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Molecular and cellular biology of proprotein convertases: Analysis of carboxy-terminal domain function

by

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A thesis submitted to the Faculty of Graduate Studies and Research

in fulfillment of the requirements for the degree of

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À mes parents, avec toute ma tendresse

Abstract

The convertases are a recently discovered family of mammalian serine proteases related in structure to the bacterial subtilisins and to the yeast enzyme kexin. These proteases share with the subtilisins the presence within their protein structure of a signal peptide, a prosegment, and a conserved catalytic region. However, they also possess additional P and COOH-terminal domains, which are not observed in subtilisins, but are present in their eucaryotic yeast counterpart, kexin. While the function of the P domain has been investigated, that of the COOH-terminal domain, excepting transmembrane and cytosolic tail structures of some convertases, remains unresolved.

In the present manuscript, the convertases' COOH-terminal domain function was investigated through the studies of two models of multiple convertase isoforms. These isoforms, which arise through differential splicing, are structurally identical, except within their COOH-terminal region. Studies by protein overexpression in cultured mammalian cells of multiple Drosophila furin isoforms and analysis of the cleavage products generated by these enzymes in coexpression with different substrates permitted to demonstrate that structural modifications of the COOH-terminal domain do not affect the cleavage specificity of the adjoined protease. However, particular COOH-terminal assemblages may affect the processing efficiency displayed by these enzymes. Further cellular localization studies by immunofluorescence and electron microscopy of two PC5 isoforms demonstrated that specific structural motives present in the COOH-terminal domain of these proteins are indeed implicated in the subcellular localization of these endoproteases, a fact which may ultimately affect their cleavage efficiency and substrate selectivity.

In conclusion, in some convertases' COOH-terminal domains, distinctive structural motives are involved in the sorting of these endoproteases to the appropriate subcellular compartment(s), and likely influence the trafficking of these proteins through the secretory pathway.

Sommaire

Les convertases constituent une famille de sérine protéases des mammifères, apparentées par leur structure aux subtilisines bactériennes et à l'enzyme de levure kexine. Ces endoprotéases possèdent, tout comme les subtilisines, un peptide signal, un segment pro et une région catalytique. Par contre, elles présentent également deux domaines supplémentaires, nommés domaines P et C-terminal, qui sont absents chez les subtilisines mais se retrouvent chez l'enzyme de levure kexine. Alors que la fonction du domaine P a été étudiée, celle du domaine C-terminal, à l'exception des régions transmembranaire et cytoplasmique de certaines convertases, demeure peu connue.

Dans le présent ouvrage, la fonction du domaine C-terminal des convertases fut étudiée à travers deux modèles de multiple isoformes. Ces isoformes, qui sont engendrées par un épissage alternatif, sont traduites en des enzymes identiques, sauf par la structure de leur région C-terminale. Des études par biosynthèse et radioimmunoétalonnage, par le biais de la surexpression de ces isoformes des convertases dans un système de cellules en culture, ont permis de déterminer que les modifications structurales du domaine C-terminal n'affectaient pas la spécificité de clivage de ces enzymes. Par contre, ces mêmes modifications peuvent jouer un rôle dans l'efficacité de clivage déployée par ces endoprotéases. Des études de localisation intracellulaire par immunofluorescence et microscopie électronique ont ensuite démontré que certains motifs de la région C-terminale uniques à chacune des isoformes de la convertase PC5 étaient impliqués dans la localisation subcellulaire distincte de chacune de ces protéines.

En conclusion, la présence du domaine C-terminal chez les convertases semble être une adaptation particulière de ces protéines de mammifères par-devant les subtilisines, permettant à ces protéases de naviguer à travers l'appareil de sécrétion, et d'atteindre le compartiment d'activité approprié à leur fonction spécifique.

Preface

This thesis is submitted to the McGill University Faculty of Graduate Studies and Research. Two options are admissible to candidates for the format of thesis submission. The first is the conventional composition format, while the second is in the form of published or submitted papers. The present work is following the second format, according to the guidelines of the Faculty of Graduate Studies and Research.

Candidates have the option of including, as part of the thesis, the text of one or more papers submitted or to be submitted for publication, or the clearly-duplicated text of one or more published papers. These texts must be bound as an integral part of the thesis.

If this option is chosen, connecting texts that provide logical bridges between the different papers are mandatory. The thesis must be written in such a way that it is more than a mere collection of manuscripts; in other words, results of a series of papers must be integrated.

The thesis must conform to the other requirements of the "Guidelines for Thesis Preparation". The thesis must include: a Table of Contents, an abstract in English and French, an introduction which clearly states the rationale and objectives of the study, a review of the literature, a final conclusion and summary, and a thorough bibliography or reference list.

Additional material must be provided where appropriate (e.g. in appendices) and in sufficient detail to allow a clear and precise judgment to be made of the importance and originality of the research reported in the thesis.

In the case of manuscripts co-authored by the candidate and others, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. Supervisors must attest to the accuracy of such statements at the doctoral oral defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of all the authors of the co-authored papers.

Two publications are presented in chapters C and D respectively. The one presented in chapter C is entitled **"Processing specificity and biosynthesis of the *Drosophila melanogaster* convertases dfurin1, dfurin1-CRR, dfurin1-X and dfurin2"**, by Isabelle De Bie, Diane Savaria, Anton J.M. Roebroek, Robert Day, Claude Lazure, Wim J.M. Van de Ven and Nabil G. Seidah, and has been published in the Journal of Biological Chemistry, 1995; 270:1020-1028. The second paper, which constitutes chapter D, is entitled **"The isoforms of proprotein convertase PC5 are sorted to different subcellular compartments"**, by Isabelle De Bie, Mieczyslaw Marcinkiewicz, Daniela Malide, Claude Lazure, Kazuhisa Nakayama, Moïse Bendayan, and Nabil G. Seidah, and has appeared in the Journal of Cell Biology 1996; 135:1261-1275.

The contribution of the co-authors to these publications is gratefully acknowledged:

Dr. Wim Van de Ven, director of the Laboratory for Molecular Oncology, Center for Human Genetics, University of Leuven, Belgium, and Dr. Anton Roebroek, provided the cDNAs and specific antibodies to the four Drosophila furin analogs which they had cloned and isolated. Dr. Kazuhisa Nakayama of the Institute of Biological Sciences and Gene Experiment Center, University of Tsukuba, Japan, provided the cDNA to the B isoform of PC5 which he isolated. Dr. Moïse Bendayan of the Department of Anatomy, Université de Montréal, and Dr. Daniela Malide, a postdoctoral fellow in Dr. Bendayan's laboratory, performed the electron microscopic detection of PC5 in the pancreatic A cells, a key experiment is the determination of the cellular localization of PC5.

Dr. Mieczyslaw Marcinkiewicz, assistant professor at the J.A. De Sève Laboratory of Molecular Neuroendocrinology, granted his extensive expertise and knowledge of immunofluorescence microscopy to the determination of the cellular localization of the PC5 isoforms in transfected AtT-20 cells. Dr. Claude Lazure, director of the Neuropeptides Structure and Metabolism Laboratory at the Clinical Research Institute of Montréal, performed the amino acid sequence analysis of both dfurin1 and PC5-A products by microsequencing, and unequivocally identified the protein forms being studied. Dr. Robert Day, associate investigator to Dr. Nabil G. Seidah, provided the recombinant vaccinia virus of dynorphin and assisted with the isolation procedures and characterization of the dynorphin products, a molecule he has extensively studied. Diane Savaria, a skillful cell culture technician in the Laboratory of Molecular Neuroendocrinology, isolated and purified the four recombinant vaccinia viruses expressing the Drosophila furin analogues, and provided assistance for cell culture procedures.

Dr. Nabil G. Seidah, director of the J.A. De Sève Laboratory of Biochemical Neuroendocrinology, acted as thesis supervisor. Fulfillment of this work could not have been possible without his insight, counseling and advice.

Both papers presented in this thesis have for theme the functional definition of the COOH-terminal domain of convertases, a family of recently discovered endoproteases (for reviews see Van de Ven et al., 1993; Seidah, Chrétien and Day, 1994; Rouillé et al., 1995; Seidah et al., 1996). Contrary to the other structurally defined regions of the convertases, such as the pro, catalytic and P domains, the COOH-terminal segment displays the least homology of amino acid sequence among the members of this family of enzymes (see Figure 1).

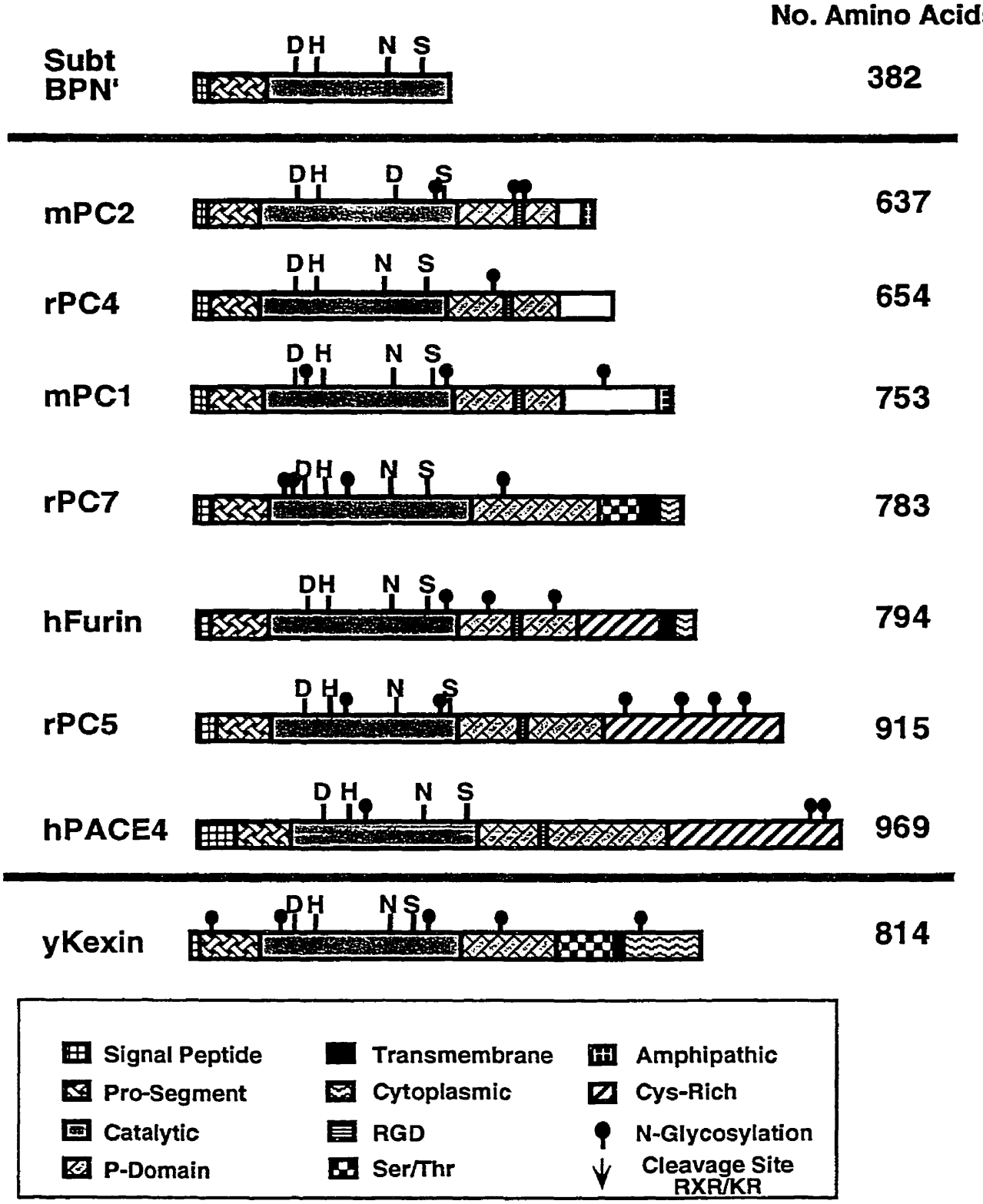

Figure 1:
Schematic representation of the mammalian members of the convertase family, with yeast kexin and bacterial subtilisin as comparison.

Functions have been assigned to all the other structural domains of the convertases, but no evident purpose has been defined for the COOH-terminal region, excepting the transmembrane anchor and cytosolic tail of some convertases. Our objective was to determine the role of this structurally variable domain in convertases, a segment which, incidentally, is absent in the related bacterial subtilisins.

The working hypothesis was that the COOH-terminal domain could either affect :
- The cleavage specificity or cleavage site recognition of the enzyme; or
- The cellular localization of the convertase, and therefore the substrate-enzyme combination of these endoproteases.

Initial studies accomplished with truncated forms of the convertase furin demonstrated that the COOH-terminal domain could be completely removed without affecting the processing capability of the enzyme in *ex vivo* coexpression experiments (Hatsuzawa et al., 1992). However, they The cleavage efficiency or processing kinetics of the different forms of furin on the substrates tested were not examined. Other investigations, this time of PC2 COOH-terminally deleted forms (Shennan Taylor and Docherty, 1994), provided evidence that the COOH-terminal domain of this convertase is not essential to the membrane-association and possible sorting of this protein to the regulated secretory pathway. However, recent studies of furin-PC2 chimeras (Creemers et al., 1996) contradict these results by demonstrating that the presence of the P and COOH-terminal domains of PC2 can redirect furin chimeras towards the regulated secretory pathway. Similarly, experiments with truncated forms of the convertase PC1 yielded contradictory results with regards to the role of the COOH-terminal domain in the sorting of this convertase to secretory granules (Zhou, Paquet and Mains, 1995; Zhou et al., 1995).

In our investigations, we undertook to answer both aspects of the potential function of the COOH-terminal domain by two related approaches:

First, the possible influence of the COOH-terminal domain on the convertases cleavage specificity was examined. This was achieved by the study of three isoforms of a Drosophila furin analog (Roebroek et al., 1993) which differ solely by the structure of their COOH-terminal domain (see Figure 2). These enzymes, as well as a the product of a second furin-like Drosophila gene (Roebroek et al., 1992, 1995), were coexpressed with three prototype substrates each exhibiting a distinct cleavage site pattern (Bresnahan et al., 1992). Enzymes and substrates were cloned into a recombinant vaccinia virus overexpression system. The recombinant viruses were used to infect several types of cultured mammalian cells. The protein products of both substrates and enzymes were analyzed by biosynthesis, immunoprecipitation and SDS-PAGE, or by radioimmunoassay. Dfurin1 protein forms were also defined by NH_2-terminal amino acid sequencing. The results obtained from these studies demonstrated that:

1.- In coexpression studies, the three dfurin1 isoforms, as well as dfurin2, were capable of cleaving three different substrates each exhibiting a distinct cleavage sequence recognition site, and to process these precursors into peptide products similar to those generated by mammalian furin. All forms of dfurins demonstrated an identical cleavage specificity towards the three categories of substrates tested.

2.- Conversely, the dfurins did not display the same potency of cleavage towards these three different substrates. The range of cleavage efficacy among the dfurins seemed to correlate the extent of proregion removal, although this was not invariably the case for each enzyme.

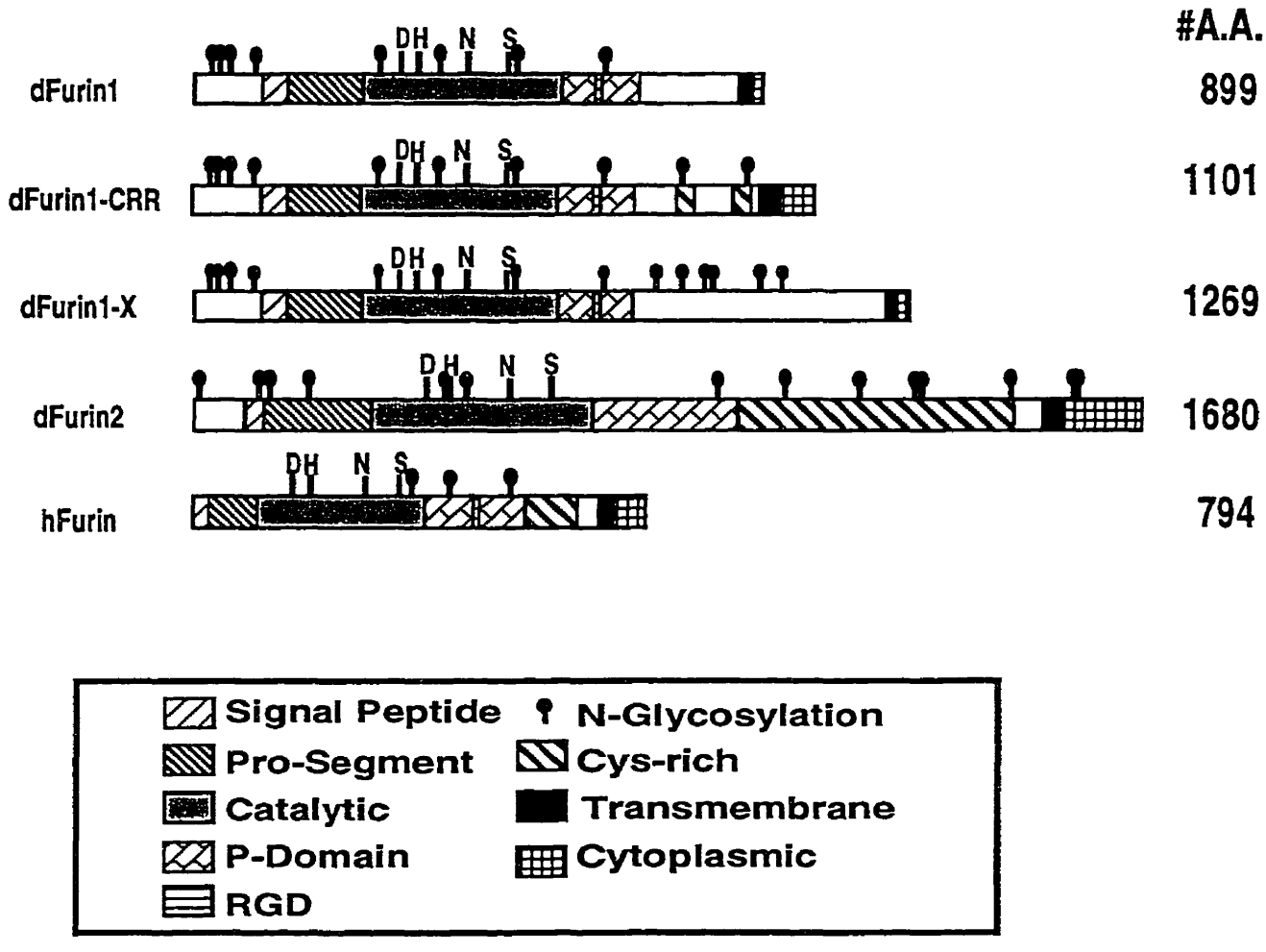

Figure 2:

Schematic representation of the three dfurin1 isoforms as well as the product of the *Dfur2* gene, with mammalian furin as comparison.

3.- The two dfurin enzymes endowed with a cysteine-rich COOH-terminal region, a motif also observed in mammalian furin, PACE4 and PC5, demonstrated a capacity to lose their prosegment in earlier compartments of the secretory pathway, as assessed by transport block studies.

4.- In all cell types tested, the membrane-bound dfurins underwent shedding of their transmembrane domain. In LoVo cells, a constitutive human cell type devoid of active furin, this shedding was an early event and was not due to the action of a protease highly dependent on calcium.

5.- While both pro- and zymogen-cleaved forms of the dfurins were released in the media in constitutive cell types, in cells possessing a regulated secretory pathway, only dfurin forms which had lost their prosegment were detectable extracellularly. In addition, the forms possessing cysteine-rich repeats seemed to accumulate in the media of these previous cells, while no notable difference could be observed among the dfurins in constitutively secreting cells.

The technical steps of this work consisted in:

— Transfer of the four dfurins cDNAs to vaccinia virus expression vectors by selection of the proper DNA restriction enzyme cleavage sites, ligation of vector and insert, amplification and purification of the new constructs.
— Transfer of the cDNA constructs to wild-type vaccinia virus to create recombinant viruses expressing the proteins of interest.
— Expression of the recombinant proteins by infection of cultured cells.
— Analysis of the protein products, either by radiolabeling of infected cells, biosynthesis, specific immunoprecipitation, separation by SDS-PAGE, and fluorogenic detection on film; or gel chromatographic separation of the products obtained by infection, and radioimmunoassay of the fractions.

The dfurin1 products were additionally characterized by microsequencing of radiolabeled proteins to determine their NH_2-terminal amino acid sequence. This required the isolation and purification of the radiolabeled products by immunoprecipitation, SDS-PAGE, elution from the gel, and microsequencing of the purified protein.

Analysis of the dfurin model permitted to conclude that structural modifications of the COOH-terminal region of the convertases do not affect the cleavage specificity of these enzymes. However, changes within this segment appear to influence the efficiency of proregion removal, and by consequence, substrate cleavage efficacy. This could reflect a different rate of progression along the secretory pathway for these proteins, which in turn could be considered in the light of the interaction of the COOH-terminal domain with the cellular sorting machinery.

In the continuity of this work, a study of two PC5 isoforms, which also displayed structural differences solely in their COOH-terminal domain (see Figure 3), was undertaken, and aimed this time at defining if the variations in COOH-terminal domain structure could affect the cellular residence and traffic of these convertases. For these studies, an overexpression system by stable transfection was preferred to the recombinant vaccinia virus method. Vaccinia virus infection has been demonstrated to alter not only host cell protein synthesis but also the cytoskeleton of infected cells (Bablanian, 1984; Schneider, 1987; Cudmore et al., 1995). While this fact has little potential impact on coexpression studies where cleavage specificities of enzymes are examined, it could, however, impair protein traffic by diminishing the physiological levels of some sorting factors. The infection method was thus judged unsuited to the achievement of sorting studies.

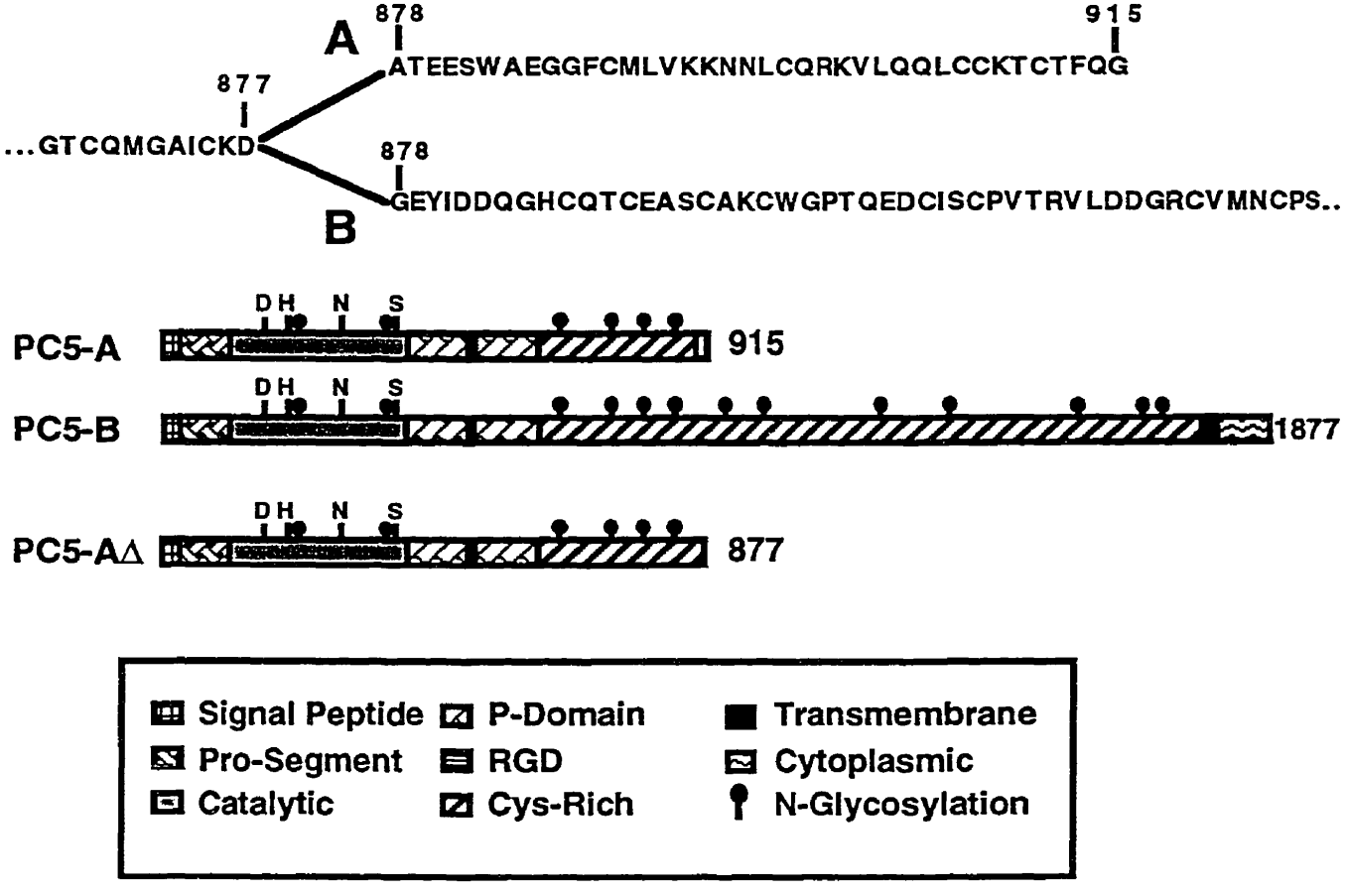

Figure 3:

Schematic representation of mammalian proprotein convertase isoforms PC5-A and PC5-B, as well as a PC5-AΔ mutant construct lacking the COOH-terminal region unique to PC5-A.

The possible influence of the COOH-terminal domain on the subcellular sorting of convertases was examined through the investigation of two mouse PC5 isoforms named PC5-A and PC5-B, which differ exclusively by the composition of this structural region (Lusson et al., 1993; Nakagawa et al., 1993a,b). PC5-B is predicted to be membrane-anchored, while PC5-A is expected to be a soluble protein. A truncated form of PC5-A lacking the COOH-terminal region unique to this isoform was also constructed to analyze the potential differences in biosynthesis and sorting between this form and the wild-type PC5-A (see Figure 3). These three convertases were overexpressed by stable transfection in AtT-20 cells, a cell type possessing both constitutive and regulated secretory pathways. The biosynthetic transformations and subcellular localization of each protein form was examined by biosynthesis, immunoprecipitation and SDS-PAGE, as well as by immunofluorescence microscopy. In addition, PC5-A products were characterized by amino acid analysis and sequencing, and electron microscopic studies of pancreatic A cells were performed to determine the compartment of residence of PC5 in a physiological model. These studies demonstrated that:

1.- Both PC5-A and PC5-B, when expressed in AtT-20 cells, were detected in several molecular forms. Three forms of PC5-A were observed, which were determined to be proPC5-A, zymogen-cleaved PC5-A and a COOH-terminally truncated form of PC5-A. For PC5-B, two forms were detected, one membrane bound and one soluble, this last one released in the medium probably as a result of cleavage NH_2-terminal to the transmembrane domain. Sulfation studies demonstrated that both PC5-B forms reach the TGN. Only PC5-A and not proPC5-A was observed to be sulfated.

2.- The soluble forms of PC5-A and PC5-B were shown to leave the cell through different secretory pathways. Studies of stimulated protein exocytosis revealed that PC5-A was stored in the secretory granules of AtT-20 cells and could be released upon stimulation with cAMP, while the secretion of the soluble form of PC5-B was not affected by this treatment.

3.- The subcellular localization of PC5-A and PC5-B in transfected AtT-20 cells was determined by double immunofluorescence studies in combination with specific secretory pathway markers. PC5-A was found to predominate in the secretory granules and Golgi of AtT-20 cells, while PC5-B exhibited colocalization with TGN38, a marker of the TGN.

In contrast to wild type PC5-A, the mutant PC5-AΔ lacking the COOH-terminal region unique to PC5-A could only be detected throughout the Golgi region, and not in secretory granules. Stimulation of secretion studies agreed with these results, since the release of PC5-AΔ was not significantly increased in the presence of cAMP, indicating that this protein does not accumulated in the secretory granules of AtT-20 cells.

4.- In pancreatic A cells, electron microscopic studies demonstrated that PC5 immunoreactivity is detectable within the granules of these cells. PC5 was observed to colocalize with both glucagon and PC2 within this organelle.

The technical steps of this work consisted in:

— Creation of a mutant PC5-A cDNA, using PCR to insert a stop codon at the end of the region common to both PC5-A and PC5-B isoforms.
— Transfection of AtT-20 cells with constitutive eucaryotic expression vectors containing the inserts of either PC5-A, PC5-AΔ or PC5-B.

Isolation of several stably transfected clones for each cell line. Analysis of protein expression levels in these clones by Western blotting and immunoprecipitation.

— Analysis of the protein products by radiolabeling of transfected cells, biosynthesis in several different conditions, using either chemical agents like Brefeldin A or 8Br-cAMP, or temperature block, followed by specific immunoprecipitation, separation by SDS-PAGE, and fluorogenic detection on film. The PC5-A products were additionally characterized by microsequencing of the radiolabeled products to determine their NH_2-terminal amino acid sequence. Kinetics of basal release were determined for each PC5 forms by quantitation at multiple time points of the proteins accumulated in the media. This was performed by biosynthesis and immunoprecipitation, followed by SDS-PAGE, fluorogenic detection, and scanning analysis of the films with the Macintosh NIH Image 1.55f program.

— The subcellular distribution of PC5 proteins in transfected cells was determined by double immunofluorescence labeling with specific markers of the secretory pathway.

— The distribution of PC5 in pancreatic A cells was determined by electron microscopy. Double labeling studies were performed to demonstrate the colocalization of PC5 with either PC2 or glucagon in the secretory granules of the pancreatic A cells.

Analysis of the PC5 model allowed us to conclude that structural modifications of the COOH-terminal region of the convertases are implicated in the determination of their subcellular localization and progression along the secretory pathway. The COOH-terminal region unique to PC5-A seems to be involved in the sorting of this protein to the secretory granules, while other signals may be responsible for the Golgi localization of PC5-B.

The fact that two isoforms issued from the same convertase gene can be redirected to different subcellular compartments through the structural modification of their COOH-terminal domain can ultimately result in the formation of distinct enzyme-substrate pairs. Indeed, the intracellular site and duration of contact between an enzyme and its substrate, as well as the cleavage efficacy of the convertase, will vary according to their subcellular localization and residence time within a particular compartment.

In conclusion, the studies presented in this thesis demonstrate that variations in the structure of the COOH-terminal region of convertases do not affect their cleavage site recognition, but can influence their subcellular localization.

Presentation of this manuscript proceeds as follows:

Chapters A and B constitute a comprehensive review of the most recent literature pertinent to this thesis. Chapter A covers the relevant aspects of protein biosynthesis, trafficking and sorting along the secretory pathway, while Chapter B reviews the subject of proprotein convertases, from their discovery to the most recent reports.

Chapters C and D present an abstract, introduction, materials and methods, discussion and reference sections for each publication. These publications treat different aspects of the same theme, the potential function of the COOH-terminal region in the convertases. Chapter C deals with the possible impact of this domain on the cleavage site recognition of the isoforms of the Drosophila furin homologues, while Chapter D examines the role of this region in directing the sorting of two PC5 isoforms to the distinct subcellular compartments.

A general conclusion is provided in Chapter E. This thesis concludes with claims to original research.

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List of abbreviations

aa	amino acid
Arf	ADP-ribosylation factor
ACTH	adrenocorticotropin hormone
ACTH*	glycosylated adrenocorticotropin hormone
ADP	adenosine diphosphate
AP	adaptor protein
ATP	adenosine triphosphate
bp	base pair; kbp, kilo base pair
BiP	Immunoglobulin heavy chain binding protein
8Br-cAMP	8Bromo-cyclic adenosine monophosphate
BFA	brefeldin A
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
°C	degree Celsius
Ca2+	calcium
Cg	chromogranin
Ci	curie(s); μCi, microcurie(s)
CB	cathepsin B
CGN	*cis*-Golgi Network
CKII	casein kinase II
CNS	central nervous system
COOH-terminal	carboxy-terminal
COP	coat protein
CPE	carboxypeptidase E
CPH	carboxypeptidase H
CRR	cysteine-rich region
dfurin	Drosophila furin
D	dalton; kD, kilo dalton
DME	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
DPP	decapentaplegic protein
EndoH	endoglycosidase H
EDTA	ethylenediaminetetracetic acid
EM	electron microscopy
END	endorphin
ER	endoplasmic reticulum
ERGIC	endoplasmic reticulum-Golgi intermediate compartment
Fc	Immunoglobulin G fragment, crystalline
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
g	gram(s); mg, milligram (s); μg, microgram(s)
Glc	glucose
GlcNac	N-acetylglucosamine
GDP	guanosine diphosphate
GPI	glycosylphosphatidylinositol
GTP	guanosine triphosphate
h	hour(s)
hfur	human furin
^{3}H	tritiated
HVEM	high voltage electron microscopy
HSP	heat shock protein
Ig	immunoglobulin

IGF	insulin-like growth factor
l	liter(s); ml; microliter(s); µl, microliter(s)
LDL	low density lipoprotein
LPH	lipotropin hormone
[^{35}S]met	[^{35}S]methionine
min	minute(s)
m7B2	mouse 7B2
mPC1	mouse PC1
mPC2	mouse PC2
mPC5	mouse PC5
M	molar; mM, micromolar;µm, micromolar
Man	mannose
Mr	relative molecular mass
MDBK	Madin-Darby bovine kidney
MDCK	Madin-Darby canine kidney
MHC	major histocompatibility complex
MSH	melanocyte-stimulating hormone
NH_2-terminal	amino terminal
NEM	N-ethyl maleimide
NSF	N-ethyl maleimide-sensitive fusion factor
pfu	plaque forming unit
PACE	paired basic amino acid cleaving enzyme
PAGE	polyacrylamide gel electrophoresis
PAM	peptidylglycine alpha-amidating monooxygenase
PBS	phosphate buffered saline
PC	proprotein convertase
PCR	polymerase chain reaction; RT-PCR, reverse-transcriptase PCR
PDI	protein disulfide isomerase
PMSF	phenylmethylsulfonyl fluoride
POMC	proopiomelanocortin
rdynorphin	rat dynorphin
RIA	radioimmunoassay
RNA	ribonucleic acid; hnRNA, heteronuclear RNA; mRNA, messenger RNA
sRNP	small nuclear ribonucleoprotein particle
SD	standard deviation
SDS	sodium dodecyl sulfate
SNAP	soluble NSF attachment protein
SNARE	soluble NSF attachment protein receptor
SRP	signal recognition particle
Tris	tris(hydroxymethyl)aminomethane
TGN	*trans*-Golgi Network
TMD	transmembrane domain
TRITC	tetramethylrhodamine isothiocyanate
vol	volume
vWF	von Willebrand factor
VIP36	36 kD vesicular integral membrane protein
VSV	vesicular stomatitis virus
VV	vaccinia virus
wt	wild type
Y-XX-Ø	tyrosine-aa-aa-hydrophobic aa

Introduction

In eucaryotes, many proteins are initially synthesized as inactive polypeptide precursors which require proteolytic cleavage to acquire their bioactivity. These proteins include several neuropeptides and peptide hormones, as well as growth factors and their receptors. Insight on the enzymes accomplishing the maturation of these proteins is fundamental to the understanding of the production of biological peptides and the regulation of their activity.

A family of endoproteases implicated in the processing of some of these polypeptide precursors has recently been identified. These enzymes, called proprotein convertases (PCs), are involved in several crucial physiological and pathological cellular processes, such as the processing of protein precursors into regulatory polypeptides in a tissue and cell-specific manner, and the cleavage of viral envelope glycoproteins. However, little is yet known about the functional regulation of these proteases.

In this manuscript, I describe how the biosynthesis, cellular localization and activity of some of these proteinases can be affected by variations within a precise structural region of convertase proteins, the COOH-terminal sequence. This is achieved through the biosynthetic analysis of the multiple products generated by differential splicing of two members of the convertase gene family, *Dfur1* and *PCSK5*. These studies demonstrate that, in the case of the Drosophila furin analogs, the COOH-terminal domain of the convertases is not involved in defining cleavage site specificity. However, studies with the isoforms of convertase PC5 illustrate that variations of the COOH-terminal sequence can affect both the cellular localization and exocytosis of these enzymes.

These investigations demonstrate that sequence modifications of the convertases' COOH-terminal domain resulting from alternative splicing of their precursor hnRNA, a phenomena observed for several members of this endoprotease family, can be implicated in the determination of their subcellular localization.

CHAPTER A

Biosynthesis and transport of secretory and membrane proteins

A.1 Alternative splicing and protein diversity

Different cell types performing specific functions within a tissue or organism synthesize particular sets of proteins. Several mechanisms exist to ensure this cell type-specific protein expression. One of these mechanisms is the processing of primary heteronuclear RNA (hnRNA) transcripts. Excision of introns and splicing of mRNA precursors not only affects the message encoded by these molecules, but also their transport out of the nucleus, stability in the cytoplasm, as well as efficiency of translation (Maniatis, 1991; Newman, 1994; Sharp, 1994).

For a number of eucaryotic genes, primary hnRNAs can be spliced in several alternative fashions, thus generating multiple transcripts. This eventually leads to the production of different proteins issued from a single gene. In many cases, splicing can be cell-type specific or regulated. Different cells (or a single cell) can thus synthesize different protein isoforms in response to specific cues. Introns are spliced out of hnRNA precursors by the precise recognition of intron-exon junctions in primary RNA transcripts. These sequences are conserved in all eucaryotes and consist of a particular structural motif which begins with the consensus sequence AG**GU** at the 5' exon-intron junction site, and ends with C**AG**G at the 3' intron-exon junction site (Hodges and Bernstein, 1994). This motif is recognized by a group of small nuclear RNAs and proteins called sRNPs (small nuclear ribonucleoprotein particles) which in turn assembles with the precursor mRNA to form a spliceosome complex. Spliceosomes catalyze the excision of introns and thus carry out splicing of mRNA precursors (Sharp, 1994). Examples of secretory and membrane-bound proteins encoded by multiple mRNAs include growth hormone (Moore et al., 1982), calcitonin (Amara et al., 1982), enkephalin and dynorphin (Garrett et al., 1989), and the convertases PACE4 (Kiefer et al., 1991), dfurin1 (Roebroek et al. 1993), PC4 (Seidah et al., 1992; Mbikay et al., 1994), and PC5 (Nakagawa et al. 1993a,b; Seidah et al., 1994; Mbikay et al., 1995).

A.2 Translation and translocation into the endoplasmic reticulum

In eucaryotes, once an mRNA has been modified to its final form by processing, addition of a 5'end cap and 3'end poly A tail, it is transported out of the nucleus. In the cytosol, translation is initiated by association with ribosomes. The nascent proteins emerging from ribosomes contain topogenic signals which determine their sorting to distinct cellular compartments. For proteins transported through the secretory pathway, a specific leader sequence permits anchoring of ribosomes to receptors at the surface of the endoplasmic reticulum membrane, and subsequent translocation into this compartment. This NH_2-terminal peptide signal interacts with a cytosolic signal recognition particle (SRP), which targets the ribosome to the surface of the rough ER by interacting with a specific SRP receptor, and subsequently allows the entry of the protein into the lumen of this organelle through a protein translocation pore, called translocon (Walter and Blobel, 1982; Millman and Andrews, 1997).

Signal sequences range in length from 15 to 70 amino acids, and the amino terminal side of the signal contains at least one positively charged residue. The core of the signal peptide consist of a stretch of hydrophobic amino acids, typically 15 to 20 residues long (von Heijne, 1990; Zheng and Gierasch, 1996). Some proteins contain and internal stop transfer sequence instead of an amino terminal signal peptide, while others contain no typical signals and are still translocated into the lumen of the endoplasmic reticulum through an alternative or salvage pathway (Ng et al., 1996).

For most secretory pathway proteins, transport to the ER lumen occurs cotranslationally, during polypeptide chain elongation. After the signal peptide has guided the protein to the ER membrane, it stays associated with the translocon, behaving as a start-transfer signal, while the remainder of the protein is threaded through the membrane.

Once the carboxy-terminal end of the protein has migrated through the membrane, the signal peptide is discharged from the translocation pore and is rapidly cleaved by a signal peptidase, while the rest of the protein is released in the lumen of the ER. For proteins which remain anchored to the membrane, the translocation process is stopped at one point during synthesis by an hydrophobic segment within the polypeptide chain. This peptide region acts as a stop-transfer signal and maintains the protein in the lipid bilayer by forming an alpha-helical membrane-spanning domain (Mothes et al., 1997).

It is in the ER that proteins become N-glycosylated by the transfer of an initial $[glucose]_3$-$[mannose]_9$-$[N\text{-}acetylglucosamine]_2$ oligosaccharide core to an asparagine residue within the sequence Asn-X-Ser/Thr, from a glycolipid containing dolichol phosphate carrier (Lennarz, 1987). This oligosaccharide structure is trimmed of its glucose moieties in the ER, then further modified while the protein is transported through the Golgi apparatus (Kornfeld and Kornfeld, 1985). Inhibitors of N-glycosylation such as tunicamycin, can block the addition of N-acetylglucosamine to dolichol phosphate, the first step in the formation of the core oligosaccharide. When N-glycan addition to proteins is prevented, the majority of non-glycosylated forms are retained in the ER were they aggregate (Olden et al., 1982). This in turn can result in an increased degradation of glycoproteins in the ER (Weintraub et al., 1985; Lippincott-Schwartz et al., 1988; Amara, Lederkremer, and Lodish, 1989; Bonifacino and Lippincott-Schwartz, 1991; Stafford and Bonifacino, 1991; Bonifacino, 1996; Kopito, 1997).

Proteins attain their tertiary configuration in the ER by interacting with resident protein chaperones. Chaperones assist protein folding and ensure protein assembly, as well as the proper formation of secondary structures encoded by the amino acid chain, such as alpha helices,

beta pleated sheets and beta turns. There are several classes of chaperones present in the ER: class I includes the immunoglobulin heavy chain binding protein BiP, which is part of the heat shock protein 70 (HSP70) family. Class I chaperones interact with hydrophobic patches on the nascent protein to permit the partial folding of polypeptide precursors and prevent their aggregation (Landry and Gierasch, 1991; Buchner, 1996). Class II chaperones comprise two lectin-like proteins, membrane-bound calnexin (Ou et al., 1993; Ware et al., 1995) and soluble calreticulin (Nauseef et al., 1995; Peterson et al., 1995), which recognize carbohydrate portions of proteins and ensure the final folding of glycoproteins before they can be exported from the ER (Fielder and Simons, 1995; Hebert, Foellmer, and Helenius, 1995). If the protein is not properly folded, glucose will be added back to the N-linked oligosaccharide. Proteins which retain a glucose moiety remain bound to calnexin and are unable to exit this compartment. When the protein has ultimately reached its proper conformation, glucosylation ceases, and rapid trimming of the remaining glucose moieties permits migration out of the ER (Bergeron et al., 1994; Hammond and Helenius, 1995; Williams, 1995). Thus, glycosylation can play an important role in the proper folding of glycoproteins. Finally, some ER enzymes also assist in this process. Protein disulfide isomerase (PDI) ensures the correct formation of disulfide bonds, while prolyl and lysyl hydroxylases perform the hydroxylation of prolines and lysines, and peptidyl-prolyl isomerases catalyze the cis/trans isomerization of X-Pro bonds (Gething and Sambrook, 1992; Helenius, Marquart, and Braakman, 1992; Martin and Hartl, 1994; Hendrick and Hartl, 1995; Buchner, 1996).

Some proteins also acquire in the ER a covalently attached glycosylphosphatidylinositol (GPI) anchor at their COOH-terminal end, and thus become capable of binding to the exterior of the plasma membrane (Ferguson, 1992; Takeda, and Kinoshita, 1995).

This complex system of folding assistance by chaperones and enzymes ensures that only correctly folded and modified proteins proceed from the ER to the Golgi apparatus, and ultimately leave the early biosynthetic compartments. For many proteins, an additional chaperone-like system is incorporated within their structure. These proteins are synthesized as larger precursors, which possess an internal proregion, usually as an additional NH_2-terminal sequence. Pro segments can perform multiple functions, ranging from targeting signals to protease inhibitors. Most importantly, they can play a critical role in the proper folding and processing of polypeptide precursors. Most proteases, for example subtilisins, as well as other proteins, have evolved a folding mechanism dependent on the presence of a prosegment. Propeptide segments facilitate the rate of folding of their associated mature domains by stabilizing their partially folded state and lowering the energy barrier between unfolded and folded states. They are then cleaved once folding is complete, to prevent catalysis of the reverse reaction (Baker, Shiau, and Agard, 1993; Sinde and Inouye, 1995).

Table A-I: ER-specific modifications

1. Signal peptide cleavage
2. Chaperone-assisted folding
3. Addition of N-linked oligosaccharides
4. Disulfide bonds formation
5. Hydroxylation of proline or lysine residues
6. Oligomerization
7. Addition of GPI anchor

Post-translational modifications:

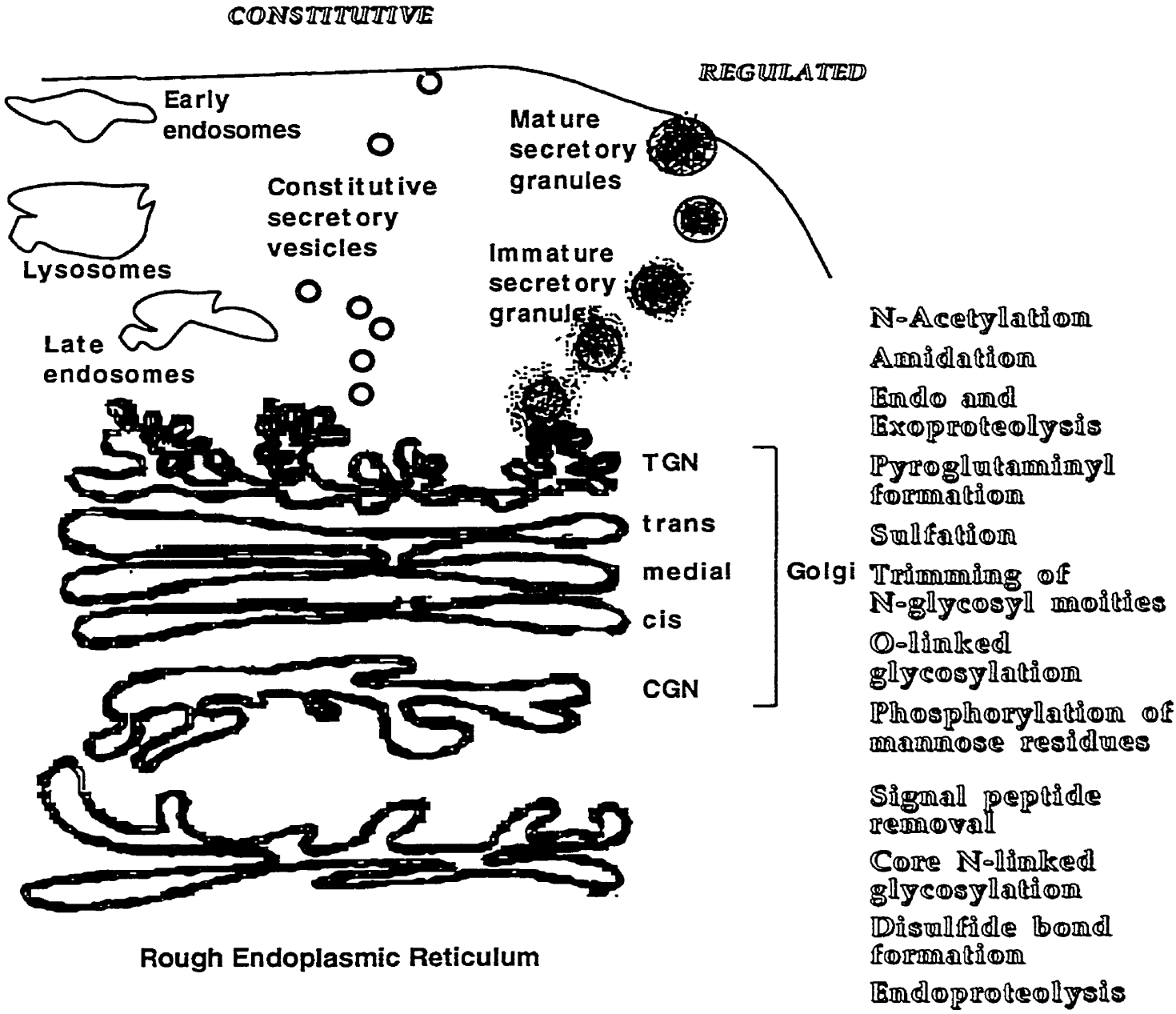

Figure A-1:

Schematic representation of secretory pathway, with *cis*-Golgi Network (CGN), *trans*-Golgi Network (TGN), constitutive vesicles, secretory granules, endosomes, lysosomes, and biosynthetic transformations taking place in each of these compartments.

A.2.1 ER retention signals

While proteins which have attained their proper conformation are released from the early secretory pathway, ER resident proteins recycle back to this latter compartment from Golgi saccules. Specific signals ensure that these proteins are maintained predominantly in the proper section of the secretory pathway.

Several types of retrieval signals have been characterized for ER resident proteins. First, a carboxy terminal tetrapeptide KDEL is found in many lumenal proteins, such as BiP and PDI. Proteins exhibiting this motif are capable of escaping the ER, but can be retrieved, sometimes all the way from the *trans*-Golgi Network (Misenböck and Rothman, 1995), and are returned to their compartment of residence by interaction with a sorting receptor (Munro and Pelham, 1987; Lewis and Pelham, 1990; Lewis, Sweet, and Pelham, 1990; Semenza et al., 1990; Vaux, Tooze, and Fueller, 1990; Tang et al., 1993).

Some ER integral membrane proteins also maintain themselves in this compartment through a retrieval system. These membrane-bound proteins, usually exhibiting a type I topology (transmembrane domain at the COOH-terminus of the molecule), have within their COOH-terminal cytosolic tail a retrieval signal consisting of two lysines (KKXX or KXKXX, where X can be any amino acid) (Jackson, Nilsson, and Peterson, 1993). A similar signal (RKPRRE) is present within the COOH-terminal cytosolic tail of calnexin (Bergeron et al., 1994). Deletion of this segment causes the redistribution of calnexin towards the Golgi and cell surface (Rajagopalan, Xu, and Brenner, 1994). For some type II proteins (transmembrane domain at the NH_2-terminus of the molecule), this signal is replaced by two arginines (RR), which must be found within the first five amino-terminal residues of the protein (Schutze, Peterson, and Jackson, 1994).

Table A-II: ER localization signals
1. NH_2-terminal **signal peptide**
2. COOH-terminal **KDEL**
3. Cytosolic tail **KKXX** or **KXKXX**
4. Cytosolic NH_2-terminal segment **RR**

A.3 Transport through the Golgi apparatus

Proteins exit the endoplasmic reticulum and are transported to the Golgi apparatus, an organelle composed of multiple cisternae or stacks. Small vesicles permit the transit of proteins from the ER to the Golgi and between the different Golgi laminae. The Golgi complex consists of three functional regions, the cis, medial and trans portions, in which distinct biochemical transformations of proteins take place, including elongation and modification of oligosaccharides (Farquhar, 1985) (see Figure A-1). Each Golgi cisterna has two distinct surfaces: an entry or cis face, and an exit or trans face. The cis- and trans-most cisternae of the Golgi are adjoined to special compartments composed of interconnected tubulo-reticular complexes, called the *cis*-Golgi and *trans*-Golgi Networks.

Transport vesicles leaving the ER first enter a vesicular tubular cluster, also called sorting exosomes, salvage or ER-Golgi intermediate compartment (ERGIC). These vesiculo-tubular extensions are thought to be connected to the *cis*-Golgi Network (Balch et al., 1994; Harter and Wieland, 1996), although they are sometimes considered as an organelle structurally distinct from the Golgi apparatus (Saraste and Kuismanen, 1984; Schweizer et al., 1990; Hauri and Schweizer, 1992). This vesiculo-

tubular network exhibits transitional elements which are contiguous with the ER and the *cis* Golgi stacks (Hauri and Schweizer, 1992; Mellman and Simons, 1992; Rothman and Orci, 1992; Balch et al., 1994; Aridor and Balch, 1996). Proteins which recycle back to the ER are thought to be sorted from those which continue along the secretory pathway in this compartment and/or the *cis*-Golgi Network (Hsu et al., 1991; Saraste and Svensson, 1991; Pelham 1995). Temperature blocks of 15°C prevent proteins from leaving the salvage compartment (Saraste and Kuismanen, 1984; Lippincott-Schwartz et al., 1990; Schweizer et al., 1990) after their exit from the ER. A marker of this compartment, ERGIC-53, has been suggested to perform a function similar to those of calnexin and calreticulin in the quality control of protein folding, in that it behaves as a lectin with a specific affinity for mannose sugars (Arar et al., 1995; Itin et al., 1996; Ponnembalam and Banting, 1996).

Proteins leave the intermediate compartment to enter the *cis*-Golgi Network (CGN). Biochemically, the CGN is defined as the compartment where the first step of phosphorylation of mannose residues in lysosomal enzymes occurs (Lazarino and Gabel, 1988). Retrieval of ER resident proteins from the CGN indicates that transfer of proteins can occur in either directions between these two organelles. Receptors for ER retention signals are not only found in the CGN but at all levels of the Golgi apparatus, as far as the *trans*-Golgi Network (Misenböck and Rothman, 1995), suggesting that the recycling pathway extends all the way to the later Golgi compartments. This recycling pathway is affected by the drug Brefeldin A (BFA), which causes the diffusion of Golgi stacks into the ER and redistribution of Golgi enzymes in this later compartment. BFA prevents assembly of the coat proteins necessary for the formation of budding vesicles, blocking the forward vesicular transport while leaving the backward microtubule-dependent transport intact (Lippincott-Schwartz et al., 1989, 1990, 1991; Pelham, 1991;

Klausner, Donaldson, and Lippincott-Schwartz, 1992; Robinson and Kreis, 1992). In contrast, components of the *trans*-Golgi Network (TGN) do not redistribute to the ER, but intermix with the endosomal recycling system. Thus, BFA inhibits the forward movement of proteins beyond the mixed ER/Golgi system.

N-linked oligosaccharide moieties which have been added to proteins in the ER are modified as the glycoproteins travel along the Golgi apparatus. Glycoproteins can undergo several different types of modifications and achieve three different types of structures: high-mannose, complex, or hybrid (Kornfeld and Kornfeld, 1985). High-mannose oligosaccharides contain two N-acetylglucosamines and several mannose residues, while complex oligosaccharides can contain more than two N-acetylglucosamines, a variable number of galactose and sialic acid residues, and sometimes fucose. After the removal of five mannose residues by mannosidases I and II in the early Golgi compartments, the core N-acetylglucosamines as well as all subsequent structures in the glycosylation pathway become resistant to the attack of a specific endoglycosidase, named Endoglycosidase H (EndoH). This enzyme is thus used to differentiate glycoprotein transformations as proteins travel along the secretory pathway.

The different oligosaccharide transformations are performed by various mannosidases, glycosidases and glycosyltransferases. These enzymes are enriched in specific Golgi compartments in the order in which they act, permitting sequential processing of oligosaccharides (Dunphy and Rothman, 1985; Roth, 1991). Proteins thus undergo several rounds of trimming and modifications as they pass through the different Golgi cisternae. They can also undergo addition of O-linked sugars in the Golgi by the action of glycosyl transferase enzymes (Jentoft, 1990), or addition of sulfate groups on tyrosine residues or sialic acid components of oligossacharides, a transformation performed by

sulfotransferases specifically located within the trans Golgi cisternae and the TGN (Baeuerle and Huttner, 1987; Hart, 1992). Some additional modifications such as endoproteolysis, exoproteolysis, COOH-terminal amidation, NH_2-terminal acetylation, phosphorylation, and pyroglutaminyl formation are also performed in this compartment (Bennett, 1985; Eipper et al., 1987; Mains et al., 1990; Pohl et al., 1991; Lasa-Benito et al., 1996; Lasa, Marin and Pinna, 1997; Vegh and Varro, 1997).

After undergoing some or all of these modifications within the Golgi apparatus, proteins reach the TGN. In this last compartment, they are sorted and segregated to leave in transport vesicles towards either the cell surface, endosomes/lysosomes or secretory granules. It has been suggested that the TGN is composed of functionally distinct subdomains, with each domain housing different types of modifying enzymes, such as sialyl transferases and sulfo transferases, or each involved in the segregation of proteins in different types of transport vesicles destined for secretory granules, the plasma membrane or the endosomal/lysosomal system (Ladinsky et al., 1994) (see Figure A-1).

Table A-III: Golgi-specific modifications

1. N-linked oligosaccharide modifications:
- Trimming of mannoses
- Addition of N-acetylglucosamine, galactose, sialic acid and fucose
2. O-linked glycosylation
3. Sulfation
4. Phosphorylation
5. COOH-terminal amidation
6. Endoproteolysis
7. Exoproteolysis
8. Pyroglutaminyl formation

Vesicles carrying proteins between the ER-Golgi systems are precisely targeted from one organelle to the other. The cytosolic surface of these vesicles is covered by a set of coat protein subunits, α-, β-, β'-, γ-, δ-, ε-, and ζ-COPs (for coat proteins), which form a protein complex named coatamer (Waters, Serafini, and Rothman, 1991; Rothman and Orci, 1992, 1996; Kreis and Pepperkok, 1994; Rothman, 1994; Schekman and Orci, 1996). Retrograde transport towards the ER is mediated by COPI coatamer-coated vesicles, which is required for the Golgi retrieval of specific ER-resident proteins exhibiting the KKXX retrieval motif (see Figure A-2 (a)) (Cosson and Letourneur, 1994; Letourneur et al. 1994). The anterograde budding is handled by ER-derived COPI and COPII transport vesicles, two distinct populations of ER-derived transport vesicles (Orci et al., 1991a, 1994; Waters, Seraphini, and Rothman, 1991; Hicke, Yoshihisa, and Rothman, 1992; Bednarek et al., 1995; Salama and Schekman, 1995; Schekman and Orci, 1996). COPI is also involved in intra-Golgi transport, while no such role could so far be attributed to COPII (see Figure A-2 (a)) (Waters, Serafini, and Rothman, 1991; Kuge et al., 1994). It has been suggested that in mammalian cells, COPII permits sorting and concentration of proteins upon export from the ER, while COPI directs retrieval from the intermediate compartment to recycle resident proteins back to the ER (Aridor and Balch, 1996). Brefeldin A, which prevents coat assembly, affects the membrane attachment of the β-COP subunit to Golgi membranes (Donaldson et al., 1990). This results from its action on another cytosolic factor, the small GTP-binding protein Arf (ADP-ribosylation factor), which provides binding sites for coatamer on membranes (Donaldson and Klausner, 1994; Elazar et al., 1994; Boman and Kahn, 1995). Animal cells exhibit several different Arf proteins with distinct cellular distributions (Cavenagh et al., 1996), but only the role of Arf1 has so far been well characterized (Kahn et al., 1991). BFA blocks Arf binding to Golgi, therefore preventing coatamer assembly and budding of coated vesicles. This in turn causes the release of Arf and coatamer from Golgi membranes and collapse of Golgi cisternae into one another (Orci et al., 1991b).

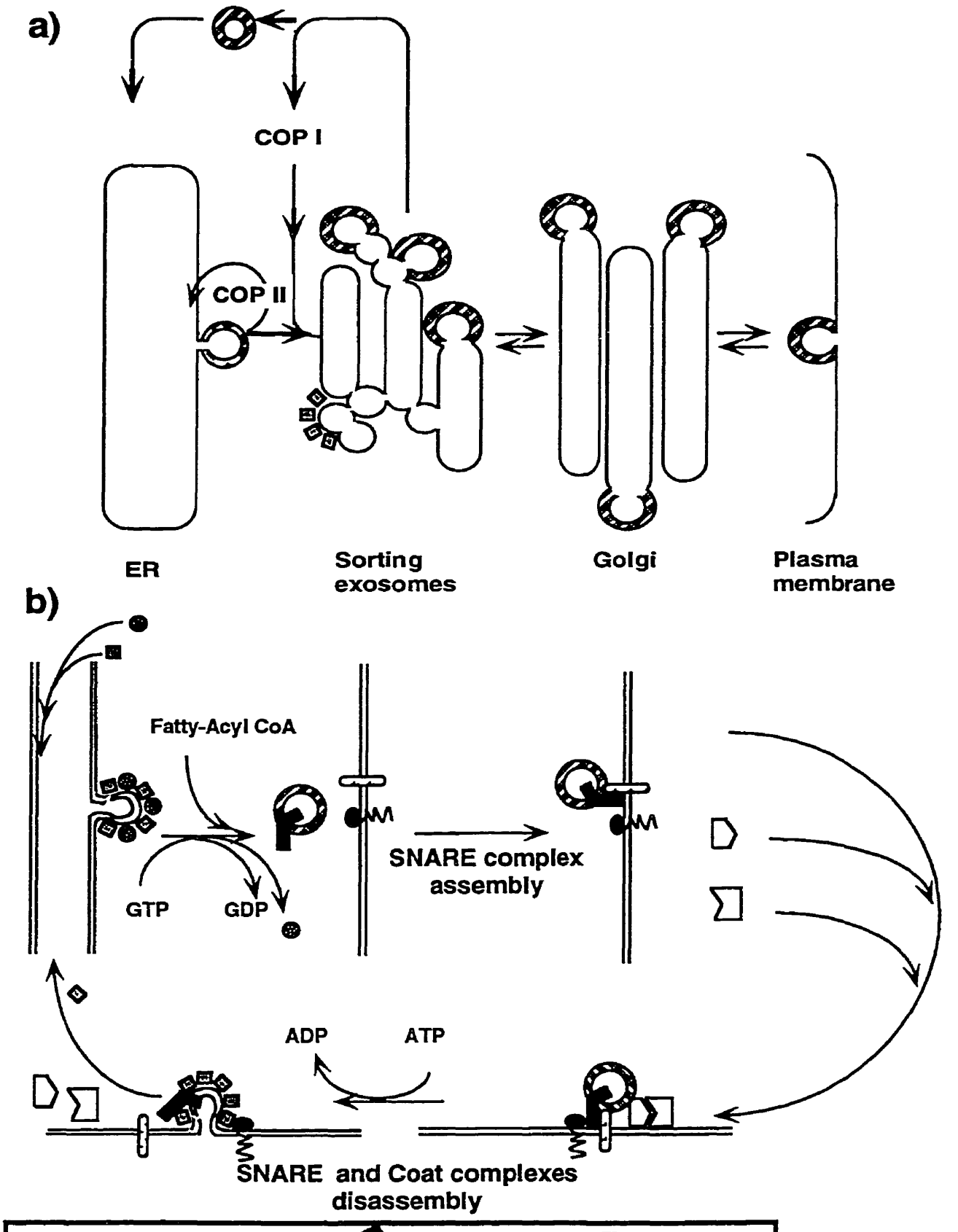
a)
COP I
COP II
ER
Sorting exosomes
Golgi
Plasma membrane
b)
Fatty-Acyl CoA
GTP
GDP
SNARE complex assembly
ADP
ATP
SNARE and Coat complexes disassembly
Coat proteins
Coatamer COP I
Coatamer COP II
v-SNARE
t-SNARE
rab
α-SNAP
NSF
ARF

Figure A-2:

a) COPI- and COPII-mediated sorting events at the level of the Golgi and ER (adapted from Salama and Schekman, 1995; Aridor and Balch, 1996). Protein export from the ER is mediated by COPII coat complex. Cargo is subsequently delivered to the sorting exosomes, where COPI will execute the retrieval of KKXX-containing proteins to the ER. Anterograde transport of proteins to the Golgi requires segregation from ER-recycling proteins and association of additional membrane components with COPI.

b) Vesicle formation and transport mediated by SNARE complex assembly, targeting and disassembly (adapted from Rothman and Orci, 1992; Rothman, 1994; Bennett, 1995; Schekman and Orci, 1996; Goda, 1997). Vesicle formation is initiated by recruitment of coat proteins to the membrane surface by ARF. GTP hydrolysis is thought to cause a conformational switch between a membrane-bound and soluble form of ARF, inducing the release of vesicles. Targeting of vesicles to the appropriate compartment is thought to require the recognition of the vesicle v-SNARE with the target membrane t-SNARE, as well as the binding of cytosolic SNAPs and NSF. See text for details.

While Arf provides binding sites for coatamer and initiates budding, coatamer binding and assembly drives vesicle budding. It is thought that coated vesicles fuse with their target membrane following ATP hydrolysis by Arf, causing the release of the coatamer from vesicles. Fusion is assisted by the N-ethyl maleimide (NEM)-sensitive fusion factor (NSF) and several other cytosolic components. These soluble NSF attachment proteins (SNAPs), recognize specific vesicular receptors called v-SNAREs (vesicle-associated SNAP receptor). Three SNAPs isoforms have so far been isolated. While the α and γ isoforms are ubiquitous, β-SNAP is specific to brain (Rothman, 1994). It is thought that vesicles locate their specific targets by the pairing of complementary receptors, a v-SNARE for the transport vesicle and a t-SNARE (target membrane associated-SNAP receptor) for the target compartment (see Figure A-2 (b)) (Söllner et al., 1993; Bennett and Scheller, 1994; Pevsner and Scheller, 1994; Rothman, 1994; Rothman and Warren, 1994; Banfield, Lewis, and Pelham, 1995; Bennett, 1995; Mellman, 1995; Lewis and Pelham, 1996; Goda, 1997), but homotypic vacuolar fusion has also been reported (Nichols et al., 1997).

A family of rab proteins has also been implicated in vesicle targeting and fusion (Zerial and Stenmark, 1993; Rothman and Söllner, 1997; Lupashin and Waters, 1997). The different members of this family of small monomeric ras-like GTPases are specific in their membrane localization (Novick and Brennwald, 1993; Pfeffer, 1994; Tavitian, 1994). The association of rab proteins with their proper intracellular membrane target is mediated by a signal present within the COOH-terminal hypervariable domain (Chavrier et al., 1991). Rab proteins assist in the precise delivery of transport vesicles to target membranes (Rothman, 1994). A member of this family has recently been described to function as timer for endocytic membrane fusion (Rybin et al., 1996).

Heterotrimeric G-proteins are also involved in the regulation of different transport steps of vesicular traffic, although their function is not as precisely defined as those of Arf and rab proteins (Melançon et al., 1987; Balch, 1990; Barr, Leyte, and Huttner, 1992; Bomsel and Mostov, 1992; Leyte et al., 1992; Pfeffer, 1992; Schwaninger et al., 1992; Melançon, 1993; Ohashi and Huttner, 1994).

A.3.1 Residence signals in Golgi proteins

In contrast to known ER-resident proteins which return to this compartment in view of the presence of a recycling signal, most Golgi glycosylation enzymes possess genuine retention signals. These integral membrane proteins exhibit a type II topology, with their uncleaved signal peptide acting as a transmembrane anchor. No cytosolic sorting domains have been detected in these proteins. The presence of a single short transmembrane domain of about 17 amino acids, and part of its flanking regions, is sufficient to promote Golgi localization (Machamer, 1991, 1993; Munro, 1991, 1995; Nilsson et al., 1993, 1994; Nilsson and Warren, 1994). The mechanisms suggested for the retention of Golgi membrane proteins comprise aggregation through the membrane anchoring and lumenal domains, also called kin-protein aggregation (Machamer, 1993; Pelham, 1995), localization determined by transmembrane domain length (Munro, 1995), and bilayer-mediated sorting (Pelham and Munro, 1993). Medial Golgi enzymes have been shown to associate with each other *in vivo* and form oligomers (Nilsson et al., 1993), a fact which supports the aggregation sorting mechanism. However, another hypothesis suggests that the transmembrane domains of these proteins act as sorting signals by mediating a lipid-based segregation due to their unique amino acid composition and physical properties (Munro, 1991; Bretscher and Munro, 1993). The length of the transmembrane anchor would permit a lipid-based partitioning of proteins, dependent on membrane thickness, composition and rigidity, which would follow a putative cholesterol gradient across the Golgi stacks (Bretscher and Munro, 1993; Nilsson and Warren, 1994; Munro, 1995; Simons and Ikonen, 1997). The transmembrane anchor length of the different Golgi enzymes would thus determine their partitioning across the Golgi cisternae. These two proposed mechanisms are not mutually exclusive and could well be complementary in mediating the retention of Golgi membrane proteins (Munro, 1995).

A.3.2 Sorting within the *trans*-Golgi Network

The *trans*-Golgi Network, like the *cis*-Golgi Network, is a specialized compartment where proteins undergo sorting to attain their organelles of residence (Orci et al., 1987). Proteins being sorted in the TGN can access several possible destinations: the lysosomes, the plasma membrane, or the secretory granules, in the specialized cells which contain these organelles. As for the CGN, restrictive temperatures, this time close to 20°C, can prevent the progression of proteins beyond the TGN (Matlin and Simons, 1983; Saraste and Kuismanen, 1984; Griffiths and Simons, 1986). Contrary to Golgi enzymes, TGN resident proteins are not retained in this section, but instead cycle between this compartment and other subcellular regions. Two examples of proteins which are retrieved from the plasma membrane to the TGN are TGN38, an integral membrane protein specified to reside in this organelle (Bos, Wraight, and Stanley, 1993; Humphrey et al., 1993; Wong and Hong, 1993; Ponnambalam et al., 1994; Wilde et al., 1994), and the processing enzyme furin, also a membrane-bound protein (Bosshart et al., 1994; Chapman and Munro, 1994; Molloy et al., 1994; Jones et al., 1995; Schäfer et al., 1995; Takahashi et al., 1995; Voorhees et al., 1995). These proteins were demonstrated to have TGN-localization signals within their cytosolic tail, which resemble the internalization signals of membrane receptors. TGN38 and furin exhibit a tyrosine-based motif, consisting of the tetrapeptide Y-XX-Ø (Ø= hydrophobic amino acid). This motif is both necessary and sufficient to retrieve integral membrane proteins from the cell surface to the TGN (Trowbridge, Collawn, and Hopkins, 1993). Mutation or deletion of this signal results in loss of TGN-localization and in the accumulation of TGN38 and furin at the cell surface. In addition to the tyrosine-based motif, furin displays a di-leucine pattern, a motif which has been characterized to act as an internalization signal in other proteins than furin (Johnson and Kornfeld, 1992), and a cluster of acidic amino acids which can be phosphorylated at serines residues by a caseine kinase II (CK-II)-like enzyme. This last motif plays an important

modulatory role in the retrieval of furin to the TGN (Jones et al., 1995; Takahashi et al., 1995). Recently, a putative Golgi-endosome sorting receptor has been cloned (Petersen et al., 1997), which presents homology to the lysosomal sorting receptors. Although this protein presents several interesting features, including a potential casein kinase II phosphorylation site, dileucine signal and tyrosine based motif, as well as an NH_2-terminal furin cleavage site, its expression pattern is restricted to certain tissues, and its exact function has not yet been established.

Soluble enzymes destined to reside in lysosomes which exit the TGN are tagged with mannose-6-phosphate residues added to N-linked oligossacharides. This signal is recognized by two specific receptors localized in the TGN (von Figura, 1991). These receptors are involved in packaging lysosomal enzymes in clathrin-coated vesicles which are targeted to the late endosomes, and then to lysosomes. Mannose-6-phosphate receptors dissociate from their cargo due to the lower pH in late endosomes, and are recycled back to the TGN (Méresse et al., 1993).

Lysosomal membrane-bound proteins (such as the mannose-6-phosphate receptors) are sorted to this compartment due to the presence of signals within their cytoplasmic tails (Peters et al., 1990; Johnson and Kornfeld, 1992; Lehman et al., 1992; Mathews, Martinie and Fambrough, 1993), which also permit the recycling of these proteins from the plasma membrane or the TGN to the lysosomes. This signal can consist of a tyrosine-based motif surrounded by polar or basic residues on either side of the tyrosine (Luzio and Banting, 1993), or of adjacent leucine and/or isoleucine residues (Johnson and Kornfeld, 1992; Letourneur and Klausner, 1992). The targeting of proteins to post-Golgi compartments by these two signals has recently been demonstrated to depend on two distinct and saturable sorting mechanisms (Marks et al., 1996).

The recycling of proteins from the plasma membrane is performed through endocytosis of clathrin-coated pits. Clathrin-coated vesicles are only found to bud from the plasma membrane or TGN, and both

populations or vesicles can be involved in endocytosis. Clathrin-coats, consisting of basket-like triskelion structures formed by heavy and light clathrin chains, assemble on TGN or plasma membranes by interacting with protein complexes called adaptors. Three adaptor protein complexes (AP) have been identified. While, AP2 is associated with the plasma, AP1 is coupled with the TGN (Robinson, 1987, 1994; Ahle et al., 1988; Hunziker, Simmen and Honing, 1996; Mallet and Brodsky, 1996; Traub et al., 1996; Rapoport et al., 1997). The recently discovered AP3 adaptor is ubiquitously distributed and is not associated with clathrin, but seems to be linked to lysosomes (Dell'Angelica, 1997; Simpson et al., 1996, 1997). These adaptor complexes are heterotetramers composed of two proteins called adaptins (α and β for the plasma membrane and β' and γ for the TGN; μ3 and β3B). Another cytosolic component, called dynamin, facilitates the formation of coated vesicles through a GTP-GDP cycle (Hesrkovits et al., 1993; Van der Bliek et al., 1993).

Several internalization signals towards endosomes have been determined in membrane-bound receptors such as those of transferrin or low-density lipoprotein. These signals consist of a short stretch of 4 to 6 amino acids forming a tight turn (Collawn et al., 1990; Trowbridge, 1991; Gruenberg and Maxfield, 1995). Mannose-6-phosphate receptors on the other hand require the presence of a tyrosine or aromatic residue within this short amino acid cluster (Canfield et al., 1991). A novel endocytosis signal was also recently described, which resembles the KKXX ER-retrieval signal (Itin et al., 1995).

Proteins which are endocytosed are sent to the early endosomes, from which they can recycle either towards the cell surface, the TGN, or lysosomes. The clathrin-coated pits and AP2 adaptor complex proteins which perform internalization consist of a distinct population from those mediating transport between the Golgi and early endosomes, called AP1 (Trowbridge, Collawn and Hopkins, 1993; Robinson, 1994). However, tyrosine-based cytoplasmic signals which mediate recycling of integral membrane proteins in the endocytic or secretory pathway both interact with clathrin-associated proteins (Ohno et al., 1995). Other components

such as rab proteins, Arf, heterotrimeric G proteins and phosphoinositides, are also involved in the recycling of proteins through endosomes (Gruenberg and Maxfield, 1995; De Camilli et al., 1996; Kearns, et al., 1997; Martin, 1997).

In unpolarized cells, proteins without specific sorting motives are believed to be delivered by default from the TGN to the cell surface through the constitutive pathway. These proteins are released from the TGN in a steady stream, called constitutive or "bulk flow" secretion. In polarized epithelia, proteins can be released towards two different membrane surfaces which exhibit distinct cell-surface components: the apical and basolateral membranes (see Figure A-3). The apical surface is usually responsible for the epithelial-specific functions of the cell, such as nutrient absorption. In contrast, the basolateral surface membrane usually resembles that of nonpolarized cells, and is involved in the fundamental processes such as cell-surface attachment and growth. Proteins destined to reach the apical or basolateral membranes are synthesized in the ER and transported to the TGN, but are then directed to distinct cell surfaces. Additionally, proteins can be exchanged between these two surfaces by a process of internalization and endocytosis to the opposite surface, called transcytosis. During this process, apical and basolateral proteins are sorted in two distinct endosome populations. Incidentally, work with viral glycoproteins recently demonstrated that constitutively released proteins can be sorted in different vesicles in the TGN, both in polarized and non-polarized cells (Much et al., 1996). This suggests that non-polarized cells are capable of discriminating between constitutively secreted proteins before export from the TGN, implying that these cells may have the necessary machinery to differentiate apical and basolateral signals, but have not undergone transformation towards polarization.

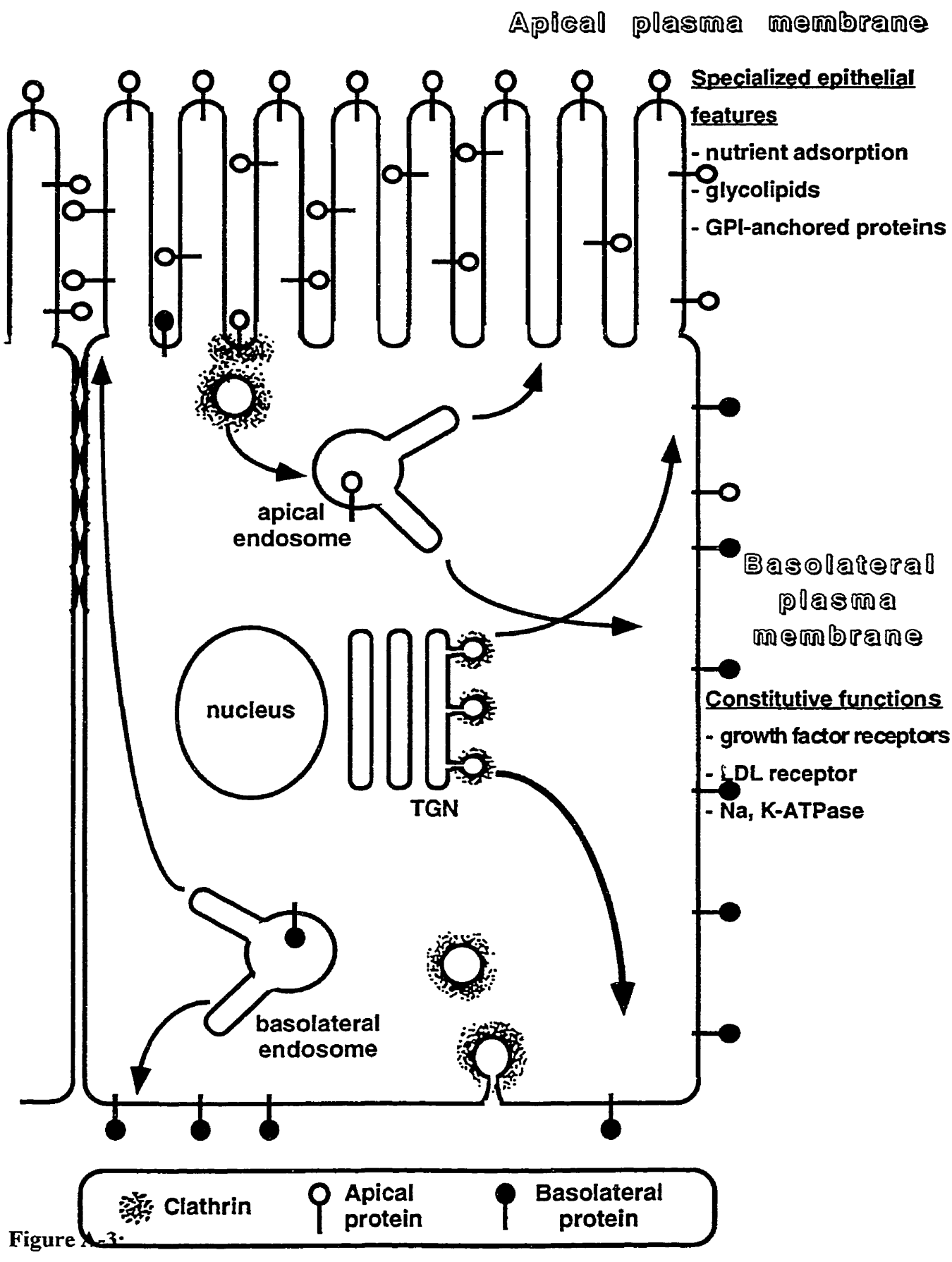

Figure A-3:

Sorting events in polarized cells (adapted from Matter and Mellman, 1994).

Contrary to unpolarized cells, transport of proteins to the apical and basolateral surfaces in epithelial cells seems to rely on selective targeting mediated by specific signals instead of bulk flow delivery. One such signal is the glycosylphosphatidylinositol (GPI) anchor (Lisanti et al., 1989; Lisanti and Rodriguez-Boulan, 1990; Simon and Ikonen, 1997). GPI-linked proteins are enriched in the apical membrane of several epithelial cell types, and their sorting to this surface may occur by a mechanism similar to that of Golgi-membrane proteins, by selective aggregation and interaction with glycosphingolipids of the apical surface (Brown, Crise, and Rose, 1989; Simons and Wandinger-Ness, 1990; Brown and Rose, 1992; Matter and Mellman, 1994; Simons and Ikonen, 1997).

N-glycans have also been suggested to play a role as potential sorting signals of glycoproteins in epithelial cells, mostly in apical membrane sorting (Rodriguez-Boulan and Powell, 1992; Fiedler and Simons, 1995; Scheiffele, Peränen, and Simons, 1995; Ponnambalam and Banting, 1996). Additionally, a lectin-like protein isolated from purified apical vesicle populations from MDCK cells called VIP36 was suggested to play a role in the apical sorting of O-glycosylated proteins (Fiedler et al., 1994; Fiedler and Simons, 1995, 1996).

Two types of basolateral targeting motives have so far been described. The first type is a tyrosine-based signal similar to the rapid endocytosis signals of some surface receptors such as that of the LDL receptor (Brewer and Roth, 1991; Hunziker et al., 1991; Mostov et al., 1992). Similarly, deletion or mutation of a di-leucine signal in the Fc receptor causes the surface expression of this protein in MDCK to shift from basolateral to apical (Hunziker et al., 1991; Matter, Yamamoto, and Mellman, 1994), demonstrating that presence of this signal is essential for targeting to the basolateral membrane.

Another type of basolateral sorting signal was uncovered from studies of the polymeric IgA receptor (Casanova, Apodaca, and Mostov, 1991; Matlin, 1992; Mostov et al., 1992). A 17 amino acid region within the cytosolic tail of this protein bears its targeting signal to the basolateral surface.

Recently, the intracellular loop of the glucose transporter isoforms GLUT1 and GLUT5 was demonstrated to play a pivotal role in the apical/basolateral sorting of these twoproteins in the polarized CaCo2 cell line ((Inukai et al., 1997).

So far, only one signal has been positively identified as being necessary for transcytosis. The phosphorylation of a particular serine residue in the cytosolic tail of the polymeric immunoglobulin receptor signals the transport of this protein to the apical surface (Casanova et al., 1990; Casanova, Apodaca, and Mostov, 1991).

Neuronal cells also display polarity by the division of their membrane in axons and dendrites. In these cells, GPI-anchored proteins such as Thy-1 are found only in axons, while viral glycoproteins, such as those of the vesicular stomatitis virus, are detected over the cell body and dendrites (Dotti and Simons, 1990; Dotti, Parton, and Simons, 1991).

Neuronal cells and some endocrine cells exhibit an additional level of complexity, by storing non-peptide neurotransmitters in synaptic vesicles. These vesicles form a regulated pathway distinct from that of secretory granules (Kelly, 1991, 1993a,b; Bennett and Scheller, 1993; Mundigl and De Camilli, 1994; Pesvner and Scheller, 1994; Südhof, 1995), and originate from a compartment continous with the plasma membrane and devoid of transferrin receptor (Schmidt, Hannah, and Huttner, 1997).

A.4 Regulated secretion

Apart from neuronal cells and some endocrine cells which display a specialized secretory pathway for non-peptide neurotransmitters, two routes exist for the release of protein products (Burgess and Kelly, 1987). All cell types possess a constitutive release pathway which, as was discussed earlier, is not subject to external stimuli regulation and permits the unrestrained flow of proteins towards the cell surface. Constitutive vesicles are small and do not possess a clathrin coat. The release of their content is not dependent on intracellular calcium concentration changes. Growth factors and integral membrane receptors follow this route to the cell surface. However, some cells, such as endocrine cells, possess another exocytosis pathway (Gumbiner and Kelly, 1982; Burgess and Kelly, 1987; Rivas and Moore, 1989). This path is called regulated, since the products conveyed through it do not flow freely towards the surface, but instead are selectively stored and accumulated into secretory granules, from which they are released upon stimulation. Regulated pathway secretion can be modulated by secretagogues such as hormones or glucose, to immediately release the granule protein stores upon reception of the appropriate physiological signal. When external stimuli provoke a transient increase in the intracellular calcium concentration, fusion of the secretory granules with the cell membrane occurs, ultimately causing the rapid discharge of their content in the extracellular space.

Some cells exhibit multiple types of secretory granules, with respect to their content. For example, the acidophilic cells of bovine pituitary have been observed to store three proteins, namely growth hormone, prolactin and secretogranin II, in three distinct kinds of secretory granules (Hashimoto et al., 1987). Another example is seen in the marine mollusk *Aplysia californica*, where distinct peptide products

arising from the proteolytic processing of a unique polypeptide precursor, the egg-laying hormone, are packed into different secretory granules which end up in opposite parts of the cell (Fisher et al., 1988; Jung and Scheller, 1991).

The membrane of secretory vesicles holds several specific proteins. Some are needed for the docking and fusion of the granules, while others are proton pumps and vacuolar ATPases which are necessary to establish and maintain the proper pH gradient across the granule membrane. Regulated secretory granules, unlike constitutive vesicles, are initially covered with a clathrin coat as they leave the TGN. At this stage, they are called immature secretory vesicles. During an enlargement process which is thought to occur through the fusion of several granules, condensation of their content and progressive acidification (Tooze and Huttner, 1990; Tooze et al., 1991; Grimes and Kelly, 1992), regulated secretory vesicles shed their clathrin coat, fuse together and become large and dense.

Although it is generally assumed that the TGN is the last "sorting station" before proteins are conveyed to the cell surface (Orci et al., 1987) either through constitutive or regulated vesicles, it has been suggested that the immature granule could also act as a sorting compartment (Arvan and Castle, 1987, 1992; Von Zastrow and Castle, 1987). During granule maturation, some constituents are excluded from the dense core of the mature granule and are released through a post-granular "constitutive-like" pathway (Arvan et al., 1991; Matsuuchi and Kelly, 1991; Kuliawat and Arvan, 1992, 1994). In normal cells, sorting of vesicular contents through this path accounts for no more than 10% of granule content. In tumor-derived cell lines however, secretion through this route can add to as much as 50% of proteins released from granules (Moore, Gumbiner, and Kelly, 1983; Matsuuchi and Kelly, 1991; Milgram, Eipper and Mains, 1994).

A.4.1 Secretory granules sorting signals

What is the molecular basis for the sorting of proteins to secretory granules? The mechanism by which proteins are selectively targeted to this path as they leave the TGN has not yet been conclusively established. While constitutively secreted proteins are thought to leave the cell by a non-selective bulk flow mechanism, peptides destined to reach secretory granules tend to aggregate in a calcium- and pH-dependent manner (Kelly, 1985; Chanat and Huttner, 1991; Ma et al., 1995). Accordingly, two mechanisms have been proposed for the sorting of proteins to secretory vesicles (Chidgey, 1993): a passive aggregation-mediated mechanism (Burgess and Kelly, 1987), and an active receptor-mediated system (Kelly, 1985). In aggregation-mediated sorting, proteins destined to the regulated secretory pathway form aggregates in the TGN which exclude other proteins destined to the constitutive pathway. It was recently demonstrated that certain prohormones aggregate in the mildly acidic conditions thought to reproduce those of the secretory granules (Colomer, Kicska, and Rindler, 1996). In the receptor-mediated sorting mechanism, specific signals present on the regulated secretory proteins are recognized by receptors which promote targeting to the regulated pathway. From the following, it will appear that these two mechanisms are not mutually exclusive and may function together.

A three-dimensional structure obtained upon comparison of different sequences, consisting mostly of relatively short NH_2-terminal stretches of polypeptide chains implicated in protein sorting to the regulated pathway, was postulated to act as a sorting signal (Kizer and Tropsha, 1991). For proteins in which the sorting signal resides in the NH_2-terminal segment, it is predicted that an alpha helical hydrophobic region will behave as the targeting motif (Gorr and Darling, 1995).

However, no definite and universal structural pattern has yet been conclusively established for the sorting of regulated secretory proteins. Some proteins exhibit sorting signals within their proregions. This is the case for several prohormones, such as POMC (Chevrier et al., 1993; Cool et al., 1995) and prosomatostatin (Sevarino et al., 1989; Stoller and Shields, 1989; Sevarino and Stork, 1991), which present several targeting signals within these regions. Secretory granule targeted membrane-bound proteins possess sorting information either within their cytosolic tail, as is the case for P-selectin (Disdier et al., 1992; Koedam et al., 1992; Subramaniam, Koedam, and Wagner, 1993; Norcott, Solari, and Cutler, 1996), or within their lumenal domain, as is seen for the amidating enzyme PAM (Milgram, Mains, and Eipper 1993, 1996). Other proteins require the presence of a particular structural motif, such as chromogranin B, the sorting of which is dependent on a unique disulfide loop (Chanat et al., 1993; Chanat, Weiss, and Huttner, 1994), while parotid-proline-rich proteins depend on their proline-rich domain for storage into secretory granules (Stahl et al., 1996). Most of these proteins, when transferred to a regulated cell type in which they are not normally expressed, will find their way to the secretory granules (Burgess, Craik, and Kelly, 1985; Moore and Kelly, 1987; Gombau and Schleef, 1994), although this is not always the case (Seethaler et al., 1991). Others, like pro-van Willebrand factor, will induce the production of the proper organelle of storage. Pro-vWF, when expressed in AtT-20 cells, does not enter ACTH-containing granules but instead induces the formation of a novel organelle with the morphological characteristics of Weibel-Palade bodies (Wagner et al., 1991). These organelles do not appear if the NH_2-terminal propeptide of vWF is deleted.

The multitude of sorting signals so far uncovered for proteins destined to the regulated secretory pathway makes the possibility of a receptor-mediated sorting mechanism less likely, although some

candidate receptor proteins have been proposed (Chung et al., 1989; Cool et al., 1997). Either an expansive number of different receptors would be necessary to sort each type of secretory protein, or if only a few receptors were indeed involved, the affinity of these receptors would need to be very broad to accommodate the multitude of targeting structures observed among secretory granule-sorted proteins. However, a recent report has claimed to have uncovered such a regulated secretory pathway receptor (Cool et al., 1997). This receptor is the membrane-associated form of carboxypeptidase E, an enzyme involved in the removal of COOH-terminal basic amino acids from cleaved polypeptides. Down regulation of CPE expression using antisense RNA caused missorting of POMC to the constitutive pathway of Neuro-2a cells. In addition, *Cpe*^{fat}/*Cpe*^{fat} mice in which this gene has undergone a point mutation resulting in the rapid degradation of this protein, exhibit constitutive release of the POMC precursor in the pituitary. While the effect of decreased CPE expression on POMC missorting was demonstrated, it still remains to be established that CPE can act as a common sorting receptor towards other secretory granule targeted proteins.

A family of proteins have been show to be consistently associated with secretory granules. These acidic proteins, called granins, are calcium-binding proteins found in secretory granules of neuroendocrine cells, which tend to aggregate in the appropriate pH conditions (Huttner, Gerdes, and Rosa, 1991; Scammel, 1993; Huttner and Natori, 1995; Ozawa and Takawa, 1995). A role has been suggested for the members of the granin family in assisting the sorting of peptide hormones, by the formation of specific aggregates in the TGN. The granins initiate their aggregation in TGN (Gerdes et al., 1989; Chanat and Huttner, 1991) and the aggregates formed exclude various constitutive proteins. The granins, which are widely distributed, could nucleate an aggregation process

which would include the proteins destined for granules but would exclude constitutively secreted proteins (Tooze et al., 1993). Indeed, chromogranin B has been demonstrated to promote the sorting of POMC-derived products to secretory granules (Natori and Huttner, 1996). Additionally, the granins could be important for the interaction of granule content with the membrane during budding, as it has been demonstrated that chromogranin B strongly binds to membranes (Pimplikar and Huttner, 1992). The granins could therefore be considered as the soluble "receptors" of secretory granule proteins, which promote the aggregation and condensation process leading to sorting.

Whether aggregation- or receptor-mediated, sorting to secretory granules is pH-dependent (Moore, Gumbiner, and Kelly, 1983) and seems to require clathrin (Tooze and Tooze, 1986), although in this latter instance, the localization of proteins which travel through the constitutive pathway of yeast has also been demonstrated to be dependent on the presence of clathrin (Payne and Schekman, 1989; Seeger and Payne, 1992).

Table A-IV: Summary of know sorting signals within the exocytic pathway	
ER	Soluble proteins and type II membrane proteins COOH-terminal KDEL Type I membrane proteins Cytoplasmic tail KKXX or KXKXX Type II membrane proteins Cytoplasmic tail RR
Golgi	Type II (and possibly type I) membrane proteins Transmembrane domain
TGN	Type I membrane proteins Cytoplasmic Y-XX-Ø, cluster of acidic amino acids, Di-leucine signal
Lysosome	Soluble enzymes Mannose-6-phosphate residues Type I membrane proteins Cytoplasmic tyrosine-containing sequence, Di-leucine signal
Apical surface	GPI anchor
Basolateral surface	N-glycans Type I membrane proteins Short cytoplasmic sequence resembling endocytic signal
Endocytosis	Type I and II membrane proteins Cytoplasmic β-turn with an essential tyrosine residue
Secretory granules	N-terminal hydrophobic alpha-helix Proline-rich region Disulfide bond ... and more

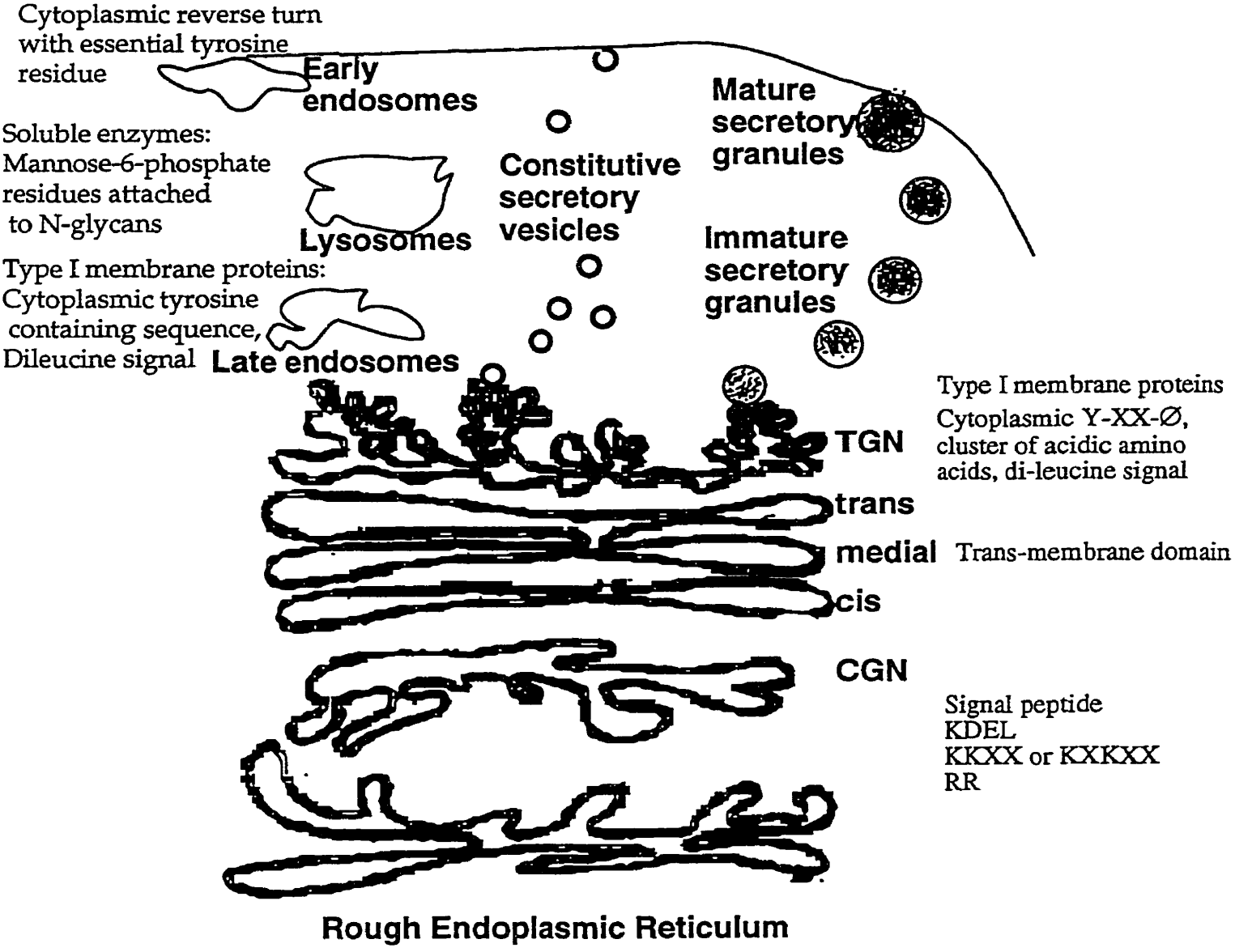

Figure A-4:

Sorting events at the level of the *cis*-Golgi Network and *trans*-Golgi Network, as well as recycling within endosomes.

A.5 Post-translational proteolytic maturation

Apart form the removal of the NH_2-terminal signal peptide in the endoplasmic reticulum, proteins traveling through the secretory pathway can undergo other proteolytic transformations which are initiated in the TGN and/or granules, and can even, in certain circumstances, continue in the extracellular space. For some proteins, removal of the signal peptide in the endoplasmic reticulum is the only necessary proteolytic transformation, which yields the final and mature active product. However, some other polypeptides, like prohormones, exist as intermediate inactive precursor species. These precursors require additional proteolytic conversions to yield the mature active peptides or protein.

Precursor polypeptides can undergo several late posttranslational modifications (Bennett et al., 1993). Production of a mature and active peptide usually requires that several sequential enzymatic steps are performed on the precursor polypeptide: 1) endoproteolysis at paired (or sometimes single) basic amino acids, 2) removal of the exposed basic amino acids by action of a carboxypeptidase, 3) amidation of COOH-terminal glycine residue by the α-amidating enzyme PAM, and/or acetylation of the NH_2-terminal amino acid by N-acetyl transferase, and 4) formation of NH_2-terminal pyroglutamic acid from glutamine. These modifications are usually essential for the peptides to acquire their bioactivity (see Table A-V).

For most precursor proteins, endoproteolysis occurs at basic amino acid residues which often constitute a pair. These cleavage signals have recently been demonstrated to be recognized by a family of endoproteases called proprotein convertases (PCs), which will be the discussed in detail in chapter B.

All possible pairs of basic amino acids, and sometimes monobasic amino acids, mostly arginine (Benoit, Ling, and Esch, 1987; Devi, 1991; Nakayama et al., 1992), can be cleaved at their COOH-terminal end to yield product peptides. However, proteolysis occurs most frequently at KR pairs, followed by RR and RK sequences, with relatively few cleavages at KK pairs. The recognition of these basic amino acid pairs is dependent on the primary sequence as well as on the secondary conformation of the precursor polypeptide in which they are included. It has been suggested that pairs of basic amino acids which are recognized by endopeptidases are present within a stretch of amino acid sequence predicted to form a β-turn or random coil (Rholam, Nicolas, and Cohen, 1986; Gomez et al., 1989; Harris, 1989; Paolillo et al., 1992; Brakch et al., 1993), or an omega loop (Bek and Berry, 1990). Immediate upstream and downstream amino acids can sometimes have a crucial impact on the choice of cleavage site (Watanabe et al., 1992; Bresnahan et al., 1992; Watanabe, Murakami and Nakayama, 1993; Rholam et al., 1995; Ledgerwood et al., 1996). It is also important to note that proteases mediating these processing steps are dependent on the presence of calcium and on appropriate pH conditions. Thus, the selection of a cleavage site relies not only on the physical properties of the substrate but also on those of the enzyme, and on the potential modifications undergone by these proteins under certain pH and/or calcium conditions.

Following the endoproteolytic cleavage of peptide precursors at monobasic or dibasic sites, the exposed COOH-terminal basic amino acids are removed by the action of a carboxypeptidase, such as carboxypeptidase E (CPE) also called carboxypeptidase H (CPH) (Fricker, 1991; Fricker et al., 1991). Carboxypeptidases remove the terminal basic amino acid residues without further degradation of the peptide product. The soluble CPE is present within the secretory granules of many

neuroendocrine tissues, including adrenal medulla and pituitary, while other members of the carboxypeptidase family, such as carboxypeptidase M (Tan et al., 1989), are membrane-bound and could be involved in the processing of precursors traversing the constitutive secretory pathway. The importance of carboxypeptidase actions in the generation of bioactive peptides have recently been demonstrated in a transgenic model of obesity and hyperglycemia, the Cpe^{fat}/Cpe^{fat} mouse (Naggert et al., 1995). In these mice, CPE is modified by a point mutation in a highly conserved region. The peptide processing efficiency of this enzyme is consecutively decreased by 60 to 90% in pancreatic islets, and this ultimately results in high levels of circulating diargynyl COOH-terminally extended insulin, which demonstrates a greatly reduced bioactivity.

The acetylation of NH_2-terminal amino acids by acetyl transferases can selectively increase or decrease peptide biological activity. For example, peptides derived from POMC can be acetylated at two sites: at the NH_2-terminal serine of αMSH, and at the NH_2-terminal tyrosine of βendorphin (Bennett, 1985; Mains et al., 1983). The analgesic properties of acetylated βendorphin are greatly reduced (Akil, Young, and Watson, 1981), while the potency of diacetylated αMSH is enhanced five-fold (Rudman et al., 1983). Additionally, an N-acetyl group can confer protection against the degradative action of aminopeptidases.

Many bioactive peptide hormones are α-amidated at their COOH-terminus. This transformation, which is performed by peptidylglycine alpha-amidating monooxygenase by the conversion of COOH-glycine residues into COOH-terminal amides (Eipper, Stoffers,

and Mains, 1992; Eipper et al., 1993), is often essential to the bioactivity and stability of these peptides.

Finally, glutaminyl cyclase promotes the formation of pyroglutamate by the cyclization of an NH_2-glutamine. This transformation can also provide some protection against aminopeptidase degradation (Pohl et al., 1991).

Table A-V: Post-translational proteolytic modifications of polypeptides

1. Endoproteolysis
2. Exoproteolysis: by Carboxypeptidases
3) NH_2 and COOH-termini modifications by:
— a) COOH-terminal Peptidylglycine alpha-amidating monooxygenase
— b) N-Acetyl transferase
— c) N-Glutaminyl cyclase

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Chapter B

Proprotein convertases

B.1 Discovery of the proprotein convertases (PCs)

Proteolytic transformation of polypeptide precursors was first described about thirty years ago, following the studies of two precursor hormones, proinsulin (Steiner, 1967; Steiner et al., 1967; Steiner and Oyer, 1969) and POMC (Chrétien and Li, 1967). The enzymes performing this processing step, however, were only recently identified. The discovery of a yeast enzyme, named kexin, was the breakthrough which uncovered the first member of this endoprotease family in eucaryotes (Julius et al., 1984) (see Figure B-1). The product of the *KEX2* gene is responsible for the cleavage of the α-mating factor polypeptide precursor at multiple pairs of basic amino acids, releasing four identical copies of the mature hormone. Kexin, a calcium-dependent serine proteinase, is structurally related to bacterial subtilisins (Fuller et al., 1988, 1989), particularly by the conservation of the catalytic Asp/His/Ser triad accomplishing the enzyme's charge-transfer relay (see Figure B-1).

In 1989, the quest for the mammalian counterparts of kexin was concluded, when computer database searches established sequence identity between kexin and the product of the c-*fes/fps* oncogene, called furin (Roebroek et al., 1986; Van de Ouweland et al., 1990). Alignment of the sequences of furin and kexin demonstrated a high degree of homology in certain segments, especially in the regions adjoining the catalytic triad. Based on the concept of sequence conservation around the active site residues of serine proteinases, the amplification of mRNA by the polymerase chain reaction (reverse-transcriptase PCR or RT-PCR) permitted the isolation of two other mammalian homologues of kexin, named PC1 (also called PC3) (Seidah et al., 1990, 1991a,b; Smeekens et al., 1991) and PC2 (Seidah et al., 1990; Smeekens and Steiner, 1990), soon followed by those of PACE4 (Kiefer et al., 1991), PC4 (Nakayama et al., 1992a; Seidah et al., 1992), PC5 (also called PC6) (Lusson et al., 1993; Nakagawa et al., 1993), and more recently PC7 (also called PC8, SPC7, or LPC) (Bruzzaniti et al., 1996; Meerabux et al., 1996; Seidah et al., 1996c) (see Figure B-1).

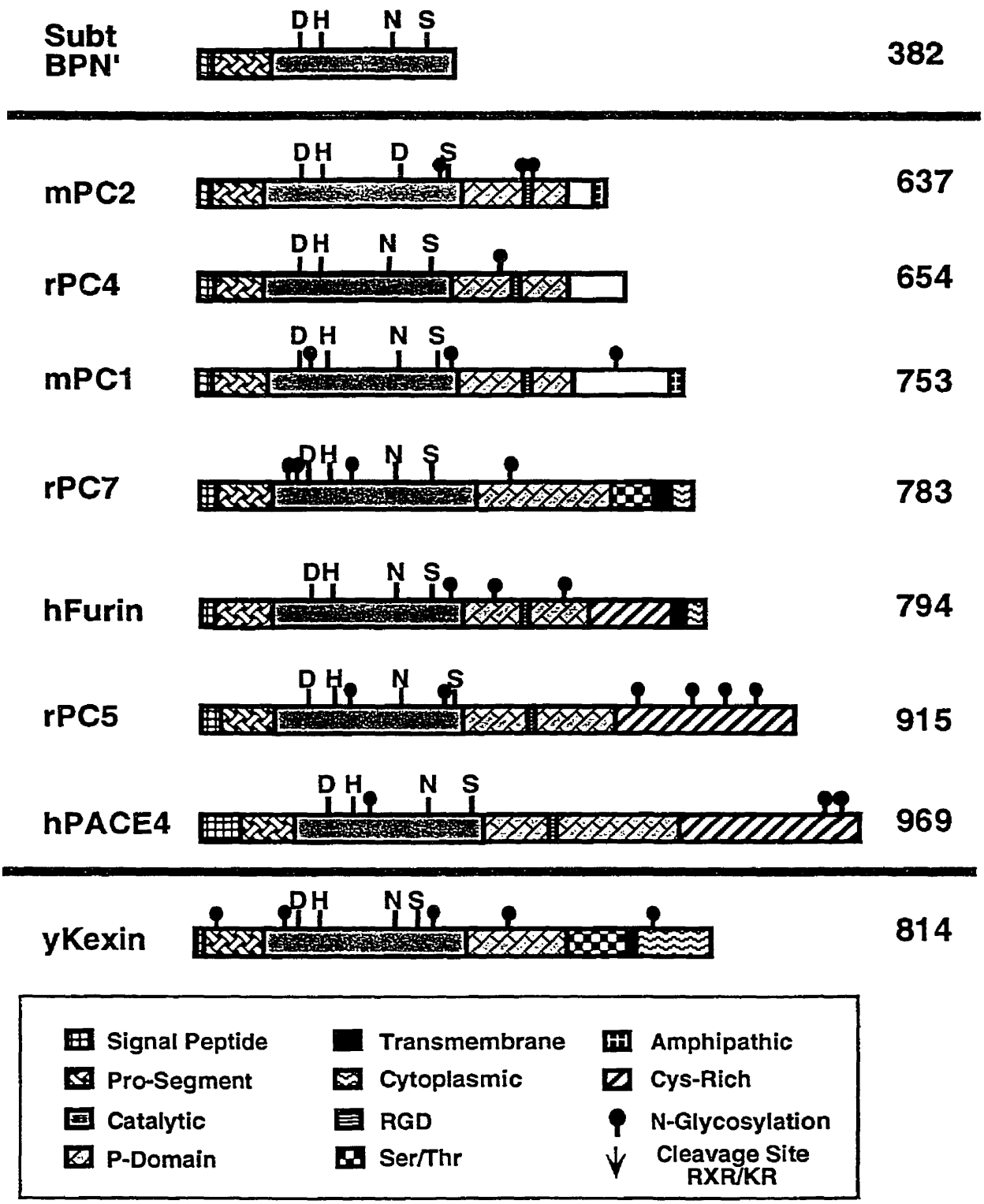

Figure B-1:
Schematic representation of the mammalian members of the convertase family, with yeast kexin and bacterial subtilisin as comparison.

Apart from furin and PACE4, each convertase has a unique chromosomal localization (see Table B-I). The general exon-intron organization sustains very little variations among convertase genes, suggesting that these proteases may have evolved from a common ancestral origin. However, each member of the family has a unique promoter region, which may hint at a specific regulation of the expression and transcription of each convertase.

While the mouse PC7 gene (*Pcsk7*) is located on chromosome 9 (Seidah et al., 1996c), in human, this member of the convertase family has been assigned to chromosome 11, at a translocation breakpoint occurring in lymphomas (Meerabux et al., 1996). No information is yet available on the promoter region of PC7.

Both furin and PACE4 are localized on chromosome 7 in mouse (Mbikay et al., 1995) (chromosome 15 in human (Roebroek et al., 1986; Barr et al., 1991; Kiefer et al., 1991)). While the promoter region of PACE4 has not yet been investigated, that of furin has been characterized (Ayoubi et al., 1994). The expression of furin is driven by alternative promoters containing SP1 elements and combinations of regulated TATA-containing and house-keeping GC-rich regions. Their differential use may modulate furin expression levels, as well as the production of multiple mRNA forms differing at their 5' non-coding regions.

Neither promoter regions of PC1 (Ftouhi et al., 1994; Hanabusa et al., 1994; Jansen et al., 1995), PC2 (Ohagi et al., 1992) nor PC4 (Mbikay et al., 1994) possess TATA-containing segments. Except for that of the human PC1, these convertases' promoters are quite GC-rich. All contain multiple transcription initiation sites. The human PC1 promoter contains two cAMP-response elements, which may mediate the cAMP-mediated hormonal transcriptional regulation of the PC1 gene (Jansen et al., 1995).

Convertase	human chromosomal location	mouse chromosomal location
PC1	5q15-q21	13[C1-C3]
PC2	20p11.1-p11.2	2[F3-H2]
PC4	19	10
furin	15q25-q26.1	7[D1-E2]
PACE4	15	7
PC5	9	19
PC7	11	9

Table B-I:

Chromosomal location of convertases genes. (according to Seidah, Chrétien, and Day, 1994; Mbikay et al., 1995; Seidah et al., 1996c).

B.2 Structure of the members of the convertase family

The members of the convertase family share substantial structural homology. All convertases possess a signal peptide or pre region, which permits entry into the secretory pathway and is cleaved cotranslationally as these proteins are being threaded through the ER membrane. This segment is followed by a propeptide of about 80 to 105 amino acid residues, which ends with the established cleavage motif -R-X-K/R-R↓. The prosegment must be removed to permit the activation of the cognate convertase. For PC1 (Goodman and Gorman, 1994), PC2 (Matthews et al., 1994), furin (Leduc et al., 1992), and kexin (Germain et al., 1992a), prosegment cleavage is an autocatalytic process, which could be intermolecular for PC2, while it is intramolecular for PC1, furin, and kexin. Except for PC2, proregion cleavage of the convertases is an early event, which takes place in the endoplasmic reticulum (Wilcox et al., 1991; Benjannet et al., 1993; Molloy et al., 1994; Creemers et al., 1995). Mammalian members of the convertase family exhibit two potential

cleavage sites composed of paired or multiple basic amino acids at the junction of the pro and catalytic regions. Even kexin shows two KR pairs at this position, one of which is the authentic proregion cleavage site. Only PC7 possesses a single multibasic site. In the case of the convertases furin and PC7, the proregion has been established to act as a specific inhibitor of the respective convertase (Anderson et al., 1995,1 997; Zhong, Munzer, and Seidah, in preparation). The prosegment is believed to remain bound to the catalytic region of the convertase, until the convertase's compartment of residence is attained. It has been suggested that (auto)cleavage of the second paired basic amino acid site of the furin prosegment could contribute to detach this peptide from the convertase, and permit full activation of the protease when it attains the TGN, its compartment of activity (Anderson et al., 1995, 1997).

While PC1, furin and kexin undergo proregion cleavage in the ER, PC2 is so far the only known convertase for which this processing step takes place in the TGN (Guest et al., 1992 Benjannet et al., 1993; Shen, Seidah and Lindberg, 1993). This cleavage is believed to be an intermolecular autocatalytic event, as studies with an active site mutant PC2 demonstrated that this mutated PC2 could still undergo proregion cleavage in the presence of wild type PC2 (Matthews et al., 1994). PC2 is also unique in the fact that it is the only known convertase for which this process is regulated by a specific binding protein, which remains affixed to the convertase until it attains the proper compartment of zymogen cleavage. This binding protein, called 7B2 (Hsi et al., 1982; Seidah et al., 1983), is a secretory granule component of neuroendocrine cells (Mbikay et al., 1989; Marcinkiewicz et al., 1993b). 7B2 was described as a specific intracellular binding protein of the convertase PC2, which interacts temporarily with this convertase in the secretory pathway (Braks and Martens, 1994; Benjannet et al., 1995a). 7B2 is a bifunctional molecule, which facilitates the maturation of proPC2 in neuroendocrine cells through its NH_2-terminal domain, and is necessary for the generation of enzymatic

activity (Benjannet et al., 1995a; Braks and Martens, 1995; Zhu and Lindberg, 1995). However, 7B2 can also act as a potent inhibitor of PC2 through its COOH-terminal region (Martens et al., 1994; van Horssen et al., 1995). The dissociation of 7B2 and PC2 is not dependent on proPC2 maturation (Braks, van Horssen, and Martens, 1996), but an internal cleavage of the 31 amino acids COOH-terminal segment of 7B2 followed by removal of the dilysine pair by CPE is believed to neutralize the inhibitory effect of this binding protein on PC2 by promoting the detachment of the two proteins (Zhu et al., 1996).

The catalytic domain of the convertases, a segment of about 240 amino acids, is the region where the highest homology of structure is observed among the mammalian members of this family. This resemblance is particularly strong between PC5 and PACE4, which share 74% identity of the catalytic region (see Table B-I). PC2 shows the least homology to the other mammalian members, especially since it is the only convertase with an aspartic acid residue instead of an asparagine within the oxyanion hole region responsible for the stabilization the oxyanion transition state. It has been suggested that this substitution at the oxyanion hole could permit PC2 to acquire a narrower and more discriminate substrate specificity (Brenner, Bevan and Fuller, 1993; Seidah, Day and Chrétien, 1993). Coexpression studies employing a PC2 mutant in which the Asp was replaced by an Asn did not demonstrate differences in kinetics of proregion cleavage or substrate cleavage, with regards to the substrate chosen, POMC, although the oxyanion hole-mutant PC2 was no longer secreted (Zhou, Paquet and Mains, 1995). However, the specific substrate selectivity of the wild-type and mutated PC2 were not investigated. Recently, the PC2 oxyanion hole Asp residue was demonstrated to be essential for the binding of proPC2 to pro7B2 within the ER (Benjannet et al., 1995b). For furin, substitution of the Asn by an Ala blocks substrate processing but not furin proregion removal, and does not significantly affect the

subcellular distribution of this enzyme (Creemers et al., 1993b, 1995), while substitution of the Asn for Asp in kexin resulted in reduced catalytic activity, but unchanged proregion cleavage (Brenner, Bevan and Fuller, 1993). This oxyanion hole-mutated kexin was shed to the same extend as wild-type kexin from the cells in which they were expressed, demonstrating that it is not retained in the ER, where it could still undergo proregion cleavage, but would not have access to its substrates (Brenner, Bevan and Fuller, 1993).

Apart from the conserved catalytic triad and the important oxyanion asparagine (aspartic acid for PC2), the convertases present a conserved cysteine within their catalytic site, four residues downstream of the histidine residue. This cysteine is presumed to function as a free thiol group, a fact which would explain the inhibitory effects of heavy metals such as zinc or mercury on the convertase enzymes (Jean et al., 1993).

Convertase	hPC1	hPC2	rPC4	hfurin	hPACE4	rPC5	rPC7
hPC1							
hPC2	54%						
rPC4	58%	55%					
hfurin	62%	56%	68%				
hPACE4	62%	51%	61%	68%			
rPC5	59%	51%	62%	67%	74%		
rPC7	45%	43%	44%	47%	50%	48%	
ykexin	46%	45%	45%	45%	45%	44%	43%

Table B-II:

Percentage sequence identity of the catalytic domains of the convertases human PC1 (hPC1), human PC2 (hPC2), rat PC4 (rPC4), human furin (hfurin), human PACE4 (hPACE4), rat PC5, (rPC5), rat PC7 (rPC7), and yeast kexin (ykexin) (according to Seidah et al., 1994, 1996c).

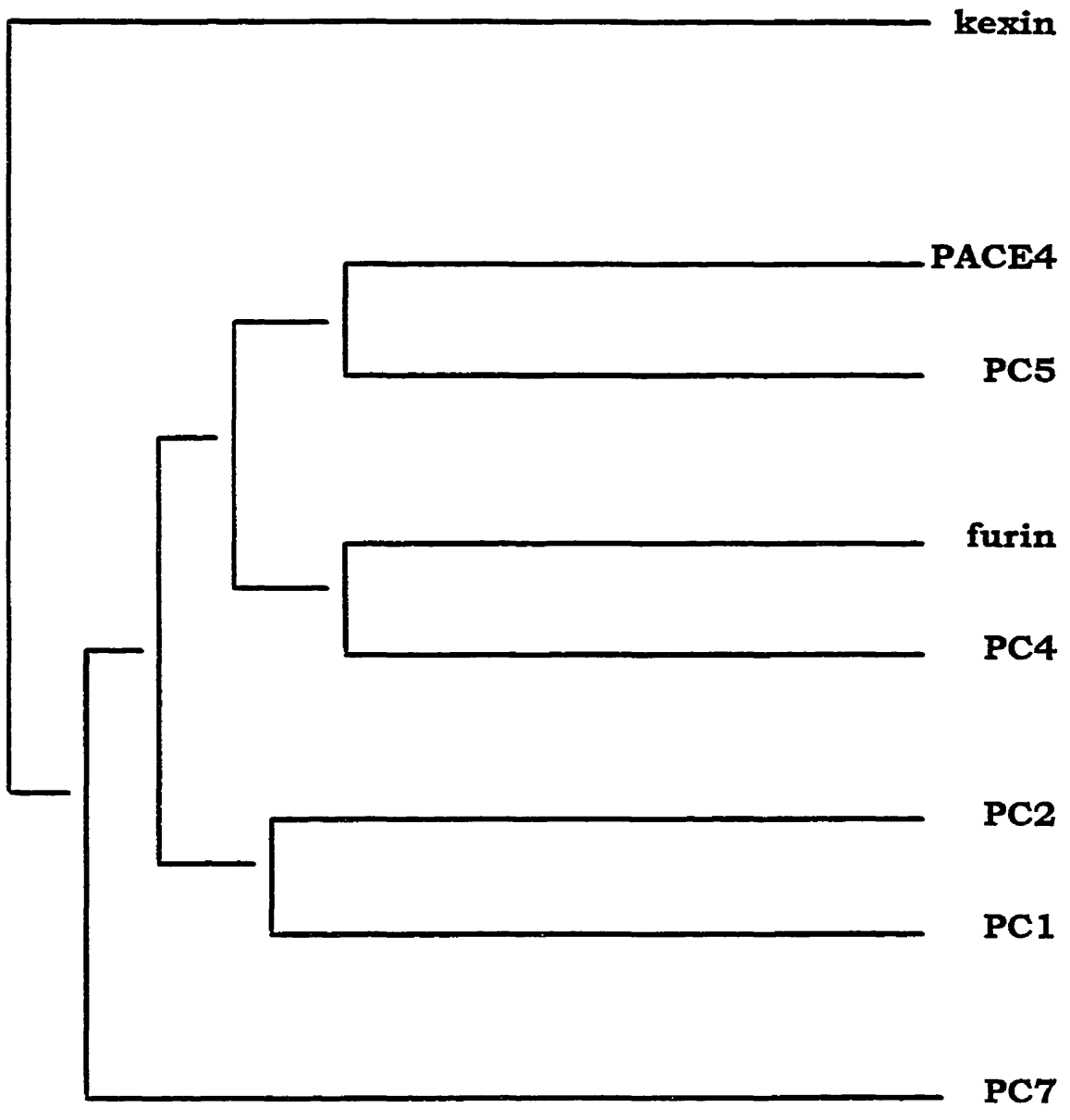

Figure B-2:

Dendogram representing the phylogenic tree of the mammalian convertases (according to Seidah et al., 1996c).

Phylogenically, PC7 is the mammalian convertase most homologous to kexin, while the pairs PC1/PC2, PACE4/PC5 and furin/PC4 form equivalence subclasses of their own (Seidah et al., 1996c) (see Figure B-2). It has been suggested that PC7 may be the ancestral mammalian convertase, as it appears to be the closest in structure to yeast kexin.

Following the conserved subtilisin-like catalytic region, all members of the convertase family share the presence of a P domain (also called homo B), a segment which, as for the COOH-terminal domain, is absent from the related bacterial subtilisin enzymes (see Figure B-1). It has been suggested that this segment could play an important role in the proper folding of the convertases. Truncation studies with both kexin and furin demonstrated that this region is essential for enzymatic activity (Fuller, Brake and Thorner, 1989; Wilcox and Fuller, 1991; Hatsuzawa et al., 1992). Recent studies with the furin-defective LoVo cell line demonstrated that a single point mutation within the P domain of this convertase can prevent autocatalytic prosegment cleavage, and ultimately result in processing deficiency (Takahashi et al., 1993, 1995b). Deletion of the P domain in kexin also blocks proregion cleavage, and prevents transformation of the zymogen into the active enzyme (Fuller et al., 1991; Gluschankof and Fuller, 1994).

It is within the P domain that a conserved R-**RGD**-L motif is observed. This motif has been established to act as a recognition signal for integrins in several extracellular matrix proteins involved in cell surface adhesion (Ruoslahti and Pierschbachel, 1986; Blobel and White, 1992; Gan et al., 1993). It was suggested that this motif could play a similar role in the convertase enzymes (Seidah, Day and Chrétien, 1993). Of all the known mammalian members of the convertase family, only PC7 exhibits a slightly different motif, with R**RG**SL in place of R**RG**DL. It is possible that the serine residue within this sequence, when phosphorylated, would

substitute for the negatively charged aspartic acid (Seidah et al., 1996c). In non-mammalian members of the convertase family, several variations of the RGD motif are observed. *Xenopus* prohormone convertases PC2 (Braks et al., 1992) and furin-like *Xen-14* and *Xen-18* gene products (Korner et al., 1991), as well as amphioxus PC1/PC3 (Oliva, Steiner and Chan, 1995) and the *Caenorhabditis elegans bli-4* gene furin-like products (Thacker et al., 1995), comprise the archetypal **RG**DL motif, while the Drosophila *Dfur1* gene products (Roebroek et al., 1991, 1993; Hayflick et al., 1992), and Limulus kexin (Kawabata, Saeki and Iwanaga, 1996), display an R**RG**DI motif. Another *Caenorhabditis elegans* Kex2-like product exhibits an R**RG**DT (Gomez-Saladin, Wilson and Dickerson, 1994), while the PC1/PC3 convertase from hydra (Chan et al., 1992), and the PC2-like product of amphioxus feature an R**RG**DV motif, respectively (Oliva, Steiner and Chan, 1995). However, other non-mammalian convertases show unrelated sequences at the corresponding RGD position: an **RG**NL sequence is observed in the *Dfur2* gene product (Roebroek et al., 1992), while the *Lymnaea stagnalis Lfur2 and LPC2* gene products exhibit sequences of **RG**NV (Smit et al., 1994) and **RG**CV (Smit, Spijker and Geraerts, 1992) respectively. As for the *Aplysia californica* convertases, the products of *APC1A* and *APC1B* display respectively an Q**RG**S and R**RG**N motif, while *AFUR* and *APC2* products exhibit sequences of R**RG**E and Y**RG**C (Chun et al., 1994) (see Table B-III). While some of the aforementioned motifs present conserved amino acid substitutions relative to the mammalian R**RG**DL motif, the fact that the RGD sequence is not preserved across all species could mean that this motif may be cryptic in convertases, or that the conserved **[R-G**-charged-**aliphatic]** amino acids in fact compose the important sequence.

Species	**RG**D-like motif	References
Mammals		
Mammalian convertases, excepting PC7	R**RG**DL	
Human and rat PC7	R**RG**SL	Bruzzaniti et al., 1996; Meerabux et al., 1996; Seidah et al., 1996c
Amphibians		
Xenopus laevis		
PC2	R**RG**DL	Braks et al., 1992
furin-like *Xen-14, Xen-18*	C**RG**DL	Korner et al., 1991
Cephalochordates		
Amphioxus		
Branchiostoma californiensis		
PC1/PC3	R**RG**DL	Oliva, Steiner and Chan, 1995
PC2-like	R**RG**DV	
Arthropods		
Insects		
Drosophila melanogaster	R**RG**DI	Roebroek et al., 1991, 1993;
Dfur1		Hayflick et al., 1992
Dfur2	P**RG**NL	Roebroek et al., 1992
Merostomateae		
Limulus		
Tachypleus tridentatus kexin	R**RG**DI	Kawabata, Saeki and Iwanaga, 1996
Annelids		
Caenorhabditis elegans		
furin-like *bli-4*	K**RG**DL	Thacker et al., 1995
Kex2-like product	R**RG**DT	Gomez-Saladin, Wilson and Dickerson, 1994
Mollusks		
Gastropods		
Aplysia californica		
APC1A	Q**RG**SV	Chun et al., 1994
APC1B	R**RG**NI	
AFUR	R**RG**EI	
APC2	Y**RG**CV	
Lymnaea stagnalis		
Lfur2	S**RG**NV	Smit et al., 1994
LPC2	T**RG**CV	Smit, Spijker and Geraerts, 1992
Cnidariae		
Hydrazoae		
Hydra vulgaris		
PC1/PC3	R**RG**DV	Chan et al., 1992

Table B-III:
Comparison of the RGD motif in convertases of several species.

Downstream of the P domain, each convertases displays a unique COOH-terminal region. This segment exhibits the least homology of structure among the members of the convertase family. Not only do each convertase possess a unique COOH-terminal domain structure, but for some of them, multiple spliced mRNA variants are generated, giving rise to COOH-terminally modified forms of these proteins. This is the case for mammalian PC4 (Seidah et al., 1992; Mbikay et al., 1994), PC5 (Nakagawa, Murakami, and Nakayama, 1993) and PACE4 (Kiefer et al., 1991; Tsuji et al., 1994; Zhong et al., 1996; Mori et al., 1997), but also for the Drosophila products of the *Dfur1* gene (Roebroek et al., 1993), as well as the furin-like convertase of C. elegans (Thacker et al., 1995), and the PC1-like products of Hydra (Chan et al., 1992). Each of the multiple splice forms of these convertases exhibit their own unique COOH-terminal features, without any modification of the other structural domains, except for some PACE4 isoforms which display in addition to COOH-terminal modifications, variations or truncations of the P domain sequence (Kiefer et al., 1991; Tsuji et al., 1994; Zhong et al., 1996; Mori et al., 1997). Each of the isoforms of Dfurin1 (Roebroek et al., 1993), PC5 (Nakagawa, Murakami, and Nakayama, 1993; Seidah, Chrétien, and Day, 1994; De Bie and Seidah, unpublished results) and PACE4 (Nagamune et al., 1995) exhibit a distinct tissue distribution.

Some of the structural features which can be observed within the COOH-terminal region of convertases are: potential amphipatic helices for PC1 and PC2, transmembrane domains and cytosolic tails for kexin, furin, PC7 ,the B isoform of PC5 and the E isoform of PACE4, a region of cysteine-rich repeats in furin, PACE4 and PC5, and a serine/threonine/proline rich region in PC7 and kexin (see Figure B-1).

The amphipatic helices of PC1 and PC2 were postulated to serve as membrane-anchors for these convertases, and eventually to assist in the sorting of these two proteins to the secretory granules (Seidah et al.,

1991a; Smeekens and Steiner, 1992), as was established for the COOH-terminal α-helical region of carboxypeptidase E (Fricker, Das, and Angeletti, 1990; Mitra, Song, and Fricker, 1994). However, this sorting function of the COOH-terminal domain of PC1 or PC2 has not yet been conclusively demonstrated, although a role of the COOH-terminal domain in the sorting of these convertases to the regulated secretory pathway has recently been hinted at (Zhou, Paquet, and Mains, 1995; Creemers et al., 1996).

During the biosynthesis of PC1, the formation of a COOH-terminal truncated form is observed. The processing step which generates this 66 kD PC1 product, which lacks most of its COOH-terminal domain, was demonstrated to take place in granules (Vindrola and Lindberg, 1992; Benjannet et al., 1993; Zhou and Lindberg, 1993, 1994). This PC1 COOH-terminal truncation product exhibits a greater potency of cleavage towards fluorogenic substrates than the full length 87 kD zymogen-cleaved PC1. It is, however, chemically less stable, and exhibits a narrower pH optimum (between 5.0 and 5.5). The carboxy-terminal cleavage of PC1 results in several alterations of PC1 enzymatic properties. This processing step may therefore have a significant impact on prohormone processing. It is possible to imagine that the COOH-terminal region of PC1 could act as an intramolecular convertase-specific inhibitor, much like 7B2 does for PC2 in an intermolecular fashion, thus permitting the full delivery of enzymatic activity in the appropriate cellular compartment upon separation of the COOH-terminal domain from the rest of the PC1 endoprotease (Jutras et al., 1997).

All membrane-bound members of the convertase family possess a cytosolic tail adjoining their transmembrane anchor. Certain signals within this region have been demonstrated to play a role in the proper cellular localization of the convertases furin (Bosshart et al., 1994; Chapman and Munro, 1994; Molloy et al., 1994; Jones et al., 1995;

Schäfer et al., 1995; Takahashi et al., 1995a; Voorhees et al., 1995), and kexin (Fuller, Brake and Thorner, 1989; Wilcox et al., 1992).

Both furin (Rehemtulla et al., 1992; Vidricaire, Denault, and Leduc, 1993; Molloy et al., 1994; Vey et al., 1994) and kexin (Germain et al., 1992b; Wilcox et al., 1992) undergo shedding of their transmembrane anchor to release a soluble form of these enzymes in the extracellular space. PC7, on the other hand, does not undergo shedding of its transmembrane anchor (Munzer et al., 1997), and is so far the only membrane-bound convertase to sustain palmytoylation of its cytosolic tail (Van de Loo et al., submitted).

The serine/threonine/proline rich region of kexin is O-glycosylated (Wilcox and Fuller, 1991), but no such posttranslational modification has yet been demonstrated for the related domain of PC7.

All mammalian convertases display several predicted N-glycosylation sites. PC1, PC2 and furin undergo such modification, but while PC1 (Benjannet et al., 1993) and furin (Molloy et al., 1994) attain complex N-glycosylation structures, PC2 remains with hybrid sugars, even as a mature enzyme (Benjannet et al., 1993).

Finally, half of the known mammalian convertases and several convertase-like products from other species possess a cysteine-rich region. This cysteine-rich region is composed of several repetitions of a particular motif which was first identified for the product of the Drosophila *Dfur2* gene (Roebroek et al., 1992), and resembles that found in the tumor necrosis factor receptor (Ward, Hoyne, and Flegg, 1995). While dfurin2 presents 10 cysteine-rich repeats, hfurin (Van Den Ouweland, 1990) and dfurin1-CRR (also called dKLIP-1) (Hayflick et al., 1992; Roebroek et al., 1993) display only 2, PACE4 (Kiefer et al., 1991) and PC5 (Lusson et al., 1993; Nakagawa et al., 1993) both exhibit 5, while the PC5-B isoform (Nakagawa, Murakami, and Nakayama, 1993) displays the longest such region with 22 repeats of the cysteine-rich motif. No function has so far

been assigned to this structural segment. The complete deletion of mfurin COOH-terminal domain, including the cysteine-rich region, had no effect on the cleavage efficiency of this enzyme (Hatsuzawa et al., 1992).

The cysteine-rich motif of the convertases has been found to be most closely related in sequence to that of the laminin family members, especially that of tenascin proteins. The tenascins are molecules with proliferative, adhesive and anti-adhesive properties. Each of these properties are associated with specific domains. It has been demonstrated that the cysteine-rich region of tenascin-R and tenascin-C has anti-adhesive properties which are RGD- and β1 integrin-independent (Pesheva et al., 1994). Another laminin-related protein which exhibits a cysteine-rich domain with some homology to that of the convertases is dystrophin. The cysteine-rich region of dystrophin has been demonstrated to be responsible for the interaction of this protein with the muscle membrane through association with a glycoprotein complex (Ibraghimov-Beskrovnaya, 1992; Suzuki, 1992; Blake et al., 1995). It is therefore possible that the cysteine-rich region of convertases has some protein-protein interaction properties, which may regulate the cellular localization of these enzymes or interactions with other molecules of the secretory pathway.

Table B-IV :Structural features observed within the convertases COOH-terminal domain

Amphipatic helix structure
Cysteine-rich repeats
N-glycosylation sites
Serine/threonine/proline-rich segment
Transmembrane domain and cytosolic tail, with possible phosphorylation or palmytoylation

B.3 Tissue and cellular distribution

Each mammalian member of the convertase family exhibits a distinct tissue and cellular distribution, which, for most convertases, has been determined by Northern blotting and/or *in situ* hybridization.

PC1 and PC2 exhibit a restricted tissue distribution, being detected only in brain and in the neuroendocrine system, or in cells derived from these tissues. PC5 and PACE4, on the other hand, demonstrate a widespread tissue expression pattern. Only furin and PC7 are ubiquitously expressed in all organs, with some regions of notable abundance, such as the liver for furin, and the spleen, colon and thymus for PC7. All cell lines so far examined express furin, PC7, and to some extend PACE4. Most cell lines with a regulated secretory pathway express PC1 and PC2, except the pheochromocytoma PC12 and somatomammotroph GH4C1 cell lines.

Convertase PC4 exhibits the most restricted tissue distribution of all known mammalian convertases, being only detected in germinal tissues, especially in rat and mouse testes (Nakayama et al., 1992a; Seidah et al., 1992; Torii et al., 1993), and more specifically in spermatocytes and round spermatids. No cell line has so far been demonstrated to express PC4.

Recently, PC4 knock-out mice have been generated, which demonstrated a clear phenotype and viable null-homozygous pups (Mbikay et al., 1997). These mice exhibit greatly impaired fertility, especially in males, with lower than expected incidence of null-homozygosity. The exact processing defect generating this phenotype has not yet been determined.

PC2 knock-out mice, like PC4 knock-outs, are viable, but exhibit a reduced growth rate, and are frailer and smaller than wild type mice (Steiner et al., in preparation). These mice present multiple endocrine defects, in particular the absence of glucagon processing, mild hypoglycemia, and proinsulin processing decreased by 60%. The pancreas of PC2 -/- mice exhibits enlarged alpha cells, with a larger number of delta

cells than wild type mice and a reduced number of beta cells. These pancreatic cells also present a greater number of immature granules than those of wild type mice.

Furin knock-out mice are not viable and die during early embryogenesis (Roebroek and Van de Ven, unpublished). This embryonic lethality is also observed in mutants of the furin-like C. elegans *bli-4* gene product (Peters, McDowall, and Rose, 1991). However, some C. elegans furin mutants are viable, and present an interesting phenotype of cuticle blistering (Thacker et al., 1995), which may reflect an important function of the bli-4 furin-like product in the processing and/or assembling of cuticle components.

Although furin is ubiquitously expressed in all cells and tissues and seems to be essential to the growth and development of organisms, two cell lines have been isolated which lack furin activity. The first cell line to be characterized is an endoprotease-deficient Chinese hamster ovary (CHO) cell line, called RPE.40. These cells display inefficient insulin proreceptor and low-density-lipoprotein receptor-related protein (LRP) processing (Moehring et al., 1993; Robertson, Moehring, and Moehring, 1993; Inocencio, Moehring, and Moehring, 1994; Willnow et al., 1996). Expression of mouse furin in RPE.40 cells restores normal processing of both precursor proteins.

LoVo cells, a human colon carcinoma, also demonstrate a lack of furin activity, even though the furin mRNA is detectable in these cells. However, it was established that in LoVo cells, both furin alleles have undergone a point mutation within the P domain, possibly preventing the processing of the furin zymogen to yield an active enzyme form (Takahashi et al., 1993, 1995b). Even without the presence of an active furin enzyme, LoVo cells do grow and proliferate. These cells express PC5, PACE4 and PC7 (Seidah, Chrétien, and Day, 1994; Seidah et al., 1996c). It is thus

possible that functional redundancy among convertase enzymes may account for the survival of this cell line.

The latest mammalian member of the convertase family, PC7, is especially prominent in the lymphoid tissues such as spleen and thymus (Bruzzaniti et al., 1996; Seidah et al., 1996c). In view of this distinctive tissue distribution, it was postulated that PC7 could have a predominant role in the processing of the GP160 viral glycoprotein of the HIV-1 virus into the mature GP120 product. However, in *ex vivo* coexpression studies, furin remains the most efficient convertase to perform this particular maturation step, and further cleaves gp160 to gp77/gp53 (Decroly et al., 1997).

PC5 is most abundant in the adrenal cortex and small intestine, even though it is also detected in a variety of other tissues (Lusson et al., 1993; Nakagawa et al., 1993a; Seidah, Chrétien, and Day, 1994; Dong et al., 1995; Mercure et al., 1996). It is solely in the cells of the adrenal cortex and of the small intestine, and at lower levels in lung, that the B isoform of PC5 is detected (Lusson et al., 1993; Nakagawa, et al., 1993a,b; Seidah, Chrétien, and Day, 1994; De Bie and Seidah, unpublished results). In these tissues, PC5-B could eventually have a function distinct from that of PC5-A. PC5-A and other PC5 mRNA forms are expressed at low levels in most cell lines, the BSC40 monkey and As4.1 mouse kidney cell lines, the rat insulinoma Rin m5F, mouse adrenal cortex Y1 and human colon adenocarcinoma LoVo cells being the most abundant sources (Nakagawa et al., 1993a; Seidah, Chrétien, and Day, 1994; De Bie and Seidah, unpublished results). PC5-B may be present in NB-1 human neuroblastoma and COS-7 African green monkey kidney cells (Nakagawa et al., 1993b), although these cell lines have not been examined with a PC5-B-specific probe. No other cell line were found to express PC5-B (Seidah, Chrétien, and Day, 1994; Laframboise et al., 1997; De Bie and Seidah, unpublished results).

In the mammalian central nervous system, PC1 and PC2 are detected solely in neuronal cells. There, PC2 is expressed more abundantly than PC1, especially in neuropeptide-rich regions such as the hippocampus, hypothalamus and cerebral cortex (Schäfer et al., 1993). Furin is found in all brain cell types, with certain areas presenting "hot spots" (Schäfer et al., 1993). PACE4 expression is enriched the cerebellum, while PC5-A is particularly abundant in the hippocampus, cortex and hypothalamus (Dong et al., 1995). The PC5-B isoform is not detected in brain tissues (Lusson et al., 1993; Nakagawa et al., 1993a,b; Seidah, Chrétien and Day, 1994). The distinct distribution of each member of the convertases family in the central nervous system hints at a unique function of each of these endoproteases towards potential neuropeptide products or receptors. PC1 and PACE4 are particularly profuse in the anterior lobe of the pituitary (Marcinkiewicz et al., 1993a; Dong et al., 1995; Johnson et al., 1995; Mains et al., 1997), while PC2 is present but much less abundant. In the intermediate lobe, however, the presence of PC2 is prominent over that of the other convertases.

	mPC1	mPC2	rPC4	rPC5-A	rPC5-B	hPACE4	hfurin	rPC7
pituitary								
anterior lobe	++	+	-	+	-	++	(+)	+
intermediate	++	++	-	(+)	-	-	+	(+)
posterior lobe	-	-	-	-	-	(+)	(+)	+
adrenals			-			-	(+)	+
cortex				++	++			
medulla	(+)	(+)						
brain			-					
cerebral cortex	++	++		+	-	(+)	++	+
hippocampus	++	++		+	-	(+)	(+)	+
cerebellum	-	+		(+)	-	++	+	+
hypothalamus	++	+		+	-	(+)	(+)	+
heart	(+)	(+)	-	+	-	++	+	+
pancreas	+ islets	+ islets	-	+ α-cells	-	+	+	+
liver	-	-	-	+	-	+	++	+
thymus	(+)	-	-	(+)	-	-	+	+
spleen	+	-	-	+	-	-	+	++
lung	(+)	-	-	+	(+)	(+)	+	+
kidney	-	-	-	+	-	++	++	+
intestine	(+)	(+)	-	++ ileum	++ ileum	++	++	++ colon
thyroid	++	++	-	+	-	(+)	+	+
testis	-	-	++	+	-	-	(+)	+
ovaries	-	-	(+)	++	-	+	+	+

Table B-V:

Tissue distribution of the known mammalian convertases. Expression levels are shown as high [++], moderate [+], low [(+)], or undetectable [-]. (according to Marcinkiewicz et al., 1993a, 1994, 1996; Schäfer et al., 1993; Halban and Irminger, 1994; Seidah, Chrétien and Day, 1994; Beaubien et al., 1995; Dong et al., 1995; Day and Dong, 1996; Seidah et al., 1996c).

B.4 Cleavage specificities

Most polypeptide precursors exhibit multiple potential dibasic cleavage sites. Not all these sites, however, are recognized by convertases or cleaved to yield bioactive peptides. The convertases preferentially recognize and cleave certain basic sequences. These enzymes comply to the following established criteria to select the appropriate cleavage site (Barr, 1991; Bresnahan et al., 1992; Watanabe et al., 1992, 1993):

- Most cleavages take place as dibasic KR or RR sites, although monobasic, tribasic, tetrabasic or multibasic sites can also be utilized.
- Aliphatic residues present at the P_1' position are not favored.
- No cysteines are found within the region P_8 to P_3' surrounding the cleavage site.
- In the case of monobasic cleavages, another basic amino acid is usually found at position P_4, P_6, or P_8 preceding the cleavage site, an arginine being more favorable than a lysine (Nakayama et al., 1992b).
- Aromatic residues are not encountered in position P_2 preceding the cleavage site (Devi, 1991).

The types of cleavage sites recognized by convertases can be subdivided in four classes:

- The simple dibasic site, which is mostly found in prohormones and propeptides, and which so far seems to be more readily recognized by PC1 and/or PC2.
- The P_4, P_2-P_1 multibasic site, -R-X-[K/R]-R, which is preferred by furin, and is usually representative of precursors of growth factors, receptors and viral glycoproteins (Hosaka et al., 1991; Oda et al., 1991).
- The monobasic site, which, as discussed above, is preceded by a second basic amino acid at an even number of amino acids away from the cleavage site.

Precursor protein	Cleavage site sequence P_8 P_7 P_6 P_5 P_4 P_3 P_2 P_1 ↓ P_1' P_2'
Type I precursors:	
von Willebrand factor (human)	-Ser-His-**Arg**-Ser-**Lys**-**Arg** ↓ Ser-Leu
Insulin receptor (human)	-Pro-Ser-**Arg**-Lys-**Arg**-**Arg** ↓ Ser-Leu
7B2 (human)	-Glu-Arg-**Arg**-Lys-**Arg**-**Arg** ↓ Ser-Val
HIV-1 GP160	-Val-Gln-**Arg**-Glu-**Lys**-**Arg** ↓ Ala-Val
CONSENSUS	- X - X - **R** - X - **K/R**-**R** ↓ X- X
Type II precursors:	
POMC(mouse)	
(JP/ACTH)	-Pro-Arg-Glu-Gly-**Lys**-**Arg** ↓ Ser-Tyr
(ACTH/βLPH)	-Pro-Leu-Glu-Phe-**Lys**-**Arg** ↓ Glu-Leu
(αMSH/CLIP)	-Pro-Val-Gly-Lys-**Lys**-**Arg** ↓ Arg-Pro
(γLPH/βEND)	-Pro-Pro-**Lys**-Asp-**Lys**-**Arg** ↓ Tyr-Gly
Proinsulin (human)	
(B/C chain)	-Gly-Ser-Leu-Gln-**Lys**-**Arg** ↓ Gly- Ile
(C/A chain)	-Thr-Pro-**Lys**-Thr-**Arg**-**Arg** ↓ Glu-Ala
CONSENSUS	- X - X - X - X - **K/R**- **R** ↓ X - X
Type III precursors:	
Pro-dynorphin (rat) (C-peptide)	-**Arg**-Gln-Phe-Lys-Val-Val-Thr-**Arg** ↓ Ser-Gln
Pro-Sommatostatin (human) (SS-28)	-Glu-Met-**Arg**-Leu-Glu-Leu-Gln-**Arg** ↓ Ser-Ala
CONSENSUS	- (**B**)-X - (**B**)- X - (**B**)- X - X - **R** ↓ X - X
Type IV precursors:	
Müllerian inhibiting substance (rat)	-Gly-**Arg**-Gly-**Arg**-Ala-Gly-**Arg** ↓ Ser-**Lys**
Protein tyrosine phosphatase μ receptor (human)	-Glu-Glu-Glu-**Arg**-Pro-Arg-**Arg** ↓ Thr-**Lys**
CONSENSUS	- (**B**)-X - (**B**)- X - (**B**)- X - X - **R** ↓ X - **K**

Table B-VI:

Examples of polypeptide precursor types cleaved by members of the convertase family {(B)= basic amino acid}.

- Finally, a fourth type of substrates seems to be favored by the convertase PC5-A. These precursor polypeptides present a monobasic arginine cleavage site, preceded by another basic amino acid in position P4, P6 or P8, in addition to a lysine at position P2' (Campan et al., 1996; Nachtigal and Ingraham, 1996).

Based on their respective cleavage specificity and distinctive tissue distribution, the convertases can be divided in three subclasses of processing enzymes:

- Furin (Bresnahan et al., 1990; Wise et al., 1990; Denault and Leduc, 1996), PACE4 (Creemers et al., 1993a; Rehemtulla et al., 1993; Seidah et al., 1996a,b; Vollenweider et al., 1996; Zhong et al., 1996; Mori et al., 1997), PC5-B (Seidah et al., 1996a,b; Vollenweider et al., 1996) and probably PC7 (Munzer et al., 1997), are involved in the processing of precursor polypeptide being transported through the constitutive secretory pathway, such as growth factors and viral glycoproteins precursors.
- PC1, PC2, and PC5-A (Benjannet et al., 1995a) are responsible for the maturation of several precursor hormones and neuropeptides, and more generally of polypeptide precursors which products end up being accumulated in secretory granules, such as POMC (Benjannet et al., 1991; Thomas et al., 1991; Zhou, Bloomquist, and Mains, 1993; Zhou and Mains, 1994; Schmidt and Moore, 1995), proinsulin (Davidson et al., 1988; Bailyes, Bennett, and Hutton, 1991; Bailyes et al., 1992; Bennett et al., 1992; Rhodes, Lincoln, and Shoelson, 1992; Neerman-Arbez et al., 1994), proglucagon (Rouillé et al., 1994, 1995a,b) and prodynorphin (Dupuy et al., 1994). PC5-A is also capable of processing other types of polypeptide precursors, such as phosphatase receptors (Campan et al., 1996), Müllerian inhibiting substance (Nachtigal and Ingraham, 1996), pro-lactase-phlorizin hydrolase (Keller et al., 1995), and possibly bone morphogenic proteins (Constam, Calfon, and Robertson, 1996), gp160 (Miranda et al., 1996; Vollenweider et al., 1996), or prorenin (Mercure et al., 1996).

- PC4 is in a class of its own, since its processing specificity has not yet been determined. No substrates have yet been identified for this convertase, which is exclusively found in germ cells.

The cleavage specificities of furin, PC1 and PC2 have been extensively studied, and will be discussed in further detail in the following section, taking examples of coexpression studies performed with archetypal polypeptide precursors.

Furin:

The first studies on furin cleavage specificity relied on the coexpression of enzyme and substrate in cultured mammalian cells through transient transfection (Van de Ven et al., 1990; Wise et al., 1990). In COS-1 cells in which human pro-von Willebrand factor and hfurin were cotransfected, the precursor substrate was cleaved at the appropriate Arg-Ser-Lys-Arg$_{763}$ site, while a mutated pro-vWF with a glycine in position 763 remained unprocessed (Van de Ven et al., 1990). Subsequent studies took advantage of another coexpression system based on infection of cultured cells with recombinant vaccinia viruses, and demonstrated that hfurin can process pro-βNGF in BSC40 cells (Bresnahan et al., 1990). Since then, more than 30 precursor substrates have been established to be cleaved by furin, including growth factors (Denault et al., 1995; Dubois et al., 1995), prohormones (Hendy et al., 1995), bacterial toxins (Klimpel et al., 1992; Molloy et al., 1992; Tsuneoka et al., 1993; Gordon et al., 1995), viral glycoproteins (Stieneke-Gröber et al., 1992; Garten et al., 1994), coagulation factors (Wasley et al., 1993), protease zymogens (Pei and Weiss, 1995) as well as other types of precursor proteins (Chen et al., 1996) (for review see Denault and Leduc, 1996). Most of these substrates present a typical Arg-X-Lys/Arg-Arg cleavage motif, or sometimes an Arg-X-X-Arg sequence, which seems to be sufficient for cleavage recognition by furin (Molloy et al., 1992). Various mutagenesis studies were undertaken to demonstrate that the P_4 amino acid preceding the cleavage site is important for furin recognition (Yoshimasa et al., 1990;

Hosaka et al., 1991; Oda et al., 1991; Molloy et al., 1992). The following rules were then ascertained as being indispensable for ideal furin cleavage recognition (Watanabe et al., 1992, 1993):

- The presence of the P_1 Arg is essential, as well as that of a basic amino acid at position P_4 or P_6.

-The presence of a P_2 basic amino acid is dispensable, as long as another basic amino acid is present at the previously mentioned positions.

- An hydrophobic residue in position P1' is particularly unfavorable to furin processing.

However, it has recently been demonstrated that furin (Galanopoulou et al., 1993; Brakch et al., 1995b) can also recognize monobasic cleavage sites in the rat prosomatostatin precursor to yield somatostatin 1-28. This suggests that, even though furin presents a marked preference for the presence of an R-X-K/R-R motif at the cleavage site (Hosaka et al., 1991; Bresnahan et al., 1992), this motif is not absolutely necessary for recognition of a substrate by this enzyme.

Mutagenesis of the furin active site residues was also undertaken (Creemers et al., 1993b). Through these studies, it was demonstrated that the presence of negatively charged residues in or near the substrate binding region of furin is crucial to the cleavage activity and specificity of this enzyme. A model of the active site binding pocket of furin has been proposed based on homology modeling with the subtilisins (Siezen et al., 1991, 1994; Creemers et al., 1993b), since no crystalline structure of the convertase enzymes is yet available. This model is fairly similar to that proposed for PC1 (Lipkind et al., 1995; Rouillé et al., 1995a), with both enzymes presenting negatively charged residues within the S1, S2 and S4 subsites of the active site binding pocket.

One of the most exciting studies pertaining to furin cleavage specificity concerns the precursor of HIV-1 glycoprotein gp120. The human immunodeficiency virus (HIV) envelope glycoprotein launches the infection of CD4+ cells by mediating fusion of the viral envelope with the target cell membrane. This fusion requires that the precursor of the HIV-1

cell surface receptor, gp160, is proteolytic cleaved into gp120 and gp41. This cleavage occurs at a site containing several arginine and lysine residues (Hallenberger et al., 1992). The presence of furin has been demonstrated in T-lymphocytes, which are host cells for the HIV virus (Kamoshita et al., 1995; Decroly et al., 1996; Miranda et al., 1996). Since furin is localized in the TGN and presents a neutral pH optimum (Molloy et al., 1992, 1994), it was proposed that this enzyme may be involved in the processing of the HIV-1 glycoprotein, which travels through the constitutive secretory pathway before attaining the cell surface. Indeed, coexpression of HIV-1 gp160 glycoprotein with furin leads to processing of this precursor to gp120. GP120 is then further cleaved by furin within the V3 loop (Decroly et al, 1996; Vollenweider et al., 1996), a region believed to be essential to gp120/CD4+ interaction (Morikawa et al., 1993). The cleavage of gp160 by furin was demonstrated to be inhibited by substances specific to the furin active site, such as chloromethyl ketones (Hallenberger et al., 1992) or a mutated variant of alpha 1-antitrypsin (alpha 1-AT), called anti-trypsin Portland (alpha 1-PDX) (Anderson et al., 1993) engineered to contain within its reactive site the sequence -Arg-X-X-Arg-. These agents also prevented the formation of syncytia in infected cells, an assay used to measure virus infectivity (Hallenberger et al., 1992; Anderson et al., 1993).

However, several studies have since then established that the processing of gp160 can efficiently take place in the absence of furin, and that this protease is therefore not essential to this processing step (Gu et al., 1995; Kamoshita et al., 1995). Other convertases are expressed in CD4+ lymphoid cells (Decroly et al. 1996; Miranda et al., 1996), the most prominent being PC7, which is also the most abundant convertase present in the immune system (Seidah et al., 1996c, 1997). Some convertases other than furin have demonstrated the capacity to cleave gp160 (Ohnishi et al., 1994; Brakch et al., 1995a; Decroly et al., 1996; Miranda et al., 1996; Vollenweider et al., 1996). Therefore, one or more enzymes other than furin may be required for the cleavage of GP160 within CD4+ cells.

PC1 and PC2:

PC1 and PC2, contrary to furin, do not remain in the TGN but can attain the secretory granules of endocrine cells, in which they are stored in their active form. Both PC1 (Zhou and Lindberg, 1993) and PC2 (Shennan et al., 1995) present mildly acidic pH optima and mM requirements for calcium, a fact consistent with the conditions thought to exist within secretory granules. However, it was demonstrated that the pH and calcium conditions within the TGN can also permit PC1 to initiate processing of precursor substrates in this compartment (Schnabel, Mains and Farquhar, 1989; Benjannet et al., 1991; Schmidt and Moore, 1995).

One of the most prominent example of precursor differentially cleaved by PC1 and PC2 is POMC (Benjannet et al., 1991). POMC is a multipeptide precursor protein synthesized in the pituitary and the brain. In the anterior lobe corticotroph cells, POMC is processed into four major products: ACTH, β-LPH, a joining peptide (JP) and an NH_2-terminal glycopeptide (N-POMC) (for review see Chrétien and Seidah, 1981; Bennett, 1985; Eipper et al., 1987; Mains et al., 1990). In the intermediate lobe, however, POMC undergoes a different processing pattern, which yields, through more extensive cleavage of the precursor, smaller peptide products such as α-MSH, CLIP (for corticotropin-like intermediate lobe peptide), γ-LPH and β-endorphin.

Both PC1 and PC2 are present within the pituitary, PC1 being more abundant in the anterior lobe, while PC2 is the dominant convertase in the intermediate lobe. Both proprotein convertases colocalize with POMC in the adult intermediate pituitary lobe, but only PC1 is present in the corticotrophs. During embryonic development, the time and levels of expression of PC1 and PC2 correlate with those of the corticotropin precursor POMC (Marcinkiewicz et al., 1993a). PC1 and PC2 expression levels are coregulated with those of POMC in physiological aging situations (Joshi et al., 1995) as well as following administration of certain substances, such as dopamine (Birch et al., 1991; Day et al., 1992).

Through coexpression studies in cultured pituitary cell lines, PC1 and PC2 were demonstrated to mediate the accurate processing of POMC, yielding processing patterns resembling either those of the anterior pituitary or of the intermediate lobe (Benjannet et al., 1991; Thomas et al., 1991; Zhou, Bloomquist, and Mains, 1993; Zhou and Mains, 1994; Schmidt and Moore, 1995). PC1 cleaves POMC into the larger peptide products such as ACTH and β-LPH, while PC2 processes this precursor into the smaller peptide products such as β-endorphin and α-MSH (see Figure B-3). Thus, coexpression of POMC with PC1 generates a processing pattern similar to that observed in the anterior pituitary, while PC2 coexpression results in the maturation of the POMC precursor into peptides observed in the intermediate lobe. The implication of PC1 in POMC processing was further demonstrated through antisense studies in AtT-20 cells (Bloomquist, Eipper and Mains, 1991). AtT-20 cells, a tumor cell line derived from the adenohypophysis corticotrophs, express high levels of POMC and PC1. When an antisense PC1 RNA is introduced in these cells, the processing of POMC into the larger products is blocked, confirming the role of PC1 in the processing of POMC. On the other hand, when PC2 is introduced into AtT-20 cells, additional cleavages of POMC are observed, which correspond to the processing pattern observed in the intermediate pituitary lobe (Zhou and Mains, 1994). Studies on the cleavage specificity of PC1 and PC2 towards POMC demonstrated that only PC2 was capable of recognizing the Lys-Lys doublet at the COOH-terminal end of β-endorphin (Zhou and Mains, 1994), as well as the Lys-Arg doublets of α-MSH and CLIP (Benjannet et al., 1991), which can presumably be explained by a wider cleavage site selectivity of PC2 towards POMC.

Thus, the unique distribution of PC1 and PC2 in the pituitary results in a cell-specific differential maturation of POMC.

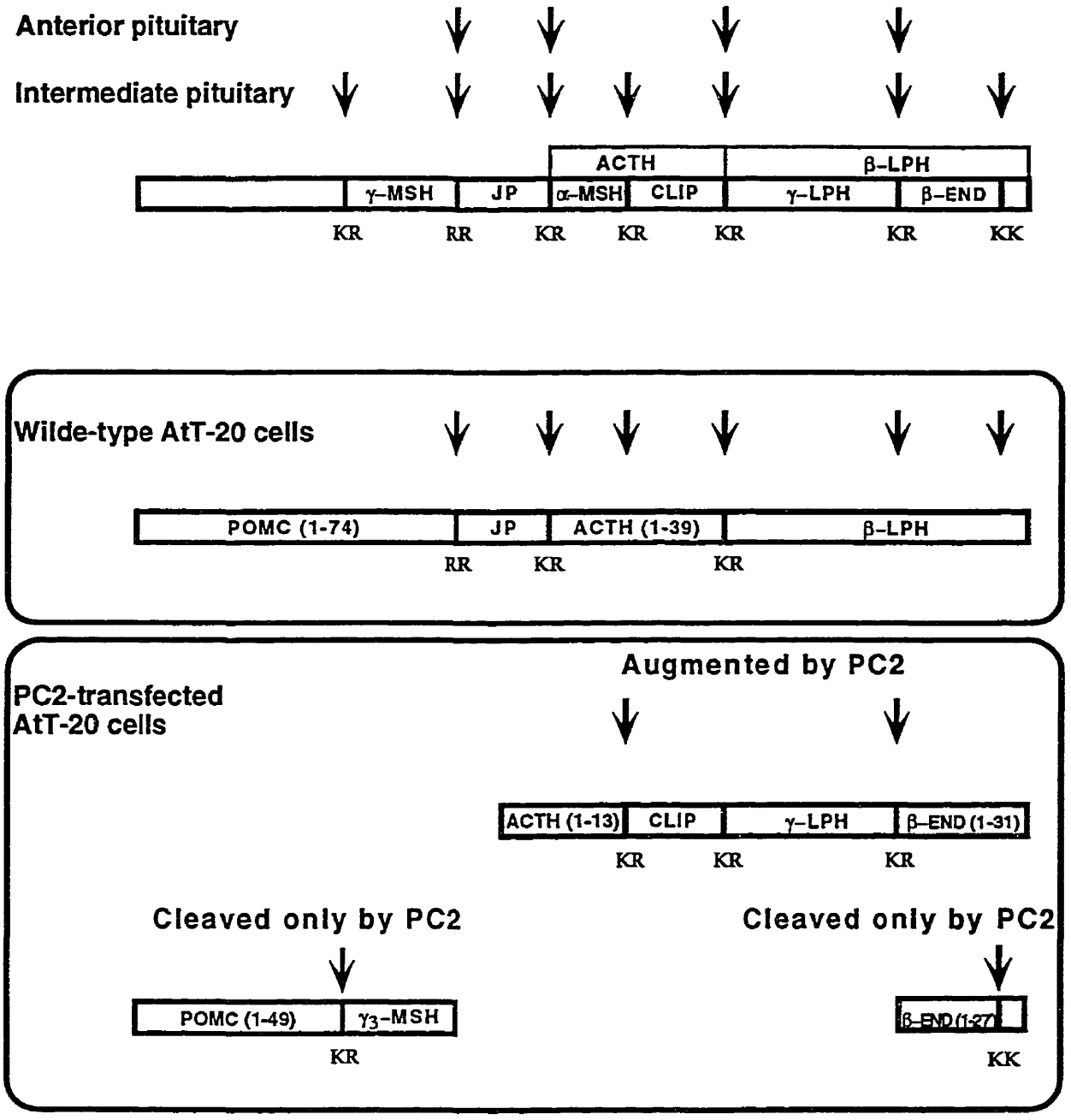

Figure B-3:

Differential processing of POMC by PC1 and PC2 (according to Benjannet et al., 1991; Halban and Irminger, 1994).

Another example of a prohormone substrate which is cleaved by both PC1 and PC2 is the proinsulin precursor. The A and B chains of insulin are connected within the polypeptide precursor by a connecting peptide (C-peptide), which is flanked on both NH_2- and COOH-terminal sides by basic amino acid doublets. Mature insulin is obtained after excision of the C-peptide, while the A and B chains remain attached through two interchain disulfide bonds.

Two enzymatic activities isolated from pancreatic β-cells, named Type I and Type II, were demonstrated to cleave respectively the Arg-Arg C-peptide/B chain junction, and the Lys-Arg A chain/C-peptide junction (Davidson, Rhodes, and Hutton, 1988; Docherty et al., 1989). Since then, it has been demonstrated that the Type I proinsulin cleaving activity corresponds to PC1, while Type II corresponds to PC2 (Davidson et al., 1988; Bailyes, Bennett, and Hutton, 1991; Bailyes et al., 1992; Bennett et al., 1992; Rhodes, Lincoln, and Shoelson, 1992; Neerman-Arbez et al., 1994). The two cleavages which liberate the mature insulin occur in a precise order, the C-peptide/B chain junction being processed by PC1 before PC2 cleaves the A chain/C-peptide bond (Rhodes and Alarcón, 1994). Expression of both PC1 and PC2 is correlated with that of proinsulin in pancreatic β-cells (Marcinkiewicz et al., 1994; Malide et al., 1995).

Coexpression studies performed in overexpression systems permitted to establish the exact cleavage specificities of PC1 and PC2 towards proinsulin (Smeekens et al., 1992; Vollenweider et al, 1992; Kaufman, Irminger, and Halban, 1995; Irminger, Meyer, and Halban, 1996). While PC1 can process human proinsulin at both dibasic sites, it exhibits a marked preference for the C-peptide/ B chain junction. PC2 on the other hand, shows specificity for the A chain/C-peptide junction.

In normal rat islets, the synthesis of PC1 but not that of PC2 seems to be glucose-regulated in parallel with that of proinsulin (Alarcón, Lincoln, and Rhodes, 1993). In contrast, in the hyperglycemic (ob/ob)

mouse islets, biosynthesis of PC1, PC2, and proinsulin is up-regulated by glucose (Martin et al., 1994). Since the islets of (ob/ob) mice contain a much greater ratio of β- to α-cells, it has been assessed that the biosynthesis of PC2 is indeed upregulated by glucose in the β-cells of normal islets, but that a concomitant downregulation of PC2 expression in α-cells may mask this effect.

Proglucagon represents another example of pancreatic polypeptide precursor which is differentially processed by PC1 and PC2 (Rouillé et al., 1994, 1995a,b). This precursor is synthesized both in the pancreatic α cells as well as in the intestinal L cells. In these tissues, proglucagon is differentially processed to yield distinct peptides (Mojsov et al., 1986), glucagon being only observed in pancreatic α-cells, while the L-cells produce the larger peptides such as glicentin, GLP-1, GLP-2 and IP-2. While PC2 can process proglucagon into glucagon (Rouillé et al., 1994, 1995a,b), the cleavages yielding the larger glicentin product as well as GLP-1 and GLP-2 can be performed by PC1 (Rouillé et al., 1994; Rothenberg et al., 1995, 1996; Rouillé, Martin and Steiner, 1995b; Dhanvantari, Seidah, and Brubaker, 1996). Other proteases could eventually mediate glicentin production or the mono Arg cleavage yielding the biologically active GLP-1 (7-37) (Rouillé, Martin, and Steiner, 1995b; Dhanvantari, Seidah, and Brubaker, 1996; Rothenberg et al., 1996). Indeed, several proglucagon processing cell lines have been demonstrated to express PC5 (Blache et al., 1994), while in the human colon, a rich source of PC5, fully processed GLP-1 (7-37) peptide is observed (Deacon, Johnsen and Holst, 1995).

B.5 Sorting signals

So far, the subcellular localization of only three of the mammalian convertases has been ascertained. PC1 and PC2 are detected in the Golgi and secretory granules of cells in which they are naturally expressed (Bennett et al., 1992; Guest et al., 1992; Marcinkiewicz et al., 1993, 1994, 1996; Lindberg, Ahn, and Breslin, 1994; Friedman et al., 1995; Malide et al., 1995; Rothenberg et al., 1995; Scopsi et al., 1995; Wetsel et al., 1995; Paquet , Massie, and Mains, 1996; Tanaka et al., 1996), as well as in those cells possessing a regulated secretory pathway in which these convertases are overexpressed (Dittié and Tooze, 1995). Only the mature forms of these enzymes are stored in the regulated secretory granules.

The COOH-terminal regions of PC1 and PC2 were initially presumed be responsible for the sorting of these two proteins to secretory granules. Potential amphipatic helices at the extreme COOH-terminal end of PC1 and PC2 were postulated to mediate the sorting of these two proteins to the secretory granules by acting as membrane-anchors (Smeekens and Steiner, 1992).

Initial studies of PC2 COOH-terminally deleted mutants claimed that the COOH-terminal domain of this convertase was not essential for membrane-association of this protein, an interaction which could potentially mediate the regulated secretory pathway sorting of this convertase (Shennan, Taylor, and Docherty, 1994). This statement was based on the observation that COOH-terminal domain deletion did not prevent the association of pro-PC2 with membranes, whereas a synthetic propeptide segment was capable of competing with pro-PC2 membrane association. However, more recent investigations of furin-PC2 chimeras demonstrate that the P and COOH-terminal domains of PC2 can redirect furin chimeras towards the regulated secretory pathway (Creemers et al., 1996). Similarly, studies of a carboxyl-terminally truncated PC1 mutant (with a stop codon in place of glycine$_{592}$) expressed in PC12 cells demonstrated that the release of this protein could still be increased upon

membrane depolarization with potassium chloride, indicating that this truncated PC1 mutant was stored in secretory granules and that the COOH-terminal domain of PC1 is therefore not required for the sorting of this enzyme to the regulated secretory pathway (Zhou et al., 1995). On the contrary, investigations using a PC1 COOH-terminal mutant truncated at aspartic $acid_{616}$ showed that this protein exhibited a more rapid rate of basal release from transfected AtT-20 cells than wild type PC1. Moreover, the release of this mutant PC1 could barely be augmented in the presence of secretagogues, demonstrating that the majority of this protein never reached mature secretory granules, and thus that the COOH-terminal domain of PC1 is needed for the efficient sorting of this convertase to secretory granules (Zhou, Paquet and Mains, 1995). Indeed, this mutant displayed a decreased cleavage potency towards POMC as compared to wild type PC1. It is worth noting that treatment of cells with potassium chloride will promote the release of proteins stored both in mature and immature secretory granules. Thus, the truncated PC1-Gly_{592} could have been released from immature secretory granules, since the exact subcellular localization of this mutant was not investigated by immunofluorescence microscopy. The potential sorting function of the COOH-terminal domain of PC1 and PC2 remains to be conclusively established.

The membrane-bound convertase furin has been demonstrated to cycle between the TGN, endosomes, and the cell surface (Chapman and Munro, 1994; Bosshart et al., 1994; Molloy et al., 1994; Sariola, Saraste, and Kuismanen, 1995; Schäfer et al., 1995; Voorhees et al., 1995). Membrane-bound kexin exhibits a somewhat similar localization to that of furin, being located within the yeast organelle equivalent to the mammalian Golgi system (Redding, Holcomb and Fuller, 1991).

The subcellular localization of furin is affected by particular signals present within its cytosolic tail (Bosshart et al., 1995; Jones et al., 1995;

Schäfer et al., 1995; Takahashi et al., 1995; Voorhees et al., 1995). The first signal consist of a Tyr-containing tetrapeptide consensus sequence, Y-XX-Ø, where Ø= an hydrophobic an amino acid, which functions as a retrieval signal from the cell surface for membrane-bound proteins (Trowbridge et al., 1993). Mutation or deletion of this signal results in the loss of furin TGN-localization and in its accretion at the cell surface. The other motives found within the cytosolic tail of furin are a cluster of acidic amino acids, within the sequence CPSDSEEDEG, where the phosphorylation of both serines by a caseine kinase II (CK-II)-like enzyme is essential to the retrieval of furin from the plasma membrane to the TGN (Jones et al., 1995; Takahashi et al., 1995). Two adjacent leucine, termed di-leucine signal, are also present within furin's cytosolic tail, and could act as an internalization motif (Johnson and Kornfeld, 1992). Kexin displays similar motives to those of furin, with the presence of an essential tyrosine-based sequence within its cytosolic tail (Wilcox et al., 1992), as well as an acidic amino acid stretch and a dileucine-motif, the functions of which have not yet been investigated.

Since no antibodies against PACE4 and PC4 have so far been developed, information on the cellular sorting and localization of these two convertases is not available.

In conclusion, convertases PC1 and PC2, which are expressed in neuroendocrine cells, can be sorted to the secretory granules of these cells, where they each proceed to perform the specific cleavage of prohormones and proneuropeptides. Membrane-bound convertase furin is present in all cell types, and is localized within the TGN. This advantageous subcellular location at the last sorting station before constitutive or regulated secretory pathways, or endosomes, gives furin access to a wider yet distinct substrate pool than PC1 and PC2.

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Chapter C

Processing specificity and biosynthesis of the *Drosophila melanogaster* convertases dfurin1, dfurin1-CRR, dfurin1-X and dfurin2.

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Processing Specificity and Biosynthesis of the *Drosophila melanogaster* Convertases dfurin1, dfurin1-CRR, dfurin1-X, and dfurin2*

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Pro-protein and pro-hormone convertases are subtilisin/kexin-like enzymes implicated in the activation of numerous precursors by cleavage at sites mostly composed of pairs of basic amino acids. Six members of this family of enzymes have been identified in mammals and named furin (also called PACE), PC1 (also called PC3), PC2, PACE4, PC4, and PC5 (also called PC6). Multiple transcripts are produced for all the mammalian convertases, but only in the cases of PC4, PACE4, and PC5 does differential splicing result in the modification of the C-terminal sequence of these enzymes. A similar molecular diversity is also observed for the convertases of *Hydra vulgaris*, *Caenorhabditis elegans*, and *Drosophila melanogaster*. In the third species, two genes homologous to human furin called *Dfur1* and *Dfur2* have been identified. The *Dfur1* gene undergoes differential splicing to generate three type I membrane-bound proteins called dfurin1, dfurin1-CRR, and dfurin1-X, which differ only in their C-terminal sequence. By using recombinant vaccinia viruses that express each of the dfurin proteins, we investigated the potential effect of the C-terminal domain on their catalytic specificities. For this purpose, these enzymes were coexpressed with the precursors pro-7B2, pro-opiomelanocortin, and pro-dynorphin in a number of cell lines, and the processed products obtained were characterized. Our studies demonstrate that these proteases display cleavage specificities similar to that of mammalian furin but not to that of PC2. In contrast, we noted significant differences in the biosynthetic fates of these convertases. All dfurins undergo rapid removal of their transmembrane domain within the endoplasmic reticulum, resulting in the release of several truncated soluble forms. However, in the media of cells containing secretory granules, such as GH4C1 and AtT-20, dfurin1-CRR and dfurin2 predominate over dfurin1, whereas dfurin1-X is never detected. While pro-segment removal occurs predominantly in the *trans*-Golgi network for all the dfurins, in the presence of brefeldin A, only dfurin1-CRR and dfurin2 can undergo partial zymogen cleavage. The conclusions drawn from the results of this study may well be applicable to the mammalian convertases PC4, PACE4, and PC5, which also display C-terminal sequence heterogeneity.

Post-translational endoproteolysis of precursor proteins is one of the mechanisms by which cells increase the diversity of their biologically active products. Initial cleavage of pro-proteins usually occurs at well defined sites consisting generally of pairs of basic amino acids, frequently Lys-Arg or Arg-Arg, but also at specific monobasic sites usually occupied by a single Arg (1–3). Recently, a number of mammalian genes and cDNAs encoding subtilisin-like enzymes have been identified, and these candidate processing enzymes were proposed to be responsible for the cleavage at dibasic and monobasic sites of precursor proteins (for reviews see Refs. 3–6). The substrate precursors include those of polypeptide hormones, neuropeptides, growth factors, growth factor receptors, certain plasma proteins, and viral envelope glycoproteins. Human furin, which is encoded by the *fur* gene, is ubiquitously expressed in all tissues and represents the first identified mammalian member of this family of convertases (7, 8). The other mammalian convertases are the neural and endocrine-specific PC1 (Refs. 9 and 10; also called PC3 in Ref. 11), PC2 (9, 12), the widely distributed PACE4 (13), PC5 (Ref. 14, also called PC6 in Ref. 15), and the testis-specific PC4 (16, 17). Several of these mammalian enzymes were found to have counterparts in other species, such as the PC1-like (or PC3-like) protein from *Hydra vulgaris* (18) and anglerfish (19), the PC1, PC2, and furin-like cDNAs of *Aplysia californica* (20, 21), the PC2 and furin-like structures of *Lymnaea stagnalis* (22), the furin-like *bli-4* gene product of *Caenorhabditis elegans* (23), Xen-14 and Xen-18 of *Xenopus laevis* (24), and the furin-like enzymes of *Drosophila melanogaster* (25–28).

The process of alternative splicing with the consequent production of several mRNA transcripts has been demonstrated for several members of the pro-protein convertase family, including human (h)[1] PACE4 (13, 29), rat (r) and mouse (m) PC4 (16), mPC5 (also called PC6) (14, 30), *Hydra* PC1 (also called PC3) (18), and *Drosophila* (d) dfurin1 (28). In *D. melanogaster*, the *fur*-like sequences dfurin1 and dfurin2 were reported to originate from two distinct genes (27, 28). In addition, Northern blot analysis of *Drosophila* embryos revealed that expression of the *Dfur1* gene generated four different sizes of transcripts encoding three proteins differing in their C-terminal sequence, which were called dfurin1, dfurin1-CRR, and dfurin1-X (28). The predicted structural characteristics of the dfurin1-related enzymes are shown in Fig. 1, where they are compared to those of dfurin2, hfurin, mPC5 (also called PC6),

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[1] The abbreviations used are: h, human; POMC, pro-opiomelanocortin; r, rat; m, mouse; d, *Drosophila*; pfu, plaque-forming unit; PAGE, polyacrylamide gel electrophoresis; TMD, transmembrane domain; ER, endoplasmic reticulum; TGN, *trans*-Golgi network; β-LPH, β-lipotropin hormone; β-END, β-endorphin.

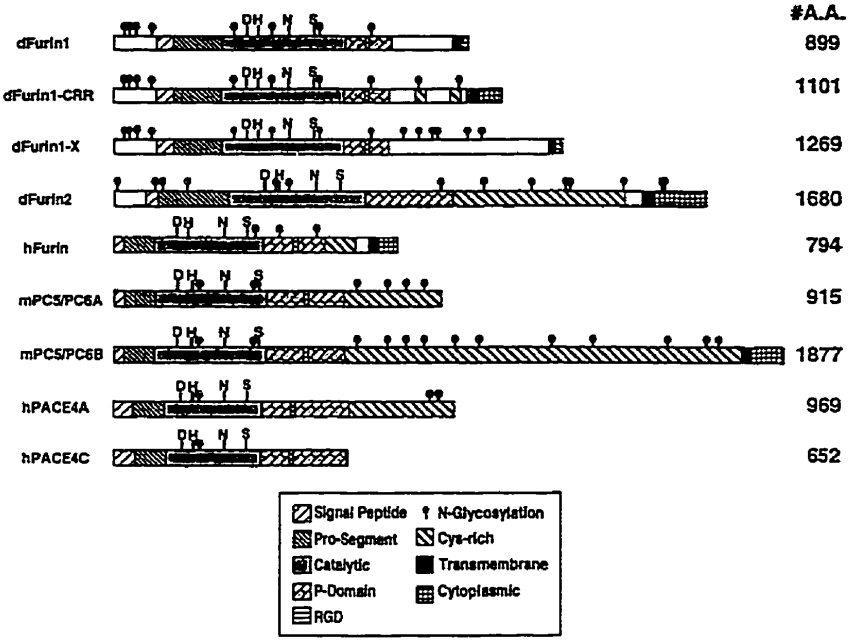

FIG. 1. **Alignment of *Drosophila* and mammalian pro-hormone convertases.** Legend to protein domains is depicted at the *bottom*, and the amino acid length of each convertase is given at the *right* of the picture.

and hPACE4. The dfurin1 isoforms are identical in structure from their N terminus up to their catalytic region but exhibit different structural domains in their C-terminal segments. Notably, the dfurin1-CRR isoform possesses a cysteine-rich domain that is also seen in dfurin2, hfurin, hPACE4, and mPC5 (also called PC6) (Fig. 1). Conservation of the Cys-rich motif in these various convertases suggests an important function for this segment of the molecule. However, complete deletion of this domain in mfurin did not seem to affect the capacity of this enzyme to intracellularly cleave coexpressed mutated ($M2R^{-4}$) mouse pro-renin (31). In an attempt to understand the functional importance of the C-terminal diversity of dfurin1-related enzymes, Roebroek *et al.* (28) coexpressed each of these convertases with either pro-von Willebrand factor or pro-β_A-activin as substrates. In that study, no significant differences with regard to the cleavage specificity of dfurin1, dfurin1-CRR, and dfurin1-X were observed. In contrast, although dfurin2 was able to efficiently process pro-von Willebrand factor, it was highly limited in its capacity to cleave pro-β_A-activin (28). So far, aside from dfurin1 and dfurin2, no other convertases have been reported in *Drosophila*. In addition, the dfurin1 isoforms revealed non-overlapping tissue distribution during the various stages of embryonic development (28) and were found to be expressed within various organs, including the central nervous system and hindgut (26, 28). This suggests widespread but distinct functions and/or cellular localization for each gene product and its isoforms. However, the endogenous *Drosophila* substrates are not yet known.

In this study, in order to define the cleavage selectivity of each convertase and its isoforms, we compared their catalytic properties to those of the mammalian enzymes PC1, PC2, and furin by cellular coexpression of vaccinia virus recombinants of dfurins with selected precursor substrates. This allowed us to probe whether the C-terminal variable segments of the dfurin1 isoforms can affect their cellular cleavage selectivity. Our selection of representative substrates was based on the classification proposed by Bresnahan *et al.* (32), where precursors are subdivided into three categories: Type I precursors contain the consensus Arg-Xaa-(Lys/Arg)-Arg↓ sequence at their cleavage site. These pro-proteins include growth factors and proteins usually processed within cells expressing furin and possibly PACE4 and/or PC5 (7). Type II precursors exhibit a pair of basic residues at the site of cleavage but no Arg residue at the P4 position. Representative examples include most pro-hormones, such as pro-opiomelanocortin (POMC), which is found in cells containing secretory granules and is now known to be processed *in vivo* by PC1 and PC2 (33). Finally, type III precursors are cleaved at a monobasic site usually represented by a single Arg↓ such as the C-peptide cleavage site of pro-dynorphin (34). Aside from defining the cleavage selectivity of each enzyme, we also characterized the biosynthetic products of each dfurin and defined their kinetics of synthesis and post-translational modifications.

MATERIALS AND METHODS

Construction of Recombinant Vaccinia Viruses—The dfurin1, dfurin1-CRR, and dfurin1-X cDNA inserts were excised from the plasmid pGEM11Zf(+) by initial *Xba*I digestion. The dfurin2 cDNA was excised from pGEM-3Zf(+) by digestion with *Rca*I. All of these linearized digestion products were blunted and subsequently digested with *Hin*dIII. These inserts were then ligated into a vaccinia virus transfer vector pMJ601 (35) linearized with *Sma*I/*Hin*dIII. The Dfur1-X cDNA insert was also excised from a pSVL vector and cloned into a pVV3-derived transfer vector (36). Expression of dfurin1-X-PVV and the expression of dfurin1-X-PMJ are driven by a vaccinia late promoter (36) and a synthetic promoter (35), respectively. The vectors containing the dfurin inserts were then used to generate recombinant vaccinia viruses (VV:dfur1, VV:dfur1-CRR, VV:dfur1-X, VV:dfur1-X.PVV, and VV:dfur2) as previously reported (33).

Cellular Infections by Vaccinia Virus Recombinants—In this work we have used four types of cells, two of which do not have secretory granules and two of which contain dense core secretory granules: LoVo human colon carcinoma cells (American Type Tissue Collection), which do not express a functional furin (37) and do not contain secretory granules; BSC40 African green monkey kidney cells, which also do not contain secretory granules; and GH4C1 rat somatomammotroph cells and AtT-20 mouse anterior pituitary cells, both of which contain dense core secretory granules. Aside from the VV:dfurins, the other vaccinia viruses used in the present studies consisted of either the wild type virus (VV:wt) or the recombinants VV:mPC1, VV:mPC2 (33), VV:hfur (38, 39), VV:mPOMC, VV:pro-m7B2 (40), and VV:rdynorphin (41). All infections and coinfections were performed at a multiplicity of infection of 1 plaque forming unit (pfu)/cell for each virus used as described previously (33).

Cellular Expression and Radiolabeling Studies—Biosynthetic analyses were performed as described previously (33, 38, 39). Briefly, 17 h postinfection, cells were washed and then switched for 1 h to a methionine-free medium (RPMI 1640, Life Technologies, Inc.) supplemented with 0.5% fetal calf serum. Subsequently, cells were either pulse-

labeled with [^{35}S]methionine (100 μCi/ml) for 1 or 2 h or for 8 or 15 min and then chased for 45 or 60 min or for 120 min, respectively, in the presence of excess unlabeled methionine. In temperature block experiments, the cells were preincubated without methionine at 37 °C and then pulse-labeled with [^{35}S]methionine for 2 h at either 37 or 20 °C. In experiments using brefeldin A or tunicamycin, cells were preincubated and incubated with either brefeldin A or tunicamycin as described before (39). In experiments with ionophore A23187, infected cells were preincubated in RPMI 1640 medium lacking methionine for 1 h and subsequently incubated in calcium-free medium containing 1 μl/ml of 5 mM A23187 dissolved in ethanol. At the end of the incubation period, the media were removed and cells were disrupted in lysis buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, and 20 μg/ml phenylmethylsulfonyl fluoride) by incubation on ice for 20 min. The media and cell lysates were immunoprecipitated with various antisera, and the precipitates were analyzed by electrophoresis on SDS-polyacrylamide gels (SDS-PAGE) followed by autoradiography.

Immunoprecipitations and SDS-PAGE Analyses—All immunoprecipitations were performed as described before (33). For dfurin1 and dfurin2 (28), 10 μl of the corresponding antibodies were used per ml of either medium or cell lysate. For POMC-derived peptides, the polyclonal antibodies used recognize either adenocorticotropin hormone (ACTH) (AT-1) or β-endorphin (β-END) (AT-2) containing peptides (33). The N-terminal human furin polyclonal antibody was obtained by immunization of rabbits using an octopus-branched synthetic peptide approach already used for the PC1 N-terminal antibody (38, 39). The peptide chosen consisted of the sequence Pro-Asp-Val-Tyr-Gln-Glu-Pro-Thr-Asp-Pro-Lys-Phe-Gln, representing residues 108–120 of rat furin (42). The 7B2 polyclonal antibody used was directed against the conserved 23–39 sequence of human 7B2 (43). For each coexpression experiment, the levels of convertases synthesized as well as the secreted products of either POMC or pro-7B2 were evaluated in parallel by specific immunoprecipitations followed by analysis by SDS-PAGE. The immunoprecipitation products were resolved by SDS-PAGE on either 6% gels, for dfurins, or 15% gels, for mPOMC and m7B2 products, followed by autoradiography. For preparative purposes, immunoprecipitated dfurin1 proteins were resolved on 6% SDS-PAGE gels, which were sliced (1 mm). The eluted radiolabeled proteins were subjected to microsequence analysis on an Applied Biosystems model 470A sequenator as described (39, 40).

Chromatography and Radioimmunoassays—Chromatography and radioimmunoassays were performed as described previously (34, 41). Briefly, 17 h postinfection, LoVo cells coinfected with VV:rdynorphin, and each of the VV:dfurins were incubated in Ham's F-12 medium (Life Technologies, Inc.) for 3 h. The media (1.5 ml) were deposited on a Sephadex G-50 (Pharmacia) column (1.5 × 90 cm) equilibrated with 1% formic acid and 0.1% bovine serum albumin, as reported earlier (34). The collected fractions (2 ml) were dried, resuspended in 1 ml of 1:1 methanol: HCl (v/v), and assayed for C-peptide immunoreactivity by a specific radioimmunoassay using a C-terminally directed antiserum (34). Using trypsin-digested pro-dynorphin, which releases the C-peptide quantitatively, we estimated that this antiserum recognizes the free C-peptide about 35-fold better than when it is attached to pro-dynorphin.

RESULTS

Comparative Cleavage Specificity of dfurin Convertases—In order to compare the cleavage selectivity of the three dfurin1 isoforms with each other and with dfurin2, we selected three representative precursor substrates, pro-7B2 (type I), POMC (type II), and pro-dynorphin (type III), which were coexpressed with the dfurins using vaccinia virus as a cellular expression system. For pro-7B2 and pro-dynorphin, coinfection studies were performed in a constitutively secreting human colon carcinoma LoVo cell line (37). This cell line, which is devoid of endogenous active furin, was chosen because we have recently shown that pro-7B2 is rapidly cleaved into 7B2 by furin (44) and that pro-dynorphin is synthesized and processed in adrenal cortex cells, which lack secretory granules (45). For POMC, a precursor exclusively synthesized in regulated cells, we chose the rat somatomammotroph cell line GH4C1, in which we previously demonstrated the capacity of PC1, PC2, and furin to process POMC (39). For each coexpression experiment, the amounts of convertases synthesized were evaluated by specific immunoprecipitations (see Figs. 5 and 8).

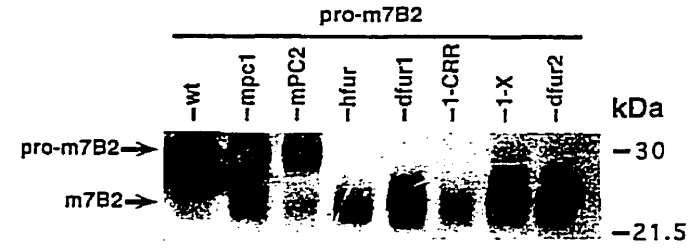

FIG. 2. **Analysis of proteolytic processing of pro-m7B2 by the dfurins.** LoVo cells were coinfected with 1 pfu of VV:pro-m7B2 and 1 pfu of VV:wt (*wt*), VV:mPC1 (*mpc1*), VV:mPC2 (*mPC2*), VV:hfurin (*hfur*), VV:dfurin1 (*dfur1*), VV:dfurin1-CRR (*1-CRR*), VV:dfurin1-X (*1-X*), or VV:dfurin2 (*dfur2*). Cells were pulsed for 15 min with [^{35}S]methionine and chased for 45 min in the presence of excess unlabeled methionine. Media were then immunoprecipitated and resolved by electrophoresis as described under "Materials and Methods." Molecular mass markers and the relative positions of pro- and mature m7B2 are indicated.

Coexpression of pro-m7B2 and dfurins in LoVo Cells—The cleavage site of pro-m7B2 contains a pentabasic sequence Arg-Arg-Lys-Arg-Arg^{155} ↓ (40, 46) and fits the Arg^{-4}-Xaa-(Lys/Arg)-Arg^{-1} ↓ consensus type I precursor cleavage site (32). As shown in Fig. 2, although PC2 is not capable of processing pro-m7B2 (30 kDa) into its mature form m7B2 (23 kDa), furin, and to a much lesser extent PC1, is able to do so as reported earlier (44). In turn, each dfurin was as efficient as hfurin in completely converting pro-m7B2 into m7B2 (Fig. 2). These data demonstrate that the recombinant vaccinia virus of each dfurin expresses an active enzyme that resembles mammalian furin in its activity more than either PC1 or PC2. However, the overexpression of each dfurin with pro-m7B2 did not reveal a difference in their cleavage preference toward this typical type I precursor.

Coexpression of mPOMC and dfurins in GH4C1 Cells—To further evaluate potential differences in cleavage site specificity of the dfurins, we coexpressed each of these enzymes with mPOMC as a representative type II precursor substrate. In this type of precursor, the known *in vivo* cleavage sites contain simple pairs of basic residues such as Lys-Arg^{-1} and Arg-Arg^{-1} without an Arg^{-4}. As previously demonstrated, mPOMC is cleaved into distinct peptide products by PC1 as compared with PC2 (33). ACTH and β-lipotropin (β-LPH) are produced by PC1 cleavage, whereas PC2 is needed to generate β-END and α-melanotropin from the same precursor (33), together with the trimming enzymes carboxypeptidase E (47) and peptidylglycine α-amidating monooxygenase (48). In addition, overexpression of furin and mPOMC in GH4C1 cells generated products similar to those obtained with PC1 (39). Thus, mPOMC was a useful model for discriminating activities among the dfurins that are similar to either PC1/furin or PC2. GH4C1 cells were coinfected with recombinant vaccinia viruses expressing mPOMC and each of the dfurins (Fig. 3). Following infection, the cells were pulse-labeled with [^{35}S]methionine for 2 h. The media were then immunoprecipitated with anti-β-END and anti-ACTH antibodies, and the products were separated by SDS-PAGE. The autoradiograms showed that, similar to hfurin and mPC1, the three dfurin1 isoforms and dfurin2 were capable of cleaving mPOMC into β-LPH (Fig. 3*A*) and ACTH (Fig. 3*B*), albeit with varying efficiencies. As was established with pro-von Willebrand factor and pro-β_A-activin by Roebroek *et al.* (28), dfurin1 seems to be the most efficient of the *Drosophila* convertases at cleaving mPOMC, followed by dfurin1-CRR and dfurin2, whereas dfurin1-X demonstrated a very weak POMC processing capability. The low activity of dfurin1-X can be explained in part by its lower expression levels under the infection conditions used, even with the PMJ construct, which

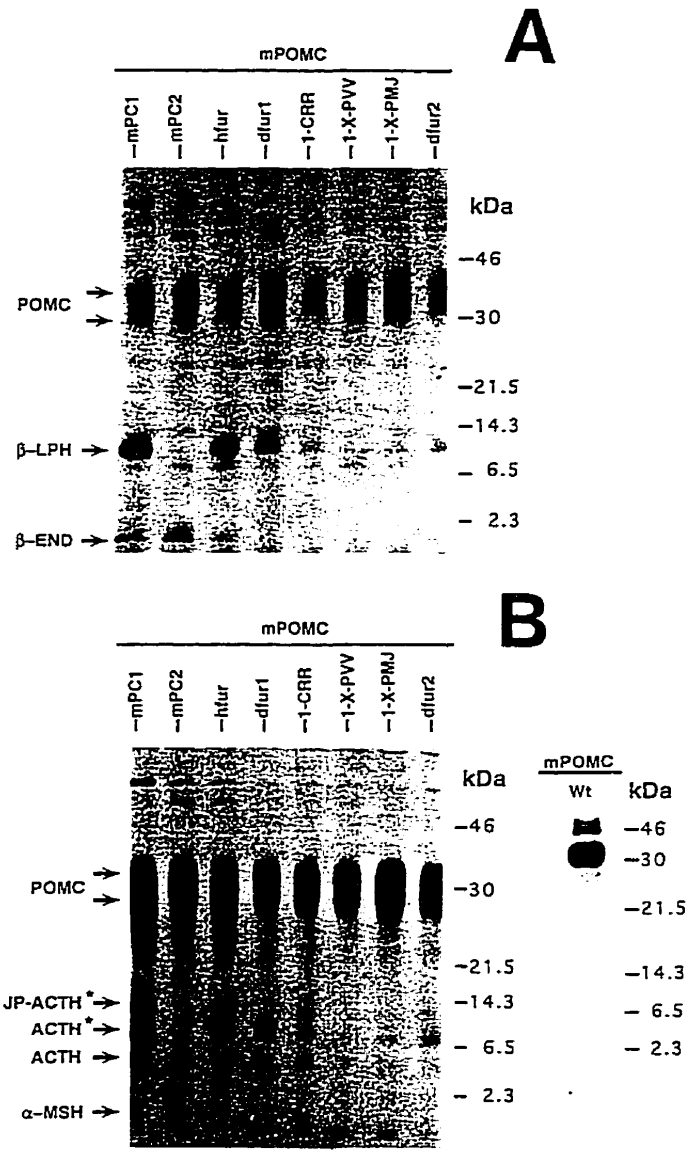

FIG. 3. **Analysis of proteolytic processing of mPOMC by the dfurins.** GH4C1 cells were coinfected with 1 pfu of VV:mPOMC and 1 pfu of VV:mPC1 (*mPC1*), VV:mPC2 (*mPC2*), VV:hfurin (*hfur*), VV:dfurin1 (*dfur1*), VV:dfurin1-CRR (*1-CRR*), VV:dfurin1-X-PVV (*1-X-PVV*), VV:dfurin1-X-PMJ (*1-X-PMJ*), or VV:dfurin2 (*dfur2*). Cells were pulsed for 2 h with [^{35}S]methionine. Media were then immunoprecipitated with either anti-β-END antibody (AT-1) (*A*) or anti-ACTH antibody (AT-2) (*B*) and resolved by electrophoresis as described under "Materials and Methods." Molecular mass markers and the relative positions of POMC, β-LPH, and β-END (*A*), and ACTH, glycosylated ACTH (*ACTH**), joining peptide-ACTH intermediary processing product (*JP-ACTH**), and α-melanotropin-like peptide (*α-MSH*) (*B*) are indicated. Wt/mPOMC negative control coinfection was also performed and immunoprecipitated with both anti-β-END antibody (AT-1) and anti-ACTH antibody (AT-2). Only the anti-ACTH immunoprecipitation is shown here.

expresses about 3–4-fold higher amounts of enzyme activity (Fig. 3, *A* and *B*) and protein (data not shown) *versus* the dfurin1-X-PVV construct. Our results show that dfurin2 is equivalent to dfurin1-CRR in its cleavage selectivity and ability to produce β-LPH (Fig. 3*A*) and ACTH (Fig. 3*B*). Quantitation of the various products was obtained by scanning the relative band intensities and normalizing the values with respect to the known number of methionines in each product (*e.g.* 3 for mPOMC and 1 for both β-LPH and β-END). The results showed that β-LPH represented 38, 22, 7, and 25% of the total immunoprecipitated POMC-related molecules produced by dfurin1, dfurin1-CRR, dfurin1-X-PMJ, and dfurin2, respectively. These values are lower than those obtained with mPC1 and hfurin, where β-LPH represented 79 and 75% of the total immunoprecipitated proteins, respectively (Fig. 3*A*). Similarly, quantitation of the data in Fig. 3*B* revealed that glycosylated ACTH migrating as an 11-kDa peptide (33) represents 51, 32, 12, 4, 2, and 8% of the total ACTH immunoreactivity produced by mPC1, hfurin, dfurin1, dfurin1-CRR, dfurin1-X-PMJ, and dfurin2, respectively. This order of cleavage efficiency is similar to that deduced from the β-LPH immunoprecipitations (Fig. 3*A*). In contrast to PC2, which produced 37% β-END and an 11% α-melanotropin-like peptide, none of the dfurins produced either of these typical PC2-generated peptides (Fig. 3). Taken together, these data demonstrate that dfurins exhibit similarities to furin/PC1, but not to PC2, in their cleavage selectivity of POMC. As shown in Fig. 8, a semi-quantitative evaluation of the protein levels of the convertases by specific immunoprecipitation suggests that the degree of cleavage observed correlates with the levels of the convertases in which the pro-segment has been excised.

Coexpression of Rat Pro-dynorphin and dfurins in LoVo Cells—Finally, the potential discriminative cleavage specificities of the dfurins toward monobasic (type III) sites were investigated using rat pro-dynorphin as a substrate and a C-terminally directed antibody that recognizes preferentially the free C-peptide. The latter peptide, which represents the last 15 amino acids of pro-dynorphin, is generated by cleavage post of a single Arg↓ residue from the C terminus of pro-dynorphin in the sequence Val-Val-Thr-Arg^{241}↓Ser (34, 41). Media of LoVo cells in which rat pro-dynorphin was coexpressed with each of the dfurins were separated on Sephadex G-50 and assayed for C-peptide immunoreactivity (Fig. 4*A*). Quantitation of the results (Fig. 4*B*) revealed that the relative efficiency of C-peptide cleavage by the various convertases as compared with mPC1 is 1, 1.8, 1.6, 1.9, 0.6, and 0.6 for mPC1, hfurin, dfurin1, dfurin1-CRR, dfurin1-X, and dfurin2, respectively. These data showed that dfurin1 and dfurin1-CRR exhibited similar efficiencies as compared with hfurin toward cleavage at the monobasic site of pro-dynorphin, whereas dfurin1-X and dfurin2 were about 3-fold less efficient.

Biosynthesis and Molecular Forms of the dfurins

Biosynthesis of dfurins in Constitutive LoVo and BSC40 Cells—Both LoVo and BSC40 cells were infected with VV:dfurin1, VV:dfurin1-CRR, VV:dfurin1-X, or VV:dfurin2, with VV:wt as control, and metabolically labeled with [^{35}S]methionine 17 h postinfection. The cell extracts and media were immunoprecipitated with either dfurin1- or dfurin2-specific antibodies (28). The precipitates were then resolved by SDS-PAGE on a 6% polyacrylamide gel (Fig. 5). Except for dfurin1-X, all dfurins exhibited the presence of more than one immunoprecipitable protein both intracellularly (Fig. 5*A*) and in the medium (Fig. 5*B*). The estimated apparent molecular masses of the major bands vary slightly between both cell types and are 119 and 95 kDa for dfurin1, 153, 140, and 117 kDa for dfurin1-CRR, 163 kDa for dfurin1-X, and 200 and 178 kDa for dfurin2 in LoVo cells, while in BSC40 cells the molecular masses calculated were of 110 and 90 kDa for dfurin1, 147, 130, and 110 kDa for dfurin1-CRR, 155 kDa for dfurin1-X, and 191 and 162 kDa for dfurin2. The molecular masses given were calculated as averages of the values deduced following linear regression analysis of at least two immunoprecipitation SDS-PAGE analyses. It is important to note that the apparent molecular masses are similar in both the media and the cells and that all forms observed intracellularly are secreted. This suggests that, in LoVo and BSC40 cells, these proteins are not anchored to membranes and represent C-terminally processed products of the various dfurins.

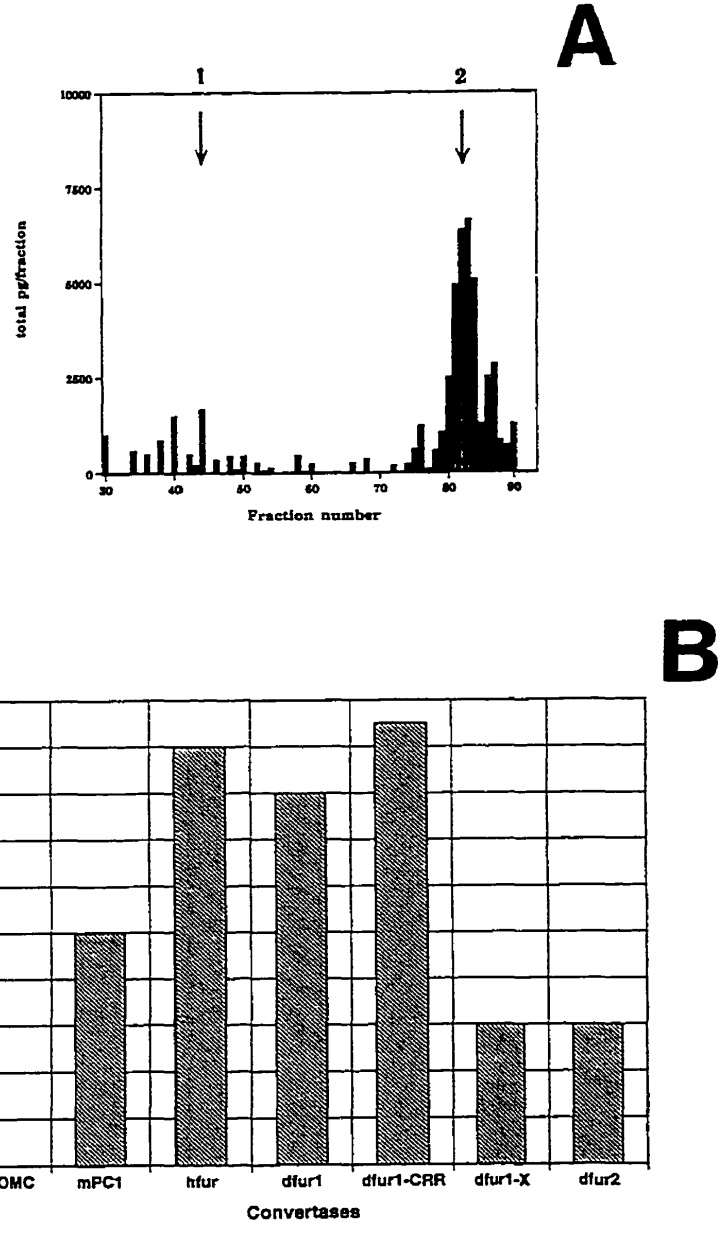

FIG. 4. **Analysis of proteolytic processing of rat pro-dynorphin by the dfurins.** As shown in *B*, LoVo cells were coinfected with 1 pfu of VV:pro-rdynorphin and 1 pfu of VV:mPOMC (*mPOMC*), VV:mPC1 (*mPC1*), VV:hfurin (*hfur*), VV:dfurin1 (*dfur1*), VV:dfurin1-CRR (*dfur1-CRR*), VV:dfurin1-X (*dfur1-X*), or VV:dfurin2 (*dfur2*). 17 h postinfection, cells were incubated for 3 h with unsupplemented Ham's F-12 medium. Media were collected and resolved by chromatography on Sephadex G-50, and fractions were assayed by radioimmunoassay for C-peptide immunoreactivity as described under "Materials and Methods." An example of the G-50 elution profile assayed by radioimmunoassay is depicted in *A*, where fractions of the coinfection medium of VV:pro-rdynorphin and VV:dfurin1-CRR separated on G50 were assayed for C-peptide immunoreactivity. *Arrows* indicate the elution positions of rat pro-dynorphin (*1*) and C-peptide (*2*). In *B*, quantitation of the percent processing into C-peptide for each rat pro-dynorphin coinfection was determined by dividing the sum of the picograms/fraction immunoreactivity found in the C-peptide peak by the total immunoreactivity due to both pro-dynorphin and C-peptide. These percentages were then divided by the mPC1 percent of cleavage value, yielding relative cleavage efficiency values for the dfurin convertases with respect to mPC1.

In order to further define some of the protein forms observed in LoVo cells, we undertook pulse-chase experiments using short pulse periods to determine potential precursor-product relationships for dfurin1- and dfurin1-CRR-immunoprecipitated proteins (Fig. 6). As shown in Fig. 6*A*, after a pulse of either 1 or 8 min, we saw the formation of at least two forms of dfurin1, migrating with apparent molecular masses of 123 and 119 kDa. As shown in the *upper panel* of Fig. 6*A*, the immunoprecipitation of the dfurin-1 products is specific, because no bands were observed when we used a normal rabbit serum. Also, after only 1 min of pulse, we saw the formation of both the 123- and the 119-kDa forms, with the former disappearing within 30 min of chase. In the bottom panel, this 123 kDa form, which is absent after a 1-h chase, could represent the precursor of dfurin1 still containing a transmembrane domain (TMD), because it is never detected in the medium (data not shown). Similarly, the 119-kDa form would represent the precursor form lacking its TMD, because it is secreted into the medium (see Fig. 5*B*). Furthermore, the difference of 4 kDa observed between the 123- and 119-kDa forms cannot be due to an N-terminal truncation of the pro-segment, because we should have expected a variance of about 17 kDa between the calculated masses of pro-dfurin1 (88 kDa) and dfurin1 (71 kDa), assuming about 1.5 kDa/*N*-glycosylation site (25). We note the slow migration of the putative TMD-containing pro-dfurin1, which travels with an apparent molecular mass overestimated by about 35 kDa (123 *versus* 88 kDa). Such abnormal migration on SDS-PAGE has previously been observed with a number of proteins, including human pro-furin and furin, which migrate with apparent molecular masses about 20 kDa higher than expected from their amino acid sequence (8, 49). The microsequence of 2×10^6 cpm of the 119-kDa form of dfurin1 (in LoVo cells) labeled in [^{35}S]methionine did not reveal the presence of methionine residues within the first 20 cycles, in agreement with its tentative assignment as pro-dfurin1 (starting at residue 152). However, microsequencing of the protein equivalent to the 95-kDa form of dfurin1 (obtained from AtT20 cells; see Fig. 8) revealed methionines at positions 5, 15, and 17 in agreement with a protein sequence starting at residue 310 of dfurin1. This result demonstrates that the smallest dfurin1 form detected represents the mature enzyme obtained following the removal of its TMD and cleavage of the pro-segment at

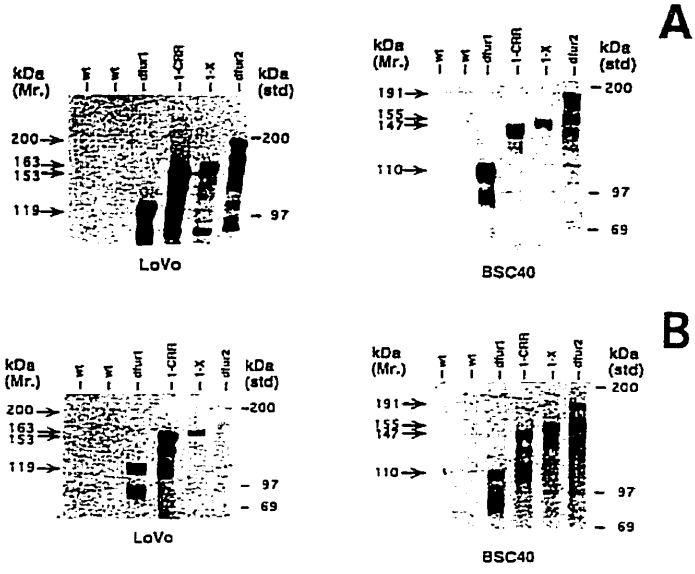

FIG. 5. **Biosynthesis of *Dfur1*- and *Dfur2*-encoded proteins in LoVo and BSC40 cells.** LoVo and BSC40 cells were infected with either VV:wt (*wt*), VV:dfurin1 (*dfur1*), VV:dfurin1-CRR (*1-CRR*), VV:dfurin1-X (*1-X*), or VV:dfurin2 (*dfur2*). Cells were pulse-labeled with [^{35}S]methionine and immunoprecipitated with rabbit anti-dfurin1 (the first *wt* lane, *dfur1*, *1-CRR*, and *1-X*) or anti-dfurin2 (the second *wt* lane and *dfur2*) antisera as described under "Materials and Methods." Both cells (*A*) and media (*B*) were immunoprecipitated. Molecular mass markers and the positions of the highest molecular forms detected of the dfurins are indicated.

the Arg-Ser-Lys-Arg^{309} ↓ site.

When dfurin1-CRR was pulse-labeled for 8 min followed by a chase of 1 and 2 h (Fig. 6A), we observed the formation of a major 153-kDa form and the transient production of a minor 162-kDa protein that disappears after a chase of 1 h. Using similar arguments to those used for dfurin1, the 162-kDa form likely represents the pro-dfurin1-CRR with its TMD, whereas pro-dfurin1-CRR lacking the TMD may be represented by the 153-kDa form. Here also, the 11-kDa difference in masses between these forms (average of four separate experiments) is smaller than that expected from the loss of the N-terminal pro-segment, for which a shift of about 17 kDa would be expected (113 and 96 kDa for pro-dfurin1-CRR and dfurin1-CRR, respectively) (28).

As shown in Fig. 6*B*, treatment of VV:dfurin-infected BSC40 cells with tunicamycin reveals that all dfurins are *N*-glycosylated. The apparent molecular masses of the major forms are 105, 139, 145, and 178 kDa for dfurin1, dfurin1-CRR, dfurin1-X, and dfurin2, respectively. Accordingly, in the presence (Fig. 6*B*) and the absence (Fig. 5A) of tunicamycin, the observed molecular masses of the major intracellular forms differ by about 5, 8, 10, and 13 kDa for dfurin1, dfurin1-CRR, dfurin1-X, and dfurin2, respectively. It is interesting to note that in the presence of tunicamycin, we also detected small amounts of larger molecular forms migrating at 118, 150, and 157 kDa for dfurin1, dfurin1-CRR, and dfurin1-X, respectively. These may represent the pro-forms still containing the TMD. In addition, we note that prevention of *N*-glycosylation causes the appearance of excessive degradation products, as was originally reported for PC1 and PC2, for which such degradation was shown to occur within the endoplasmic reticulum (ER) (39).

Finally as shown in Fig. 6*C*, in BSC40 cells infected with VV:dfurins in the presence of the Ca^{2+} ionophore A23187, only the putative pro-dfurins lacking the TMD are detectable by immunoprecipitation, because the molecular masses of the major forms are virtually the same as those seen in a similar 1-h

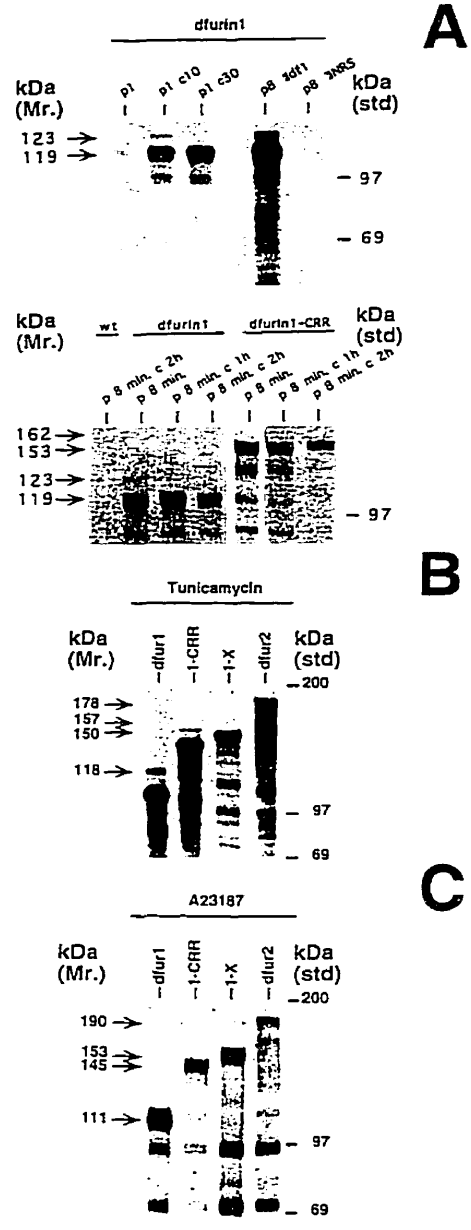

FIG. 6. *A*, pulse-chase analysis of *dfurin1* and *dfurin1-CRR*. In the *top panel*, LoVo cells were infected with VV:dfurin1 (all *lanes*), and some cells were pulse-labeled with [^{35}S]methionine for 1 min (*p1*, *p1 c10*, and *p1 c30*) and then chased for 10 (*p1 c10*) or 30 min (*p1 c30*). Other cells were pulsed for 8 min (*p8 ādf1* and *p8 āNRS*). In *p1*, *p1 c10*, *p1 c30*, and *p8 ādf1*, cells were immunoprecipitated with rabbit anti-dfurin1 antiserum, whereas in *p8 āNRS*, VV:dfurin1-infected cells were immunoprecipitated with normal rabbit serum. In the *bottom panel*, LoVo cells were infected with VV:wt (*wt*), VV:dfurin1 (*dfurin1*), or VV:dfurin1-CRR (*dfurin1-CRR*). Cells were pulse-labeled with [^{35}S]methionine for 8 min. Some cells were chased for 1 (*p 8 min. c 1h*) or 2 h (*p 8 min. c 2h*). All cells were then immunoprecipitated with rabbit anti-dfurin1 antiserum as described under "Materials and Methods." Molecular mass markers are indicated. *B*, biosynthesis of dfurins in the presence of tunicamycin. BSC40 cells were infected with VV:dfurin1 (*dfur1*), VV:dfurin1-CRR (*1-CRR*), VV:dfurin1-X (*1-X*), or VV:dfurin2 (*dfur2*). Cells were pulse-labeled with [^{35}S]methionine in the presence of tunicamycin for 1 h and immunoprecipitated with rabbit anti-dfurin1 antiserum (*dfur1*, *1-CRR*, and *1-X*) or rabbit anti-dfurin2 antiserum (*dfur2*) as described under "Materials and Methods." Molecular mass markers are indicated. *C*, biosynthesis of dfurins in the presence of ionophore A23187. BSC40 cells were infected with VV:dfurin1 (*dfur1*), VV:dfurin1-CRR (*1-CRR*), VV:dfurin1-X (*1-X*), or VV:dfurin2 (*dfur2*). Cells were pulse-labeled with [^{35}S]methionine in a calcium-free medium containing A23187 for 1 h and immunoprecipitated with rabbit anti-dfurin1 antiserum (*dfur1*, *1-CRR*, and *1-X*) or rabbit anti-dfurin2 antiserum (*dfur2*) as described under "Materials and Methods." Molecular mass markers are indicated.

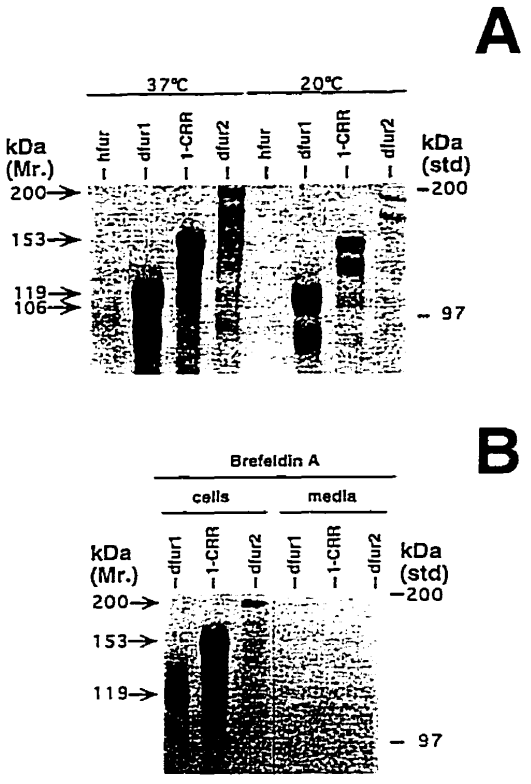

FIG. 7. *A*, comparative biosynthesis of *Dfur1*- and *Dfur2*-encoded proteins at 37 and 20 °C. LoVo cells were infected with VV:hfurin (*hfur*), VV:dfurin1 (*dfur1*), VV:dfurin1-CRR (*1-CRR*), or VV:dfurin2 (*dfur2*). Cells were pulse-labeled with [^{35}S]methionine for 2 h at either 37 or 20 °C as indicated and immunoprecipitated with anti-hfurin (*hfur*), anti-dfurin1 (*dfur1* and *1-CRR*), or anti-dfurin2 (*dfur2*) antisera as described under "Materials and Methods." Molecular mass markers are indicated. *B*, biosynthesis of *dfurin1*, *dfurin1-CRR*, and *dfurin2* in the presence of brefeldin A. LoVo cells were infected with VV:dfurin1 (*dfur1*), VV:dfurin1-CRR (*1-CRR*), or VV:dfurin2 (*dfur2*). Cells were pulse-labeled with [^{35}S]methionine in the presence of brefeldin A for 1 h and immunoprecipitated with anti-dfurin1 (*dfur1* and *1-CRR*) or anti-dfurin2 (*dfur2*) antisera as described under "Materials and Methods." Both *cells* and *media* were immunoprecipitated. Molecular mass markers are indicated.

pulse in the absence of this Ca^{2+}-depleting agent (compare Figs. 5*A* and 6*C*). Because we could not detect the forms that presumably lack their pro-segment in the presence of A23187, this suggests that the cleavage of the pro-sequence is largely inhibited under conditions of low Ca^{2+} concentrations. In contrast, because we did not observe the higher molecular mass forms still containing the TMD, this implies that A23187 does not significantly affect the removal of this C-terminal domain.

In order to define whether the shorter forms of the dfurins are produced within the ER/Golgi stacks, we repeated the pulse labeling of LoVo cells infected with the various vaccinia virus recombinants of dfurins both at the permissive 37 °C and restrictive 20 °C temperature, as well as in the presence of the fungal metabolite brefeldin A. It is well known that at 20 °C, the transport of membrane glycoproteins from the *trans*-Golgi network (TGN) to the cell surface is severely retarded (50), whereas brefeldin A causes the redistribution of the *cis*- and medial Golgi stacks to the ER (51), thus preventing traffic from this compartment to the TGN. The data in Fig. 7*A* demonstrates that after a 2-h pulse with [^{35}S]methionine performed at 37 or 20 °C, the processing pattern of the dfurin1 isoforms and dfurin2 is very similar at both temperatures. No immunoreactive dfurin1- or dfurin2-related proteins are detected in the medium at 20 °C, confirming that secretion is blocked at this temperature (data not shown). This suggests that all observed processing events occur while the proteins transit from the ER

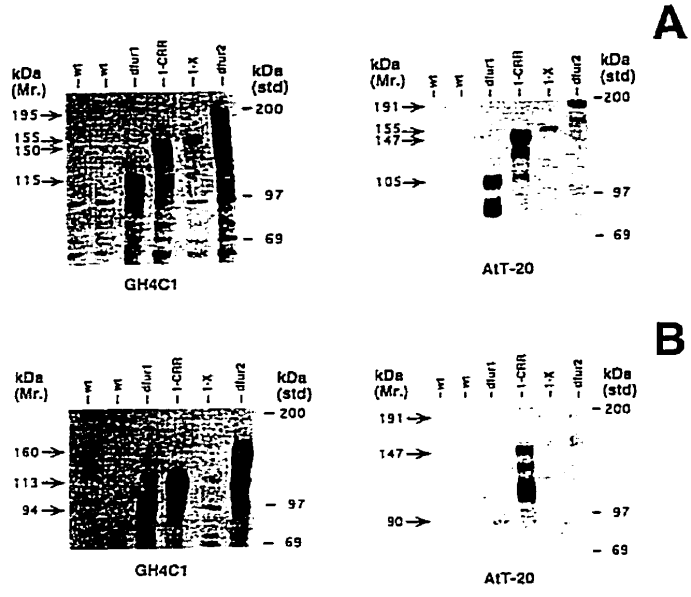

FIG. 8. **Biosynthesis of *Dfur1*- and *Dfur2*-encoded proteins in GH4C1 and AtT-20 cells.** GH4C1 and AtT-20 cells were infected with VV:wt (*wt*), VV:dfurin1 (*dfur1*), VV:dfurin1-CRR (*1-CRR*), VV:dfurin1-X (*1-X*), or VV:dfurin2 (*dfur2*). 17 h postinfection, cells were preincubated in medium lacking methionine and then pulse-labeled with [^{35}S]methionine for 2 h and immunoprecipitated with rabbit anti-dfurin1 (the first *wt* lane, *dfur1*, *1-CRR*, *1-X*) or anti-dfurin2 (the second *wt* lane and *dfur2*) antisera as described under "Materials and Methods." Both cells (*A*) and media (*B*) were immunoprecipitated. Molecular mass markers and the positions of the highest forms of dfurins detected are indicated.

up to the TGN and not during or after secretion. However, in the presence of brefeldin A (Fig. 7*B*), the protein forms detected are mostly the high molecular mass pro-forms lacking the TMD with small amounts of shorter forms of dfurin1-CRR and dfurin2 detectable, suggesting that the removal of the pro-segment of the dfurins occurs in the TGN but can also happen earlier for dfurin1-CRR and dfurin2.

Biosynthesis of dfurins in GH4C1 and AtT-20 Cells—In order to also define the molecular forms of dfurins obtained in regulated cells and compare them with those previously observed in constitutive cells, GH4C1 and AtT-20 cells infected with either VV:dfurins or VV:wt were pulse-labeled for 2 h with [^{35}S]methionine. Fig. 8 depicts the autoradiogram of the SDS-PAGE separation of the immunoprecipitated products obtained from the cell extracts (Fig. 8*A*) and media (Fig. 8*B*). The results show that in GH4C1 and AtT-20 cell extracts, the processed products of each dfurin are similar in size to those detected in BSC40 and LoVo cells (compare Figs. 8*A* and 5*A*). In AtT-20 and GH4C1 cells, we detect several molecular forms of respective masses: (105, 115) and (90, 94) kDa for dfurin1, (147, 150), (130, 133), and (110, 113) kDa for dfurin1-CRR, 155 kDa for dfurin1-X, and (191, 195) and 160 kDa for dfurin2. In the media of GH4C1 cells, only the 94-, 113-, and 160-kDa forms of dfurin1, dfurin1-CRR, and dfurin2 are observed, respectively, whereas no dfurin-X products were detected. Similarly, in AtT-20 media, the proteins detected are: the 90-kDa form of dfurin1, the 110-kDa form of dfurin1-CRR (with smaller amounts of the 147- and 130-kDa forms), no dfurin1-X, and mostly the 160-kDa form of dfurin2 with very small amounts of the 191-kDa form. Therefore, in contrast to the results obtained with constitutive cells (Fig. 5*B*), in the media of GH4C1 and AtT-20 cells we observed only the smaller forms of the dfurins (Fig. 8*B*).

DISCUSSION

Although it has been established that multiple mRNA forms exist for each one of the six known mammalian convertases

belonging to the kexin/subtilisin family (3–6, 52, 53), so far only the predicted protein sequences of PACE4 (13, 29), PC4 (16, 54), and PC5 (also called PC6) (30) have been reported to be affected by this diversity. The physiological advantage provided by this C-terminal diversity is not yet known, especially because the complete deletion of the C-terminal Cys-rich segments of mfurin (31) does not seem to affect the intracellular cleavage capability of this enzyme in the TGN. Conceivably, the different C-terminal sequences present in the various isoforms may impart a specific cellular address, as is the case for the amidation enzyme isoforms (55). Alternatively, the rate of processing of potential substrates or the cleavage selectivity of the various isoforms for different precursors may be affected by their C-terminal diversity. The availability of three different dfurin1 isoforms (28) allowed us to address this question in the context of mammalian cells. The identification of a second furin-like enzyme in *Drosophila*, dfurin2 (27), gave us the opportunity to compare the cleavage selectivity of dfurin1 and dfurin2 convertases with three types of precursors, each exhibiting a different processing motif. Our results using pro-7B2 and those of Roebroek *et al.* (28) using pro-von Willebrand factor, both of which contain the consensus cleavage motif Arg^{-4}-Xaa-(Lys/Arg)-$Arg^{-1}\downarrow$, show that all dfurins are able to process type I precursors. Our data extend the characterization of the dfurins to show that these convertases exhibit cleavage selectivities closer to those of mammalian furin or PC1, but not PC2, toward either type II (POMC) or type III (pro-dynorphin) precursors. Although quantitative differences in the amounts of generated products were noted between each dfurin1 isoform, we cannot exclude the fact that some of these variations are not due to the expression of different levels of these enzymes, especially in the case of dfurin1-X. Indeed, as shown in Figs. 3 and 8 as well as in the microsequencing results, it appears that the degree of processing of POMC can be directly correlated with the levels of immunoprecipitated convertases in which the pro-segment has been excised. In addition, coexpression studies with pro-7B2 (Fig. 2) and pro-dynorphin (Fig. 4) show that dfurin1 and dfurin1-CRR are closer to mammalian furin rather than to PC1 in their substrate cleavage efficacy. Our data with pro-7B2 (Fig. 2) and POMC (Fig. 3) demonstrated that dfurin2 exhibits similar cleavage efficiency and selectivity to dfurin1 and dfurin1-CRR. In contrast, the extent of cleavage of pro-dynorphin by dfurin2 is similar to that of dfurin1-X and about 40% of that observed for dfurin1 and dfurin1-CRR (Fig. 4). This difference in cleavage efficiency of dfurin1-X and dfurin2 toward rat pro-dynorphin compared with pro-m7B2 is substrate-determined and not cell type-determined, because both studies were conducted in LoVo cells. A similar lower efficiency of cleavage of pro-β_A-activin by dfurin1-X as compared with the two other dfurin1 isoforms was reported earlier (28), whereas dfurin2 did not cleave this substrate but processed pro-von Willebrand factor (28).[2] Thus, the C-terminal domain of the dfurin1 isoforms does not affect their cleavage selectivity. In no case did these convertases exhibit a PC2-like processing pattern. It is therefore unlikely that the protein diversity generated by the differential splicing of the *Dfur1* gene can generate an enzyme with a similar cleavage preference as that of PC2, suggesting that the true *Drosophila* PC2-like convertase has yet to be identified. In this regard, a PC2-like convertase was recently cloned from *C. elegans*, an organism more evolutionarily ancient than *D. melanogaster* (56).

Because the C-terminal variability of the dfurin1 proteins does not affect their cleavage specificity, is it possible that this domain influences the biosynthetic transformations undergone by the dfurins? To answer this question, we examined the fate of these proteins in several cell types. Originally, Roebroek *et al.* (28) reported two forms of dfurin1 of approximate molecular masses of 115 and 110 kDa in COS cells transfected with a dfurin1 cDNA. In the two constitutive cells used in this vaccinia virus infection study, LoVo and BSC40, using the same antibodies as Roebroek *et al.* (28), we were able to detect up to three forms of dfurin1 with molecular masses of 123 (Fig. 6*A*), 119 and 95 kDa (Fig. 5*B*), with the longest form only observed when short pulse periods were used (Fig. 6*A*). It is likely that the two shorter forms are equivalent to those reported in the COS cells study (28). Because our data show that these smaller two forms are secreted into the medium and that microsequencing confirmed their assignment as pro-dfurin1 and dfurin1, the 123-kDa form should represent pro-dfurin1 with its TMD, a form that rapidly disappears during the chase (Fig. 6*A*). The proposed cleavage of the 123-kDa form of dfurin1 generating the soluble smaller proteins and occurring N-terminal to the TMD seems to be unaffected by the presence of either the Ca^{2+} ionophore A23187 (Fig. 6*C*) or brefeldin A (Fig. 7*B*). This suggests that the C-terminal TMD cleavage occurs early along the biosynthetic pathway, most probably within the ER. Because treatment with A23187 inhibits the formation of dfurin1, the Ca^{2+} dependence of the TMD cleavage reaction is not the same as that of the pro-dfurin1 to dfurin1 processing. The same conclusion was drawn for the other dfurins.

Similar to the results with dfurin1, our results with dfurin1-CRR demonstrate the production of a transient cellular 162-kDa form (Fig. 6*A*), which we believe represents pro-dfurin1-CRR with its TMD, whereas the other soluble forms represent pro-dfurin1-CRR, dfurin1-CRR, and a shorter C-terminally truncated form, respectively, all lacking the TMD because they can be detected extracellularly. Both the dfurin1-X and dfurin2 proteins that immunoprecipitated were likewise found in the media of infected cells. This implies that the TMD of all dfurins is rapidly removed, yielding a pro-dfurin form that is then further processed to dfurin. Only in the case of dfurin1-CRR did we observe a third smaller form in both cells and media. Although the processing sites that result in TMD cleavage of the dfurins are difficult to define in view of their abnormal migration on SDS-PAGE, we observed that this shortening of the dfurins is not Ca^{2+}-dependent and can occur in LoVo cells, which are devoid of endogenous hfurin activity (37). When hfurin and mPC6-B (30) are overexpressed in cell lines, soluble forms are also observed arising from the partial loss of their TMD. However, this shedding event occurs after the exit of hfurin and mPC6-B from the ER, possibly within the TGN or at the cell surface (49, 57).[3]

The brefeldin A and temperature block experiments demonstrate that the processing of pro-dfurins to dfurins occurs in the TGN, whereas the zymogen cleavage of mammalian pro-furin to furin has been demonstrated to occur in the ER (49). Only in the case of dfurin1-CRR and dfurin2 did we observe some processing in the presence of brefeldin A (Fig. 7*B*). Could it be that the furin-like convertases endowed with Cys-rich motifs can undergo an earlier pro-segment removal, as is the case with dfurin1-CRR, dfurin2, and mammalian furin, whereas those lacking this structural characteristic, such as dfurin1, dfurin1-X, and PACE4C, undergo later processing? Future work on the definition of the functional role of the Cys-rich motif in convertases will undoubtedly shed more light on this question.

In regulated cells, such as AtT-20 and GH4C1, we observed an intracellular pattern of immunoprecipitated proteins (Fig.

[2] Roebroek, A. J. M., Ayoubi, T. A. Y., Creemers, J. W. M., Pauli, I. G. L., and Van de Ven, W. V. M. (1995) *DNA Cell Biol.*, in press.

[3] S. Benjannet and N. G. Seidah, unpublished data.

8A) similar to that seen in the constitutive cells. In contrast, in the media of AtT-20 and GH4C1 cells, the smaller forms of dfurins are predominantly secreted (Fig. 8B), especially for dfurin1-CRR and dfurin2 (26–28). Thus, the presence of the Cys-rich motif may not only confer to dfurin-CRR and dfurin2 the ability to undergo earlier zymogen processing along the secretory pathway but also an increased probability of C-terminal cleavage and secretion in regulated cells.

In conclusion, the data presented in this work showed that the three isoforms of dfurin1 and dfurin2 have similar characteristics to mammalian furin in terms of both catalytic activity and loss of their TMD. The differences in the C-terminal structure of the dfurin1 isoforms do not seem to influence their catalytic activity but may affect the rate and cellular site of processing of their pro-segment and ultimately influence their residence in different organelles. It is therefore possible that each isoform that is expressed in a tissue-specific manner at different stages of the *Drosophila* embryonic development (26, 28) may exert actions on specific substrates, which are coordinately expressed. Some of the possible *Drosophila* precursor substrates that are cleaved at paired basic residues include those related to the transforming growth factor-β family, such as the decapentaplegic protein (58), the integrin α-chain (59), and insulin-like pro-receptor (60). The conclusions drawn from the results of this work may well be applicable to the mammalian PC5 (also called PC6) (14, 30), PACE4 (29), and PC4 (16), because these proteins also exhibit several isoforms, some of which contain the same Cys-rich motif as that found in dfurin1-CRR (26, 28) and dfurin2 (27). The results obtained from this study should help to broaden our understanding of the role of differentially spliced forms of pro-protein convertases both in mammalian and non-mammalian systems, where such diversity has also been reported (for reviews see Refs. 4, 5, and 52). Future work will undoubtedly lead to a more detailed definition of the roles of the C-terminal domains of these varied pro-protein and pro-hormone convertases.

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The three type-I membrane-bound proteins called dfurin1, dfurin1-CRR, and dfurin1-X arising from differential splicing (Roebroek et al., 1993) exhibit identical structural features from their NH_2-terminal to their catalytic region, but each possess distinct COOH-terminal domains.

Study of the multiple isoforms of the *Dfur1* gene was aimed at answering several questions:

- What is the physiological advantage conferred to convertases by generating multiple isoforms through alternative splicing, a process observed for several members of this family, including mammalian PACE4, PC4, PC5/6, hydra PC1/3 and *C. elegans* furin-like bli-4.

- What is the possible influence of the variable COOH-terminal domain on the cleavage selectivity or cleavage site recognition of the convertases.

To answer these questions, the cleavage selectivity of each dfurin protein was defined, by comparing their catalytic properties to those of the mammalian enzymes PC1, PC2 and furin. This was achieved by cellular coexpression of recombinant vaccinia viruses each expressing one of the dfurin proteins with the precursors pro7B2, proopiomelanocortin (POMC) and prodynorphin, since no endogenous Drosophila substrates have yet been determined.

Substrates were selected based on the classification proposed by Bresnahan et al. (1992), where precursors are subdivided into three categories (see Table B-VI):

- Type I precursors comprise the consensus Arg-X-(Lys/Arg)-Arg↓ sequence at their cleavage site, as observed in the precursor of 7B2.

- Type II precursors present a pair of basic residues at the cleavage site, but no Arg residue at the P4 position, such as POMC.
- Type III precursors are processed at a monobasic site, usually a single Arg, such as the C-peptide cleavage site of pro-dynorphin.

Apart from the cleavage selectivity of each enzyme, the biosynthetic products of each dfurin as well as their post-translational modifications were also characterized.

In coexpression studies, all dfurins were capable to process the three types of precursor substrates, and demonstrated an identical cleavage specificity towards the three categories of substrates investigated. The dfurins exhibited cleavage selectivities resembling those of mammalian furin or PC1, but not that PC2, towards either type II (POMC) or type III (prodynorphin) precursors. However, these enzymes did not display the same efficacy of cleavage towards these three different substrates. Differences in the amounts of products generated from POMC and prodynorphin were noted between the dfurin proteins. The range of cleavage efficacy among the dfurins seemed to correlate the extent of proregion removal, although this was not invariably the case for each enzyme. These variations may also have been due to potential differences in expression levels of these enzymes.

Although differences in cleavage specificity were not observed among the dfurins, these enzymes displayed differences in their biosynthetic fates.

The membrane-bound dfurins underwent shedding of their transmembrane domain in all cell types tested. In the furin-deficient LoVo cells, this shedding was an early event and was not due to the action of a highly calcium-dependent protease. Mammalian furin also undergoes shedding of its transmembrane domain to release a soluble

form of the enzyme. In this case, however, the process occurs in the TGN and is thought to be autocatalytic (Vey et al., 1994).
In constitutive cell types, both pro- and zymogen-cleaved forms of the dfurins were released in the media. In regulated cells, only zymogen-cleaved dfurin forms were detected extracellularly. Protein forms possessing cysteine-rich repeats seemed to accumulate in the media of these previous cells, while no notable difference could be observed in constitutively secreting cells.

Although no PC1 or PC2-like convertases have so far been isolated from Drosophila, the furin-like isoforms of this species could not replace these other convertases in their specific processing functions. The biosynthetic and processing studies of the dfurin isoforms model clearly demonstrated that no differences among the cleavage specificities of the dfurin convertases could be observed. All dfurin1 isoforms, as well as dfurin2, presented furin-like processing specificities. However, differences in compartment of proregion cleavage were observed between dfurin1 and its cysteine-rich isoform, as well as with dfurin2, which also possesses a cysteine-rich region. It appears that dfurin proteases endowed with a cysteine-rich region, a motif also observed in mammalian furin, PACE4 and PC5, are capable of undergoing proregion cleavage in early secretory compartments, while dfurin1 undergoes this processing step only after reaching the TGN. The Cys-rich motif may thus confer to dfurin-CRR and dfurin2 the ability to undergo earlier zymogen processing along the secretory pathway, as well as a greater probability to undergo COOH-terminal cleavage and secretion in regulated cells.

Structural modifications of the COOH-terminal region of the dfurin1 isoforms do not seem to influence their catalytic specificity, and thus the

protein diversity generated by differential splicing does not generate enzymes with different cleavage specificities. It may, however, affect the rate and cellular site of prosegment processing of these enzymes and at last influence their residence in different organelles.

Since no physiological Drosophila substrates were available to test the cleavage specificity of these endoproteases, we had to rely on an artificial system of overexpression in mammalian cells, through recombinant vaccinia virus infection. These mammalian cells may not have had the appropriate cofactors necessary for the Drosophila enzymes proper function, because of species differences. The presence of a mammalian enzyme with no counterpart in Drosophila could eventually explain the very precocious cleavage of the transmembrane domain observed for all dfurin proteins, while mammalian convertases only undergo shedding after reaching the TGN.

If the Dfurin1 isoforms do not display different cleavage specificities, they do, however, display distinct tissue distributions (Roebroek et al., 1993). For the two isoforms for which specific probes could be obtained, dfurin1-CRR and dfurin1-X, Northern blot and *in situ* analyses demonstrated that these two products exhibit specific expression at distinct developmental stages, as well as different tissue expression patterns. This isoform-specific tissue distribution is also observed for mouse and rat PC5 (Seidah, Chrétien, and Day, 1994; Nakagawa et al., 1993; De Bie and Seidah, unpublished results) and rat PACE4 isoforms (Nagamune et al., 1995). It is therefore possible that convertase isoforms could mediate distinct substrate cleavages, not because of distinct processing specificity, but because of distinct tissue and/or cellular or subcellular distribution.

The possible influence of COOH-terminal domain on the cellular localization of convertase was therefore investigated through the study of two mouse PC5 isoforms, which also displayed structural differences solely in their COOH-terminal domain (see Figure D-1). One of these isoforms is predicted to be membrane-bound, while the other should be soluble. The possibility that these two proteases could be directed to distinct subcellular compartments trough COOH-terminal structural modifications imparted by splicing was therefore investigated. The respective subcellular distribution and biosynthesis of these two PC5 isoforms in stably transfected AtT-20 cells was examined. AtT-20 cells were chosen for they have been extensively characterized in multiple sorting studies, and because their endogenous PC5 levels are almost undetectable. AtT-20 cells present two paths through which proteins can attain the cell surface, which are the constitutive and regulated pathways. The sorting and biosynthesis of COOH-terminally modified PC5 isoforms in a stable transfection model could thus be compared to establish if COOH-terminal structures can affect the sorting and/ or biosynthesis of convertases.

Chapter D

The isoforms of proprotein convertase PC5 are sorted to different subcellular compartments.

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The Isoforms of Proprotein Convertase PC5 Are Sorted to Different Subcellular Compartments

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Abstract. The proprotein convertase PC5 is encoded by multiple mRNAs, two of which give rise to the COOH-terminal variant isoforms PC5-A (915 amino acids [aa]) and PC5-B (1877 aa). To investigate the differences in biosynthesis and sorting between these two proteins, we generated stably transfected AtT-20 cell lines expressing each enzyme individually and examined their respective processing pattern and subcellular localization. Biosynthetic analyses coupled to immunofluorescence studies demonstrated that the shorter and soluble PC5-A is sorted to regulated secretory granules. In contrast, the COOH-terminally extended and membrane-bound PC5-B is located in the Golgi. The presence of a sorting signal in the COOH-terminal 38 amino acids unique to PC5-A was demonstrated by the inefficient entry into the regulated secretory pathway of a mutant lacking this segment. EM of pancreatic cells established the presence of immunoreactive PC5 in glucagon-containing granules, demonstrating the sorting of this protein to dense core secretory granules in endocrine cells. Thus, a single PC5 gene generates COOH-terminally modified isoforms with different sorting signals directing these proteins to distinct subcellular localization, thereby allowing them to process their appropriate substrates.

THE mammalian subtilisin/kexin-like convertases are proprotein and prohormone proteinases implicated in the processing of numerous precursors. Seven members of this family of enzymes have so far been identified in mammals (for reviews see Seidah et al., 1994, 1996; Van de Ven et al., 1993). These proteins share a conserved catalytic domain, containing the typical Asp/His/Ser triad of subtilisin-like serine proteinases. They also exhibit the presence of a prosegment and a P domain (also called homo B). Apart from these conserved regions, each convertase possesses a distinct COOH-terminal domain exhibiting diverse structural motifs, such as a transmembrane anchor and cytosolic tail, cysteine-rich repeats, or potential amphipathic structures. Furthermore, for three of the mammalian convertases, a differential splicing mechanism leads to the production of COOH-terminally modified isoforms (Seidah et al., 1992; Kiefer et al., 1991; Tsuji et al., 1994; Lusson et al., 1993; Nakagawa et al., 1993*a*,*b*; Mbikay et al., 1995). Multiple molecular forms of the convertases are also observed in *Aplysia californica* (Chun et al., 1994), *Hydra vulgaris* (Chan et al., 1992), *Lymnaea stagnalis* (Smit et al., 1992), *Caenorhabditis elegans* (Thacker et al., 1995), and *Drosophila melanogaster* (Roebroek et al., 1993).

While functions have been proposed for the pro, catalytic, and P domains of the convertases, the role of the COOH-terminal segment is less understood. It was postulated that the latter could be involved in cellular sorting, or that it could modulate the enzymatic activity of the adjoined catalytic domain. In this respect, ex vivo coexpression studies demonstrated that the COOH-terminal truncation of furin did not affect the cellular enzymatic activity of this convertase on renin or its mutants (Hatsuzawa et al., 1992). It was also reported that the three isoforms of *Drosophila* furin1 (dfurin1, dfurin1-CRR, and dfurin1-X) did not display significant differences in either their catalytic activity or cleavage specificity (De Bie et al., 1995). Thus, the proposed involvement of the COOH-terminal segment in cellular sorting remains open to investigations.

To test the hypothesis that the COOH-terminal domain influences the cellular transport of some convertases, we compared the cellular traffic of soluble PC5-A and membrane-bound PC5-B (Lusson et al., 1993; also called PC6-A

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and PC6-B, Nakagawa et al., 1993*a*,*b*) (see Fig. 1). These COOH-terminal variant isoforms of the mouse convertase PC5 exhibit distinct tissue distributions: while expression of PC5-A is widespread, being especially abundant in intestine and adrenals, that of PC5-B is only detected in these two tissues and lung (Lusson et al., 1993; Nakagawa et al., 1993*a*,*b*; Seidah et al., 1994). These isoforms were used as models to determine if both would be sorted to the same organelle, or would reside in different compartments of the secretory pathway. To answer this question, stable AtT-20 cell lines expressing either PC5-A or PC5-B were established. This allowed both the comparison of the respective biosynthesis and subcellular localization of each isoform and the demonstration of the critical role of the COOH-terminal domain on the cellular traffic of these proteins.

Materials and Methods

Cell Culture and Transfection

AtT-20 cells were grown in DME supplemented with 10% FCS. Stable cell lines overexpressing both A and B isoforms of mouse PC5 (Lusson et al., 1993; Nakagawa et al., 1993*b*) were established using 40–60 μg of RcCMV constructs in which these cDNAs were inserted downstream of the human cytomegalovirus promoter. Cells were transfected using lipofectin (GIBCO BRL, Gaithersburg, MD) according to the manufacturer's instructions. After transfection, cells were selected with 0.5 mg/ml G418(neomycin)-containing medium for 2–3 wk. Drug-resistant cells lines were isolated and characterized by Western blotting and immunoprecipitation of radiolabeled PC5. For both A and B isoforms, at least six positive clones were selected and analyzed. Initial experiments were performed using several clones for each cell line. Eventually, single clones were used for all experiments (clones A5 for PC5-A and B1 for PC5-B). Cell lines were maintained in 0.2 mg/ml G418-containing medium, passaged weekly, and were stable for several months.

A PC5-AΔ construct obtained by PCR (sense primer from bp 2,326–2,348 containing a unique DraIII site, and antisense primer introducing a stop codon and XbaI site at base 2,700 [Lusson et al., 1993]), in which the COOH-terminal 38 amino acids unique to PC5-A were removed, was similarly transferred to AtT-20 cells to compare its sorting with that of the wild-type PC5-A.

Antibodies

The NH_2- and COOH-terminal mouse PC5 polyclonal antibodies were obtained by immunization of rabbits using an octopus branched synthetic peptide approach already used for the PC1 NH_2-terminal antibody (Basak et al., 1995; Benjannet et al., 1993). The peptides chosen consisted of the sequences Asp-Tyr-Asp-Leu-Ser-His-Ala-Gln-Ser-Thr-Tyr-Phe-Asn-Asp-Pro-Lys, representing residues 116–132 consisting of the PC5 NH_2-terminal sequence after the potential activation site Arg-Thr-Lys-Arg, and Pro-Pro-Gly-His-Tyr-His-Ala-Asp-Lys-Lys-Arg-Cys-Arg-Lys, representing residues 677–690 of mouse PC5.

Other antibodies used were as follows: anti-TGN38 raised in guinea pig (Schäfer et al., 1995), guinea pig anti–ACTH (Peninsula Laboratories, Inc., Belmont, CA), rabbit anti–cathepsin B (Lee et al., 1995), rabbit anti–PC2 (Basak et al., 1995; Benjannet et al., 1993), and rabbit anti–glucagon (Incstar Co., Stillwater, MN).

Biosynthetic Labeling, Immunoprecipitations, and SDS-PAGE Analyses

Biosynthetic analyses were performed as previously described (Benjannet et al., 1993). Briefly, cells that had reached 80% confluence were washed with PBS, and then switched for 1 h to a methionine- or sulfate-free medium (RPMI 1640) (GIBCO BRL) supplemented with 0.5% FCS. Subsequently, cells were either labeled with [^{35}S]methionine (100 μCi/ml) or [^{35}S]Na_2SO_4 (sodium sulfate) (200 μCi/ml) (Mandel Scientific Co., Ontario, Canada). In temperature-blocking experiments, cells were preincubated in absence of methionine at 37°C, and then labeled with [^{35}S]methionine for 2 h at either 37°C or 20°C. In experiments performed with brefeldin A (Cedarlane Laboratories, Ltd., Ontario, Canada), the drug was used throughout the preincubation and labeling period at a final concentration of 5 μg/ml, as described before (Benjannet et al., 1993). Stimulation of secretion was performed by adding 8Br-cAMP (Sigma Chemical Co., St. Louis, MO) at a final concentration of 5 mM to the incubation medium. At the end of the incubation period, the media were removed and cells were disrupted in lysis buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, and 20 μg/ml PMSF) by incubation on ice for 20 min. The media and cell lysates were precleared in two steps using normal rabbit serum and protein A–agarose, and then immunoprecipitated. All immunoprecipitations were performed as described before (Benjannet et al., 1993). Endoglycosidase H (Oxford Glycosystems, Ltd., Rosedale, NY) digestions were performed according to the manufacturer's instructions. The immunoprecipitation products were resolved by electrophoresis on 8% polyacrylamide gels (SDS-PAGE) followed by treatment with Entensify (Dupont-New England Nuclear, Wilmington, DE) and autoradiography. For preparative purposes, immunoprecipitated proteins were resolved on 6% SDS-PAGE gels, which were sliced (1 mm). The eluted radiolabeled proteins were subjected to microsequence analysis on a sequenator (model 470A; Applied Biosystems, Foster City, CA) as described (Benjannet et al., 1993).

Quantification of Bands in Autoradiograms

Gels were exposed to XAR-5 film (Eastman Kodak Co., Rochester, NY) at −80°C for multiple time periods. Films were then read by flatbed scanning and analyzed with the Macintosh NIH Image 1.55f program (Apple Computer Inc., Cupertino, CA) by measuring the intensity of each band above background. This measurement permitted the calculation of the amount of labeled proteins secreted in the presence or absence of 8Br-cAMP and to establish the release kinetics of PC5.

Immunofluorescence

Localization of PC5 proteins in transfected cells was determined by indirect immunofluorescence. Typically, cells were grown on polylysine-coated (Sigma Chemical Co.) chamber slides (Nunc, Inc., Roskilde, Denmark) for 72 h, rinsed once in PBS, and fixed in 4% formaldehyde/0.1% picric acid in 0.1 M phosphate buffer, pH 7.2, for 1 h at 15°C. Cells were then washed several times over 48 h with PBS containing 0.01% Triton X-100 at 4°C and subsequently reacted overnight with the primary antibody diluted in 10% normal goat serum. The PC5 COOH-terminal antiserum was used at a dilution of 1:250. Guinea pig anti–TGN38 antibody was used at a 1:50 dilution, and the guinea pig anti–ACTH was used at 1:75, while the rabbit anti–cathepsin B antibody was diluted at 1:2,000. After rinsing with PBS, cells were incubated for 30 min at 37°C with TRITC-conjugated goat anti–rabbit IgG diluted 1:15 in 10% normal goat serum (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). When primary antibodies were from guinea pig species, the immunoreaction was revealed using a FITC-labeled secondary antibody raised in goat (Jackson ImmunoResearch Laboratories, Inc.) diluted 1:10 in 10% normal goat serum. The combination of antibodies raised in rabbit with those raised in guinea pig led to a double green and red labeling that permitted us to perform colocalization studies. Displacement of PC5 immunoreaction was performed by blocking antibodies with an excess (>5 × 10^{-6} M) of the multiple antigenic peptides used for immunization, which was done by preincubation overnight at 4°C with 100 μl of the 1:100 diluted antibody. Samples were examined using a microscope with standard epifluorescence attachment (Carl Zeiss, Inc., Thornwood, NY), equipped with a Plan-Neofluor ×40/0.75 objective.

Electron Microscopy

Pancreatic tissue from five normal Sprague-Dawley rats was fixed by immersion in 1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for 2 h at room temperature, dehydrated in graded methanol, and embedded in either Lowicryl K4M at −20°C, as previously described (Bendayan, 1984), or in Unicryl (British Biocell Int., Cardiff, UK) at −20°C (Scala et al., 1992; Malide et al., 1995). Thin-sections were cut, mounted on nickel grids with or without Parlodion-coating, and processed for postembedding colloidal gold immunocytochemistry. For immunolabeling, tissue sections were first transferred for 30 min on a drop of 0.15 M PBS, pH 7.2, containing 0.1% ovalbumin, and then incubated overnight at 4°C with the following anti-

bodies at a dilution of 1:100: rabbit anti-PC2, rabbit anti-PC5, and rabbit anti-glucagon (Incstar Co.). Sections were then rinsed with PBS and incubated for 30 min at room temperature with the protein A–gold complex prepared with 10 nm gold particles (OD_{520} = 0.5), as previously described (Bendayan, 1989; Park and Bendayan, 1992). The grids were then washed with PBS and distilled water, dried, and stained with uranyl acetate before examination. For double immunogold labeling, both sides of the tissue sections were used, according to Bendayan (1982). Combinations of anti-PC2/anti-PC5, anti-PC2/anti-glucagon, and anti-PC5/anti-glucagon antibodies were performed on different faces of the grids in conjunction with protein A–gold complexes formed with 5, 10, or 15 nm gold particles. Several control experiments were carried out to assess the specificity of the labeling obtained. For these controls, incubation was performed with normal serum or specific antibodies preadsorbed overnight with an excess of their corresponding antigens.

Results

Expression of PC5-A and PC5-B in Stably Transfected AtT-20 Cells

To test the hypothesis that the PC5 isoforms (Fig. 1) could be sorted to different compartments of the secretory pathway, stable transfectants of PC5-A or PC5-B were established in the mouse corticotroph AtT-20 cell line. This cell line was chosen since it has been extensively studied in multiple investigations on the sorting of proteins within the constitutive and regulated pathways (Burgess and Kelly, 1987; Matsuuchi and Kelly, 1991).

The PC5 forms in transfected AtT-20 cells were initially examined by labeling for 2 h with [^{35}S]sulfate followed by immunoprecipitation. As shown in Fig. 2, PC5-A–expressing cells produce a major immunoreactive 117-kD protein detected in cell extracts and medium. Immunoprecipitates of the PC5-B–expressing cells revealed the presence in the cell lysate of two PC5-B–specific proteins migrating with an apparent M_r of 210 and 170 kD, the latter form also being detected in the medium (Fig. 2). A similar result was obtained after a 4-h pulse in which the 210-kD form of PC5-B was more evident in the cell extracts and was not detected in the medium (data not shown). These data suggest that the sulfated 210-kD form that reached the TGN (Hart, 1992; Baeuerle and Huttner, 1987) is not secretable, indicating that it represents membrane-bound PC5-B. The presence of a shed 170-kD immunoreactive form of PC5-B is reminiscent of the fate of another type I membrane-bound convertase, furin (Rehemtulla et al., 1992), suggesting that COOH-terminal cleavage of the membrane-anchored PC5-B occurs along the secretory pathway. The two bands detected in the cell extracts of an M_r of ~95–105 kD are considered nonspecific, as they are also detected in control AtT-20 cells transfected with the RcCMV expression vector alone.

Precursor–Product Analysis of PC5-A

The proprotein convertases are initially synthesized as zymogens and must undergo excision of their prosegment before they can be activated (see Fig. 1). To define the organelle in which prosegment cleavage of pro-PC5-A to PC5-A occurs, pulse-chase analysis of the biosynthetic fate of PC5-A in AtT-20 cells labeled with [^{35}S]methionine was undertaken. As shown in Fig. 3, after a 1-min pulse, two immunoreactive PC5 products migrating with an apparent M_r of 126 and 117 kD are detected intracellularly. Progressively upon chase, both bands diminish in intensity with the appearance of a 65-kD form 60 min later. At the same time, the secretion of a major 117-kD protein is observed in the medium, as well as that of a relatively minor 65-kD product that becomes more visible after a 120-min chase. Since an NH_2-terminally directed PC5 antibody is used to perform immunoprecipitations, the 65-kD form represents a COOH-terminally shortened fragment of PC5-A. Quantitative scanning of the autoradiogram revealed that in the medium, the ratio of 65 to 117 kD progressively increases from 0.30 to 0.45 during 2–4-h chase periods, respectively (data not shown).

Protein sequencing was then performed to unequivocally establish the identity of the PC5-A protein forms. As shown in Fig. 4, [^{3}H]Tyr^3 and [^{3}H]Tyr^{2,11} protein sequences were deduced for the 126-kD and the 117-kD forms, respectively. Based on the reported cDNA sequence and the predicted PC5 primary structure (Lusson et al., 1993), it can be concluded that the 126-kD form is

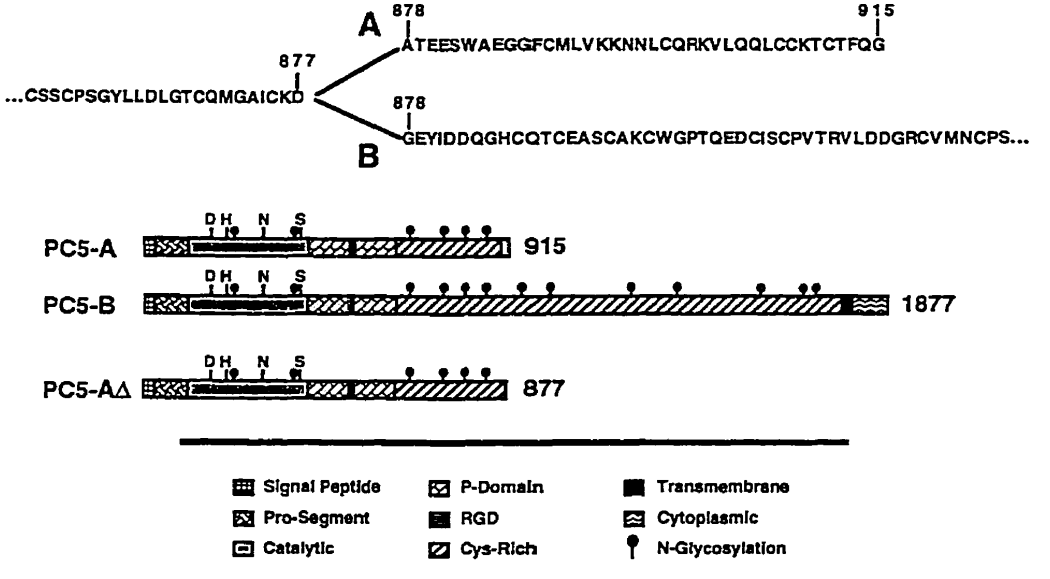
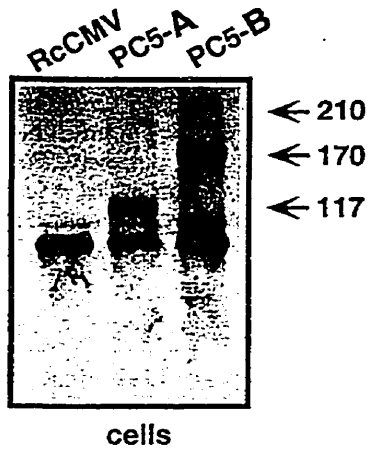
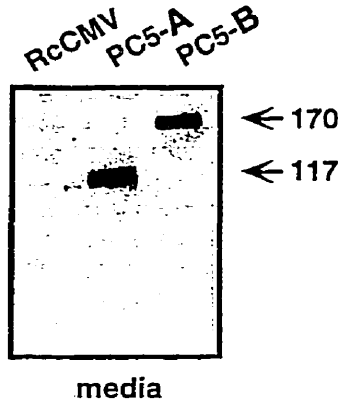


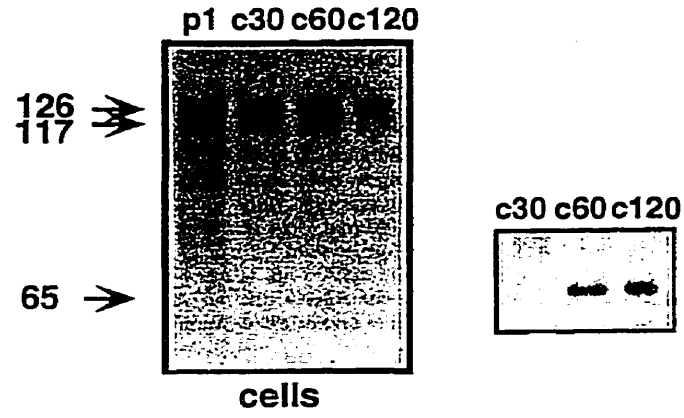
Figure 1. Schematic representation of mammalian proprotein convertase isoforms PC5-A and PC5-B. Legend to protein domains is depicted at the bottom. Amino acid length is given for each convertase. The sequence at the end of the COOH-terminal region common to PC5-A and PC5-B and the one unique to each isoform is given at the top. A schematic representation of the mutant PC5-AΔ construct used in this work is also shown.

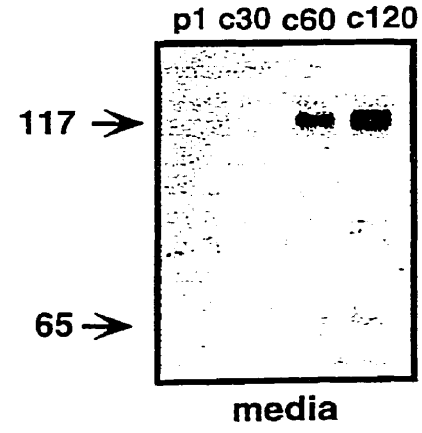




[35S]sulfate

Figure 2. Sulfate-labeling of PC5-A– and PC5-B–encoded proteins. PC5-A– or PC5-B–transfected cells were labeled for 2 h with [^{35}S]sulfate, followed by immunoprecipitation of both cells and media with an anti–NH_2-terminal PC5 antiserum and resolution on 8% SDS-PAGE gels. Control cells transfected with the RcCMV vector alone were similarly treated. Molecular masses are given in kD.





[35S]methionine

Figure 3. Endoproteolytic transformations of PC5-A. PC5-A–transfected cells were pulse labeled with [^{35}S]methionine for 1 min (*p1*), and then chased (*c*) for 30, 60, or 120 min, followed by immunoprecipitation with a polyclonal rabbit anti–NH_2-terminal PC5 antiserum and resolution on 8% SDS-PAGE gels. A more contrasted exposure is given to show the intracellular production of the 65-kD fragment. Molecular masses are given in kD.

pro-PC5-A, the sequence of which begins after the predicted signal peptidase cleavage site CysArgThr↓ArgVal-TyrThrAsnHis. The 117-kD protein represents PC5-A, which is produced after cleavage of the 82–amino acid prosegment at the sequence ValValLysLysArgThrLys-Arg_{82}↓AspTyrAspLeuSerHisAlaGlnSerThrTyrPheAsnAsp-ProLys. Sufficient amounts of the 65-kD form could not be accumulated to obtain an unambiguous sequence. However, since this fragment is immunoreactive to the NH_2-terminal antibody that was raised against the above 15–amino acid peptide starting at the -AspTyrAsp . . .- sequence, it likely represents a COOH-terminally truncated product of the 117-kD PC5-A form.

To define whether intracellular zymogen cleavage of the 126-kD pro-PC5-A is an early event, the protein was blocked in anterior secretory pathway compartments using the fungal metabolite brefeldin A (BFA)[1]. This agent causes the disassembly of the Golgi complex and fusion of the cis-, medial-, and trans-Golgi (but not the TGN) with the ER (Lippincott-Schwartz et al., 1989, 1991). In the presence of BFA, the conversion of the 126-kD pro-PC5-A into the 117-kD PC5-A is still observed (Fig. 5 *a*), but COOH-terminal truncation generating the 65-kD product is no longer detected. Thus, NH_2-terminal prosegment processing can occur in early compartments of the secretory pathway, but not COOH-terminal truncation. Furthermore, the absence of sulfation of the 126-kD form (Fig. 2) is taken as evidence that pro-PC5-A does not reach the TGN, where sulfation is known to take place (Hart, 1992; Bauerle and Huttner, 1987). Endoglycosidase H digestions were also performed and demonstrated that the 126-kD pro-PC5-A was digested by endoglycosidase H during chase times of up to 4 h, while the intracellular and

1. *Abbreviations used in this paper*: BFA, brefeldin A; POMC, pro-opiomelanocortin; TMD, transmembrane domain.

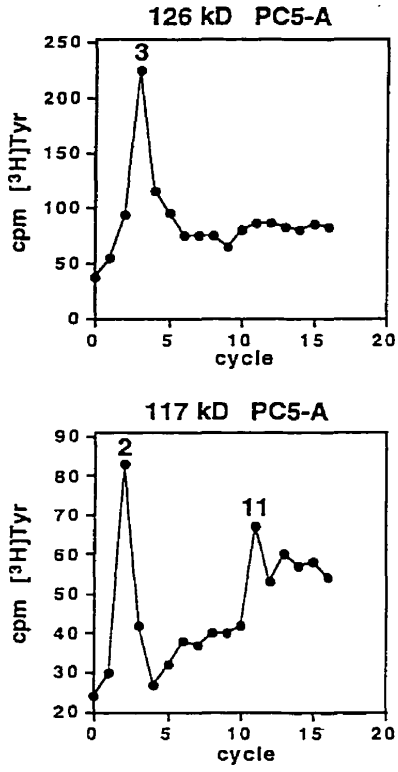


Figure 4. Microsequencing of PC5-A 126- and 117-kD products. The deduced sequence positions of released tritiated residues are indicated, clearly establishing the identity of the 126-kD product as pro-PC5-A and the 117-kD product as zymogen-cleaved PC5-A.

secreted 117- and 65-kD forms were resistant to this treatment after a 1-h chase (data not shown).

Since the COOH-terminal truncation generating the 65-kD product does not occur in the presence of BFA (Fig. 5 *a*), this cleavage must occur in a late compartment of the secretory pathway. To further substantiate this hypothesis, an experiment at a restrictive temperature of 20°C, which traps secretory proteins at the level of the TGN (Matlin and Simons, 1983), was performed. The formation of the 65-kD product was not observed at 20°C (Fig. 5 *b*). Therefore, COOH-terminal truncation of PC5-A occurs after egress of the 117-kD form out of the TGN.

Cellular Processing of PC5-B

As already shown in Fig. 2, in AtT-20 cells stably transfected with PC5-B, the sulfated protein forms that reach the TGN have an apparent M_r of 210 and 170 kD. Since only the 170-kD form is secreted in the medium, this suggests that the 210-kD sulfated protein is retained intracellularly via the transmembrane domain (TMD). In a similar time course to PC5-A (Fig. 3), pulse labeling of these cells with [^{35}S]methionine for 1 min followed by chase times of up to 2 h demonstrated that the 170-kD COOH-terminally truncated form is first detected in the cell extracts and medium after a 60-min chase period (Fig. 6 *a*). Since the 170-kD product is detected in the cell extracts, COOH-terminal truncation of PC5-B therefore occurs intracellularly. To determine whether the production of the 170-kD form is an early or late event along the secretory pathway,

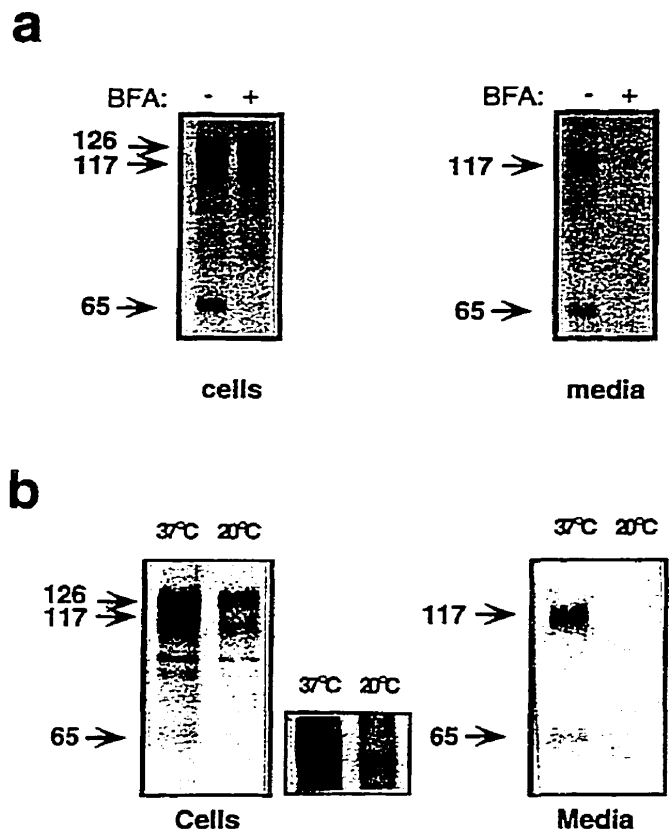


Figure 5. (*a*) Biosynthesis of PC5-A in the presence of brefeldin A. PC5-A–transfected AtT-20 cells were labeled with [^{35}S]methionine for 2 h in the presence or absence of brefeldin A and immunoprecipitated with an anti-NH_2-terminal PC5 antiserum, followed by resolution on 8% SDS-PAGE gels. Molecular masses are given in kD. (*b*) Effect of 20°C temperature blockade on PC5-A processing. PC5-A–transfected AtT-20 cells were labeled with [^{35}S]methionine at either 20° or 37°C for 2 h and immunoprecipitated with an anti-NH_2-terminal PC5 antiserum. A more contrasted exposure is given to clearly establish that the intracellular production of the 65-kD fragment is absent at 20°C. Molecular masses are given in kD.

AtT-20 cells expressing PC5-B were labeled with [^{35}S]methionine for 2 h in the presence or absence of BFA. Results shown in Fig. 6 *b* demonstrate that the 170-kD form is not detected in the presence of BFA, suggesting that loss of the TMD occurs in a late secretory pathway compartment.

PC5-A Enters Secretory Granules While PC5-B Is Localized in the Golgi

Biosynthetic studies. Since both 117- and 65-kD PC5-A (Fig. 3) and the 170-kD PC5-B (Fig. 6) are secreted in the medium, it was essential to determine whether each of these forms was released through the same secretory pathway. The basal and stimulated release of PC5-A and PC5-B in several of our clonal cell lines was thus compared by labeling of the cells for 2 h with [^{35}S]sulfate in the presence (+) or absence (−) of 8Br-cAMP. In Fig. 7 *a*, the release of the 117-kD PC5-A is seen to increase up to threefold in the presence of the secretagogue. Since the 65-kD PC5-A product is not sulfated, the experiment was

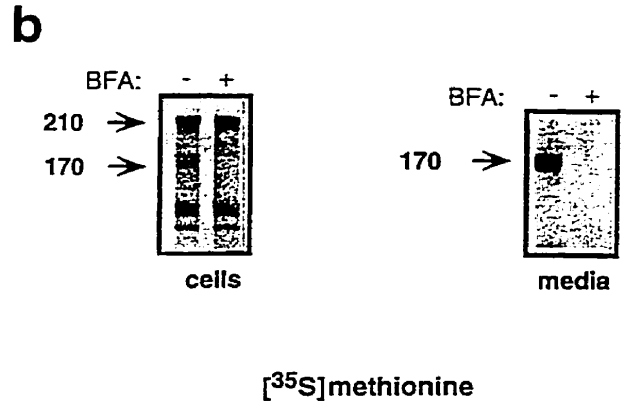
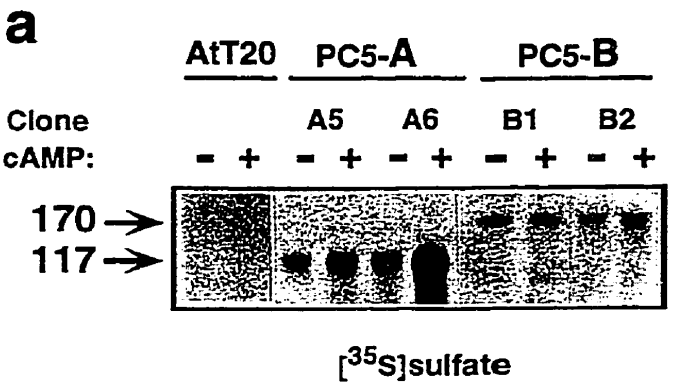


Figure 6. (*a*) Analysis of PC5-B transformations by pulse-chase experiments. PC5-B–transfected cells were pulse labeled with [^{35}S]methionine for 1 min (*p1*), and then chased (*c*) for 30, 60, or 120 min, followed by immunoprecipitation. Molecular masses are given in kD. (*b*) Biosynthesis of PC5-B in the presence of brefeldin A. PC5-B–transfected AtT-20 cells were labeled with [^{35}S]methionine in the presence or absence of brefeldin A for 2 h and immunoprecipitated. Molecular masses are given in kD.



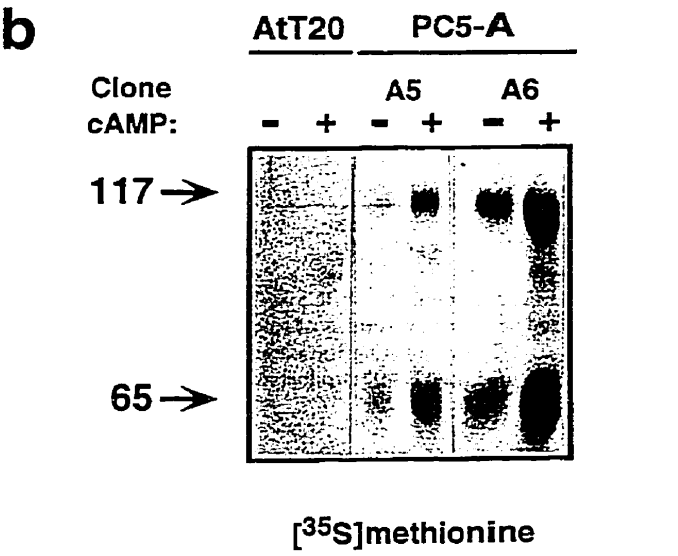


Figure 7. (*a*) Comparative basal and cAMP-stimulated release of sulfate-labeled PC5-A– and PC5-B–encoded proteins: PC5-A is stored in secretory granules, while PC5-B shed form is released through the constitutive pathway. PC5-A– or B–transfected cells were labeled for 2 h with [^{35}S]sulfate and treated with (+) or without (−) 8Br-cAMP. Media were immunoprecipitated with an anti–NH_2-terminal PC5 antiserum and resolved by SDS-PAGE. Molecular masses are given in kD. (*b*) Comparative basal and cAMP-stimulated release of methionine-labeled PC5-A–encoded proteins: both intact and COOH-terminally truncated products are stored in secretory granules. PC5-A–transfected cells were labeled for 2 h with [^{35}S]methionine and treated with (+) or without (−) 8Br-cAMP. Media were immunoprecipitated with an anti–NH_2-terminal PC5 antiserum and resolved by SDS-PAGE. Molecular masses are given in kD.

repeated using [^{35}S]methionine labeling. Results shown in Fig. 7 *b* demonstrate that the release of the 65-kD PC5-A form is also stimulated in presence of 8Br-cAMP. In contrast, the level of the secreted 170-kD PC5-B remains virtually unchanged upon this treatment (Fig. 7 *a*). The ratio of stimulated over nonstimulated bands was calculated by scanning and quantification of the autoradiograms, and it was found to be 0.90 for PC5-B and 2.5 for PC5-A (average values, see Table I). This suggests that while the 117- and 65-kD forms of PC5-A can enter secretory granules, the shed form of PC5-B (170 kD) exits from the cell via the constitutive secretory pathway (Burgess and Kelly, 1987).

To provide further evidence that the PC5-A sorting signal to secretory granules resides in its COOH-terminal region, a mutant PC5-A construct was produced, in which the last 38 amino acids unique to PC5-A were deleted (see Fig. 1). This mutant PC5-AΔ exhibits a biosynthetic pattern similar to that of PC5-A in pulse-chase experiments, with the presence of 122- and 113-kD forms in the cell extracts (Fig. 8). A notable exception is the production of the 65-kD fragment that, at the 2-h chase period in the medium, is reduced by half as compared with wild-type PC5-A (compare Figs. 3 and 8 *a*). The release of this PC5-AΔ mutant was not stimulated by 8Br-cAMP to the same extent as that of the wild-type PC5-A, showing only a 1.2-fold increase, while PC5-A release could be augmented by up to threefold in the presence of this secretagogue (Fig. 8 *b* and Table I).

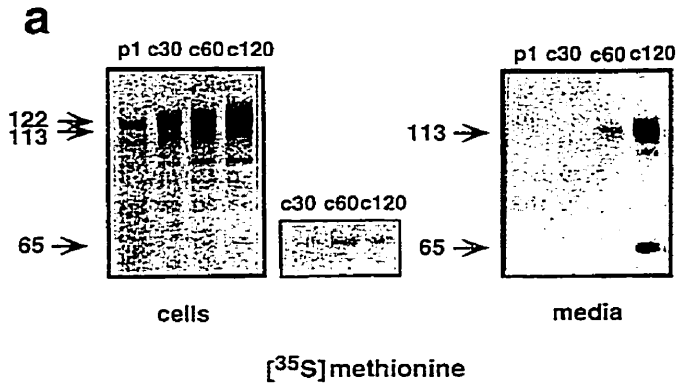
To clarify the type of release of each PC5 protein, secretion kinetics experiments were performed (Fig. 9). PC5-A–, PC5-AΔ–, and PC5-B–expressing cells were pulsed for

Table I. Ratio of 8Br-cAMP–stimulated Secretion to Basal Release for PC5 Proteins

	Ratio of stimulated secretion to basal release (Band intensity + 8Br-cAMP/ Band intensity − 8Br-cAMP)	SD
PC5-A	2.50	± 0.17
PC5-B	0.90	± 0.11
PC5-AΔ	1.21	± 0.19

Autoradiographs were quantified as described in Materials and Methods. The average ratio of stimulated release over basal release for 2-h labeling periods was calculated for each PC5 protein, taking values of five such experiments.

30 min with [^{35}S]methionine and chased for the times indicated. Their respective accumulation in the medium was calculated for each chase time by scanning and quantification of the autoradiograms as described in Materials and Methods (Fig. 9). While PC5-A demonstrates secretion kinetics representative of a protein released through the regulated pathway (Grimes and Kelly, 1992; Arvan and Castle, 1987; Chavez et al., 1996), PC5-B and PC5-AΔ exhibit notably distinct basal secretion patterns.



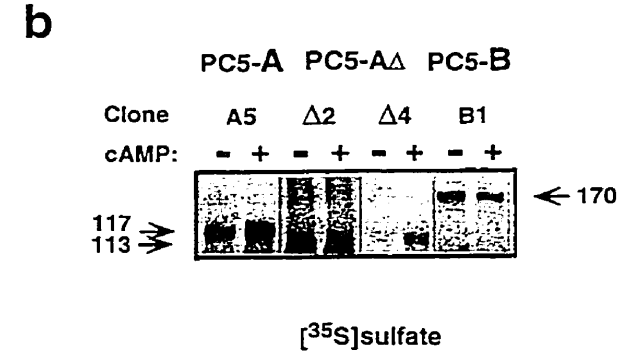


Figure 8. (*a*) Analysis of PC5-AΔ transformations by pulse-chase experiments. PC5-AΔ–transfected cells were pulse labeled with [^{35}S]methionine for 1 min (*p1*), and then chased (*c*) for 30, 60, or 120 min, followed by immunoprecipitation with a polyclonal rabbit anti–NH_2-terminal PC5 antiserum. A more contrasted exposure is given to show the intracellular production of the 65-kD fragment. Molecular masses are given in kD. (*b*) Comparative basal and cAMP-stimulated release of sulfate-labeled PC5-A–, PC5-B–, and PC5-AΔ–encoded proteins. PC5-transfected cells were labeled for 2 h with [^{35}S]sulfate and treated with (+) or without (−) 8Br-cAMP. Media were immunoprecipitated with an anti–NH_2-terminal PC5 antiserum and resolved by SDS-PAGE. Molecular masses are given in kD.

Immunofluorescence Analysis. To define more precisely the subcellular localization of PC5 proteins, AtT-20 transfected cells were analyzed by immunofluorescence microscopy (Marcinkiewicz et al., 1996). The data in Fig. 10 *a* show that PC5-A immunoreactivity exhibits a pattern of punctate staining observed in the cytoplasm and at the tips of cellular extensions, known to contain secretory granules (Matsuuchi et al., 1988), and immunostaining at paranuclear positions, which correspond to the Golgi apparatus. Weak immunoreaction could also be seen in the presumptive perinuclear ER. By double immunofluorescence, PC5-A is demonstrated to colocalize with ACTH in secretory granules and Golgi apparatus (Fig. 10, *a* and *b*). The PC5-B labeling pattern (Fig. 10 *c*) is quite different from that observed for either PC5-A or ACTH (Fig. 10, *a* and *b*). Immunofluorescence staining of PC5-B (Fig. 10 *c*) overlaps with that of TGN38 (Fig. 10 *d*), a TGN-resident protein (Luzio et al., 1990). A mutant PC5-AΔ, lacking the PC5-A–specific COOH-terminal segment (*PC5-Adelta*; Fig. 10 *e*) also colocalizes with TGN38 by double immunofluorescence (Fig. 10, *e* and *f*), showing that this mutant PC5-A seems to reside primarily in compartments anterior to the secretory granules. The specificity of immunolabeling is confirmed by the absence of PC5 immunoreactivity in AtT-20 cells transfected with the RcCMV expression vector alone (Fig. 10 *g*), and by the displacement of the labeling reaction with excess antigen (data not shown). We note that the labeling pattern of cathepsin B (*CB*; Fig. 10 *h*), a marker of lysosomal compartments, is distinct from that observed for PC5 in all our transfected cell lines, although in this case colabeling could not be achieved since both antibodies are from rabbit species.

Immunocytochemical Evidence of PC5 Localization in Pancreatic Glucagon-secreting Cells by EM

To demonstrate that the sorting of PC5-A into secretory granules is not fortuitous or the result of overexpression in AtT-20 cells, the cellular localization of PC5 in pancreas was examined by EM. In single-labeling experiments, glucagon immunogold labeling was present over the electron-dense structure of secretory granules, in cells displaying the characteristics of the A cells (Park and Bendayan, 1992) (Fig. 11 *a*). Using antibodies against the convertases PC2 and PC5 in conjunction with protein A–gold complexes, the gold particles were shown to be associated with the secretory granules of the A cells (Fig. 11, *b* and *c*). Double-labeling experiments revealed the colocalization of both PC2 and PC5 within the same granules of the A cells (Fig. 11 *d*). Double labeling using antibodies to PC2 or PC5 together with a glucagon-specific antibody demonstrated that the majority of glucagon–rich granules also display PC2 or PC5 immunoreactivity (Fig. 11, *e* and *f*).

Discussion

In this work, the biosynthesis and transport of PC5-A and PC5-B, two isoforms generated from a single gene (Mbikay et al., 1995), were investigated. Specifically, their respec-

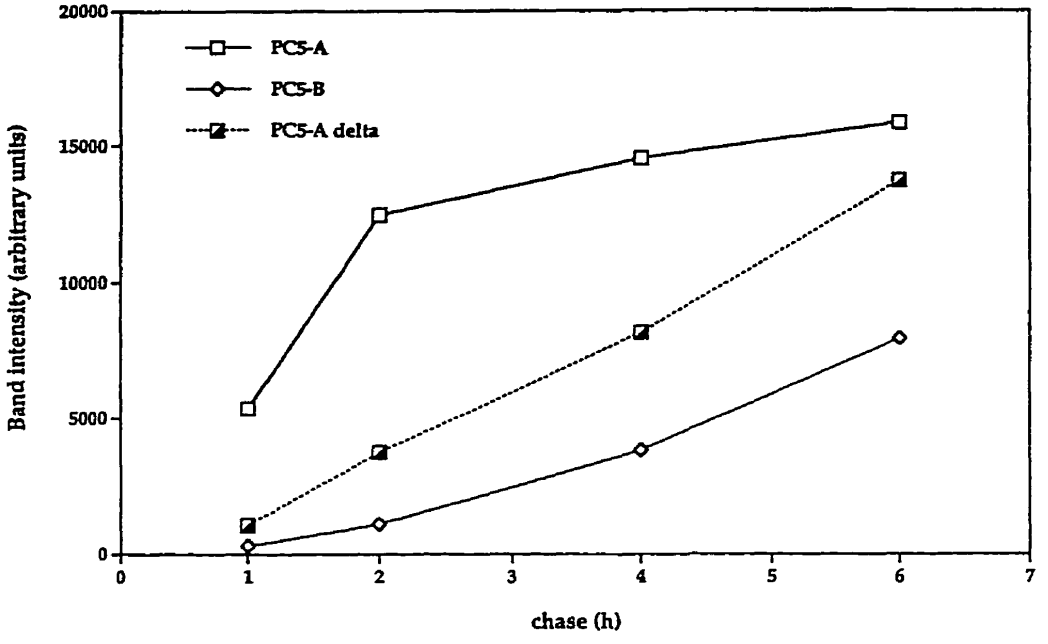


Figure 9. AtT-20–transfected cells were labeled for 30 min with [^{35}S]methionine and chased for the times indicated. Media were immunoprecipitated and resolved by SDS-PAGE. Autoradiographs of these gels were analyzed as described in Materials and Methods to permit quantification of the accumulation of released proteins in the medium at each time point. The calculated band intensities are given in arbitrary units.

tive biosynthetic pattern, sorting, and subcellular localization in stably transfected AtT-20 cells were compared. This work revealed that each isoform resides in a different intracellular compartment, suggesting their distinct functional destinations and the presence within their individual COOH-terminal domains of specific sorting signals.

Biosynthetic Fates of PC5-A and PC5-B

Initially, since data were not available on the biosynthesis of PC5, analysis in cells that endogenously express relatively high levels of its mRNA, namely BSC40 cells (Nakagawa et al., 1993*a*), was attempted. However, detection of sufficient amounts of [^{35}S]methionine-labeled PC5-A above background was unsuccessful. This approach was thus unsuitable to undertake extensive biosynthetic studies on PC5. Hence, the isolation of stable transfectants overexpressing PC5 was preferred, allowing in addition the comparison of the biosynthetic fate and transport of the isoforms PC5-A and PC5-B.

Pulse-chase analysis performed in transfected cells demonstrated that PC5-A is initially produced as pro-PC5-A, and rapidly processed to PC5-A (within a 1-min pulse) in early compartments of the secretory pathway, in a fashion similar to that of the granule-associated convertase PC1 (Benjannet et al., 1993). Pro-PC5-A predominates intracellularly, even after chase periods of up to 2 h (Fig. 3). It is possible that the intracellular predominance of pro-PC5-A over PC5-A at all chase times may be due to its relatively high expression levels in transfectant clones. As assessed by its persistent sensitivity to endoglycosidase H digestion, pro-PC5-A remains in a compartment where transformation and trimming to complex sugar types does not occur (data not shown). With the added observation that pro-PC5-A is not sulfated (Fig. 2), whereas PC5-A is, this suggests that the zymogen remains in the ER, and only the NH_2-terminally processed PC5-A is allowed to exit from this compartment. This is in contrast to PC2, which is processed in the TGN/granules (Benjannet et al., 1993). PC5-A also undergoes COOH-terminal truncation into a 65-kD product, presumably within immature secretory granules. This is again similar to the fate of PC1 (87 kD), which is COOH-terminally cleaved into a 66-kD form, also within granules (Vindrola and Lindberg, 1992; Benjannet et al., 1993). Based on its size and the PC5-A sequence (Lusson et al., 1993), and by homology to the re-

Figure 10. PC5-A colocalizes with the secretory granules and Golgi apparatus marker ACTH, while PC5-B colocalizes with the TGN marker TGN38. (*a*) Immunofluorescence analysis of PC5-A intracellular distribution shows density throughout the cytoplasm and at the tips of cellular extensions containing secretory granules (*thin arrows*) and in paranuclear position, corresponding to a presumptive Golgi apparatus (*thick arrow*). The pattern of PC5 pancellular distribution much resembles that of ACTH shown in *b*. Here, as typically seen in AtT-20 cells, ACTH immunoreactivity is found to be spread out over the cell and at the tips of cellular extensions (*thin arrows*). (*c*) Intracellular distribution of PC5-B (*thick arrows*), as detected by immunofluorescence, is comparable to that of the TGN marker TGN38 (*d*) (*thick arrows*). The empty arrow depicts lack of PC5 immunoreaction at the tip of the cellular extensions of PC5-B–transfected cells. PC5-AΔ immunofluorescence (*e*) (*thick arrows*) is also found to colocalize with TGN38 (*f*) (*thick arrows*). (*g*) Lack of PC5 immunostaining in control AtT-20 cells transfected with the RcCMV vector is depicted by the empty arrows. (*h*) Cathepsin B (*CB*) labeling (*curved arrows*) is markedly different from PC5 labeling observed in PC5-A–, PC5-B–, or PC5-AΔ–transfected cells. Bar, 20 μm.

a
c
e
g

cently described COOH-terminal cleavage site of PC1 (Zhou and Lindberg, 1994), the probable cleavage site generating this 65-kD fragment is at the sequence His_{646}TyrHisAlaAspLysLysArg$_{653}$↓Cys.

Pulse-chase analysis of the isoform PC5-B was then undertaken to compare its biosynthetic fate to that of PC5-A. This protein is present in transfected AtT-20 cells as a membrane-bound 210-kD form and a shed 170-kD product (Figs. 2 and 6, *a* and *b*). The truncated 170-kD product is detected after a 1-h chase both in the cell extracts and medium (Fig. 6 *a*), which suggests that COOH-terminal cleavage takes place in an intracellular compartment. Production of the PC5-B 170-kD truncation product is prevented in the presence of BFA, pointing to a late Golgi compartment as the location where COOH-terminal processing takes place. Similarly, the COOH-terminal truncation of membrane-bound furin was demonstrated to take place in the TGN (Vey et al., 1994). It should be mentioned that the extent of cleavage of the 210-kD form into the 170-kD PC5-B is somewhat dependent on protein expression levels. For example, in clones where expression levels are high, the ratio of the intracellular 210-kD to 170-kD form is in favor of the latter species, whereas this ratio is reversed in moderately expressing clones. This observation may be relevant to the different tissues that express this isoform. For example, in the ileum in which PC5-B mRNA is abundant, production of the 170-kD form would be expected to be more substantial than in lower expressing tissues, such as lung. The membrane-bound convertases furin (Molloy et al., 1994; Rehemtulla et al., 1992), the *Drosophila* furins (De Bie et al., 1995), and kexin (Germain et al., 1992) also exhibit shedding of their TMD, allowing the secretion of active enzymes. This is not only observed in conditions of overexpression (Rehemtulla et al., 1992), but also for the endogenous furin of Madin-Darby bovine kidney (MDBK) cells (Vey et al., 1994). Secretion of truncated PC5-B may therefore be physiologically important for the processing of extracellular substrates. A smaller truncation product of 65 kD, such as the one detected for PC5-A, is never observed for PC5-B, even though the cleavage site is present within the region common to both isoforms. Since it was demonstrated that this PC5-A COOH-terminal truncation segment is produced in immature granules, it can be concluded that PC5-B does not enter this compartment.

Although sequencing of the 170-kD form of PC5-B labeled with [^{3}H]tyrosine was attempted several times, it was not possible to obtain unambiguous sequence data, probably as a result of the difficulty of directly sequencing such a large protein (data not shown). The 170-kD protein could be derived from the 210-kD PC5-B by an NH_2- and/or COOH-terminal truncation. Since the loss of 40 kD is much greater than that expected for the 9 kD prosegment, it is likely that the 170-kD form is generated by COOH-terminal truncation of the 210-kD PC5-B. Also, the 170-kD product is not retained intracellularly, as would be expected of a form having lost its transmembrane anchor. However, the secreted 170-kD form is presumed to lack the NH_2-terminal prosegment as well, since zymogen forms of either PC1, PC2, or furin are not released from AtT-20 cells (Benjannet et al., 1993). Also, PC5-B activity obtained from recombinant vaccinia virus–infected cells could be detected in vitro in culture media using the fluorogenic substrate pGlu-Arg-Thr-Lys-Arg-methylcoumarin amide (Jean et al., 1993), concomitant with the observation of a 170-kD protein (Seidah, N.G., unpublished results).

Differential Intracellular Sorting of PC5-A and PC5-B

When expressed in AtT-20 cells, the two PC5 isoforms were sorted to distinct compartments of the secretory pathway. While the short and soluble PC5-A isoform entered the secretory granules, the membrane-bound PC5-B remained in the Golgi. Immunofluorescence images (Fig. 10 *a*) agree with the results obtained by biosynthetic labeling (Fig. 7), both of which demonstrate the presence of PC5-A in secretory granules. PC5-A (Fig. 10 *a*), like ACTH (Fig. 10 *b*) and PC1 (Marcinkiewicz et al., 1996), exhibits a dual Golgi/granule localization. Since cAMP stimulates the release of both 117-kD and 65-kD forms of PC5-A (Fig. 7 *b*), the punctate immunofluorescence found throughout the cytoplasm (Fig. 10 *a*) is presumed to correspond with granules carrying both products. In contrast, immunofluorescence data demonstrate that PC5-B resides in a compartment where it colocalizes with the TGN marker TGN38 (Luzio et al., 1990) (Fig 10, *c* and *d*).

To further demonstrate that the sorting of PC5-A to secretory granules is neither an artifact nor the result of overexpression in AtT-20 cells, we performed EM studies to demonstrate the presence of PC5 in the granules of an endocrine tissue. The data (Fig. 11) clearly demonstrate that PC5 colocalizes with both glucagon and PC2 within the granules of the pancreatic A cells. The presence of PC5 in the Golgi could not be established, as a result of the scarcity of this organelle in these cells. Further undertakings are needed to demonstrate the presence of PC5 in the Golgi of pancreatic A cells at the EM level and to determine which form of PC5 prevails in this cellular compartment.

The pancreatic A cells provide a physiological model for the sorting of PC5 in an endocrine tissue. In agreement, in two glucagon-expressing cell lines, the 65-kD PC5-A product has been detected by Western blotting (Blache et al., 1994). One of these cell lines, namely αTC1-6, is derived from the pancreatic A cells (Hamaguchi and Leiter, 1990). This strongly suggests that PC5-A, and not PC5-B, is the species detected in the A cells of the pancreas.

The principal sorting signal that permits PC5-A to enter secretory granules seems to reside within its unique COOH-terminal 38 amino acids. Indeed, a truncation mutant of PC5-A in which this segment was removed was no longer detected in the secretory granules of AtT-20 cells by immunofluorescence microscopy (Fig. 10 *e*). Moreover, 8Br-cAMP stimulation of the release of this protein only resulted in a 1.2-fold increase of secretion in the medium, while that of wild-type PC5-A could be augmented by up to three times (Table I). Therefore, PC5-AΔ seems to have lost its capacity to efficiently enter secretory granules.

The secretion kinetics of PC5-AΔ are also quite distinct from those of PC5-A. In Fig. 9, where the basal releases of PC5-A, PC5-AΔ, and soluble PC5-B are compared, PC5-A demonstrates a secretion pattern typical of proteins released through the regulated secretory pathway of trans-

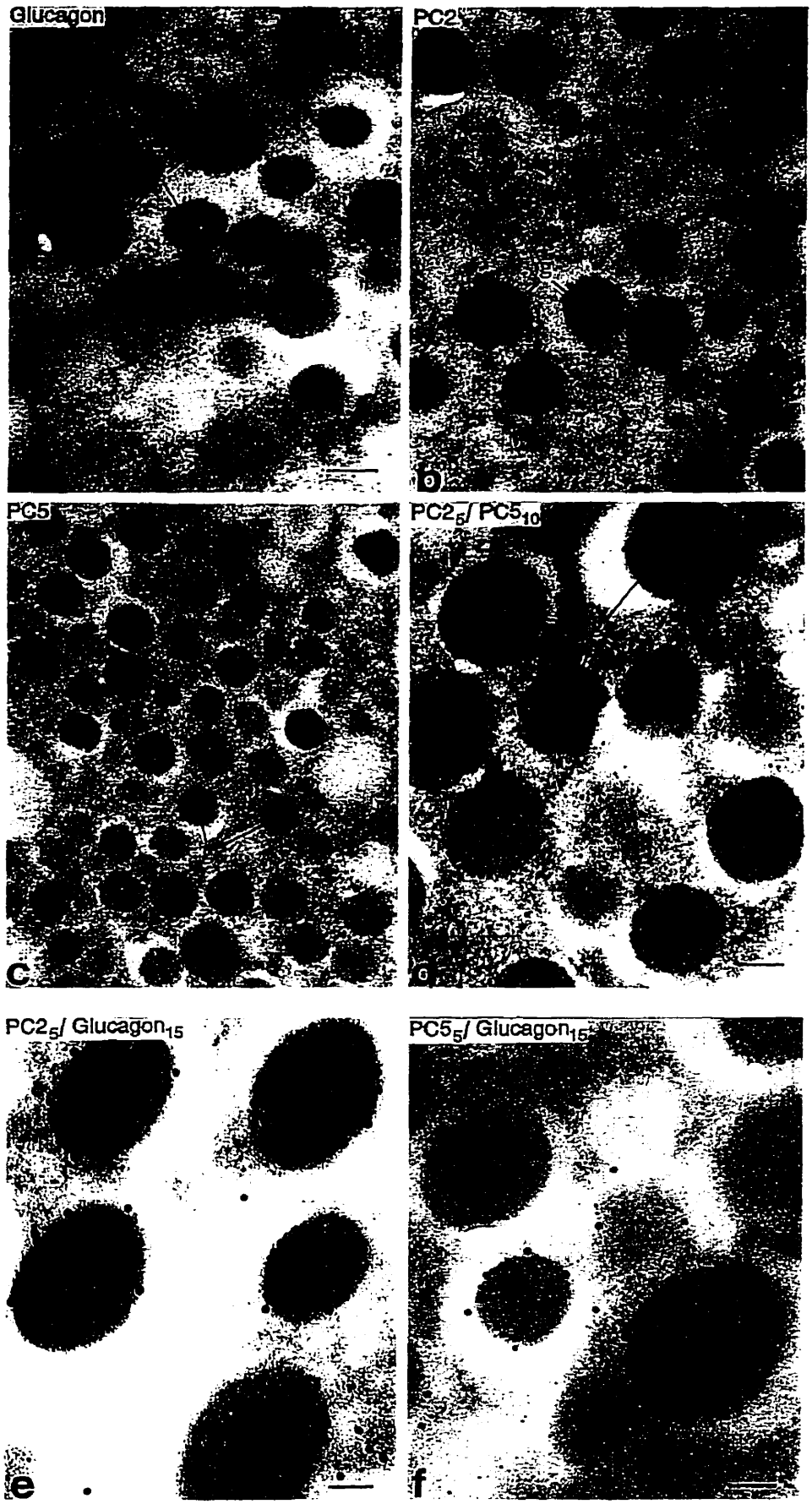


Figure 11. EM immunocytochemical detection of PC5 and PC2 in pancreatic glucagon-secreting cells. Pancreatic A cells were immunolabeled with the following antibodies complexed to protein A–gold: anti-glucagon (*a*, *e*, and *f*), anti-PC2 (*b*, *d*, and *e*), and anti-PC5 (*c*, *d*, and *f*). In *a*, the gold particles complexed to the glucagon antibody are detected over the secretory granules (*g*). (*b*) The secretory granules (*g*) of the A cell are labeled with the PC2 antibody complexed to protein A–gold. (*c*) Labeling of the A cell with the PC5 antibody complexed to protein A–gold is seen over the majority of secretory granules (*g*). (*d–f*) Sections of glucagon-secreting cells were then processed by double immunogold labeling: (*d*) PC2 (5-nm particles) and PC5 (10-nm particles) are colocalized in the secretory granules (*g*). (*e*) PC2 (5-nm particles) is colocalized with glucagon (15 nm particles) in many of the secretory granules. (*f*) PC5 (5-nm particles) colocalizes with glucagon (15-nm particles) in many granules of the A cell. Bars: (*a* and *b*) 0.2 μm; (*c–f*) 0.1 μm.

formed cell lines, while PC5-AΔ and soluble PC5-B exhibit linear constitutive releases. Tumor-derived endocrine cell lines have been demonstrated to have a high rate of basal, unstimulated release of granule contents. This is especially true of AtT-20 cells (Matsuuchi and Kelly, 1991; Milgram et al., 1994), which release about half of their granule content of ACTH in an unstimulated fashion (Moore et al., 1983). Therefore, Fig. 9 depicts this unstimulated granule exocytosis of PC5-A, which is initially rapid and thought to occur through immature granules, and then reaches a plateau as the protein accumulates in the mature secretory granules.

The basal release of PC5-AΔ and the soluble form of PC5-B, on the other hand, is believed to occur through constitutive vesicles, as their content increases linearly with time in the extracellular medium. The observed rate of release of PC5-B is the lowest of the three PC5 forms, probably because this protein must first undergo excision of its transmembrane domain before being released into the extracellular medium. The fact that the initial rate of release of PC5-A is higher than that of the mutant PC5-AΔ may be due (*a*) to the fact that the expression levels of PC5-A in the cell lines analyzed are higher than those of PC5-AΔ, and therefore the PC5-A overflow from granules is more important than the constitutive release of the mutant; or (*b*) to the release of PC5-AΔ from the cell being delayed by the slower rate of exit of this protein out of the ER, as the mutation introduced in the COOH-terminal domain could affect the proper folding of this protein.

The small increase of PC5-AΔ release in the presence of 8Br-cAMP (Fig. 8 *b*; Table I) could be explained by the entry of this protein in immature secretory granules, as these organelles can release their content upon stimulation (Tooze et al., 1991). This is further supported by the production of a 65-kD truncation product at 37°C, but not at 20°C, by both PC5-A (Fig. 5 *b*) and PC5-AΔ (data not shown). In comparison, PC5-B is never processed into this smaller truncation product, even though the cleavage site is present within the region common to both isoforms. The differential sorting of PC5-A and PC5-AΔ could therefore occur in immature granules (Arvan and Castle, 1992; Bauerfeind and Huttner, 1993), from which PC5-AΔ could be released through the constitutive-like pathway (Kuliawat and Arvan, 1994). Alternatively, PC5-AΔ could enter the immature granules, but then be recycled from this compartment back to the TGN to be released through constitutive vesicles.

Sequence-specific Sorting Signals of PC5-A and PC5-B

PC5-A. In an attempt to uncover a potential granule-sorting signal, the sequence unique to PC5-A was tentatively aligned with those of other proteins known to enter the secretory granules, namely chromogranin B and pro-opiomelanocortin (POMC). A consensus sequence emerged that conformed to the signals already defined for chromogranin B (Chanat et al., 1993) and POMC (Cool et al., 1995). This consensus sequence consists of two cysteines, which in the cases of chromogranin B (Chanat et al., 1993) and POMC (Bennett et al., 1986) have been demonstrated to form a disulfide bond, and conserved hydrophobic residues, especially a central leucine (Fig. 12 *a*). This motif is also observed in chromogranin A (Benedum et al., 1987) and in the convertase PACE4-A (Kiefer et al., 1991). We may therefore have identified a potential granule-sorting signal for PC5-A, which could also be functional in the

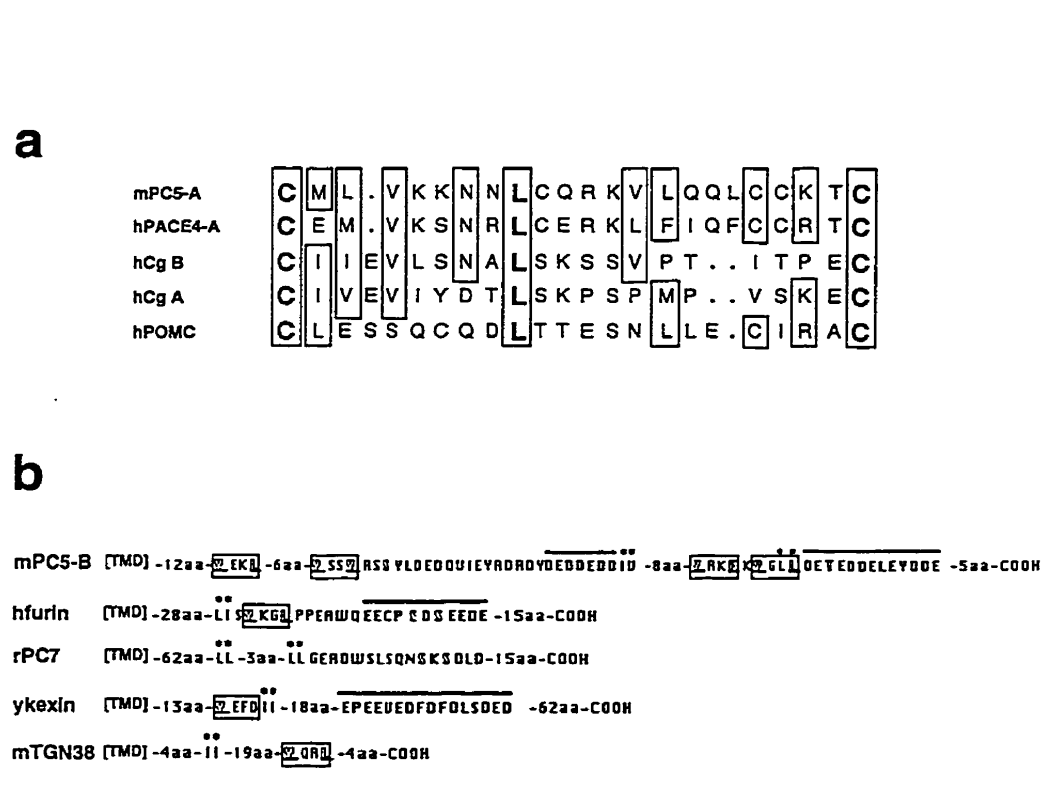


Figure 12. (*a*) Alignment of amino acid sequences showing homology between PC5-A, PACE4-A, chromogranin B, chromogranin A, and POMC. Conserved amino acid sequences of mouse mPC5-A, human hPACE4-A, human chromogranin B (*hCg B*), human chromogranin A (*hCg A*), and human hPOMC are aligned. Conserved residues are boxed, and the important cysteines and central leucine printed in bold. (*b*) Alignment of PC5-B cytosolic tail sequence with those of furin, kexin, TGN38, and PC7. Known and potential TGN-localization motives are emphasized. The motif Y-X-X-(hydrophobic) is boxed. Acidic stretches are topped with a bar. Potential casein kinase II phosphorylation sites are outlined. Dileucine motives are topped with an asterisk (*). *m*, mouse; *h*, human; *y*, yeast; *r*, rat.

convertase PACE4-A. Further studies are needed to demonstrate whether this signal is a true sorting domain and can act on constitutively secreted proteins, as was accomplished with chimeras of the prosegment of anglerfish somatostatin-1 and α-globin (Stoller and Shields, 1989). We conclude that the COOH-terminal 38–amino acid sequence is involved in the sorting of PC5-A to the regulated pathway. This segment may be necessary for the presumed calcium-induced aggregation of PC5-A, allowing its entry into mature secretory granules (Burgess and Kelly, 1987; Tooze et al., 1993). This does not exclude the possibility that additional sorting signals are present in other PC5 domains. Studies with both POMC (Chevrier et al., 1993) and somatostatin (Sevarino and Stork, 1991) demonstrated that multiple sorting signals to the regulated secretory pathway exist in these molecules.

PC5-B. Potential signals permitting the retention of PC5-B in Golgi compartments were likewise investigated, by comparing its COOH-terminal–specific sequence to those of other membrane-bound proprotein processing enzymes.

Several trafficking signals governing TGN localization have been recently defined to reside within the cytoplasmic tail of the convertase furin (Molloy et al., 1994; Chapman and Munro, 1994; Voorhees et al., 1995; Schäfer et al., 1995; Jones et al., 1995; Takahashi et al., 1995; Bosshart et al., 1995). These include (*a*) a cluster of acidic amino acids in the sequence CPSDSEEDEG, where the phosphorylation of both serines by a caseine kinase II–like enzyme plays an important modulatory role in the retrieval of furin to the TGN (Jones et al., 1995; Takahashi et al., 1995); (*b*) a Tyr-containing motif, where the tetrapeptide consensus sequence Y-XX-(hydrophobic) is a necessary and sufficient cytoplasmic domain signal to retrieve integral membrane proteins from the cell surface to the TGN (Trowbridge et al., 1993); and (*c*) adjacent leucine and/or isoleucine residues, termed dileucine signal, which has been described as an internalization motif (Johnson and Kornfeld, 1992).

All three signals are present in the cytosolic tail of PC5-B (Fig. 12 *b*). Two acidic clusters are seen in mouse PC5-B, within the sequence (Nakagawa et al., 1993*b*): TMD-28aa-SYLDEDQVIEYRDRDYDEDDEDD-19aa-DETEDDE-LEYDDE-5aa-COOH, with two potential (Ser and Thr) casein kinase II phosphorylation sites. Both acidic clusters and the casein kinase II phosphorylation site at the serine residue are conserved in rat PC5-B (GenBank accession number: Pcsk5 V47014). The cytosolic sequence of PC5-B also exhibits four Tyr-containing motifs: TMD-12aa-YEKL-6aa-YSSY-35aa-YRKF-1aa-YGLL-18aa-COOH, which could be potentially functional. Interestingly, PC5-B (LeuLeu, at two positions), furin (LeuIle), and PC7 (LeuLeu, at two positions) all contain a dileucine motif within their cytoplasmic tail. Future internalization studies aimed specifically at detecting PC5-B at the cell surface and its predicted retrieval to intracellular compartments should assess the implication of the Tyr-containing and/or dileucine signals in this process. Alignment of the known cytosolic tail localization motives of furin, PC7, kexin (Wilcox et al., 1992), and TGN38 (Humphrey et al., 1993; Bos et al., 1993; Wong and Hong, 1993; Ponnambalam et al., 1994; Wilde et al., 1994), all membrane-bound protein residents of the Golgi or TGN, with the motives seen in PC5-B, is shown in Fig. 12 *b*.

In conclusion, PC5-A and PC5-B constitute the first example for the convertase family of processing enzymes, in which two active forms originating from a single gene exhibit distinct cellular localization. This may represent a mechanism to regulate bioactive enzymes by directing them to different subcellular destinations to process unique sets of precursor substrates. Since the convertases exhibit overlapping tissue distributions as well as in vitro or ex vivo cleavage specificities, the formation of the appropriate convertase/substrate combinations could ultimately be regulated by their particular intracellular localization.

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Proprotein convertase PC5 is encoded by multiple mRNAs, two of which give rise to the COOH-terminal variant isoforms PC5-A (915 aa) and PC5-B (1877 aa).

Study of these PC5 isoforms was conducted to determine the potential influence of COOH-terminal structure variations on the cellular traffic and biosynthesis of these proteins.

The differences in biosynthesis and sorting between two mouse PC5 isoforms named PC5-A and PC5-B, which differ exclusively by the composition of their COOH-terminal structural region (Lusson et al., 1993; Nakagawa et al., 1993a,b), and a truncated form of PC5-A lacking the COOH-terminal segment unique to this isoform (see figure D-1), were investigated. The three convertases were overexpressed by stable transfection in AtT-20 cells, a cell type possessing both constitutive and regulated secretory pathways. The respective processing pattern and subcellular localization of each PC5 protein was examined by biosynthetic analyses coupled to immunofluorescence studies, to determine if all PC5 proteins would be sorted to the same organelle, or if each would reside in different compartments of the secretory pathway. Additionally, electron microscopic studies of pancreatic A cells were performed to determine the compartment of residence of PC5 in a physiological model.

Several molecular forms of PC5-A and PC5-B expressed in AtT-20 cells are observed, three for PC5-A, which were determined to be proPC5-A, zymogen-cleaved PC5-A and a COOH-terminally truncated form of PC5-A, and two for PC5-B, one membrane-bound and one soluble, this last form probably resulting from a cleavage NH_2-terminal to the

transmembrane domain. Both PC5-B forms are sulfated and thus reach the TGN, while only PC5-A and not proPC5-A is sulfated.

Soluble forms of PC5-A and PC5-B exit the cell through different secretory pathways. Stimulated protein release demonstrated that PC5-A was stored in the secretory granules of AtT-20 cells and could be liberated upon stimulation with cAMP, while secretion of the soluble form of PC5-B did not change upon this treatment.

Double immunofluorescence studies permitted to determine the subcellular localization of PC5-A and PC5-B in transfected AtT-20 cells. PC5-A was found to reside in the secretory granules and Golgi of AtT-20 cells, while PC5-B colocalized with TGN38, a marker of the TGN.

The mutant PC5-AΔ lacking the COOH-terminal region unique to PC5-A, contrary to wild type PC5-A, could only be detected throughout the Golgi region, and not in secretory granules of At-T20 cells. The release of PC5-AΔ was not significantly increased in the presence of cAMP, indicating that this protein does not accumulated in the secretory granules of AtT-20 cells.

Electron microscopic studies of pancreatic A cells established that PC5 immunoreactivity is detected within the granules of these cells. PC5 colocalizes with both glucagon and PC2 within this organelle, demonstrating the sorting of this protein to dense core secretory granules in endocrine cells.

The studies of the PC5 isoforms demonstrated the critical role of the COOH-terminal domain on the cellular traffic of convertases. Each isoform was found to reside in a different intracellular compartment, suggesting the presence of specific sorting signals within their individual COOH-terminal domains. This model established that structural modifications of the COOH-terminal region of some convertases are

implicated in the determination of the subcellular localization and traffic along the secretory pathway. The presence of a sorting signal in the COOH-terminal 38 amino acids unique to PC5-A was demonstrated by the inefficient entry into the regulated secretory pathway of a mutant lacking this segment, while other signals are probably responsible for the Golgi localization of PC5-B. Thus, from a single PC5 gene, several COOH-terminally modified isoforms are generated, each with different sorting signals directing these proteins to distinct subcellular localization.

Chapter E

General Conclusion and claims to original research

CONCLUSIONS

The mammalian subtilisin/kexin-like proprotein convertases, which cleave protein precursors intracellularly within specific subcellular secretory pathway compartments, possess additional carboxy-terminal structures as compared to the related bacterial subtilases, which function in the extracellular space. What is the advantage conferred to convertases over subtilisins by the presence of these additional domains?

The first of these two structural domains, which immediately follows the catalytic region, is the P domain (Gluschankof and Fuller, 1994). This region is indispensable to the proper maturation of convertases. Kexin (Gluschankof and Fuller, 1994) and furin (Hatsuzawa et al., 1992; Creemers et al., 1993) in which this region has been deleted or truncated past a critical Gly which marks the end of the structural homology between kexin and the mammalian convertases, do not undergo proregion cleavage and are retained intracellularly. Similarly, isoforms of the convertase PACE4 in which this region has been modified by splicing do not undergo proregion cleavage, are retained in the ER and are processing-deficient (Zhong et al., 1996). It has been suggested that the P domain of convertases could act as a scaffold to assist in the proper folding of the adjoined catalytic domain and in the positioning of the proregion for autocatalytic removal (Sinde and Inouye, 1995). However, despite numerous efforts, the exact functional purpose of the P domain has not yet been elucidated.

The other region which immediately follows the P domain is the COOH-terminal domain, where the most structural diversity is observed among the members of the convertase family. In mammalian convertases, this region is dispensable to the catalytic activity of these enzymes. COOH-terminally truncated forms of kexin (Fuller, Brake and Thorner, 1989; Germain et al., 1992; Gluschankof and Fuller, 1994) or

furin (Hatsuzawa et al., 1992; Creemers et al., 1993) are still capable of cleaving the appropriate substrates, while the multiple COOH-terminally variant dfurin1 isoforms do not display differences in cleavage specificity (this work). However, certain regions within the COOH-terminal domain of convertases were recently demonstrated to be involved in the determination of the intracellular residence of these enzymes.

The lumenal COOH-terminal domains of PC1 (Zhou, Paquet, and Mains, 1996), PC2 (Creemers et al., 1996), and now PC5-A (this work), are involved in directing these convertases to the regulated secretory pathway. Certain structural motifs within PC5-A affect the sorting of this protein to regulated secretory granules, although the exact signal has not been unequivocally characterized. Gene-transfer studies, in which the putative COOH-terminal sorting segment would be attached to a constitutively secreted protein, such as albumin or α-antitrypsin, are needed to demonstrate that this region can divert a protein from the constitutive to the regulated pathway. It is possible that this COOH-terminal signal requires the additional presence of the P domain, as furin-PC2 chimeras were recently demonstrated to be more efficiently targeted to the regulated secretory pathway when both P and COOH-terminal domains of PC2 were attached to the furin catalytic domain (Creemers et al., 1996). We have suggested that the signal within PC5-A COOH-terminal segment, which is also found in convertase PACE4, may also affect the sorting of this latter convertase to secretory granules. Indeed, a recent report demonstrated that PACE4 resides in the secretory granules of transfected AtT-20 cells (Mains et al., 1997). A truncated form of this convertase was generated to yield a product similar to the 66 kDa truncated from of PC1, which is only found in granules. While both PC1 ($NDRR_{591}\downarrow GV$, Zhou et al., 1995) and PC5-A ($RFRYSR_{587}\downarrow VE$, this work), exhibit potential autocatalytic cleavage sites at about 100 amino acids downstream of the RRGD motif, no such site is found in PACE4 (Kiefer et al., 1991). Thus, no natural 66 kDa PACE4 product is observed in biosynthetic studies (Zhong et al., 1996; Mains et

al., 1997), and the truncated 66 kDa product generated by mutagenesis does not accumulate in secretory granules (Mains et al., 1997). While full-length PACE4 still enters immature granules, the fact that its COOH-terminal domain is not processed as those of PC1 and PC5-A may prevent PACE4 from maintaining itself in the regulated secretory pathway. Since the COOH-terminal sorting signal remains attached to the rest of the molecule, PACE4 could be continually cycling between the immature granules and Golgi through the interaction of its COOH-terminal domain with a putative sorting receptor.

For membrane-bound convertases, signals within the cytosolic tails of furin (reviewed in Rouillé et al., 1995), kexin (Wilcox et al., 1992), and possibly PC5-B (this work), are involved in maintaining these convertases in the constitutive secretory pathway. The presence of a tyrosine-based signal for both kexin and furin, and casein kinase II phosphorylation sites for furin, are needed for the proper localization of these proteins in the Golgi-endosomal system. Although the transmembrane domain of furin was not found to contribute to the TGN localization of this enzyme (Voorhees et al., 1995), it was suggested that additional localization signals towards the lysosomal/endosomal system may reside within its lumenal domain (Bosshart et al., 1994).

The potential TGN-localization signals within the cytosolic tail of PC5-B require additional investigation by mutagenesis, to demonstrate that these amino acid sequences, which are equivalent to those of furin, are indeed functional.

All membrane-bound convertases so far discovered, except PC7, (Munzer et al., 1997; Seidah et al., 1997) undergo shedding of their transmembrane domain to release a soluble form of the enzyme in the extracellular medium. What is the reason of this shedding, and what protease mediates this cleavage, which so far has not been demonstrated to be autocatalytic?

It has been suggested that generation of soluble counterparts of membrane-bound proteins could be a common way for cells to multiply protein functions with a minimum of energy and structural complexity involvement (Ehlers and Riordan, 1991). Multiple forms of the same protein can be obtained either through alternative splicing of pre-mRNA (PC5), transcription of closely related but distinct genes (furin and PACE4), or posttranslational hydrolysis of the extracellular membrane-bound domain (furin, kexin, and PC5-B). Several membrane-bound proteins, including angiotensin converting enzyme (Soubrier et al., 1988; Ehlers, Chen and Riordan, 1991) and TNF-α (Arribas et al., 1996; Black et al., 1997; Moss et al., 1997), undergo shedding of their transmembrane domain. Recently, a metalloproteinase belonging to the ADAMS family was demonstrated to mediate the shedding of several membrane-bound surface proteins (Arribas et al., 1996; Black et al., 1997; Moss et al., 1997). This protease, called TACE, for TNF-α converting enzyme, recognizes the motif P-XXX-A↓. Incidentally, this motif is present within the lumenal domain of all three shed convertases. Furin presents two such sites at positions Pro_{714}-XXX-Ala_{718} and Pro_{598}-XXX-Ala_{700}. Cleavage at the second proposed site would yield a product with the molecular mass of furin Δ704 (Hatsuzawa et al., 1992), which is closest in molecular weight to shed furin. Furin Δ713 (Vidricaire, Denault and Leduc, 1993) exhibits a slightly higher molecular mass than shed furin. The P-XXX-A motif is also present at four different positions in PC5-B: Pro_{1756}-XXX-Ala_{1760}; Pro_{1478}-XXX-Ala_{1482}; $\underline{Pro_{1340}\text{-XXX-}Ala_{1345}}$; and Pro_{1294}-XXX-Ala_{1299}, the underlined site being the most likely to yield the observed 170 kDa shed PC5-B product. A slightly different motif is observed in kexin, with Pro_{673}-XX-Ala_{676}, or $\underline{Pro_{651}\text{-XXXX-}Ala_{656}}$, the second site being the most likely to yield shed kexin, since the Kex2Δp 666 construct closely resembles this kexin product (Germain et al., 1992). Another proposed

candidate shedding enzyme is the signal peptidase (Ehlers and Riordan, 1991), which could promote the early shedding of dfurin proteins in ER.

It is interesting to note that the cytosolic tails of membrane-bound dfurin1-CRR and dfurin1-X do not display the same motives (Roebroek et al., 1993). If these proteins had kept their transmembrane domain beyond the ER in biosynthetic studies, would they have been localized to different compartments? Are there other signals within the lumenal domain which affect the cellular localization of the convertases, since we did observe differences in efficiency of proregion cleavage and substrate processing among the dfurins?

One of the motives which is found in the lumenal COOH-terminal region of several convertases is the cysteine-rich repeat. No function has yet been assigned to this region in convertases. It is possible that the CRR region could be involved in mediating protein-protein interactions. These interactions could be homophilic, and mediate the formation of enzyme multimers. This type of homophilic clustering has been observed for the TGN protein TGN38 (Anderson, Sullivan, and Stow, 1995), although in this case, cysteine-rich regions are not involved. Alternatively, the CRR region could mediate heterophilic interactions with other secretory pathway proteins interacting with the convertases. Incidentally, the shedding metalloprotease TACE also possesses a cysteine-rich region.

Finally, the physiological substrates of PC5-A, PC5-B and the Drosophila furins still remain to be determined. While the Drosophila furins and PC5-B may cleave substrates related to growth factors and receptors, a role for PC5-A in the cleavage of a regulated secretory protein remains to be established. This may well be accomplished with the demonstration of the cleavage of pro-neurotensin by PC5-A, a protein stored in chromaffin cells granules of the adrenal medulla (Barbero et al., submitted).

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CLAIMS TO ORIGINAL RESEARCH

In the present work, we have taken advantage of the existence of multiple COOH-terminally variant isoforms of two convertases, dfurin1 and PC5, to assess the potential influence of different COOH-terminal structures on the cleavage specificity and subcellular localization of these proteinases. Through two distinct overexpression systems, one by recombinant vaccinia virus infection of cultured cells for the dfurin isoforms, and one by stable transfection of PC5 proteins in the cell line AtT-20, we established the cleavage specificity, biosynthetic transformations, and subcellular localization of these different proteins.

Claims pertaining to research presented in Chapter C:

- We are the first to demonstrate that structural changes within the COOH-terminal region of the dfurin1 convertase isoforms do not affect the cleavage specificity of these enzymes.

- All three possible cleavage site recognition motives were examined, through the coexpression of the dfurin enzymes with three different precursor substrates to unequivocally establish that Drosophila furins indeed exhibit furin-like activity and not PC1-like or PC2-like activity.

- The Drosophila furins undergo biosynthetic transformations similar to those of the mammalian furin, including proregion cleavage, glycosylation, and shedding of the transmembrane domain. This event, however, occurs in early secretory compartment for the Dfurins, contrary to mammalian furin for which this event requires a slightly acidic milieu.

Claims pertaining to research presented in Chapter D:

- Our studies are the first to establish the biosynthetic transformations of both cloned PC5 isoforms. Both PC5-A and PC5-B undergo rapid removal of their NH_2-terminal proregion. Both protein forms are then further cleaved within their COOH-terminal domain. PC5-B undergoes a truncation of its transmembrane domain which permits the release of a soluble form of the enzyme. PC5-A is also cleaved to yield a COOH-terminally shortened form which is only present in regulated secretory granules.

- We are the first to establish the subcellular localization of both PC5-A and PC5-B isoforms in transfected AtT-20 cells. While PC5-A can enter regulated secretory granules, where, like PC1, it undergoes COOH-terminal truncation to yield a shortened form, PC5-B is found to colocalize intracellularly with the TGN marker TGN38. The soluble PC5-B form does not enter regulated secretory granules, as its release cannot be stimulated by cAMP. Thus PC5-B remains excluded from the regulated secretory pathway while PC5-A can follow this route.

- Our studies are the first to establish with a model of natural isoforms and a deletion mutant, that some structures present within the COOH-terminal domain of the isoforms of convertase PC5 are implicated in their subcellular localization. While both natural isoforms exhibit distinct cellular localization, the mutant PC5-AΔ lacking the COOH-terminal region unique to this convertase can no longer access the regulated secretory pathway. PC5-AΔ, like PC5-B, colocalizes with TGN38, and its release is not significantly influenced by cAMP. We therefore may have identified a secretory granule sorting

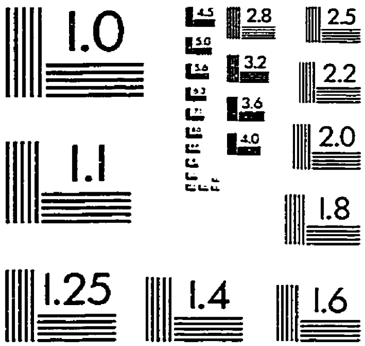
signal within the COOH-terminal region unique to PC5-A through the comparison of PC5-A wild type versus PC5-AΔ localization and biosynthesis.

- For the first time, the presence of PC5 in the glucagon-producing cells is established, providing a physiological model for the expression of this protein.

Claims pertaining to both Chapters C and D:

Our studies have permitted for the first time to establish the functional significance of the presence of multiple spliced forms for the same convertase. While these multiple spliced forms display identical catalytic specificities, by targeting them to different subcellular compartments, cells can access a more diverse array of precursor substrates.

IMAGE EVALUATION TEST TARGET (QA-3)



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