a PDK phosphorylation motif (Figs. 39, 40, and 43). The conserved phosphorylation motif shared by both mammalian and *S. pombe* calnexins may represent a conserved function in both organisms.

Calnexin was originally co-purified along with three integral membrane proteins from isolated canine pancreatic microsomes (Wada *et. al.*1991). They are TRAPα (previously known as pgp35 or SSRα), TRAPβ (previously known as gp25H or SSRβ) and gp25L (Prehn *et. al.*1990; Wada *et. al.*1991; Hartmann *et. al.*1993; Dominguez *et. al.*1998). TRAPα and TRAPβ are components of a tetrameric complex (Hartmann *et. al.*1993). The association of calnexin with components of TRAP complex (TRAPα and TRAPβ) envisages a possible interaction between calnexin and the translation and translocation machinery. The availability of calnexin in the vicinity of the translocon would enhance the interaction between calnexin and nascent N-linked glycoproteins

during their translocation and/or upon their release from the translocon. Indeed, it was demonstrated in our *in vitro* system that calnexin interacts with ribosomes and the interaction is regulated by calnexin phosphorylation (Fig. 33). Furthermore, the interaction between calnexin and ribosome was shown to be regulated by the activity of MAPK cascade *in vivo* (Chevet *et. al.*1999). Calnexin phosphorylation may increase its concentration to the translocon to facilitate glycoprotein synthesis and translocation into the ER.

TRAPα (or SSRα) is another kinase substrate for CK2 in isolated microsomes *in vitro* (Ou *et. al.*1992). Primary sequence analysis of TRAPα revealed a Ser-Pro-Arg (SPR) sequence, which is the same phosphorylation motif identified in calnexin, in addition to a CK2 phosphorylation motif (Fig. 36). Our *in vitro* study with kinase inhibitors showed that the phosphorylation of TRAPα, by microsomal associated kinases, is subjected to a similar level of inhibition as calnexin phosphorylation (Fig. 30B). The function of TRAPα is still unclear. Similar to calnexin, TRAPα was found to associate with ribosomes (Gorlich *et. al.*1992; Chevet *et. al.*1999). Proteoliposomes reconstituted from a detergent extract that had been immunodepleted of TRAPα showed unimpaired translocation activity (Migliaccio *et. al.*1992). Phosphorylation and dephosphorylation may regulate the *in vivo* function of TRAPα. It is probable that the phosphorylation function of both calnexin and TRAPα is subjected to a common regulatory mechanism and their functions are tightly coordinated and regulated. It remains to be determined whether the interaction between the ribosome and the TRAPα is also regulated by its phosphorylation.

Phosphorylation of microsomal membrane proteins by PKC was recently shown to enhance the translocation efficiency *in vitro* (Gruss *et. al.* 1999). Three PKC phosphorylated microsomal proteins were identified by immunoprecipitation and they are TRAM (translocating chain associated membrane protein), Sec61β and docking protein α (DPα or signal sequence particle receptor α subunit) (Gruss *et. al.* 1999). Interestingly, primary sequence analyses of the available TRAM protein sequence from human (Accession no. CAA45218), canine (Accession no. CAA45217) and *C. elegans*

(Accession no. CAA18772) showed that all three TRAM proteins contain a potential Ser-Pro-Arg (SPR) phosphorylation motif at their carboxyl termini. These cytosolic domains of TRAM, however, do not contain any putative CK2 phosphorylation motif. Neither Sec61β nor DPα contains a proline-directed kinase (PDK) phosphorylation motif from the available sequences on the database. TRAM was originally identified as a 36 kDa polytopic glycoprotein of the ER that cross-linked to translocating nascent peptides (Gorlich *et. al.* 1992). TRAM has been shown to be essential for the integration of two tested proteins, preprolactin and prepro-α-factor, in reconstituted proteoliposomes (Gorlich *et. al.* 1993). It has been suggested that TRAM might serve to orient the signal sequence for translocation (Gorlich *et. al.* 1992). A common signaling cascade may regulate the SPR phosphorylation motifs of TRAM, TRAPα, and calnexin. It is probable that the phosphorylation of the SPR motifs of TRAM, TRAPα, and calnexin may allow a rapid recruitment for them to interact with ribosomes and facilitate nascent protein synthesis and translocation across the ER. Dephosphorylation by a phosphatase at the SPR motif may allow a rapid dissociation of calnexin, TRAM, and TRAPα from the translocation machinery (Fig. 45A).

Calnexin was recently found to interact with SERP1 (stress-associated ER protein 1) (Yamaguchi *et. al.* 1999). The cDNA sequence of SERP1 revealed that it is identical to RAMP4 (ribosome-associated membrane protein 4). SERP1/RAMP4 is a type I integral membrane protein consisting of 66 amino acids (Gorlich *et. al.* 1993). Hypoxia or stress that induced the UPR pathway also enhanced the expression of SERP1. Overexpression of SERP1/RAMP4 has been shown to suppress aggregate formation (Yamaguchi *et. al.* 1999). SERP1/RAMP4 was found to interact with nascent integral membrane proteins and facilitate their glycosylation (Yamaguchi *et. al.* 1999)(Schroder *et. al.* 1999). RAMP4 may act in concert with calnexin to facilitate the glycosylation and folding of integral membrane glycoproteins.

The retention exerted by the lectin binding of calnexin may act positively to facilitate glycoprotein folding and assembly and negatively to mediate the disposal of

misfolded proteins by retrograde translocation and degradation by the cytosolic proteasome (Chevet *et. al.*1999; Ellgaard *et. al.*1999).

Calnexin was found in a complex with ubiquitinated apoB and the translocon subunit, Sec61β. We also showed that only full-length apoB molecules are associated with calnexin (Fig. 4D). In our study, calnexin associated apoB100 may be ubiquitinated by its smearing appearance (Fig. 4D) which is similar to previously reported by other investigators (Yeung *et. al.*1996; Benoist and Grand-Perret 1997; Fisher *et. al.*1997; Zhou *et. al.*1998; Du *et. al.*1999; Sakata and Dixon 1999). This may suggest a role of calnexin in mediating the retrograde translocation for apoB degradation by the cytosolic proteasome machinery (Chen *et. al.*1998). Calnexin was also suggested to participate in the ubiquitination of the PiZ variant of α_1-antitrypsin. In this study, calnexin was found to be ubiquitinated (Qu *et. al.*1996). Retrograde translocation of the truncated and soluble Ribophorin I_{332} (RI_{332}) or its release into the cytosol for cytosolic proteasomal degradation was prevented under ubiquitination-deficient condition. Under this condition, the truncated RI_{332} remained membrane bound and protease resistant prior ubiquitination. By co-immunoprecipitation, RI_{332} was found in an association with calnexin and sec61β. Ubiquitination was suggested to trigger the release of RI_{332} from calnexin and mediate the retrograde translocation of RI_{332} to the cytoplasm (de Virgilio *et. al.*1998). Calnexin binding to RI_{332} may provide an opportunity for it to fold since the removal of its N-glycosylation site accelerates its degradation (de Virgilio *et. al.*1999). Alternatively, calnexin binding may provide the membrane anchorage to retain the misfolded glycoproteins in the close proximity to the translocon and mediate the retrograde translocation.

The translocon has been proposed to act as a passive channel (Matlack *et. al.* 1998)(Romisch *et. al.* 1999). The translocon associating proteins were suggested to modulate the direction of protein trafficking via the translocon. Signal sequence targeting of nascent polypeptides and ribosome binding to the translocon may direct protein translocation into the ER channel (Matlack *et. al.* 1998). Der1p, Hrd1p, and Der1p/Hrd3p are potential candidates that could modulate and direct the retrograde

translocation of misfolded proteins in yeast (Knop *et. al* 1996)(Plemper *et. al.* 1999)(Bordallo *et. al* 1998). Mannose trimming was also suggested in signaling and/or directing the degradation of numerous misfolded glycoproteins both in yeast and in mammalian cells (Liu *et. al.*1997; Jakob *et. al.*1998; Yang *et. al.*1998). It is possible that G_1M_8 modified glycoprotein binding to calnexin may induce calnexin phosphorylation, hypothetically, at its CK2 motifs. Hence, phosphorylation of calnexin may regulate the interaction between the channel and accessory molecules in the cytosol or in the ER membrane to facilitate the retrograde transport of misfolded proteins into the cytosol. CK2 phosphorylation may exert this effect since CK2 phosphorylated calnexin was shown to have a negative binding to ribosome (Fig. 33). CK2 phosphorylated calnexin may prevent the ribosome from binding to the translocon and thus prevent obtrusion for the misfolded proteins to enter the cytosol from the ER lumen.

Hypothetically, SPR phosphorylation of calnexin, TRAM, and TRAPα may enhance the association between ribosomes and the translocon directly and subsequently enhance protein translocation into the ER. Removal of the phosphate moiety from the SPR motif by a phosphatase from the triply phosphorylated calnexin would disengage the ribosome binding to the translocon. CK2 phosphorylated calnexin may recruit other accessory proteins to the translocon and mediate the retrograde translocation of misfolded protein for cytosolic degradation. TRAPα may or may not act in concert with calnexin in the disposal of misfolded proteins (Fig. 45A).

Reconstituted proteoliposomes consisting of Sec61 complex, SRP receptor and TRAM were the minimal requirement for protein translocation (Gorlich et. al 1993). It is probable that the calnexin is not essential for the translocation process but rather this process may be facilitated by calnexin. Protein synthesis and the expression of MHC class I molecules were not affected in a calnexin-deficient cell line, CEM-NRK (Scott and Dawson 1995). Transient transfection of calnexin did not alter the resistance of CEM-NRK cells towards the cytotoxicity of natural killer cells. Calnexin expression however was shown to correlate with the increased expression of two adhesion molecules, CD44 and LFA-1α, examined in CEM-NRK cells (Malyguine *et. al.* 1998).

Microsomes from both CEM and calnexin-transfected cells could be isolated to examine the role of calnexin in ERAD *in vitro*. The fate of *in vitro* translated substrates such as null Hong Kong variant α_1-antitrypsin and T cell receptor alpha subunit can be followed in the reconstituted proteoliposomes. Null Hong Kong variant of α_1-antitrypsin and unassembled TcR alpha subunit are known substrates for proteasomal degradation (Liu, *et. al.*1997; Yang *et. al.*1998). A modified ERAD assay employed by McCracken and Brodsky (McCracken and Brodsky 1996) and Xiong *et. al.* (Xiong *et. al.* 1999) could be used to dissect the role of calnexin here. Calnexin-deficient microsomes are likely translocation competent based on the observation with CEM-NRK cells (Scott and Dawson 1995) and reconstituted proteoliposome (Gorlich *et. al.* 1993). The fate of *in vitro* translated and translocated substrates could be assessed by the incubation with either ATP, cytosol or proteasomal inhibitors. Hypothetically, plasmids encoding calnexin phosphorylation mutants could be transfected into the CEM cells to assess the role of calnexin phosphorylation in protein folding and in ERAD.

In addition to the molecular chaperone function, calnexin may participate in the ER signaling and the maintenance of ER calcium homeostasis.

Calnexin and TRAPα are the two major calcium-binding proteins of the ER membrane (Wada *et. al.*1991). Calnexin expression has been shown to be induced with calcium ionophore treatment in *S. pombe* (Jannatipour and Rokeach 1995; Parlati, *et. al.*1995). Calnexin phosphorylation may participate in the maintenance of the ER calcium concentration that was suggested by the study conducted by Roderick *et. al.* (Roderick *et. al.*1998). In their system, they showed that co-expression of calnexin, calmegin, or calreticulin exerted an inhibitory effect on SERCA2b, a Ca^{2+}-ATPase that is responsible for the uptake of calcium into the ER lumen (Meldolesi *et. al.* 1998). The inhibitory effect of calreticulin was dependent on its lectin binding to SERCA2b (John, *et. al.*1998). The inhibitory effect exerted by both calnexin and calmegin was abolished when the consensus PKC phosphorylation site at their most carboxyl termini was removed (Roderick *et. al.*1998). In mammalian calnexin, the PKC phosphorylation motif is also a potential PDK phosphorylation motif with a Ser-Pro-Arg (SPR) sequence (Fig.

23). It is possible that calnexin may detect the calcium level within the ER in concert with other calcium-binding proteins including calreticulin, BiP and GRP94 (Michalak *et. al.* 1998)(Michalak *et. al.* 1999)(Chevet *et. al.*1999). Calcium binding to the luminal domain of canine calnexin has been shown to induce a conformational change as determined by its sensitivity towards protease K digestion (Ou *et. al.*1995; Vassilakos, *et. al.*1998). At a low calcium concentration, calnexin binding to monoglucosylated oligosaccharides was abolished *in vitro* (Vassilakos *et. al.*1998). Calcium has been shown to be required for calnexin binding to murine MHC class I molecules *in vivo* (Capps and Zuniga 1994) and α_1-antitrypsin *in vitro* (Le *et. al.*1994). Furthermore, the luminal domain of calnexin was found in an oligomeric complex *in vitro* in the presence of low calcium content (Ou *et. al.*1995; Vassilakos *et. al.*1998). It is unclear whether the effect of calcium on calnexin also exists *in vivo* although hetero-oligomeric formation between the full-length and truncated calnexins has been observed *in vivo* (Ho *et. al.*1999). It is probable that at a high ER calcium concentration, calnexin may exist primarily as monomers in association with nascent glycoproteins (Fig. 45B, high ER calcium panel, and pathway 1). Substrate associated calnexin is still phosphorylated at its SPR motif (Figs. 15 and 16). Dephosphorylation of calnexin at its CK2 motifs did not abolish the association with its substrates (Figs. 15 and 16). SPR-phosphorylated calnexin may exert an inhibitory effect on SERCA2b to maintain the level of ER calcium (Fig. 44B, high ER calcium panel, and pathway 2). Disturbance of luminal ER calcium storage with thapsigargin, which inhibits the Ca^{2+}-ATPase (SERCA), or ionophore, which depletes the ER calcium pool, has been shown to block protein processing and causing partially folded proteins to accumulate [Kuznetsov, 1993 #548]. The accumulation of unfolded proteins activates the UPR pathway and enhances the transcription of ER chaperone genes (Sidrauski *et. al.*1998; Kaufman 1999). Loss of ER Ca^{2+} also induces eIF-2α phosphorylation causing a general inhibition of translation effects that are attenuated by prior induction of ER stress proteins (Hinnebusch 1994). In low ER calcium concentrations resulting from the disturbance of the ER or the induction of thapsigargin and/or calcium ionophore, calnexin binding to glycoproteins is

presumably abrogated or prevented (Capps and Zuniga 1994; Le *et. al.*1994; Vassilakos, Michalak *et. al.*1998). Non-calcium binding and substrate free calnexin may oligomerize and dephosphorylate at its SPR motif. Alternatively, oligomerization of free calnexin may prevent calnexin from being phosphorylated at its SPR motif in a low calcium concentration environment. Non-SPR phosphorylated calnexin is not associated with ribosomes (Fig. 33). The removal of calnexin from the translocation machinery may act in accordance with eIF-2 α phosphorylation that inhibits protein synthesis (Fig. 45B, low ER calcium panel, and pathway 1). Non-SPR-phosphorylated calnexin may remove its inhibitory effect on SERCA2b and the level of ER calcium will return to the norm with the uptake of calcium by SERCA2b (Fig. 45B, low ER calcium panel, and pathway 2). Calnexin phosphorylation may act in concert with UPR pathway. When calnexin binding was inhibited by castanospermine, numerous calnexin substrates were found to form complex with BiP (Hammond and Helenius 1994; Balow *et. al.*1995; Gaudin 1997; Zhang *et. al.*1997; de Virgilio *et. al.*1999) (Fig. 46B, low ER calcium panel, and pathway 3). This may indirectly activate the UPR pathway. The ligand for Ire1p and PERK, the unfolded protein sensor of the UPR pathway, is presently unknown. The amount of free BiP has been suggested to be essential for the activation of UPR pathway (Fig. 45B, pathway 3). It has been suggested that the UPR pathway containing a sensing mechanism to detect the changes either in the concentration of complexes formed between BiP and unfolded proteins or in the concentration of free BiP (Chapman *et. al.*1998). Overexpression of BiP has been shown to down-regulate the UPR pathway in both cultured CHO cells and in yeast (Sidrauski *et. al.*1998). In a low ER calcium concentration depicted here, the amount of free BiP is presumably reduced by complexing with calnexin substrates and the amount of BiP complexes formed between BiP and unfolded protein is increased. The induction of BiP expression has been observed when the generation of monoglucosylated substrates was hindered by the lack of glucosidase II activity in mammalian cells (Balow *et. al.*1995). An induction of BiP expression was also observed in *S. pombe* when either the activity of GII or UGGT was missing (D'Alessio *et. al.*1999)(Fernandez *et. al.* 1996). Activation of the UPR pathway will increase the

synthesis of ER chaperone proteins via Ire1p (Shamu and Walter 1996; Tirasophon *et. al.*1998) and will halt protein synthesis to alleviate stress by eIF-2α phosphorylation via PERK (Harding *et. al.*1999) (Fig. 45B, low ER calcium panel, and pathway 3). Hence, ER stress induced by perturbation of calcium homeostasis may be combated in a coordinated manner by calnexin phosphorylation. Dephosphorylation of calnexin at its SPR motif may (1) reduce the efficiency of protein translocation into the ER to prevent further stress to the ER, (2) remove its inhibitory effect on SERCA2b to re-fill the ER calcium store, and (3) indirectly activate the UPR pathway (Fig. 45B). The restoration of ER calcium homeostasis, potentially by dephosphorylation of calnexin at its SPR motif, would allow the interaction among the ER chaperones to confront the accumulation of unfolded proteins previously induced by calcium depletion. At the replenished calcium concentration, calnexin will presumably resume its monomeric structure and assist protein folding or mediate the degradation of misfolded proteins incurred during the period of low ER calcium concentration. Regulation of the SERCA2b activity by calnexin phosphorylation may represent a prompt response toward stress condition. It would be of interest to determine whether TRAPα also participates and/or acts in concert with calnexin in maintaining the ER calcium homeostasis and activation of UPR pathway.

The stretch of positively charged amino acid residues (^{551}RKPKAEEDEI LNRSPRNRKPRRE^{573}, Fig. 13) at the carboxy-terminal of the mammalian calnexin displays a reminiscence to the bipartite nuclear localization sequence (NLS) observed in some nuclear localized proteins including *S. cerevisiae* transcription factor SW15 (Jans, *et. al.* 1995)(Jans *et. al.* 1996). SW15, which is involved in yeast mating type switching, is excluded from nuclear by cyclin dependent kinase (cdk) CDC28 phosphorylation. Mutation of the two cdk phosphorylation serines to alanines, one of which is found within the spacer of the bipartite NLS, of SW15 has been shown to be sufficient to render its constitutive nuclear localization. It was suggested that the phosphorylation of SW15 by cdk prevents the nuclear translocation of SW15 (Jans, *et. al.* 1995)(Jans *et. al.* 1996). The localization of SV40 T-Ag has been suggested to be regulated by dual kinase

activities in the vicinity of its NLS. The nuclear transport of SV40 T-Ag is enhanced by the phosphorylation at its CK2 motif. Phosphorylation of SV40 T-Ag at its cdk site reduces its nuclear accumulation (Jans, *et. al.* 1995)(Jans *et. al.* 1996). Calnexin has been observed to associate with the nuclear envelope (Hochstenbach *et. al.*1992; Gilchrist and Pierce 1993). It has been suggested that calnexin may participate in the maintenance of nuclear matrix calcium concentration (Gilchrist and Pierce 1993). It remains to be determined whether the observed nuclear localization of calnexin is phosphorylation regulated.

By yeast genetics, calnexin is essential in *S. pombe* (Jannatipour and Rokeach 1995; Parlati *et. al.*1995). Calnexin phosphorylation does not appear to be essential in *S. pombe* (Chapter VI) and this may correlate with the earlier observation that the cytosolic domain of Cnx1p is dispensable (Parlati *et. al.*1995). Calreticulin has been shown to affect the embryonic development of cardiac system in mice (Mesaeli *et. al.*1999). It would be of interest to determine the role of calnexin and/or calnexin phosphorylation in early embryonic development. One could replace the endogenous calnexin with non-phosphorylated calnexin in the embryonic stem cells to assess the role of calnexin phosphorylation in embryonic development and cellular function.

Here, we have characterized the *in vivo* phosphorylation of calnexins from both cultured mammalian cells and *S. pombe*. We found that the proline-directed serine phosphorylation site is utilized in both organisms. Proline-directed calnexin phosphorylation may represent a conserved function in both organisms. Calnexin in both organisms may participate in the chaperone function and the phosphorylation of calnexin may participate in detecting the stress anticipated by the ER. We have also identified that ERK-1 is a potential kinase for mammalian calnexin phosphorylation at the SPR motif. Calnexin phosphorylation may participate in the above-mentioned function. It would be of interest to examine whether calnexin phosphorylation participates in its trafficking along the secretory pathway. We have attempted to examine the possibility of calnexin trafficking employing cultured dorsal root ganglia. Calnexin was shown to be phosphorylated in both cell bodies and neurite enriched fractions (Fig. 25B). It is also

possible that the calnexin may be dephosphorylated by the phosphatase presence in the neurite-enriched fraction (Fig. 25). The phosphatase(s) that regulates calnexin phosphorylation have not yet been identified although okadaic acid was shown to increase calnexin phosphorylation *in vivo* (Schue *et. al.* 1994). Hence, neurite enriched fraction is a potential source for the purification and identification of the phosphatase(s) that is responsible for calnexin dephosphorylation. The identification calnexin phosphatase(s) will allow us to examine the role of calnexin phosphorylation in the above mentioned functions.

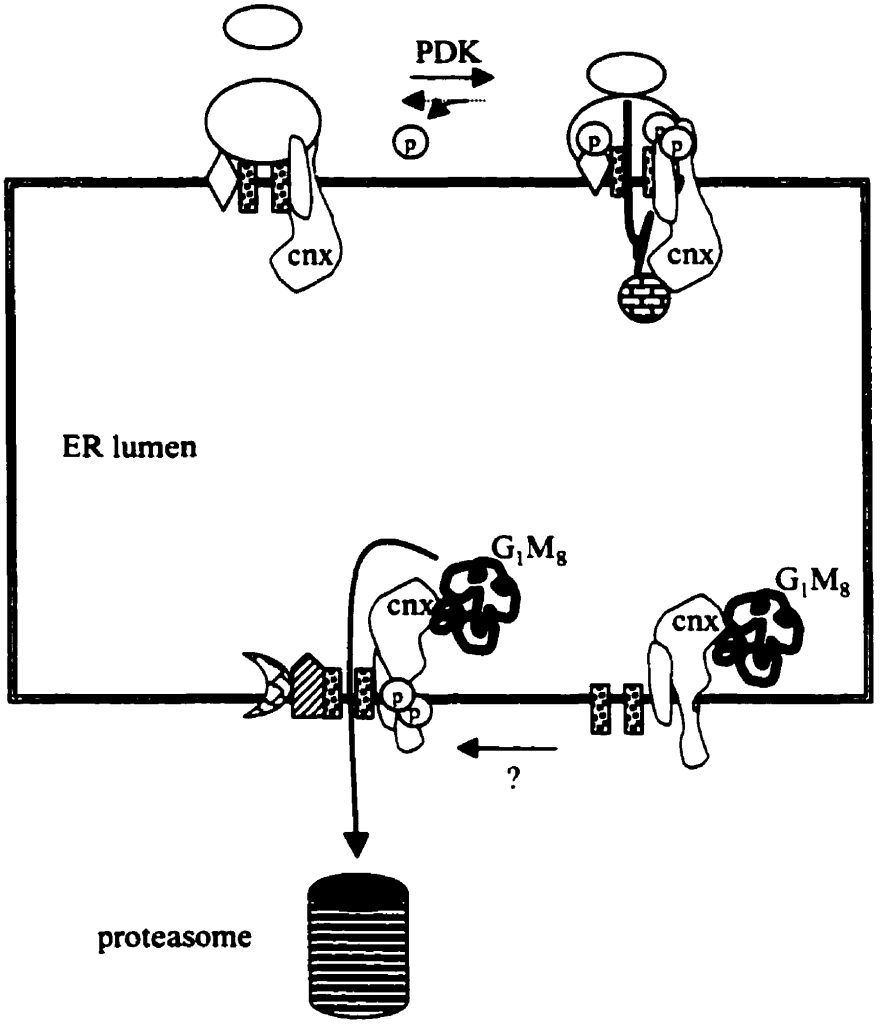
Calnexin phosphorylation may represent a constituent of the UPR pathway or a novel ER signaling cascade that is involved in both the quality control mechanism of protein folding and the calcium homeostasis. The elucidation of the regulatory mechanism that triggers calnexin phosphorylation will allow us to gain new insights into the intricacy and complexity of the ER functions.

Figure 45: Potential functions of calnexin phosphorylation.

A. Calnexin phosphorylation may modulate the direction of protein trafficking across the ER membrane. Phosphorylation of calnexin, TRAPα, and TRAM at their SPR motifs may recruit ribosomes to the translocon and facilitate nascent glycoprotein translocation into the ER lumen. Dephosphorylation of calnexin, TRAPα, and TRAM at their SPR motifs may disengage the ribosomes at the cytosolic surface of the ER. Phosphorylation of calnexin (and TRAPα) by protein kinase such as CK2 may recruit other unidentified factors to the translocon and modulate its direction of the traffic. The binding to G_1M_8 glycoprotein (G_1M_8) that signals for degradation may induce calnexin (and TRAPα) phosphorylation by CK2 or vice versa. Misfolded proteins may then be presented for degradation by cytosolic proteasome.

B. Calnexin may act as a modulator of ER stress caused by the disturbance of ER calcium homeostasis. At a filled ER calcium store, calcium-binding calnexin interacts with nascent glycoproteins and facilitate its folding along with components of the calnexin cycle including ERp57, glucosidase II, UGGT, and other chaperones within the ER (not shown here). Calnexin is presumably phosphorylated at its SPR motif. Calnexin interacts with SERCA2b and inhibits its calcium intake. At a low ER calcium store, which could be induced by calcium ionophore, calnexin binding to glycosylated substrates is abrogated (1). Calcium- and substrate-free calnexin may oligomerize and may dephosphorylate at its SPR motif. These two events could happen independent of each other or calnexin oligomerization may not occur at all. Dephosphorylated calnexin at its SPR motif may remove its inhibitory effect on SERCA2b and may increase calcium intake to bring calcium level back to normal (2). Partially folded substrates from calnexin may interact with BiP (3). This will reduce the amount of free BiP in the ER and potentially activate the UPR cascade. Activation of Ire1p will increase the synthesis of ER chaperone and activation of PERK will phosphorylate eIF-2α to halt protein synthesis to alleviate stress. As the intake of calcium by SERCA2b and the removal of ionophore bring the calcium level to the fill-stored level, calnexin may resume its monomeric form and interacts with monoglucosylated glycoprotein. Misfolded glycoproteins would be targeted for degradation as depicted in A. The level of free BiP is resumed and UPR pathway is halted. Calnexin may resume its inhibitory effect on SERCA2b.

A


PDK
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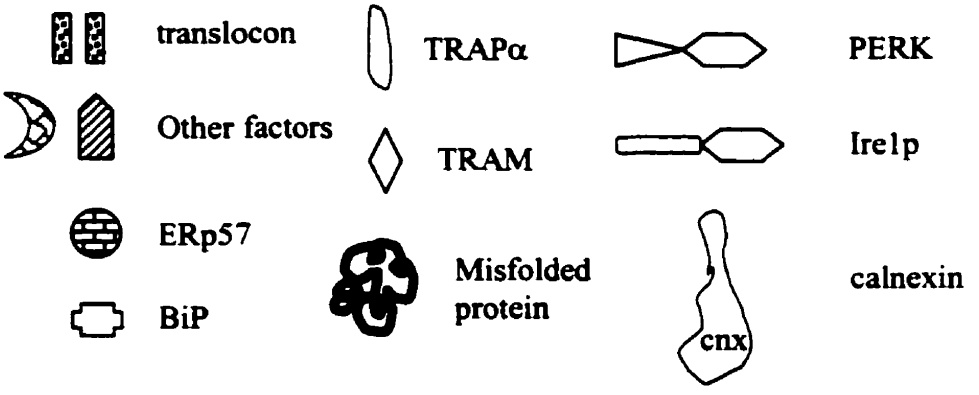
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TRAM
Misfolded protein
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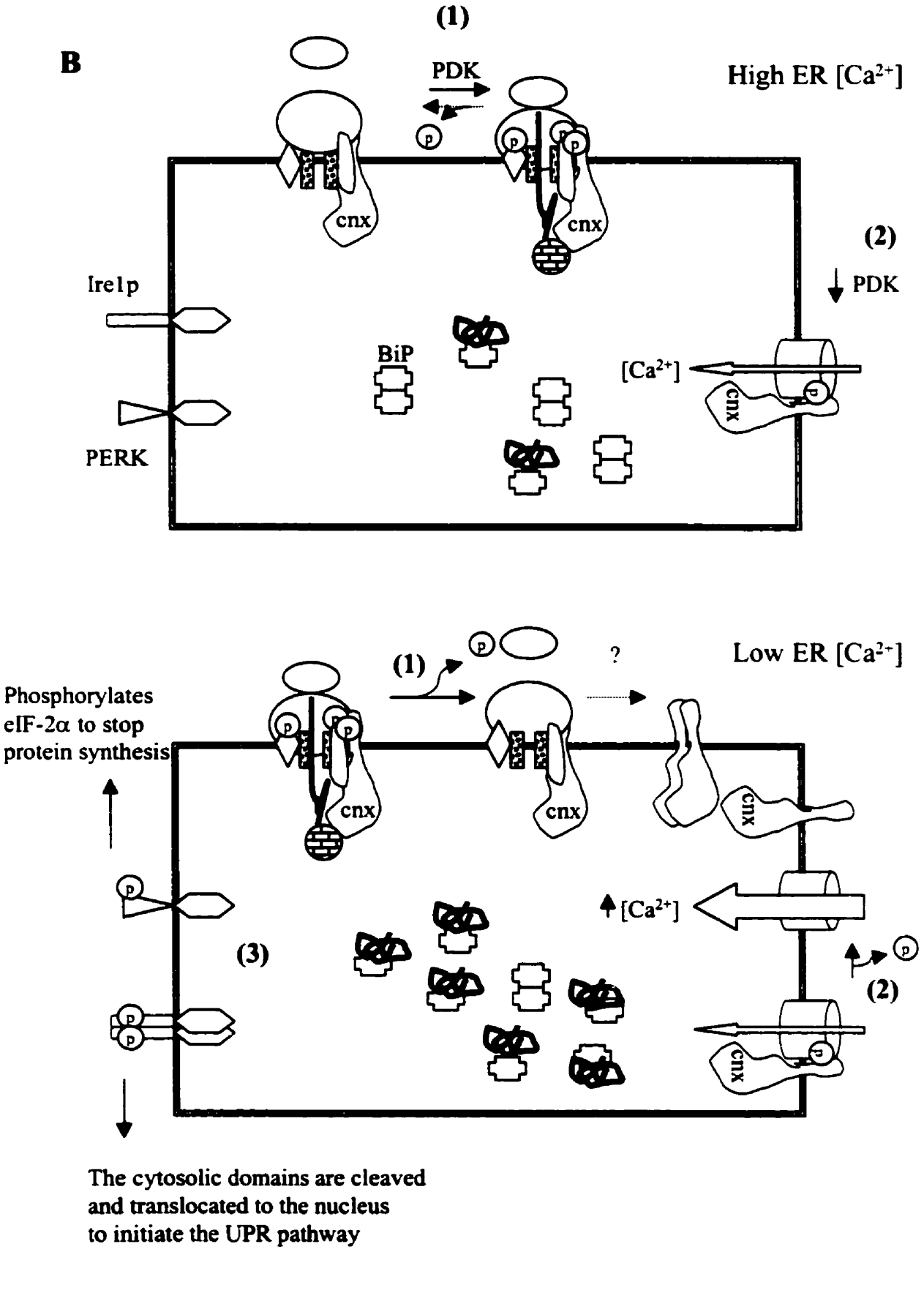

The cytosolic domains are cleaved
and translocated to the nucleus
to initiate the UPR pathway

Original Contributions

In this work, we have characterized calnexin phosphorylation in both cultured mammalian cells and in the fission yeast, *Schizosaccharomyces pombe*. The significance of calnexin phosphorylation was studied in isolated canine pancreatic microsomes. The following original observations are the result of our study.

1. This is the first work demonstrating that both mammalian and *S. pombe* calnexins are exclusively *in vivo* phosphorylated on serine residues.
2. This is the first work identifying the *in vivo* phosphorylation sites of mammalian calnexin by mass spectral analyses. Both human and canine calnexins are *in vivo* phosphorylated on three serine residues that are conserved among mammalian calnexins. The serines are within motifs that could be potentially recognized and phosphorylated by protein kinase CK2, PKC and/or PDK. This study was also the first to show that both mammals and S. pombe use a conserved serine phosphorylation PDK motif of calnexin.
3. This is the first reporting that calnexin could be *in vitro* phosphorylated on the same *in vivo* phosphorylation sites by kinases associated with isolated canine pancreatic microsomes. The microsomal associated kinase activities responsible for calnexin phosphorylation could be affected separately by various kinase inhibitors. Hence, isolated canine pancreatic microsomes represent an attractive model to study the function of calnexin phosphorylation.
4. This is the first reporting that ERK-1 is a possible physiological relevant kinase for calnexin phosphorylation.
5. This is the first demonstration that calnexin interacts with ribosomes and the interaction is regulated by calnexin phosphorylation. Synergic actions of both CK2 and ERK-1 enhance calnexin interaction with ribosomes *in vitro*.
6. This is the first reporting that calnexin phosphorylation is not modulated by O-linked GlcNAc modification.

7. This is the first study indicating that calnexin is *in vivo* phosphorylated in cultured rat dorsal root gangalia.
8. This is the first reporting that the cytosolic domain of *S. pombe* calnexin interacts with a protein with an apparent molecular mobility of approximately 150 kDa by SDS-PAGE.
9. This is the first reporting that the oleic acid increases the rate of dissociation of full-length apoB from calnexin.

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