Compartmentalization of signal transduction from the epidermal growth factor and insulin receptors in rat liver parenchyma

by

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Pour ma Famille, en remerciement pour tout.

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ABSTRACT

The liver parenchyma is the major target organ enriched in EGF and insulin receptors. Following the administration of a receptor saturating dose of EGF, there was a rapid internalization of receptor coincident with its tyrosine phosphorylation. In the endosome, the adaptor protein SHC was recruited to and tyrosine phosphorylated by the EGF receptor. GRB2 and the Ras guanine nucleotide exchange factor, SOS, was primarily associated with the EGF receptor in endosomes. This is believed to contribute to a cytosolic pool of a complex of tyrosine phosphorylated SHC-GRB2/SOS. The activation of these molecules lead to the initiation of the mitogenic Ras-MAPK cascade as assessed by mobility shifts of Raf-1 and MAPK by SDS-PAGE. This culminated in the increase in mRNA levels for the early response genes, c-fos, c-jun, c-myc.

Upon internalization into the endosome, insulin dissociates from its receptor and is rapidly degraded. The role of the endosomal acidic insulinase (Authier *et al.*, 1994) in the regulation of signaling from the insulin receptor was investigated using an insulin analog, H2, which binds more tightly to the insulin receptor and was less susceptible to endosomal degradation. In response to H2 stimulation, the level of receptor phosphorylation at the plasma membrane was lower than that in response to insulin. While the insulin-activated receptor became partially dephosphorylated upon internalization, the H2-activated receptor maintained its state of phosphorylation. Insulin stimulation resulted in a higher level of IRS-1 phosphorylation than H2 stimulation, but here was no observable SHC or Ras-MAPK cascade activation in response to either of these ligands. However, H2 stimulation resulted in an increase in the mRNA levels of c-jun and c-myc transcripts. The kinetics and extent of internalization of the receptor were the same in response to both ligands, however, in the endosomal fraction, a transient accumulation of the H2-stimulated receptor was observed.

These findings demonstrate specificity of signaling from the mitogenic EGF receptor and the metabolic insulin receptor tyrosine kinases and support the hypothesis that signaling and the regulation of signaling from these receptors can take place at the level of the endosome in rat liver in vivo.

RESUME

Le foie est l'organe qui exprime le plus grand nombre de récepteurs pour le facteur de croissance EGF et pour l'hormone insuline dans le rat. Après l'injection intraveineuse d'une dose saturante d'EGF, le récepteur de l'EGF est rapidement autophosphorylé et internalisé dans le compartiment endosomal. Au niveau endosomal, la protéine SHC, une molécule servant d'adapteur, s'associe au récepteur de l'EGF pour être ensuite phosphorylée sur ses résidus tyrosine. De même, la protéine GRB2, une molécule servant d'adapteur pour SOS (un facteur favorisant l'échange des nucléotides GDP en GTP associés à Ras) est pareillement associée au récepteur dans l'endosome. L'activation de ces molécules conduit à la stimulation de la voie de la MAP-kinase, qui se traduit par une variation de la mobilité électrophorétique de l'enzyme Raf-1 et MAP-kinase détectable par électrophorèse en gel SDS-PAGE. Finalement, cette voie conduit à une augmentation de l'expression des ARN messagers des gènes c-fos, c-jun et c-myc.

Après translocation dans l'endosome, l'insuline internalisée se dissocie de son récepteur pour être ensuite rapidement dégradée dans la lumière des vésicules d'endocytose. Le rôle de l'insulinase endosomale acide dans la régulation de la signalisation du récepteur de l'insuline internalisé a été étudié en utilisant un analogue de l'insuline (dénommé H2). Cet analogue H2 se lie au récepteur de l'insuline avec plus d'affinité que l'insuline native mais cependant est moins protéolysé par l'insulinase endosomale. Après l'injection intraveineuse de l'analogue H2, la phosphorylation du récepteur de l'insuline au niveau de la membrane plasmique est diminuée comparativement à l'injection d'insuline native. De plus, alors qu'une déphosphorylation du récepteur de l'insuline se produit rapidement après l'injection d'insuline, cela ne ce produit pas après l'injection de l'analogue H2. De plus, la phosphorylation de la molécule IRS-1 est plus importante après l'injection d'insuline que de l'analogue H2. Aucun des deux ligands ne conduit à une stimulation des molécules SHC ainsi qu'à une stimulation de la voie de la MAP-kinase. Au niveau moléculaire, l'analogue H2 provoque une augmentation de l'expression des messagers des gènes c-jun et c-myc.

En conclusion, ces résultats démontrent une spécificité dans la signalisation du récepteur de l'EGF conduisant à son effet mitogène ainsi que dans celle du récepteur de l'insuline conduisant à ses effets métaboliques. De plus, ces travaux soulignent l'importance potentielle du compartiment endosomale hépatique dans la signalisation et la dissémination intracellulaires du signal hormonal générés par ces deux types de récepteurs.

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ABBREVIATIONS

AP-1	activator protein 1
AP-2	adaptor protein 2
ATP	adenosine triphosphate
BSA	bovine serum albumin
cDNA	complementary DNA
CHO	chinese hamster ovary cells
cpm	counts per minute
CsCl	cesium chloride
DEPC	diethyl pyrocarbonate
EAI	endosomal acidic insulinase
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
FGF	fibroblast growth factor
GAP	GTPase activating protein
GAPDH	glyceraldehyde phosphate dehydrogenase
GE	Golgi endosome fraction
GRB2	growth factor receptor binding protein 2
GTP	guanosine triphosphate
HBS	HEPES buffered saline
HGF	hepatocyte growth factor
HPLC	high pressure liquid chromatograpy
HEPES	N-(2-Hydroxyethyl)-1-piperazine N'-2-ethane sulfonic acid
HRP	horse radish peroxidase
IGF-1(R)	insulin-like growth factor-1(receptor)
IP	immunoprecipitation
IPTG	isopropyl β-D-thiogalactopyranoside
IRS-1	insulin receptor substrate-1
kDa	kilo Dalton
KIU	kallikrein inhibitor units
MAPK	mitogen activated protein kinase
mSOS	mouse homolog of Son-of -sevenless
Na_3VO_4	sodium orthovanadate
NGF	nerve growth factor
OD	optical density
ODC	ornithine decarboxylase
OsO_4	osmium tetrachloride
PEPCK	phosphoenolpyruvate carboxy kinase
PBS	phosphate buffered saline
PH	pleckstrin homology domain
PI3'-kinase	phosphoinositol 3' kinase
PKC	protein kinase C
PM	plasma membrane
PMSF	phenylmethylsulfonyl fluoride
PTB	phosphotyrosine binding domain
RTK(s)	receptor tyrosine kinase(s)
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SH2 / 3	src homology 2 /3 domain

SHC	SH2 containing / homologous to collagen protein
SHPTP	SH2 containing phosphotyrosine phosphatase
SIE	serum inducible element
SRE/SRF	serum response element / serum response factor
TCF	ternary complex factor
TGFα	transforming growth factor α
TP	total particulate fraction
Tris	2(hydroxymethyl) 2-amino-1,3 propanediol
Tween-20	polyoxyethylenesorbitan monolaurate

Introduction

Many polypeptide hormones and growth factors, including insulin and epidermal growth factor (EGF), mediate their biological effects by binding to and activating the intrinsic tyrosine kinase activity of their respective cell surface receptors. Ligand binding leads to the activation of the receptors and the initiation of signal transduction pathways which control diverse physiological processes, including cell metabolism, differentiation and proliferation. Because of the importance of these processes in normal development and maintenance of homeostasis, as well as the role they play in developmental disorders and neoplasia, it is important to determine the specific signal transduction pathways leading from these receptors and the regulation of these pathways in a physiological context.

The major organ responsible for whole body homeostasis is the liver, which is composed primarily of quiescent and highly differentiated parenchymal cells; the hepatocytes. These cells carry out the specialized functions of the liver, including production of the majority of plasma proteins, and regulation of carbohydrate, urea, fatty acid and cholesterol metabolism. In addition, the liver displays the remarkable and unique capacity to undergo hyperplasia and to repopulate lost tissue and cells resulting from physical, infectious and toxic injury (reviewed in Steer, 1995 and Diehl and Rai, 1995). The liver expresses receptors for insulin and EGF, both of which have been implicated in these processes. The liver therefore provides a relevant physiological model for the study of the metabolic and mitogenic signal transduction pathways from these two receptor tyrosine kinases.

The Biological Actions of Insulin in Hepatocytes

Under normal conditions, hepatocytes are regularly exposed, via the portal circulation, to major increases in insulin concentration in response to food intake. The liver removes >45% of the insulin reaching it in one pass (Jaspan *et al.*, 1981). The functions of insulin on the regulation of glucose metabolism in the liver are well established. In response to insulin stimulation lipogenesis is increased and gluconeogenesis decreases. Insulin can affect the regulation of these pathways by modulating the expression of key enzymes (rev. Messina, 1989). For example, the rate limiting step in gluconeogenesis is the conversion of oxaloacetate to phosphoenolpyruvate, which is catalyzed by the cytosolic enzyme phosphoenolpyruvate carboxy kinase (PEPCK). Insulin acts to inhibit the transcription of the PEPCK gene both in intact liver as well as in cultured hepatoma cells. In addition, insulin (in the presence of glucose) results in the increase of glucose kinase gene expression, which catalyses the first step (glucose to glucose-6-phosphate) of the glycolytic pathway in liver (Doiron *et al.*, 1994). Furthermore, insulin stimulates

glyceraldehyde 3'-phosphate dehydrogenase (GAPDH; a glycolytic enzyme) gene expression in H35 hepatoma cells (Alexander *et al.*, 1988). These actions of insulin are involved in the regulation of blood glucose levels. The transcription of albumin, a major plasma protein synthesized and secreted by the liver, is also subject to regulation by insulin (rev. Messina, 1989).

Although the metabolic effects represent the major physiological actions of insulin, its growth promoting actions may play an important role in fetal growth, organogenesis and tissue repair and regeneration. The mitogenic responses to insulin include the stimulation of DNA synthesis and promotion of cell growth. However, many of the studies observing the activation of a mitogenic pathway in response to insulin have used cells overexpressing the insulin receptor (Drazin, 1993; Medema, 1993) or with respect to liver, hepatoma cell lines. These cells may not represent a physiologically significant model for insulin action. In addition, many cell types express the insulin-like growth factor 1 (IGF-1) receptor. Insulin has the ability to cross react with the IGF-1 receptor at high hormone concentrations (like those used in many cultured cell studies). The binding of insulin to the IGF-1 receptor results in the activation of a kinase that is 10 times more active as a mitogen than the insulin receptor kinase, in 3T3 mouse fibroblasts (rev. Taylor *et al.*, 1991). As a consequence, some of the insulin mediated mitogenic responses observed in culture may in fact be carried out by insulin-activated IGF-1 receptors. Interestingly, experiments carried out by Osterop *et al.* (1992) revealed that the higher the number of transfected insulin receptors in Chinese hamster ovary (CHO) cells, the lower the level of [^{3}H]-thymidine incorporation in response to insulin. Although these results suggest a possible role for insulin in mitogenic signaling, they may not reflect the signal transduction pathways of the insulin receptor in liver *in vivo*. Indeed, this organ receives insulin regularly and in large doses but does not appear to undergo a mitogenic response. Acting alone, insulin is not an initiator of hepatocyte proliferation (Bucher *et al.*, 1977). However, the infusion of anti-insulin serum into rats after partial hepatectomy, impaired the regenerative capacity of the liver, supporting the role of insulin as a co-mitogen during hepatic regeneration.

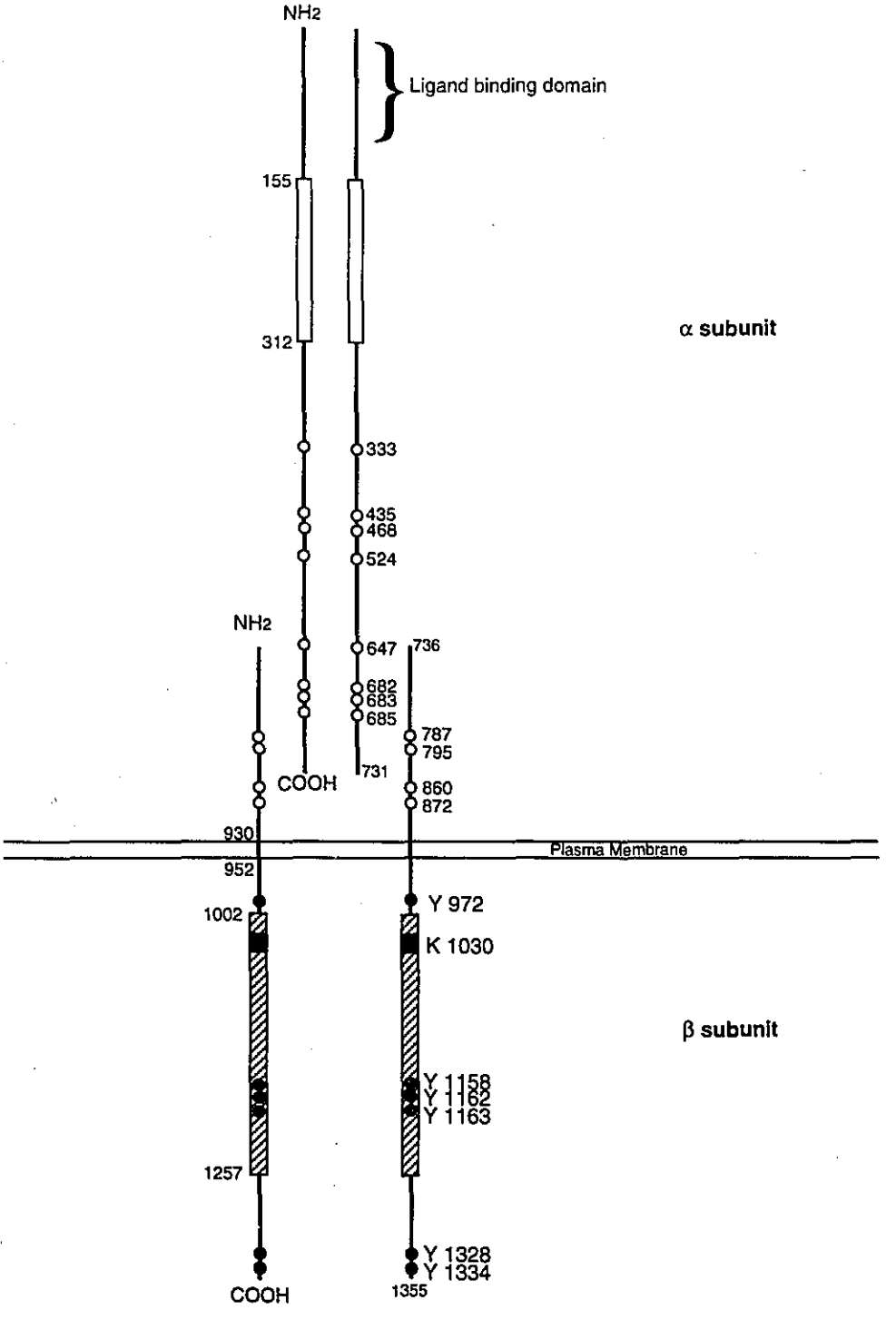
Many of the effects observed in response to insulin stimulation are cell and tissue specific and involve a discrete subset of proteins. Responses to insulin stimulation are also dependent on the dose and length of exposure. Evidence exists indicating that the insulin receptor exerts its metabolic and mitogenic effects through divergent signal transduction pathways but the point of divergence is unknown. In both cases however, it appears that protein phosphorylation plays a central role (Saltiel, 1994).

Figure 1

Schematic representation of the Insulin receptor

The insulin receptor exists as an $\alpha_2\beta_2$ heterotetramer (α subunit, 130 kDa; β subunit, 94 kDa). The insulin binding site is indicated and postulated to reside between residues 1-120. The amino acid positions of the cysteine residues involved in the formation of disulfide bonds between the two α subunits and the α and β subunits of the insulin receptor are indicated (o). The tyrosine kinase activity of the receptor is activated upon ligand binding, resulting in the autophosphorylation of select residues in the juxtamembrane domain, the kinase domain as well as at the C-terminus. The sites of tyrosine phosphorylation (●) and ATP binding (■) and the intrinsic tyrosine kinase domain of the receptor (▨) are indicated. The cysteine rich domains (▭), typical of these receptors, and the amino acid residues corresponding to the transmembrane domain are also indicated.

The numbering system for the residues of the insulin receptor, takes into account the 12 amino acids of exon 16 which are expressed in the hepatic form of the receptor.


NH2
Ligand binding domain
155
312
α subunit
333
435
468
524
NH2
647
736
682
683
685
787
795
731
COOH
860
872
930
Plasma Membrane
952
Y 972
1002
K 1030
β subunit
Y 1158
Y 1162
Y 1163
1257
Y 1328
Y 1334
COOH
1355

The Insulin Receptor

Although the insulin receptor is ubiquitously distributed in mammalian tissues, one of the major target organs for insulin, based on receptor number, is the liver (Kahn *et al.*, 1974) where $>10^5$ insulin receptors are expressed per hepatocyte.

The insulin receptor is a type I transmembrane glycoprotein derived from a precursor that is proteolytically cleaved to yield an α (135 kDa) and β (94 kDa) subunit which are covalently linked by disulfide bonds. Two such dimers are covalently linked, by disulfide bonds, to form the functional heterotetrameric ($\alpha_2\beta_2$) insulin receptor. The α subunit is entirely extracellular, while the β subunit contains the transmembrane domain, as well as the cytosolic kinase domain, internalization motifs and autophosphorylation sites (rev. Taylor *et al.*, 1991).

I The Extracellular Domain and Ligand Binding

The α subunit contains the insulin binding region of the receptor. There is strong evidence that insulin binds to a domain very near the N-terminus (amino acids 20-120) of the α subunit (DeMeyts *et al.*, 1990), although other regions in the α subunit, such as the cysteine rich domain, may be involved in determining the specificity of ligand binding (Taylor *et al.*, 1991). The insulin receptor can bind to insulin-like peptides including the insulin-like growth factors, IGF-I and IGF-II, but the affinity for these ligands is approximately 100-fold less than that for insulin. There is an alternative splicing site surrounding exon 11 of the insulin gene, which results in two possible receptor isoforms differing by 12 amino acids near the C-terminus of the α subunit (Mosthaf *et al.*, 1990; White and Kahn, 1994). In the liver, as well as in muscle and adipose tissue, the insulin receptor expresses the whole of exon 11 and therefore the longer receptor isoform (rev. Taylor *et al.*, 1992). The presence of the 12 extra amino acids causes a 2-fold reduction in the affinity of the receptor for insulin binding (Mosthaf *et al.*, 1990; McClain, 1991). With respect to the liver, the presence of the lower affinity receptor isoform may be explained as an evolutionary adaptation to the 2-3 fold higher doses of insulin seen by this organ due to its anatomical relationship with the pancreas.

When insulin binding data are plotted, a curvilinear Scatchard plot is obtained. This was suggested, initially, as representing a heterogeneous population of insulin receptors; one consisting of a low number of high affinity receptors and the other a high number of low affinity receptors (Kahn *et al.*, 1974). Subsequently, the curvilinear Scatchard plot was explained by assuming negative cooperative interactions between insulin binding sites.

This is consistent with the observation that the insulin receptor dimer ($\alpha\beta$) demonstrates a linear Scatchard plot with an affinity corresponding to the low affinity state of the tetrameric receptor ($\alpha_2\beta_2$). The assembly of two dimers into the tetrameric form is believed to cause a conformational change, increasing the receptors' affinity for insulin binding (Taylor *et al.*, 1991; Lee *et al.*, 1993).

II The Catalytic Domain

Upon insulin binding to its receptor, a conformational change is transmitted to the intracellular domain, which results in the activation of the tyrosine-specific protein kinase activity of the receptor. The catalytic domain of the insulin receptor is delineated by residues 1002-1257. This domain is highly conserved in all receptor tyrosine kinases (RTKs) (Ullrich *et al.*, 1985) and contains a consensus sequence, gly-X-gly-X-X-gly-$X_{(15\text{-}20)}$-lys, which functions as a part of the ATP binding site (Taylor *et al.*, 1991). This lysine residue is critical and its replacement completely abolishes tyrosine kinase activity (Chou *et al.*, 1987). While kinase activity is dispensable for receptor expression and cell surface targeting of RTKs, including the insulin receptor, it is indispensable for efficient internalization, signal transduction and the induction of both early and late cellular responses initiated by ligand binding.

III Insulin Receptor Internalization

In the rat liver hepatocyte, insulin receptors in their unbound state are preferentially associated with surface microvilli, with the exclusion of insulin receptors from coated pits being clearly demonstrated (Bergeron *et al.*, 1979). Studies by Carpentier and McClain (1995) reveal that the C-terminus of the insulin receptor functions to anchor the unoccupied receptors to the microvillar membrane. Insulin binding to the receptor results in the activation of the receptor tyrosine kinase which initiates internalization by releasing this constraint. Precisely how this takes place remains to be resolved. The route of internalization i.e. through the intermediate of clathrin coated pits and vesicles or noncoated vesicles remains unclear. In H35 hepatocytes, occupied receptors appeared to be excluded from coated pits (rev. Knutson, 1991). Likewise, in HepG2 hepatoma cells, where coated pit formation was inhibited by the depletion of potassium under hypotonic conditions, insulin / insulin receptor complexes were internalized normally even in the absence of clathrin coats. While, these observations were carried out in conditions of high receptor occupancy, under more physiological conditions, low insulin concentrations and

low receptor occupancy, internalization was inhibited by potassium depletion suggesting a coated pit mechanism. These results, and those derived from CHO cells, suggest that the insulin-receptor complex may be internalized by both smooth and coated pits in a manner that may be related to receptor occupancy (Backer *et al.*, 1991; McClain and Olefsky, 1988).

An alternative route of endocytosis, through caveolae, has been proposed. However calveolin, an integral membrane protein which serves as a marker for these structures, was undetectable in liver (Scherer *et al.*, 1994). Thus, it is unlikely that receptor internalization in the liver takes place via this pathway.

Mechanism of Internalization

Insulin mediated endocytosis requires specific amino acid residues found in the juxtamembrane domain (encoded by exon 16) of the receptor. This region, capable of inducing endocytosis of an insulin receptor lacking exons 17-22 (Carpentier and McClain, 1995), contains two endocytic sequences; a strong code, $GPLY^{965}$ and a weak code, $NPEY^{972}$ (Rajagopalan *et al.*, 1991). Mutation of these codes to APLA and APEA, respectively reduced internalization to 32% and 87% respectively, as compared to wild type insulin receptor internalization. Both codes form a tight β turn structure exposing a tyrosine residue, an essential recognition motif which is present in the internalization codes of many receptors. In addition to these endocytic codes, efficient internalization of the insulin receptor requires the activation of the receptor tyrosine kinase and tyrosine phosphorylation (Backer *et al.*, 1991). An active kinase may be required to induce a conformational change which could result in the exposure of internalization codes, as suggested for the EGF receptor (Cadena *et al.*, 1994). Alternatively, an active tyrosine kinase receptor may be needed to phosphorylate substrates involved in mediating receptor internalization.

pp120/HA4

Formisano *et al.* (1995) investigated the role of pp120/HA4, a substrate of the insulin receptor that is predominantly expressed in the liver, in receptor internalization. The transfection of antisense pp120/HA4 cDNA into H35 hepatoma cells inhibited insulin-induced receptor internalization 2-3 fold, implicating this protein in the regulation of endocytosis. The effect of this protein in increasing internalization appeared to be specific for the insulin receptor. Based on the observation that pp120/HA4 shared some homology with sequences thought to be important recognition elements for AP-2 adaptor proteins (implicated in EGF receptor internalization), it was proposed to be part of a complex contributing to the interaction of the activated insulin receptor with clathrin coated pits.

However, immunolocalization of pp120/HA4 in rat liver revealed that this protein was associated exclusively to the bile canalicular membrane (Margolis *et al.*, 1988), and consequently unlikely to be directly involved in insulin receptor internalization. Thus, the role of this protein in mediating insulin receptor internalization *in vivo* remains unresolved. This study demonstrates the caution which must be used in extrapolating data obtained from cell culture to *in vivo* systems, where cells are subject to defined cell-cell and cell-extracellular matrix interactions and display different topological arrangements.

Enigma

Using the yeast two hybrid system, a protein which specifically interacts with the internalization region of the insulin receptor, encoded for by exon 16, was uncovered (Wu and Gill, 1994). This protein, termed enigma, contains two LIM domains in its C-terminus. LIM domains were first identified as regions of homology in the homeodomain proteins lin-11, isl-1 and mec-3, involved in *D. melanogaster* development. These domains have been proposed to function in protein-protein interactions and have been identified in proteins that act in the cytoplasm and with the cytoskeleton and focal adhesions (Schmeichel and Beckerle, 1994). Enigma interacts with insulin receptor exon 16 through the second LIM domain, exclusively. Mutation of the strong endocytic code (GPLY^{965} to APLA) almost completely abolished this interaction, whereas the mutation of NPEY^{972} to APEA decreased this interaction to 60%. These results were in accordance with the decreased internalization rates seen for these mutant receptors (see above) and demonstrate that LIM domains are capable of interacting with the tyrosine tight turn motif of these endocytic codes. Enigma failed to interact with the endocytic codes of the constitutively internalizing low density lipoprotein receptor and transferrin receptor or the EGF receptor (^{996}QQGFF), revealing the specificity in the recognition of enigma for internalization motifs (Wu and Gill, 1994).

IV The Endosomal Apparatus

Once sequestered from the plasma membrane, the coated vesicles lose their clathrin coats and the receptor-ligand complex becomes concentrated into a heterogeneous nonlysosomal population of tubulovesicular structures referred to as the endosomal apparatus (reviewed by Bergeron *et al.*, 1985). The endosomal apparatus is positioned both temporally and physically between the plasma membrane (PM) and the lysosome. The endocytic apparatus has been subdivided into two main compartments, namely early and late endosomes, based on the time required for an internalized receptor to accumulate in

these compartments. Initially, receptor-ligand complexes are delivered into early endosomes (within 2-5 min) which are located at the cell periphery and consist of weakly acidic tubular elements (pH 6-6.5) (Wall and Hubbard, 1985; Mellman *et al.*, 1986). For many ligand-receptor complexes this endosomal acidification results in the dissociation of the ligand from its receptor. Ligand degradation may occur in this compartment, as is the case for insulin (Authier *et al.*, 1994). It appears that it is early in the endosomal pathway that a mechanism exists which sorts the receptors to be recycled to the PM from those targeted to the lysosome for degradation (Lai *et al.*, 1989a). This mechanism appears to be dependent, at least to some extent, on receptor occupancy levels. Late endosomes which are moderately acidic (pH 5-6), fill more slowly (10-20 min) and consist of tubulovesicular structures of varying sizes located in the Golgi-lysosome area of the cell (Dunn and Hubbard, 1984; Wall and Hubbard, 1985). Some receptor recycling may occur at this point as well. The precise fate of ligand-receptor complexes within the endosomal apparatus varies with each particular ligand and receptor, and the mechanisms which control their fates remain to be determined.

Fate of Internalized Insulin and Insulin Receptors

Internalization of the insulin-receptor complex constitutes the major mechanism of insulin degradation and down-regulation of cell surface receptors. The acidic pH of the endocytic compartment causes the dissociation of insulin from its receptor. Several studies have demonstrated that the endosome is a major site of degradation of insulin (Doherty et al, 1990; Backer et al, 1990; Authier *et al.*, 1994). Insulin degradation is initiated in the early endosome, as rapidly as 1 min after the injection of insulin into rats. The degradation of insulin appears to be carried out by an endosomal acidic insulinase, found in the lumen of hepatic endosomes (Authier *et al.*, 1994). The insulin protease characterized in cell-free endosomes demonstrates optimal activity between pH 5.0 and 5.5. Because acidification augments the release of insulin from the receptor, the question arises, is the insulin protease active on receptor bound insulin, or does the enzyme require free insulin? The data of Doherty *et al.* (1990) indicate that the inhibition of insulin release from the receptor reduces degradation of insulin. Thus it appears that dissociated insulin is degraded, driving further dissociation and degradation, resulting in a receptor free of bound insulin.

While insulin is degraded in the endosome, the insulin receptor may recycle back to the plasma membrane or translocate into lysosomes for degradation. Prolonged insulin stimulation, or receptor saturating doses of insulin, appears to cause the degradation of the internalized receptor in rat liver (Lai *et al.*, 1989a; Backer *et al.*, 1990; Doherty *et al.*, 1990).

V Insulin Receptor Kinase Activation and Phosphorylation

In the absence of ligand binding, the extracellular domain of the insulin receptor has a strong inhibitory effect on kinase activity (rev. Taylor *et al.*, 1992). Insulin binding removes this inhibitory influence and the kinase is activated, probably as the result of conformational changes which are transmitted to the intracellular domain. To become activated, however, the receptor must be in its $\alpha_2\beta_2$ form. Although insulin can bind to the $\alpha\beta$ form, binding does not activate the tyrosine kinase of this form of the receptor. Thus, receptor kinase activation as well as tyrosine autophosphorylation appears to occur through a *trans*-mechanism in which insulin binding to one $\alpha\beta$ dimer results in the activation and phosphorylation of the adjacent, covalently bound β subunit (Lee *et al.*, 1993).

The insulin receptor cytosolic tail is poorly homologous to other RTK C-termini, indicating the presence of insulin receptor specific sequences. Upon ligand binding and receptor tyrosine kinase activation, the receptor undergoes autophosphorylation. There are three domains in the β subunit containing autophosphorylation sites; the juxtamembrane region (Y^{972}), the regulatory region within the kinase domain (Y^{1158}, Y^{1162}, Y^{1163}), and the C-terminus (Y^{1328}, and Y^{1334}) (White and Kahn, 1994). These major sites of receptor autophosphorylation have been shown to become phosphorylated in response to insulin in rat hepatoma cells (Tornqvist *et al.*, 1988). However, the exact sites which are phosphorylated at any time during the receptors' transit from the plasma membrane to the endosome, and possibly back to the plasma membrane, remains to be clarified. Indeed, partial dephosphorylation of the insulin receptor in the endosome has been observed (Burgess *et al.*, 1992; Faure *et al.*, 1992). This information would be useful in determining the role of individual tyrosine residues in regulating receptor internalization, trafficking and signaling.

Insulin receptor activation correlates temporally with phosphorylation of the residues in the kinase domain, rather than those in the C-terminus. Autophosphorylation of all three tyrosine residues in the regulatory region stimulates kinase activity 10-20 fold (White *et al.*, 1988). Mutant insulin receptors, which contained phenylalanine instead of Y^{1162} or both Y^{1162} and Y^{1163}, transfected into CHO cells exhibited decreased kinase activity (Ellis *et al.*, 1986). In many cell types, the regulatory region is only bisphosphorylated, possibly limiting the amplitude of the insulin response, however, in rat hepatocytes, trisphosphorylation predominates suggesting a tissue specific mechanism for up-regulation of the insulin response (Issad *et al.*, 1991). Upon phosphorylation, a

secondary conformational change is believed to occur which makes the receptor capable of substrate phosphorylation. In fact, once autophosphorylated, the insulin receptor has been shown to remain active even in the absence of ligand.

The two tyrosine residues (Y^{1328}, and Y^{1334}) in the C-terminus undergo rapid ligand induced autophosphorylation. Deletions of the C-terminus which remove these two tyrosine phosphorylation sites have no effect on the insulin stimulated autophosphorylation in other regions, receptor kinase activity or endocytosis (Myers *et al.*, 1991; Thies *et al.*, 1989). However, cells expressing these truncated receptors were inefficient in stimulating glucose transport and glycogen synthase, but exhibited increased mitogenic activity in response to insulin. This suggests that the C-terminus may be involved in metabolic signaling and as an inhibitory regulator of mitogenic signaling. Mutation of these two tyrosine residues to phenylalanine, results in normal metabolic effects but leads to an increase transmission of the mitogenic signal. This suggests that the C-terminus tyrosine residues play no role in metabolic signaling but normally exert an inhibitory effect on the mitogenic properties of the insulin receptor (Takata *et al.* , 1991). Thus it appears that different regions of the insulin receptor C-terminus regulate different biological effects.

The juxtamembrane region of the insulin receptor β-subunit, encoded by exon 16, is essential for signal transmission. This region contains Y^{972}, which resides in an NPXY motif. Replacement of this tyrosine with phenylalanine results in impaired signaling despite the fact that autophosphorylation of the other regions is normal and the kinase activates fully *in vitro* (White *et al.*, 1988). This mutation impaired the ability of the receptor to phosphorylate an endogenous 185 kDa protein substrate (White and Kahn, 1994), suggesting that phosphorylation of this tyrosine residue is involved in insulin receptor substrate recognition.

VI Internalization, Phosphorylation and Receptor Kinase Activity

Insulin administration *in vivo* results in the tyrosine phosphorylation of the insulin receptor and the rapid endocytosis of the receptor-ligand complex into hepatic endosomes. The level of phosphotyrosine per β subunit of the insulin receptor at the plasma membrane was shown to exceed that in the endosome (Khan *et al.*, 1989; Burgess *et al.*, 1992), although the specific tyrosine residues which are dephosphorylated during internalization have not been identified. However, examination of the receptor kinase activity revealed a higher kinase activity in the endosomal insulin receptor, with respect to both autophosphorylation (Khan *et al.*, 1986) and exogenous substrate phosphorylation. These data are consistent with a selective dephosphorylation of inhibitory sites by an endosomal

receptor tyrosine phosphatase (Faure *et al.*, 1992), in a manner analogous to the regulation of the src kinase family members. In addition, the endosomal kinase appeared to have a lower K_m for synthetic substrates, suggesting that it may be a more efficient enzyme for potential cytosolic substrates present at low concentrations.

The insulin receptors at the PM and in endosomes displayed different patterns of phosphorylation, the endosomal receptor containing only phosphotyrosine, the plasma membrane receptor containing both phosphotyrosine and phosphoserine residues (Khan *et al.*, 1989). Studies by Pang *et al.* (1985), using intact hepatoma cells demonstrated that insulin induced tyrosine phosphorylation of the insulin receptor reached maximal levels within 2 min of insulin administration. Serine phosphorylation of the insulin receptor, however, required 10 min to attain maximal levels. Serine phosphorylation has been shown to decrease insulin stimulated tyrosine kinase activity, as well as reducing the affinity of the receptor for insulin. Thus, the presence of insulin induced serine phosphorylation of the receptor at the PM may be a way of preventing or attenuating signaling from this site, subsequent to receptor internalization. The enzymes that phosphorylate the insulin receptors on ser/thr residues are unclear, although protein kinase C (PKC) and a specific insulin receptor serine kinase have been implicated in this process (rev. Knutson, 1991).

VII Protein Domains Involved in Signal Transduction

The Pleckstrin Homology Domain

Pleckstrin homology (PH) domains were originally recognized as a repeat in the protein pleckstrin and later found in various signaling and cytoskeletal proteins, including several phospholipase C (PLC) isoforms, growth factor receptor binding protein 7 (GRB7), Ras GTPase-activating protein (GAP), insulin receptor substrate 1 (IRS-1), and the guanine nucleotide exchange factor son-of-sevenless (SOS) (rev. Musacchio *et al.*, 1993). Although the sequence (~100 amino acid) which comprises PH domains is poorly conserved, a common structure is formed composed of a seven stranded β-barrel and an α helix at the C-terminus. This domain is believed to be involved in protein-protein and/or protein-lipid interactions. Several PH domains have been shown to bind to the βγ-complexes of heterotrimeric G proteins (Touhara *et al.*, 1994). In addition, the PH domain has been shown to bind phosphatidylinositol-4,5-bisphosphate, primarily through ionic interactions (Hyvönen *et al.*, 1995), a mechanism which may mediate the membrane localization of these proteins, many of which are found to be membrane associated.

The SH2 and SH3 Domains

Src homology 2 domains (SH2) were first recognized as a region of homology between the c-fps and c-src gene products (Sadowski *et al.*, 1986). These noncatalytic regions, present in a wide variety of signaling proteins, are involved in mediating protein-protein interactions. SH2 domains consist of approximately 100 amino acid residues which bind to phosphorylated tyrosine residues (Mayer and Baltimore, 1993). Specificity in SH2 recognition of phosphotyrosines was realized from the observation that not all tyrosine phosphorylated receptors bound the same subset of SH2 containing molecules. It has been determined that the specificity of SH2 recognition is carried by the amino acids on the carboxyl side of the phosphotyrosine (Marengere *et al.*, 1994, Songyang and Cantley, 1995).

SH3 domains are small protein domains (55-70 amino acids) that are found in a number of proteins that associate with and regulate the cytoskeleton and in signal-transduction proteins (rev. Pawson and Gish, 1992). SH3 binding sites appear to be proline rich motifs of approximately 10 amino acids. Two classes of binding sites can be defined; class I sites, arg-X-X-pro-X-X-pro, (X - proline or hydrophobic residue) and class II sites, whose consensus sequence appears in the opposite orientation (Mayer and Eck, 1995). These two classes may confer specificity to the SH3 - proline region interaction. There is also evidence for a role for SH3 domains in directing the cellular localization of signaling molecules (Bar-Sagi *et al.*, 1993).

It is possible to divide proteins bearing these motifs into two main classes. Type I defines proteins that, in addition to SH2 and/or SH3 domains, contain enzymatic activities. These include PLC-γ, pp60$^{c\text{-}src}$, Ras-GAP and SOS (Carpenter, 1992). These proteins are believed to become activated upon phosphorylation and/or by virtue of their association with neighboring proteins. The second class of src homology domain containing proteins includes those which are composed almost entirely of SH2 and SH3 domains and have no apparent catalytic function. These include GRB2, SHC, IRS-1 and the p85 subunit of phosphoinositol (PI) 3'-kinase. These proteins function as adaptors, mediating the association between cellular proteins, or as regulatory subunits of enzymes, effectively linking activated receptors to downstream signaling molecules (rev. Mayer and Baltimore, 1993).

The PTB Domain

Among the several domains identified involved in protein-protein interactions, the phosphotyrosine binding (PTB) domain has been identified most recently (Kavanaugh *et*

al., 1995). This domain is present in several signal transduction molecules. As the name suggests, the PTB domain binds specifically to phosphotyrosine residues (van der Geer and Pawson, 1995). Unlike the SH2 domain, the specificity of PTB binding is conferred by the amino acids which are located N-terminal to the phosphorylated tyrosine (Kavanaugh *et al.*, 1995). For example, it has been shown that four to six residues N-terminal to the tyrosine (especially those at position -5, and -1) are necessary for the high affinity binding of the SHC PTB domain (Trüb *et al.*, 1995).

VIII The IRS-1 Signal Transduction Pathway

Upon intravenous infusion of insulin into the rat, the most prominent tyrosine phosphorylated protein, in adipose tissue, muscle and liver, is the insulin receptor substrate, IRS-1. IRS-1 is a minor cytoplasmic protein which was purified and cloned from rat liver and found to be highly conserved and expressed in most cell types and tissues (Rothenberg *et al.*, 1991, Sun *et al.*, 1991). IRS-1 protein levels appear to be differentially regulated in target tissues such as muscle and liver (Saad *et al.*, 1992). Gene knock out experiments have concluded that there exists both IRS-1 dependent and IRS-1 independent signal transduction pathways leading from the insulin receptor. In the livers of IRS-$1^{-/-}$ animals, the presence of a tyrosine phosphorylated protein can be found in anti-PI3'-kinase immunoprecipitates after insulin stimulation. This protein was found to be related to IRS-1 and so termed IRS-2 (Araki *et al.*, 1994). Most tissues appear to express both IRS-1 and IRS-2, but the ratio of these two proteins differs depending on the cell type, which may be involved in the fine tuning of signaling from the insulin receptor.

The predicted open reading frame in the cDNA of IRS-1 encodes a 131 kDa protein, however IRS-1 migrates between 165-170 kDa in resting cells and 175-185 kDa in stimulated cells (rev. White and Kahn, 1994), probably due to secondary structure and the increase in ligand-induced tyrosine phosphorylation, respectively. Although there is no transmembrane spanning region, based on primary sequence analysis, the N-terminus (residues 13-115) of IRS-1 contains a conserved PH domain. This domain, also called the IRS homology domain 1 (IH1), may function to target IRS-1 to the membrane compartment where it is in proximity of activated receptor tyrosine kinases (Myers *et al.*, 1994). The IRS-1 PH is 62% identical to the PH domain of IRS-2, a difference which may serve to target the IRS isoforms to different subcellular locations. Evidence suggests that the PH domain of IRS-1 is not required for the engagement of downstream signals but rather is one of the elements that mediates the efficient coupling with the insulin receptor.

Indeed, insulin stimulated IRS-1 phosphorylation is greatly reduced if the PH domain is deleted (Myers *et al.*, 1995).

IRS-1 contains a region immediately downstream from the PH domain which has also been implicated in the interaction between the insulin receptor and IRS-1. This region, called IH2, resembles a PTB domain (Sun *et al.*, 1995), and may bind the $NPXY^{972}$ motif found in the juxtamembrane region of the activated insulin receptor (O'Neill *et al.*, 1994). The phosphorylation of IRS-1 appears to require the phosphorylation of this tyrosine residue (Kaburagi *et al.*, 1993), as receptors which contain substitutions or deletions of the $NPXY^{972}$ motif are unable to mediate the phosphorylation of IRS-1(O'Neill *et al.*, 1994), the activation of PI3'-kinase or glucose transporter translocation in transfected CHO cells (White *et al.*, 1988). However, while a small proportion of IRS-1 can be shown to be in association with activated insulin receptor in CHO cells transfected with both insulin receptors and IRS-1, and a direct interaction between the N-terminus of IRS-1 and phosphorylated Y^{972} has been demonstrated using the yeast two hybrid system (O'Neill *et al.*, 1994), this has not been demonstrated in non transfected cells. In these cells, IRS-1 is expected to bind to the catalytic domain of the receptor, forming an enzyme / substrate complex, but this is expected to be transient and unstable and the dissociation of the phosphorylated IRS-1 should be favored. Tyrosine phosphorylation of $NPXY^{972}$ may be important for substrate selection since receptors which lack this motif do not phosphorylate IRS-1. Thus the high efficiency and specificity of interaction between the insulin receptor and IRS-1 requires both membrane localization of IRS-1 and IRS-1 recognition sites on the receptor.

IRS-1 does not possess catalytic activity but instead acts as an adaptor protein. It is known that IRS-1 contains at least 21 potential tyrosine phosphorylation sites, some of which are phosphorylated in response to insulin stimulation. Six of these are located in YMXM motifs, which are known to interact with the SH2 domains of various signal transduction molecules such as PI3'-kinase, GRB2, and the tyrosine phosphatase, SHPTP (Pawson and Gish, 1992). In this way, IRS-1 may act to bring proteins in proximity to the insulin receptor which can then phosphorylate and activate them or to cause, through induction of conformational changes, the activation of these proteins.

PI3'-Kinase

PI3'-kinase is a heterodimeric enzyme composed of a p85 regulatory subunit, containing one SH3 and two SH2 domains, and a p110 subunit which is a dual specificity kinase with both lipid and serine kinase activity (Panayotou *et al.*, 1992; Lam *et al.*, 1994).

Insulin injection in mice resulted in the ~6 fold increase in IRS-1 associated PI3'-kinase activity in the liver (Araki *et al.*, 1994). The p85 subunit does not appear to be phosphorylated in response to insulin stimulation in many cultured cells (Kelly and Ruderman, 1993; Backer *et al.*, 1992). The two SH2 domains of the p85 subunit bind strongly to phosphorylated Y^{939} and Y^{608} (both in YMXM motifs) of IRS-1, in response to insulin stimulation (Myers *et al.*, 1994; Backer *et al.*, 1992). Upon binding to phosphotyrosine containing peptides, the p85 subunit of PI3'-kinase undergoes a conformational change and activates the p110 subunit (Panayotou *et al.*, 1992). The association with IRS-1, which accounts for as much as 70% of total cellular PI3'-kinase in stimulated cells (Backer *et al.*, 1992) may serve to recruit PI3'-kinase to the plasma membrane which may be important for the interaction with lipid substrates. The role of 3'-phosphorylated phosphoinositols generated by PI3'-kinase activation remains to be shown although several lines of evidence suggest a role for PI3'-kinase in the regulation of intracellular trafficking of proteins (Stephens, 1995; Shepherd *et al.*, 1996).

PI3'-kinase has been proposed to function upstream of Ras and Raf-1 in mediating the insulin signal transduction pathway leading to the transcriptional activation of the c-fos serum response element, in CHO-IR cells (Yamauchi *et al.*, 1993). It is not clear how this interaction results in the activation of Ras, but suggests an alternative to GRB2/SOS interactions with Ras as a mechanism for Ras activation. On the other hand, Rodriguez-Viciana *et al.* (1994) propose that Ras can regulate PI3'-kinase activity. The p110 subunit of PI3'-kinase was shown to co-immunoprecipitate with Ras. This interaction appeared to involve the effector region of Ras and was highest when Ras was bound to GTP.

While the exact role of PI3'-kinase in insulin signaling remains unclear, the importance of PI3'-kinase in insulin action has been demonstrated. Wortmannin, a specific inhibitor of PI3'-kinase (both lipid and serine kinase activities) blocks the antilypolytic action of insulin on adipocytes (Okada *et al.*, 1994), the insulin induced glucose uptake and glycogen synthesis, insulin induced inhibition of glycogen synthase kinase-3 (GSK-3) activity (Quon *et al.*, 1995; Shepherd *et al.*, 1995; Yamamoto-Honda *et al.*, 1995), as well as insulin-induced inhibition of PEPCK gene expression (Sutherland *et al.*, 1995).

IRS-1 in Mitogenic Signaling

Although IRS-1 overexpression has been reported in hepatocellular carcinoma and its expression is important during liver regeneration (Ito *et al.*, 1996), most of the studies addressing the potential role of IRS-1 in mitogenic responses to insulin have been carried out in cell lines overexpressing the insulin receptor. Microinjection of anti-IRS-1 antibodies inhibited insulin and IGF-1 stimulated DNA synthesis and cell growth in HIRcB

fibroblasts. This implicated IRS-1 in the mediation of mitogenic signaling from the insulin receptor (Rose et al, 1994; Myers *et al.*, 1994) and was postulated to occur as a result of the association of IRS-1 with GRB2 linking SOS (Ras guanylnucleotide exchange factor) and the Ras pathway to IRS-1. However, insulin stimulated association of SOS or SOS activity with IRS-1 could not be detected in fibroblasts expressing high numbers of insulin receptors (Pronk *et al.*, 1994; Sasaoka *et al.*, 1994a), which suggested that the association of SOS with IRS-1 did not contribute significantly to the activation of the Ras-mitogen activated protein kinase (MAPK) pathway in response to insulin. The level of expression of IRS-1 was shown to activate or inhibit insulin signaling depending upon the cell context. In CHO cells expressing high levels of insulin receptors, increased expression of IRS-1 resulted in the attenuation of insulin stimulated DNA synthesis and c-fos gene transcription (Yamauchi *et al.*, 1994a). Thus the role of IRS-1 in insulin induced mitogenic signaling remains unclear, although the specific concentrations of both the insulin receptor and IRS-1 as well as the cell type appear to be involved in determining downstream signaling.

Serine/Threonine Phosphorylation of IRS-1

IRS-1 becomes phosphorylated on additional serine and threonine residues after insulin stimulation (Tanti *et al.*, 1994). There are over thirty potential ser/thr phosphorylation sites in IRS-1. Several kinases, including MAPK, casein kinase II (CKII) (Tanasijevic *et al.*, 1993) and PI3'-kinase (Lam *et al.*, 1994), have been proposed to phosphorylate serine and/or threonine residues in IRS-1 in an insulin dependent manner. The phosphorylation of these residues may be involved in the regulation of signal transduction. Indeed, it has been shown that the ser/thr phosphorylation of IRS-1 by okadaic acid caused a decrease in the ability of the insulin receptor to phosphorylate IRS-1 and a reduction in the ability of proteins such as PI3'-kinase to dock to IRS-1 (Tanti *et al.*, 1994). This may also be the result when MAPK phosphorylates IRS-1 on residues located near those which are known to recruit the SH2 domains of PI3'-kinase. Thus, the regulation of signal transduction by ser/thr phosphorylation of IRS-1 may be twofold; interfering with the binding of downstream effectors to IRS-1 and/or as a negative feedback mechanism which ensures a limited duration of signal transduction. The precise nature of the regulation would be dependent on the subset of kinases which are activated by insulin treatment in a given cell type.

The large number of insulin receptors expressed in liver parenchyma is indicative of the important role insulin plays in this organ, as a regulator of metabolism and potentially, mitogenesis. The hepatocyte of the male rat possesses an equally large number of EGF receptors as insulin receptors. Activation of EGF receptors, in many cell types, is often associated with a mitogenic response.

The Biological Actions of EGF in Hepatocytes

EGF has been demonstrated to be a primary mitogen for hepatocytes and hepatoma cells in culture, stimulating limited hepatocyte DNA synthesis (Michalopoulos, 1990) and ornithine decarboxylase activity in hepatoma cells (Moriarity *et al.*, 1981; Bucher *et al.*, 1977). EGF induced glucose (GLUT-1) transporter mRNA expression in primary hepatocyte cultures (Mischoulon *et al.*, 1992). These effects have all been implicated in EGF induced cell proliferation.

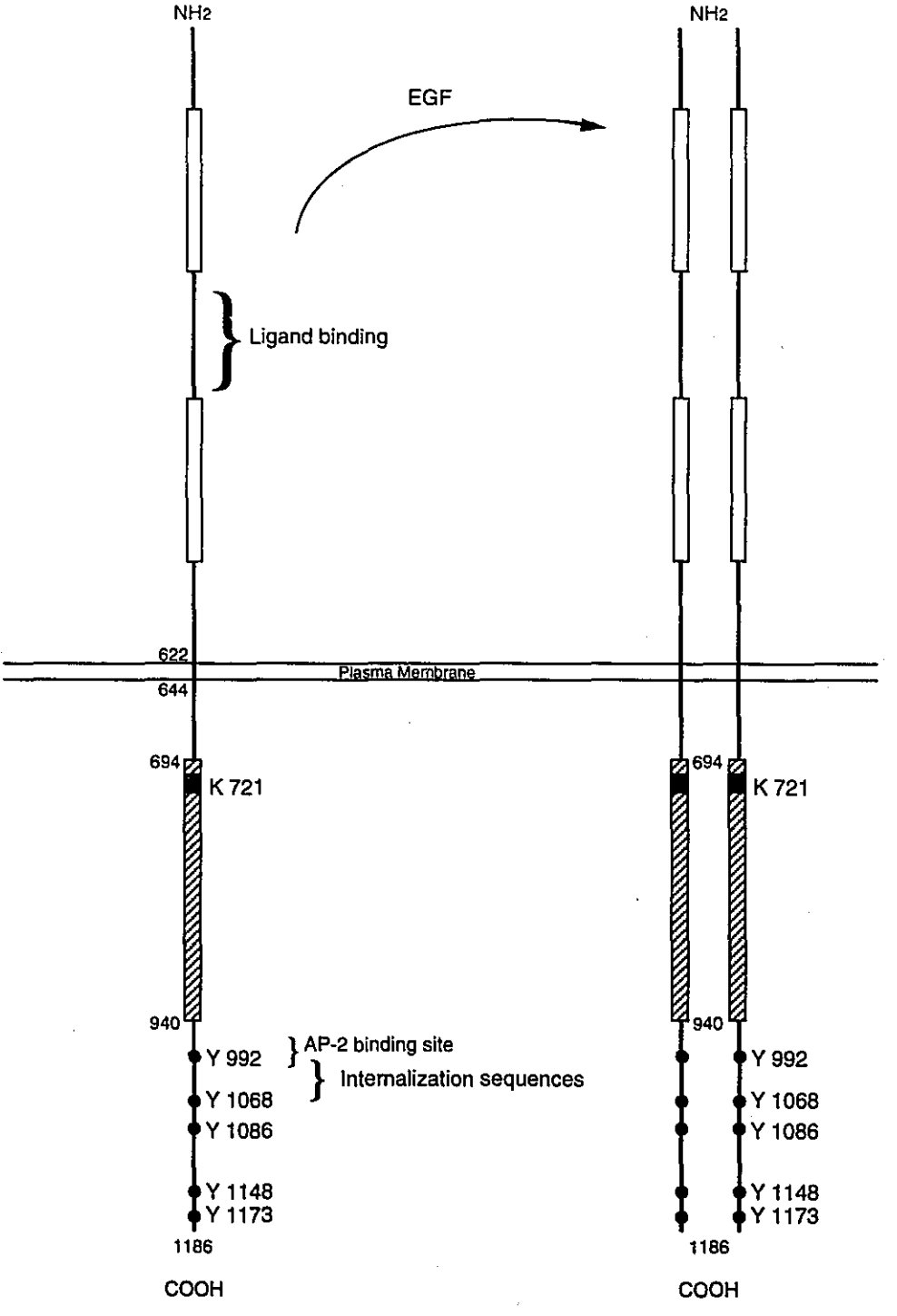
EGF has been implicated as a hepatotrophic factor during liver regeneration. Indeed, circulating levels of EGF, transforming growth factor (TGF) -α, hepatocyte growth factor (HGF) increase dramatically after partial hepatectomy, a procedure which results in large scale hepatocyte proliferation (rev. Diehl and Rai, 1995; Steer, 1995). These are strong inducers of DNA synthesis in cultured hepatocytes. The EGF receptor may be involved in liver regeneration and development through the agency of the EGF-like molecule TGF-α. TGF-α has 35% homology to EGF and binds the same receptor. EGF and TGF-α generally exert the same effects on cell proliferation and function in a variety of cell types. However, TGF-α is often a more potent agonist than EGF, for example TGF-α is a more potent promoter of hepatocyte growth than EGF . TGF-α exerts its effects via an autocrine loop. TGF-α synthesis is stimulated in liver cells in culture as well as *in vivo* by the activation of the EGF receptor by TGF-α itself or by EGF (rev. Fausto *et al.*, 1995). TGF-α is expressed at high levels in early postnatal life (up to week 1) and is associated with hepatocyte proliferation during this time. The decline in replication, which occurs at the same time that the liver reaches adult levels in the liver weight / body weight ratio is accompanied by an abrupt decline in TGF-α (rev. Fausto *et al.*, 1995). This observation suggests that environmental cues provide a mechanism which inhibit signal transduction from the activated EGF receptor and shut down TGF-α synthesis.

There is some data which suggests that EGF can act as a metabolic regulator of hepatocyte function. In both perfused livers and isolated hepatocytes from fasted rats, EGF has been shown to cause a rapid but transient increase in gluconeogenesis (Soler and Soley, 1993). In hepatocytes derived from fed rats, however, EGF, like insulin,

Figure 2

Schematic representation of the EGF receptor

The EGF receptor is a glycosylated, transmembrane protein with an apparent mobility on SDS-PAGE corresponding to 170 kDa. This receptor, present as monomers at the cell surface, dimerizes upon ligand binding, which leads to the activation of the intrinsic tyrosine kinase activity and the trans-phosphorylation of specific tyrosine residues in the cytosolic tail of the receptor. The ligand binding region (amino acids 321-367) and the location of the adaptor protein (AP-2) binding site and the internalization sequences are labeled. The sites of tyrosine phosphorylation (●) and ATP binding (■) and the intrinsic tyrosine kinase domain of the receptor (▨) are indicated, as are the cysteine rich domains (▭), and the amino acid residues corresponding to the receptor transmembrane domain.


NH2
NH2
EGF
Ligand binding
622
644
Plasma Membrane
694
K 721
940
Y 992
AP-2 binding site
Internalization sequences
Y 1068
Y 1086
Y 1148
Y 1173
1186
COOH
694
K 721
940
Y 992
Y 1068
Y 1086
Y 1148
Y 1173
1186
COOH

stimulated glycogen synthase activity and glycogen synthesis (Bosch *et al.*, 1986). Other studies suggest that EGF counteracts the glycogenic effects of insulin (Peak and Agius, 1994). Furthermore, EGF alone inhibited glycogen deposition and stimulated glycolysis in isolated hepatocytes (Quintana *et al.*, 1995). The precise role of EGF in the regulation of metabolism *in vivo* remains unclear, but these data suggest that the metabolic actions of EGF are dependent on precise physiological conditions in which the hepatocytes find themselves.

The EGF Receptor

Hepatocytes take up more than 90% of an injected dose of iodinated-EGF in the first pass though the liver (St. Hilaire *et al.*, 1983). This is carried out by high affinity, specific binding sites for EGF which have been demonstrated on isolated rat liver membranes (Cohen and Savage, 1974), isolated liver parenchymal cells (Moriarity and Savage, 1980), as well as by binding studies (O'Keefe *et al.*, 1974). Approximately 1.5 x 10^5 EGF receptors are expressed per hepatocyte.

The EGF receptor, 170 kDa glycoprotein, is composed of a single subunit which is targeted to the plasma membrane. The extracellular domain of receptor tyrosine kinases contains the most distinct feature of the receptor and is composed of combinations of sequence motifs. The EGF receptor can bind EGF and related molecules such as TGF-α. The affinity constants of TGF-α, and EGF are very similar or identical (Ebner and Derynk, 1991). The ligand binding domain for EGF and TGF-α maps to a region located between residues 321-367 of the receptor, however, it appears that the binding sites for these ligands do not perfectly overlap (Winkler *et al.*, 1989). This may be responsible for the apparent differences in biological responses in response to these ligands. The ligand binding domain is flanked by two cysteine-rich domains (Lax *et al.*, 1988) whose function has been proposed to maintain receptor structural integrity rather than being involved in ligand recognition.

I EGF Receptor Internalization

In untreated livers, the EGF receptor has been localized exclusively to the sinusoidal and lateral surfaces of the plasma membrane (Dunn *et al.*, 1986; Lai *et al.*, 1989b). Upon ligand binding, the receptor is redistributed to coated pits and rapidly endocytosed. Chen *et al.* (1989) suggested that a region (residues 1022-1186) in the C-terminus of the unoccupied EGF receptor masks the internalization sequences. Upon

receptor activation and autophosphorylation of the tyrosine residues in this inhibitory domain, a conformational change results which releases this inhibition, and permits endocytosis. Tyrosine kinase activity is required for ligand induced EGF receptor internalization (Glenney *et al.*, 1988), and results in an internalization rate >5 fold higher than that of the kinase negative receptor (Chen *et al.*, 1989).

The internalization sequences required for ligand induced endocytosis and down-regulation of the EGF receptor were mapped to residues 973-1022. This region, conferring both calcium regulation and receptor endocytosis, was called the CaIn domain (Chen et al, 1989). Further analysis revealed that these two EGF dependent functions were in fact mediated by distinct regions in the CaIn domain (Chang *et al.*, 1991). The endocytic motifs of the EGF receptor (^{996}QQGFF and ^{973}FYRAL) were predicted to display features of a tight turn, a structure present in insulin receptor internalization codes as well (Chang *et al.*, 1993). The three NPXY sequences in the EGF receptor tail, unlike those in the insulin receptor, do not appear to be essential for internalization, since their deletion does not impair this process (Chang *et al.*, 1993).

The specificity found in sequences required for internalization suggests that the mechanisms of internalization (as well as intracellular trafficking) are controlled by receptor specific endocytosis molecules. The adaptor protein 2 (AP-2) complex, identified because of its ability to induce the formation of clathrin coats at the PM (reviewed in Pearse and Robinson, 1990), has been implicated in this role for the EGF receptor.

AP-2

Entrapment of the EGF receptor into coated pits is postulated to occur because an AP-2 complex, already localized at the PM, recognizes and associates with sorting signals on activated EGF receptors (Boll *et al.*, 1995). This interaction would cause an increase in the affinity of AP-2 for clathrin and result in the rapid assembly of the clathrin coated pits (Pearse and Robinson, 1990).

The EGF receptor appeared to be capable of recruiting AP-2, in an EGF dependent manner, without receptor activation or autophosphorylation. However, this interaction was found to be 6-8 fold less than in the wild-type receptor (Sorkin and Carpenter, 1993; Boll *et al.*, 1995). This correlates with the reported ability of inactive EGF receptor to internalize and/or down regulate in a ligand dependent manner, but at decreased rates compared with wild-type receptors. Although AP-2 lacks the SH2 domain needed to interact directly with phosphorylated tyrosine residues, receptor autophosphorylation appears to be required for high levels of AP-2 binding (Nesterov *et al.*, 1995a). Phosphorylation may be involved in causing a conformational change in the receptor tail

leading to the exposure of AP-2 binding motifs. These motifs have been mapped to residues 970-991, in the regulatory C-terminus of the EGF receptor (Boll *et al.* 1995; Sorkin *et al.*, 1995; Nesterov *et al.*, 1995a), located within a larger region known to be involved in endocytosis of the receptor (see above). The identified AP-2 binding domain is not conserved in the insulin or transferrin receptors. Deletion of this AP-2 binding determinant, abolishes complex formation, however, this mutant receptor is indistinguishable from the wild type receptor in internalization and down regulation kinetics (Nesterov *et al.*, 1995b). This suggests that processes other than AP-2 binding regulate receptor endocytosis.

There is evidence for a role for AP-2 later in the endocytic process. The presence of a significant population of endocytic membranes and vesicles coated with adaptors but devoid of clathrin, has been demonstrated by double immunofluorescence microscopy and biochemical analyses (Guagliardi *et al.* 1990; Chakrabarti *et al.*, 1993). A specific role for AP-2 in the fusion of early endosomes has been proposed (Beck *et al.* 1992). In addition, microinjection of anti-AP-2 antibodies limits the accumulation of internalized ligand to late perinuclear compartments (Chin *et al.*, 1989). Since perinuclear localization is the culmination of a sequential transport of internalized receptors through multiple compartments (early to late endosomes) this observation implies a possible role for AP-2 at a number of steps in the endocytic pathway.

Fate of Internalized EGF and EGF Receptor

The initial step in internalization after the binding of ligand is the clustering of receptor into coated pits which, with the aid of the GTPase dynamin, pinch off to form coated vesicles. Soon after their formation, these vesicles loose their clathrin coats and undergo a series of fusion events to form the early endosomal compartment. It is in this compartment that the receptors are sorted and their fate determined. Whether an internalized receptor is degraded or recycled has implications on the magnitude and duration of signaling. EGF does not dissociate from its receptor in the endosomal compartment, but it does undergo partial proteolysis as it traffics from early endosomes to the lysosome (Renfrew and Hubbard, 1991a). If this plays a role in the modulation of the EGF receptor activity remains to be determined. It is generally believed that the vast majority of EGF receptor undergoes degradation in the lysosomal compartment (Renfrew and Hubbard, 1991b). However, Lai *et al.* (1989a) demonstrated that EGF receptor down regulation (degradation) in rat liver fractions, occurred only when >50% of the receptors were occupied. At lower receptor occupancy levels, the receptor was internalized and was subsequently recycled to the PM. These observations imply that intracellular trafficking is

a function of intracellular ligand levels. This hypothesis is supported by the studies on TGF-α induced EGF receptor internalization.

Although both EGF and TGF-α follow similar kinetics of internalization, TGF-α dissociates from the EGF receptor at a higher pH (half maximal dissociation at pH 6.9) (Ebner and Derynk, 1991). This suggests that during endocytosis and the acidification of the endosome, TGF-α dissociates from its receptor in an early endosome, while EGF dissociates only in the lysosome or a prelysosomal compartment.. At physiological concentrations, more TGF-α than EGF recycles back to the PM, and fewer EGF receptors are degraded, resulting in a stronger cell stimulus, and a shorter lag before restimulation can take place (Ebner and Derynk, 1991).

Evidence indicates that endosomal sorting is controlled by tyrosine kinase activity (Honegger *et al.*, 1987; Felder et al, 1990). French *et al.* (1994) have concluded that kinase activity was not crucial for lysosomal targeting although it enhanced downregulation, possibly by stabilizing interactions with the endocytic apparatus (see AP-2; Herbst *et al.*, 1994). A model was proposed, for postendocytic compartmentation, where lysosomal targeting of the EGF receptor is mediated by specific and saturable components that interact with the cytoplasmic tail of the occupied receptor (French *et al.*, 1994) .

II Ligand Binding and Receptor Dimerization

All known growth factor receptors appear to undergo receptor dimerization upon ligand binding (rev. Schlessinger and Ullrich, 1992). This phenomenon was first demonstrated for the EGF receptor where dimerization was shown to be a crucial early event in response to EGF stimulation; dimerization is required for kinase activation. Dimerization is presumed to occur due to conformational changes in the extracellular domain (Greenfield *et al.*, 1989), which stabilize the interactions between two occupied receptor molecules (Lax *et al.*, 1991; Hurwitz *et al.*, 1991). The precise regions involved in stabilizing this conformation remain to be identified. Two properties of the EGF receptor essential to its function have been attributed to the dimeric form of the receptor, including the presence of high affinity EGF binding sites and the activation of the tyrosine kinase activity (Sorokin *et al.*, 1994).

III Kinase Activation and Receptor Phosphorylation

The ligand induced receptor dimerization results in the proximity of the two cytosolic tails and a subsequent conformational change. This conformational change is

believed to result in the stimulation of the catalytic activity and the consequent transphosphorylation of the receptor molecules (Schlessinger and Ullrich, 1992; Cadena *et al.*, 1994). All five autophosphorylation sites in the EGF receptor are located in the C-terminus of the receptor (Margolis *et al.*, 1989; 1990; Walton *et al.*, 1990), unlike the insulin receptor which contains three regulatory tyrosine residues in the kinase domain.

The importance of the autophosphorylation sites, in the regulation of EGF receptor signaling, is demonstrated by the fact that C-terminal deletions of the EGF receptor result in enhanced transforming ability of the receptor (Yarden and Ullrich, 1988, Khazaie *et al.*, 1988). Further studies on receptors with deletions or point mutations in autophosphorylation sites demonstrated that while the mutant receptors exhibited normal V_{max}, they demonstrated a lower K_m for the phosphorylation of exogenous substrate (Honegger *et al.*, 1988). It has been proposed that autophosphorylation sites serve to fine-tune kinase activity by acting as competitive inhibitors for exogenous substrate phosphorylation (Honegger *et al.*, 1988). The autophosphorylation sites are first to be phosphorylated upon kinase activation due to the proximity of these sites in adjacent dimerized receptors. By competing with exogenous substrates for binding to the substrate binding region of the kinase, the autophosphorylation sites prevent the phosphorylation of substrates with high K_m, and thus further modulate signal transduction.

The major *in vitro* sites of EGF receptor autophosphorylation were identified as Y^{1068}, Y^{1148} and Y^{1173} (Downward *et al.*, 1984). In vivo, however, Y^{1173} is the site preferentially phosphorylated, while Y^{1148} and Y^{1068} are phosphorylated to a lesser extent. Two additional minor sites at Y^{992} and Y^{1086} were later identified in the EGF receptor (Margolis *et al.*, 1989; Walton *et al.*, 1990). The extent of phosphorylation of these sites *in vivo* i.e. in a physiological context, is not clear but differences may account for tissues -specific variations in the signal transduction pathways initiated.

IV Internalization, Phosphorylation and Kinase Activity

Internalization reduces the number of receptors at the cell surface and relocates the receptor to a different signaling environment. Morphological and biochemical evidence demonstrates that the EGF receptor remains associated with its ligand and is capable of dimerization within the endosome (Carpentier *et al.*, 1987; Nesterov *et al.*, 1990; Lai *et al.*, 1989a; Sorkin *et al.*, 1991; Sorkin and Carpenter, 1991), suggesting that the receptor kinase remains active in this compartment. McCune and Earp (1989) showed, in WB 344 rat liver epithelial cells, that peak receptor autophosphorylation activity was found at times when the majority of receptors had been internalized but not yet degraded. A greater extent

of EGF receptor phosphorylation in endosomes compared to that in the PM was also demonstrated in rat liver *in vivo* (Wada *et al.*, 1992). Limited proteolysis of isolated endosomes revealed that, in these structures, the EGF receptor kinase domain and phosphorylation sites were cytoplasmically oriented and thus remained accessible to signal transduction molecules (Lai *et al.*, 1989b; Renfrew and Hubbard, 1991b; Wada *et al.*, 1992). The presence of an active EGF receptor tyrosine kinase in the endosome of rat liver was demonstrated by Kay *et al.* (1986). These data imply that the internalized EGF receptor remains active and may continue to phosphorylate substrates and carry out signal transduction. Indeed, studies where the endocytosis of the EGF receptor was inhibited showed that internalization was required for the tyrosine phosphorylation of a specific subset of proteins (McCune and Earp, 1989). The ability of the EGF receptor localized within multivesicular endosomes to phosphorylate annexin I, an endogenous substrate, supports the presence of an active receptor, even late in the endocytic pathway (Futter *et al.*, 1993).

V Signal Transduction Molecules Involved in EGF Signaling

SHC

In 1992, Wada *et al.* and Donaldson and Cohen, identified a 55 kDa protein as the major tyrosine phosphorylated substrate for the EGF receptor in liver. Subsequently, Pelicci *et al.* (1992) characterized the mammalian gene which encoded this protein and two related proteins of 46 and 66 kDa. These proteins were named SHC based on several characteristic motifs; a C-terminal SH2 domain and an adjacent glycine/ proline rich region which has homology to the α1 chain of collagen. More recently, an N-terminal PTB domain has been identified which contributes to the specific protein-protein interactions of SHC (Trüb *et al.*, 1995; van der Geer *et al.*, 1995; Batzer *et al.*, 1995). The SHC proteins are highly conserved, ubiquitously expressed and are tyrosine phosphorylated in response to the activation of a wide variety of receptor tyrosine kinases (Ruff-Jamison *et al.*, 1993; Yokote *et al.*, 1994; Pronk *et al.*, 1993; Sasaoka *et al.*, 1994b).

SHC proteins do not possess catalytic activity, instead, they act as adaptor proteins. SHC is able to interact with several proteins via its SH2 domain which appears to bind preferentially to YMXM motifs. In addition, the SHC PTB domain can interact with EGF receptor NPXY motifs, of which there are several (i.e. NPVY^{1086} and NPDY^{1148})(van der Geer *et al.*, 1995). There is evidence, *in vitro*, that the PTB domain of SHC binds to the EGF receptor more strongly than the SHC SH2 domain. The binding of SHC to the EGF receptor seems to be mediated mainly by the interaction of the PTB domain with Y^{1148},

which may be further stabilized by the interaction of SHC SH2 domain with Y^{1173} (Batzer *et al.*, 1995). This may explain the high stoichiometry of association between SHC and the EGF receptor.

The direct interaction between SHC and the phosphorylated EGF receptor is proposed to result in the phosphorylation of SHC. The principle site of tyrosine phosphorylation in SHC (Y^{317}) is found within a Y(L/I/V)NV sequence which corresponds with the consensus GRB2 SH2 binding motif (Songyang *et al.*, 1993). SHC phosphorylation results in the association of SHC with GRB2 and SOS (a guanine nucleotide exchange factor) which implicates SHC in the Ras signaling pathway. However, the direct binding of SHC to the EGF receptor does not appear to be essential for SHC phosphorylation and association with GRB2, in an EGF dependent manner (Gotoh *et al.*, 1994).

SHC and Insulin Receptor Signaling

Although the insulin receptor contains an $NPEY^{972}$ motif in the juxtamembrane domain, the SHC PTB domain binds with low affinity to this motif. This may be due to the fact that the sequence surrounding this tyrosine does not contain a hydrophobic residue at the -5 position (Trüb *et al.*, 1995). Indeed, there is no evidence that SHC binds to the insulin receptor in living cells. In addition, SHC did not bind strongly to any potential IRS-1 phosphorylation sites (Sasaoka *et al.*, 1994a; Ward *et al.*, 1996).

Treatment of fibroblasts or CHO cells overexpressing the insulin receptor, resulted in the rapid tyrosine phosphorylation of SHC (Pronk *et al.*, 1993; Sasaoka *et al.*, 1994a). However, no association could be detected between the activated receptor and SHC. Microinjection of anti-SHC antibodies into Rat 1 fibroblasts (expressing EGF, insulin and IGF-1 receptors) reduced the ability of the three ligands to stimulate DNA synthesis (to roughly the same extent) leading to the conclusion that functional SHC is required for mitogenic signal transduction from these three receptors (Sasaoka *et al.*, 1994b). The high concentration of receptors in these cells may result in the reduced specificity and abnormal regulation of signaling from the insulin receptor.

The studies of Ohmichi *et al.* (1994a) were carried out in a Pheochromocytoma (PC)-12 cell line which expressed equal numbers of receptors for both EGF and insulin. In these cells activation of the EGF receptor resulted in the tyrosine phosphorylation of SHC and its association with GRB2, while insulin receptor activation did not, regardless of the insulin dose administered or the time of incubation. Furthermore, insulin administration was not effective in activating Ras and the MAPK cascade in these cells (Ohmichi *et al.*, 1994b). Thus, it appears that the ability of the insulin receptor to access

the MAPK pathway is cell type dependent and there exist mechanisms which serve to selectively uncouple the insulin receptor from this pathway.

GRB2/SOS

GRB2 is a 26 kDa adaptor protein, which consists entirely of an SH2 domain flanked by two SH3 domains. The SH2 domain binds tyrosine phosphorylated residues in a number of proteins, including the EGF receptor and SHC (Rozakis-Adcock *et al.*, 1992). The SH3 domains bind to the proline rich region in the C-terminus of the guanine nucleotide exchange factor, SOS (Egan *et al.*, 1993; Li *et al.*, 1993; Rozakis-Adcock *et al.*, 1993).

Mammalian cells contain two related but distinct SOS proteins (150 kDa), SOS1 and SOS2, which are widely expressed during development and in adult tissues (Bowtell *et al.*, 1992). A region of 117 amino acids in the C-terminus, which differs between SOS1 and SOS2, imparts an increase in apparent binding affinity of SOS2 towards GRB2, suggesting that the two SOS isoforms may contribute differently to receptor-mediated Ras activation (Yang *et al.*, 1995). The GRB2/ SOS complex is present in untreated as well as growth factor treated cells (Cussac et al, 1994). In addition, Buday and Downward (1993) observed an EGF dependent increase in the number of GRB2/SOS complexes.

GRB2 (associated with SOS) binds with directly and with high affinity to phosphorylated (P) Y^{1068}, to a lesser extent to PY^{1086} of the activated EGF receptor. The GRB2/SOS complex may also bind to PY^{1148} and PY^{1173} of the receptor indirectly, via the adaptor SHC (Batzer *et al.*, 1994; Okutani *et al.*, 1994). Studies by Cussac *et al.* (1994) reveal that the GRB2 SH2 domain has the highest affinity for the SHC tyrosine phosphorylated site, followed by EGF receptor sites. The SHC/GRB2/SOS complex that forms upon EGF stimulation has *in vitro* nucleotide exchange activity toward Ras, suggesting a similar complex formed *in vivo* can lead to Ras activation (Pronk *et al.*, 1994). Some studies, however, suggest the guanine nucleotide exchange activity is not measurably affected by growth factor stimulation, and binding of GRB2/SOS to phosphotyrosine residues (Buday and Downward, 1993; Cussac *et al.*, 1994). GRB2/SOS translocation seems to be the important function, since the targeting of SOS to the PM is sufficient to cause the activation of Ras (Aronheim *et al.*, 1994; Lemmon *et al.*, 1994; Buday and Downward, 1993). Indeed, SOS can be seen to translocate to the particulate fraction upon EGF stimulation (Buday and Downward, 1993).

EGF treatment of cells results in a mobility shift of SOS as seen by SDS-PAGE, presumably due to the increase in ser/thr phosphorylation levels (Burgering *et al.*, 1993). This phosphorylation occurs relatively late compared to the formation of the SHC/ GRB2/

SOS complex and Ras activation, which suggests that SOS phosphorylation is not involved in the initial activation of Ras. Instead, this modification may be a negative regulatory mechanism (Pronk *et al.*, 1994).

GRB2/SOS and Insulin Receptor Signaling

The putative GRB2 binding sites on the insulin receptor have very low affinity for the GRB2 SH2 domain and consequently, GRB2 does not bind this receptor directly (Skolnik *et al.*, 1993b; Pronk *et al.*, 1994; Cussac *et al.*, 1994). However, GRB2 may be involved in insulin receptor signaling through its interaction with tyrosine phosphorylated IRS-1 (preferentially Y^{895}) and/or SHC (Y^{317}) (Baltensperger *et al.*, 1993; Skolnik *et al.*, 1993b). While SOS could be found associated with IRS-1 by these groups, the presence of SOS in GRB2 / IRS-1 complexes could not be detected in fibroblasts overexpressing the insulin receptor (Pronk *et al.*, 1994). One possible explanation for this discrepancy may be the fact that Baltensperger *et al.* were using cell lines transfected with SOS, which may affect the degree of intermolecular associations. Most evidence suggests that it is the interaction of GRB2/SOS with phosphorylated SHC which results in the activation of Ras and the MAPK cascade in response to insulin (Skolnik *et al.*, 1993b; Sasaoka *et al.*, 1994a). IRS-1 and SHC appear to compete for a limited pool of GRB2 and the extent of GRB2 associated with SHC correlates with downstream mitogenic signaling (Yamauchi *et al.*, 1994). Thus, SHC appears to be involved in mitogenic signaling from the activated insulin receptor while IRS-1 may function in the metabolic pathway through PI3'-kinase activity.

Ras GTPase

The Ras genes encode a family of related, 21 kDa guanosine triphosphatases (GTPase), localized to the inner leaflet of the plasma membrane due to a series of C-terminal modifications (Hancock *et al.*, 1990; Bokoch and Der, 1993). A wide variety of stimuli can induce the activation of Ras including the activation of receptor tyrosine kinases such as the insulin and EGF receptor (Lowy and Willumsen, 1993). When Ras proteins are activated, they induce a number of cellular responses such as activation of serine-threonine kinases, the expression of early response genes and proliferation. While Ras activation is most commonly thought of as promoting mitogenic responses, in some cell types it is involved in the induction of differentiated phenotypes. The differentiation of 3T3-L1 fibroblasts to adipocytes is dependent on Ras activation, as is the differentiation of PC12 cells in response to nerve growth factor (NGF). The final outcome of the activation of Ras is most likely a consequence of the tissue-specific differences in Ras isoform

expression (Lowy and Willumsen, 1993) and the type and state of differentiation of the cell.

The biological activity of Ras is determined by the bound nucleotide; Ras-GDP being the inactive form and Ras-GTP, the active form (Bourne *et al.*, 1991). The majority of Ras in quiescent fibroblasts is found in the inactive GDP bound form. When Ras binds GTP, it undergoes a conformational change which is necessary to initiate downstream signals (Pai *et al.*, 1989). The ratio of GTP-bound to GDP-bound forms becomes elevated in growth-factor stimulated cells (Satoh *et al.*, 1990; Gibbs *et al.*, 1990). Growth factor stimulation may increase the ratio of GTP to GDP bound Ras by either activating or recruiting guanine nucleotide exchange factors (GNEF) (see GRB2/SOS), or by inactivating Ras GTPase activating proteins (GAP).

Regulation of Ras Activity by GRB2/SOS

The intrinsic rate at which guanine nucleotides exchange on and off Ras is very slow (Bourne *et al.*, 1991). SOS interacts with regions distal to the guanine nucleotide binding site of Ras, bringing about a decrease in the affinity toward the bound nucleotide (Segal *et al.*, 1993). Thus SOS facilitates the dissociation of GDP which results in the binding of GTP, since the levels of GTP in the cytosol exceed those of GDP. Thus activation of SOS in response to growth factor administration results in the activation of Ras.

Regulation of Ras Activity by GAP

The intrinsic GTPase activity of Ras limits the lifetime of the active GTP-bound form by the slow hydrolysis of GTP to GDP. In vitro, the half-life of conversion of GTP to GDP is greater than 1 hour. However, the half life of Ras-GTP in quiescent cells is just a few minutes. The GTPase activating protein, GAP was the first protein identified to be implicated in the control of Ras activity (Trahey and McCormick, 1987). GAP is as a cytoplasmic 120 kDa protein that appears to be ubiquitously expressed. The amino terminus of GAP contains two SH2 and one SH3 domain, while the active domain of GAP can be localized to the C-terminal portion of the protein (Lowy and Willumsen, 1993). GAP binds to the phosphorylated tail of the EGF receptor via its SH2 domain (Ellis *et al.*, 1990) and becomes tyrosine phosphorylated in response to EGF stimulation (Liu and Pawson, 1991). GAP, which binds preferentially to Ras in the GTP bound form (Vogel *et al.*, 1988), functions catalytically to accelerate Ras' intrinsic GTPase activity by up to five orders of magnitude (Gideon *et al.*, 1992). Thus, GAP functions as a potent negative regulator of normal Ras activity.

Thus, the extent and duration of activation of Ras in response to growth factor stimulation is determined, in part, by the extent and duration of interaction with these two regulatory enzymes.

The MAPK Cascade

Ras controls the activation of the mitogen-activated protein kinase (MAPK) cascade, in response to growth factor stimulation (Marshall, 1995a). The MAPK signaling cascade modulates important cellular processes such as transcription and protein synthesis (Davis, 1993). Raf-1 functions directly downstream of Ras and is the first component of the MAPK cascade (Seger and Krebs, 1995).

Raf-1

Raf-1 is a 72-74 kDa cytoplasmic protein that is ubiquitously expressed in all adult tissues and displays a considerable degree of evolutionary conservation (Storm *et al.*, 1990). It is comprised of an N-terminal regulatory region and a C-terminal domain that has ser/thr kinase activity (Avruch *et al.*, 1994). The regulatory region (CR1) contains a zinc finger motif and multiple serine and threonine residues (CR2) (Chow *et al.*, 1995). Deletion of the N-terminal domain activates the oncogenic potential of Raf-1 suggesting that this domain functions to suppress the catalytic activity of Raf-1 in unstimulated cells.

Raf-1 was shown to interact directly with Ras-GTP both *in vivo* and *in vitro*, an interaction mediated by sequences found in the CR1 domain of Raf-1 and the effector region of Ras (Vojtek *et al.*, 1993; Zhang *et al.*, 1993; Chuang *et al.*, 1994). Recent studies suggest that the role of Ras activation may be to target Raf-1 to the cell surface. Raf-1, modified by the addition of a C-terminal CAAX motif was localized to the plasma membrane (PM), and increased MAPK activity 20 fold without Ras activation (Leevers *et al.*, 1994; Stokoe *et al.*, 1994). However, several lines of evidence suggest that Raf-1 requires more than recruitment to the PM to become fully activated, in fact, Raf-1 constitutively localized to the PM can be further activated by treatment with EGF. Evidence further suggests that once Raf-1 is activated the interaction with Ras is no longer required (Leevers *et al.*, 1994; Stokoe *et al.*, 1994).

The precise mechanism of Raf-1 activation at the PM remains unclear. In response to EGF stimulation Raf-1 became phosphorylated on serine residues (Baccarini *et al.*, 1991). The highly phosphorylated Raf-1 has increased protein ser/thr kinase activity (Morrison *et al.*, 1988; 1989). The kinase responsible for Raf-1 serine phosphorylation is unknown, although kinases downstream in the MAPK cascade have been shown to phosphorylate Raf-1 (Anderson *et al.*, 1991). While PKC is able to phosphorylate Raf-1

in vitro, it does not appear to be involved in Raf-1 phosphorylation in response to EGF (Baccarini *et al.*, 1991; Macdonald *et al.*, 1993). The activation of Raf-1 is dependent on the phosphorylation of specific sites.

MEK

MEK is the first physiologically relevant Raf-1 substrate (Kyriakis *et al.*, 1992; Macdonald *et al.*, 1993). MEK is part of an evolutionary conserved family of protein ser/thr kinases (Seger and Krebs, 1995), which are activated by serine and threonine phosphorylation by Raf-1 (Alessi *et al.*, 1994). Although several other kinases can function as MEK activators, for example MEKK, the Raf family members play an important role in response to growth factor stimulation. Activated MEK is highly specific for downstream components of the MAPK cascade, $p44^{MAPK}$ and $p42^{MAPK}$(Seger and Krebs, 1995). MEK is capable of phosphorylating the regulatory threonine and tyrosine residues of the MAPKs. Therefore, it belongs to the family of dual specificity protein kinases, that also includes the MAPKs (Seger *et al.*, 1991).

MAP Kinase

Three forms of MAPK (also known as extracellular signal-regulated kinases or ERKs) have been identified, $p44^{MAPK}$ and $p42^{MAPK}$, and an alternative spliced form $p40^{MAPK}$ (Seger and Krebs, 1995). The MAPKs are proline directed ser/thr kinases, which are activated as a result of the phosphorylation of threonine and tyrosine residues in a TXY motif. This is carried out by MEK1 and MEK family members (Cobb *et al.*, 1991; Ray and Sturgill, 1988). Activated MAPK have been shown to phosphorylate substrates upstream in the kinase cascade for example, MEK (Matsuda *et al.*, 1993), Raf-1 (Anderson *et al.*, 1991), and T^{669} in the juxtamembrane domain of the EGF receptor (Northwood *et al.*, 1991). This may be a mechanism of negative feedback regulation, designed to limit the activation time of the MAPK pathway. Following activation, MAPK is able to translocate to the nucleus where it phosphorylates and activates key transcription factors such as Myc (Seth *et al.*, 1991), Jun (Baker *et al.*, 1992) and $p62^{TCF}$/Elk-1 (Gille *et al.*, 1992).

Nuclear Transcription

Quiescent cells respond to extracellular signals such as EGF and insulin stimulation, at the level of the nucleus, by the transcriptional induction of early response genes. This include the nuclear proto-oncogenes c-fos, c-jun and c-myc (Greenberg and Ziff, 1984; Quantin and Breathnack, 1988). Induction of expression of these genes is rapid (within 30 min) and independent of new protein synthesis, suggesting that the

induction is a direct consequence of post-translational modifications of pre-existing transcription factors. Resting cells express low levels of transcription factors, such as Jun, Fos, Myc and $p62^{TCF}$/Elk-1, which are maintained in inactive states(Boyle *et al.*, 1991). When activated, transcription factors regulate the transcription of various cellular genes, thereby playing a crucial role in cell growth, differentiation, and development.

c-Fos and c-Jun

Rapid and transient induction of c-fos and c-jun gene transcription is observed in rat hepatic cells in response to insulin (Messina, 1990; Gurney *et al.*, 1992), as well as in regenerating liver, 10-60 min after partial hepatectomy (Kruijer *et al.*, 1986; Mohn *et al.*, 1990). The c-fos gene has several regulatory sequences in its promoter region, including the serum response element (SRE), the sis-inducible element (SIE) and the AP-1 binding site. The SRE is a significant component of the c-fos promoter that contributes to mitogen-induced c-fos gene expression (Treisman, 1992; 1995). The factors that bind this element to form a ternary complex include the serum response factor $p67^{SRF}$ and the ternary complex factor, $p62^{TCF}$/Elk-1. Mitogen stimulated transcription of c-fos can be accounted for, in part, by the phosphorylation and activation of $p62^{TCF}$/Elk-1 by MAPK (Gille *et al.*, 1992; Marais *et al.*, 1993). The 5' promoter region of c-jun has a putative AP-1 site (Kitabayashi *et al.*, 1990) and has been shown to be positively regulated by Jun activation in response to growth factors (Angel *et al.*, 1988). The c-jun promoter region does not contain an SRE but does contain an RSRF (related to serum response factor) binding site which appears to be an important element for EGF induction of the promoter (Han *et al.*, 1992). Induction of c-jun occurs mainly at the transcriptional level as demonstrated by nuclear run on assays. However, regulation of transcription appears to be vastly more complicated as many regulatory promoter sequences are being identified.

Fos and Jun proteins can form heterodimers which have enhanced affinity for DNA target sites relative to Jun homodimers (Angel and Karin, 1991; Curran and Franza, 1988). These complexes (called AP-1) bind DNA at specific AP-1 sites (consensus sequence: TGACTCA) and regulate transcription through these elements (Ransone and Verma, 1990). Many genes have been found to contain AP-1 sites in their promoter regions including the c-jun and c-fos genes themselves. AP-1 is a positive regulator of ornithine decarboxylase (ODC) gene transcription, but this has been demonstrated to be cell type dependent (Wrighton and Busslinger, 1993). AP-1 has been implicated in the down regulation of several genes, including c-fos (Sassone-Corsi *et al.*, 1988), c-myc (Hay *et al.*, 1989), PEPCK (Gurney at al., 1992) and the albumin gene in hepatocytes (Hu and Isom, 1994).

c-myc

Regulation of transcription of the proto-oncogene c-myc plays a role in cellular proliferation (reviewed in Marcu *et al.*, 1992). The increased transcription of the c-myc gene has been demonstrated in several hepatic cell lines, in response to factors such as EGF and insulin, as well as after partial hepatectomy (Goyette *et al.*, 1984; Makino *et al.*, 1984; Kruijer *et al.*, 1986; Morimura *et al.*, 1990). However, the observation that transfection of c-myc into primary hepatocytes did not initiate DNA synthesis (Skouteris and Kaser, 1992) suggests that other components are required for this to take place. In addition, several c-myc transgenic mouse strains expressing high levels of Myc do not form malignant hepatic carcinomas, which are found in mice containing both c-myc and Ras transgenes (Sandgren *et al.*, 1989). Normal rat liver epithelial cells transfected with a c-myc gene were not transformed, but c-myc in combination with the Raf-1 gene proved efficient in causing liver cell transformation (Garfield *et al.*, 1988). Thus evidence suggests that Myc functions in association with components acting downstream of the Ras pathway to cause hepatic cell proliferation.

The c-myc proto-oncogene product is a nuclear protein that functions as a transcription factor (Kato and Dang, 1992). It binds as a heterodimer with Max, to the DNA sequences CACGTG (Blackwell *et al.*, 1990; Cole *et al.*, 1991; Amati *et al.*, 1992). The major phosphorylation site on Myc *in vivo* (S^{62}), a site of phosphorylation by MAPK *in vitro* (Seth *et al.*, 1991), is associated with enhanced transcription factor activity. The ODC gene is a transcriptional target of Myc (Peña *et al.*, 1993; Bello-Fernandez *et al.*, 1993). Myc is also capable of negatively autoregulating its transcription, adding another level to transcriptional control to the actions of Myc (Penn *et al.*, 1990).

It appears that the activation of the Ras-MAPK pathway results in changes in gene transcription which prepare the hepatocyte for proliferation, i.e. the decrease in albumin gene expression and the increase in ODC levels (Nakamura *et al.*, 1983). In addition, EGF stimulation of primary hepatocytes and partial hepatectomy results in the induction of Ras mRNA transcription (Kost and Michalopoulos, 1990; Goyette *et al.*, 1984).

Much of the work carried out on receptor tyrosine kinase signal transduction has been carried out in cultured cells. The high degree of control over the cellular environment and the ability to genetically manipulate these cells is essential for the identification of novel proteins, protein-protein interactions and pathways involved in signal transduction. However, removing these cells from their normal physiological environment results in the loss of structural cues and hormonal regulation on which 'normal' cells rely. Nearly all cell lines maintained in long-term culture are transformed and therefore, may not represent the *in vivo* state of the cell. In addition, overexpression of proteins or transfection of new proteins into cells places these proteins in a cellular context which may not be genetically programmed to regulate them. Given that cell-specific factors dictate the final cellular response to any extracellular signal, and that developmental and environmental factors also play a role, it is hazardous to extrapolate from one cell type to another and from cell culture to intact organs. Although a variety of proteins have been shown to serve as substrates *in vitro* for receptor tyrosine kinases, only a few are actually *in vivo* substrates in a given cell type. In order to elucidate the signal transduction pathways used by the EGF and insulin receptor tyrosine kinases and the regulation, if any, of these pathways at the level of the endosome, the rat liver model was used as a more physiologically representative model.

Experimental Procedures

Materials

Receptor-grade EGF was purchased from Collaborative Research Inc. (Lexington, MA), insulin was obtained from Eli Lilly (Indianapolis, IN) and the insulin analog H2 was obtained from Dr. G. Danielsen (Novo Nordisk, Denmark). Nitrocellulose membrane (BA85) was obtained from Xymotech (Mt. Royal, PQ). [^{125}I]-goat anti-rabbit IgG (8.70 μCi/μg), [^{125}I]-goat anti-mouse IgG (19.9 μCi/μg) were obtained from NEN-Mandel (St. Laurent, PQ) and ICN Biomedicals (Mississauga, Ont.) respectively. HRP-conjugated goat anti-rabbit IgG, and protein-A-HRP were purchased from BioRad Laboratories (Mississauga, Ont.) while the HRP-conjugated goat anti-mouse IgG was purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). ECL Detection kit was obtained from Amersham (Oakville, Ont.). Protein A and Protein G sepharose were obtained from Pharmacia Biotech Inc. (Baie d'Urfé, PQ) as were restriction enzymes. The GeneClean II Kit for insert purification was purchased from Bio101 Inc. (La Jolla, CA). [α-^{32}P] dCTP (3000 Ci/mmol) was purchased from NEN-Mandel (St. Laurent, PQ). The Random Primer DNA Labeling System was purchased by GIBCO BRL (Burlington, PQ). Hybond-N membrane was obtained from Amersham (Oakville, Ont.). Kodak XAR-5 OMAT x-ray film was purchased from PM. Canada Inc. (Montreal, PQ). Reagents for SDS-PAGE and protein determination were from BioRad Laboratories (Mississauga, Ont.).

Unless otherwise indicated, all other reagents and chemicals were obtained from Sigma Chemical Company (St. Louis, MO), Boehringer Mannheim, Fisher Scientific (Montreal, PQ), and Anachemia (Lachine, PQ). Reagents for molecular biology were of nucleic acid grade (DNase and RNase free).

Antibodies used for immunoprecipitation and Western blotting are described in Table I. The cDNA probes used for Northern blot experiments are described in Table II

Animals

Male Sprague-Dawley rats (100-120 g body weight) were obtained from Charles River Laboratory (St. Constant, PQ). Lab Chow and water were available ad libitum until 14-16 hours prior to administration of growth factor or hormone, when they were fasted. New Zealand White rabbits for use in the preparation of antisera were obtained from Charles River Laboratory (St. Constant, PQ).

Methods

I Subcellular Fractionation

Fasted rats were anesthetized with an injection of sodium pentobarbital (100 µl/ 100 g body weight) (Somnotol, MTC Pharmaceuticals, Cambridge, Ont.) Animals were injected, via the hepatic portal vein, with 10 µg/100 g body weight of EGF or 15 µg/100 g body weight of insulin or the insulin analog, H2. At various times (0.5, 5, 15, 30, or 60 min) after injection, animals were sacrificed and their livers were rapidly removed and minced in ice cold homogenization buffer (0.25 M sucrose, 20 mM Tris base, pH 7.4, 1 mM $MgCl_2$) containing the following protease and phosphatase inhibitors; 5 mM iodoacetamide, 165 Kallikrein inhibitor units (KIU)/ml aprotinin, 0.5 mM phenymethylsulfonyl fluoride, 4 mM NaF, 100 mM Na_3VO_4, 5 mM sodium molybdate, 10 mM β-glycerophosphate, 2 mM benzamidine, 5 mM p-nitrophenyl phosphate. Aprotinin, PMSF, and iodoacetamide were added to the buffer immediately before use. All subsequent steps were carried out at 4°C and all buffers contained the above inhibitors.

The isolation of plasma membranes (PM) was carried out essentially as described by Kay *et al.* (1986). To isolate plasma membranes, livers were homogenized using a Dounce homogenizer (B pestle) to a final concentration of 5 ml/g liver. Homogenates were centrifuged at 280 x g for 5 min (Sorvall SS-34 rotor) to obtain a pellet (P1) and a supernatant (S1). The pellet was resuspended in half the original volume using the Dounce homogenizer and centrifuged at 280 x g for 5 min to obtain P2 and S2. S2 and S1 were combined and centrifuged at 1500 x g for 10 min to obtain P3 and S3. Pellets P2 and P3 were combined, resuspended and the molarity was adjusted to 1.42 M sucrose. This homogenate was overlaid with 0.25 M sucrose and centrifuged at 82 000 x g for 1 hour (Beckman SW-28 rotor). The pellicule at the 0.25 M and 1.42 M interface was collected, the molarity was adjusted to 0.39 M sucrose and centrifuged at 1500 x g for 10 min (Sorvall SS-28). The resulting pellet (PM) was resuspended in homogenization buffer.

To obtain the total particulate fraction (TP), one ml of the homogenate (5 mg/g liver) was centrifuged at 100 000 x g (Beckman rotor TLA 100.2) for 1 hour. The pellet was resuspended in 2 ml of homogenization buffer.

The GE fraction was prepared as described by Khan *et al.* (1989) with minor modifications. Livers were homogenized using a Potter-Elvehjem homogenizer to generate

20% liver homogenates. These were then centrifuged for 10 min at 1500 x g (Sorvall SS-34) and the supernatant collected and centrifuged at 200 000 x g for 30 min (Beckmann 60 Ti rotor). The supernatant was used as the cytosolic (Cyt) fraction. The microsomal pellet was resuspended to a molarity of 1.15 M sucrose and overlaid with 1.00 and 0.25 M sucrose cushions and centrifuged at 200 000 x g for 1.5 h (Beckman SW28 rotor). The GE fraction was collected at the 0.25 M and 1.00 M sucrose interface.

Proteins concentration of subcellular fractions was measured using the Bradford method (1976) and BSA as a standard. The yield (mg protein / g liver) of each fraction was calculated and is indicated in Table III. The yields obtained for the PM and GE fractions (Table III) were consistent with the yields described by Kay *et al.* (1986) and by Wada *et al.* (1992), respectively. Fractions were aliquoted and stored at -70°C.

Recoveries of Subcellular Fractions

The EGF receptor content in the TP, PM and GE fractions was determined by quantitative immunoblotting using phosphoimager analysis. In the TP, it was determined to be 2.54 ± 0.26 U/mg cell fraction protein and was unchanged from 0 to 60 min after EGF injection. The EGF receptor content of the PM at 0 and 15 min after EGF injection was found to be 12.2 ± 3.4 and 2.40 ± 0.02 U/mg cell fraction protein, respectively. For the GE at 0 and 15 min after EGF injection, the values were 12.8 ± 2.5 and 57.8 ±4.1 U/mg cell fraction protein, respectively. Using the yields for each fraction (see Table III), recoveries were calculated to be 19.2% for the PM and 16.2 % for the GE.

II Electron Microscopy of Subcellular Fractions

100 micrograms of plasma membrane and GE fraction, freshly isolated, were fixed in 2.5% gluteraldehyde, 100 mM sodium cacodylate (pH 7.4) at 4°C overnight. Samples were then filtered under N_2 onto Millipore nitrocellulose filters (pore size of 0.8 µm)(Millipore-Waters, Mississauga, Ont.) using the filtration apparatus described by Baudhuin *et al.* (1967). Filters were washed 3 x 20 min and once overnight with 0.1 M sodium cacodylate buffer. Filters were then post-fixed in 2% OsO_4, 100 mM sodium cacodylate buffer (pH 7.4) and stained with 1% tannic acid, pH 7.4 (Simionescu and Simionescu, 1976), and then 2% uranyl acetate, 100 mM maleic buffer, pH 5.7 (Karnovsky, 1967) and processed for routine electron microscopy.

III Generation of anti-SHC Antibodies

Protein Expression and Purification

E. Coli transformed with the pGEX vector containing the SHC SH2 domain insert were obtained from Drs. Jane McGlade and Tony Pawson (Samuel Lunenfeld Research Institute, University of Toronto). A single colony was used to inoculate 200 ml of LB broth (Sambrook *et al.*, 1989) containing 40 µg/ml of ampicillin. The liquid culture was grown overnight at 37°C in a shaking incubator. The culture was then diluted into 1 liter of fresh LB/ampicillin and grown for an additional hour. 100 mM IPTG was added, to a final concentration of 0.1 mM, for 4 hours to induce fusion protein expression. Cells were pelleted by centrifugation at 5000 x g for 10 min and then resuspended in 30 ml ice-cold PBS/ 0.5 mM PMSF. Cells were lysed, 1 ml at a time, using a 5 mm diameter probe sonicator (setting 100; 50 watts), twice for 15 seconds. 10% Triton X-100 was added, to a final concentration of 1%, to the lysed cells, which were then incubated on ice for 30 min. The cells were then centrifuged at 10 000 x g (Beckman JA-20 9500) for 10 min at 4°C to remove insoluble material. The supernatant was collected and 2 ml 50% glutathione-agarose bead slurry was added and the mixture rotated for 1 hour at 4°C. The beads are then washed 3 times with 50 ml of ice-cold PBS with intervening 10 sec centrifugations at 500 x g. The fusion protein was eluted by adding 1 ml 50 mM Tris (pH 8) / 10 mM reduced glutathione for 15 min at 4°C. The eluant was collected after centrifugation. A second elution was carried out, but the recovery was minimal.

Immunization and Bleeds

Prior to injection of antigen, 10 ml preimmune blood was taken from the central ear artery of 2-2.5 kg female New-Zealand white rabbits. The rabbits were then injected intramuscularly with 2 mg (500 µl) of the purified GST-SHC SH2 fusion protein and an equal volume of Freund's complete adjuvant. The Boosters (1 mg fusion protein/ Freund's incomplete adjuvant), were given 30 and 60 days after the initial immunization, and 25 ml blood was taken each week. The blood was incubated for 30 min at 37°C, and then overnight at 4°C. Serum was obtained by centrifugation at 2200 rpm (Beckman, JS-4.2) for 30 min at 4°C.

Serum obtained was used for the affinity purification of SHC antibodies and was carried out according to Pelicci *et al.* (1992).

IV Immunoprecipitation

Immunoprecipitations were carried out on the cytosolic, PM and GE fractions. Cytosolic fractions were solubilized in immunoprecipitation buffer (1% Triton X-100 / HBS (50 mM HEPES pH 7.5, 150 mM NaCl, 10% glycerol) for 30 min at 4°C. PM and GE fractions were solubilized in immunoprecipitation buffer containing 0.5% deoxycholate. The samples were centrifuged at top speed for 5 min in an Eppendorf microfuge. 10 µg of monoclonal EGFR antibody (IgG 151 BH-6), affinity purified anti-SHC antibodies or anti-phosphotyrosine (PY) antibody were added to the supernatants and incubated for 2 h at 4°C. The immune complex was precipitated using 100 µl of a 20% protein A sepharose slurry (α-SHC, α-IRS-1) or 75 µl of 20% protein G sepharose slurry (α-EGFR, α-PY, α-960, α-enigma) for 1 hour at 4°C with rotation. The beads were then washed three times with immunoprecipitation buffer and once with HBS alone. Immunoprecipitated proteins were removed from the beads by the addition of Laemmli sample preparation buffer (Laemmli, 1970).

V Immunoblotting and Competition protocol

Samples were boiled in Laemmli sample preparation buffer for 5 min, electrophoresed on 8% SDS-polyacrylamide gels (10% resolving gel for proteins less than 30 kDa) and transferred onto nitrocellulose for one hour (0.5 hr for GRB2 immunoblots). The blots were then incubated in 5% skimmed milk (SM) in TNT buffer (0.15 M NaCl, 0.05% Tween-20, 10 mM Tris, pH 7.5) three times for 30 min. α-PY immunoblots were incubated in 2% BSA/TNT. Blots were then incubated with primary antibodies overnight at room temperature and then washed with 0.5% SM/TNT or 0.5% BSA/TNT three times for five minutes. The blots were then incubated with [^{125}I]-conjugated (200 000 cpm/ml) or HRP-conjugated secondary antibodies (1:5000 for HRP-protein A or 1:10000 for HRP-goat anti-mouse), in 0.5% SM/TNT or 0.5% BSA/TNT, for 45 min and then washed for 25 min with 5 changes. The chemiluminescence reaction, with the HRP-conjugated secondary antibodies, was carried out as described by the manufacturer. For visualization, the blots were exposed to Kodak X-OMAT X-ray film. Quantitation of [^{125}I] signals was carried out using a BAS 200 Fuji Bio-Imaging Analyzer (Fuji BioMedical Systems, Inc., Bethesda, MD), with the gradation, resolution and sensitivity settings as described by the manufacturer for immunoblots.

When experiments were carried out in triplicate, for quantitation, Western blots were carried out simultaneously and the blots were all exposed to phosphoimager analysis at the same time.

For the enigma competition studies, blots were incubated with the anti-enigma antibody (1:1000 in 5% SM/TNT) which had been preincubated with 20 µg of either enigma N-terminal peptide or enigma C-terminal peptide (obtained from Dr. G.N. Gill; UCSD, LaJolla, CA), for 1 hr at room temperature. After overnight incubation, the blots were processed as described above and visualized by chemiluminescence.

VI HPLC Analysis of Insulin and H2 Degradation by Endosomal Fractions

Endosomes were isolated as described above (I. Subcellular Fractionation), in the absence of any protease or phosphatase inhibitors. The soluble extract from the endosomes was isolated by freeze/ thawing in 5 mM sodium phosphate (pH 7.4), and disruption in the same hypotonic medium with a small Dounce homogenizer (15 strokes with a tight type A pestle) followed by centrifugation at 300 000 x g for 30 min. The soluble proteins (~0.01 µg) were incubated with 10^{-6} M insulin or the insulin analog, H2, in 0.25 ml of 62.5 mM citrate-phosphate buffer (pH 5.0) for 2 hr. The samples were then acidified with acetic acid (20% final) and immediately loaded onto a reverse-phase HPLC column (Waters, µBondapak C18). A 0-45% linear acetonitrile gradient was established in 60 min. The absorbance was read at 214 nm. The control incubations were carried as described above, in the absence of endosomal fraction proteins.

VII RNA Isolation

RNA was isolated from liver using Acid Guanidinium Thiocyanate/ Phenol/ Chloroform extraction as described by Chomczynski *et al.* (1987). Rats were fasted for 14-16 hours prior to administration of growth factor or hormone via hepatic portal vein injection. Male animals were injected with 10 µg/100 g body weight of EGF or 15 µg/100 g body weight of Insulin or H2. Animals were sacrificed at various times (0, 15, 30, or 60 min) after injection and their livers were rapidly removed. Livers were stored, frozen in liquid nitrogen, or immediately homogenized in 1 ml/100 mg liver of ice-cold denaturing solution (4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% sarcosyl, 0.1 M β-mercaptoethanol) with 100 µl of Antifoam A emulsion, using a Brinkmann Polytron (Brinkmann Instruments, Westbury, NY) at setting 8 for 5-15 seconds. After

homogenization, 0.1 ml/100 mg liver of 2 M sodium acetate (pH 4), 1 ml/ 100 mg liver of water-saturated phenol, and 0.2 ml/100 mg liver of chloroform-isoamyl alcohol (49:1 vol/vol) was added with thorough mixing by inversion after addition of each reagent. The suspension was incubated 15 min at 4°C and then centrifuged at 10 000 x g (Sorvall SS-34 rotor) for 20 min at 4°C. The aqueous phase was transferred to a fresh tube and the RNA precipitated with 1 vol of 100% isopropanol overnight at -20°C. Samples were centrifuged at 10 000 x g for 20 min at 4°C. The resulting pellets were dissolved in 0.3 ml denaturing solution and the RNA was precipitated with 1 ml 100% isopropanol for 2 h at -20°C. Samples were then centrifuged for 10 min at 4°C in a microfuge and the resulting pellets were resuspended in 100 µl 75% ethanol to remove trace amounts of guanidinium contaminants. Samples were centrifuged for 5 min at 4°C in a microfuge and the pellet was dried under vacuum. RNA was dissolved in DEPC-treated water and were stored at -70°C until use.

Concentration, Yield and Purity Determination

The concentration of RNA in the samples was determined by spectrophotometric measurement of the absorbance of 4 µl of the RNA solution in 996 µl water at λ of 260 nm. The yield of total RNA isolated from rat liver was calculated to be 7.09 ± 1.14 µg/mg liver (n=58). The purity of the isolated RNA was determined by calculating the ratio of OD 260/280 and was determined to be 1.80 ± 0.11. The integrity of the isolated was tested by separating the RNA samples on denaturing agarose / formaldehyde gels to detect the relative amounts of the 28S and 18S ribosomal RNA bands.

VIII Probe preparation

Transformation of E.Coli

Plasmid DNA (0.5-1.0 µg) was added to 20 µl competent E.Coli. The culture was placed on ice for 10 min, heat shocked at 42°C for 1.5 min, and placed on ice. 180 µl of 2YT medium (Sambrook *et al.*, 1989) containing 0.2% glucose was added and the cells were incubated at 37°C for 30-60 min. The cells were streaked onto 9 mm 1.5% agar / 2YT plates (0.2% glucose, 100 µg/ml ampicillin) and incubated overnight at 37°C. A single colony was used to inoculate 10 ml LB medium (Sambrook *et al.*, 1989) (40 µg/ml Ampicillin) and incubated overnight at 37°C. The transformed bacteria were stored in media and a final concentration of 15% glycerol at -80°C.

Plasmid isolation

Plasmid isolation was carried out using large scale preparation based on the Birnboim method (1979). Transformed E.Coli were streaked onto a 9 mm LB agar / antibiotic plate (40 µg/ml Ampicillin or 10 µg/ml Tetracycline) and incubated overnight at 37°C. A single colony was used to inoculate a 50 ml LB broth culture containing the appropriate antibiotic (see Table II). The culture was incubated overnight with aeration, at 37°C. The cells were pelleted by centrifugation at 4000 rpm (Sorvall GS3) for 10 min. The supernatant was removed and the pellet resuspended in 1.28 ml of freshly prepared Solution I (2 mg/ml lysozyme, 5 mM glucose, 10 mM EDTA, 25 mM Tris-HCl (pH 8.0)). The suspension was incubated on ice for 15 min, after which 2.56 ml of Solution II (0.2N NaOH, 1% SDS) was added. The tube was gently vortexed and maintained on ice for 5 min. Subsequently, 1.92 ml of Solution III (3 M sodium acetate (pH 4.8)) was added and after gently vortexing, the solution was incubated on ice for 20 min. The mixture was centrifuged at 4000 rpm (Sorvall GS3) for 20 min. 5.6 ml of ice cold isopropanol was added to the supernatant. The plasmid DNA was allowed to precipitate for 2 hr at -20°C and then pelleted at 5000 rpm (Sorvall GS3) for 20 min. The pellet was dissolved in 1 ml of TA buffer (0.1 M sodium acetate / 0.05 M Tris-HCl (pH 8.0)) and reprecipitated with 2 ml of cold ethanol for 1 hr at -70°C. The plasmid was pelleted and the air dried pellet resuspended in 1 ml of 150 mM NaCl and 50 mM Tris-HCl (pH 7.5). 100 µg of RNase A was added to this solution which was then incubated for 1 hr at 37°C, then 200 µg of proteinase K was added and the mixture incubated for 1 hr at 37°C. Subsequently, three extractions were performed using equal volume of phenol-chloroform mixture. After the final extraction, the DNA in the aqueous layer was precipitated for 1 hr at -70°C using 2.5 vol of cold ethanol. The pellet was dissolved in 4 ml TE buffer (10 mM Tris·Cl, 1 mM EDTA; pH 8.0) and exactly 4.3 g CsCl and 0.3 ml of Ethidium Bromide (10 mg/ml) was added. The solution was transferred to a Beckmann Quick-Seal tube and centrifuged at 50 K rpm for 16 hr (Beckmann, VTi65 rotor). The plasmid band was collected as described in Sambrook *et al.* (1989). The ethidium bromide was removed from the plasmid DNA by multiple extractions with equal volume of SSC saturated isopropanol (1 vol 20 x SSC and 2 vol isopropanol). After the final extraction, 2 vol of water and 0.1 vol of 3 M sodium acetate was added and the plasmid DNA precipitated with 0.7 vol of cold isopropanol for 2 hr at -20°C. The plasmid DNA was pelleted, resuspended in 0.4 ml of TA buffer and precipitated with 1 ml ethanol for 1 hr at -70°C. The final pure plasmid DNA was obtained

by centrifugation in a microfuge at maximum speed for 10 min and was resuspended in water and stored at -20°C.

Insert Isolation and Labeling

Plasmid solution was incubated for 2 h at 37°C with the appropriate restriction enzymes (Table II) as described by the manufacturer. The reaction was stopped with the addition of EDTA (pH 7.5) to a final concentration of 10 mM. DNA loading buffer type III was added to the sample (Sambrook *et al.*, 1989). The mixture run on a 1.2% agarose gel / 1 X TAE buffer (40 mM Tris-acetate, 1 mM EDTA (pH 8.0)) at 95 V for 1.5 h to separate the insert from the vectors and undigested plasmid. The insert DNA band, visualized by ethidium bromide staining under UV light, was excised and cleaned using the Gene Clean II Kit as described by the manufacturer. Purified inserts were dissolved in water and stored at -20°C. The inserts were labeled with [α-^{32}P] dCTP by oligolabeling with Klenow DNA polymerase and hexanucleotides as primers using the Random Primer Labeling System as described by the manufacturer. The specific activity was 6.53 x 10^8 cpm/ mg cDNA fragment.

IX Northern Blotting

The samples (20 µg total RNA per lane) were loaded onto a 1.2% (w/v) agarose gel containing 0.66 M formaldehyde / 1 x HEPES-EDTA (pH 7.8) and the electrophoresis was carried out at 25 V for 15 h. The gel was subsequently washed 2 x 30 min with 10 x SSC (1.5 M NaCl, 0.15 M sodium citrate, pH 7). The RNA was transferred to Hybond-N nylon membranes using the LKB.VacuGene XL (Pharmacia Canada Ltd. Baie D'Urfe, PQ) vacuum apparatus at 50 cm H_2O for 1.5 h. The blots were then prehybridized in hybridization buffer (50% deionized formamide, 5 x SSC, 20 mM Tris-HCl (pH 7.5), 1 x Denhardt's solution, 10% Dextran Sulfate, 0.1% SDS, 200 mg/ml denatured salmon sperm DNA), without probe, overnight at 45°C. [α-^{32}P] dCTP-labeled cDNA probe was hybridized (10^6 cpm / ml) to the filters at 45°C for 2-3 h in fresh hybridization buffer. The blots were then washed 2 times 15 min (1 x SSC, 0.1% SDS) at room temperature, and 3 times 20 min (0.2 x SSC, 0.1% SDS) at 68°C. Quantitation of [α-^{32}P] signals was carried out using a BAS 200 Fuji Bio-Imaging Analyzer (Fuji BioMedical Systems Inc., Bethesda, MD), with the gradation, resolution and sensitivity settings as described by the manufacturer for immunoblots. The blots are exposed to film for up to 3 days at -70°C.

Table I Antibodies used for Immunoprecipitation and Western Blotting

Antibody	Raised to:	Type	Specificity	Dilution[1]	Source
anti-AP-2 (AC1-MII)	Hydroxylapatite grp II 100 kDa proteins	monoclonal	~ 100 kDa α-adaptin	1:200	Dr. M.S. Robinson
anti-EGF Receptor (IgG 151 BH6)	Extracellular domain of the EGFR	monoclonal	170 kDa EGFR	1:200 - 1:500*	Lai et al. 1989
anti-EGF Receptor	Synthetic peptide corresponding to residues 1164- 1176 of the EGFR	polyclonal	170 kDa EGFR	1:1000	Wada et al. 1992
anti-enigma	N-terminus of the LIM domain protein enigma	polyclonal	~ 55 kDa enigma	1:1000	Dr. G.N. Gill
anti-GRB2	Full length murine GRB2	polyclonal	23 kDa GRB2	1:500	UBI
anti-Insulin Receptor (α 960)	Synthetic peptide to residues 942-969 of the β-subunit of the insulin receptor	polyclonal	94 kDa β-subunit of the insulin receptor	1:500 1:200*	Burgess et al. 1992
anti-IRS-1	Synthetic peptide to residues1221-1235 of C-terminus of IRS-1	polyclonal	185 kDa IRS-1	1:500 1:100*	Dr. M.F. White
anti-MAP Kinase	Synthetic peptide corresponding to residues 333-367 of rat erk 1	polyclonal	44, 43 and 42 kDa isoforms of MAPK	3µg/ ml	UBI
anti-Phosphotyrosine	Phosphotyrosine-BSA	monoclonal	Phosphotyrosine containing proteins	1:1000	Sigma
anti-Raf-1	Peptide to 12 C-terminal residues of Raf-1	polyclonal	74 kDa Raf-1	1:1000	Santa Cruz
anti-SHC	SH2 domain of SHC (residues 366-473)	polyclonal	46, 55, and 66 kDa isoforms of SHC	1:1000 1:250 - 1:500*	Dr. T. Pawson
anti-mSOS 1	C-terminal region of murine SOS1	polyclonal	175 kDa mSOS1	4µg/ml	UBI

1 - Dilutions used for Western Blotting * - Dilutions used for Immunoprecipitations

Table II cDNA probes used for Northern Analysis

Probe	Plasmid	Plasmid size (kb)	Resistance	Restiction Site	Insert Type	Probe Size (kb)	Source
c-myc	pSP64 c-myc	4.90	Amp+	Hind III	murine cDNA	1.90	Dr. N. Sonenberg
c-fos	pSAEM 1-cfos	7.10	Amp+	BamHI	mouse genomic DNA	3.90	Dr. R. St. Arnaud
c-jun	RSV-cJ	5.45	Amp+	Hind III / Pst I	cDNA	0.90	Dr. P. Jolicoeur
GAPDH	pBR322-GAPDH	5.46	Tet+	Pst I	rat cDNA	1.10	Piecheczyk et al., 1984

Table III Protein yield of subcellular fractions

Fraction	Yield (mg/g liver)	n
Cytosol	53.78 ± 5.51	62
Total Particulate	85.10 ± 12.06	38
Plasma membrane	2.77 ± 1.22	47
GE	0.51 ± 0.15	54

Rat liver homogenates were fractionated into cytosol and total particulate fractions, and purified plasma membranes or GE fractions. The yield of protein (mean ± SD) is indicated as well as the number of fractionations (n).

Chapter I

EGF and Insulin Receptor Internalization

Rat liver was chosen as an *in vivo* model system for elucidating receptor tyrosine kinase (RTK) signal transduction specificity, and the regulatory role, if any, of internalization and trafficking. The adult liver, composed predominantly of parenchymal cells, controls critical metabolic processes, including glucose homeostasis. In addition, the liver processes the unique ability to regenerate after injury. Hepatocyte growth responses are of particular interest since they involve cells which are normally quiescent and constitute highly regulated processes (Steer *et al.*, 1995). A variety of factors acting through RTKs, including insulin and EGF, have been implicated in the regulation of hepatocyte function and growth.

The liver is positioned to remove a wide range of molecules from the circulating blood and may clear more than 80% of an injected dose of either EGF or insulin from the circulation in one pass through the liver (Lai *et al.*, 1989a; Jaspan *et al.*, 1981). This is accomplished by means of high affinity, specific, saturable receptor mediated endocytosis. The liver parenchyma enriched in receptors for both insulin and EGF is the major target organ for their respective ligands (O'Keefe *et al.*, 1974; Kahn *et al.*, 1974). Both EGF and insulin receptors are expressed in large numbers ($\sim 1 \times 10^5$) and in roughly equal amounts in hepatocytes of male rats (Lai *et al.*, 1989a; Kahn *et al.*, 1974, Dunn and Hubbard, 1984).

In untreated livers, these receptors are distributed diffusely over the plasma membrane. Upon ligand binding the receptor-ligand complexes have been postulated to cluster into clathrin-coated pits and be rapidly internalized into the endocytic compartment (Bergeron *et al.*, 1985). The progressive acidification of the endosomal compartment may lead to ligand / receptor dissociation, as is the case for insulin. Receptors may be recycled to the cell surface and the ligand degraded. However, in specific situations, for example in the case of ligand overload, both the ligand and the receptor are targeted to the lysosome for degradation (Lai *et al.* 1989a; Backer *et al.*, 1990). The mechanisms and molecules involved in directing internalization and the fate of specific ligand / receptor complexes remain unclear, although candidate molecules have been proposed. The clathrin associated AP-2 complex has been implicated in ligand mediated EGF receptor internalization (Boll *et al.*, 1995) while the LIM domain protein enigma is proposed to function in this capacity for the insulin receptor (Wu and Gill, 1995).

The objective of this chapter is to use the previously characterized rat liver system, in particular the subcellular fractionation techniques which generate purified plasma membranes and endosomes, to study the internalization process of RTKs at both these sites. The kinetics and specificity of internalization of the EGF and insulin receptors, as well as the association of these receptors with putative internalization molecules will be assessed in this physiological system.

Results

Established protocols were used to prepare plasma membrane (PM) and endosomal fractions (Kay *et al.*, 1986; Wada *et al.*, 1992). Electron microscopy, carried out using a methodology to ensure random sampling, demonstrated that all domains of the hepatic plasma membrane were present, with little contamination from other cellular organelles (Fig.3A). The sinusoidal, lateral and bile canalicular membrane components of the hepatocyte were present as were the coated pits and coated vesicles associated with the sinusoidal surface (Fig.3B). Several coated vesicles appeared to be in close association with the numerous microfilaments (ca 6 nm) observed in this domain. Electron microscopy of the endosomal (GE) fraction revealed large intact endosomal structures with intraluminal lipoprotein particles (Fig.4). Although Golgi saccules could be found in this fraction, no cross contamination with PM was demonstrated by marker enzyme analysis (Bergeron *et al.* 1986; Khan *et al.*, 1986; Lai *et al.*, 1989a,b). After morphologically confirming the identity of the fractions, cell fraction protein profiles after SDS-PAGE and Coomassie blue staining were established (Fig.5). Three polypeptides of high molecular weight (190, 200 and 234 kDa) were found to be diagnostic of purified PM.

The effect of ligand administration on total cellular EGF and insulin receptor levels was determined. Within the time frame of the experiments (up to 60 min post ligand injection) there was no significant loss of either the EGF or the insulin receptor from the total particulate (TP) fraction (Fig.6A and B, respectively).

Receptor / ligand internalization into endosomes is one of the immediate-early responses to ligand administration (Bergeron *et al.*, 1985). To study this response in the rat liver model, subcellular fractionation was carried out in order to follow the receptor from the plasma membrane into the endocytic compartment. The results showed a rapid redistribution of EGF receptors (in response to EGF) and insulin receptors (in response to insulin) from the PM into the GE compartment (Fig.7A and B, respectively). Quantification of [^{125}I] signals for the EGF and insulin receptors in the PM, revealed that the extent of receptor internalization differed between these two receptors (Fig.8A). Whereas ~75% of the plasma membrane EGF receptors were internalized after EGF injection, only ~34% of the insulin receptors internalized in response to insulin. However, in < 1 min, one half of total internalizing receptors (both EGF and insulin receptors) had already been internalized. The kinetics of the EGF and insulin receptor accumulation into the endosomal compartment appeared to differ slightly (Fig.8B); while the maximal accumulation of the EGF receptor occurred at 15 min post-injection, the insulin receptor

appeared to reach maximal receptor levels in the endosome by 5 min. The disappearance of both receptors from the GE appeared to take place with similar kinetics.

To determine the specificity of EGF receptor internalization, EGF receptor internalization was assessed after EGF and insulin stimulation. The results show that no significant internalization of the EGF receptor was observed in response to insulin stimulation (Fig.9).

The AP-2 adaptor protein complex has been implicated in EGF receptor internalization as a consequence of the ability of AP-2 to induce clathrin coats (Pearse and Robinson, 1990) and the ligand dependent association of AP-2 with the EGF receptor (Sorkin and Carpenter, 1993; Boll *et al.*, 1995; Nesterov *et al.*, 1995a). To determine the role of AP-2 in receptor internalization in the liver, the distribution of AP-2 in PM and GE fractions was assessed. In response to EGF stimulation (Fig.10A), AP-2 was observed to redistribute to the GE fraction. The increase of AP-2 at 5 min after ligand injection coincided with the appearance of the EGF receptor into this fraction (see Fig.8A). No corresponding redistribution of AP-2 was observed in response to insulin stimulation (Fig.10B), consistent with the idea that AP-2 does not associate with the insulin receptor *in vitro*. To determine if the presence of AP-2 in the endosomes was due to its *in vivo* association with the EGF receptor, anti-EGF receptor immunoprecipitations were carried out (Fig.11). Negligible amounts of AP-2 were detected in association with the EGF receptor at the PM, however, from 5-30 min post-EGF injection AP-2 could be seen in physical association with the endosomally located EGF receptor (Fig.11B).

The cytosolic LIM domain containing protein, enigma has been proposed to function in insulin receptor internalization based on the observation that it binds with high affinity to the internalization sequences of the insulin receptor (Wu and Gill, 1994). The subcellular distribution of enigma was determined in response to insulin stimulation (Fig.12A). The anti-enigma antibody (raised to the N-terminal of enigma) recognized several polypeptides in the rat liver fractions. In the cytosol, a polypeptide of approximately 93 kDa was identified, but the expected 55 kDa band corresponding to enigma was not detected. In the total particulate and the PM fraction, however, a 55 kDa polypeptide was strongly recognized by this antibody. In the PM, an additional band was detected at approximately 67 kDa. No bands were detected in the endosome by Western blotting. To ensure that the bands detected were specifically recognized by the anti-enigma antibody, a competition study was performed (Fig.12B). When the antibody was preincubated with enigma N-terminal peptide (lanes 5-8), binding to the three major polypeptides (55, 67 and 93 kDa) was competed for. Preincubation with enigma C-terminal peptide did not compete for antibody binding (lanes 9-12). Since the yeast two

hybrid system demonstrated an association between enigma and exon 16 of the insulin receptor (Wu and Gill, 1994), immunoprecipitation of the insulin receptor from PM and GE fractions (isolated after insulin injection) and immunoblotting with anti-enigma was carried out to determine if this interaction occurred in our *in vivo* system (Fig.13). Only the 67 kDa polypeptide was observed to be in association with the insulin receptor at 5 min after insulin injection. Surprisingly, this association occurred both in the PM and the GE fraction at this time (Fig.13B). Immunoprecipitation of enigma from the GE fraction, followed by immunoblotting with anti-enigma antibodies, demonstrated that the 67 kDa polypeptide was present in this fraction, at low levels undetectable by Western blotting.

Discussion

A physiological target of insulin action is liver parenchyma with the endocrine pancreas positioned anatomically to deliver the initial bolus of insulin to hepatocytes enriched with insulin receptors. Remarkably, liver parenchyma is also an anatomical site, expressing high concentrations of EGF receptors (O'Keefe *et al.*, 1974). The high efficiency clearance of both EGF and insulin (> 80% of an injected dose) by the liver after intraportal injections results in minimal dilution of the administered ligand by other tissues (St. Hilaire *et al.*, 1983; Lai *et al.*, 1989a). Although endothelial and Kupffer cells are also present in the liver, it is the hepatocyte which harbors the highest concentration of EGF and insulin receptors which bind EGF and insulin specifically (Bergeron *et al.*, 1979; Dunn and Hubbard, 1984, Bergeron *et al.*, 1985). The nearly equal numbers of these two receptors in the hepatocytes of male animals is of additional significance by making comparative studies between EGF and insulin action possible in a physiological context.

Hepatocytes of adult rodents and humans have long life spans and rarely divide under normal conditions (less than 1:1000 hepatocytes is in S-phase at any time) (Fausto *et al.*, 1995) making it possible to observe any potential EGF or insulin induced stimulation of mitogenic responses. The ability of the liver to respond to mitogenic stimuli such as, partial hepatectomy and EGF stimulation appears to be diminished in aged rats as compared to young adults (Liu *et al.*, 1996). As a consequence of these observations, the rats used in this study are young adults readily responsive to EGF as well as insulin treatment.

One of the most rapid responses to ligand stimulation, is the redistribution of the ligand/ receptor complexes from the cell surface to the endosomal compartment. The role of internalization and the mechanisms involved in regulating internalization remain unclear. The existence of established methods of isolating PM and endosomes from the liver (Kay *et*

al., 1986; Wada *et al.*, 1992) provides us with the means to study the internalization of the EGF and insulin receptors both in its initial stages at the PM and during its passage through the endosomal compartment. Although several methods were available for the isolation of PM from rat liver the procedure that was employed in this study was established by Hubbard *et al.* (1983). This preparation, unlike the others results in a high percentage of sinusoidal PM. This is advantageous since it is on this surface (in addition to the lateral membrane) that the EGF and insulin receptors are localized. Since coated pits and vesicles are present in this preparation, then our studies should be relevant to the mechanisms whereby receptors may be targeted to these initial stages of the endocytic system. The procedure used for GE isolation yields both early and late endosomal structures. In order to facilitate the isolation of this compartment, the animals were fasted overnight, to deplete liver glycogen stores which interfere with isolation procedures.

The kinetics of EGF and insulin receptor loss from the PM appears to be similar based on the $t_{1/2}$ of internalization. The rapid loss of cell surface receptors (~50% of internalizing receptors in < 1 min), in response to ligand binding, suggests that these receptors are 'primed' for internalization. It is unlikely that this represents receptor preclustering in coated pits since this has been excluded with both the EGF and insulin receptors in liver parenchyma and cultured cells (Bergeron *et al.*, 1985). An alternate possibility is that the cell surface receptors are in close proximity to molecules involved in mediating internalization. Lending support to this idea is the fact that the molecules postulated to function in mediating the internalization of the EGF and insulin receptors, AP-2 and enigma respectively, are found constitutively at the PM (Fig.10 and Fig.12A) and thus in a position to interact rapidly with the activated receptors.

Although the kinetics of internalization are the same for both receptors under study, the very different extent of internalization (~75% for EGF receptor, ~34% for insulin receptor) suggests that ligand-mediated receptor endocytosis occurs through a high affinity endocytic system that is both specific and saturable. If this is the case, then neither AP-2 nor enigma can be regulating the extent of internalization since they are not completely redistributed to the endosome in association with the receptors. In addition, several studies have demonstrated a late time of association between the EGF receptor and AP-2 in response to EGF stimulation in cultured cells. A small amount of adaptins were present in EGF receptor immunoprecipitations obtained from A431 cells incubated at 4°C with EGF. A temperature shift to 37°C resulted in an increase amount of alpha adaptins in the immunoprecipitates. Association was found 2-3 min of the start of the 37°C incubation, reached a maximum at 10-14 min., and then declined. Clathrin was not found in EGF receptor immunoprecipitations (Sorkin and Carpenter, 1993). Studies done on NIH 3T3

cells expressing the human EGF receptor indicate a maximal association of AP-2 with the immunoprecipitated EGF receptor occurred at 6-8 min following a shift to 37°C (Sorkin *et al.* 1995). Boll *et al.* (1995) did similar experiment using mouse B82L cells transfected with the human EGF receptor and demonstrated that the maximal association between the EGF receptor and AP-2 occurred after 20-60 min of rewarming. These data are consistent with the present observation that it is in the endosomal fraction that AP-2 association with the EGF receptor is greatest (Fig.11B). Such late times of association between AP-2 and the receptor raises the question; what stage of internalization is represented by the association of the EGF receptor and AP2? These observations suggest that AP-2 may play a role later in the endocytic process, possibly in vesicle fusion (Beck *et al.*, 1992) or receptor sorting (Chin *et al.*, 1989).

The LIM domain protein enigma has been demonstrated to bind the internalization domain of the insulin receptor, i.e. exon 16, in yeast two hybrid system (Wu and Gill, 1994). Our data demonstrates an association, but not of the expected 55 kDa protein, corresponding to enigma, but to a specific 67 kDa protein recognized by the antibody to enigma. The association is seen at 5 min in both the PM and endosomal fractions and is inconsistent with a role early in endocytosis. The precise function of AP-2 and enigma in EGF and insulin signaling remains to be elucidated.

Following the loss of the receptor from the plasma membrane, is the accumulation of the receptors into the GE fraction. The level of receptor in this fraction, consisting of early and late endosomes, is at its highest between 15-30 min for EGF and 5-30 min for insulin. Between 30 and 60 min, about 40% of the internalized receptors are lost from endosomes which are isolated by this procedure. There is no observable receptor recycling i.e. return of the receptor to the plasma membrane (Fig.8A) which is consistent with studies by Lai *et al.* (1989a) which have shown that receptor saturating doses of EGF resulted in receptor downregulation and not recycling. However, over the time course of our study (60 min) the receptor content of the TP fraction was unchanged. Therefore, the loss of receptors from the GE fraction beginning at 15 min suggests that the receptors have moved on to another pre-lysosomal compartment which does not co-fractionate with the GE fraction. Thus these is a substantial duration in which both the EGF and insulin receptors are present in a structure, the endosome, from which they may carry out further signaling.

Figure 3

Electron micrograph of liver plasma membrane fraction

Freshly isolated rat liver plasma membranes (PM) were processed for electron microscopy as described in Experimental Procedures. This fraction (panel A) illustrates that plasma membranes were from liver parenchyma and consists of the three functionally and morphologically distinct domains found in these cells, i.e. the sinusoidal surface (sin. PM), the lateral surface (lat. PM), as characterized by intracellular junctions (arrowheads), and the bile canalicular surface (BC). A contaminating lipid droplet (L) is indicated. The bar represents 1.0 μm.

A higher magnification (panel B) of a portion of the sinusoidal plasma membrane reveals the numerous coated pits and vesicles (arrows) associated with this plasma membrane domain. Microfilaments (ca 6 nm in diameter) are prominent (arrowheads). The bar represents 0.5 μm.

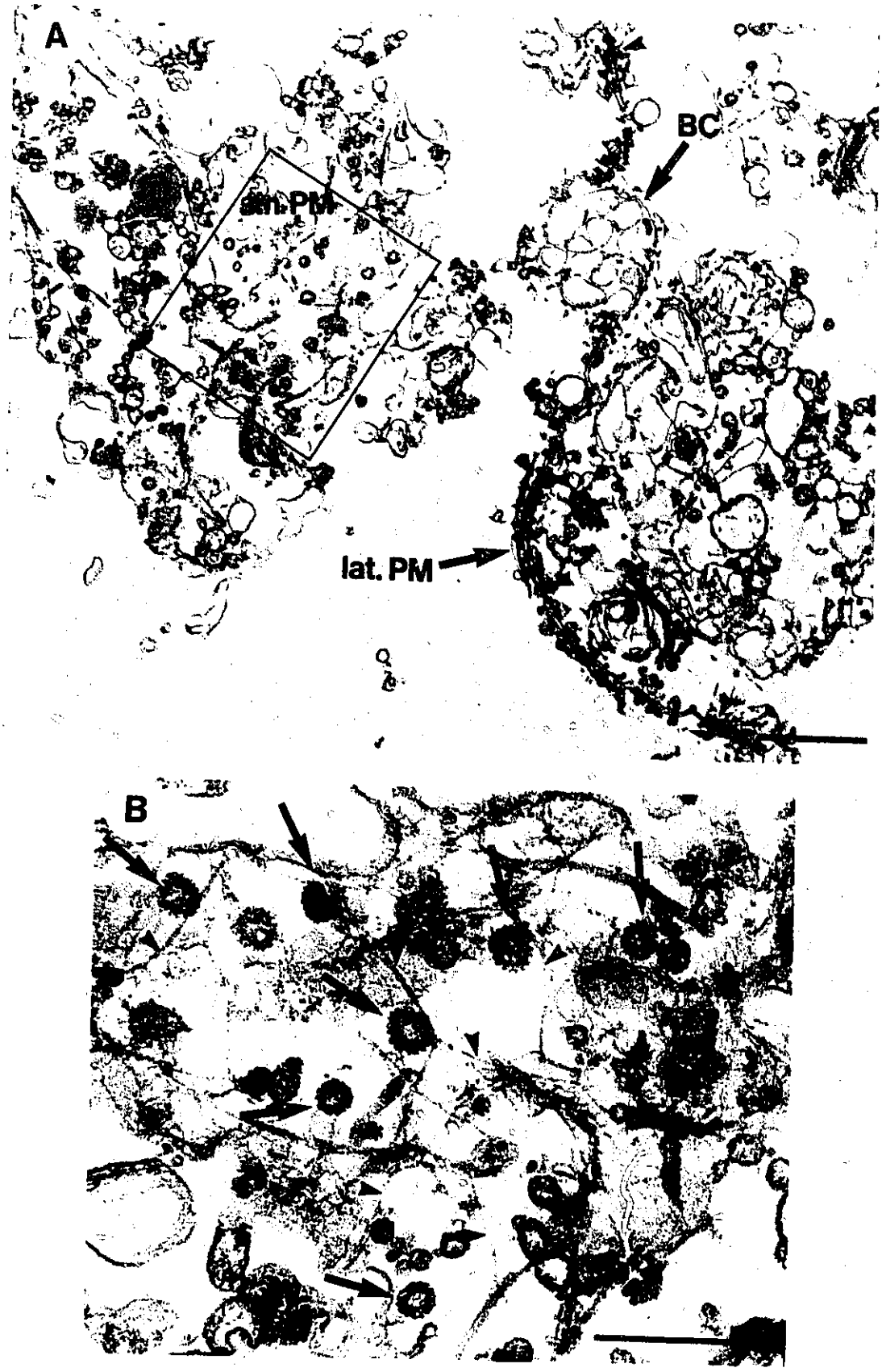

A
BC
lat. PM
B

Figure 4

Electron micrograph of the liver endosomal fraction

The endosomal fraction (GE) was isolated from rat liver and was immediately processed for electron microscopy as described in Experimental Procedures. This fraction consists primarily of endosomes (E) filled with lipoprotein particles and Golgi saccules (Gs). The bar represents 0.5 μm.

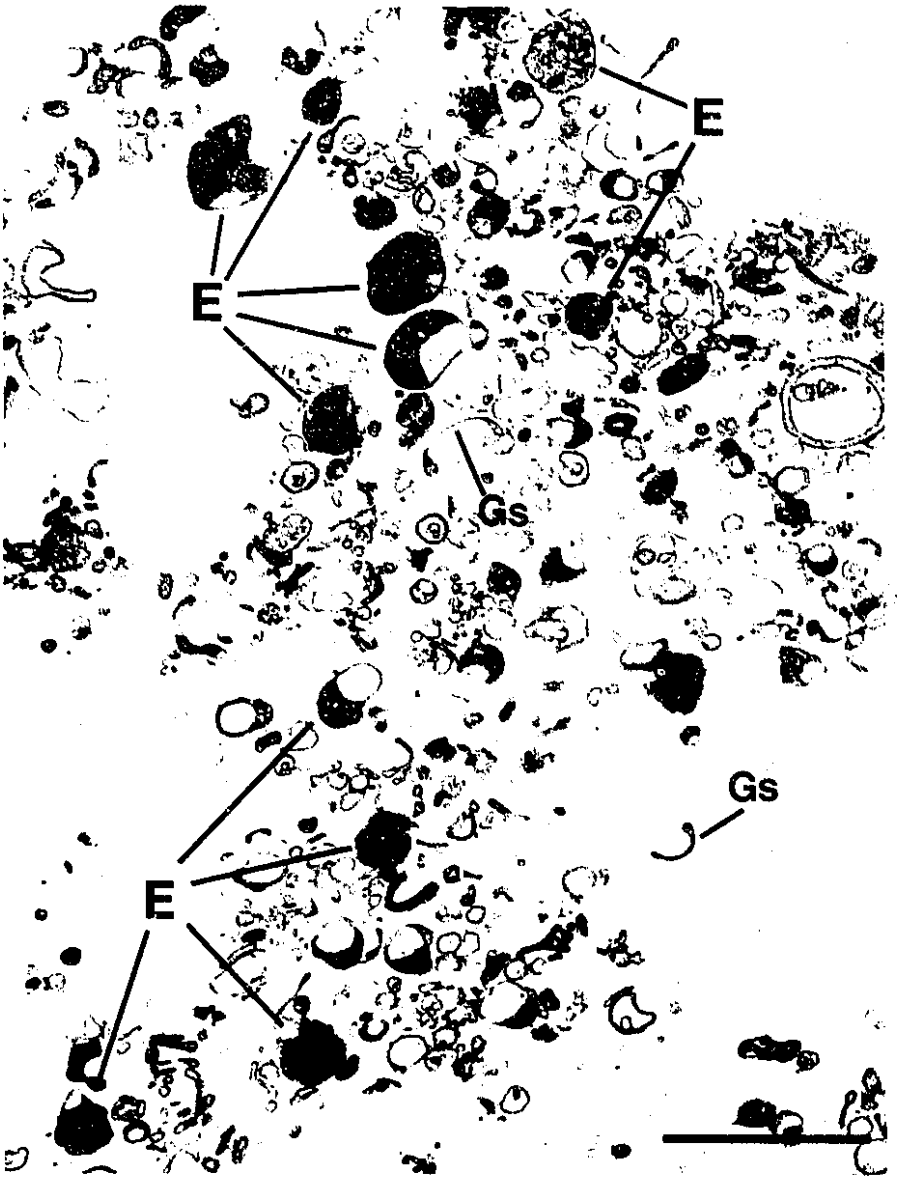

E
E
E
Gs
Gs

Figure 5
SDS-PAGE of isolated rat liver fractions

Proteins (25 μg) from isolated liver fractions were resolved by SDS-PAGE on an 8% resolving gel. Polypeptides were visualized by Coomassie blue staining. Lane 1, homogenate; lane 2, cytosol; lane 3, total particulate; lane 4, plasma membrane; lane 5, endosomes. The arrowheads indicate high molecular weight polypeptides (mobilities of approximately 190, 200 and 234 kDa) which were used as diagnostic indicators of purified PM. Molecular mass markers are indicated on the left.

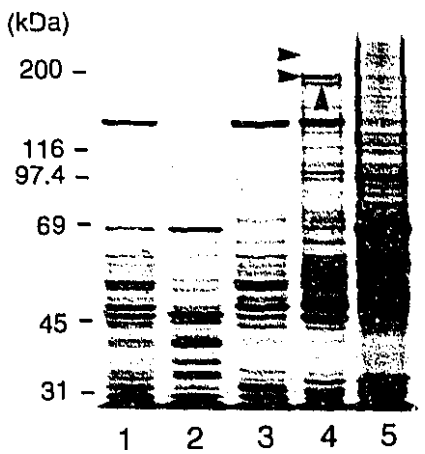
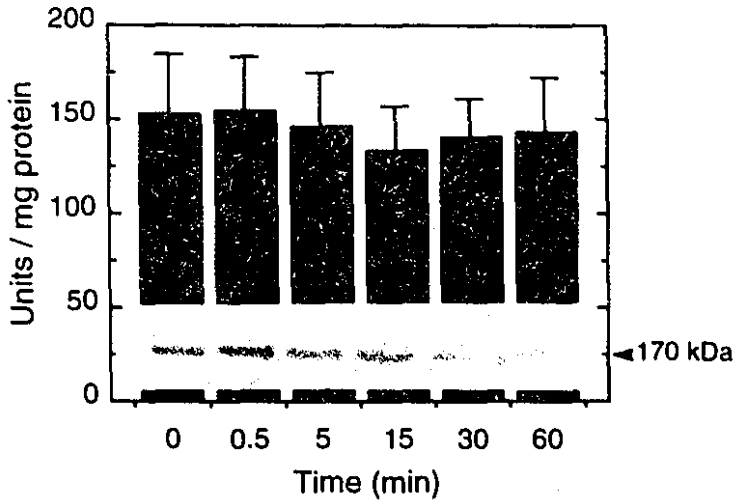

(kDa)
200 –
116 –
97.4 –
69 –
45 –
31 –
1
2
3
4
5

Figure 6

Receptor content in total particulate fractions after ligand injection

Total particulate fractions were isolated from rat liver homogenates at the indicated times after the injection of either EGF (panel A) or Insulin (panel B). Two hundred μg of cell fraction protein was applied to each lane and resolved by SDS-PAGE. After transfer to nitrocellulose, Western blots were done using anti-EGF receptor antibodies (panel A) or anti-Insulin receptor antibodies (panel B). These bands were visualized using [^{125}I]-labeled secondary antibodies and exposed to X-ray (insets). The mobilities of the EGF receptor (170 kDa; panel A) and the β-subunit of the insulin receptor (94 kDa; panel B) are indicated on the right. The radioactive EGF and Insulin receptor bands were quantified using phosphoimager analysis. The data represents the mean of 3 separate experiments ± SD.

A. EGF receptor



B. Insulin receptor

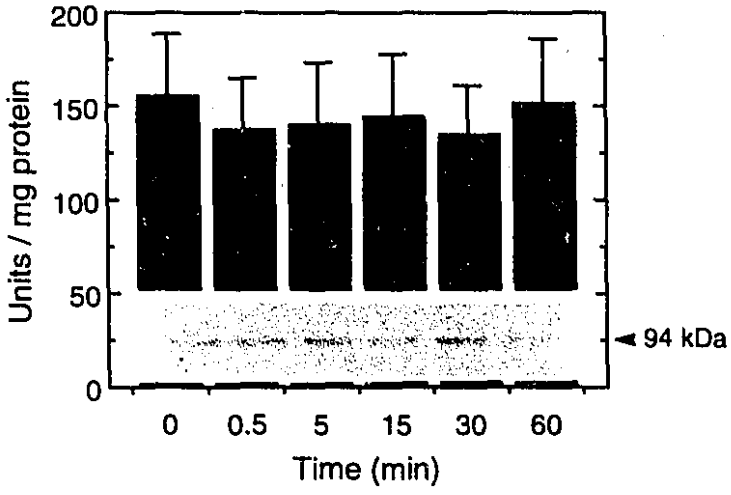
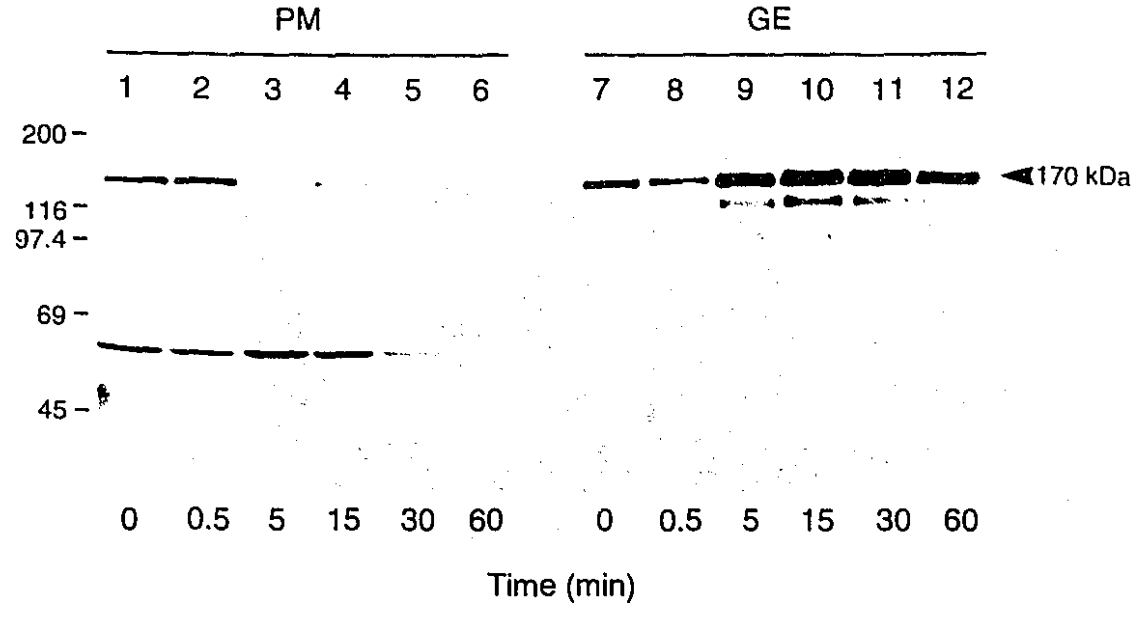


Figure 7

Receptor internalization in response to ligand stimulation

Plasma membrane (PM) and endosomes (GE) were isolated at the indicated times after the portal vein injection of 10 μg/100 g body weight EGF (panel A) or 15 μg/100 g body weight Insulin (panel B). 50 μg protein of PM and 25 μg protein of GE were resolved by SDS-PAGE on an 8% resolving gel, transferred to nitrocellulose and Western blots were performed using anti receptor antibodies. In panel A, anti-EGF receptor antibodies were used and the arrow indicates the 170 kDa band corresponding to the mobility of the EGF receptor. In panel B, antibodies recognizing the β-subunit of the insulin receptor were used. The arrow points to the 94 kDa β-subunit of the insulin receptor. Molecular mass standards are indicated at the left.

A. EGF receptor



B. Insulin receptor

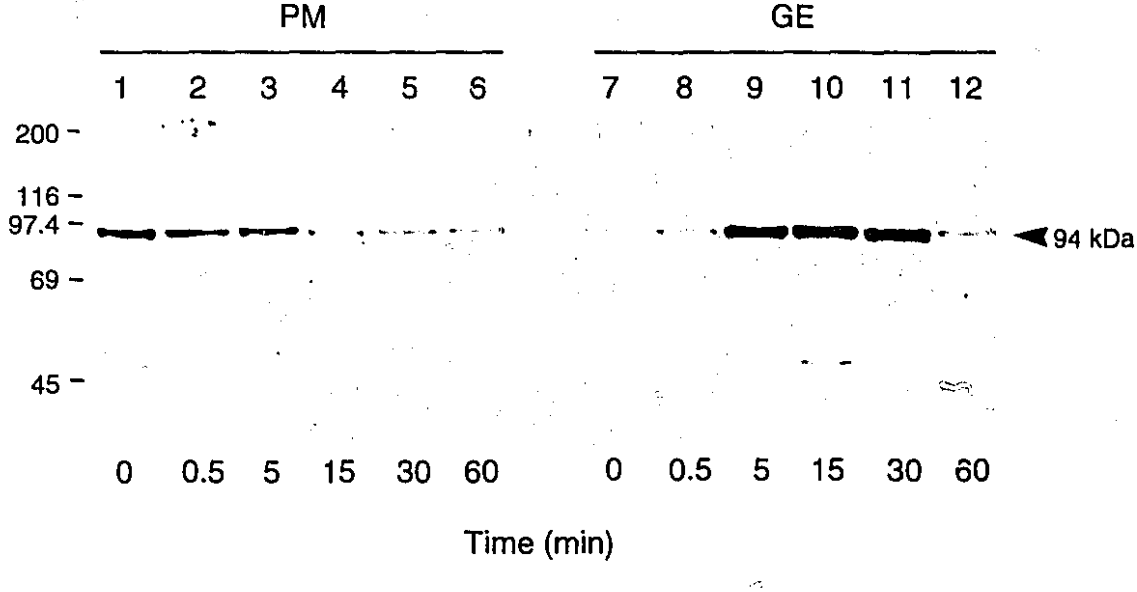
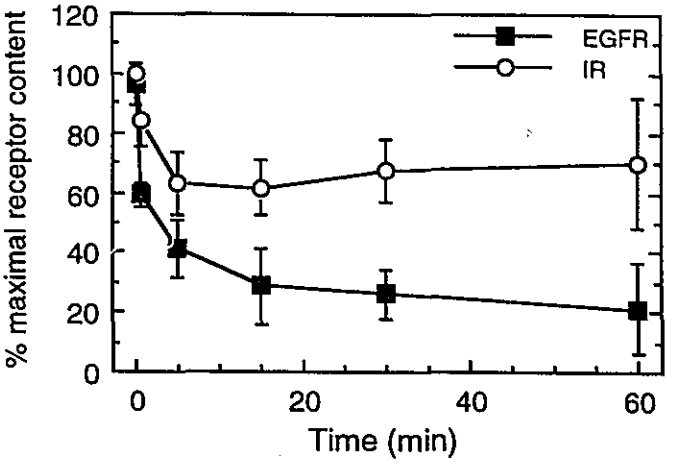


Figure 8

Quantification of receptor redistribution in response to ligand stimulation

Western blotting of liver plasma membrane (PM; panel A) and endosomal (GE; panel B) fractions were carried out as described in Figure 7. The [^{125}I]-labeled bands of 170 kDa and 94 kDa, corresponding to the EGF receptor (—■—) and the β-subunit of the insulin receptor (—o—), respectively, were quantified by phosphoimager analysis. The values are expressed as a function of maximal receptor content, found at 0 time in the PM and at 15 min in the GE fraction. The data represents 4 separate experiments ± SD for the EGF receptor, and 3 separate experiments ± SD for the insulin receptor.

A. Receptor content in PM



B. Receptor content in GE

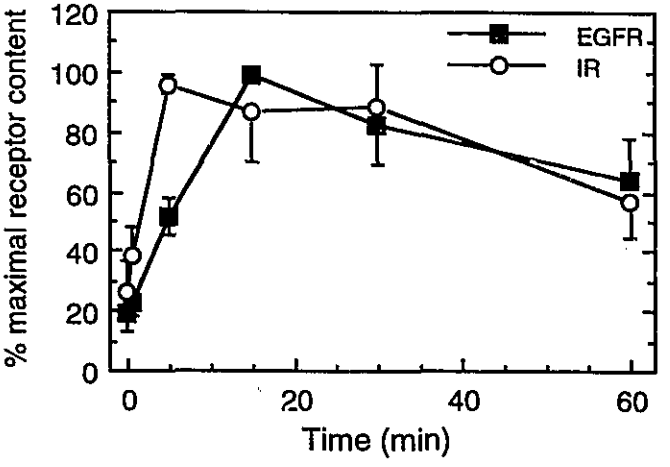
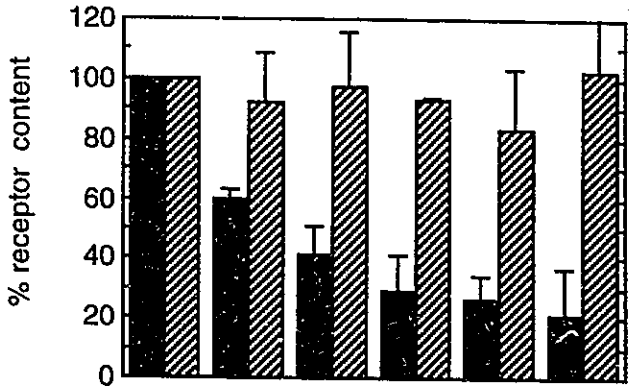


Figure 9

EGF receptor redistribution in response to EGF and Insulin stimulation

Plasma membrane (PM; panel A) and endosomal (GE; panel B) fractions were isolated at the indicated times after EGF (■) or insulin (▨) injection. 50 µg protein of PM and 25 µg protein of GE were resolved by SDS-PAGE on an 8% resolving gel, transferred to nitrocellulose and immunoblotted using anti-EGF receptor antibodies. The 170 kDa [^{125}I]-labeled band corresponding to the EGF receptor was quantified by phosphoimager analysis and the results expressed as a percentage of receptor content in the control fraction (0 time). The data represent the mean ± SD (n=4 for EGF; n=3 for insulin).

A. PM


% receptor content
120
100
80
60
40
20
0

B. GE

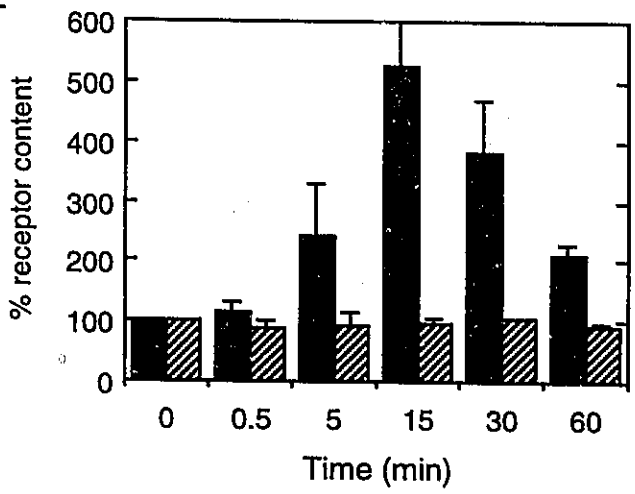
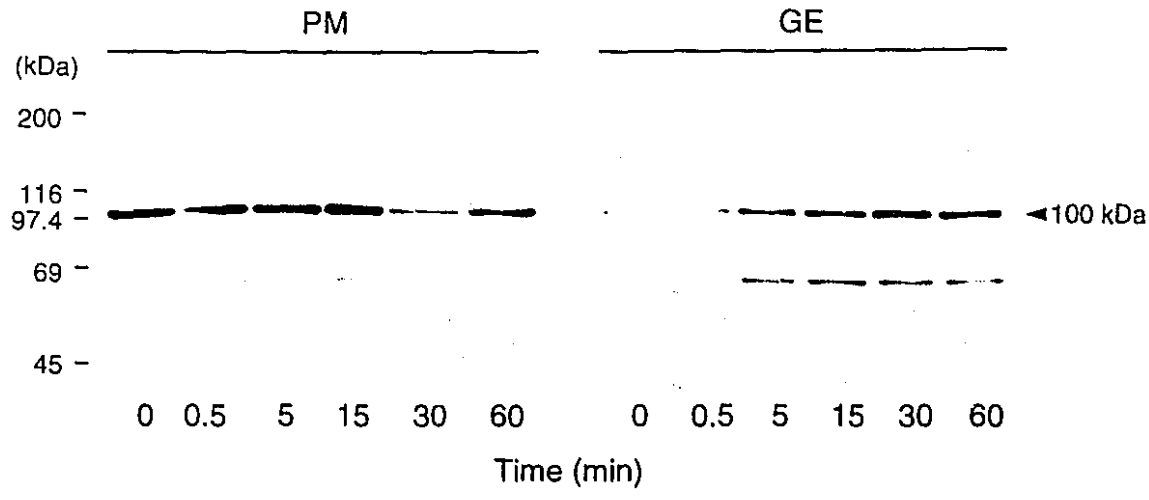

% receptor content
600
500
400
300
200
100
0
0
0.5
5
15
30
60
Time (min)

Figure 10

Distribution of the adaptor protein, AP-2, in response to ligand stimulation

Plasma membrane (PM) and endosomal (GE) fractions were isolated at the indicated times after the portal vein injection of 10 μg/100 g body weight EGF (panel A) or 15 μg/100 g body weight Insulin (panel B). 50 μg protein of PM and 25 μg protein of GE were resolved by SDS-PAGE on an 8% resolving gel and transferred to nitrocellulose. Western blots using an anti-AP-2 antibody (AC1-M11; Robinson, 1987) were carried out. The band corresponding to the expected mobility of AP-2 (~100 kDa) is indicated by the arrowhead. Molecular mass markers are indicated on the left.

A. EGF injected



B. Insulin injected

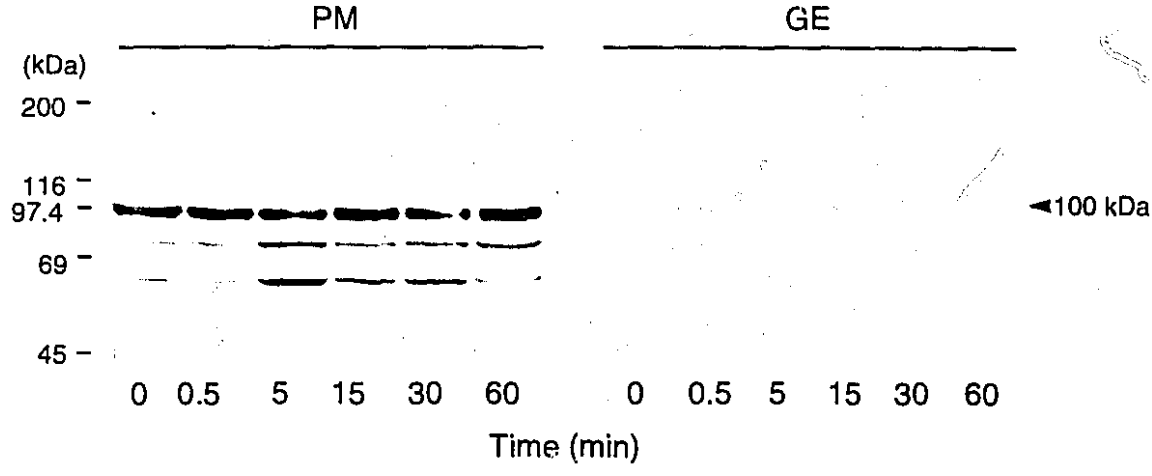
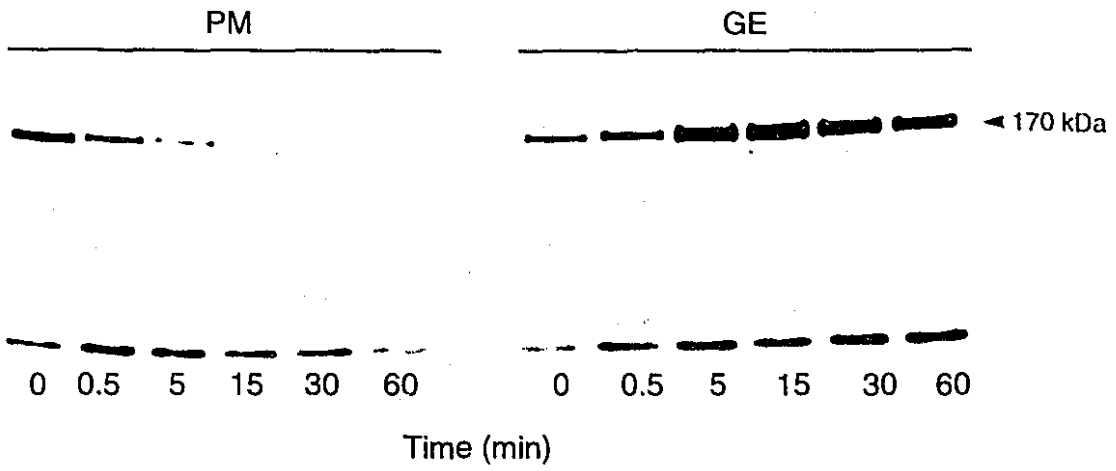


Figure 11

Association of AP-2 with the EGF receptor

Plasma membrane (PM) and endosomes (GE) were isolated at the indicated times after the portal vein injection of 10 μg/100 g body weight of EGF. The EGF receptor was immunoprecipitated from 500 μg of PM and 200 μg of GE fraction using the IgG 151 BH6 monoclonal anti-EGF receptor antibody (Lai et al., 1989). The immunoprecipitated proteins were resolved by SDS-PAGE and transferred to nitrocellulose. The membranes were blotted with anti-EGF receptor antibodies (panel A) or anti-AP-2 antibodies (panel B). The mobility of the EGF receptor (170 kDa; panel A) and the AP-2 (~100 kDa; panel B) are indicated.

A. IP: α-EGF receptor
Blot: α-EGF receptor



B. IP: α-EGF receptor
Blot: α-AP-2

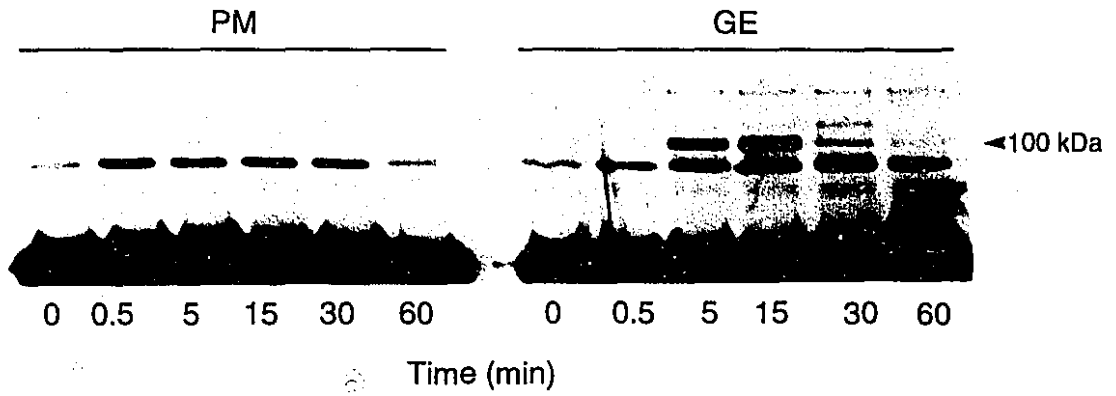


Figure 12

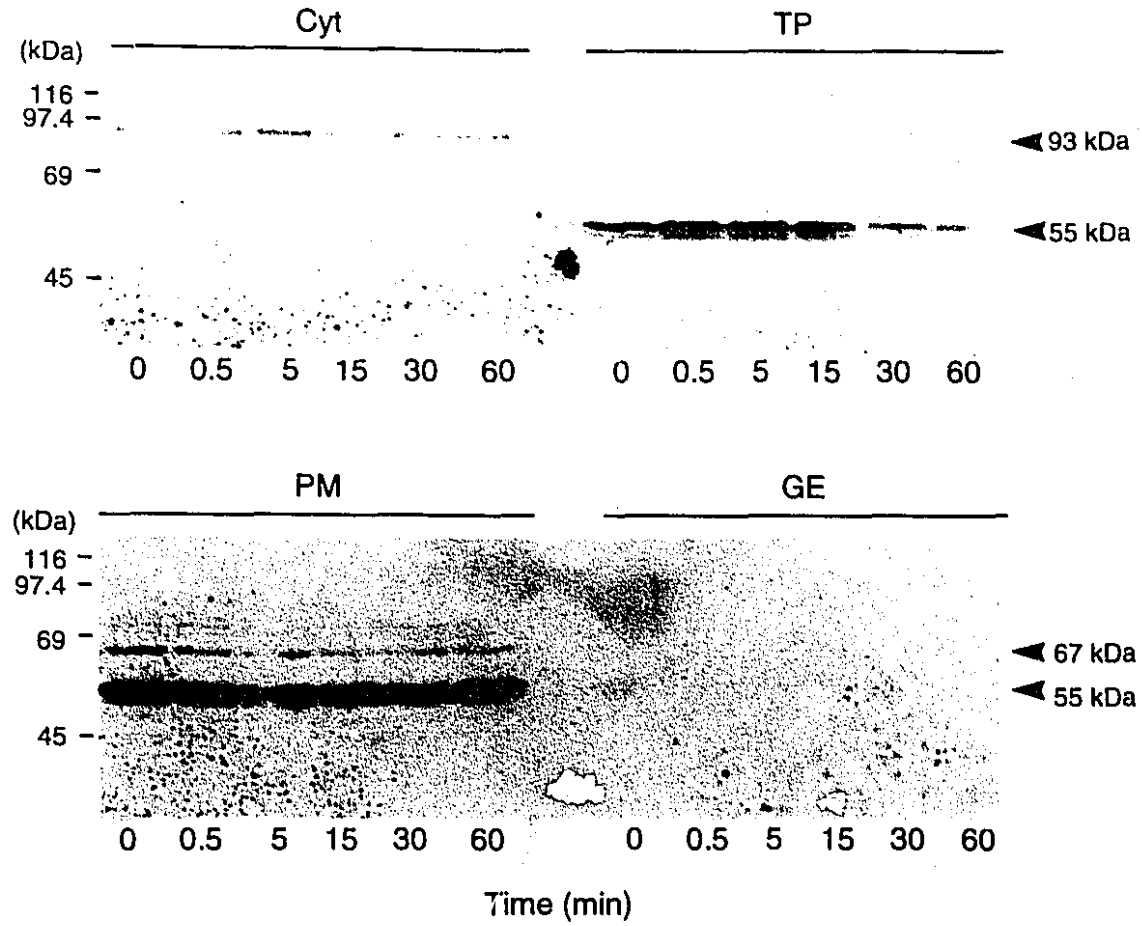
Distribution of the LIM domain protein, enigma, in response to insulin stimulation

Rat liver fractions, cytosol (Cyt), total particulate (TP), plasma membrane (PM) and endosomes (GE), were isolated at the indicated times after the portal vein injection of 15 μg/100 g body weight of insulin. The proteins of the Cyt, TP, PM and GE fractions (300 μg, 200 μg, 50 μg, and 25 μg, respectively) were resolved by SDS-PAGE on 8% resolving gels. An antibodies raised to the N-terminus of enigma was used for immunoblotting (panel A). The bands recognized by this antibody (Cyt, 93 kDa; TP, 55 kDa; PM, 55 and 67 kDa) are indicated on the right.

To identify non-specific interactions between the anti-enigma antibody and polypeptides in rat liver fractions, blots of the fractions described above (Cyt, TP, PM, GE) were immunoblotted with anti-enigma antibody preincubated in the absence (panel B, lanes 1-4) or presence of 20 μg of enigma N-terminal peptide (panel B, lanes 5-8) or enigma C-terminal peptide (panel B, lanes 9-12) as described in Experimental Procedures. Cyt - lanes 1, 5 and 9; TP - lanes 2, 6 and 10; PM - lanes 3, 7 and 11; GE - lanes 4, 8 and 12.

The molecular mass standards are indicated on the left in both panels.

A


Cyt
TP
(kDa)
116
97.4
69
45
93 kDa
55 kDa
0 0.5 5 15 30 60
0 0.5 5 15 30 60
PM
GE
(kDa)
116
97.4
69
45
67 kDa
55 kDa
0 0.5 5 15 30 60
0 0.5 5 15 30 60
Time (min)

B

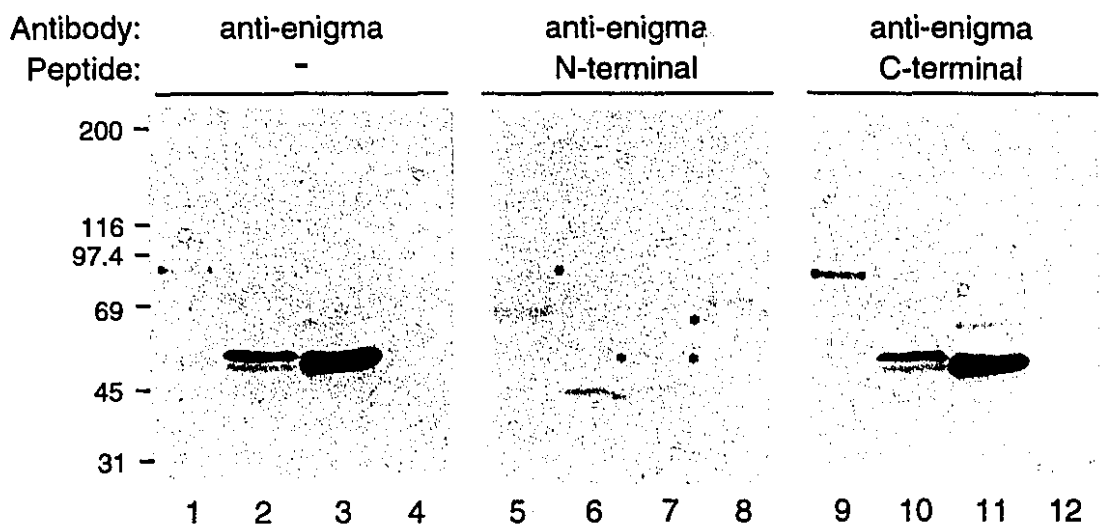
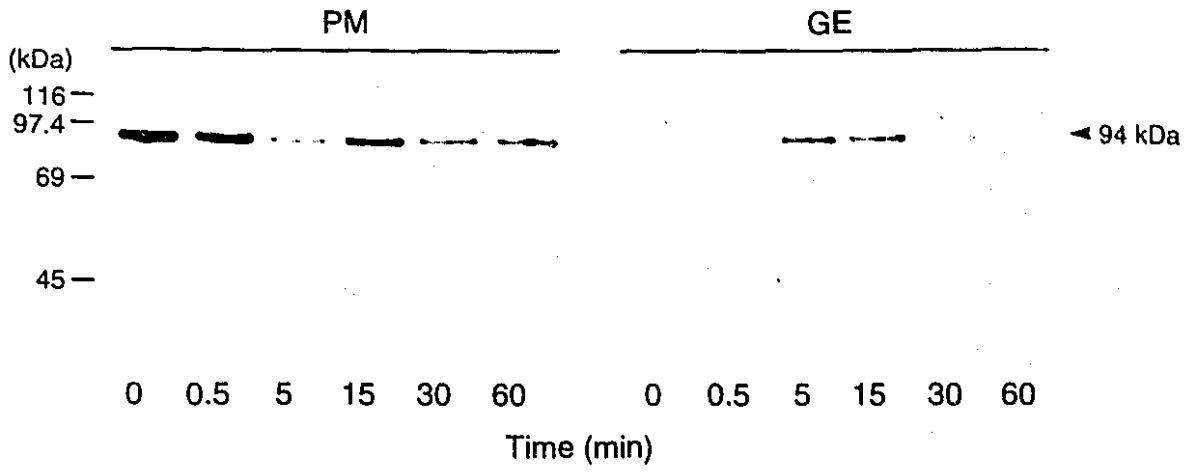

Antibody:
Peptide:
anti-enigma
-
anti-enigma
N-terminal
anti-enigma
C-terminal
200
116
97.4
69
45
31
1 2 3 4 5 6 7 8 9 10 11 12

Figure 13

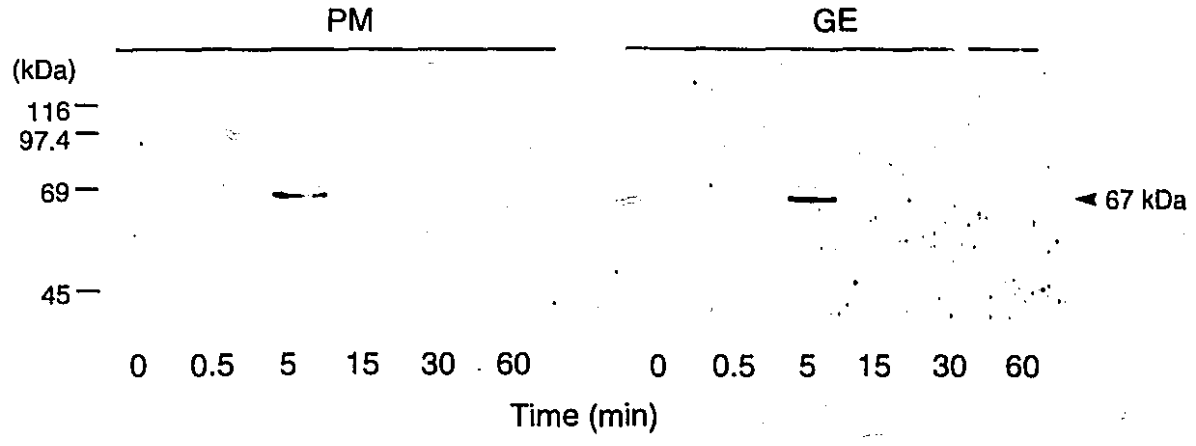
Association of LIM domain protein, enigma, with the insulin receptor

PM (500 μg) and GE (200 μg) fractions isolated after the injection of insulin were immunoprecipitated with anti-insulin receptor antibodies. The immunoprecipitated proteins were resolved by SDS-PAGE, and Western blotted with anti-insulin receptor antibody (panel A). The membrane was stripped and reprobed using the anti-enigma antibody raised to the N-terminus (panel B). The 94 kDa β-subunit of the insulin receptor and the 67 kDa protein recognized by the anti-enigma antibody are indicated on the right of panel A and B, respectively. Molecular mass markers are indicated on the left.

A. IP: α-Insulin Receptor
Blot: α-Insulin Receptor



B. IP: α-Insulin Receptor
Blot: α-Enigma (N-terminal)



Chapter II

The EGF Receptor - Signaling and Compartmentalization

The interaction of EGF or insulin with their respective cell surface receptors results in a number of rapid events, including receptor internalization (Chapter I) and the activation of the receptors' intrinsic tyrosine kinase. The activated receptor tyrosine kinase catalyzes auto-phosphorylation, creating binding sites for downstream signal transduction molecules containing SH2 and/or PTB domains. These proteins serve as second messengers to transduce signals initiated at the plasma membrane.

The protein SHC was identified as a major physiological substrate of the EGF receptor (Wada *et al.*, 1992; Donaldson and Cohen, 1992) and appears to be involved in mitogenic signal transduction from the activated EGF receptor. Overexpression of SHC has been shown to lead to cellular proliferation and transformation (Pelicci *et al.*, 1992), and co-immunoprecipitation studies has uncovered a link between SHC tyrosine phosphorylation and ras activation though the adaptor protein GRB2 and the associated guanine nucleotide exchange factor SOS (Rozakis-Adcock *et al.*, 1992). With respect to insulin action, the major insulin receptor substrate, IRS-1 has been identified and purified from liver parenchyma (Rothenberg *et al.*, 1991). Using CHO cells (Skolnik *et al.*, 1993b), COS-1 cells (Baltensperger *et al.*, 1991) and fibroblasts (Pronk *et al.*, 1993), transfected with the insulin receptor, receptor induced IRS-1 phosphorylation has also been linked to the ras pathway, through IRS-1 recruitment of GRB2 and SOS. The involvement of SHC has also been demonstrated in these cells following insulin receptor activation.

The convergence of the signal transduction pathways from these two receptor tyrosine kinases is remarkable since, *in vivo*, their physiological responses in the liver are quite different. The acute actions of insulin in the adult liver are related primarily to metabolic effects such as the regulation of glycogen formation and the consequent maintenance of blood glucose homeostasis (see Introduction). Although the role of EGF is less well understood, EGF is believed to be involved in organ repair through increased mitogenesis (see Introduction),

A second rapid response to receptor activation is the internalization of the receptor/ ligand complex into endosomes. This has generally been believed to represent a mechanism of ligand clearance and degradation and signal attenuation. On the other hand, internalization may be relevant to receptor bioactivity. The rapidity of internalization, and the fact that there may be a significant lag before receptor degradation and deactivation, suggests that signal transduction and regulation may take place in the endosome prior to entry into the lysosome.

To evaluate the relationship between signaling and compartmentalization *in vivo*, isolated hepatic subcellular fractions were evaluated for the presence of ligand dependent tyrosine phosphorylated and/or receptor associated signaling molecules.

Results

The injection of a single bolus EGF or insulin into the portal circulation of young adult rats leads to the rapid autophosphorylation of their respective receptor kinases in the liver plasma membranes. This increase in phosphorylation was coincident with the rapid internalization of the receptors into the intracellular compartment (Khan *et al.*, 1989; Lai *et al.*, 1989b; Wada *et al.*, 1992; see Chapter I). Despite the similarity in rapidity of receptor internalization (Chapter I) and the receptor saturating doses used in both cases (10 μg/100 g body weight EGF and 15 μg/100 g body weight insulin), clear differences were observed in the tyrosine phosphorylated proteins induced by ligand stimulation in the rat liver plasma membrane (PM) and endosomal (GE) fractions (Fig.14). The administration of EGF resulted in the strong tyrosine phosphorylation of a 170 kDa protein and a 55 kDa protein, within 30 sec of ligand administration (Fig.14A). The 170 kDa protein has been previously been shown to be the EGF receptor (Wada *et al.*, 1992). In the GE fraction, these proteins were found to be maximally phosphorylated at 15 min. Other less prominent proteins (66, 46 and 42 kDa) appeared to be phosphorylated in the GE fraction in response to EGF (asterisks). In contrast, insulin administration led to the rapid (within 30 sec) tyrosine phosphorylation of the 94 kDa β-subunit of the insulin receptor, but this level of phosphorylation was not maintained in the GE as the receptor was internalized (Fig.14B).

The major protein tyrosine phosphorylated in response to EGF appears to be of 55 kDa which corresponds to the molecular mass of the adaptor protein SHC. Hence, SHC recruitment to hepatic plasma membranes and endosomes was evaluated as a function of EGF and insulin administration *in vivo* (Fig.15). Low levels of two of the SHC isoforms, 46 and 55 kDa, were found to associate with the PM in control animals (at 0 time) while only the 55 kDa form was found in association with control endosomes (0 time). In response to EGF stimulation, the 55 kDa isoform of SHC appears to be recruited to the PM. In the GE fraction, EGF stimulation resulted in a stronger and prolonged signal for the 46 and 55 kDa SHC isoforms, and the additional recruitment of the third, 66 kDa isoform of SHC (Fig.15A). In contrast, the recruitment of SHC to plasma membrane following insulin stimulation was markedly lower, and no recruitment could be observed in the GE (Fig.15B).

The SH2/SH3 containing adaptor protein GRB2 has been demonstrated to associate with tyrosine phosphorylated SHC (Rozakis-Adcock *et al.*, 1992). In addition, GRB2 has been shown to associate with the motif at phosphotyrosine (PY)1068, and to a lesser extent PY^{1086}, of the tyrosine phosphorylated EGF receptor (Batzer et al, 1994). As a

consequence, the recruitment of GRB2 to the PM and GE fractions was determined in response to EGF and insulin injection *in vivo*. After EGF stimulation, GRB2 was found to be recruited primarily to the endosomal fraction, with very little association found at the PM (Fig.15C). In response to insulin no detectable GRB2 was found in either of the membrane compartments (Fig.15D).

To demonstrate a physical association between the phosphorylated EGF receptor, SHC, GRB2 and SOS in the endosome, EGF receptor co-immunoprecipitation studies were undertaken (Fig.16). The EGF receptor was clearly shown to be phosphorylated (A), and in association with the phosphotyrosine modified 55 kDa isoform of SHC (B) as well as GRB2 (C) and mSOS (D), in an EGF dependent manner. It is interesting to note that while all isoforms of SHC appear to be in association with the EGF receptor, only the 55 kDa form becomes significantly tyrosine phosphorylated (compare Fig.16A, lane 4 with 16B, lane 4).

Phosphoimager analysis was carried out on the EGF receptor, SHC and GRB2 in the plasma membrane and endosomal fractions to determine the quantitative contribution of these compartments to signal transduction by the EGF receptor (Fig.17). The results are expressed in phosphoimager unit / g liver which takes into account the yields and recoveries of the respective subcellular fractions (see Experimental Procedures). Ligand dependent translocation of the EGF receptor from the cell surface to endosomes was rapid and extensive, with greater than 60 % of the receptors internalizing. The level of EGF receptor present in the endosome remains high (between 2 and 4 fold that seen at 0 time) up to 60 min post injection (Fig.17A and Chapter 1). The phosphotyrosine content of the EGF receptor in the PM peaked briefly at 30 sec (Fig.17B). The majority of tyrosine phosphorylated EGF receptor was found in the endosomal fraction, where peak phosphorylation occurred at 15 min and remained above that found in the PM up to 60 min.

The kinetics of EGF dependent recruitment of the 46 and 55 kDa isoforms of SHC appeared identical and occurred primarily at the PM within 30 sec of EGF administration (Fig.17C,E). From 15-60 min after EGF injection, the content of SHC in endosomes and PM was identical. However, the phosphorylation of the 55 kDa isoform of SHC appeared higher in the GE fraction (Fig.17D). Determination of the extent of tyrosine phosphorylated of the 55 kDa isoform of SHC at peak times of receptor phosphorylation in the PM (30 sec) or in endosomes (15 min) revealed a significantly higher ($p<0.001$) phosphotyrosine content of SHC in the endosomal fraction (Fig.18). Quantitation of the GRB2 recruitment to the membrane compartment revealed that recruitment and association was largely with endosomes as opposed to PM (Fig.17F).

Although the EGF and insulin receptors are exclusively membrane bound, many of the components of signal transduction pathways, including SHC and GRB2/SOS are primarily cytosolic (although some SHC isoforms associated constitutively with the membranes; Fig.15A). Anti-phosphotyrosine immunoblots were carried out to determine the effect of ligand stimulation on the tyrosine phosphorylation of cytosolic signal transduction molecules (Fig.19). Two polypeptides (55 kDa and 120 kDa) were seen to be phosphorylated in response to EGF within 30 sec of ligand administration. These polypeptides were not phosphorylated in response to insulin stimulation. Following immunoprecipitation of the cytosol with anti-SHC antibodies, this 55 kDa polypeptide was shown to correspond to the 55 kDa isoform of SHC (Fig.20C). Phosphoimager analysis of the 55 kDa isoform of SHC (Fig.20A) and phosphorylated SHC (Fig.19, lanes 1-6) in the cytosol was carried out to determine the extent of phosphorylation of SHC in this compartment in response to EGF stimulation. A three fold increase in the amount of phosphorylated SHC was seen and was maintained at an elevated level up to 60 min (Fig.20B). Immunoprecipitation of the cytosolic fractions with anti-SHC antibodies and immunoblotting with antibodies to GRB2 and SOS (Fig.20D,E) revealed that these signal transduction molecules were present as an EGF-dependent complex with phosphorylated SHC, and that this cytosolic complex persisted up to 60 min.

Discussion

The assessment of compartmentalization during receptor signaling in the rat liver has revealed specific regulation of EGF and insulin induced signal transduction *in vivo* during the early phases (0-15 min) of EGF receptor internalization. Interestingly, despite the equal numbers of receptors for EGF and insulin on the rat hepatocyte (Burgess et al, 1992; Wada et al,, 1992), and the receptor saturating doses of ligand used, both the extent of receptor internalization (Chapter I) and tyrosine phosphorylation of receptor and substrates observed were very different between these two receptors.

In response to EGF stimulation, rapid (within 30 sec) tyrosine phosphorylation and internalization of the receptor was observed (Fig.17 and Chapter I). The level of receptor phosphorylation was maintained in the endosomal compartment suggesting that substrate recruitment could continue at this site. Indeed, SHC was rapidly recruited to the PM (46 and 55 kDa forms of SHC) and internalized along with the receptor into the endosomal compartment, where further recruitment (in particular the 66 kDa form) appeared to take place. Because of the differential use of translation initiation sites (46 and 55 kDa isoforms) and alternate splicing(66 kDa form)(Pelicci *et al.*, 1992), the proteins differ in

their amino terminal sequences. However, even the 46 kDa version of SHC contains the PTB domain and is capable of interacting with the phosphorylated EGFR. The precise role of the various isoforms of SHC in EGF receptor signaling still remains to be determined, although the specific recruitment of the 66 kDa isoform to the endosome suggests a function in this compartment.

The 55 kDa isoform of SHC was found to be the major substrate of the EGF receptor *in vivo* (Wada *et al.*, 1992; Donaldson and Cohen, 1992; Ruff-Jamison *et al.*, 1993) and although a proportion of SHC proteins appeared to be tyrosine phosphorylated at the PM, the level of SHC phosphorylation was highest in the endosome (Fig.18). The low level of phosphorylated SHC found at the PM may be the result of the rapid dissociation of the phosphorylated SHC from the receptor. Indeed, a cytosolic pool of phosphorylated SHC can be observed at this time. The high levels of SHC tyrosine phosphorylation in the endosome is consistent with the continued phosphorylation of SHC by the EGF receptor during receptor internalization. There is evidence that the EGF receptor retains functional activity in early endosomes and prelysosomal compartments (McCune and Earp, 1989; Kay *et al.*, 1989; Wada *et al.*, 1992). The prolongation of receptor kinase activity and high degree of SHC phosphorylation in this compartment suggests that new SH2 and PTB containing proteins may be recruited during internalization. This may lead to increased signaling and/or the initiation of other signal transduction pathways at this time and location.

The adaptor protein GRB2, in association with SOS, has been shown to be involved in linking the EGF receptor to the Ras signaling pathway (Rozakis-Adcock *et al.*, 1993). In response to EGF stimulation *in vivo*, GRB2 was primarily recruited to the endosomal compartment where it appeared in a complex with phosphotyrosine modified SHC, SOS, and the activated EGF receptor. The low levels of GRB2 recruitment to the PM is surprising since a direct physical interaction between activated receptors, GRB2/SOS and Ras has been proposed as the mechanism by which Ras is activated in response to growth factors, including EGF. The lack of GRB2 recruitment to the PM may be explained in several ways. It is possible that the preferred site of GRB2 SH2 binding to the EGF receptor (Y^{1068}) is not phosphorylated at the PM, or that only the lower affinity site (Y^{1086}) is phosphorylated, which could explain the low levels of GRB2 observed at the PM in response to EGF stimulation. A second possibility is that although the binding sites on the EGF receptor are present, the presence of a clathrin cage around the activated receptor in both the coated pits and vesicles may interfere with the recruitment of the GRB2/SOS complex. On the other hand, GRB2 has been postulated to be able to associate indirectly with the EGF receptor via its interaction with phosphorylated SHC. Indeed,

studies have demonstrated that the GRB2 SH2 domain displayed a higher affinity for the SHC phosphotyrosine site than either of the EGF receptor sites (Cussac *et al.*, 1994). SHC is recruited to the PM and although a portion is phosphorylated at this site, the major site of SHC phosphorylation is endosomal. Thus the pattern of recruitment of GRB2 to the membrane compartments harboring activated EGF receptor may be a consequence of SHC phosphorylation. Further studies will have to be undertaken to distinguish between these possibilities.

The function of GRB2/SOS recruitment to the endosome is unclear since there is no Ras, the downstream target of SOS action, at this site. However, a cytosolic complex of activated SHC in association with GRB2/SOS was observed within 30 sec of EGF administration and was maintained up to 60 min after EGF injection. It is possible that the association of GRB2 with the EGF receptor, via binding to tyrosine phosphorylated SHC, could lead to the generation of this cytosolic pool of tyrosine phosphorylated SHC/GRB2/SOS complexes free to access and activate Ras at the PM. This proposed function for the cytosolic complex provides a rational for the adaptor protein SHC. Thus endosomal EGF receptor activity would serve to extend signal transduction to Ras, beyond that possible at the PM. In addition, the presence of an active, phosphorylated EGF receptor in the endosome may serve to recruit or phosphorylate other downstream signal transduction molecules, further modulating signaling from the EGF receptor.

Tyrosine kinase activity of the EGF receptor has been demonstrated to regulate whether internalized EGF receptor in the endosomal membrane is recycled back to the PM or targeted into the intravesicular content of multivesicular endosomes for down-regulation. Felder *et al.* (1990) showed that a kinase negative point mutant of the receptor, although capable of accessing the endosome, was not able to gain access the intraluminal vesicle population of multivesicular endosomes. They postulated that the tyrosine phosphorylation of an endosomal substrate by the EGF receptor would regulate this sorting event. The presence of GRB2/SOS may be relevant in the above process, as well as having a role in signal transduction.

In addition to the phosphorylation of SHC in response to EGF stimulation, a 120 kDa polypeptide was observed to be tyrosine phosphorylated in the cytosol. The phosphorylation of this protein was evident within 30 sec of ligand administration. The Ras GTPase activating protein (GAP) is a protein of 120 kDa which has been shown to become phosphorylated and activated in response to epidermal growth factor stimulation (Liu and Pawson, 1991). GAP has been shown to be involved in the deactivation of Ras, by stimulating Ras' GTPase activity (Trahey and McCormick, 1987), and may in this way negatively regulate the mitogenic pathway activated by the EGF receptor and GRB2/SOS.

GAP has been shown to bind to the phosphorylated EGF receptor (Ellis *et al.*, 1990), however, no 120 kDa polypeptide was observed in the PM. The identity of the 120 kDa phosphoprotein as GAP remains to be confirmed.

Insulin binding to its receptor at the PM resulted in the activation of the receptor β-subunit tyrosine kinase within 30 sec and the equally rapid internalization of the complex into endosomes (Chapter I). Burgess *et al.* (1992) demonstrated that insulin receptor internalization resulted in the partial dephosphorylation of the receptor, as confirmed here. However, the kinase activity of the receptor in the endosomal compartment of the liver remained active, suggesting that signaling from the endosomal insulin receptor could be possible (Khan *et al.*, 1989).

Despite the equal numbers of receptors for insulin and EGF in the male rat liver and the receptor saturating doses of ligand injected, SHC phosphorylation and association with GRB2/SOS was not observed in response to insulin stimulation *in vivo*. This may be due to the fact that the SHC PTB domain has a lower affinity for the insulin receptor PTB binding site than that of the EGF receptor (Trüb *et al.*, 1995) making it a less likely to be an *in vivo* substrate for the insulin receptor than for the EGF receptor. This lack of SHC activation, is contrary to the results obtained in studies employing a variety of cell types transfected with insulin receptors. In these cells, insulin was demonstrated to cause the rapid phosphorylation of SHC, the activation of GRB2 and the initiation of the Ras-MAPK pathway (Baltensperger et al, 1993; Pronk *et al.*, 1993; Skolnik *et al.*, 1993). The discrepancy between these results and the ones observed in liver *in vivo*, may be due to the presence of high numbers of receptors which may interfere with the specificity of signaling. In addition, these cells, being chosen for their ability to grow in culture, may be more responsive to the co-mitogenic actions of insulin, than hepatocytes in the liver, which are normally quiescent. Indeed it seems unlikely that the signal transduction pathways from the EGF and insulin receptors should converge *in vivo*, as suggested by the cell culture studies, since their physiological responses in the liver are different. This suggests that although the Ras pathway is functional in young adult hepatocytes as seen in response to EGF, the insulin receptor is unable to access it.

We postulate that mechanisms have evolved, in the hepatocyte, which regulate the specificity insulin and EGF receptor signal transduction. As a consequence of rapid receptor internalization, we suggest that the endosome plays a role in this regulation. One potential regulatory mechanism may be at the level of insulin degradation in the endosome. Liver endosomes contain a potent acidic insulinase activity (Doherty *et al.*, 1990; Authier *et al.*, 1994) which causes the rapid degradation of insulin. Backer *et al.* (1990) have observed that this enzyme is absent from CHO cells. We suggest that this accounts, at least

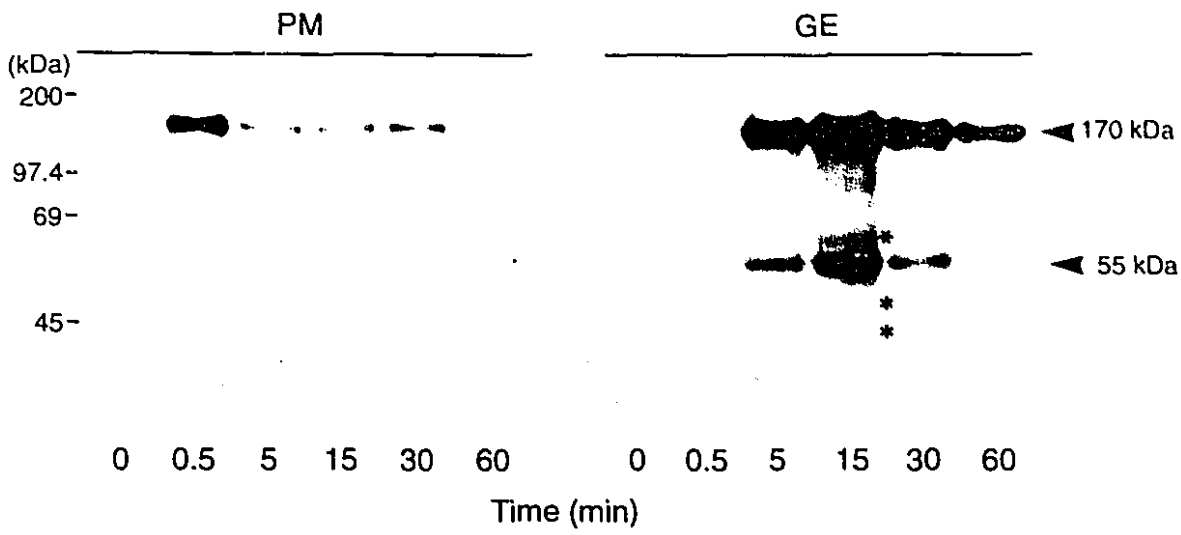
in part, for the enhanced, tyrosine phosphorylation of SHC, and the activation of the Ras pathway observed in insulin receptor transfected CHO cells. Regulation at the level of the endosome may discriminate between the metabolic and mitogenic response of insulin receptor activation.

Figure 14

Distribution of tyrosine phosphorylated proteins in response to ligand stimulation

Rat liver plasma membrane (PM) and endosomal (GE) fractions were isolated at the indicated times after the injection of 10 μg/100 g body weight of EGF (panel A) or 15 μg/100 g body weight of insulin (panel B). 50 μg protein of PM and 25 μg of GE fraction were resolved by SDS-PAGE, transferred to nitrocellulose and immunoblotted with antisera recognizing phosphotyrosine. The mobilities of the EGF receptor (170 kDa), the adaptor protein SHC (55 kDa), and the β-subunit of the insulin receptor (94 kDa) are indicated at the right. The asterisks indicate tyrosine phosphorylated proteins of 66, 46 and 42 kDa found in the GE fraction in response to EGF stimulation. Molecular mass markers are indicated on the left.

A. EGF injected



B. Insulin injected

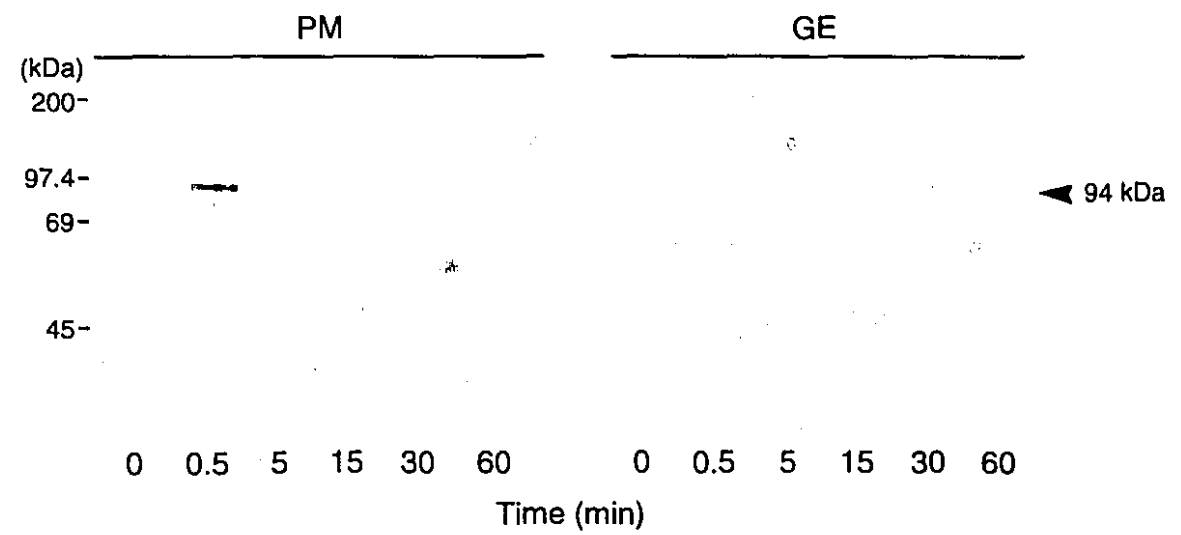


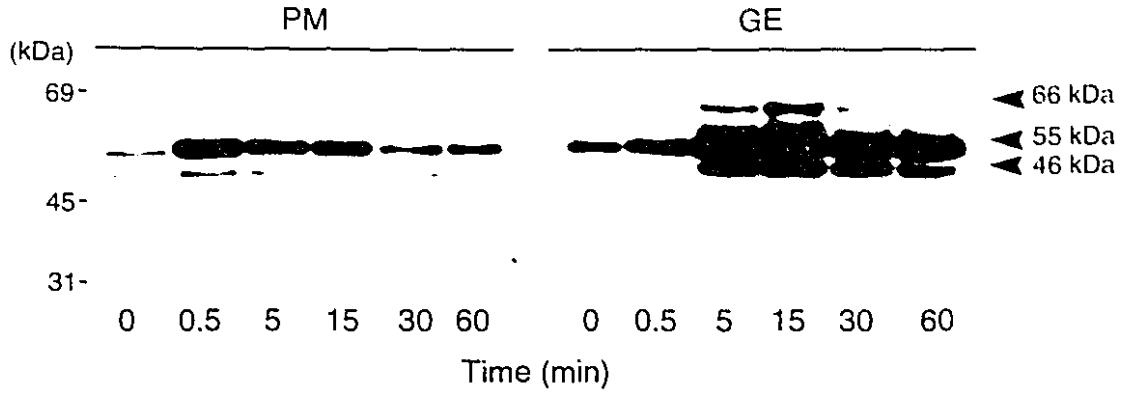
Figure 15

Recruitment of the adaptor proteins, SHC and GRB2, to membrane compartments harboring activated receptors

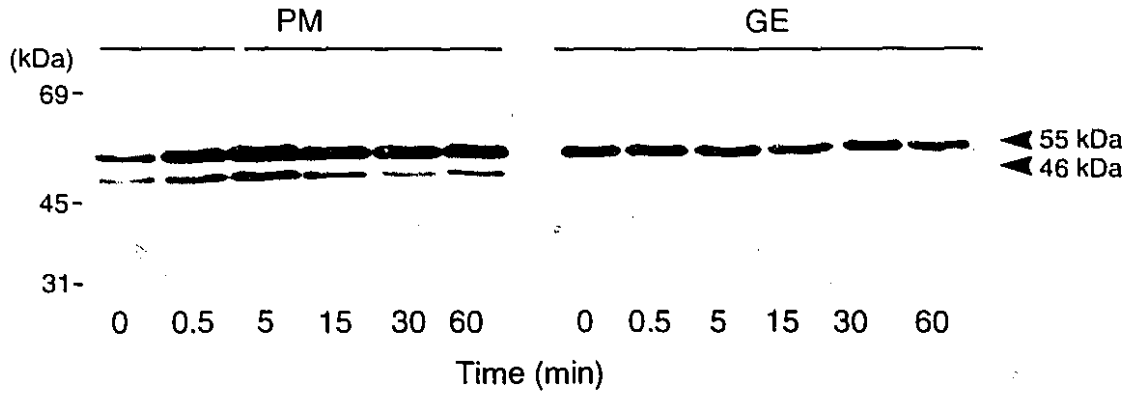
Plasma membrane (PM) and endosomes (GE) isolated at the indicated times after EGF (panel A and C) or insulin injection (panel B and D), were resolved by SDS-PAGE on 8% resolving gels for the immunoblots using antibodies to SHC (panel A and B) and 10% resolving gels for immunoblots using anti-GRB2 antibodies (panel C and D). 50 µg PM protein or 25 µg GE protein were loaded per lane. The molecular masses of the three isoforms of SHC (46, 55 and 66 kDa; panel A and B) and the apparent mobility of GRB2 (26 kDa; panel C and D) are indicated on the right. Molecular mass markers are indicated on the left.

α-SHC Blots

A. EGF



B. Insulin



α-GRB2 Blots

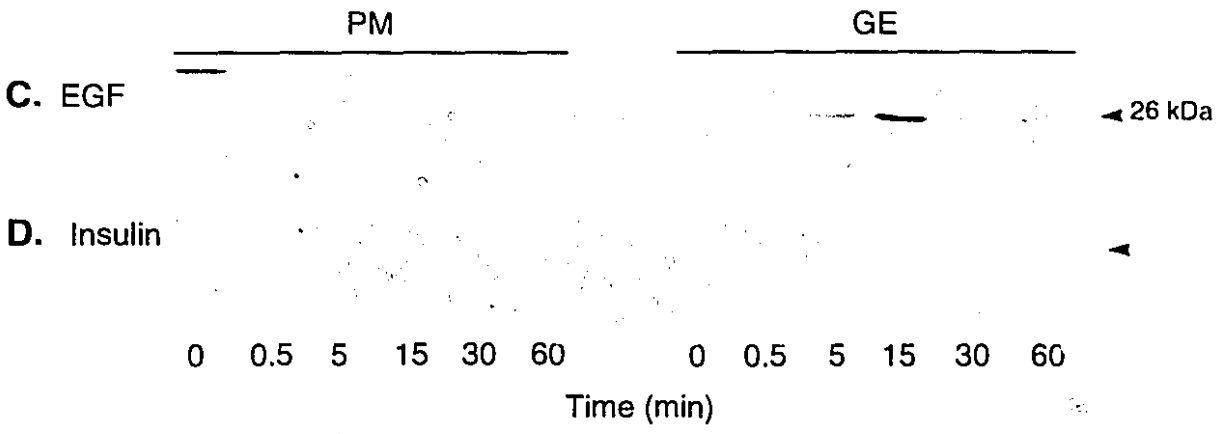


Figure 16

Association of tyrosine phosphorylated SHC, GRB2 and SOS with tyrosine phosphorylated EGF receptors, in endosomes

Endosomal fractions isolated from control rat liver homogenates (lanes 1) or from liver homogenates prepared at 15 min after EGF injection (lanes 2) were immunoblotted with antisera to phosphotyrosine (PY; panel A), SHC (panel B) or GRB2 (panel C). The bands are compared to those revealed after immunoprecipitation of the EGF receptor (anti-EGF receptor monoclonal IgG 151 BH-6 antibody) from GE fractions isolated from control rat liver homogenates (lanes 3) or from liver homogenates prepared at 15 min after EGF (lanes 4) or insulin (lane 5) injection. The immunoprecipitated GE fractions (lane 3 and 4) were also immunoblotted with antisera to SOS (panel D). The mobilities of the EGF receptor (170 kDa), the 46, 55, and 66 kDa isoforms of SHC, GRB2 (26 kDa) and SOS (150 kDa) are indicated to the right of each respective panel. The cross reactivity between immunoblotting IgG and immunoprecipitating IgG heavy chain is indicated (*).

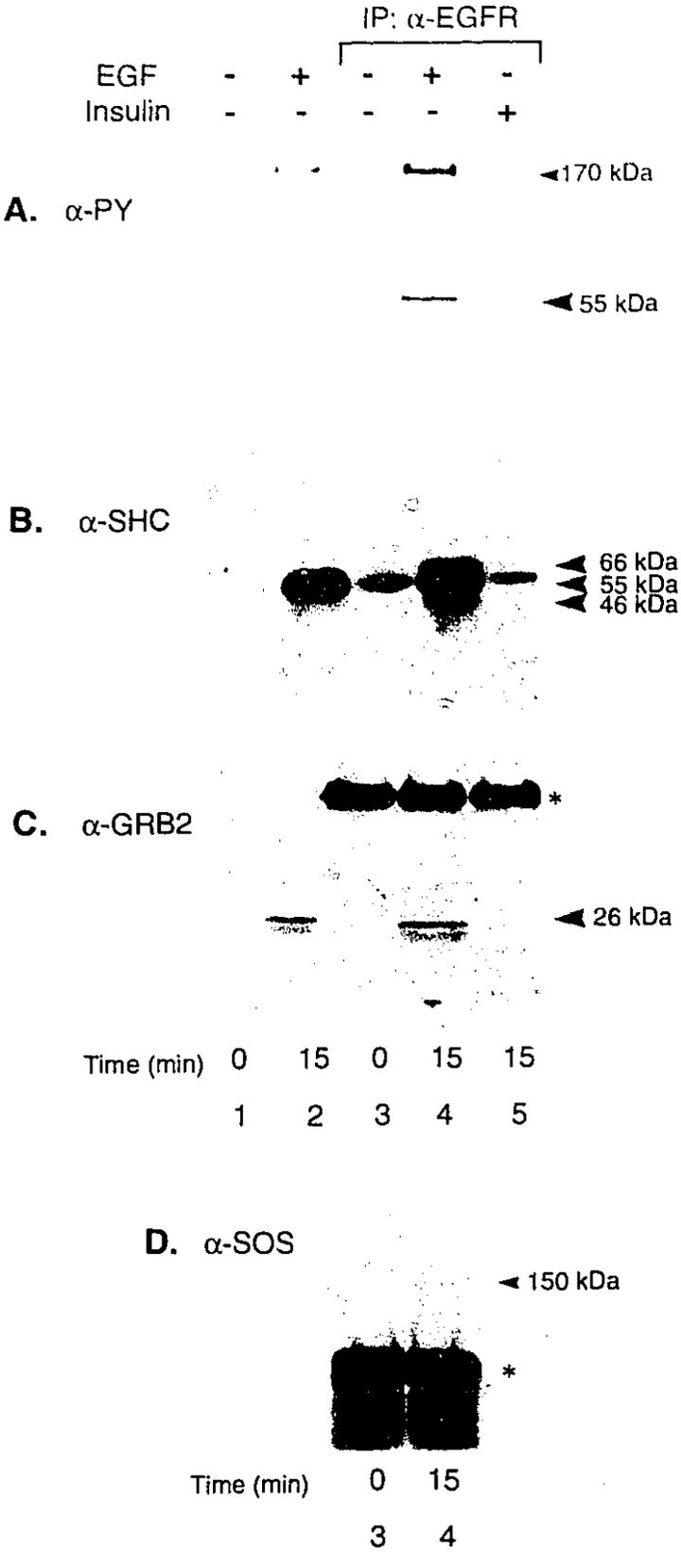
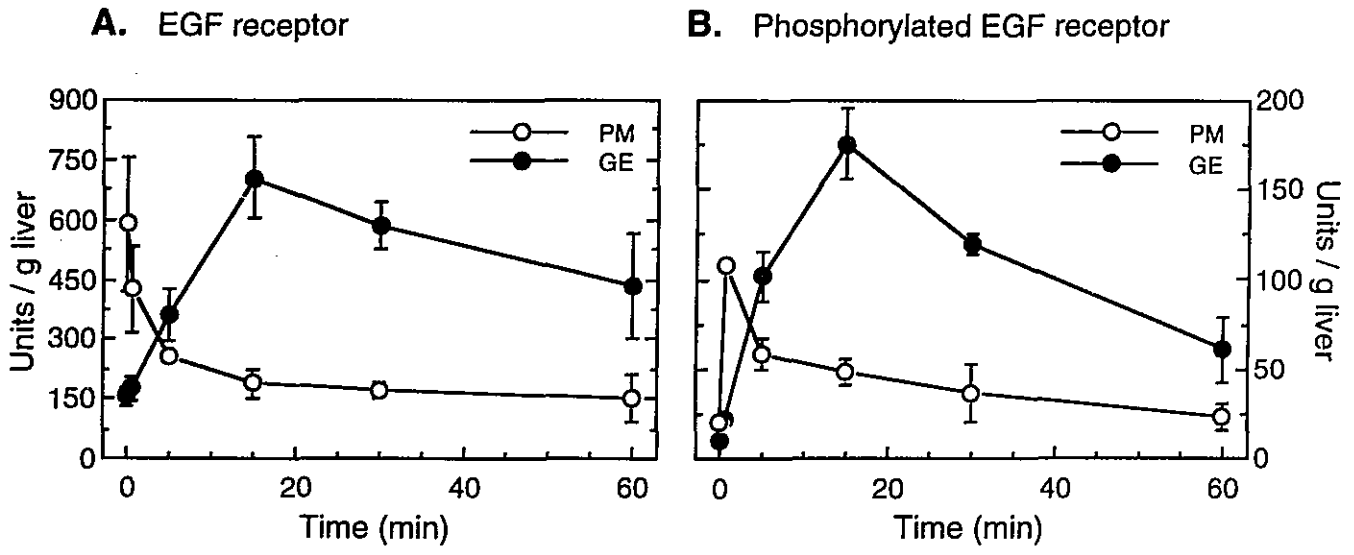

IP: α-EGFR
EGF - + - + -
Insulin - - - - +
170 kDa
A. α-PY
55 kDa
B. α-SHC
66 kDa
55 kDa
46 kDa
C. α-GRB2
*
26 kDa
Time (min) 0 15 0 15 15
1 2 3 4 5
D. α-SOS
150 kDa
*
Time (min) 0 15
3 4

Figure 17

Kinetics of EGF receptor internalization, tyrosine phosphorylation, SHC recruitment and phosphorylation, and GRB2 recruitment in membrane compartments, in response to EGF stimulation

Rat liver plasma membrane (PM) fractions (—o—) and endosomal (GE) fractions (—●—) were isolated after the injection of 10 μg/100 g body weight of EGF. 50 μg of PM and 25 μg of GE protein were resolved by SDS-PAGE, transferred to nitrocellulose and immunoblotted using antibodies to the EGF receptor (panel A), phosphotyrosine (panels B and D), SHC (panels C and E) or GRB2 (panel F). The [^{125}I] bands corresponding to the EGF receptor (panel A), the phosphorylated EGF receptor (panel B), the 55 and 46 kDa isoforms of SHC (panel C and E, respectively), the phosphorylated 55 kDa isoform of SHC (panel D) and GRB2 (panel F), were quantified by phosphoimager analysis. Each point represents the mean of three separate experiments ± SD and is expressed as arbitrary units per gram liver calculated as described in Experimental Procedures.


A. EGF receptor
B. Phosphorylated EGF receptor
Units / g liver
Time (min)
PM
GE
900
750
600
450
300
150
0
200
150
100
50
0
20
40
60

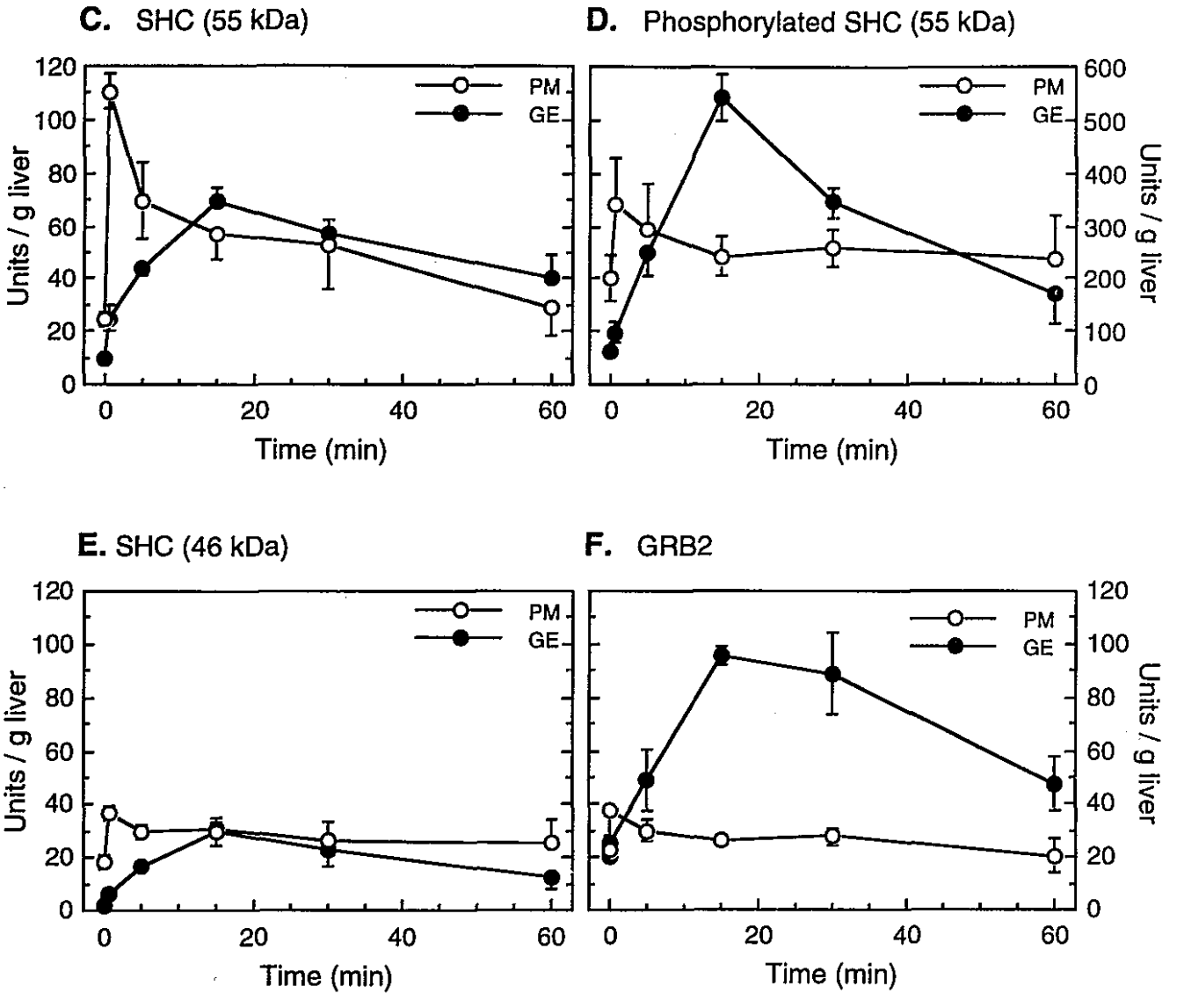

C. SHC (55 kDa)
D. Phosphorylated SHC (55 kDa)
E. SHC (46 kDa)
F. GRB2
PM
GE
Units / g liver
Time (min)
0
20
40
60
120
100
80
60
40
20
0
600
500
400
300
200
100

Figure 18

Specific concentration of phosphotyrosine modified SHC at the PM and in the GE fraction

At peak times of EGF receptor tyrosine phosphorylation (activation) at the plasma membrane (0.5 min; ■) and in the GE fraction (15 min; ⊠), the content of phosphotyrosine modified SHC and SHC were evaluated by immunoblotting followed by phosphoimager analysis. The results represent the mean ± SD for three separate experiments.

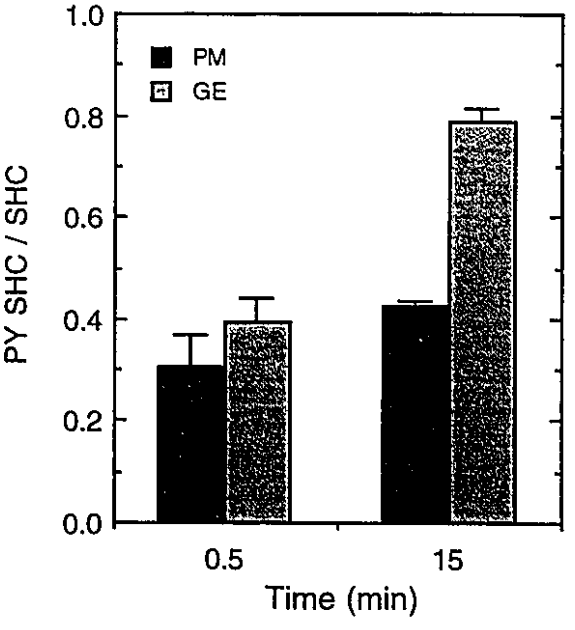

1.0
0.8
0.6
0.4
0.2
0.0
PY SHC / SHC
PM
GE
0.5
15
Time (min)

Figure 19

Tyrosine phosphorylation of cytosolic proteins in response to ligand stimulation

Rat liver cytosol was isolated at 0-60 min after the injection of EGF (10 µg/100 g body weight) or insulin (15 µg/100 g body weight). 300 µg of cytosolic protein from each time point was resolved by SDS-PAGE and immunoblotted with antisera to phosphotyrosine. The two cytosolic tyrosine phosphorylated proteins (55 kDa and 120 kDa) seen in response to EGF stimulation, are indicated on the right. Their relative mobilities were calculated from the mobilities of the molecular mass markers indicated on the left.

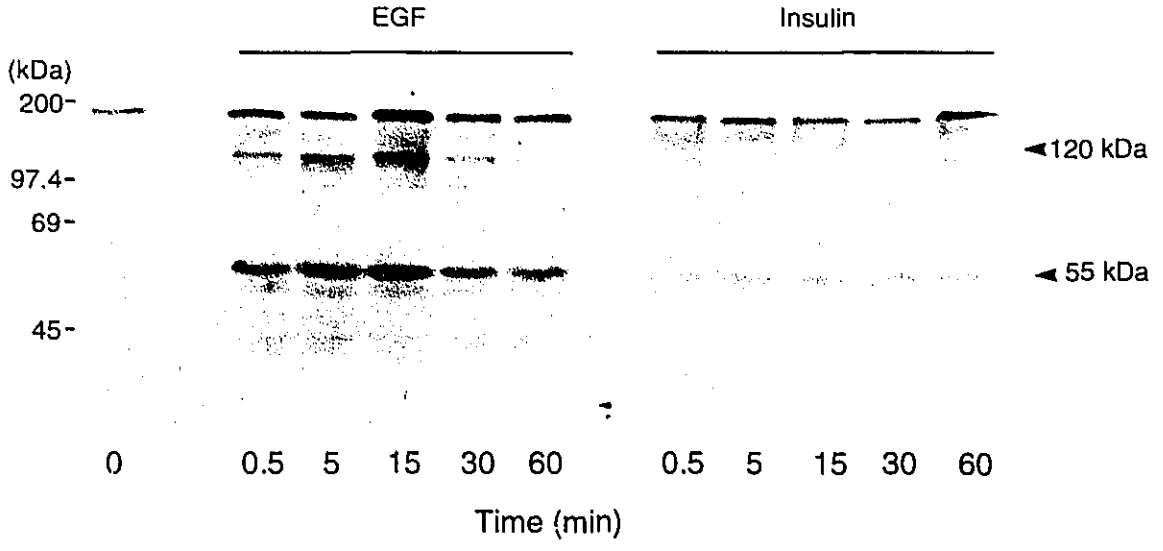
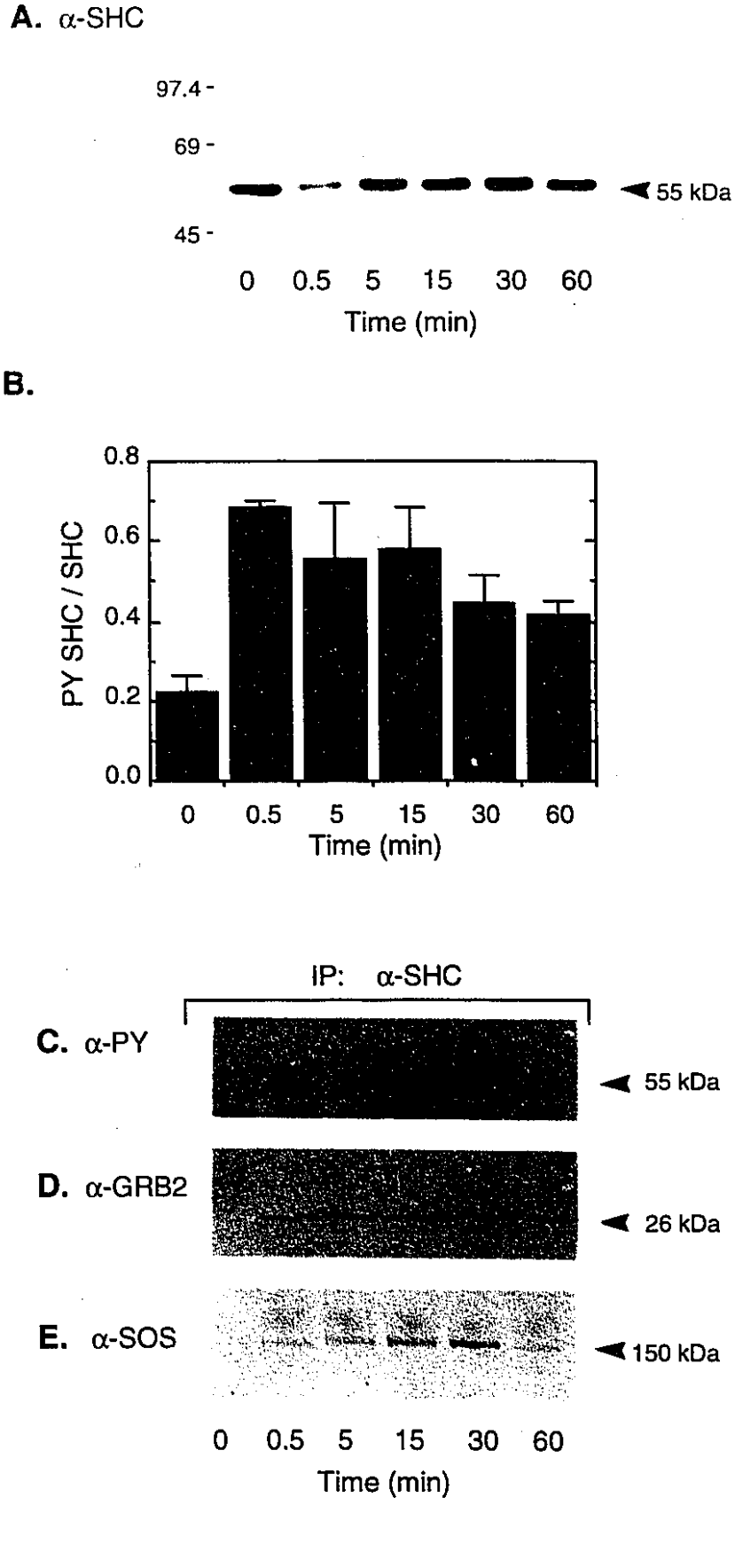

EGF
Insulin
(kDa)
200-
97.4-
69-
45-
120 kDa
55 kDa
0
0.5
5
15
30
60
0.5
5
15
30
60
Time (min)

Figure 20

EGF dependent phosphorylation of SHC and association with GRB2 and SOS in the cytosol

Rat liver cytosol was isolated at 0-60 min after the injection of EGF (10 μg/100 g body weight). Cytosolic protein (100 μg) was resolved by SDS-PAGE and immunoblotted with an antibody raised to the SHC SH2 domain (panel A; see Experimental Procedures). The [^{125}I]-labeled bands corresponding to the 55 kDa SHC isoform and to the phosphorylated 55 kDa protein seen in response to EGF stimulation (Fig.19, lanes 2-6) were quantitated by phosphoimager analysis. The results are expressed as the ratio of phosphotyrosine-modified SHC / SHC, and represent the mean ± SD for three separate experiments (panel B). Molecular mass markers are indicated on the left of panel A.

The cytosolic fractions were immunoprecipitated with affinity purified antibodies raised to the SHC SH2 domain. The immunoprecipitates were immunoblotted with antibodies specific for phosphotyrosine (panel C), GRB2 (panel D) or SOS (panel E). The mobilities of tyrosine phosphorylated SHC (55 kDa), GRB2 (26 kDa) and SOS (150 kDa) are indicated on the right.


A. α-SHC
97.4 -
69 -
45 -
55 kDa
0 0.5 5 15 30 60
Time (min)
B.
PY SHC / SHC
0.8
0.6
0.4
0.2
0.0
0 0.5 5 15 30 60
Time (min)
IP: α-SHC
C. α-PY
55 kDa
D. α-GRB2
26 kDa
E. α-SOS
150 kDa
0 0.5 5 15 30 60
Time (min)

Chapter III

The Insulin Receptor - Endosomal Regulation

The signal transduction pathways employed by the activated insulin receptor have been studied extensively. Ligand administration has been shown to result in the tyrosine phosphorylation of substrates such as IRS-1 and SHC which then function as adaptors for downstream effectors such as PI3'-kinase and GRB2/SOS. The activation of these molecules links insulin stimulation to both metabolic and mitogenic signal transduction pathways.

In addition, insulin stimulation results in the rapid internalization of the insulin-receptor complex into endosomes (Chapter I). This has generally been proposed to be a mechanism of signal attenuation. However, there is accumulating evidence suggesting that the endosome plays a role in the regulation of signal transduction beyond simply causing attenuation and receptor downregulation. The insulin receptor, although dephosphorylated in the endosome, appears to possess a higher kinase activity (V_{max}/Km) in this compartment (Khan *et al.*, 1989; Burgess *et al.*, 1992). How this is regulated is not clear, however, several endosomal mechanisms exist which may be involved in this process.

The acidic environment of the endosome causes the dissociation of insulin from its receptor, early in the internalization pathway. Insulin is then subject to degradation by a specific endosomal enzyme; the endosomal acidic insulinase (EAI; Authier *et al.*, 1994). The observation that insulin is dissociated and degraded efficiently, while for example EGF is not (Renfrew and Hubbard, 1991a), suggests that insulin degradation plays a role in the regulation of insulin receptor signaling

The endosomal environment provides another mechanism which may be involved in the regulation of signaling from internalized receptors - tyrosine phosphatases which are postulated to be associated with the endosomal membrane (Faure *et al.*, 1992). The specific dephosphorylation of the insulin receptor in the endosome results in the augmentation of the receptor kinase activity, possibly in a manner analogous to the one seen for c-src kinase activation at the PM. In addition, these phosphatases may modulate signaling by causing the dephosphorylation of specific receptor sites, preventing further interaction with select downstream molecules.

To address the role of endosomal regulation of the activated insulin receptor *in vivo*, subcellular fractionation techniques and an insulin analog, H2, were used. This analog has a dissociation rate constant (k_d) of 8.28×10^{-4} s^{-1} compared to that of insulin, 5.52×10^{-2} s^{-1}, which represents a significantly higher affinity of H2 for the insulin receptor (Hansen *et al.*, 1996). Using H2 as the ligand to activate the insulin receptor is postulated to interfere with the normal endosomal regulation of signal transduction from this receptor by preventing ligand dissociation, and result in altered signaling from the insulin receptor in response to this ligand.

Results

Insulin dissociates from its receptor early in the endosomal pathway, and is rapidly degraded by EAI (Doherty *et al.*, 1990; Authier *et al.*, 1994). Insulin degradation by this enzyme is believed to drive further insulin dissociation. The insulin analog, H2, has a high affinity for the insulin receptor and as a consequence dissociates less readily (Hansen *et al.*, 1996). To assess the ability of the liver EAI to degrade the insulin analog, H2, and thus drive dissociation from the insulin receptor, *in vitro* degradation studies were undertaken. A two hour incubation of insulin with endosomal extracts containing EAI, resulted in a ~65% degradation of insulin as determined by HPLC analysis (Fig.21A, B). The same experiment carried out with H2 resulted only in a ~35% degradation (Fig.21C, D).

To determine if the greater affinity of H2 for the insulin receptor affected receptor internalization and trafficking, the insulin receptor content at the plasma membrane (PM) and in endosomal (GE) fractions, after either insulin or H2 injection, were measured by quantitative immunoblotting (Fig.22). The insulin receptor internalized rapidly ($t_{1/2} < 1$ min), and to the same extent in response to both ligands. Approximately 34% of the total cell surface receptors internalized after a receptor saturating dose of ligand was administered (C). The receptor appeared to be internalized into the endosomal fraction with the same initial kinetics up to 5 min (D). At this time however, the insulin receptor appeared to accumulate temporarily in the endosome, in response to H2, which was not observed in response to insulin. At 15 min post injection, the receptor was lost from the endosomal compartment with the same kinetics regardless of the ligand used.

To determine if the protracted presence of the insulin receptor in the endosome affected receptor signaling, the tyrosine phosphorylation of proteins in the PM and endosomes was investigated (Fig.24). There were no proteins, other than the insulin receptor, which appeared to be tyrosine phosphorylated in these compartments in response to either ligand, as determined by Western blotting. Using phosphoimager analysis, the extent of receptor tyrosine phosphorylation was determined. The initial burst (30 sec) of receptor phosphorylation observed at the PM and the GE was greater in response to insulin than H2 (C, D), although subsequently the level of phosphorylation of the receptor in the PM was similar in response to both ligands. In the endosomal fraction however, the phosphorylation state of the receptor in response to H2, increased at 5 min and remained high up to 60 min, while that in response to insulin gradually decreased during this time (D). The highly tyrosine phosphorylated state of the insulin receptor observed in the

endosome in response to H2, raises the possibility that new substrates may be recruited to the receptor in this compartment.

The cytosolic adaptor protein SHC has been implicated in insulin receptor mitogenic signaling in cultured cells (Pronk *et al.*, 1993; Sasaoka *et al.*, 1994b). SHC has not been shown to be associated with the insulin receptor normally (Trüb *et al.*, 1995), however, it is possible that the highly tyrosine phosphorylated state of the insulin receptor in the endosome, in response to H2, results in SHC recruitment to this compartment. This idea was tested by anti-SHC immunoblotting (Fig.25). There was no observable difference in the recruitment of any isoform of SHC to either the PM or the GE fractions, in response to either insulin or H2.

SHC tyrosine phosphorylation by the insulin receptor may occur rapidly, without stable interaction between the enzyme and the phosphorylated substrate, however, this may result in the presence of tyrosine phosphorylated SHC in the cytosol. Therefore, the effect of insulin and H2 on the tyrosine phosphorylation of cytosolic proteins was investigated (Fig.26). Although no tyrosine phosphorylated polypeptide of 55 kDa was observed either in response to either insulin or H2 injection, stimulation of the insulin receptor by these ligands resulted in the phosphorylation of a polypeptide of approximately 185 kDa. The phosphorylation of this molecule could be observed within 30 sec of receptor activation.

The major substrate for the insulin receptor is IRS-1, a protein of approximately 185 kDa (rev. Myers *et al.*, 1994). This protein becomes tyrosine phosphorylated on multiple sites and acts as a docking protein for other cytoplasmic signal transduction molecules. When liver cytosol, plasma membrane and endosomal fractions were immunoblotted with anti-IRS-1 antibodies (Fig.27), the presence of a cytoplasmic pool of IRS-1 was confirmed. The results also revealed the presence of a subset of IRS-1 molecules associated with the PM, but not the endosome, in the absence of any stimulation. Neither insulin nor H2 stimulation caused a discernible redistribution of IRS-1 either to the PM or to the endosomal compartment. To confirm that the 185 kDa tyrosine phosphorylated polypeptide seen in response to insulin receptor activation was indeed IRS-1, immunoprecipitation studies were undertaken.

Immunoprecipitation of IRS-1 from cytosolic fractions isolated after the injection of either insulin, or H2 revealed a rapid ligand-induced tyrosine phosphorylation of IRS-1 in response to insulin and H2 (Fig.28). IRS-1 was phosphorylated to the same extent (roughly 8 fold) within the first 30 sec after ligand administration (C). Maximal IRS-1 phosphorylation by the H2-stimulated insulin receptor occurred at this time, however, between 0.5 and 5 min, the insulin-stimulated insulin receptor appeared to continue to phosphorylate IRS-1. After insulin stimulation, peak tyrosine phosphorylation of IRS-1

occurred at 5 min. Subsequent to peak phosphorylation , IRS-1 became dephosphorylated, but levels were still above control levels up to 60 min post ligand injection.

Discussion

Stimulation of the insulin receptor has been demonstrated to result in biological effects which can generally be classified as metabolic or mitogenic responses (see Introduction). The major substrates to be tyrosine phosphorylated, in response to insulin receptor activation of a variety of cell types, are IRS-1 and SHC (rev. White and Kahn, 1994; Pronk *et al.*, 1993). These molecules represent the initial steps in the pathways leading to insulin responses. The extent to which receptor activation results in the initiation of these pathways appears to depend on cellular context.

The distribution of IRS-1 is generally considered to be cytosolic, although a significant portion was shown to associate with intracellular membranes in 3T3-L1 adipocytes (Heller-Harrison, 1995). In rat liver, membrane-associated IRS-1 appeared to be constitutively localized to the plasma membrane and was absent from the endosomal fraction. The association of IRS-1 to membranes may be explained by the presence of a PH domain in this molecule. This domain is known to interact with phosphotidylinositols as well as $\beta\gamma$ subunits of heterotrimeric GTP binding proteins (see Pleckstrin Homology Domain). These interactions may target IRS-1 selectively to unique membranes in different cell types. The presence of IRS-1 associated with the plasma membrane may serve to increase the efficiency of interaction between the receptor and IRS-1, by increasing the local concentration of IRS-1, or by presenting IRS-1 tyrosine residues to the kinase in the proper orientation. Indeed, deletion of the PH domain of IRS-1 greatly reduces insulin stimulated IRS-1 phosphorylation (Myers *et al.*, 1995). However, tyrosine phosphorylation of the IRS-1 at the plasma membrane could not be detected by Western blotting. Instead, insulin injection into rats resulted in the rapid (within 30 sec) tyrosine phosphorylation of cytosolic IRS-1. This supports the idea that tyrosine-phosphorylated IRS-1 was released into the cytosol, as observed by Heller-Harrison *et al.* (1995) in adipocytes. The creation of SH2 and PTB binding sites in this adaptor molecule, can lead to the binding and activation of downstream molecules including PI3'-kinase and GRB2/SOS (see Myers *et al.*, 1994). Thus it appears that IRS-1 could link the insulin receptor to metabolic responses, via PI3'-kinase association and mitogenic responses through GRB2/SOS, and possibly PI3'-kinase as well.

Another adaptor molecule with which GRB2/SOS has been shown to interact, in response to insulin stimulation, is phosphorylated SHC (Pronk *et al.*, 1993; Sasaoka *et al.*,

1994a). In insulin-stimulated liver, however, no SHC phosphorylation could be observed. The lack of SHC phosphorylation could be due to the absence of SHC from the liver, however, this is not the case since EGF dependent phosphorylation of SHC could be observed in the liver (Chapter II). The specificity of insulin receptor signaling suggests the presence of a liver specific mechanism which prevents insulin induced SHC phosphorylation. For example, an insulin-activated phosphatase could rapidly dephosphorylates SHC, or an insulin dependent molecule could sequester the receptor away from SHC, thereby preventing its phosphorylation. On the other hand it is possible that the specificity of the insulin receptor kinase for the SHC SH2 domain might be low (Trüb *et al.*, 1995), resulting in a lower rate of interaction between the receptor and SHC, and the observations of SHC phosphorylation in cell culture may be a result of the overexpression of the insulin receptor. Thus it appears that in the liver, a major target organ for insulin action, the insulin receptor cannot access and phosphorylate SHC, and therefore does not access the Ras/MAPK pathway in this way.

The insulin induced activation of the mitogenic Ras-MAPK cascade in the liver seems unlikely given the metabolic responses described for insulin in this organ and the low levels of dividing cells present in the liver, which is sporadically exposed to high concentrations of endogenous insulin. Ohmichi *et al.* (1994a,b) described a PC12 cell line in which insulin stimulation resulted in receptor autophosphorylation, IRS-1 phosphorylation and PI3'-kinase activation and increase glucose, lipid and protein synthesis, but no activation of any components of the MAPK cascade. Thus it appears that the major metabolic actions of the insulin receptor can be carried out by MAPK-independent pathways.

Because of the rapid internalization of the receptor-ligand complex, the presence of an active kinase in endosomes and the efficient degradation of insulin in the endosome, this compartment was postulated to be involved in the regulation of signaling from the insulin receptor. This regulation was postulated to occur through the endosomal acidic insulinase, found in the endosomal lumen, which efficiently degrades insulin (Authier *et al.*, 1994) and/or as a consequence of receptor dephosphorylation by endosomal associated tyrosine phosphatases (Faure *et al.*, 1992).

In order to disrupt intra-endosomal receptor regulation an insulin analog which binds more strongly to the receptor than insulin and is less susceptible to endosomal degradation than insulin was used to stimulate the insulin receptor kinase. The dissociation data of Hansen *et al.* (1996) and the HPLC data presented here support the idea that H2

remains bound to the receptor during endocytosis, whereas insulin is dissociated and degraded, leaving an unoccupied receptor.

The rate and extent of insulin receptor internalization from the PM was not affected by the ligand used. However, the receptor appeared to be temporarily retained in the endosome after H2 administration. Thus trafficking seems to be affected to some extent which suggests that receptor occupancy may play a role in receptor trafficking. Interestingly, the protein enigma shown to be associated with the insulin receptor after insulin stimulation, was not associated with the receptor after H2 injection. This raises the possibility that enigma plays a role in the regulation of insulin receptor trafficking. On the other hand, enigma has recently been implicated in ret receptor signal transduction (Durick *et al.*, 1996). It is possible then that enigma may be involved in signal transduction from the insulin receptor in an as yet undefined pathway, which is disrupted in the presence of a ligand (H2) bound receptor.

Studies of insulin analogs have demonstrated an inverse relationship between the ability of these analogs to mediate mitogenic and metabolic responses and receptor dissociation rate constants (Hansen *et al.*, 1996). Insulin has been shown to be mitogenic in CHO-IR cells. In these cells H2 stimulation results in almost 30 fold increase in mitogenic potency. In adipocytes, this ligand results in a 4-5 fold augmentation in metabolic responses, such as lipogenesis, compared with insulin. It is possible that endosomal retention, as a consequence of the ligand remaining bound to the receptor, results in the prolongation of signaling and consequently a higher mitogenic and metabolic responses. A similar situation was reported in Rat-1 fibroblasts in culture expressing high levels of IGF-1 receptors. These receptors are similar in structure to insulin receptors, but their activation results in growth related rather than metabolic responses (Zapf *et al.*, 1994). In a recent study comparing the kinetics of IGF-1 internalization in Rat-1 fibroblasts with those of insulin in the same cells but overexpressing insulin receptor, a marked difference in the endosomal dissociation of the cognate ligands was uncovered. While insulin was rapidly dissociated and degraded after internalization, IGF-1 was more resistant to acid induced dissociation from its receptor and resulted in a prolonged (up to 120 min) accumulation of intracellular (endosomal) IGF-1 (Zapf *et al.*, 1994). The differences in endosomal ligand dissociation has been speculated to be involved in the regulation of the different bioeffects of the two related receptors.

The activity of the tyrosine kinase as well as its tyrosine phosphorylated state have been implicated in receptor trafficking and have been shown to be required for signal transduction. Although the kinase activity of the insulin receptor in the endosome in

response to insulin or H2 administration was not examined, the tyrosine phosphorylation state of the receptor was determined to see if any effect could be detected *in vivo*. It appears that the insulin receptor becomes dephosphorylated in the endosome after insulin stimulation. This is consistent with the literature and has been suggested to correlate with increased kinase activity in this compartment (Burgess *et al.*, 1992). Activation of the insulin receptor with H2 did not result in receptor dephosphorylation after internalization. Indeed, the level of receptor phosphorylation was maintained up to 60 min. The observation implies that the dephosphorylation of the insulin receptor in the endosome requires the dissociation of the ligand from the receptor and its degradation. This may be explained in the following way; insulin dissociation and degradation results in the presence of an unbound but phosphorylated receptor. This state may result in a conformational change in the receptor C-terminus allowing phosphatases to partially dephosphorylate the receptor. This dephosphorylation may lead to receptor activation in a manner analogous to the activation of c-src kinase by dephosphorylation. When the ligand remains bound, as is the case with H2, no conformational change can occur, and consequently no dephosphorylation or kinase activation. Thus the presence of H2 bound to the insulin receptor would suggests that the kinase activity is not increased in the endosome, although this remains to be tested more directly, by studying *in vitro* kinase activity of the endosomal receptor towards exogenous substrates.

If signal transduction from the insulin receptor can be regulated at the level of the endosome, then the modification of the insulin receptor by using H2 as a ligand, should result in a different pattern or degree of phosphorylation of substrate proteins. H2 stimulation did not appear to cause the tyrosine phosphorylation of new substrates as determined by Western blotting. This suggests that while the phosphorylation state and possibly the kinase activity of the insulin receptor in the endosome have been modified in response to H2, the specificity of signaling from the receptor has been largely retained. This is consistent with the idea that the intracellular make up of the cell plays a significant role in the determination of signal transduction pathways used by specific receptors. On the other hand, the use of H2 as a ligand for the insulin receptor resulted in a modulation of the level of phosphorylation of IRS-1. Within the first 30 sec after ligand administration, cytosolic IRS-1 was phosphorylated to the same extent in response to both ligands. Subsequently, insulin administration caused IRS-1 phosphorylation to increase and to peak by 5 min post injection, while in response to H2 IRS-1 underwent dephosphorylated at this time. This is consistent with an increase in kinase activity of the insulin-stimulated receptor, as previously suggested, and a lack of this activation response to H2. Thus it appears that the endosomal insulin receptor is capable of continuing to

phosphorylate IRS-1 at least up to 5 min post injection. Indeed this time frame is similar to that described by Klein *et al.* (1987) in adipocytes which demonstrated that the activated insulin receptors, which are internalized in a fully active state in these cells, started to deactivate within 3-4 min as they passed through low and high-density microsomal membranes.

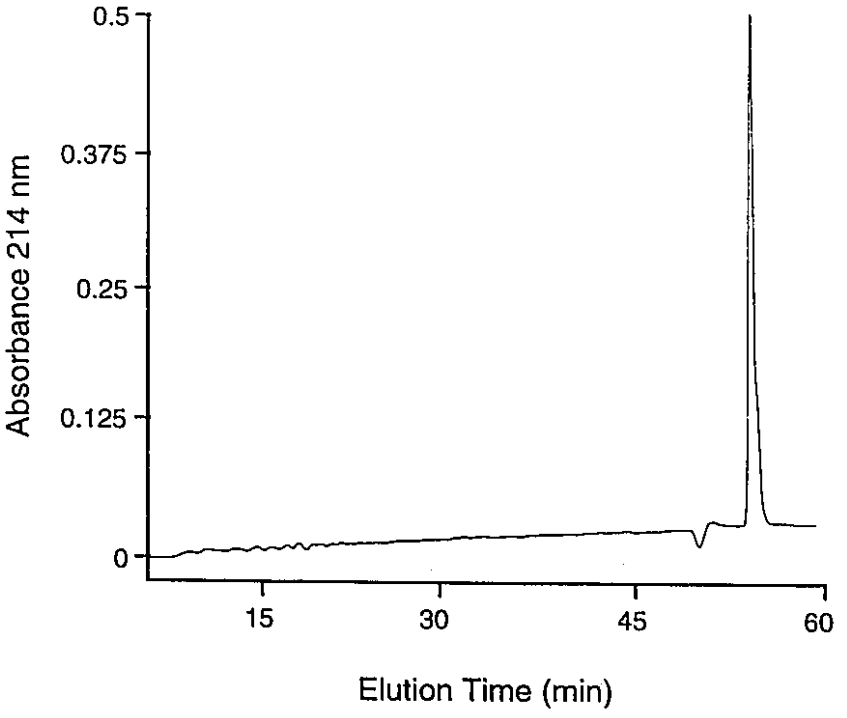
The work in this chapter has demonstrated that the endosome plays a role in the regulation of signaling by the insulin receptor. Ligand dissociation and degradation, appears to regulate subsequent dephosphorylation and activation of the receptor. When this is prevented as is the case with H2, receptor trafficking, recruitment of molecules and phosphorylation are all affected.

Figure 21

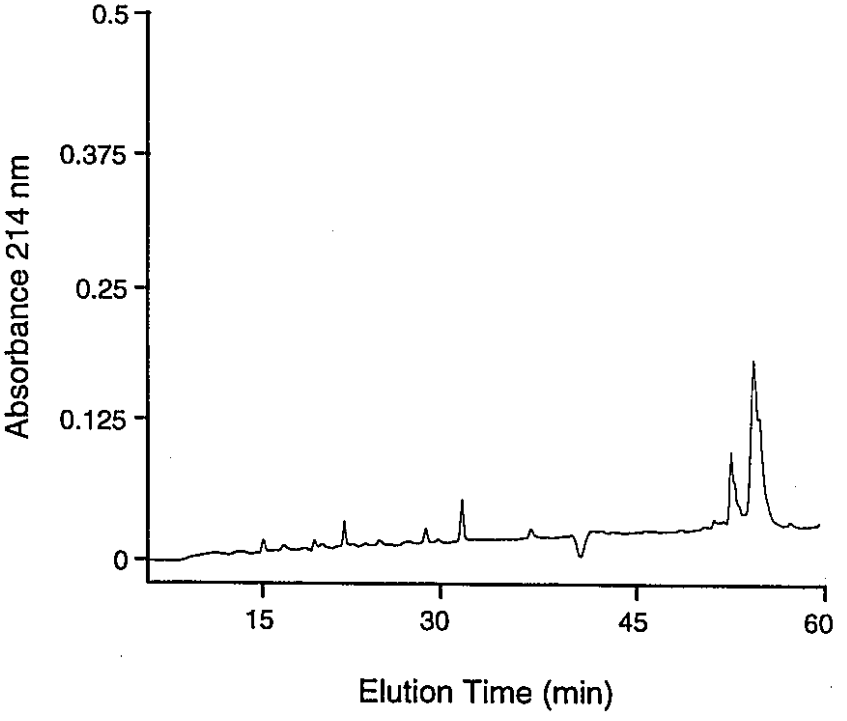
Degradation of insulin and the insulin analog, H2, by endosomal extracts

Soluble endosomal extracts were obtained from endosomes isolated from rat liver in the absence of any protease or phosphatase inhibitors. Insulin (10^{-6} M) or the insulin analog, H2 (10^{-6} M) were incubated in the absence (panel A and C, respectively) or presence of ~0.01 μg soluble endosomal proteins (panel B and D, respectively) for 2 hours at 37°C. The samples were acidified with acetic acid (20% final) and immediately loaded onto a reverse-phase HPLC column. A 0-45% linear acetonitrile gradient was established in 60 min. Absorbance was read at 214 nm, and intact insulin and H2 eluted at ~55 and 53 min respectively.

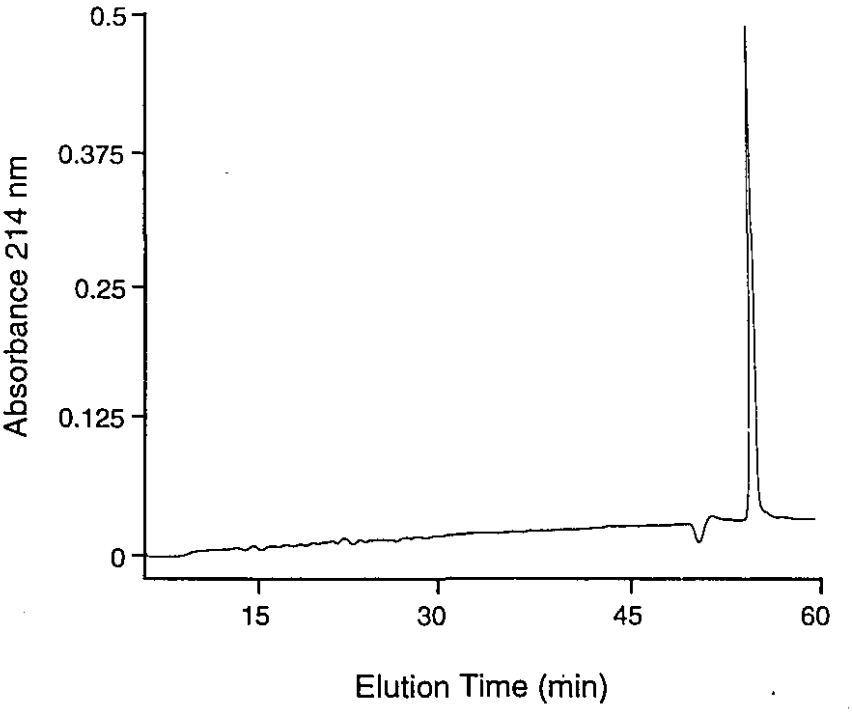
A. 10^{-6} M Insulin



B. 10^{-6} M Insulin + Endosomal Fraction
Incubation at 37°C, 2 hr



C. 10^{-6} M H2



D. 10^{-6} M H2 + Endosomal Fraction
Incubation at 37°C, 2 hr

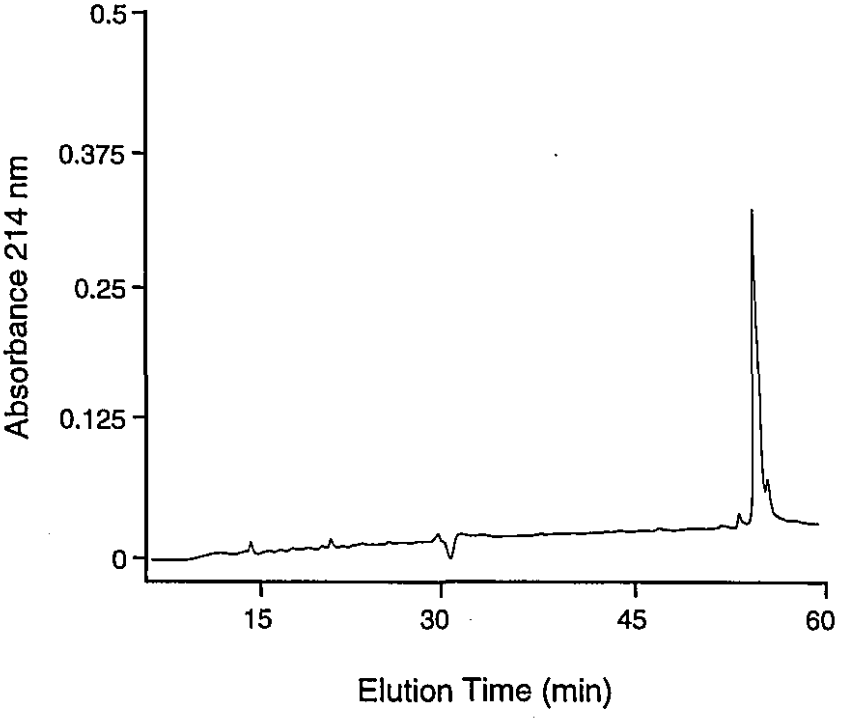
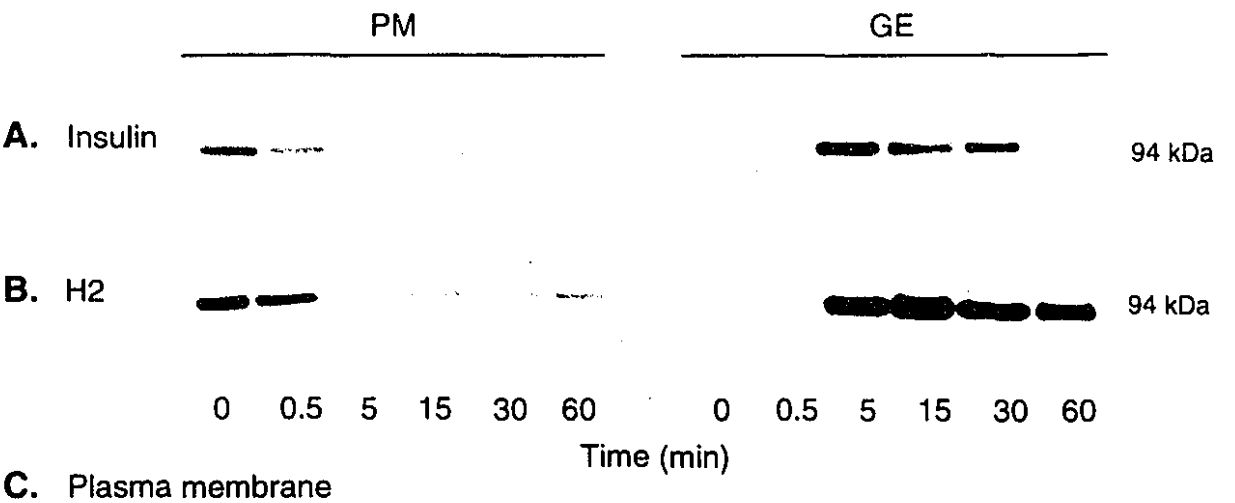


Figure 22

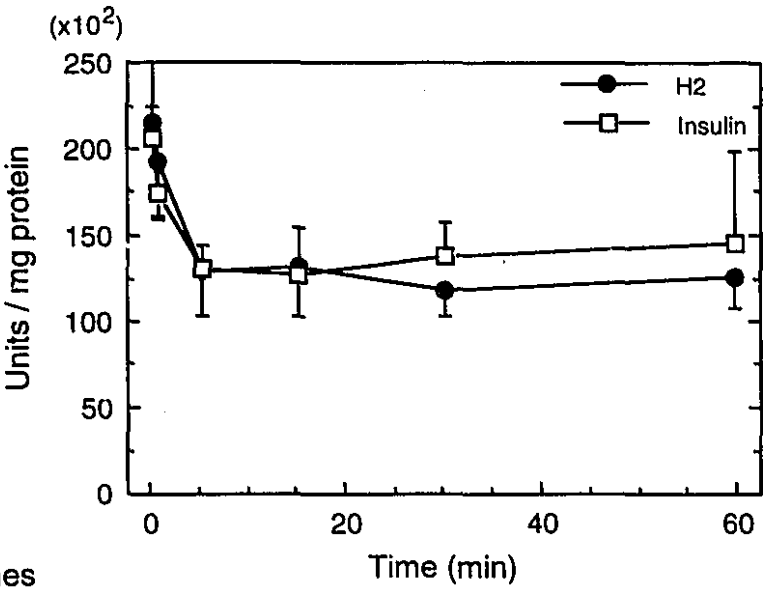
Kinetics of insulin receptor internalization in response to insulin and H2 stimulation

Plasma membrane (PM) and endosomal (GE) fractions were isolated at the indicated times after the injection of 15 μg/100 g body weight of either insulin (panel A) or the insulin analog, H2 (panel B). 50 μg of PM and 25 μg of GE fraction were resolved by SDS-PAGE on an 8% resolving gel, transferred to nitrocellulose and immunoblotted with an anti-insulin receptor antibody. The mobility of the β-subunit of the insulin receptor (94 kDa) is indicated to the right of each blot.

PM (panel C) and GE fractions (panel D) were isolated at the indicated times after the injection of either insulin (—□—) or the insulin analog, H2 (—●—). The fractions were immunoblotted as described above and the [^{125}I]-labeled band corresponding to the β-subunit of the insulin receptor, was quantitated by phosphoimager analysis. The results are expressed as arbitrary phosphoimager units / mg cell fraction protein and represent the mean ± SD of three separate experiments for both insulin and H2.



C. Plasma membrane



D. Endosomes

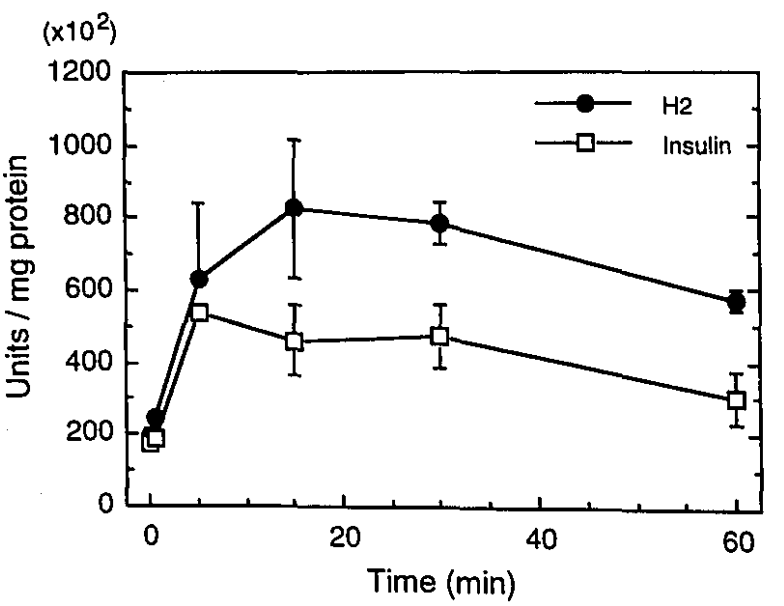


Figure 23

Association of enigma with the insulin receptor in response to insulin and H2

Plasma membrane (PM) and endosomal (GE) fractions were isolated at the indicated times after the injection of 15 μg/100 g body weight of either insulin or the insulin analog, H2. The insulin receptor was immunoprecipitated from 500 μg of PM and 200 μg of GE. The immunnoprecipitated proteins were resolved by SDS-PAGE on an 8% resolving gel, transferred to nitrocellulose and immunoblotted with an anti-insulin receptor or anti-enigma antibody. The mobility of the β-subunit of the insulin receptor (94 kDa) and the 67 kDa polypeptide recognized by the enigma antibody is indicated to the right of each blot.

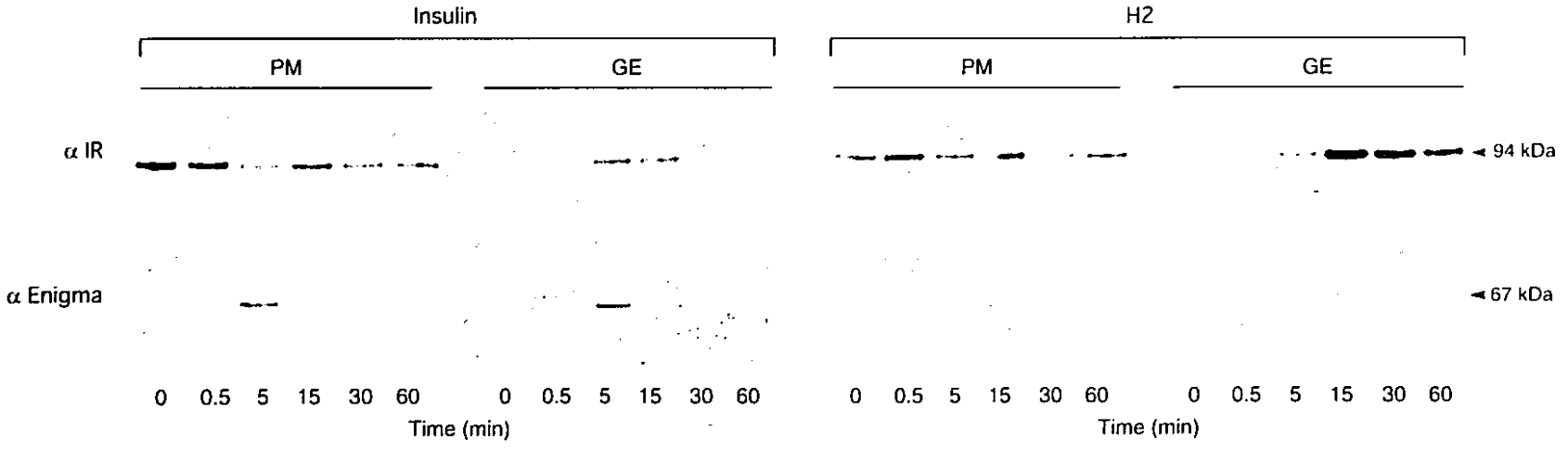
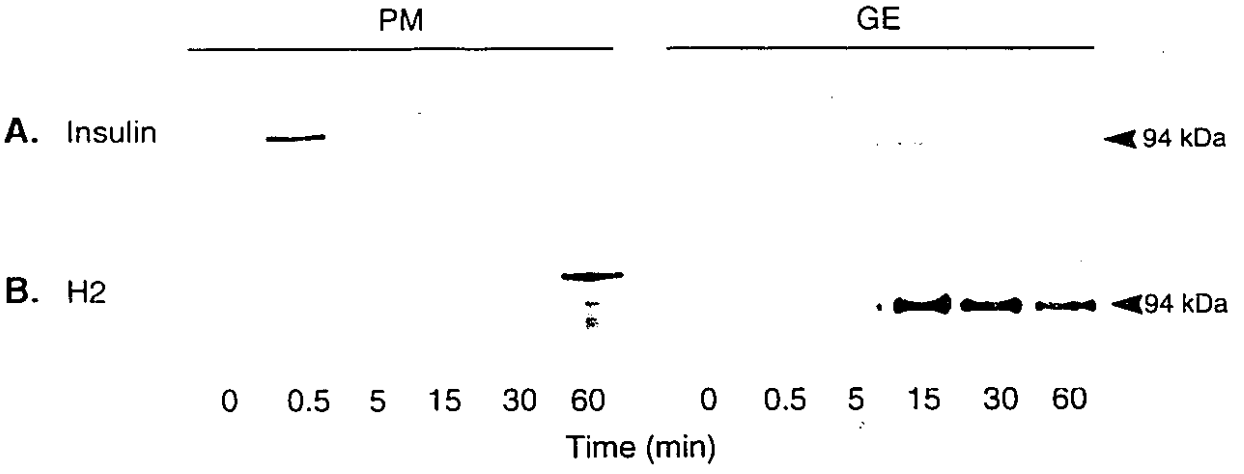

Insulin
H2
PM
GE
PM
GE
α IR
α Enigma
94 kDa
67 kDa
0 0.5 5 15 30 60 0 0.5 5 15 30 60
0 0.5 5 15 30 60 0 0.5 5 15 30 60
Time (min)
Time (min)

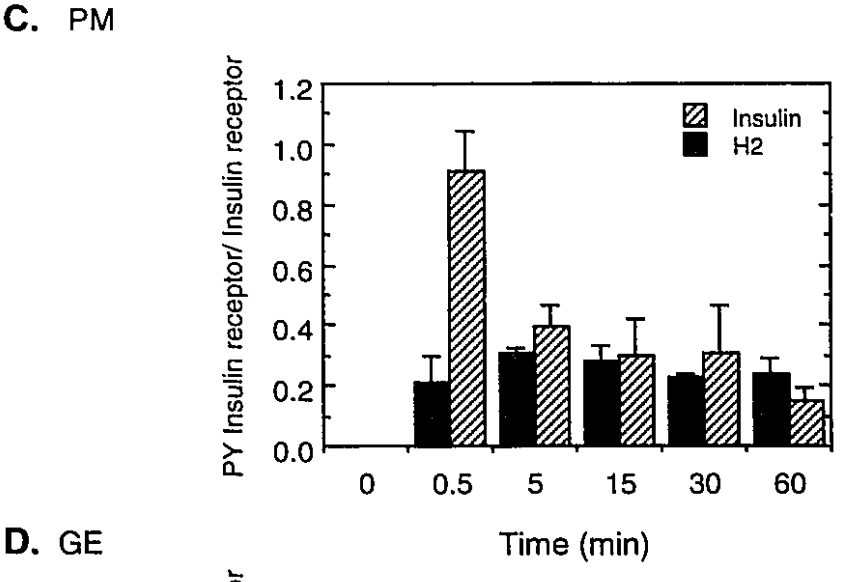
Figure 24

Insulin receptor phosphorylation in response to insulin and H2 stimulation

Plasma membrane (PM) and endosomal (GE) fractions were isolated at the indicated times after the injection of 15 μg/100 g body weight of either insulin (panel A) or the insulin analog, H2 (panel B). Proteins were resolved by SDS-PAGE and immunoblotted with anti-phosphotyrosine antibodies. The 94 kDa tyrosine phosphorylated β-subunit of the insulin receptor is indicated on the right of each blot.

PM (panel C) and GE fractions (panel D) were isolated at the indicated times after the injection of either insulin (▨) or the insulin analog, H2 (■). The fractions were immunoblotted as described above and the [^{125}I]-labeled band corresponding to the tyrosine phosphorylated β-subunit of the insulin receptor, was quantitated by phosphoimager analysis. The ratio of tyrosine phosphorylated (PY) insulin receptor / insulin receptor was calculated by dividing the PY quantitation by the amount of insulin receptor present at each respective time point (Fig.22). The bars represent the mean ± half variance of two separate experiments.


PM
GE
A. Insulin
94 kDa
B. H2
94 kDa
0 0.5 5 15 30 60 0 0.5 5 15 30 60
Time (min)


C. PM
PY Insulin receptor/ Insulin receptor
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Insulin
H2
0 0.5 5 15 30 60
Time (min)

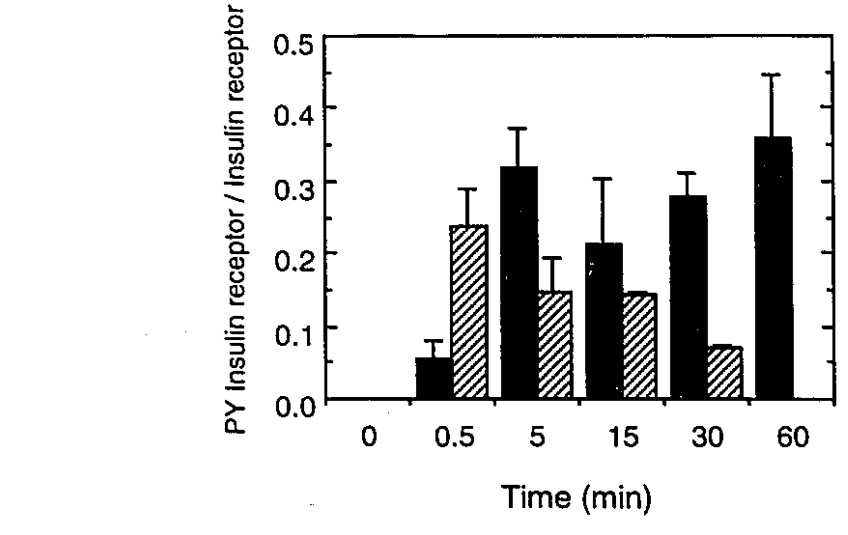

D. GE
PY Insulin receptor / Insulin receptor
0.5
0.4
0.3
0.2
0.1
0.0
0 0.5 5 15 30 60
Time (min)

Figure 25

SHC distribution in membrane compartments in response to insulin and H2 stimulation

Plasma membrane (PM) and endosomal (GE) fractions were isolated at the indicated times after the injection of 15 µg/100 g body weight of either insulin (panel A) or the insulin analog, H2 (panel B). 50 µg PM fraction protein and 25 µg GE fraction protein were resolved by SDS-PAGE and immunoblotted with antibody raised to the SHC SH2 domain. HRP-conjugated secondary antibody was used to visualize the SHC isoforms associated with the membrane fractions. The 46 and 55 kDa isoforms of SHC are indicated on the right.

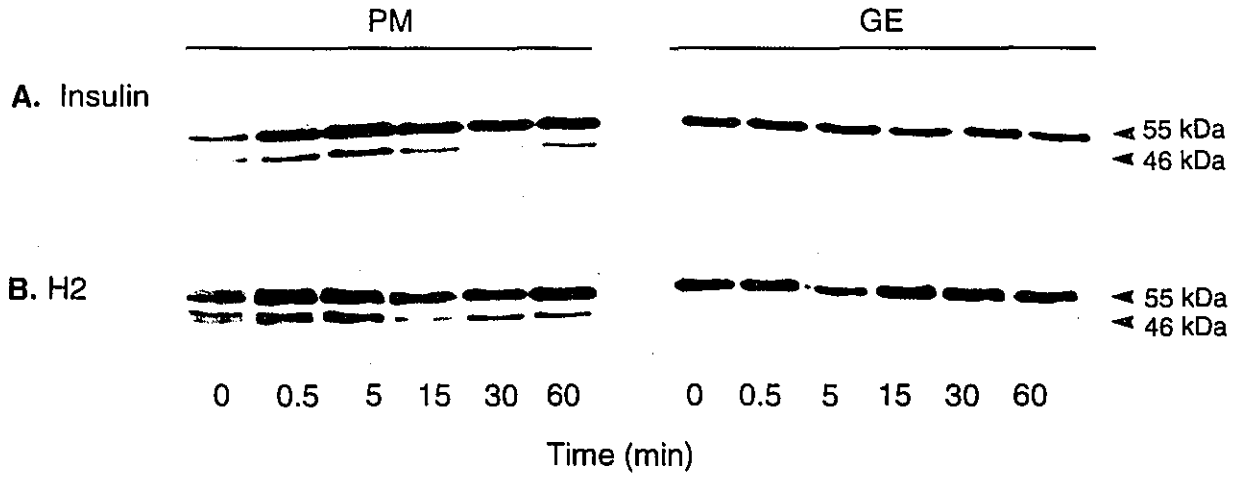

PM
GE
A. Insulin
B. H2
55 kDa
46 kDa
55 kDa
46 kDa
0 0.5 5 15 30 60
0 0.5 5 15 30 60
Time (min)

Figure 26

Tyrosine phosphorylation of cytosolic proteins, in response to insulin and H2

Rat liver cytosol was isolated at the indicated times after the injection of 15 μg/100 g body weight of either insulin or the insulin analog, H2. 300 μg of protein was resolved by SDS-PAGE on an 8% resolving gel, transferred to nitrocellulose and immunoblotted with antisera to phosphotyrosine. A 185 kDa tyrosine phosphorylated polypeptide is indicated. The molecular mass markers are indicated on the left.

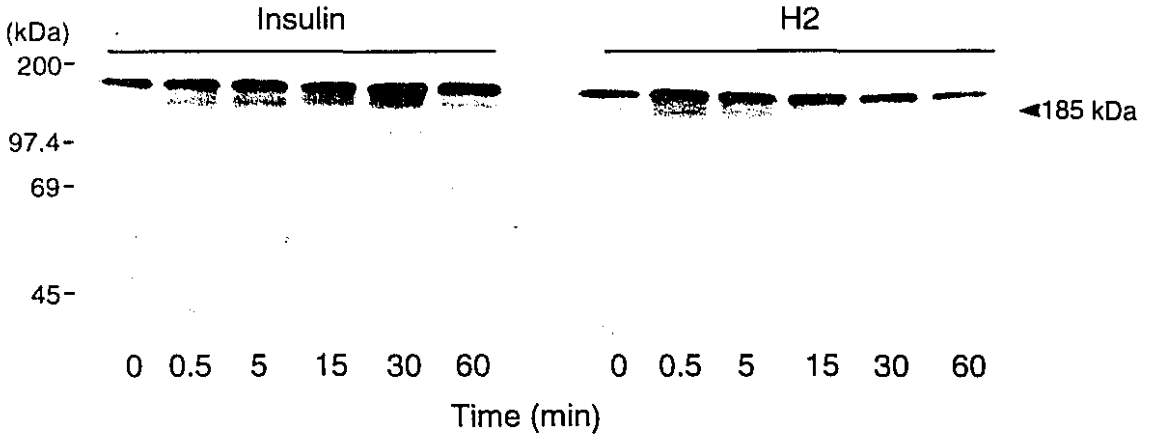

(kDa)
200-
97.4-
69-
45-
Insulin
H2
185 kDa
0 0.5 5 15 30 60
0 0.5 5 15 30 60
Time (min)

Figure 27

IRS-1 distribution in response to insulin and H2 stimulation

Cytosol (Cyt), plasma membrane (PM), endosomal (GE) fractions and were isolated at the indicated times after the injection of 15 μg/100 g body weight of either insulin (panel A) or the insulin analog, H2 (panel B). 300 μg cytosolic protein, 50 μg PM fraction protein, and 25 μg GE fraction protein were resolved by SDS-PAGE and immunoblotted with antibody raised to the insulin receptor substrate, IRS-1. Arrows on the right indicate the mobility of IRS-1 (185 kDa). Molecular mass markers are indicated on the left.

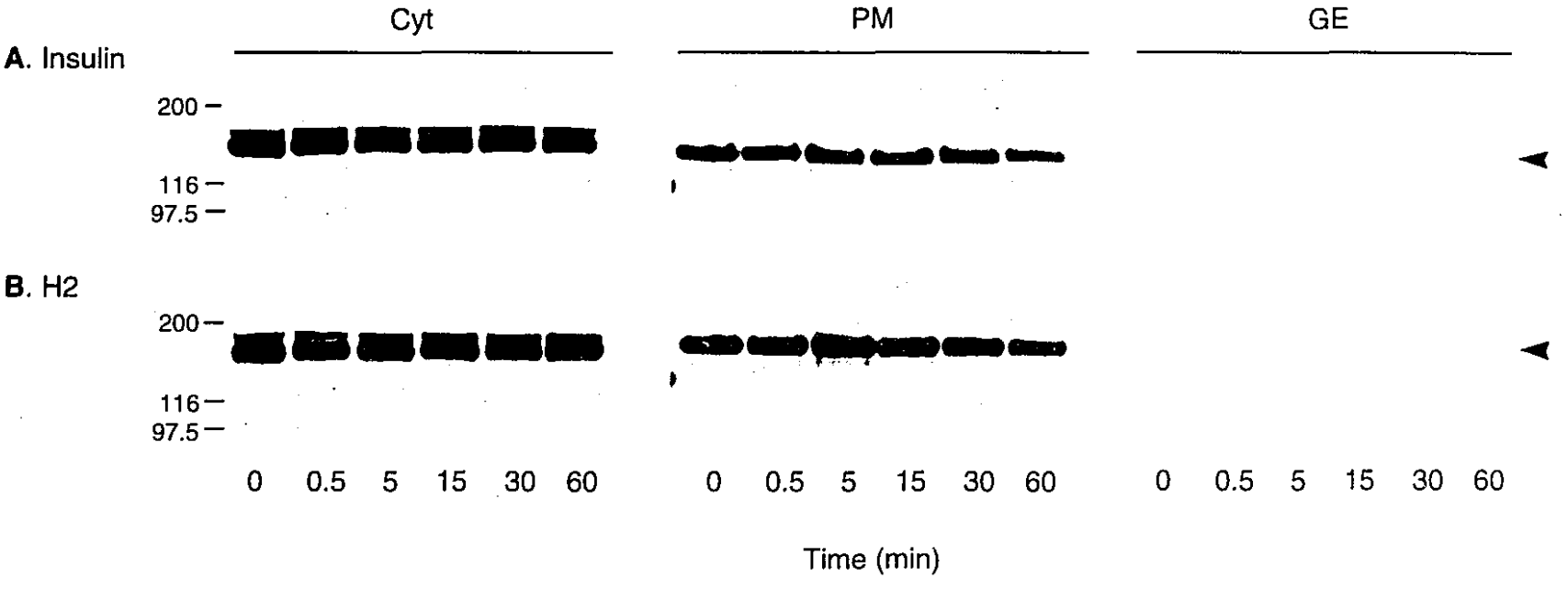
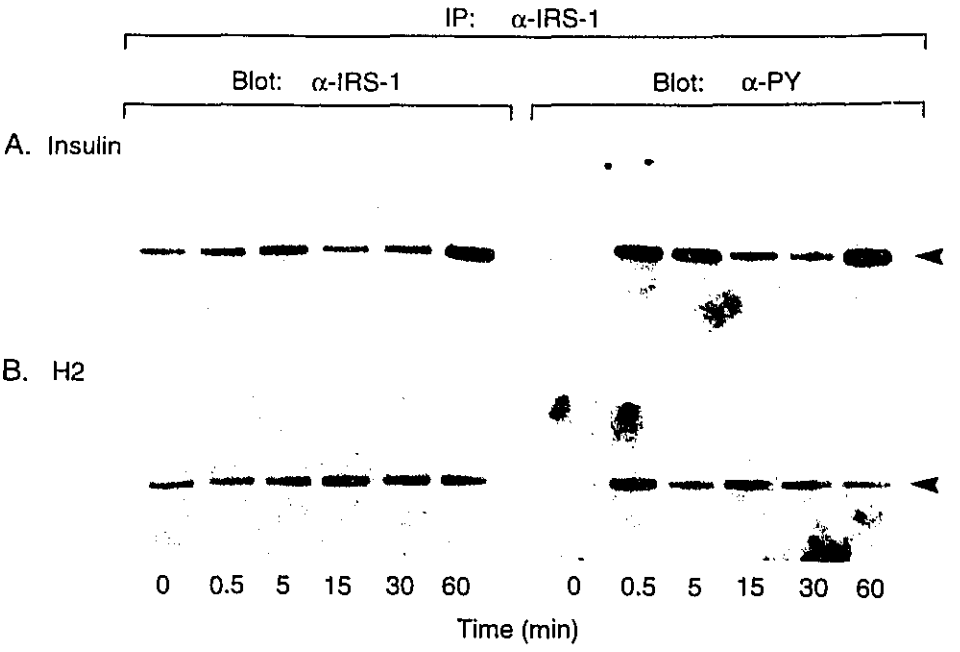

Cyt
PM
GE
A. Insulin
200
116
97.5
B. H2
200
116
97.5
0 0.5 5 15 30 60
0 0.5 5 15 30 60
0 0.5 5 15 30 60
Time (min)

Figure 28

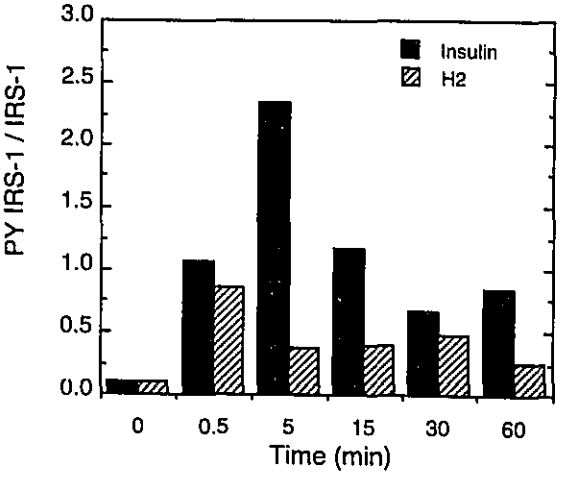
Tyrosine phosphorylation of cytosolic IRS-1 in response to ligand stimulation

Rat liver cytosol was isolated at the indicated times after the injection of 15 µg/100 g body weight of insulin (panel A) or H2 (panel B). 10 mg of cytosolic protein was immunoprecipitated with anti-IRS-1 antibodies. The immunoprecipitated proteins were resolved by SDS-PAGE and immunoblotted with anti-IRS-1 or anti-phosphotyrosine (PY) antibodies. The 185 kDa bands corresponding to IRS-1 and the phosphorylated IRS-1 are indicated to the right of each panel (arrowheads).

The [^{125}I]-labeled bands corresponding to IRS-1 and tyrosine phosphorylated IRS-1 were quantified by phosphoimager analysis and the results expressed as the ratio of tyrosine phosphorylated (PY) IRS-1 to IRS-1.


IP: α-IRS-1
Blot: α-IRS-1
Blot: α-PY
A. Insulin
B. H2
0 0.5 5 15 30 60
0 0.5 5 15 30 60
Time (min)

C.


PY IRS-1 / IRS-1
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Insulin
H2
0 0.5 5 15 30 60
Time (min)

Chapter IV

Ras-MAPK cascade and Nuclear Transcription

The activation of the EGF and insulin receptors has been shown to result in the accumulation of the active GTP-bound form of Ras in a variety of cell systems (Li *et al.*, 1993; Buday and downward, 1993). The positive regulation of Ras, i.e. the exchange of GDP for GTP, requires the action of guanine nucleotide exchange factors such as SOS (Bowtell *et al.*, 1992; Segal *et al.*, 1993). The interaction of SOS with Ras has been shown to depend on receptor activation, and the recruitment of the GRB2/SOS complex to the membrane where Ras is located. In response to EGF, the GRB2/SOS complex may bind the tyrosine phosphorylated residues of the EGF receptor itself, or that of SHC (Rozakis-Adcock et al., 1993; Sasaoka et al., 1994b). In response to insulin receptor stimulation, the GRB2/mSOS complex has been postulated to interact with phosphorylated SHC and/or IRS-1, leading to the activation of Ras (Pronk et al., 1993; Rose et al., 1994). Thus, Ras might be a connecting point for the signal transduction pathways from these tyrosine kinase receptors.

The generation of Ras-GTP leads to the activation of a cytosolic serine/threonine protein kinases cascade, involving Raf-1, MEK and MAP kinase (MAPK) (Marshall, 1995a). Raf-1 interacts directly with Ras-GTP, and when activated, phosphorylates and activates MEK, which in turn phosphorylates and activates MAPK (Seger and Krebs, 1995). Ultimately, the function of this cascade appears to be to generate activated MAPK capable of translocating into the nucleus. When in the nucleus a variety of preexisting transcription factors can be phosphorylated and activated by MAPK (Seth *et al.*, 1991; Baker *et al.*, 1992; Gille *et al.*, 1992). As a result of transcription factor activation a series of early response genes are transcribed. Among the best characterized early response genes are the c-fos, c-jun and c-myc proto-oncogenes (rev. Ransone and Verma, 1990). These genes code for proteins which are themselves transcription factors, which regulate transcription from their own promoters as well as others. Although some targets for these transcription factors are known, for example ODC and PEPCK, the regulation of these downstream genes involves other regulatory factors as well. The growth factor induced stimulation of these genes is often correlated with a mitogenic response, although this is dependent on the cell type, and the state of differentiation of the cell.

Ligand stimulated EGF receptor activation resulted in the phosphorylation of SHC in the endosomal compartment as well as in the cytosol. An association of GRB2/SOS with the EGF receptor was observed in the endosome, as well as with phosphorylated SHC in a cytosolic complex (Chapter II). In response to insulin receptor activation, either by insulin or H2, IRS-1 phosphorylation was evident, however, no SHC phosphorylation was observed (Chapter III). Thus a potential pathway to Ras-MAPK activation exists in rat liver in response to both EGF and insulin receptor activation. In the former, this may occur

though the EGF receptor or phosphorylated SHC, in the latter case, through the intermediate of IRS-1. The following studies were carried out to determine if the Ras-MAPK cascade was activated by these ligands in rat liver, and resulted in the classically observed early response gene transcription.

Results

Growth factor induced activation of the GTPase Ras is generally determined directly using [^{32}P] labeled cells in culture. However, the evaluation of the GTP content of Ras *in vivo* presents experimental difficulties with respect to the specific radioactivities of [^{32}P]-GTP pools following [^{32}P] administration. We elected therefore to assess the molecules downstream of Ras, i.e. Raf-1 and MAPK, to determine if indeed this signal transduction pathway is activated in rat parenchyma by EGF, insulin or H2 stimulation.

Raf-1 Western blots were carried out on liver cytosol after ligand administration. These results showed that a Raf-1 mobility shift could indeed be observed, but only in response to EGF stimulation (Fig.29). This shift appeared between 5 and 15 min after stimulation and was maintained up to 60 min. The time course was extended to show that Raf-1 became partially dephosphorylated by 90 min post injection.

When these cytosolic fractions were immunoblotted with anti- MAPK antibodies, a mobility shift was demonstrates only in response to EGF stimulation (Fig.30). Mobility shifts in SDS-PAGE have been proposed to reflect MAPK activation and has been attributed to phosphorylation by MEK (Cobb *et al.*, 1991; Seger and Krebs, 1995). The mobility shift was apparent 30 sec after EGF stimulation and peaked at 5 -15 min (A). No shift was observable in response to insulin stimulation (B) although a small shift was observed in response to H2 stimulation at 5 min post-injection (C).

It has been suggested that Ras, Raf-1, MEK and MAPK become associated in a large complex at the plasma membrane as a result of ligand induced receptor activation. To determine if the recruitment of MAPK could be observed in the membrane compartments as a result of EGF receptor activation, the PM and GE fractions were immunoblotted with antibodies to MAPK (Fig.31). The results showed a recruitment of MAPK to the PM within 30 sec of EGF administration, and an association with the endosomal compartment which peaked at 15 min post injection. The kinetics of association of this MAPK to the PM and the endosomal fraction appear to parallel those of the EGF receptor internalization and phosphorylation (see Chapter II, Fig.17).

Activation of the EGF and insulin receptors has been shown to result in changes in the nuclear transcription and/or the increase in messenger RNA levels of the early response

genes (Messina *et al.*, 1989, 1990; Ransone and Verma, 1990). Northern blots were carried out on total RNA isolated from rat livers after the injection of EGF, insulin or H2 to test if this effect of EGF or insulin administration could be observed *in vivo*. In response to EGF stimulation, an increase in the level of c-fos transcript was observed by 15 min. The level of c-fos mRNA peaked at 30 min (~4 fold increase) and declined thereafter (Fig.32). There was no observable effect of either insulin or H2 on the levels of this transcript. The c-fos gene product functions as a transcription factor with the assistance of the c-jun protein. Therefore, the effect of the various ligands on the levels of c-jun mRNA was examined (Fig.33). EGF stimulation resulted in a maximal ~5 fold increase in c-jun mRNA levels. This level was reached by 30 min post injection and appeared to be maintained up to 1 hr. In response to insulin the levels of transcript increased only slightly by 60 min. H2 stimulation, however, increased c-jun transcript levels ~2 fold by 30 min and ~3 fold by 60 min.

Another early response gene whose mRNA levels have been shown to be increased in response to both EGF and insulin stimulation, is the c-myc transcription factor (Kruijer *et al.*, 1986; Morimura *et al.*, 1990). EGF stimulation of rat liver, resulted in a maximal increase in transcript level of ~2 fold by 30 min which was maintained up to 60 min (Fig.34). Insulin did not appear to cause a significant increase in c-myc mRNA level, however, H2 stimulation resulted in a ~2 fold increase which could be observed by 60 min.

An increase in GAPDH (a glycolytic enzyme) gene expression has been demonstrated in response to insulin stimulation in H35 hepatoma cells (Alexander *et al.*, 1988; Messina, 1989), therefore, the level of this transcript was determined in response to ligand stimulation. The level of GAPDH mRNA did not appear to be affected by the administration of either EGF, insulin or H2, in rat liver, for the time course of the experiment (Fig.35). As a result, GAPDH mRNA levels were used as a control to ensure equal loading of RNA on the gels.

Discussion

The activation of insulin and EGF receptor tyrosine kinases has been demonstrated to result in the initiation of the Ras-MAPK cascade (Lowy and Willumsen, 1993). The first step in this cascade is the activation of the ser/thr kinase Raf-1, as a consequence of its interaction with activated GTP-bound Ras. While the regions in these two molecules required for this interaction have been identified, the exact mechanism by which Raf-1 activation takes place is unclear, although phosphorylation of Raf-1 on ser/thr residues is involved. Changes in

the phosphorylation status of Raf-1 and other proteins in the cascade, can be observed as mobility shifts in SDS-PAGE, and have been suggested to represent kinase activation states. In response to *in vivo* EGF administration, mobility shifts could be observed in both Raf-1 and MAPK. The mobility shift of Raf-1 may be a result of autophosphorylation and as thus could represent Raf-1 activation (Morrison *et al.*, 1988; 1989). On the other hand, Raf-1 mobility shift may be the result of MAPK phosphorylation in a negative feedback loop (Anderson *et al.*, 1991). The kinetics of Raf-1 phosphorylation are delayed with respect to those of MAPK which is consistent with the Raf-1 mobility shift being, at least in part, a result of phosphorylation by MAPK in a feedback loop. This may represent a negative regulatory mechanism designed to control the length of time the MAPK cascade can remain active. If this is the case, the duration of this hyperphosphorylated state of Raf-1, from 15- 60 min, may prevent a second round of signaling by this pathway before this time. This could be tested by injection of a second dose of EGF during this time to determine if MAPK, which is downstream of Raf-1, could be reactivated.

Activated MAPK may also phosphorylate other upstream components of the pathway, such as MEK1 (Matsuda *et al.*, 1993), and the EGF receptor (Northwood *et al.*, 1991; Hill and Treisman, 1995; Marshall, 1995b). The presence of phosphoserines and threonines in the EGF receptor in response to ligand stimulation may represent an additional level of negative feedback control at the level of the signal initiator, the receptor kinase activity. The observation that MAPK is recruited to the PM and the endosome, with apparently similar kinetics as the activated EGF receptor, could be consistent with the role of MAPK as an inhibitor of EGF receptor activity. However, the presence of an active EGF receptor tyrosine kinase in the endosome of rat liver was demonstrated by Kay *et al.* (1986). In addition, an increase in the phosphorylation of EGF receptor associated SHC was observed in this compartment which is consistent with continued kinases activity (Chapter II). These data imply a function other than negative regulation, for the recruitment of MAPK to the PM and the endosome.

When MAPK is activated, it translocates to the nucleus, where it phosphorylates and activates pre-existing transcription factors, such as Jun and $p62^{TCF}$/Elk (Baker *et al.*, 1992; Gille *et al.*, 1992). These factors act to regulate the transcription of specific genes for example, c-jun and c-fos and c-myc, which themselves act as transcription factors. These genes are considered early response genes as they are the first to be modulated in response to ligand treatment and do not require de novo protein synthesis to regulate their transcription. EGF stimulation of rat liver results in the increase in level of mRNA for the transcription factors c-fos, c-jun and c-myc.

The c-fos gene has several regulatory sequences in its promoter region, including the serum response element (SRE), the sis-inducible element (SIE) and the AP-1 binding site (Treisman, 1992; 1995). At the SRE, a ternary complex forms which includes the serum response factor $p67^{SRF}$ and the ternary complex factor, $p62^{TCF}$/Elk-1. $p62^{TCF}$/Elk-1 is regulated by MAPK phosphorylation in response to extracellular signals. The SIE binds the transcription factors of the STAT family which are activated in response to cytokine and growth factor stimulation. There is evidence, via genomic footprinting, that the SRE and AP-1 sites are constitutively occupied *in vivo*, while the SIE binding activity is highly regulated. The contributions of these various regulatory regions remains to be elucidated. Studies by Treisman (1995) using c-fos promoter mutants in NIH 3T3 cells indicate that EGF activated the c-fos promoter via cooperation of the SRE and the SIE, although it did not induce detectable STAT DNA binding activity. The c-jun promoter region does not contain and SRE but does contain an RSRF (related to serum response factor) binding site which appears to be an important element for EGF induction of the promoter (Han *et al.*, 1992). However, regulation of transcription appears to be vastly more complicated as many regulatory promoter sequences are being identified. There are other mechanisms, in addition to increased gene transcription, which lead to the accumulation of a specific mRNA. These include the regulation of RNA stability, an alteration of the processing of the primary transcript, controlling the stability of the transcript before its release from the nucleus, and modification of the transport of the processed mRNA out of the nucleus. The induction of the two components of the AP-1 complex through different members of the large MAPK family, including the related Jun N-terminal kinase/ stress activated protein kinase (JNK/SAPK), may ensure that the AP-1 activity responds to a wide range of stimuli (Cano and Mahadevan, 1995). It is also possible that through divergent activation of MAPKs and JNK/SAPKs, different extracellular stimuli may induce different types of AP-1 complexes thus leading to the transcription of different sets of target genes.

The proteins translated from these two mRNAs are transcription factors (Ransone and Verma, 1990). They function as Fos-Jun heterodimers or Jun-Jun homodimers to regulate gene expression. The time course of activation reveals that the maximal levels of c-fos and c-jun transcript are attained at the same time (30 min). However, while the level of c-fos transcripts rapidly declines thereafter, that of c-jun remains elevated. This may be explained by the fact that fos negatively autoregulates its promoter while jun positively autoregulates its promoter. Ultimately this will result in two different sets of transcription complexes being activated. Initially , the level of Fos and Jun will be similar and the heterodimers will form preferentially. Subsequently , Fos levels will drop while Jun levels

will remain elevated leading to the formation of homodimers. These transcription complexes interact with different affinities to the AP-1 promoter site (Curran and Franza, 1988; Angel and Karin, 1991), and as a consequence, different sets of genes will be transcriptionally regulated by these two complexes at different times after EGF stimulation. This may represents another level of control which results in a the fine tuning of further gene transcription.

High AP-1 (Fos and Jun protein complex) expression has been shown to directly suppress the activity of the albumin gene enhancer (Hu and Isom, 1994). This correlates with a loss of differentiated phenotype and enhanced hepatocyte proliferation in cultured cells (Rana *et al.*, 1994). However, while EGF is present in the blood of normal mice, it apparently acts as a hepatocyte mitogen only in regenerating livers (Fausto *et al.*, 1995), in addition, the infusion of EGF into the portal veins of healthy adult animals fails to induce a proliferative response comparable to that which follows partial hepatectomy. This limited proliferative response *in vivo* may be a result of the presence of an intact extracellular environment which may play a role in limiting the EGF induced proliferative response in a healthy animal. This implies that other factors, such as TGF-α and HGF/SF for example, are involved in the full proliferative response obtained in the liver in response to injury.

Administration of insulin has been proposed to cause the activation of the Ras-MAPK cascade. This activation has been proposed to take place as a result of the association of GRB2/SOS with phosphorylated IRS-1 or SHC (Rose *et al.*, 1994; Myers *et al.*, 1994; Sasaoka *et al.*, 1994a,b). In addition PI3'-kinase has been proposed to function upstream of Ras and Raf-1 in mediating the insulin signaling pathway leading to the transcriptional activation of the c-fos serum response element (Yamauchi *et al.*, 1993). However, the administration of receptor saturating doses of either insulin or the insulin analog H2 did not result in the tyrosine phosphorylation of SHC nor in the activation of the Ras-MAPK cascade. This implies that tyrosine phosphorylated IRS-1 did not activate GRB2/SOS, and that PI3'-kinase, if associated with IRS-1, did not function upstream of Ras in the liver.

Insulin administration did not cause the stimulation of nuclear transcription of the early response genes to the levels attained after EGF stimulation. In H411E cells, rat hepatoma cells that are known to retain the characteristics of mature hepatocytes, insulin has been shown to lead to c-myc mRNA induction within 2 hours of treatment (Morimura *et al.*, 1990). The response, however, was completely blocked by cycloheximide suggesting that this induction was dependent on the de novo synthesis of transcription factors. The primary role of insulin in the liver is to control metabolic responses, therefore the lack of activation of the Ras-MAPK pathway, which is generally believed to be a

mitogenic pathway, is consistent with this *in vivo* function of insulin in the liver. In addition, Lazar *et al.* (1995) demonstrated that MAPK activation was not required for insulin regulation of glucose metabolism, including glycogen synthesis, glucose uptake and lipogenesis in highly responsive insulin target cells, 3T3-L1 adipocytes.

The increase of c-jun and c-myc mRNA levels in response to H2 is interesting. The lack of MAPK and Raf-1 mobility shifts in response to H2 suggests that another pathway is activated by the insulin receptor, stimulated with H2, which selectively accesses c-jun but not c-fos. It is possible that the stress activated protein kinases cascade may be involved. This pathway leads to the N-terminal phosphorylation and activation of the transcriptional activity of Jun (Minden *et al.*, 1994) which may then autoregulate the AP-1 element in the c-jun promoter. This remains to be tested directly. The transfection of c-myc into primary hepatocytes did not initiate DNA synthesis in these primary hepatocytes (Skouteris and Kaser, 1992) and evidence suggests that Myc functions in association with components acting downstream of the Ras pathway to cause hepatic cell proliferation. Therefore, the increase in c-jun levels alone (without c-fos), may not be sufficient for c-myc to elicit a proliferative response in hepatocytes The effect of increasing the level of these two transcripts remains to be determined. Since the only significant difference observed in response to insulin and H2 stimulation occurred at the level of the endosome, one might look at this compartment in search of the pathways initiated which cause selective transcriptional activation in response to H2.

The stimulation of rat liver with EGF clearly results in the activation of a classical mitogenic pathway: the Ras/MAPK cascade. The involvement of SHC, GRB2/SOS were demonstrated (Chapter II) and appeared to cause the activation of the Ras/MAPK pathway, as well as the expected early responses, an increase in mRNA levels of c-fos, c-jun and c-myc, at the level of the nucleus. Although insulin stimulation in cell culture resulted in the activation of the MAPK cascade and nuclear transcription of the early response genes, this was not observed in rat liver. This suggests that although the pathway is present and functional, the liver possesses a mechanism which prevents activation of the Ras-MAPK by the insulin receptor. The mechanism which carries this out remains to be identified.

Figure 29

Raf-1 mobility shift in response to ligand stimulation

Rat liver cytosol was isolated at the indicated times after the injection of 10 µg/ 100 g body weight EGF (panel A), 15 µg/ 100 g body weight of insulin (panel B) or the insulin analog, H2 (panel C). 300 mg of cytosolic protein was resolved by SDS-PAGE and immunoblotted with anti-Raf-1 antibodies. A shift in mobility of Raf-1 was observed in response to EGF stimulation (panel A). Molecular mass markers are indicated on the left.

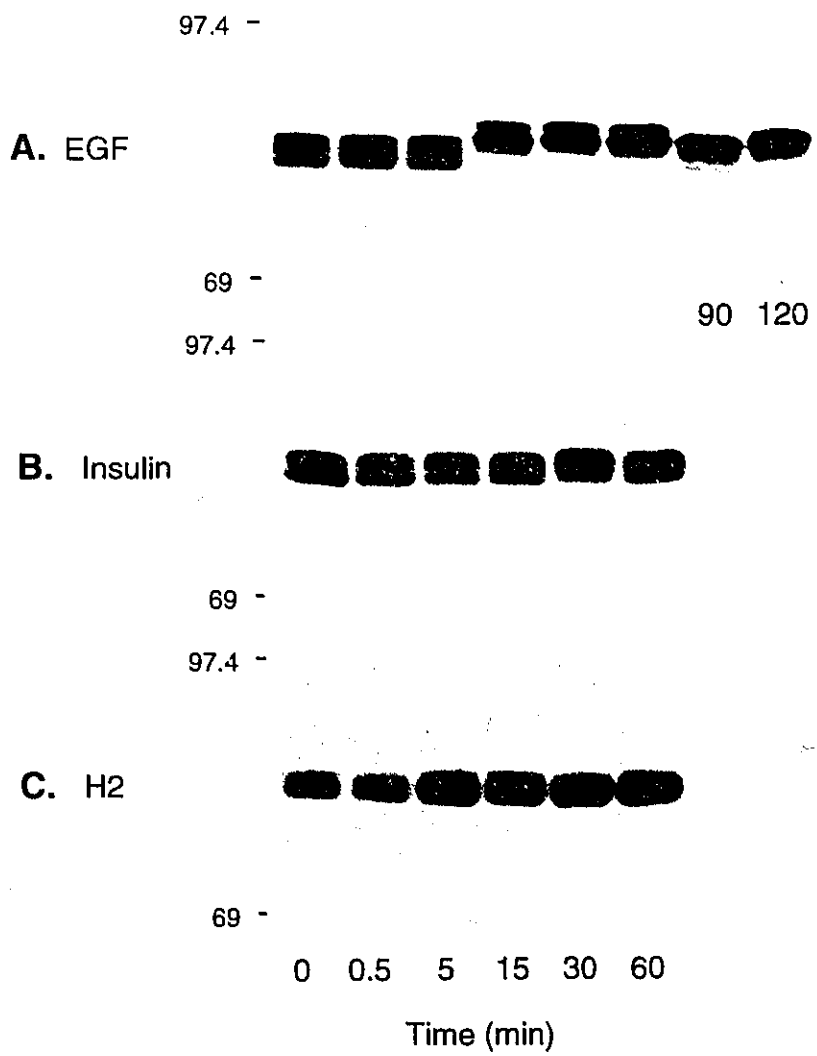

97.4
A. EGF
69
97.4
90 120
B. Insulin
69
97.4
C. H2
69
0 0.5 5 15 30 60
Time (min)

Figure 30

Cytosolic MAP kinase mobility shift in response to ligand stimulation

Rat liver cytosol was isolated at the indicated times after the injection of EGF (panel A), insulin (panel B) or the insulin analog, H2 (panel C). 300 mg of cytosolic protein was resolved by SDS-PAGE and immunoblotted with anti-MAP kinase antibodies. A shift in molecular weight of one of the MAP kinase isoforms was observed in response to EGF stimulation and is indicated on the right by an arrowhead. The molecular mass markers are indicated on the left.

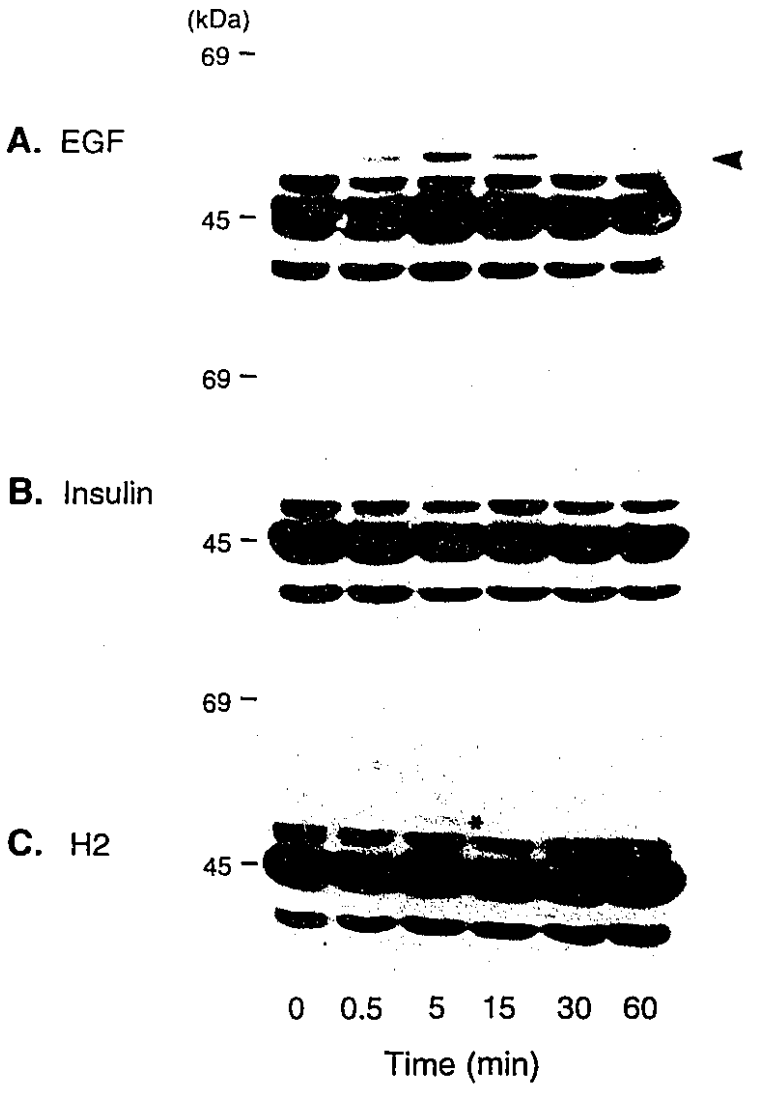

(kDa)
69
A. EGF
45
69
B. Insulin
45
69
C. H2
45
0 0.5 5 15 30 60
Time (min)

Figure 31

MAP kinase redistribution in response to EGF stimulation

Rat liver plasma membrane (PM) and endosomal (GE) fractions were isolated at the indicated times after the injection of 10 μg/100 g body weight of EGF. 100 μg of PM fraction protein and 50 μg of GE fraction protein were resolved by SDS-PAGE and immunoblotted with anti-MAP kinase antibodies. The MAP kinase recruited to the membrane fractions in an EGF dependent manner is indicated by the arrowhead on the right.

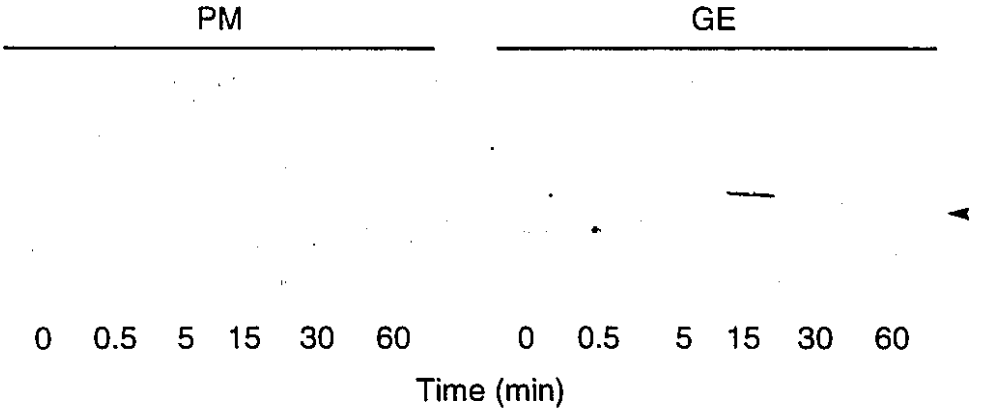
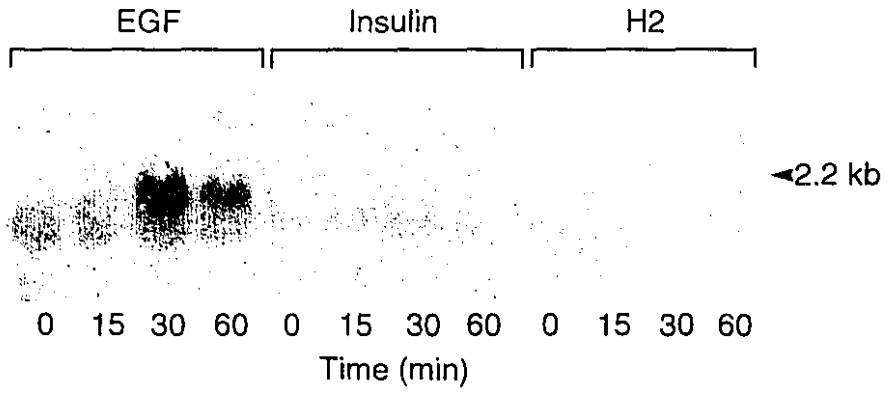

PM
GE
0 0.5 5 15 30 60
0 0.5 5 15 30 60
Time (min)

Figure 32

Northern analysis of c-fos mRNA levels in response to ligand stimulation

Total RNA was isolated from rat livers at the indicated times after the injection of 10 µg/ 100 g body weight EGF (—●—) or 15 µg/ 100 g body weight of insulin (—□—) or the insulin analog, H2 (—△—). 20 µg of RNA was resolved on a denaturing 1.2 % agarose/ formaldehyde gel. The RNA was transferred onto Nylon membrane and hybridized with a [^{32}P]-dCTP labeled cDNA probe for c-fos (panel A). The band representing the c-fos transcript (2.2 kb) is indicated on the right. The [^{32}P] band was quantified by phosphoimager analysis and expressed as arbitrary phosphoimager units (panel B). The results represent the mean ± SD of three separate experiments.

A.


EGF
Insulin
H2
2.2 kb
0 15 30 60 0 15 30 60 0 15 30 60
Time (min)

B.

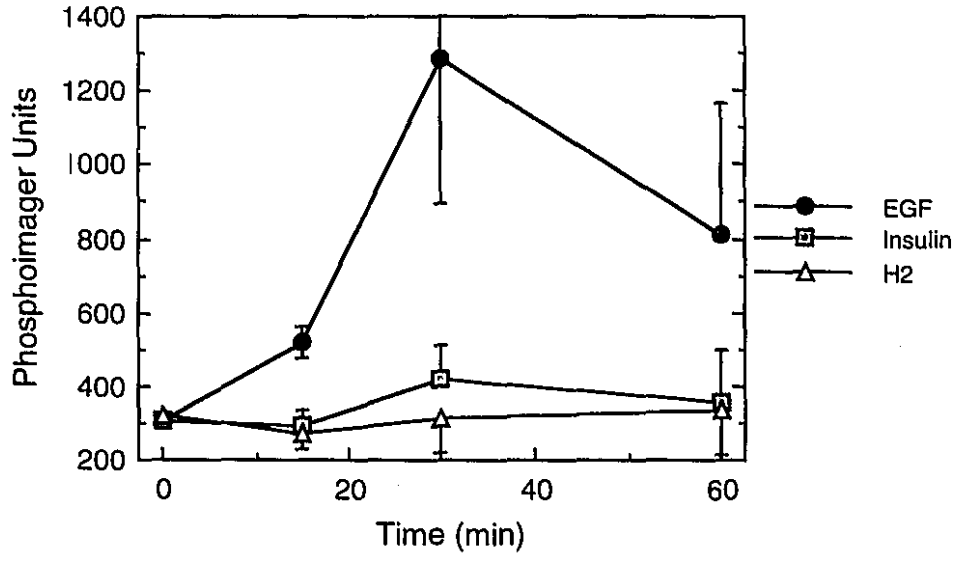
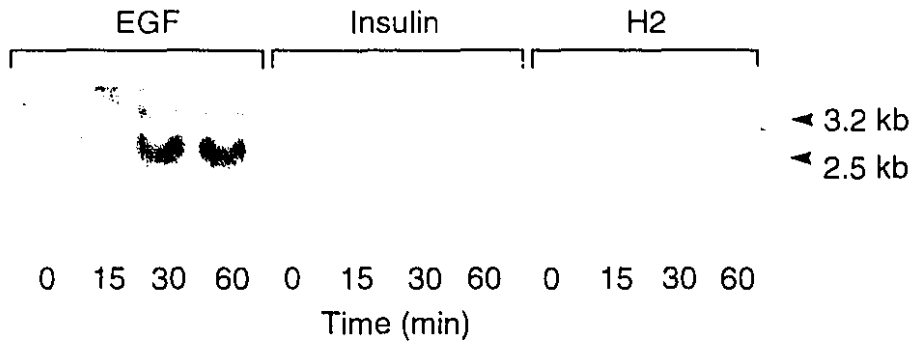

Phosphoimager Units
1400
1200
1000
800
600
400
200
0
20
40
60
Time (min)
EGF
Insulin
H2

Figure 33

Northern analysis of c-jun mRNA levels in response to ligand stimulation

Total RNA was isolated from rat livers at the indicated times after the injection of 10 µg/ 100 g body weight EGF (—●—) or 15 µg/ 100 g body weight of insulin (—■—) or the insulin analog, H2 (—Δ—). 20 µg of RNA was resolved on a denaturing 1.2 % agarose/ formaldehyde gel. The RNA was transferred onto Nylon membrane and hybridized with a [^{32}P]-dCTP labeled cDNA probe for c-jun (panel A). The bands representing the two c-jun transcripts (2.5 and 3.2 kb) are indicated on the right. The [^{32}P] band corresponding to the 2.5 kb transcript was quantified by phosphoimager analysis and expressed as arbitrary phosphoimager units (panel B). The results represent the mean ± SD of three separate experiments.

A.


EGF
Insulin
H2
3.2 kb
2.5 kb
0 15 30 60 0 15 30 60 0 15 30 60
Time (min)

B.

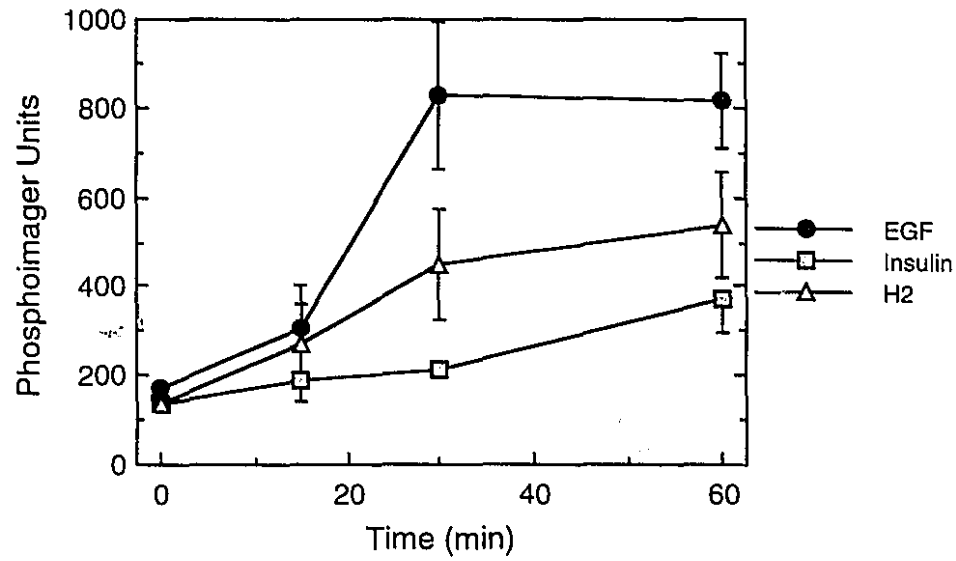
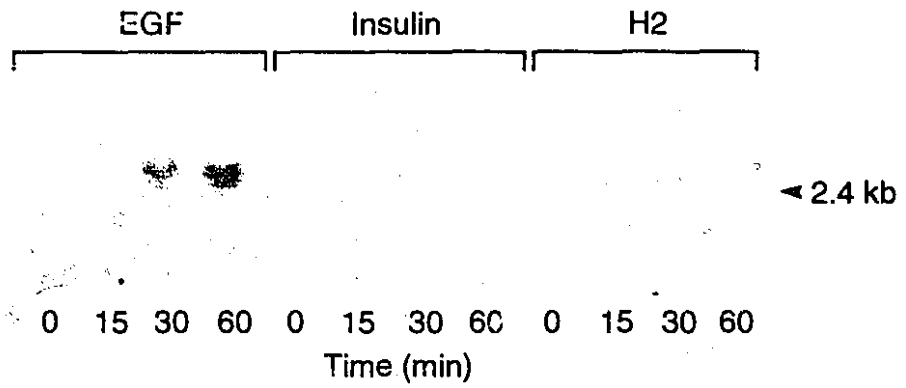

Phosphoimager Units
1000
800
600
400
200
0
0
20
40
60
Time (min)
EGF
Insulin
H2

Figure 34

Northern analysis of c-myc mRNA levels in response to ligand stimulation

Total RNA was isolated from rat livers, at the indicated times after the injection of 10 μg/ 100 g body weight EGF (—●—) or 15 μg/ 100 g body weight of insulin (—▣—) or the insulin analog, H2 (—△—). 20 μg of RNA was resolved on a denaturing 1.2 % agarose/ formaldehyde gel. The RNA was transferred onto Nylon membrane and hybridized with a [^{32}P]-dCTP labeled cDNA probe for c-myc (panel A). The band representing the c-myc transcript (2.4 kb) is indicated on the right. The [^{32}P] band was quantified by phosphoimager analysis and expressed as arbitrary phosphoimager units (panel B). The results represent the mean ± SD of three separate experiments.

A.


EGF
Insulin
H2
2.4 kb
0 15 30 60 0 15 30 60 0 15 30 60
Time (min)

B.

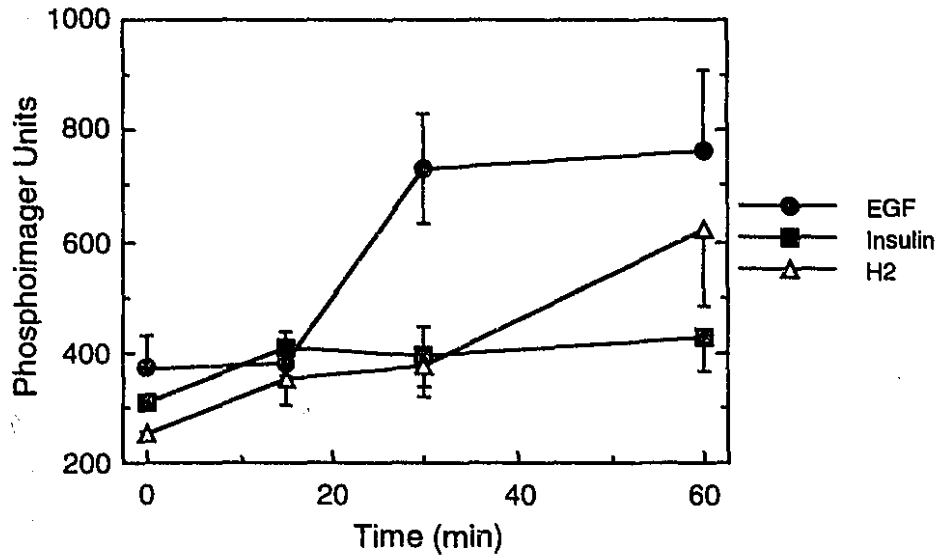
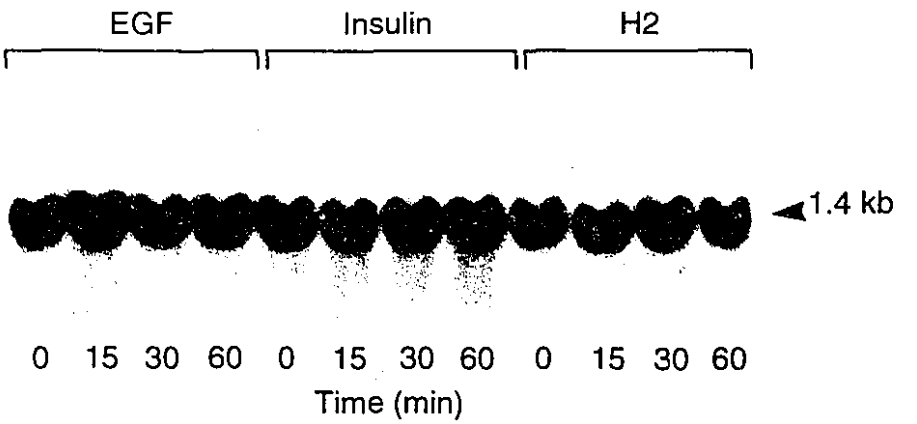

1000
800
600
400
200
Phosphoimager Units
0
20
40
60
Time (min)
EGF
Insulin
H2

Figure 35

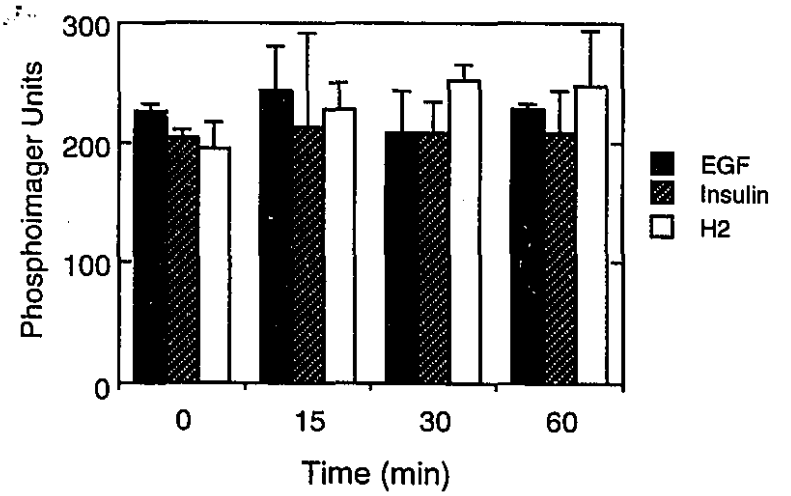
Northern analysis of GAPDH mRNA levels in response to ligand stimulation

Total RNA was isolated from rat livers at the indicated times after the injection of 10 µg/ 100 g body weight EGF (■) or 15 µg/ 100 g body weight of insulin (▨) or the insulin analog, H2 (□). 20 µg of RNA was resolved on a denaturing 1.2 % agarose/ formaldehyde gel. The RNA was transferred onto Nylon membrane and hybridized with a [^{32}P]-dCTP labeled cDNA probe for GAPDH (panel A). The band representing the GAPDH transcript (1.4 kb) is indicated on the right. The [^{32}P] band was quantified by phosphoimager analysis and expressed as arbitrary phosphoimager units (panel B). The results represent the mean ± SD of three separate experiments.

A.


EGF
Insulin
H2
1.4 kb
0 15 30 60 0 15 30 60 0 15 30 60
Time (min)

B.


Phosphoimager Units
300
200
100
0
0
15
30
60
Time (min)
EGF
Insulin
H2

Discussion

Signal transduction describes the conversion of an extracellular signal into intracellular signaling cascades that feed the information from the receptor into the nucleus where changes in gene expression are induced. These extracellular signaling molecules often exert their effects on target cells through the binding and activation of receptor tyrosine kinases (RTKs). Because signal transduction pathways from activated RTKs, such as the insulin and EGF receptors, control critical cellular functions such as metabolism, growth and differentiation, these pathways must be specific and highly regulated. The control of specificity of signal transduction pathways employed by an activated receptor, and the regulation of these pathways must occur at multiple levels in the cell. This work has attempted to gain insight into the signal transduction pathways used by the EGF and insulin receptor in a physiologically relevant system, rat liver parenchyma, and the potential role of receptor endocytosis in regulating signaling from these two receptors.

Signal transduction from the Plasma Membrane

In resting hepatocytes receptors for EGF and insulin are expressed, in equal numbers, on the cell surface. Ligand binding induces receptor dimerization, kinase activation and the transphosphorylation of receptors on defined tyrosine residues. Progress in understanding the mechanisms of receptor tyrosine kinase signal transduction has been made with the identification of multiple downstream targets of activated receptors (van der Geer *et al.*, 1994). Many of these proteins contain SH2 domains and/or PTB domains which specifically recognize phosphorylated tyrosine residues within the context of the flanking amino acids (van der Geer *et al.*, 1995). These interactions are specific and thus define the signal transduction pathways accessible to each activated, phosphorylated receptor.

The EGF Receptor

The tyrosine autophosphorylation of the epidermal growth factor (EGF) receptor at specific sites leads to the rapid recruitment (within 30 sec of injection) and stable association of several downstream signaling molecules. Although two of the SHC isoforms are present constitutively at the PM, EGF stimulation results in the further recruitment of SHC to the activated receptor. SHC contains both a PTB and an SH2 domain enabling it to bind two phosphorylated tyrosine residues of the EGF receptor (Batzer *et al.*, 1995). SHC is also a substrate for the active EGF receptor tyrosine kinase, however it appears that only a portion of the molecules present are phosphorylated by the

EGF receptor in this compartment. The reason for this is unclear, but it may be that the kinase has not reached its maximal activity at this time. Phosphorylation of SHC provides a site for the binding of the GRB2 SH2 domain and thus may serve as a mechanism to recruit SOS to plasma membrane associated Ras (Rozakis-Adcock *et al.*, 1992; Gotoh *et al.*, 1994). GRB2 may also associate with the activated, tyrosine phosphorylated EGF receptor directly. However, in the plasma membrane of rat liver cells, only very low levels of GRB2 are seen associated with the EGF receptor. This may be the consequence of the low levels of SHC phosphorylation or of the absence of GRB2 binding sites in the EGF receptor. The affinity of the GRB2 SH2 domain appears to be greater for the SHC tyrosine residue than those on the receptor (Cussac *et al.*, 1994), which suggests that if phosphorylated SHC is present, GRB2 would bind to this molecule preferentially. A possible explanation for the observations, may be the association of GRB2/SOS with phosphorylated SHC bound to the receptor and the subsequent dissociation of the SHC-GRB2/SOS complex into the cytosol. Indeed, such a cytosolic complex could be detected within 30 sec of ligand administration. The low levels of GRB2/SOS associated with the EGF receptor, in conjunction with the rapid rate of receptor internalization implies that a physical link between the receptor, the GRB2/SOS complex and Ras may not be possible. Thus, the generation of a receptor-independent cytosolic pool of phosphorylated SHC-GRB2/SOS complexes may be responsible for the activation of Ras and the subsequent phosphorylation of MAPK which is observed during this time. The identification of the precise tyrosine residues which are phosphorylated on the plasma membrane localized EGF receptor, using antibodies recognizing specific phosphotyrosine residues for example, may reveal the exact mechanism of GRB2/SOS recruitment to the plasma membrane and clarify how Ras activation by GRB2/SOS takes place.

The Insulin Receptor

When the insulin receptor is activated in rat liver it becomes autophosphorylated. However, the extent of receptor phosphorylation was greater when insulin was used as the ligand instead of the high affinity analog, H2. Why this occurred is not clear, but may be due to slight differences in receptor conformation, making tyrosine residues less accessible to the kinase. Unlike the EGF receptor, insulin receptor kinase activation and autophosphorylation of the cytosolic tail does not result in the stable association with the major downstream substrates. This may due to the nature of the tyrosine residues which are available in this receptor after autophosphorylation. These sites do not bind with high affinity to the SH2 and PTB domains of downstream signaling molecules, therefore the interactions are transient. Nevertheless, the major substrate of the insulin receptor kinase,

insulin receptor substrate-1 (IRS-1), was phosphorylated in response to insulin stimulation. In rat liver a pool of IRS-1 molecules was localized to the PM. A rapid efficient interaction between the receptor and substrate may be essential as a result of the rapid internalization of the receptor. However, no tyrosine phosphorylated IRS-1- could be detected at the PM., which is consistent with the dissociation of phosphorylated IRS-1 into cytosol. Indeed, tyrosine phosphorylated IRS-1 can be detected in the cytosol within 30 sec of ligand administration, and the extent of IRS-1 phosphorylation was not affected by the different ligands used. This suggests that the kinase is equally accessible and active towards this substrate, regardless of the state of receptor phosphorylation. Although several studies have demonstrated SHC and MAPK activation in response to insulin stimulation, no SHC or MAPK phosphorylation was observed in response to insulin receptor stimulation in rat liver parenchyma *in vivo*.

Despite the numerous potential substrate molecules and interactions described in cultured cells for the EGF and insulin receptors, only a few appear to be physiologically relevant in any given system. The specificity of EGF and insulin RTK signaling in the rat liver appears to be established early after receptor activation. Since a portion of cellular SHC and IRS-1 appeared to be localized at the PM, readily accessible to both receptors if necessary, specificity is most likely a consequence of the relative affinities of the various SH2 domains for the tyrosine phosphorylated residues present in each receptor. In addition, there may exist liver specific molecules, for example phosphatases or molecules capable of selectively sequestering downstream substrates away from the activated receptor, which serve to prevent high levels of phosphorylation of the many other potential targets or their interaction with these receptors.

Ligand Mediated Receptor Internalization

Signal transduction pathways appear to be initiated at the plasma membrane. However, in liver parenchyma, the major *in vivo* organ enriched in EGF and insulin receptors, the binding of these ligands induces the rapid internalization of their respective receptors into endosomes without an appreciable time lag (Kay *et al.*, 1986; Khan *et al.*, 1986; Lai *et al.*, 1989a).

The extent of receptor internalization demonstrates specificity with regards to the internalization mechanism. Although receptor saturating doses were used in both cases, in neither case did all the receptors internalize. In addition, the extent of EGF receptor internalization in was significantly greater than that of the insulin receptor. This observation

suggests that the regulation of the amount of receptor that is internalized is a result of the interaction of the receptor with a limited pool of receptor-specific internalization molecules. Specificity of the molecules involved is also supported by the observation that the sequences involved in receptor internalization are quite different in the EGF and insulin receptors. The molecules implicated in EGF and insulin receptor internalization, AP-2 and enigma respectively, although specific in their interaction with the receptors, are not likely to be the internalization molecules controlling the extent of receptor endocytosis. These molecules appear to be present at the plasma membrane even after receptor internalizes, i.e. they are not limiting. In addition, the late time course of association of AP-2 with the activated EGF receptor i.e. in the endosome is unexpected if AP-2 is to be a causal link to internalization.

With respect to enigma, the association seen with the insulin receptor, at 5 min both in the PM and the endosome after insulin stimulation, is absent after H2 injection. Since the kinetics of insulin receptor internalization are the same in response to these two ligands, it is reasonable to conclude that enigma is not involved in the control of insulin receptor internalization. However, the association of enigma with the endosomal insulin receptor after insulin but not H2 stimulation, and the observed difference in receptor trafficking in this compartment are consistent with a function for enigma in the proper routing of the insulin receptor. Enigma may also play a role in signal transduction from the insulin receptor as suggested by Durick *et al.* (1996) for the c-ret tyrosine kinase receptor. Further studies need to be carried out to clarify the role of enigma in insulin receptor signal transduction.

For the EGF receptor, internalization has been proposed to represent a desensitization and/or attenuation response since the rapid sequestration of the receptor into endosomes would remove it from its downstream target Ras, the majority of which is constitutively associated with the plasma membrane (Bokoch and Der, 1993). Indeed, the introduction of truncated EGF RTKs, which retained kinase activity but were internalization defective, into NR6 cells, led to enhanced mitogenesis and cellular transformation implying a relationship between internalization and attenuation (Wells *et al.*, 1990). For the insulin receptor, the rapid internalization may not be linked to attenuation since its major substrate, IRS-1, is a soluble protein (Myers *et al.*, 1994). However, in endosomes of liver parenchyma, EGF and insulin receptor tyrosine kinase signaling appears to be selectively regulated.

Endosomal Regulation of Signal Transduction

The EGF Receptor

Although the EGF receptor is eventually targeted to the lysosome for degradation, there may to be a significant period in which the receptor in the endosomal compartment may continue to signal. The C-terminal of the receptor, containing both the active tyrosine kinase domain and the autophosphorylated sites, remains cytosolically oriented and accessible to downstream signal transduction molecules in the cytosol. Although initially recruited to the PM, SHC was found to undergo further recruitment, in particular the 66 kDa isoform of SHC, to the EGF receptor in this compartment. This suggests that this SHC isoform carries out a specific, but as yet undefined function in the endosomal compartment. The tyrosine phosphorylated 55 kDa isoform of SHC was also found primarily in this compartment. As well, the EGF receptor in the endosome appeared to recruit GRB2/SOS to a significantly greater extent than at the PM. The precise role of this complex in the endosome remains to be determined although, the continued presence of the cytosolic pool of phosphorylated SHC-GRB2/SOS in the cytosol may be attributed to the release of this complex from the endosomal receptor, especially at later times (5-15 min) after EGF receptor internalization. This complex could activate Ras at the plasma membrane. Such a scenario would serve as an effective amplification mechanism for accessing Ras, over and above that in physical association, via adaptor proteins, to plasma membrane EGF receptor. This may provide an alternative explanation to the findings whereby truncated EGF RTKs are transforming (Wells *et al.*, 1990) due to the continued activation of a cytosolic pool of SHC which is itself capable of cell transformation (Pelicci *et al.*, 1992).

The adaptor protein complex, AP-2, has been proposed to function in the initial stages of internalization. However, in co-immunoprecipitation studies using purified PM fractions, AP-2 appeared to associate with the activated receptor in the endosome. Our observations are consistent with the observations of Nesterov *et al.* (1995), and support a role for AP-2 in receptor sorting or endosomal fusion, a role that has been proposed by Beck *et al.* (1992).

The endosomal apparatus is positioned both temporally and physically between the plasma membrane and the lysosome. The endosomal lumen becomes progressively more acidic and provides an environment expected to cause the dissociation of internalized ligand / receptor complexes (Bergeron *et al.*, 1985). Although EGF is resistant to dissociation from its receptor in endosomes (Lai *et al.*, 1989b), this is not the case for other EGF

receptor ligands (French *et al.*, 1995). TGF-α, a highly potent EGF agonist, binds with an identical affinity constant to the EGF receptor, but dissociates at a markedly higher pH. Although both EGF and TGF-α have similar internalization kinetics, the targeting of the receptor for degradation in lysosomes (down-regulation) was more effective by EGF than TGF-α (Ebner and Derynck, 1991). The greater biological potency of TGF-α may be due to the repeated presentation of recycled TGF-α and EGF receptor at the cell surface, resulting in several rounds of signaling and in this way bypassing the occupancy-induced down regulation of the EGF receptor by EGF (French *et al.*, 1994). Whether the tyrosine phosphorylation of and the molecules associated with the EGF receptor are altered following TGF-α mediated receptor internalization is not known. Determination of the association of AP-2 with the EGF receptor after TGF-α administration, for example, could help to clarify the role of AP-2 in EGF receptor trafficking.

The Insulin Receptor

Insulin receptor internalization is accompanied by the dissociation and degradation of insulin in the endosomal lumen, and the decrease in receptor phosphotyrosine content as compared to the plasma membrane (Burgess *et al.*, 1992). However, detailed biochemical studies revealed an increase in the activation state (i.e. the activated V_{max}/Km of tyrosine phosphorylation of *in vitro* substrates) of the purified insulin RTK in endosomes (Khan *et al.*, 1989; Burgess *et al.*, 1992), suggestive of continued signaling form this compartment. Several endosomal mechanisms for the regulation of insulin receptor signaling have been proposed; the endosomal insulin degrading enzyme within the endosome (Authier *et al.*, 1994), and on the cytosolic side of endosomes, protein phosphotyrosine phosphatase activities have been uncovered (Faure *et al.*, 1992). To determine if ligand dissociation and EAI played a role in the regulation of insulin receptor signaling from the endosome, the disruption of regulation by this enzyme was attempted by stimulating the insulin receptor with the insulin analog, H2, which has higher affinity for the receptor (Hansen *et al.*, 1996) and is less susceptible endosomal degradation than insulin. This is postulated to result in an increased number of ligand bound insulin receptors compared to the case after insulin stimulation. The results demonstrated that, contrary to the insulin-stimulated insulin receptor, receptors stimulated by H2 are not dephosphorylated in the endosome. The following conclusion can be drawn from this: the dissociation and degradation of insulin in the lumen of the endosome is required for receptor dephosphorylation, potentially as the result of a conformational change in the receptor, which makes the phosphotyrosine residues accessible to the endosomal associated phosphatases.

Insulin receptor dephosphorylation upon entry into the endosome has been suggested to result in further activation of the receptor after insulin stimulation (Burgess *et al.*, 1992). This increase in activity may be reflected by the increase in the level of tyrosine phosphorylation of cytosolic IRS-1. After H2 stimulation however, this further increase in IRS-1 phosphorylation was not observed. This is consistent with a lack of further receptor activation after H2 stimulation. However, the receptor kinase activity in the endosome after H2 stimulation still needs to be evaluated directly to confirm this hypothesis. Thus the results, based on IRS-1 phosphorylation levels, support the idea that dephosphorylation of the receptor results in increased kinase activity. This stimulation of kinase activity by dephosphorylation is transient, however. Further dephosphorylation of the receptor appears to reduce kinase activity, by 5 min as evaluated by IRS-1 dephosphorylation. Studies using the *in vivo* introduction of the potent phosphotyrosine phosphatase inhibitor bisperoxo (1,10-phenanthroline) oxovanadate anion (bpV(phen)) found a selective effect on the phosphotyrosine content of the basal level of insulin receptor constitutively present in endosomes (Bevan *et al.*, 1995). Augmentation of the phosphotyrosine content of the endosomal insulin RTK led to the enhanced phosphorylation of IRS-1 as well as other downstream effects. Although this appears contradictory at first glance, to the results obtained above, it may be explained by the fact that bpV(phen), by inhibiting phosphatases, probably results in a receptor phosphorylated for maximal activity, which cannot subsequently be deactivated due to the presence of phosphatase inhibitors. This data supports the idea that the IRS-1 phosphorylation seen in response to insulin but not H2, may be accounted for by endosomal receptor signaling.

Disruption of the state of insulin receptor phosphorylation, and possibly receptor kinase activity in the endosome, by using H2 as a ligand, did not result in the activation of the Ras-MAPK pathway. The restricted access of the insulin receptor to this pathway in liver was maintained. However, the demonstration of an effect of H2 stimulation on the level of c-jun and c-myc transcripts suggests that an alternative pathway has been activated. The nature of the pathways responsible for this effect remain to be identified, it is possible that the stress activated protein kinases cascade may be involved. This pathway leads to the N-terminal phosphorylation and activation of the transcriptional activity of Jun (Minden *et al.*, 1994) which may then autoregulate the AP-1 element in the c-jun promoter.

The selective dissociation of ligands from receptors in the endosome has been shown to affect the intracellular trafficking of the receptor as was demonstrated in the case of the EGF receptor (Ebner and Derynck, 1991; French *et al.*, 1995). In the case of the insulin receptor, H2 stimulation appeared to cause a transient accumulation, at 5 min post

injection, of the receptor in the endosomal compartment. This was not due to an increase in internalization of receptors from the PM, instead this effect could be related to the phosphorylation state and/or the kinase activity of the receptor, both of which have been implicated in endosomal sorting. Interestingly, the protein enigma, which was postulated to be involved in internalization, was associated with the endosomal insulin receptor, at 5 min, but only after insulin stimulation. Thus, the lack of association with enigma, in response to H2, raises the possibility that enigma plays a role in the regulation of sorting of the insulin receptor from the endosome. A more detailed time course of receptor association with enigma in response to insulin and H2 may shed some light on the function of enigma in response to insulin receptor activation in the rat liver.

The endosomal compartment provides regulation for signal transduction from the EGF and insulin receptor kinases, beyond merely transporting these receptors to the lysosome for degradation. The selective dissociation of ligands, and in some cases their degradation in the endosome appears to play an important role in signal regulation. This appears to be involved in the modulation of receptor phosphorylation and kinase activity as well as the regulation of receptor trafficking, which are important in the determination of the strength and duration of a signal. Both the EGF and insulin receptor kinases appear to remain active for a time within the endosomal compartment. Based on substrate phosphorylation, the EGF receptor appears to remain active for up to 15 min, the insulin receptor up to 5 min post-injection. Up until these times however, the major substrates for these receptors, SHC and IRS-1 respectively, and possibly minor substrates as well, continue to be phosphorylated. In doing this, the EGF and insulin receptors in the endosome may contribute to the presence of a cytosolic pool of activated molecules, PY SHC-GRB2/SOS and IRS-1-PI3'-kinase, respectively, which act mainly at the PM. This mechanism may have arisen as a consequence of the rapid internalization of receptors into endosomes. Internalization sequesters receptors away from plasma membrane localized effectors, and a cytosolic pool, generated by the receptor at the PM, initially, and in the endosome, after internalization, would prolong the duration of signaling. In addition, the presence of phosphotyrosine residues in endosomal EGF receptors permits the continued recruitment of substrates to this compartment and further signaling. The control of receptor trafficking also appears to reside in the compartment. Recycling vs. downregulation regulates signaling by determining the time required before a second round of signaling can take place.

Conclusion

Using subcellular fractionation of rat liver we have been able to define the specificity of signaling from the EGF and insulin receptors, with respect to the Ras-MAPK pathway and to find support for endosomal regulation of signaling from both these receptors, in physiologically relevant system. The further identification of RTK phosphorylated molecules in endosomes may not only uncover the missing molecular links in receptor traffic but also extend our knowledge of further mechanisms for the propagation of signal transduction. Indeed, the selection of differentiation or metabolic pathways as opposed to pathways involved in cell cycle and growth control for different RTKs may be a consequence of their compartmentalization. The availability of a growing number of reagents for studying RTK signal transduction, membrane traffic, and subcellular compartments suggests that this is likely to be a growing field of study.

ORIGINAL CONTRIBUTIONS

Original contributions 2, 3 and 4, were done in collaboration with Gianni Di Guglielmo, and as a consequence, I claim responsibility to the portion of the work pertaining to the endosomal and cytosolic compartment.

1. This is the first study using subcellular fractionation to assess the distribution of the putative internalization molecules, AP-2 and enigma, for the EGF and insulin receptors respectively, in rat liver. Both AP-2 and enigma were localized to the plasma membrane (PM) but absent from the endosomal compartment in unstimulated rat liver. In response to EGF stimulation, AP-2, was redistributed and associated preferentially with the EGF receptor in the endosomal compartment. In response to insulin stimulation, enigma was associated with the PM and the endosomal fraction only at 5 min post injection. In response to stimulation with the insulin analog, H2, no association with enigma could be observed.

2. This is the first detailed study carried out using subcellular fractionation of rat liver to assess activated EGF receptor association with and phosphorylation of signal transduction molecules. In response to EGF injection, SHC was recruited to the PM, but was highly tyrosine phosphorylated primarily in the endosome. GRB2 was recruited primarily to the endosomal compartment. The endosomal EGF receptor was associated with tyrosine phosphorylated SHC, GRB2 and SOS. MAPK was recruited to the PM and the endosome with kinetics similar to those of EGF receptor internalization.

3. This is the first work carried out on rat liver subcellular fractions, comparing insulin- and H2-stimulated insulin receptor internalization and phosphorylation. The kinetics and extent of internalization of the receptor were the same in response to insulin and H2. However, in the endosomal fraction, a transient accumulation of the H2-stimulated insulin receptor was observed. Insulin induced receptor phosphorylation at the PM was greater than that observed in response to H2 stimulation. Upon endocytosis, the insulin-activated receptor became partially dephosphorylated, while the H2-activated receptor maintained its state of phosphorylation.

4. This is the first study investigating the distribution and the tyrosine phosphorylation of the insulin receptor substrate, IRS-1, in rat liver subcellular fractions, in response to insulin and H2 stimulation. A portion of cellular IRS-1 was localized to the PM, but not

the endosome, in unstimulated liver. Stimulation with insulin or H2 resulted in the tyrosine phosphorylation of cytosolic IRS-1, but insulin stimulation resulted in a higher level of IRS-1 phosphorylation than H2 stimulation.

5. This is the first study comparing the EGF and insulin receptor specificity in accessing the Ras-MAPK pathway leading to nuclear transcription, in rat liver. Raf-1 and MAPK mobility shifts, were observed in response to the injection of receptor saturating doses of EGF, but not in response to insulin or H2. EGF administration caused an increase in the mRNA levels of the early response genes, c-fos, c-jun and c-myc. Insulin stimulation did not result in the significant increase in the messenger levels for these transcripts. H2 stimulation selectively induced an increase in mRNA for c-jun and c-myc, but to a lesser extent than that seen in response to EGF.

Publications

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Abstracts

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