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Kevin B. Clairmont

*University of Massachusetts Medical School*

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The Structure, Function, and Regulation of Insulin-like  
Growth Factor II/Mannose 6-Phosphate Receptor Forms

A Thesis Presented

by

Kevin Brian Clairmont

Submitted to the Faculty of the  
University of Massachusetts Graduate School of Biomedical Sciences, Worcester  
in partial fulfillment of the requirements for the degree of:

Doctor of Philosophy in Biomedical Sciences

October 1990

Biochemistry and Molecular Biology

**THE STRUCTURE, FUNCTION, AND REGULATION OF INSULIN-LIKE GROWTH  
FACTOR II/MANNOSE 6-PHOSPHATE RECEPTOR FORMS**

A Thesis

By

KEVIN BRIAN CLAIRMONT

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Department of Biochemistry and Molecular Biology  
October, 1990

*Dedicated  
to  
Family*



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## Abstract

In mammals a single receptor protein binds both insulin-like growth factor II (IGF-II) and mannose 6-phosphate (Man 6-P) containing ligands, most notably lysosomal enzymes. However, in chick embryo fibroblasts IGF-II binds predominantly to a type 1 IGF receptor, and no IGF-II/Man 6-P receptor has been identified in this species. In order to determine if chickens possess an IGF-II/Man 6-P receptor, an affinity resin (pentamannosyl 6-phosphate (PMP) Sepharose) was used to purify receptors from chicken membrane extracts by their ability to bind mannose 6-phosphate. Then  $^{125}\text{I}$ -IGF-II was used to evaluate their ability to bind IGF-II. These experiments demonstrate that nonmammalian Man 6-P receptors lack the ability to bind IGF-II, suggesting that the ability to bind IGF-II has been gained recently in evolution by the mammalian Man 6-P receptor.

The second area of study involves the serum form of the IGF-II/Man 6-P receptor. This receptor had been detected in the serum of a number of mammalian species, yet its structure, function, regulation, and origin were unknown. Initial studies, done with Dr. R. G. MacDonald, showed that the serum receptor is truncated such that the C-terminal cytoplasmic domain of the cellular receptor is removed. These studies also demonstrate a regulation of serum receptor levels with age, similar to that seen for the cellular receptor, and that the serum form of the receptor existed in several forms which appeared intact under nonreducing conditions, but as multiple proteolytic products upon reduction. Finally, these studies demonstrated that both the cellular and serum IGF-II/Man 6-P receptors are capable of binding IGF-II and Man 6-P simultaneously.

In studies on the serum form of the IGF-II/Man 6-P receptor that I have conducted independently, the regulation of the serum IGF-II/Man 6-P and transferrin receptors by

insulin has been demonstrated. In these studies, insulin injected into rats subcutaneously resulted in a time and dose dependent increase in serum receptor levels. Finally, to investigate the relationship of the serum IGF-II/Man 6-P receptor to the cellular form of the receptor, pulse chase experiments were performed. These experiments demonstrate that the soluble (serum form released into the medium) receptor is a major degradation product of the cellular receptor. Furthermore, the lack of detectable amounts of the lower Mr soluble receptor intracellularly and the parallel relationship of cell surface and soluble receptor suggest that the proteolysis is occurring from the cell surface. Finally, a number of experiments suggest that the degradation rate depends upon the conformation state of the receptor: binding of IGF-II or Man 6-P makes the receptor more susceptible to proteolysis while the presence of lysosomal enzymes prevents receptor proteolysis.

In summary, the serum form of the IGF-II receptor is a proteolytic product of the cellular form of the receptor. The rate of release depends upon the number of receptors at the cell surface and the binding state of the receptor. In circulation, the receptor retains the ability to bind both types of ligands, it thus may serve as an IGF binding protein and/or a lysosomal enzyme binding protein. These results suggest a model whereby the cellular receptor is proteolytically cleaved by a plasma membrane protease to produce a short membrane anchored fragment and the serum receptor. In vivo this pathway serves as the major degradative pathway of the IGF-II/Man 6-P receptor, with the serum form being cleared from circulation by further degradation and reuptake.

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## List of Abbreviations Used

cDNA: complementary deoxyribonucleic acid

DTT: dithiothreitol

EDTA: ethylenediaminetetraacetic acid

Hepes: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

IGF-I and II: insulin-like growth factors I and II

IGFBP: Insulin-like Growth Factor Binding Protein

Ig: immunoglobulin

Man 6-P: mannose 6-phosphate

PBS: phosphate buffered saline

PMP: pentamannosyl 6-phosphate

PMSF: phenylmethylsulfonyl fluoride

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

U: units

## Chapter I

### Background

#### A. Diabetes

Diabetes mellitus translated literally from Greek means siphoning of sugar and obtains its name from the fact that people with this condition excrete large amounts of a sugar rich urine. Until the twentieth century, people with diabetes died shortly after the symptoms appeared. At present, treatment prolongs life, but the life expectancy of a diabetic is significantly shorter than the population at large (57 for insulin dependent diabetes mellitus, 70 for non-insulin dependent diabetes mellitus, as compared to 77 for the general population (Ekoe, 1988)). Furthermore, complications diminish the quality of life of a diabetic. Among these complications are neuropathy (peripheral numbness present in 12% of diabetics upon diagnosis for diabetes), small vessel disease (such as retinopathy which can result in blindness; present in 3% upon diagnosis and eventually afflicts 92% of diabetics (Brownlee, 1985)), and nephropathy (kidney disease is present in 10% of diabetics upon diagnosis and responsible for up to 50% of deaths caused by diabetes (Ekoe, 1988)). In addition to these medical complications are less apparent biochemical changes which may be the underlying cause of the clinical complications. Among the biochemical alterations are changes in basement membranes (Cahill, 1985) and, in blood, decreased generation of cytotoxic cells (Fernandes *et al.*, 1978; Mahmoud *et al.*, 1976; Friedman and Beyer, 1977), a decrease in the affinity of hemoglobin for oxygen, decreased levels of 2, 3-diphosphoglycerate and increases in the viscosity of serum, the aggregation of red blood cells, the rate of fibrinogen consumption, and in the circulating levels of  $\alpha_2$ -macroglobulin,  $\beta$ -lipoprotein, coagulation factors V and VIII (Marble *et al.*, 1985) and lysosomal enzymes (Goi *et al.*, 1986 and 1987).

While the name is derived from the Greek, it is likely that diabetes mellitus existed before that era. The mention of polyuria on the Egyptian Ebers papyrus (dated 1550 B.C., Brothers, 1976) makes it likely that diabetes has always plagued mankind. Further references to the symptoms of diabetes have been found in the writings of the Indians, Chinese, Greeks, and Arabs (Brothers, 1976). Diabetes mellitus has now been shown to exist as two types referred to as juvenile (or insulin dependent) and adult onset (or non-insulin-dependent). Juvenile diabetes is due to the autoimmune destruction of the  $\beta$  cells of the islets of Langerhans, thereby destroying the body's source of insulin. People with certain histocompatibility antigens are prone to this disease (Todd *et. al.*, 1988). Adult onset diabetes is characterized by insulin resistance, that is the lack of responsiveness of cells to insulin. In this disease normal or elevated levels of insulin are found in serum, but glucose levels remain elevated. An understanding of the mechanism of insulin action, and the possibility of bypassing the necessity for insulin, provides the only hope for a cure to these diseases.

While its symptoms were described throughout history, the first suggestions of the cause of the disease weren't obtained until the end of the nineteenth century. Bernard demonstrated in 1858 (Bernard, 1858; Best, 1952) that a secretion of the pancreas played an important role in the digestion of carbohydrate, protein, and fat. In 1869, Langerhans gave structure to this important organ by publishing his anatomical studies (Langerhans, 1869; Best, 1952). In 1889 Oskar Minkowski and Joseph von Mering showed that removal of the pancreas from a dog induced diabetes mellitus in the animal (Houssay, 1952). These findings set the stage for the discovery of insulin, the hormone of the pancreatic islets which is responsible for regulating the metabolism of nutrients, by Banting and Best in 1922 (Banting and Best, 1922; Banting, Best, and McLeod, 1922).

## B. Insulin

Banting and Best followed up the discovery of Minkowski and von Mering by using the pancreatectomized dog as a model system. As described above, this animal became diabetic and died shortly thereafter. They collected material from a pancreas 7-10 weeks following ligation of the pancreatic ducts. This treatment caused the degeneration of the acinar, but not the insular, cells thereby allowing the purification of insulin in the absence of the proteolytic enzymes of the acinar cells. Material purified from this extract was then assayed by injecting it into pancreatectomized dogs and measuring the ability of the material to decrease glucose in urine and to keep the animals alive. Following their purification of insulin (Banting and Best, 1922; Banting, Best, and McLeod, 1922), this material was shown to decrease blood glucose levels in diabetic patients. Insulin, as initially identified and purified in 1922, remains the major method of treatment for diabetes.

Following its initial discovery, insulin has become one of the most studied polypeptides. It was first crystallized in 1926 (Abel, 1926), it was then the first protein sequenced in 1953 (Sanger and Tuppy 1951a and b; Sanger and Thompson 1953a and b), and among the first whose structure was determined using X-ray crystallography (Adams *et al.*, 1969; Blundell *et al.*, 1971; Dodson *et al.*, 1979). It is a very small protein with a molecular weight of 5715 Da (bovine). Insulin is synthesized on the rough endoplasmic reticulum as a larger precursor of Mr=11260 Da referred to as preproinsulin. Cotranslationally the signal sequence is removed to produce proinsulin (8677 Da). Proinsulin, which contains the A and B chains of mature insulin connected by the C peptide, is then packaged in secretory vesicles. Acidification of these secretory vesicles causes the cleavage of the C peptide by vesicular proteases to produce mature insulin

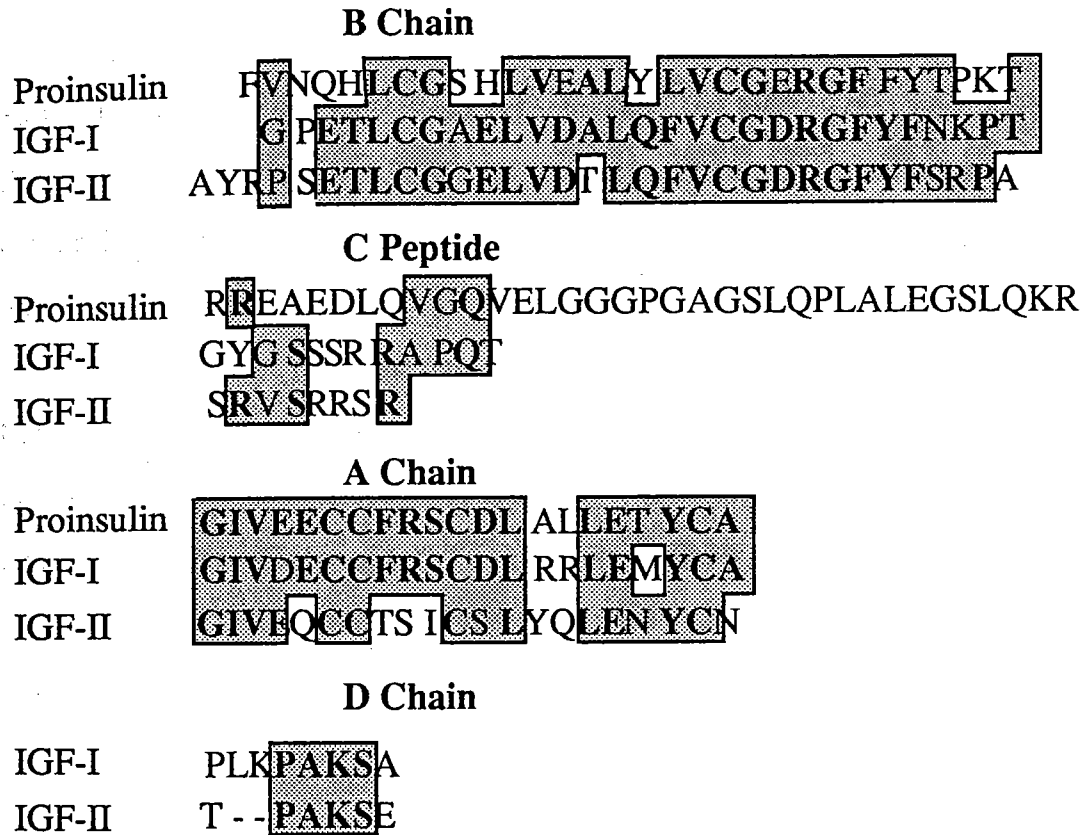
(Steiner *et al.*, 1974; Orci *et al.*, 1986). Insulin can then be released by pancreatic  $\beta$  islet cells as necessary to regulate blood glucose.



### C. Insulin-like Growth Factors

Insulin-like growth factors (IGFs) are so named due to their structural similarity to insulin. Of the 19 residues which are invariant in the insulins sequenced, 17 are conserved in the IGFs (Rinderknecht and Humbel, 1978a and b). As shown in Figure 1, IGFs consist of A and B chains which are homologous with the corresponding chains of insulin (41-43% identity; Zapf and Froesch, 1986), a longer, unrelated C chain (62% homology between IGF-I and IGF-II; Zapf and Froesch, 1986), and an additional carboxy-terminal D chain. Furthermore, IGFs can mimic some of the effects of insulin and, due to the lack of inhibition of their insulin-like effects by anti-insulin antibodies have been referred to as non-suppressible insulin-like activity (Froesch *et al.*, 1963; Rinderknecht and Humbel, 1976a and b; Zapf *et al.*, 1978). The major effects of IGF-I is thought to be as a mediator of growth hormone action on cartilage and bone growth (thus the terms somatomedin (Daughaday *et al.*, 1972) and sulfation factor (Salmon and Daughaday, 1957; van Wyk *et al.*, 1971)). Direct injection of IGF-I into animals produces a predominantly insulin-mimetic effect with little or no effect on growth (Skottner *et al.*, 1987). However, as will be discussed in the next section, IGF actions on growth may require that they be complexed to IGF binding proteins. IGF-II has also been shown to have cell replication promoting effects for which it has been termed multiplication stimulating activity (MSA) (Rechler *et al.*, 1981).

IGFs are found in many vertebrates (Zapf *et al.*, 1981; Hey, Brown, and Thorburn, 1988). IGF-II is the dominant IGF in fetal and neonatal development (Moses *et al.*, 1980, White *et al.*, 1982; Adams *et al.*, 1983; DeChiara, Efstratiadis, and Robertson, 1990) being present at 1.8-4.4  $\mu\text{g/ml}$  of serum (20-100 times the level in adults), while IGF-I plays a greater role in the maturing animal (Jansen *et al.*, 1985). IGF-

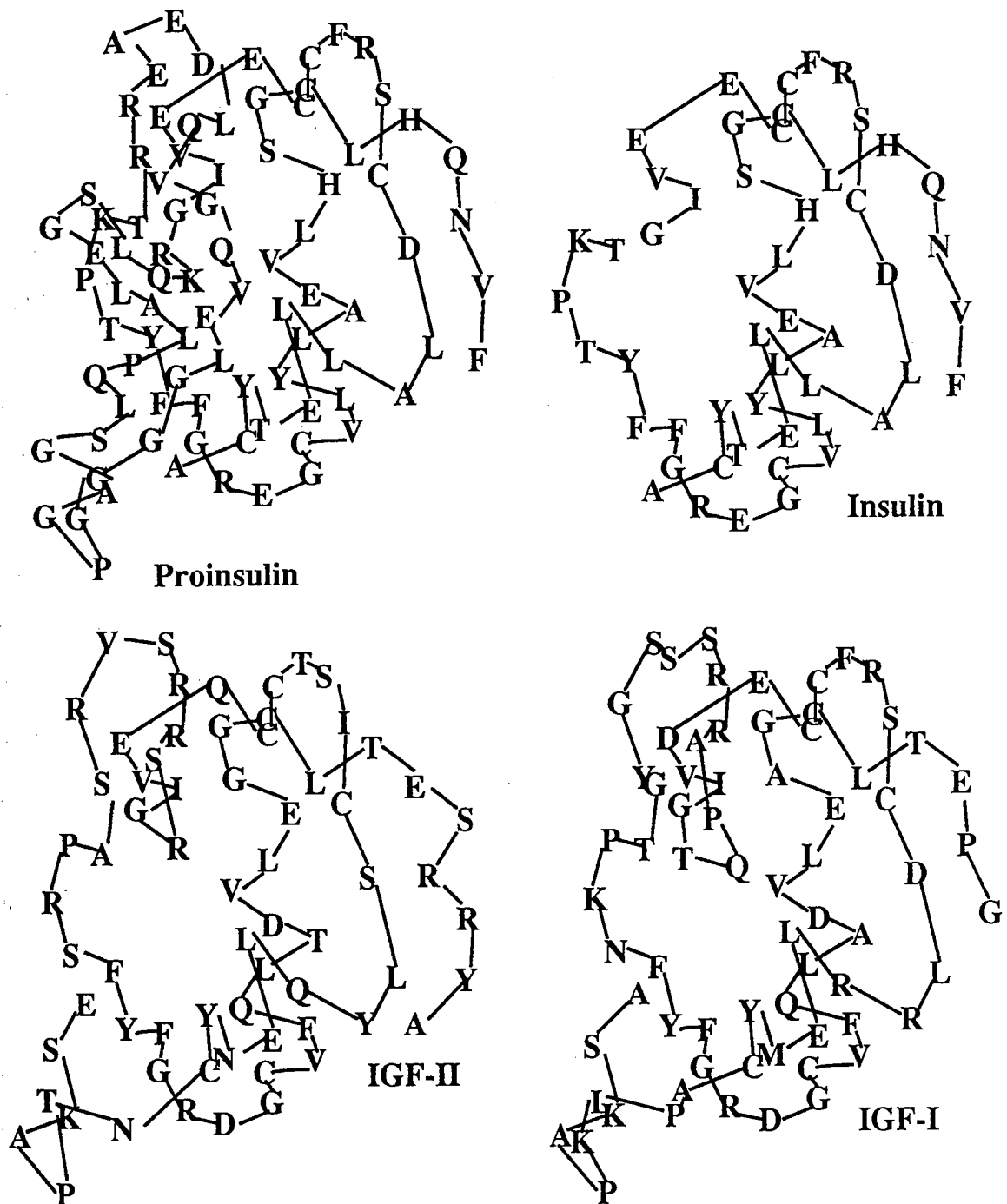


**Figure 1: Sequences of the Members of the Insulin Family.** A comparison of the sequences for proinsulin (Sanger and Tuppy, 1951a and b; Sanger and Thompson, 1953a and b; Ullrich *et al.*, 1977), insulin-like growth factor I (Rinderknecht and Humbel, 1978a), and insulin-like growth factor II (Rinderknecht and Humbel, 1978b). Amino acids are indicated by single letter codes. Identical amino acids are in bold type and conserved regions are indicated by shaded boxes.

I is predominantly secreted by liver and is growth hormone dependent, while IGF-II is produced by cells throughout the body and is not regulated by growth hormone (Mesiano *et. al.*, 1989).

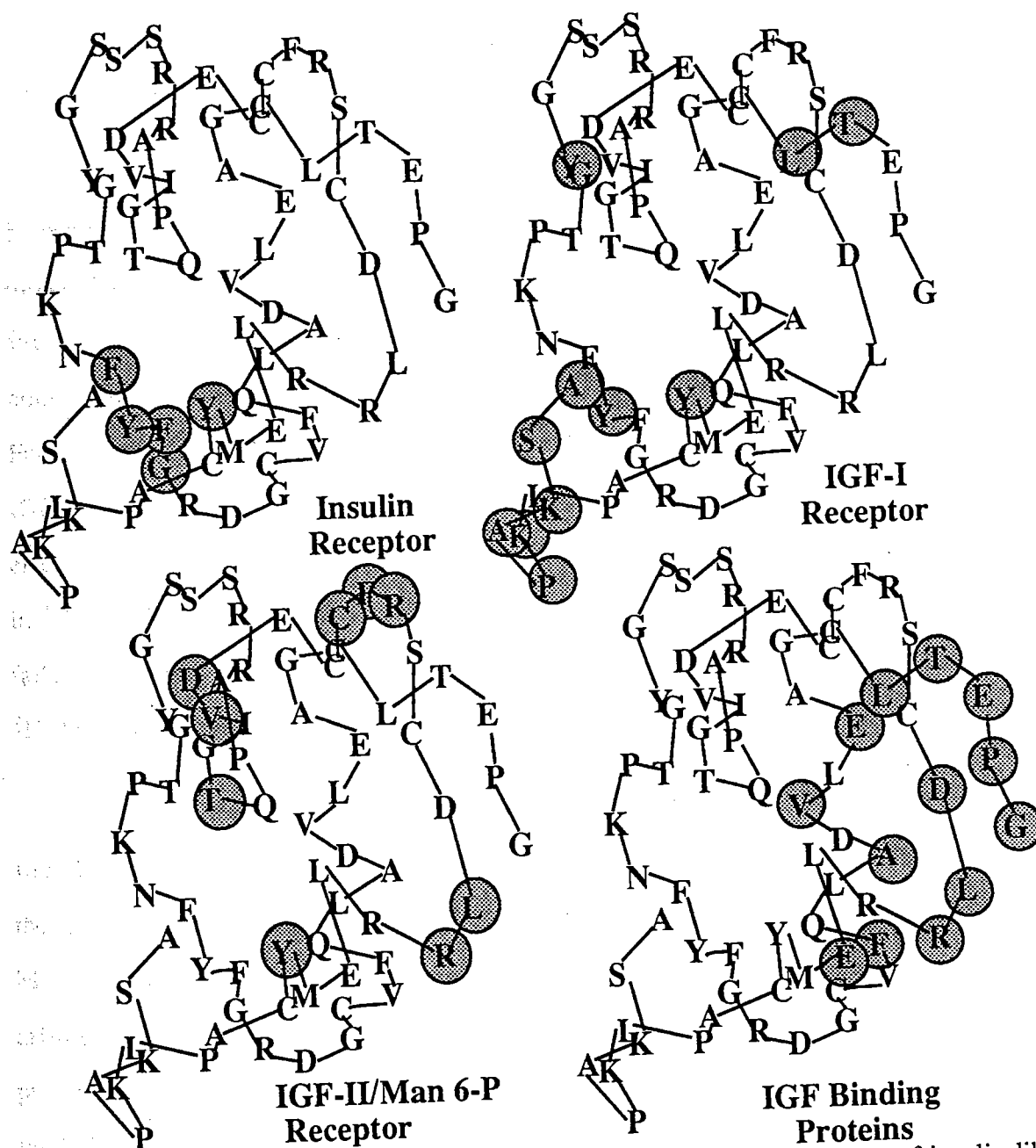
IGF-I and IGF-II are structurally very homologous (Figures 1 and 2), with molecular weights of 7649 (Rinderknecht and Humbel, 1978a) and 7471 daltons (Rinderknecht and Humbel, 1978b), respectively. There are two variations of IGF-II in mammals: the major "small" form is very similar to IGF-I, while the less abundant "big" form has the amino acids RLPG inserted in place of S29, CGR in place of S33, and a 21 amino acid C-terminal extension referred to as the E-domain (Jansen *et al*, 1985; Zumstein, Luthi, and Humbel, 1985; Zapf and Froesch, 1986). A "big" form of IGF-II containing the E-peptide has been measured in circulation to correlate with levels of total IGF-II: 253 ng/ml in neonates as opposed to 5.7 ng/ml in adults (Hylka, Kent, and Straus, 1987). The "big" IGF-II is a minor constituent whose bioactivity has not been demonstrated; therefore IGF-II will refer to only the small form hereafter.

There is a single 30 kilobase gene for IGF-II on human chromosome 11, near the gene for insulin (Dull *et al.*, 1984; Pagter-Holthuisen *et al.*, 1988), which gives rise to a number of mRNAs in most fetal and neonatal tissues through alternate splicing. The most abundant (4 kb mRNA) fails to translate leaving a 1.2 kb message as the major translatable message. These mRNAs produce a 22 kDa prepro-IGF-II when translated in vitro. No mRNAs encoding IGF-II are detected in adult liver, muscle, or intestine. The prepro-IGF-II consists of a 24 amino acid presequence (pro-IGF-II=20kDa), the coding region of 67 residues (mature=7500 Da), and an 89 amino acid C-terminal extension referred to as the E-peptide (intermediate=8700 Da).



**Figure 2: Structure of the Members of the Insulin Family** The predicted structures of insulin, proinsulin, insulin-like growth factor I, and insulin-like growth factor II (Blundell, Bedarkar, Rinderknecht, and Humbel, 1978; Blundell, Bedarkar, and Humbel, 1983).

As will be discussed in later sections, IGFs bind a number of proteins including the insulin receptor, the IGF-I receptor, the IGF-II/Man 6-P receptor, and serum carrier proteins (Rechler *et al.*, 1980). Recent experiments have used the available cDNA sequence of the IGFs to mutate regions in order to ascertain which regions of the IGF are responsible to binding which proteins (Figure 3). As would be predicted based on the ability of ligands to bind to receptors and binding proteins there is significant overlap between the binding sites for the insulin and IGF-I receptors, while the binding sites for the IGF-II/Man 6-P receptor and the IGF binding proteins are distinct. As can be seen (Figure 3), the insulin receptor binds to one quadrant of IGF-I, with the following residues being involved: G22, F23, Y24, F25 and Y60 (Cascieri *et al.*, 1988; Cascieri *et al.*, 1989; DeMeyts *et al.*, 1990). Similarly, the IGF-I receptor binds to Y24, Y60, K65, P66, A67, K68, S69, A70 in the same region of the IGFs (Cascieri *et al.*, 1988; Cascieri and Bayne, 1989) and to T4, L5, G30, and Y31 from the another portion of the molecule (Cascieri *et al.*, 1988; Cascieri and Bayne, 1989). The IGF-II/Man 6-P receptor binds to IGFs in a number of regions, supposedly interacting with amino acids which project from the molecule in three dimensions: T41, V44, D45, C48, F49, R50, S51, L54, R55, R56 and Y60 (Cascieri and Bayne, 1989; Cascieri *et al.*, 1989). Finally, the IGF binding proteins interact with a number of amino acids concentrated in the lower right quadrant: G1, P2, E3, T4, L5, E9, V11, A13, Q15, F16, D53, L54, R55, and E58 (Szabo *et al.*, 1988; Bagley *et al.*, 1989; Cascieri and Bayne, 1989; Cascieri *et al.*, 1989; Wallace *et al.*, 1989). This information can explain the affinity differences of various natural and synthetic ligands for the receptors and binding proteins and should make possible the synthesis of ligands with various desired specificities and biological activities.



**Figure 3: Binding Sites on Insulin-like Growth Factor I.** The structure of insulin-like growth factor I indicating the amino acids which have been demonstrated to be involved in ligand binding to the insulin, IGF-I, and IGF-II receptors and serum binding proteins. (Information compiled from Cascieri *et al.*, 1988; Szabo *et al.*, 1988; Bagley *et al.*, 1989; Brinkman *et al.*, 1989; Cascieri and Bayne, 1989; Cascieri *et al.*, 1989; DeMeys *et al.*, 1990)

#### D. IGFs in Circulation are Associated with Binding Proteins

IGFs circulate as complexes of much higher molecular weight than would be expected if the IGFs circulated freely. It has been demonstrated that the increase in molecular weight is due to the binding of IGFs by specific carrier proteins (Zapf, Waldvogel, and Froesch, 1975; Hintz and Liu, 1977) and that, in contrast to free IGFs, bound IGFs stimulate growth but fail to mimic insulin action (Zapf and Froesch, 1986). Furthermore, IGFs present in these complexes have a significantly longer half-life than free IGFs (Moses *et al.*, 1976). Certain IGF binding proteins may enhance the biological effects of IGF-1. For instance, in fibroblasts IGF-I alone resulted in only a 38% increase in  $^3\text{H}$ -thymidine incorporation, while addition of amniotic fluid binding protein (which failed to increase  $^3\text{H}$ -thymidine incorporation by itself) resulted in a 4.4 fold increase in  $^3\text{H}$ -thymidine incorporation (Elgin, Busby, and Clemmons, 1987).

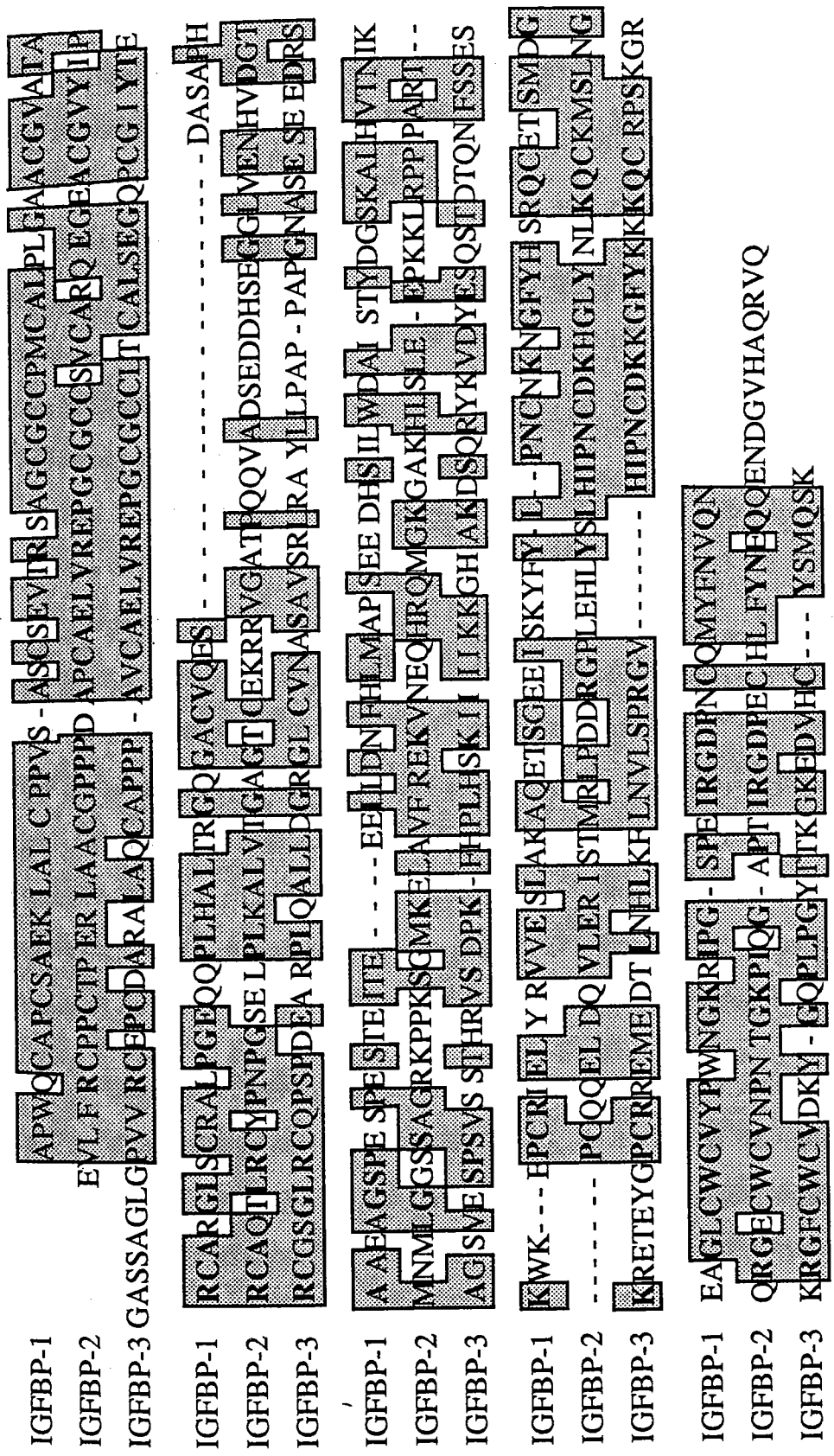
In man, IGFs circulate predominantly as 150 kDa and 40 kDa forms (Van den Brande *et al.*, 1986). In circulation, 62% of the IGF-I circulates in the 150 kDa complex, the remainder in the 40 kDa complex (Yang *et al.*, 1989). The 150 kDa form has a native Mr of 175 kDa and a stokes radius of 4.8 nm (Rapp *et al.*, 1988) and a 3-fold higher affinity for IGF-II than IGF-I (Rapp *et al.*, 1988). The 40 kDa complex has a 6-fold preference for IGF-II (Binoux *et al.*, 1986). The binding proteins can be separated from the IGFs by gel filtration chromatography at acidic pH (Van den Brande *et al.*, 1986) or by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE, Binoux *et al.*, 1986). In either case the free binding proteins are then capable of binding IGFs. In fact, the use of SDS-PAGE of serum followed by electrotransfer of the proteins from the gel onto nitrocellulose filters, then the incubation of the nitrocellulose filters in a solution of

$^{125}\text{I}$ -IGF-I or II has proven a valuable tool in the identification of IGF binding proteins (Hossenlop *et al.*, 1986).

Using these techniques, binding proteins of molecular weights 42, 39, 34, 30, and 24 kDa have been identified which differ in their affinities for IGF-I and IGF-II (Binoux *et al.*, 1986). When affinity crosslinked to IGFs, the 40 kDa complex referred to earlier is shown to have components of 38, 40, and 46 kDa (Binoux *et al.*, 1986) or 32 and 43 kDa (Yang *et al.*, 1989), while the large 150 kDa complex is composed of major constituents of 33, 37, and 49 kDa, and less abundant components of 23, 26, 46, 120, and 140 kDa (Binoux *et al.*, 1986). Uncrosslinked components of 30-32, 42, and 45 kDa (corresponding to crosslinked components of 37, 46, and 49 kDa) have been isolated from the 150 kDa complex and shown to share the same N-terminus (Zapf *et al.*, 1988) and are thus likely proteolytic products from the same glycoprotein (Yang *et al.*, 1989). An additional IGF binding protein has been identified in humans that has a molecular weight of 32 kDa and was previously identified as pregnancy associated endometrial  $\alpha_1$ -globin (Bell *et al.*, 1988). This is the major protein synthesized by the decidualized endometrium and is a predominant protein in the amniotic fluid. This protein inhibits the effects of IGFs, and its levels in maternal serum are inversely correlated with birthweight. In other species, IGF binding proteins have also been identified. In sheep, for instance, IGF-I is found in a 20-100 kDa peak in adults and in a 20-50 kDa and a 200 kDa peak in the fetus as determined by gel filtration chromatography (Butler and Gluckman, 1986).

Despite the number of insulin-like growth factor binding proteins identified by their ability to bind IGFs, only three distinct forms have been cloned and sequenced thus far (Drop and Hintz, 1989; Figure 4). Insulin-like growth factor binding protein (IGFBP)-1 is the protein discussed above as a pregnancy associated endometrial globulin. IGFBP-3 is





**Figure 4: Sequences of the Insulin-like Growth Factor Binding Proteins.** A sequence comparison of the three IGF binding proteins cloned thus far: IGFBP-1, IGFBP-2, and IGFBP-3 (Brinkman *et al.*, 1988; Brown *et al.*, 1989; Wood *et al.*, 1988; Spencer *et al.* 1989). Sequence identities are boldfaced and conserved regions in shaded boxes. The greatest degree of conservation is seen near the termini, and somewhat less in the middle of the molecules.

BP53, referred to above as the 49 kDa component of the 150 kDa complex, and is the acid stable subunit of the 150 kDa complex (Wood *et al.*, 1988; Mukku *et al.*, 1989). Finally, IGFBP-2, which is discussed below, is the only binding protein which has been cloned from a number of species. It is 33% homologous with IGFBP-3 and, like IGFBP-3, is cysteine rich at its termini. In the rat, a IGFBP-2 is a predominant binding protein of 33 kDa in fetal and neonatal serum (White *et al.*, 1982) which has been shown to be synthesized by a number of cell lines and fetal cells as a 35 kDa precursor, which, following cleavage of the signal sequence, is released as the mature 33 kDa binding protein. The cDNA for this binding protein has been cloned and sequenced (Brown *et al.*, 1989) and shown to code for a protein of 32,886 Da with a 34 residue prepeptide and a 270 amino acid mature protein with molecular weight 29,564 Da. This protein contains no N-linked glycosylation sites, but contains a C-terminal RGD sequence which may allow it to bind to the cell surface via integrin receptor (Margot *et al.*, 1989). Of the 18 cysteines in the molecule, 12 are between residues 40-115 at the N-terminus, and 5 between residues 240-285 and this pattern is conserved in all species from which this protein has been cloned (rat (Brown *et al.*, 1989), bovine (Szabo *et al.*, 1988; Ballard *et al.*, 1989), and human (Julkunen *et al.*, 1986; Brewer *et al.*, 1988)). The presence of the expected disulfides is required for biological and immunological activity (Brinkman *et al.*, 1989).

Like the IGFs, there is substantial evidence that certain agents regulate the levels of IGF binding proteins in circulation. Unlike IGF-I which is growth hormone dependent, levels of IGFBP-2 are increased significantly upon hypophsectomy (Margot *et al.*, 1989). By contrast, IGFBP-3 is increased in acromegaly and decreased in hypopituitism (Binoux *et al.*, 1986). The effect of growth hormone on IGFBP-3 levels has been demonstrated directly by hypophsectomy with or without growth hormone treatment (Moses *et al.*,

1976). In osteoblasts, both growth hormone and estradiol stimulate the release of the 150 kDa carrier protein (Schmid *et al.*, 1989).

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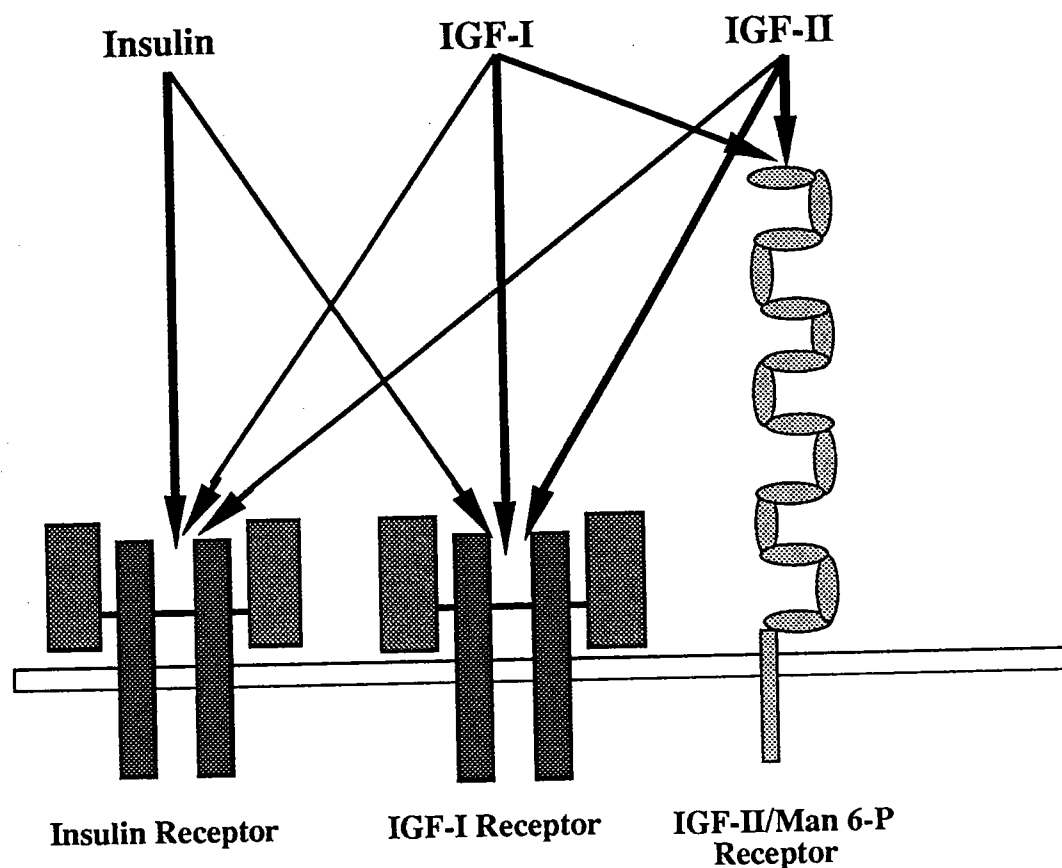
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### E. The Insulin and IGF-I Receptors

In addition to binding a number of insulin-like growth factor binding proteins while in circulation, IGFs bind to several cellular receptors. Specifically, three distinct receptors have been identified by crosslinking studies, and a fourth putative IGF receptor has been identified by its homology to known IGF receptors (Shier and Watt, 1989). By crosslinking, the receptors fall into two classes: a monomeric receptor of ~250 kDa (the IGF-II/Man 6-P receptor) and heterotetrameric receptors consisting of 2 $\alpha$  and 2 $\beta$  subunits of Mr 125-135 and 90-95 kDa, respectively, referred to as the insulin and IGF-I receptors (Czech, Massague, and Pilch, 1981; Massague and Czech, 1982; Yamaguchi *et al.*, 1986). While each of these three receptors has the greatest affinity for the homologous ligand, the ligands are also able to interact with heterologous receptors. As shown in Figure 5, insulin will bind to the insulin or IGF-I receptor, while both IGF-I and IGF-II bind to the insulin, IGF-I, or IGF-II receptors. While the heterotetrameric structure of the insulin and IGF-I receptors allows for binding of two molecules of ligand, there is negative cooperativity such that the second molecule binds with a much lower affinity than the first (Pang and Shafer, 1984).

Both the insulin and IGF-I receptors are synthesized as a large precursor (~210 kDa) containing both the  $\alpha$  and  $\beta$  subunits (Kasuga *et al.*, 1982a; Rechler and Nissley, 1986) as determined by crosslinking studies. Studies to follow the biosynthesis and processing of the insulin receptor have shown that following translation and cleavage of the signal sequence, it has an Mr=170kDa. The molecular weight increases to 190kDa following glycosylation in the ER. This is then proteolytically processed upon reaching the Golgi apparatus into 120kDa and 90kDa subunits, which are further glycosylated to the 130kDa and 95kDa mature  $\alpha$  and  $\beta$  subunits (Forsayeth, Maddux, and Goldfine, 1986).



**Figure 5: Ligand-Receptor Interactions in the Insulin Family.** Insulin and the insulin like growth factors show crossreactivity among their specific receptors. This diagram schematically represents these interactions, with the increased thickness of lines indicating increased affinity.

The cloning and sequencing of the insulin (Ullrich *et al.*, 1985; Ebina *et al.*, 1985) and IGF-I (Ullrich *et al.*, 1986) receptors have further demonstrated their similarity (Figures 6 and 7).

The similar structures of the IGF-I and insulin receptors also give rise to similar immunologic and functional properties (Rechler and Nissley, 1986). Both receptors are tyrosine kinases. Upon binding of ligand, the tyrosine kinase is activated by an increase in  $V_{max}$  for ATP hydrolysis, resulting initially in autophosphorylation of a number of sites in the cytoplasmic domains of the receptors (White *et al.*, 1988). This autophosphorylation serves to activate the kinase (Rosen *et al.*, 1983; Yu and Czech, 1984b; Herrera and Rosen, 1987) to make its elevated activity ligand independent. Removal of the phosphate by alkaline phosphatase decreases the activity of the tyrosine kinase 65% (Yu and Czech, 1984b). Furthermore, even in the absence of ligand, tyrosine phosphorylation of these sites activate the insulin receptor tyrosine kinase (Yu *et al.*, 1985). The insulin receptor can also be regulated by other agents. For instance, cAMP decreases the activity of the kinase and the affinity of the receptor for insulin (Czech, 1985) and serine phosphorylation in general can decrease receptor kinase activity (Herrera and Rosen, 1987). Streptozotocin diabetic rats had a kinase activity decreased 45% as compared to controls without a change in receptor number or affinity of receptor for insulin (Gherzi *et al.*, 1986). This resulted in an increase in the concentration of insulin required for 1/2 max activation of the kinase from 22 to 55 ng/ml (Gherzi *et al.*, 1986). Other studies of diabetic rats have confirmed that the decrease in kinase activity is proportional to the severity of diabetes even in animals with increased receptor numbers (Okamoto *et al.*, 1986).

Like the insulin receptor, the IGF-I receptor tyrosine kinase is activated by ligand (1/2 max at 0.4 nM; Morgan, Jarnigan, and Roth, 1986). The tyrosine kinase domain of

IR MGTGRRGAAAPLLVAALLLGAAGHLYP -- GÉVC - PGMDIRNNLT RLHELENCVIEGHLOQLLAFKTRPED FRDLSPFKL IMITDY LLLF  
IIR - - - - MKSGS GGGSPS LWGLLFLS AAFS LWPTSGF I CGPG I DIRNDYQQLKRL ENCTVIEGYLHILL ISK - - - AEDYRSYRFPFKLTVITEY LLLF

IR RYVGLSKDLFPNLTIVRG S RLFFNYALVIFEMVHLKELGLYNLMNITRGSVRIEKN ELCVLAT IDWSRILDSVEDNYVIVLNKDDNEECG  
IIR RVAGLESGLDLPNLTIVRGWKLFFNYALVIFEMINLKDEGLYNLRNTRG AI RIEKNADLCYLSLTVDSWLSLDA VSNNYVIVGNKPK - ECG

IR DICPGTAKGKTNCATVINGQFVB RCWTHSHCQKVCPT ICKS HGCETAGLCHSECI GNCSPQDDP TKCVACRNEY LDGR CVETCPPPYVHF  
IIR DLCPGTMBEKPMECKTINNEYRWCWTTNRQKMCPSCTCGKRACTENNECCHECCLGS CSAPDNDA CVACRHHYVAGVCPACPPNYRF

IR ODWRCVNF FQDHLHKCKNSRRQCCHQYVIHNNKC IP ECPSTYMNSS NLL - CTFCLGPCPKVCHLEGEKTTIDSVTSAQE LRGCTVINGSL  
IIR EGWRCVDRDFCAN ILS AES SDSE - - - G FVIHDGECMQBCPSGF I R NGSQSMYC IPCEGPCPKVCEBEKTKTIDSVTSAQMLQCGCTIFKGNL

IR IINIRGGNNIAAELEANLGLJEE I SGYLKIRRSYALVLSLFF RKLRLRGETLE IGNYSFYALDNQNLRLQW DWS KHNLTTQCK L FFHYNPKLCLS  
IIR LNIIRRGN IAS ELENFMGLIEVVTGYVKIRSHALVLSLFLKNLRLIGEEQLEGNYSFYVLDNQN<sup>400</sup>QQLW DWDHRNLTIKAGKMYFAFNPKLCSV

IR EHKMEEVSGTKGRQERNDIAKNTNGDQASCENELKFSYRTSF DKILLRWEPPYPPDFRDLGFMFLFYKEAPYQNVTE FDGQDA CGSNSWT  
IIR EYRMEEVGTGKRQSKGDNINRNGERASCESDVLHFTSTITSKNRI IITWHRYP PPDYRDLI SFTVY<sup>300</sup>YKEAPF KNYTEYDGDQD ACGSNSWN

IR VVD I DPPLRSNDPKSQNHQHPGLMRGLKPWTQYA I PVKTL - VTF S DERRTYGAKSDI IVVQTDATNPSVPLD<sup>300</sup>P I SVSNSSO IILKWKPPSPD  
IIR MVDVD - - L - - - PPNKDVEPG I L LHGLKPWTQYAVYVKA<sup>300</sup>AVTLTMVENDH IRGAKSE ILY IRINAS VPS IPLDVLASNSSQLIV<sup>600</sup>KWNPSPSLP

IR NGNTHLYV FWERQAE<sup>500</sup>DS ELF ELDYCLKGLKLSRTWS - P PFESEDSQKHQSEY - EDS AGECCSPKTD<sup>500</sup>S OI LKELES<sup>500</sup>SRFRKTFEDYLHN<sup>700</sup>VVFP  
IIR NGNLSYVYRWRQ<sup>500</sup>PQDGYLYRHNYCSK D-K IPIRKYADGTD IEEVT ENPKTEVC GGEKGPCCACPKTEAEKQAEKEEAEYRKYVFNFLHN<sup>700</sup>SIFVP

IR RPSRKR<sup>800</sup>RS LG DVCNVTVAVPT - VAAFPNTSSTVSPTEEHRP - FEK - VVNKESLVISGLRHTGYRIELQACNQDTP<sup>800</sup>EE<sup>800</sup>CSVAAY<sup>800</sup>VSARTMPE  
IIR RPE<sup>800</sup>KKR<sup>800</sup>DMQVANTMS SRSRNTTAADTYNTDPELETEYPP FESRVDNKERTIVISNLRPT LYRIDIH SCNHEAEKLGCSASFVEAR<sup>900</sup>TMPA

IR AKADDIVGPVT HE IFENN<sup>800</sup>VHLMWQEPKEPNGLVLYEVSRYR<sup>800</sup>GDEELHLCDTRKHFAL<sup>800</sup>E RGCRLRGLS PGNYSVIRATSLAGNGSWT<sup>800</sup>BPT  
IIR EGADDI<sup>800</sup>IPGPVTWEPR EN S IFLKWPENPNGLI LMVEIKYGSQVQDRE: CVSRQEYRKYGGAKLNRNLPNGNYTARIQA TSLSGNGSWT<sup>800</sup>DPV

IR YFYVTDYLDVPSNIA<sup>800</sup>KN<sup>800</sup>YV<sup>800</sup>NS<sup>800</sup>Y<sup>800</sup>GS<sup>800</sup>Y<sup>800</sup>AL<sup>800</sup>R<sup>800</sup>K<sup>800</sup>R<sup>800</sup>Q<sup>800</sup>D<sup>800</sup>GP<sup>800</sup>LG - - PLYAS SNPEYLSADVPFCSVYVDEWEVS REKITLRL<sup>800</sup>REI<sup>800</sup>CG<sup>800</sup>SG<sup>800</sup>FG  
IIR FFYVQAKTYENFIHL<sup>900</sup>HAL<sup>900</sup>RA<sup>900</sup>V<sup>900</sup>N<sup>900</sup>V<sup>900</sup>GG<sup>900</sup>Y<sup>900</sup>ML<sup>900</sup>Y<sup>900</sup>HR<sup>900</sup>K<sup>900</sup>R<sup>900</sup>NSR - LGNGVLYASVNPYFSAAD - - - - - VYVDEWEVAAREKIMTSRE<sup>900</sup>CG<sup>900</sup>SG<sup>900</sup>FG

IR MVYEGNARD I I KGEAETRA<sup>1000</sup>VA<sup>1000</sup>VT<sup>1000</sup>NES AS LRERIEFLNEASVMKGF<sup>1000</sup>TCHHV<sup>1000</sup>VRLLGVVSKGQPTLVVME<sup>1000</sup>LMAHGD<sup>1000</sup>LKSVL<sup>1000</sup>RS<sup>1000</sup>LRPEA<sup>1000</sup>ENNPGRP  
IIR MVEYGA<sup>1000</sup>KGVV<sup>1000</sup>KDEPETRA<sup>1000</sup>IE<sup>1000</sup>FTVNEAASMRERIEFLNEASVMKERNCHHV<sup>1000</sup>VRLLGVV<sup>1000</sup>SQGP<sup>1000</sup>TLV I MELMTRGD<sup>1000</sup>LKSVL<sup>1000</sup>RS<sup>1000</sup>LRPEMENN<sup>1000</sup>PVLA

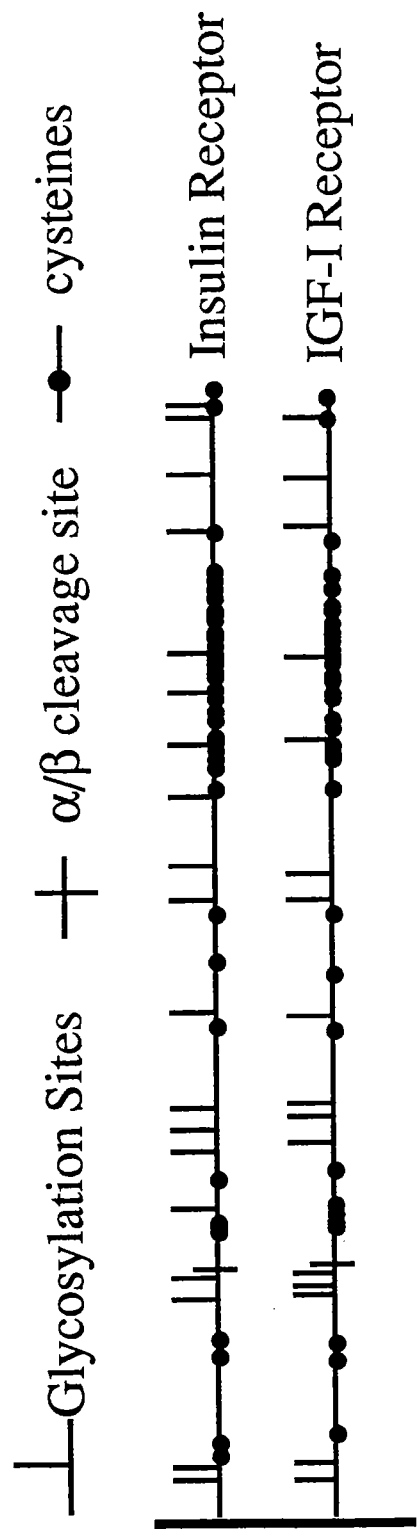
IR PPTLQEMIQMAAEIADGMA<sup>1100</sup>YLN<sup>1100</sup>AK<sup>1100</sup>KFV<sup>1100</sup>HRDLA<sup>1100</sup>ARN<sup>1100</sup>CMVA<sup>1100</sup>AHDFTVKIGDFGMTRDI<sup>1100</sup>ETD<sup>1100</sup>Y<sup>1100</sup>Y<sup>1100</sup>RK<sup>1100</sup>GGK<sup>1100</sup>GLL<sup>1100</sup>PVR<sup>1100</sup>WMA<sup>1100</sup>PESL<sup>1100</sup>KD<sup>1100</sup>GV<sup>1100</sup>FTT<sup>1100</sup>SSDM<sup>1100</sup>WSF  
IIR PPSLSKMIQMA<sup>1100</sup>GEIADGMA<sup>1100</sup>YLN<sup>1100</sup>AK<sup>1100</sup>KFV<sup>1100</sup>HRDLA<sup>1100</sup>ARN<sup>1100</sup>CMVA<sup>1100</sup>EDFTVKIGDFGMTRDI<sup>1100</sup>ETD<sup>1100</sup>Y<sup>1100</sup>Y<sup>1100</sup>RK<sup>1100</sup>GGK<sup>1100</sup>GLL<sup>1100</sup>PVR<sup>1100</sup>WMS<sup>1100</sup>PESL<sup>1100</sup>KD<sup>1100</sup>GV<sup>1100</sup>FTT<sup>1100</sup>YS<sup>1100</sup>SDV<sup>1100</sup>WSF

IR GVV<sup>1200</sup>LWEITSLAEQPYQ<sup>1200</sup>GLSNEQV<sup>1200</sup>LKFVMDG<sup>1200</sup>G<sup>1200</sup>YL<sup>1200</sup>DPD<sup>1200</sup>NC<sup>1200</sup>PER<sup>1200</sup>VDL<sup>1200</sup>MRMC<sup>1200</sup>WQ<sup>1200</sup>FN<sup>1200</sup>MR<sup>1200</sup>PT<sup>1200</sup>LE<sup>1200</sup>IVN<sup>1200</sup>LK<sup>1200</sup>DDL<sup>1200</sup>HL<sup>1200</sup>SP<sup>1200</sup>FE<sup>1200</sup>VS<sup>1200</sup>F<sup>1200</sup>FISEEN<sup>1200</sup>KAPE  
IIR GVV<sup>1200</sup>LWEIATLAEQPYQ<sup>1200</sup>GLSNEQV<sup>1200</sup>LR<sup>1200</sup>FM<sup>1200</sup>EG<sup>1200</sup>GL<sup>1200</sup>DK<sup>1200</sup>PD<sup>1200</sup>NC<sup>1200</sup>PD<sup>1200</sup>ML<sup>1200</sup>FEL<sup>1200</sup>MRMC<sup>1200</sup>WQ<sup>1200</sup>Y<sup>1200</sup>NP<sup>1200</sup>K<sup>1200</sup>MR<sup>1200</sup>PS<sup>1200</sup>LE<sup>1200</sup>I<sup>1200</sup>S<sup>1200</sup>SI<sup>1200</sup>KEEME<sup>1200</sup>PG<sup>1200</sup>FRE<sup>1200</sup>VS<sup>1200</sup>F<sup>1200</sup>Y<sup>1200</sup>SEEN<sup>1200</sup>KLPE

IR SEELEMEFEDMEN<sup>1300</sup>VPLDRSSH<sup>1300</sup>COR<sup>1300</sup>E<sup>1300</sup>AG<sup>1300</sup>GRD<sup>1300</sup>GG - - - - - SSLGFKRS<sup>1300</sup>Y<sup>1300</sup>EEH<sup>1300</sup>IP<sup>1300</sup>Y<sup>1300</sup>T<sup>1300</sup>H<sup>1300</sup>M<sup>1300</sup>G<sup>1300</sup>G<sup>1300</sup>K<sup>1300</sup>K<sup>1300</sup>NG<sup>1300</sup>R<sup>1300</sup>IL<sup>1300</sup>TL<sup>1300</sup>PR<sup>1300</sup>SN<sup>1300</sup>PS  
IIR PEELD<sup>1300</sup>LEPENMESVPLD<sup>1300</sup>PSASSS<sup>1300</sup>LP<sup>1300</sup>LP<sup>1300</sup>DRHSG<sup>1300</sup>HKA<sup>1300</sup>EN<sup>1300</sup>GP<sup>1300</sup>GV<sup>1300</sup>LV<sup>1300</sup>LRAS<sup>1300</sup>FD<sup>1300</sup>ER<sup>1300</sup>Q<sup>1300</sup>PY<sup>1300</sup>AH<sup>1300</sup>M<sup>1300</sup>NG<sup>1300</sup>G<sup>1300</sup>R<sup>1300</sup>K<sup>1300</sup>NER<sup>1300</sup>AL<sup>1300</sup>PL<sup>1300</sup>QS<sup>1300</sup>STC

Figure 6: The Insulin and Insulin-like Growth Factor I Receptor Sequences. A comparison of the insulin and IGF-I receptor sequences (Ebina *et al.*, 1985; Ullrich *et al.*, 1985; Ullrich *et al.*, 1986). Identities are indicated by boldface letters. The  $\alpha/\beta$  cleavage site is indicated by the open box, the transmembrane domain by the slashed box, and the ATP binding site by the shaded box. Asterisks indicate sites of tyrosine phosphorylation of the insulin receptor discussed in the text.

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**Figure 7: Features of the Extracellular Domains of the Insulin and IGF-I Receptors. A comparison of the structural features of the insulin and IGF-I receptor extracellular domains (Czech *et al.*, 1990).**



the two receptors are very closely related as shown by the ability of an antibody to the insulin receptor tyrosine kinase domain to inhibit the IGF-I receptor tyrosine kinase (Morgan, Jarnigan, and Roth, 1986). Upon treatment of cells which contain both insulin and IGF-I receptors with insulin, both receptor types are phosphorylated (Beguinot *et al.*, 1988) and downregulated. Similarly, treatment with low levels of IGF-I results in downregulation of both receptors although only the IGF-I receptor shows increased phosphorylation and tyrosine kinase activity (Heaton, Krett, and Gelehrter, 1986; Beguinot *et al.*, 1988). Under certain conditions, insulin receptor heterodimers can combine with IGF-I receptor heterodimers to produce mixed heterotetramers (Jonas, Newman, and Harrison, 1986; Treadway *et al.*, 1989; Moxham, Duronio, and Jacobs, 1989).

Despite the similarities between the insulin and IGF-I receptor discussed above, they are not identical. First, and most obviously, their ligand specificities differ. Second, while the insulin receptor binding requires glycosylation, the IGF-I receptor is capable of binding IGF-I in cell lines with a number of glycosylation defects (Podskalny *et al.*, 1986). There are also differences in the signal produced by the receptor tyrosine kinases (Lammers *et al.*, 1989). While both tyrosine kinase domains are equipotent in stimulating glucose transport, the IGF-I receptor possesses a more active tyrosine kinase (3-fold) and is more effective at stimulating DNA synthesis (10-fold; Lammers *et al.*, 1989).

There are also differences in insulin receptor isolated from different tissues. While functionally identical, these receptors show differences in molecular weight, the basis of which has not been determined in some cases. A comparison of receptors from muscle and liver reveals that both subunits have a increased Mr in liver (101 vs 98 kDa  $\beta$  and 135 vs 131 kDa  $\alpha$ ; Burant *et al.*, 1986). While the difference can be removed from the  $\beta$  subunits by deglycosylation with Endo H, the  $\alpha$  subunits retain the difference in Mr following this

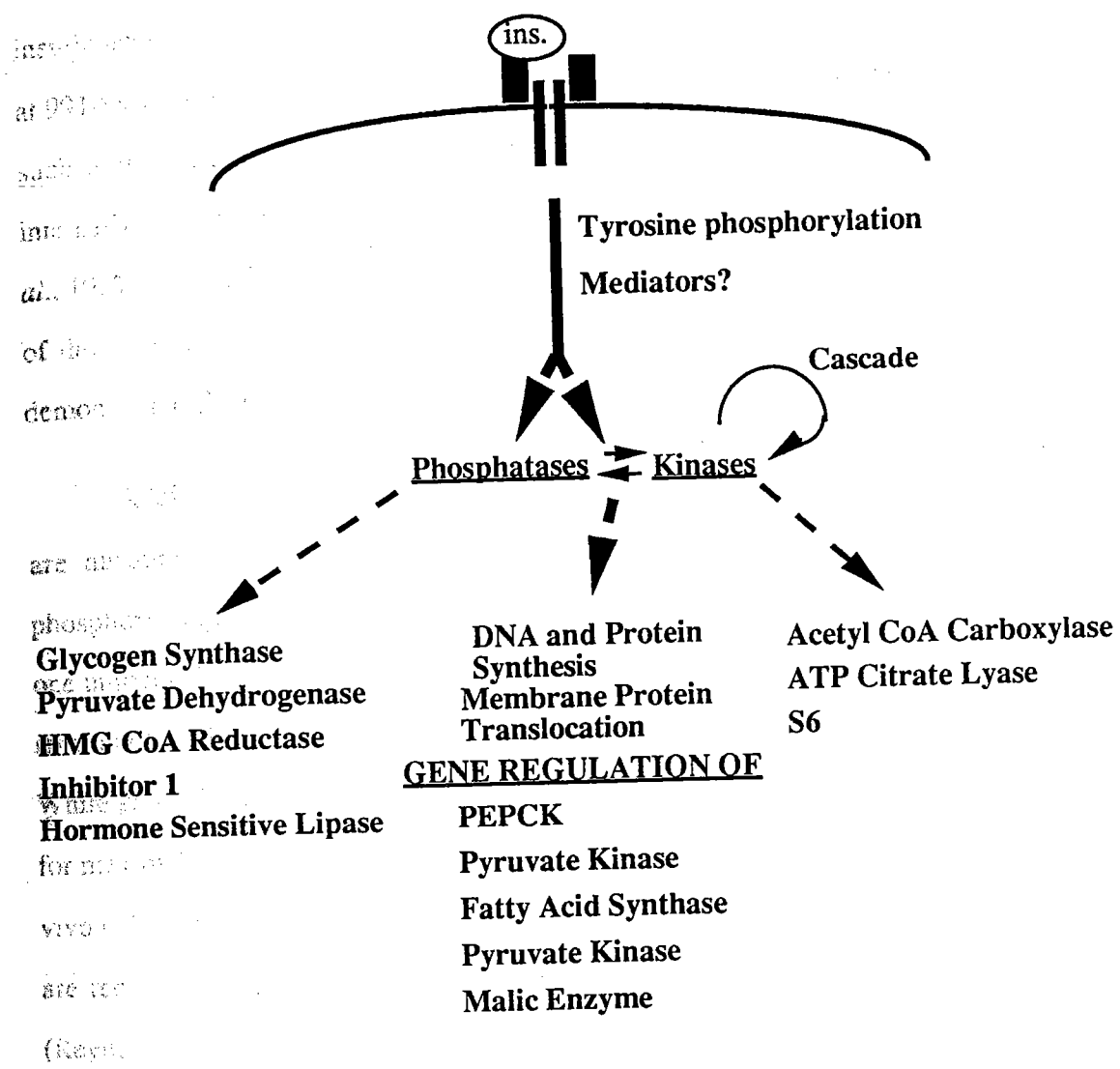
treatment (Burant *et al.*, 1986). Similarly, comparison of brain and placental insulin and IGF-I receptors demonstrate that the brain receptors are 10 kDa smaller, but that this difference can be removed by Endo F treatment (Heidenreich *et al.*, 1983; Heidenreich *et al.*, 1986).

## F. The Role of the Insulin Receptor Tyrosine Kinase in Insulin Action

Since the discovery of insulin, diabetes researchers have been trying to unlock the secrets of how insulin works. Initially this centered upon identifying target tissues, biological effects, and eventually the receptor for insulin. The main target tissues for insulin are the liver, muscle, and adipose. The precise effects of insulin differ in each of these tissues to result in a decrease in blood glucose. For example glycogenesis is increased in the liver while glucose transport and utilization are increased in adipose and muscle tissues.

The discovery of the insulin receptor marked a significant advance in the field. However, although the first steps of insulin action on a cell, that is the binding of insulin to its receptor and the activation of the insulin receptor tyrosine kinase, have been known for several years, the steps following have been more difficult to identify. An alternative approach in which a terminal biologic effect is followed back from the observed effect towards the binding of insulin to its receptor has likewise failed to significantly advance our understanding of the mechanism of insulin action. Despite, or perhaps due to, this lack of evidence, a number of models of insulin action have appeared (Figure 8). In order to evaluate the validity of these models numerous experiments have been performed. These models have been reviewed in a number of recent articles (Czech, 1981; Berger and Berchtold, 1985; Czech *et al.*, 1985)

The preferred model at present involves the insulin receptor tyrosine kinase phosphorylating and activating serine/threonine kinases, leading to a cascade of phosphorylations and dephosphorylations which activate enzymes involved in the ultimate effects of insulin. In order to test the importance of the insulin receptor tyrosine kinase in



**Figure 8: Models of Insulin Action.** Numerous models have been proposed to explain the mechanism of insulin action. This diagram depicts a number of landmarks: insulin binding to the insulin receptor as the first step, kinases and phosphatases stimulated by insulin as intermediate effects, and final effects on gene expression, membrane protein distribution, and enzymatic activities of proteins involved in carbohydrate, protein, and lipid metabolism.

insulin action, mutations have been created in the ATP binding site (Gly-X-Gly-X-X-Gly at 991-996 and Lys 1018; Goldfine, 1987) which destroy tyrosine kinase activity. Using such mutations, it has been demonstrated that tyrosine kinase activity is required for internalization of ligand, downregulation, and ligand induced receptor turnover (Russell *et al.*, 1987). Using *Xenopus* oocytes transfected with glucose transporters, the importance of the insulin receptor tyrosine kinase in regulation of glucose transport has also been demonstrated (Vera and Rosen, 1990).

Additionally, the tyrosines in the cytoplasmic domain of the insulin receptor which are autophosphorylated can be mutated or deleted. While 6 tyrosines can be phosphorylated in this region of the insulin receptor (1146, 1150, 1151, 1316, 1322 and one unidentified tyrosine phosphorylated of the remaining 8), deletion of the two most C-terminal tyrosines (1316 and 1322) does not alter kinase activity (Tornqvist *et al.*, 1988; White *et al.*, 1988). The remaining three identified phosphorylated tyrosines are required for maximal kinase activity, although only two of the three are normally phosphorylated *in vivo* (1146 and 1150 or 1151; White *et al.*, 1988). Furthermore, tyrosines 1150 and 1151 are required for insulin induced internalization and degradation of the insulin receptor (Reynet *et al.*, 1990). Yet another mutation has involved the expression of the cytoplasmic domain of the insulin receptor with or without a membrane anchor. In these experiments it was demonstrated that the membrane anchored form constitutively increased glucose transport while the construct lacking a membrane anchor was without effect (Ellis *et al.*, 1987). Taken together these results demonstrate that an intact insulin receptor tyrosine kinase plays a critical role in the transduction of the insulin signal across the cell membrane.

## G. Insulin Induces the Redistribution of Membrane Proteins

### 1. Glucose Transporters

One of the best characterized and most studied terminal responses to insulin is the increased uptake of glucose by muscle (Park *et al.*, 1959) and adipose tissue (Crofford and Renold, 1965; Czech, 1976). Little was known about the mechanism of this increase prior to the discovery of the ability of cytochalasin b to inhibit glucose transport (Levine and Goldstein, 1955; Carter, 1967; Bloch, 1973; Taverna and Langdon, 1973) by binding to the glucose transporter (Czech, 1976). By photoaffinity labelling of the glucose transporter with cytochalasin b, it was demonstrated that insulin induced a redistribution of glucose transporters from an intracellular site to the cell surface (Cushman and Wardzala, 1980; Suzuki and Kono, 1980; Karnieli *et al.*, 1981; Wardzala and Jenrenaud, 1981; Oka and Czech, 1984; Karnieli *et al.*, 1986).

The role of the redistribution of glucose transporters in the insulin induced increase in glucose transport has not yet been resolved. The increase in glucose transport is larger than the redistribution, generally being on the order of 20-fold as opposed to 4-fold, for the redistribution. The change in transporter number at the cell surface has also been demonstrated to slightly precede the increased transport (Karnieli *et al.*, 1981). Even the discovery of additional glucose transporters in insulin sensitive tissues failed to explain this discrepancy (Horuk *et al.*, 1986; James, Lederman, and Pilch, 1987; Oka *et al.*, 1988; Kayano *et al.*, 1988; Zorzano *et al.*, 1989; Charron *et al.*, 1989; James, Strube, and Mueckler, 1989; ). In addition, certain agents have been able to mimic the insulin induced increase in redistribution of glucose transporters without causing such a change in glucose transport (Horner, Munck, and Lienhard, 1987), to alter glucose transport but not glucose

transporter number (Kuroda *et al.*, 1987; Kahn and Cushman, 1987) or to prevent the insulin induced redistribution of glucose transporters but not the increase in transport (Baly and Horuk, 1987). These data demonstrate that glucose transporter number and activity can be independently regulated.

The mechanism by which insulin increases the cell surface number of glucose transporters and the rate of glucose transport has not yet been clearly defined. It has been demonstrated, however, that intracellular calcium (Klip and Ramlal, 1987b), membrane potential (Klip, Ramlal, and Walker, 1986), protein kinase C (Klip and Ramlal, 1987a; Robertson *et al.*, 1989), and phosphorylation of the glucose transporter (Gibbs, Allard, and Leinhard, 1986; Allard *et al.*, 1987) are not involved in these responses to insulin. Insulin may, however, induce a conformational change in the receptor which could play a role in its increased activity (Holmann and Rees, 1987; May, 1988).

## 2. The IGF-II/Man 6-P Receptor

Initially, the effect of insulin on the IGF-II/Man 6-P receptor was detected as a change in the apparent affinity of this receptor for IGF-II (Massague, Blinderman, and Czech, 1982; Oppenheimer *et al.*, 1983). Later, this effect was demonstrated to be due to an increase in the number of IGF-II/Man 6-P receptors at the plasma membrane and not a change in affinity (Wardzala *et al.*, 1984; Oka *et al.*, 1984; Oka, Rozek, and Czech, 1985). Like the glucose transporter, the increase in IGF-II/Man 6-P receptors at the plasma membrane is accompanied by a decrease in the number of receptors in the low density microsomes (Wardzala *et al.*, 1984; Oka *et al.*, 1984). This change in cell surface receptor number results from a change in receptor recycling which also accelerates the rate of IGF-II

degradation (Oka, Rozek, and Czech, 1985). This results in an increase in the rate of lysosomal enzymes without an interruption of intracellular sorting of newly synthesized lysosomal enzymes (Braulke *et al.*, 1990). Recent experiments have demonstrated that anti-IGF-II/Man 6-P receptor antibodies can block this response in a number of cell lines, while overexpression of the IGF-II/Man 6-P receptor increases the effect of insulin (Kovacina, Steele-Perkins, and Roth, 1989).

The While the regulation of the distribution of IGF-II/Man 6-P receptor is similar to the regulation of glucose transporters by insulin, there may be some differences. First, while there is significant evidence that the activity as well as the distribution of glucose transporters is regulated by insulin, only the subcellular distribution of the IGF-II/Man 6-P receptor is altered by insulin. Other differences between these systems include the observation that the insulin induced redistribution of IGF-II/Man 6-P receptors is chloroquine sensitive, while that of glucose transporters is not (Oka *et al.*, 1987) and the redistribution of the IGF-II/Man 6-P receptor by insulin correlates with its dephosphorylation (Corvera and Czech, 1985; Corvera *et al.*, 1988a and b). The fold change in these proteins at the plasma membrane is different as well, with the increase in glucose transporter (4-fold) being somewhat greater than the increase in IGF-II/Man 6-P receptor (~2-fold; Appell, Simpson, and Cushman, 1988). In addition, the distribution of IGF-II/Man 6-P receptors is altered by a number of agents in addition to insulin: epidermal growth factor (Braulke *et al.*, 1989), IGF-II (Braulke *et al.*, 1989), mannose 6-phosphate (Braulke *et al.*, 1989), vanadate (Kadota *et al.*, 1987), and hydrogen peroxide (Kadota *et al.*, 1987).

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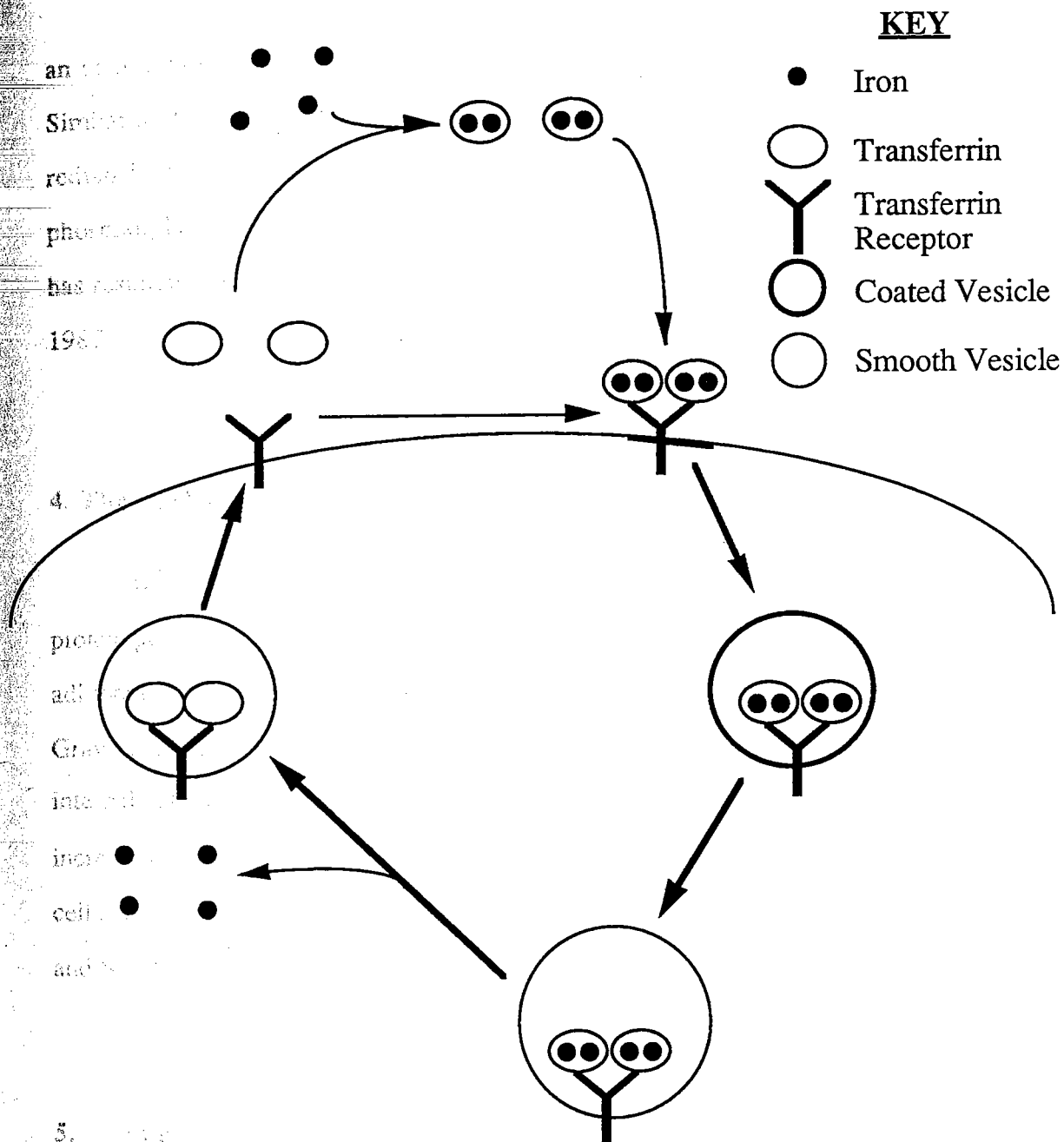
### 3. The Transferrin Receptor

The transferrin receptor is a constitutively cycling receptor with a predominantly intracellular distribution. The transferrin-transferrin receptor cycle is also very similar to the movement of the IGF-II/Man 6-P receptor (Figure 9). At the cell surface the transferrin receptor binds to transferrin which has a high affinity for receptor at neutral pH. The transferrin-transferrin receptor complex is then internalized and arrives at an acidic compartment, similar to the prelysosomal compartment, where transferrin releases the bound iron. The iron proceeds to the lysosome, from which it is released into the cell. The apotransferrin remains bound to the transferrin receptor in the acidic compartment, but is released at the neutral pH of the cell surface (Klausner *et al.*, 1983; Hopkins, 1983).

This process is regulated by insulin in a manner similar to that observed for the glucose transporters and IGF-II/Man 6-P receptor (Davis, Corvera, and Czech, 1986; Tanner and Leinhard, 1987). The regulation of the distribution of the transferrin receptor may be more general and more diverse than that of either of the proteins discussed above. In addition to insulin, the distribution of transferrin receptor can be altered by transferrin (Klausner, Harford, and van Renswoude, 1984), epidermal growth factor (Wiley and Kaplan, 1984; Davis and Czech, 1986); platelet derived growth factor (Davis and Czech, 1986), IGF-I (Davis and Czech, 1986), and phorbol esters (Klausner, Harford, and van Renswoude, 1984; May, Jacobs, and Cuatrecasas, 1984).

#### Figure 9:

The kinetic mechanism of the redistribution of transferrin receptors by insulin, IGF-I, and EGF has been studied in some detail. The insulin induced redistribution is due to an increase in the rate of externalization of receptor (Tanner and Leinhard, 1987) as is the EGF induced redistribution (Davis *et al.*, 1987), while the IGF-I induced translocation is due to



**Figure 9: The Transferrin/Transferrin Receptor Cycle.** Iron is strongly associated with transferrin at neutral pH to form diferric transferrin. The transferrin receptor binds diferric transferrin with high affinity at the cell surface. Ligand and receptor are then internalized as a unit and enter endosomes. There, the acidic pH causes the dissociation of iron from transferrin to produce apotransferrin. Despite the loss of iron from transferrin, the apotransferrin remains tightly bound to the transferrin receptor at this acidic pH. However, when the transferrin-

an alteration of both the internalization and externalization rates (Davis *et al.*, 1987). Similar to the IGF-II/Man 6-P receptor discussed above, the transferrin receptor induced redistribution, in this case caused by phorbol esters, correlates with an altered phosphorylation of the receptor. Mutagenesis of the sites of phosphorylation, however, has resulted in no change in receptor recycling or in its regulation (Davis and Meisner, 1987).

#### 4. The $\alpha_2$ -Macroglobulin Receptor

Like the proteins described above, the  $\alpha_2$ -macroglobulin receptor is a membrane protein predominantly intracellular in distribution which recycles constitutively. In 3T3-L1 adipocytes, insulin causes a redistribution of this protein to the cell surface (Corvera, Graver, and Smith, 1989). While the redistribution results in an increase in the rate of internalization of  $\alpha_2$ -macroglobulin, the internalization rate is significantly less than the increase in cell surface receptor number. This suggests that the increase in receptors at the cell surface is caused by a decrease in the receptor's internalization rate (Corvera, Graver, and Smith, 1989).

#### 5. Summary and New Avenues of Research

While a number of differences exist in the observed redistributions of receptor protein discussed above, in no case is the data inconsistent with a general mechanism which could be responsible for the growth factor induced redistribution of the proteins described above, and possibly other proteins not yet characterized. As described above, the

pathways of the IGF-II/Man 6-P receptor and the transferrin receptor are very similar. Both have been shown to be internalized through coated vesicles (Geuze *et al.*, 1984b; Stoorvogel *et al.*, 1989) as is the  $\alpha_2$ -macroglobulin receptor (Corvera, Graver, and Smith, 1989). All of these proteins normally exist predominantly in an intracellular compartment, and recent evidence suggests that a glucose transporter (Glut 1), the transferrin receptor, and the IGF-II/Man 6-P receptor coexist in the same intracellular vesicles (Tanner and Leinhard, 1989). In the case of the IGF-II/Man 6-P receptor, much of the intracellular receptor has been shown to be in the prelysosomal compartment (Fischer *et al.*, 1980; Marchase, Koro, and Hiller, 1984). Recent evidence demonstrates that clathrin coated vesicle assembly can be regulated by insulin (Corvera, 1990) and mitogens (Corvera *et al.*, 1989). This could explain the observed redistributions. The presence of kinases in coated vesicles (Bar-Zvi and Branton, 1986; Bar-Zvi, Mosley, and Branton, 1988; Pauloin, Thuriereau, and Jolles, 1988; Morris, Mann, and Ungewickell, 1990) may explain the observed correlations of phosphorylation and redistribution, and provides a testable hypothesis for the mechanism of regulation of clathrin assembly: that growth factors induce a cascade of kinases that result in the activation of coated vesicle kinases, phosphorylation of specific coated vesicle proteins, and alterations in the rate of assembly and disassembly of clathrin coats. Recent evidence (Hanson, Schook, and Puszkin, 1990) demonstrates that changes in the phosphorylation state of clathrin light chain  $\beta$  alters the phosphorylation and dephosphorylation of the clathrin associated protein AP50.

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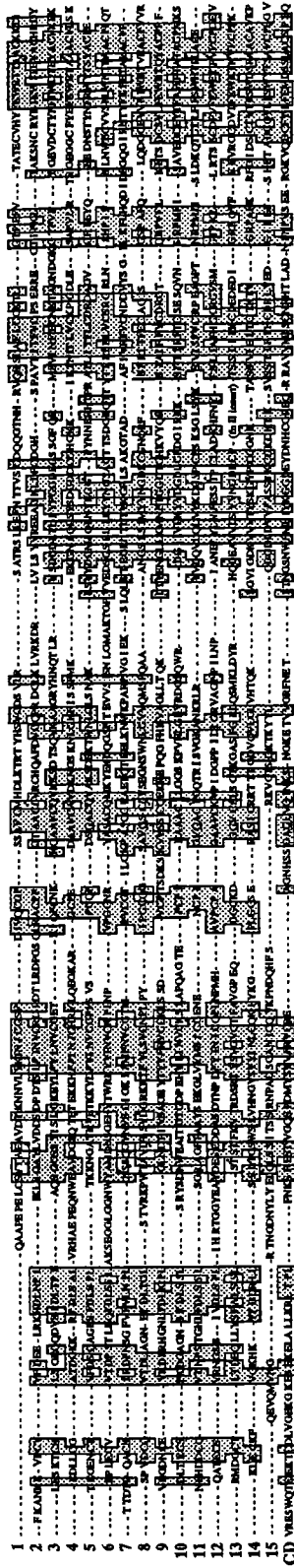
## H. The IGF-II Receptor is the Mannose 6-Phosphate Receptor

The cDNA for the human IGF-II receptor was first cloned and sequenced in 1987 (Morgan *et al.*, 1987). The sequence revealed 80% sequence identity between this receptor and the available partial sequence from the bovine mannose 6-phosphate (Man 6-P) receptor (Lobel *et al.*, 1987). The identity of the sequences was demonstrated when the human Man 6-P receptor was cloned and sequenced (Oshima *et al.*, 1988) and shown to be 99.4% identical to the human IGF-II receptor (Morgan *et al.*, 1987). Despite the sequence identity, however, none of these papers demonstrated that a single protein was capable of binding both ligands. The initial demonstrations that the same protein could bind both IGF-II and Man 6-P containing ligands (Roth *et al.*, 1987; MacDonald *et al.*, 1988) used two types of evidence: 1. A membrane extract loaded onto a PMP-Sepharose column was then eluted with mannose 1-phosphate, then glucose 6-phosphate, and finally Man 6-P. The only detectable affinity crosslinking to  $^{125}\text{I}$ -IGF-II was found in the Man 6-P eluted fraction. 2. The presence of Man 6-P increased the affinity of receptor for IGF-II two-fold, and this effect was removed by washing receptor in Man 6-P free buffer. Following this, a number of reports were published confirming this finding (Roth *et al.*, 1987; Tong, Tollefson, and Kornfeld, 1988; Kiess *et al.*, 1988), demonstrating that both ligands could bind simultaneously to different sites (Waheed *et al.*, 1988; Braulke *et al.*, 1988), or that uptake of IGF-II was increased by Man 6-P in the medium (Polychronakos and Piscina, 1988). The nature of the increased affinity also remains a puzzle, as in some reports the effect is reversible (MacDonald *et al.*, 1988) while in others it is not (Polychronakos, Guyda, and Posner, 1988)-suggesting that the effect may simply be due to removal of endogenous lysosomal enzymes by Man 6-P. Physiologically, IGF-II can inhibit

lysosomal enzyme binding to the IGF-II/Man 6-P receptor and thereby the uptake of these ligands (Kiess *et al.*, 1989).

The cloning and sequencing of the IGF-II/Man 6-P receptor (Lobel *et al.*, 1987; Morgan *et al.*, 1987; Lobel, Dahms, and Kornfeld, 1988; MacDonald *et al.*, 1988; Oshima *et al.*, 1988) revealed a protein structurally very different than the IGF-I or insulin receptors. The IGF-II/Man 6-P receptor is a 2451 amino acid monomer of 270294 Da which consists of fifteen 150 amino acid repeat regions in the extracellular domain, a single transmembrane domain of 23 amino acids, and a short 164 amino acid cytoplasmic domain (von Figura, Gieselmann, and Hasilik, 1985; Sahagian and Steer, 1985; Morgan *et al.*, 1987). The 15 repeat regions have a low homology to each other, but the positions of the cysteines within each of these regions is well conserved (Figure 10). While this protein bears no resemblance to any other protein capable of binding IGFs, it is homologous to the cation dependent mannose 6-phosphate receptor, which resembles a single repeat unit of the IGF-II/Man 6-P receptor (Figure 10; Dahms *et al.*, 1987).

A.



B.



**Figure 10: Sequence Comparison of IGF-II/Man 6-P Receptor Repeat Units and the Extracellular Domain of the Cation Dependent Man 6-P Receptor.** A. Amino acids are shown in single letter codes and identities indicated by boldface letters, and conservative regions by shaded boxes. Where more than a single amino acid is conserved in a particular position (4 or more occurrences), the second most common amino acid is italicized, and the third underlined. Similarly, conservation of a second type of amino acid is indicated by a slashed box (Morgan *et al.*, 1987; Dahms *et al.*, 1987) B. The structures of the IGF-II/Man 6-P receptor and the cation dependent Man 6-P receptor are represented as described in Figure 7 (Czech *et al.*, 1990).

## I. The Role of the IGF-II/Man 6-P Receptor in the Transport of Lysosomal Enzymes

The mannose 6-phosphate receptors are responsible for directing the transport of proteins possessing a mannose 6-phosphate recognition marker to the lysosomes. Newly synthesized lysosomal enzymes (Table 1) bind to the mannose 6-phosphate receptors in the trans-Golgi. These complexes are then localized into coated pits of the Golgi apparatus and coated vesicles are formed. These vesicles then uncoat and enter a compartment, called the prelysosomal compartment (PLC) or compartment of uncoupling of receptor and ligand (CURL), where the vesicle acidifies and lysosomal enzymes are released. A further sorting occurs in this compartment such that the lysosomal enzymes are directed to the lysosomes and the receptors return to the Golgi apparatus (von Figura and Hasilik, 1986; Geuze *et al.*, 1988; Dahms, Lobel, and Kornfeld, 1989). The precise roles of the two mannose 6-phosphate receptors in this process has not been clearly defined as yet. However, only the IGF-II/Man 6-P receptor is capable of binding to mannose 6-phosphate containing proteins at the cell surface to direct them to lysosomes (Sly and Fischer, 1982; Kyle *et al.*, 1988). Finally, a pathway not involving the mannose 6-phosphate receptors may direct many of the lysosomal membrane proteins to the lysosomes (Klionsky and Emr, 1990).

This model has developed in the last few decades based on evidence from a number of investigators. The initial evidence came from studies by Elizabeth Neufeld of I-cell disease patients' fibroblasts. These cells normally secrete lysosomal enzymes into the medium, but are capable of taking up lysosomal enzymes from other cell lines (Hickman and Neufeld, 1972). The I-cell lysosomal enzymes, however, cannot be taken up by other cell lines (Hickman and Neufeld, 1972). This led to the hypothesis that the enzymes carried a recognition marker. Further experiments identified the recognition marker to be



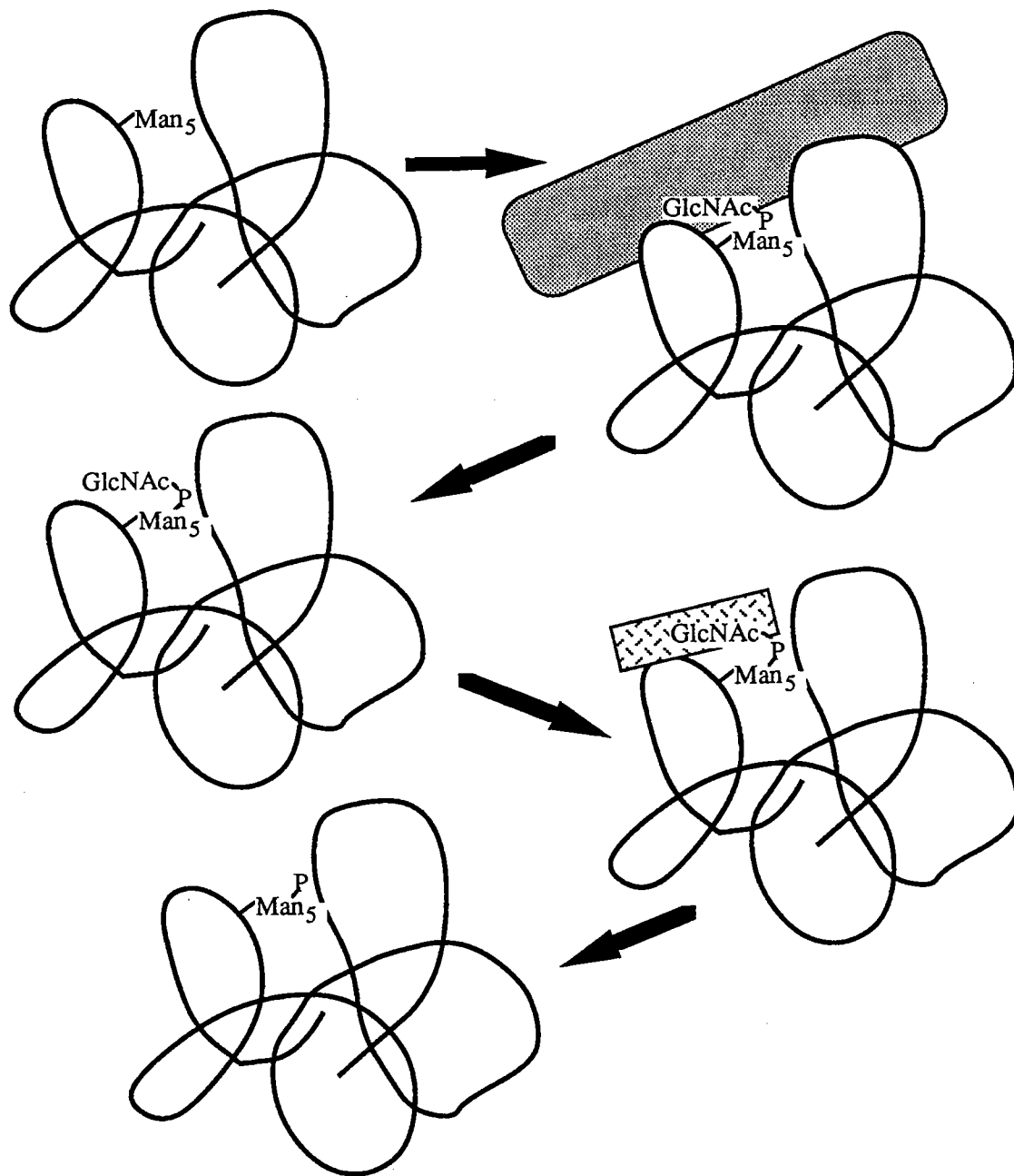
**Table 1: Lysosomal Enzymes.** The IGF-II/Man 6-P Receptor serves to sort lysosomal enzymes from the Golgi apparatus and direct them to lysosomes. The following is a list of the identified lysosomal enzymes, their function, and pH optima (From Tappel, 1969).

Enzyme	Substrate(s)	pH optimum
$\alpha$ -N-Acetylhexosaminidase	Heparan Sulfate	4.5
$\beta$ -N-Acetylhexosaminidase	glycoproteins and glycolipids	4.0-4.6
Acid Deoxyribonuclease	DNA	4.5
Acid Phosphatases	o-phosphoric monoesters	5.0-6.0
Acid Pyrophosphatase	FAD, ATP	4.0
Acid Ribonuclease	RNA	6.0-6.5
Arylamidase	amino acid arylamides	6.8-7.2
Arylsulfatase A	aryl sulfates	4.9
Arylsulfatase B	aryl sulfates	5.9
Aspartylglucosylamine Amido Hydrolase	aspartylglucosylamine link in glycoproteins	7.6
Cathepsin A	proteins and peptides	3.5-5.4
Cathepsin B	proteins and peptides	5.0
Cathepsin C	peptides	5.1
Cathepsin D	proteins	3.6-3.8
Cathepsin E	proteins	2.5
Collagenase	collagen	6.0
Esterase	fatty acid esters	3.6-4.0
$\alpha$ -Fucosidase	fucosides	5.6-5.9
Galactocerebrosidase	galactocerebrosides	6.0
$\beta$ -Galactosidase	glycolipids and glycoproteins	3.6
Glucocerebrosidase	glucocerebrosides	6.0
$\alpha$ -Glucosidase	glycogen	5.0

Enzyme	Substrate(s)	pH optimum
$\beta$ -Glucosidase	glycoproteins	5.0
$\beta$ -Glucuronidase	polysaccharides	4.5-5.2
Hyaluronidase	hyaluronic acid, chondroitin sulfate	3.5
Lysozyme	mucopolysaccharides	6.2
$\alpha$ -Mannosidase	glycoproteins	4.6-5.0
Peptidase	peptides	7.8
Phosphatidic Acid Phosphatase	phosphatidic acids	6.4
Phosphodiesterase	oligonucleotides, phosphodiesters	5.2
Phospholipase	phospholipids	4.5
Phosphoprotein Phosphatase	phosphoproteins	5.5
O-seryl-N-acetyl-galactosaminide glycosidase	seryl acetylgalactosaminide link in glycoproteins	4.4
Sialidase	glycoproteins and glycolipids	4.0-4.4
Sphingomyelinase	sphingomyelin	5.0
Triglyceride Lipase	triglycerides	4.2
$\beta$ -Xylosidase	glycoproteins, mucopolysaccharides	5.0

sensitive to oxidation (Hickman, Shapiro, and Neufeld, 1974), mannosidase treatment (Hieber *et al.*, 1976), deglycosylation (Ullrich *et al.*, 1978), and alkaline phosphatase treatment (Ullrich *et al.*, 1978). Uptake of lysosomal enzymes was inhibited by a number of agents, but most strongly by fructose 1-phosphate, mannose 6-phosphate, and phosphomannans (Ullrich *et al.*, 1978; Sando and Neufeld, 1977; Kaplan, Achord, and Sly, 1977; Kaplan *et al.*, 1977). The recognition marker was eventually identified as mannose 6-phosphate by the ability of proteins containing the marker to inhibit the uptake of  $\beta$ -galactosidase (Distler *et al.*, 1979) or by its presence on  $\alpha$ -N-Acetylglucosaminidase (von Figura and Klein, 1979) or  $\beta$ -glucuronidase (Natowicz *et al.*, 1979).

The synthesis of the mannose 6-phosphate recognition marker is a two step process (Figure 11; Lazzarino and Gabel, 1988). Briefly, N-acetylglucosaminyl 1-phosphate is transferred from UDP-N-acetylglucosamine to the C6 hydroxyl of a mannose residue by UDP-N-acetylglucosamine: lysosomal enzyme N-acetylglucosaminyl-1-phosphotransferase. The N-acetylglucosamine is then removed by N-acetylglucosamine 1-phosphodiester  $\alpha$ -N-acetylglucosaminidase to create the mannose 6-phosphate recognition marker (von Figura and Hasilik, 1986). The transferase prefers oligosaccharides of 6-9 residues and shows selectivity for lysosomal enzymes in their native conformation (Reitman and Kornfeld, 1981). While no consensus sequence for the transferase has been identified in lysosomal enzymes, the available evidence suggests that several small areas which are separated in the primary structure but near each other in the mature, folded protein may be the recognition signal for this enzyme (Faust and Kornfeld, 1989). It is this process that is defective in I-cell fibroblasts due to a defective transferase (Hasilik and Neufeld, 1980).



**Figure 11: Synthesis of the Mannose 6-Phosphate Recognition Marker.** The synthesis of the  $\text{Man}_6\text{-P}$  recognition marker is a two step process. The first step involves the addition of N-acetylglucosaminyl 1-phosphate to the end of a glycosyl chain on a lysosomal enzyme. The N-acetylglucosamine is then removed to leave the  $\text{Man}_6\text{-P}$  recognition marker.

The first receptor identified to play a role in the binding of lysosomal enzymes was the IGF-II/Man 6-P receptor. This protein was purified by affinity chromatography on immobilized lysosomal enzymes or phosphomannans (Sahagian, Distler, and Jourdian, 1981; Steiner and Rome, 1982; Sahagian, Distler, and Jourdian, 1982). Specifically, this protein recognizes mannose 6-phosphate as a phosphomonoester (Varki and Kornfeld, 1983) and requires two or more of these residues for high affinity binding (Fischer, Creek, and Sly, 1982). The presence of the cation dependent mannose 6-phosphate receptor wasn't detected in cell lines possessing the IGF-II/Man 6-P receptor. However, the existence of lysosomes in cells deficient in or lacking this protein (Gabel, Goldberg, and Kornfeld, 1983) suggested another mechanism existed to direct newly synthesized enzymes to lysosomes (Hoflack and Kornfeld, 1985b). From P388D1 macrophages, a cell line lacking the IGF-II/Man 6-P receptor, the cation dependent mannose 6-phosphate receptor was identified (Hoflack and Kornfeld, 1985a). This protein is also present in bovine liver (Hoflack and Kornfeld, 1985a). It has an Mr~46 kDa and appears to bind ligand as a dimer (Stein *et al.*, 1987a) trimer (Hoflack and Kornfeld, 1985a), or a tetramer (Stein *et al.*, 1987b), and may, in fact, bind ligand independently of cations (Junghans, Waheed, and von Figura, 1988). The cloning of this protein revealed a homology in the extracellular region to a repeat of the IGF-II/Man 6-P receptor (Dahms *et al.*, 1987).

## J. The IGF-II/Man 6-P Receptor Binds Some Prohormones and May Play a Role in Their Processing

In addition to lysosomal enzymes, several other proteins have been demonstrated to possess mannose 6-phosphate and to be capable of binding to the IGF-II/Man 6-P receptor. In general, the lysosomal enzymes possess two or more mannose 6-phosphate moieties, thus binding very tightly to the IGF-II/Man 6-P receptor, while these other proteins possess only one or two mannose 6-phosphates and bind with lower affinity (Fischer, Creek, and Sly, 1982; Dong and Sahagian, 1990). This may explain the phenomenon whereby the vast majority of lysosomal enzymes are sorted directly from the Golgi apparatus to the lysosome, while the prohormones often escape the cell and are later recaptured. Among these prohormones and growth factors are pro-transforming growth factor  $\beta 1$  (TGF- $\beta 1$ ), proliferin, and thyroglobulin (the precursor to thyroid hormones).

### 1. Pro-transforming Growth Factor $\beta 1$

The presence of two phosphorylated oligosaccharides at Asn 82 and 136 on pro-TGF $\beta 1$  has recently been determined (Purchio *et al.*, 1988) and it has been demonstrated that this protein binds to the IGF-II/Man 6-P receptor in a mannose 6-phosphate inhibitable manner (Kovacina *et al.*, 1989). The presence of a latent, precursor form of TGF $\beta$  has previously been suggested (Nilsen-Hamilton, *et al.*, 1980; Wakefield *et al.*, 1988; Miyazono *et al.*, 1988) as well as a latent form complexed to  $\alpha_2$ -macroglobulin (O'Connor-McCourt and Wakefield, 1987). It is of interest that while there are multiple forms of TGF $\beta$  (Derynck *et al.*, 1985; Obberghen-Schilling *et al.*, 1987; Ikeda, Lioubin, and Marquardt, 1987; Marquardt, Lioubin, and Ikeda, 1987; Dijke, *et al.*, 1988) only the first

form, TGF $\beta$ 1, has been shown to possess the lysosomal enzyme recognition marker mannose 6-phosphate.

The mature TGF $\beta$  is a homodimer of two 112 amino acid subunits (Marquardt, Lioubin, and Ikeda, 1987) each subunit of which is synthesized as a much larger precursor of 391 amino acids (Derynck, *et al.*, 1985). The TGF $\beta$ s bind to several cellular receptors, one of which (280 kDa) has equal affinities for TGF $\beta$ 1 and TGF $\beta$ 2, while the other two receptors (65 and 85 kDa) have higher affinity for TGF $\beta$ 1 (Cheifetz *et al.*, 1987). It is the 65 kDa receptor which appears to mediate the decrease in cellular proliferation induced by TGF $\beta$ s (Boyd and Massague, 1989).

The precise role of the TGF $\beta$ s is still being investigated (Roberts *et al.*, 1987). They appear to play a role in the production of collagen matrix and its interaction with cells (Ignotz and Massague, 1987; Montesano and Orci, 1988) and in the control of proliferation, differentiation, and transformation (Sporn *et al.*, 1986; Keski-Oja *et al.*, 1987). The physiological role of the binding of pro-TGF- $\beta$ 1 to the IGF-II/Man 6-P receptor is also not known at this time, but it may play a role in the processing of this prohormone.

## 2. Proliferin

The next growth factor that possesses a mannose 6-phosphate moiety and can bind to the mannose 6-phosphate receptor is proliferin (Lee and Nathans, 1988). Proliferin is an approximately 25 kDa glycoprotein growth factor which is secreted by mouse placental tissue and a number of growing mouse cell lines (Lee and Nathans, 1987). It is related to the placental lactogens (Lee and Nathans, 1987) and shows a 31% identity with prolactin. At this time the only receptor identified for this molecule is the IGF-II/Man 6-P receptor,

which it binds with a high affinity ( $k_d=1-2$  nM) which is dependent upon glycosylation and phosphorylation and can be inhibited by mannose 6-phosphate ( $k_i\sim 10$   $\mu$ M) or anti-IGF-II/Man 6-P receptor antibodies (Lee and Nathans, 1988). The functional role of proliferin binding to the IGF-II/Man 6-P receptor is unknown, as is the ultimate physiologic role of this molecule.

### 3. Thyroglobulin

The best studied prohormone possessing mannose 6-phosphate recognition markers is thyroglobulin (Yamamoto *et al.*, 1985; Herzog, Neumuller, and Holzmann, 1987). Thyroglobulin is a large protein which is the precursor to the thyroid hormones T3 and T4. The process by which this occurs is depicted in Figure 12. Thyroglobulin is secreted into the thyroid follicle lumen where it is iodinated and concentrated (Herzog, Neumuller, and Holzmann, 1987). The iodinated thyroglobulin then binds the IGF-II/Man 6-P receptor and is endocytosed. It then arrives in a lysosome population which may be distinct from the cells degradative lysosomes (Selmi and Rousset, 1988). The iodinated thyroglobulin is then processed into the mature thyroid hormones by sequential cleavage by cysteine proteinase I, cathepsin B, and other lysosomal proteases (Dunn and Dunn, 1988). The mature thyroid hormones thus produced can then be released as needed.

Figure

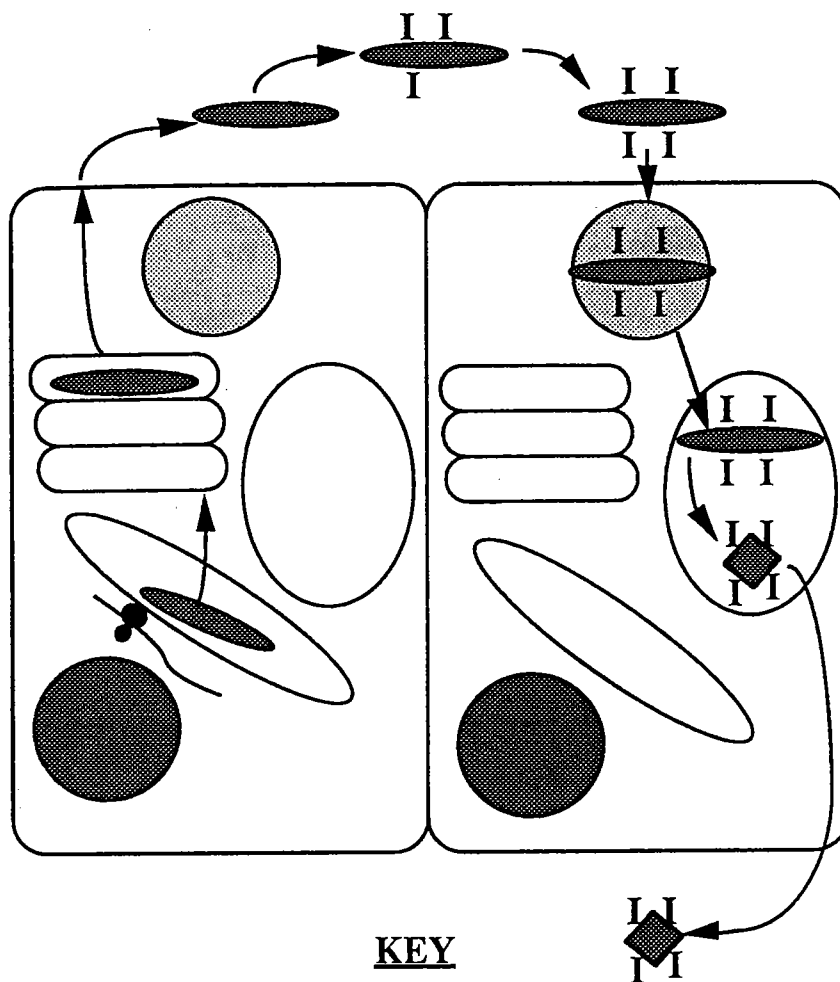
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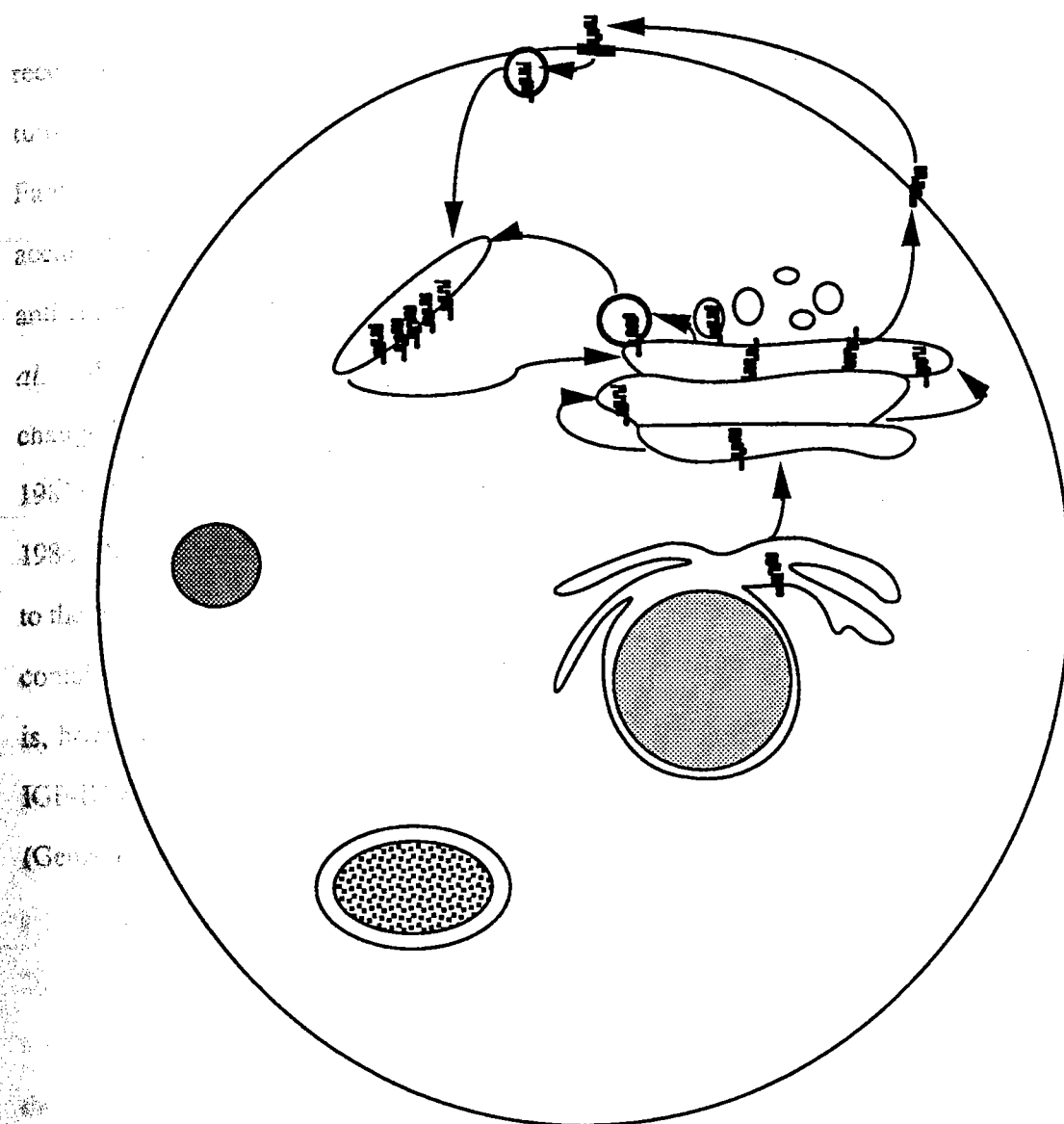


**Figure 12 Possible Model for the Processing of Thyroglobulin.** Newly synthesized thyroglobulin is exported from the cell into the thyroid follicle lumen where iodine is added to the tyrosines. Next, the iodinated thyroglobulin is bound by the IGF-II/Man 6-P receptor, internalized, and delivered to lysosomes. Thyroglobulin is then processed by lysosomal enzymes to produce the mature thyroid hormones, T3 and T4.

### K. The Subcellular Location of the IGF-II/Man 6-P Receptor

In order to direct lysosomal enzymes as described in an earlier section, it is necessary for the IGF-II/Man 6-P receptor to be present in certain intracellular compartments and absent from others (Nolan and Sly, 1987). Specifically, it must be present in the Golgi apparatus and the plasma membrane in order to bind ligand. It must then be sorted by coated pits and appear in the prelysosomal compartment (Griffiths *et al.*, 1988). Finally, it must not arrive in the lysosomes in order to be able to direct multiple lysosomal enzymes to the lysosomes (Figure 13). The mechanism by which IGF-II/Man 6-P receptors are properly sorted has only recently been addressed (Lobel *et al.*, 1989).

The presence of an IGF-II/Man 6-P receptor has been demonstrated in essentially every cell type and tissue studied, although differences in glycosylation may alter its apparent molecular weight (McElduff, Poronnik, and Baxter, 1987; Taylor, Scott, and Baxter, 1987). The cell types in which the presence of an IGF-II/Man 6-P receptor has been demonstrated include rat hepatocytes (Scott and Baxter, 1987) and liver (Valentino *et al.*, 1988), human fetal fibroblasts (Conover, Rosenfeld, and Hintz, 1987), rat kidney (Haskell, Pillion, and Meezan, 1988; Valentino *et al.*, 1988), mouse and rat neural tissue (Valentino *et al.*, 1988; Ocrant *et al.*, 1988) and rat lung, heart, fat, ovary, testes, and adrenals (Taylor, Scott, and Baxter, 1987). The IGF-II/Man 6-P receptor is normally found in a number of subcellular locations including the plasma membrane, Golgi apparatus, coated vesicles, and CURL (Geuze *et al.*, 1984a and b; Geuze *et al.*, 1985). A similar distribution is seen for the cation dependent mannose 6-phosphate receptor where 3% is seen at the cell surface, 20% in endocytic vesicles, and the remainder in the Golgi apparatus (Stein *et al.*, 1987a). Neither of the receptors is found in lysosomes (Stein *et al.*, 1987a; Brown, Goodhouse, and Farquhar, 1986). Functional studies of the movement of



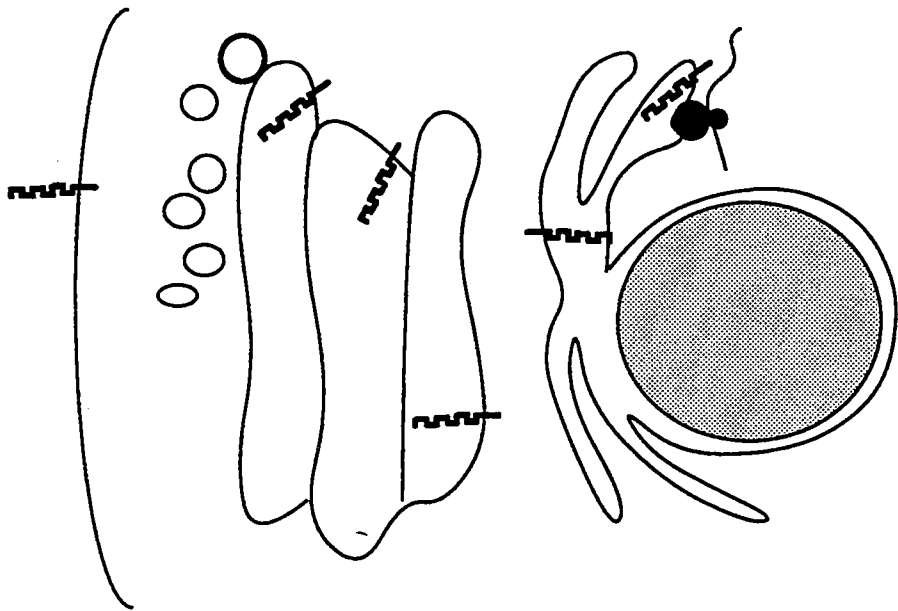
**Figure 13: Possible Model for the Subcellular Distribution and Movement of the IGF-II/Man 6-P Receptor.** The IGF-II/Man 6-P receptor resides primarily in the trans Golgi network. From this compartment the receptor can move to the prelysosomal compartment through clathrin coated vesicles or escape the coated vesicles to reach the plasma membrane. Those that reach the plasma membrane are then endocytosed through clathrin coated vesicles to reach the same prelysosomal compartment. In the prelysosomal compartment the decreased pH causes the dissociation of ligand from receptor, and the receptor is sorted back to the Golgi apparatus while the ligands go on to lysosomes.

receptors have shown that in I-cell fibroblasts (Brown and Farquhar, 1984b) or following tunicamycin treatment (Gonzalez-Noriega *et al.*, 1980; Brown, Constantinescu, and Farquhar, 1984) receptors accumulate in Golgi coated vesicles, while chloroquine causes accumulation in endosomes (Brown, Constantinescu, and Farquhar, 1984). Monensin and anti-receptor antibodies also cause the accumulation of receptors intracellularly (Braulke *et al.*, 1987; Nolan *et al.*, 1987). Although binding of ligand does cause a conformational change in the receptor as measured by protease sensitivity (Westcott, Searles, and Rome, 1987), the ligand occupancy does not appear to affect receptor movement (Oka and Czech, 1986; Braulke *et al.*, 1987). Both Man 6-P receptors cycle from the cell surface and return to the trans Golgi (Duncan and Kornfeld, 1988) and copurify in intracellular vesicles which contain the asialoglycoprotein receptor (Messner, Griffiths, and Kornfeld, 1989). There is, however, sorting of receptors such that 20-45 minutes following internalization the IGF-II/Man 6-P receptor is sorted from the transferrin and asialoglycoprotein receptors (Geuze *et al.*, 1983; Stoorvogel *et al.*, 1989).

## L. The Synthesis, Processing, and Degradation of the IGF-II/Man 6-P Receptor

The IGF-II/Man 6-P receptor is synthesized as a 2491 amino acid preprotein, which gives rise to a 2451 amino acid mature protein following removal of the presequence (Morgan *et al.*, 1987). Following synthesis, this protein undergoes a series of modifications to give rise to mature receptor (Figure 14). The first receptor form detectable in H35 cells has an Mr=245 kDa, which is converted to an Mr=250 kDa form with a half time of 2 hours (MacDonald and Czech, 1985). The 245 kDa form has 2-6 mannose side chains, and about half of the total mannoses appear in the mature form (MacDonald and Czech, 1985). Neuraminidase treatment causes the conversion of the 250 kDa form into the 245 kDa form (MacDonald and Czech, 1985). Endo H treatment causes the 245 kDa form to be converted to a 232 kDa form, a form which accumulates in the presence of tunicamycin (MacDonald and Czech, 1985). Monensin caused the accumulation of the 245 kDa form (MacDonald and Czech, 1985). BW 5147 (Goldberg, Gabel, and Kornfeld, 1983) and CHO (Sahagian and Neufeld, 1983) cells show similar processing following translation. Both the 245 kDa or the 250 kDa forms are capable of binding IGF-II, while the 232 kDa form wasn't functional in binding this ligand. No glycosylation is apparently required for binding of mannose 6-phosphate (Sahagian and Neufeld, 1983). It has been hypothesized that the lag time following synthesis of this, and other, receptors to bind ligand is due to a requirement to form the correct disulfide bonds in the endoplasmic reticulum (Olson and Lane, 1989). Like the IGF-II/Man 6-P receptor, the cation dependent mannose 6-phosphate receptor is synthesized as a precursor, then glycosylated to a mature form within 2 hours (Stein *et al.*, 1987a).

<u>Location</u>	<u>Glycosylation</u>	<u>Mr</u>
Plasma Membrane	GlcNAc <sub>2</sub> Man <sub>3</sub> GlcNAc <sub>4</sub> Gal <sub>3</sub> NANA <sub>3</sub>	250000
Trans Golgi	GlcNAc <sub>2</sub> Man <sub>3</sub> GlcNAc <sub>4</sub> Gal <sub>3</sub> NANA <sub>3</sub>	250000
Medial Golgi	GlcNAc <sub>2</sub> Man <sub>5</sub> GlcNAc <sub>2</sub>	245000
Cis Golgi	GlcNAc <sub>2</sub> Man <sub>5</sub>	245000
Endoplasmic Reticulum	GlcNAc <sub>2</sub> Man <sub>8</sub>	245000
ER-upon synthesis	GlnAc <sub>2</sub> Man <sub>9</sub> Glc <sub>3</sub>	245000
	None	232000



**Figure 14: Synthesis and Processing of the IGF-II/Man 6-P Receptor.** The IGF-II/Man 6-P receptor is coded by a single gene to produce one mRNA species. This mRNA encodes a 2491 amino acid polypeptide which is cotranslationally transported into the endoplasmic reticulum. Signal peptidase then removes a 40 amino acid presequence to produce a 2451 amino acid mature peptide. A number of glycosylation steps then occur to the polypeptide to increase this protein sequentially from 232 kDa to a mature Mr of 250 kDa (MacDonald and Czech, 1985; Morgan *et al.*, 1987). The figure indicates the probable glycosylation of the experimentally determined receptor forms.

The degradation of the receptor occurs slowly, with a half life of 24 hours in human fibroblasts (Creek and Sly, 1983). In I-cell disease the half life is somewhat longer, being 32 hours, while  $\beta$ -glucuronidase and  $\text{NH}_4\text{Cl}$  decreased the half life slightly (Creek and Sly, 1983). The degradation is nonlysosomal (Creek and Sly, 1983; Sahagian, 1984), is not inhibited by leupeptin (Creek and Sly, 1983), and may involve an extracellular protease (Sahagian, 1984). The degradation rate also appears to depend on the culture conditions as CHO cells showed a half life of receptor of 16 hours in monolayer but only 6 hours in suspension culture (Sahagian and Neufeld, 1983). One cell line, MOPC 315, possess a markedly decreased number of receptors because, although receptor is synthesized normally, it is degraded very rapidly (Goldberg, Gabel, and Kornfeld, 1983).

### M. The IGF-II/Man 6-P Receptor as a Signalling Protein

The biological role of the IGF-II/Man 6-P receptor in signalling responses to the IGFs has been under debate for a number of years. Two types of responses have been proposed to be mediated by IGF-II through the IGF-II/Man 6-P receptor: insulin-like and proliferative responses. In this section the evidence accumulated supporting and refuting a role for the IGF-II/Man 6 P receptor in mediating these responses will be presented. In the discussion section of this manuscript this data will be combined with data from this thesis project and both possibilities evaluated more critically and a conclusion reached.

The data supporting a role of the IGF-II/Man 6-P receptor in mediating some of the responses to IGF-II is supported by a number of types of evidence: 1. correlation of IGF binding to the IGF-II/Man 6-P receptor with responses; 2. the inability of these responses to be blocked by antibodies to the insulin and IGF-I receptor; 3. the ability of some responses to be blocked by antibodies which recognize the IGF-II/Man 6-P receptor; and 4. the ability of ligands for the Man 6-P binding site of the IGF-II/Man 6-P receptor to alter these responses.

IGF-II has been implicated to cause an increase in thymidine and sulfate incorporation into fetal rat costal cartilage through its own receptor based only on the ability of IGF-II to cause this response (Hill and Milner, 1984). On the basis of ligand specificity, IGF-II stimulates 2-deoxyglucose and  $\alpha$ -aminoisobutyric acid uptake in L6 myoblasts (Beguinet *et al.*, 1985), increases the activity of the  $\text{Na}^+/\text{H}^+$  exchanger in canine renal proximal tubular cells (Mellas, Gavin, and Hammerman, 1986), and promotes growth in the K562 human erythroblast cell line (Tally, Li, and Hall, 1987) and in fetal rat adrenal cells (van Dijk, Tanswell, and Challis, 1988) through the IGF-II/Man 6-P receptor.



Using antibodies which block ligand binding to the insulin receptor, the IGF-II/Man 6-P receptor has been implicated to induce increases in thymidine incorporation in human fibroblasts (King *et al.*, 1980) and to phosphorylate the insulin receptor  $\alpha$ -subunit in dog kidney basolateral membranes (Hammerman and Gavin, 1984). A combination of the dose dependence of the response, the inability of the response to be inhibited by antibodies to the insulin or IGF-I receptors, and by the ability of this response to be mimicked by antibodies to the IGF-II/Man 6-P receptor provide strong evidence that an increase in glycogen synthesis is mediated through the IGF-II/Man 6-P receptor in human hepatoma cells (Hari *et al.*, 1987). A role of the IGF-II/Man 6-P receptor is indicated in induction of inositol triphosphate (IP3) production in renal proximal tubule basolateral membranes by a 2-fold potentiation of the IGF-II effect by mannose 6-phosphate (Rogers and Hammerman, 1989).

The most studied IGF-II responsive system involves the increase in thymidine incorporation observed in Balb/c 3T3 cells made competent with PDGF then primed with EGF (Nishimoto *et al.*, 1987a and b). Corresponding with this response was a 2-fold sustained increase in  $Ca^{++}$  influx which was dependent upon extracellular  $Ca^{++}$  and inhibitable by lanthanum, cobalt, and tetramethlin but not nitrendipene (Nishimoto *et al.*, 1987a and b). Much higher concentrations of IGF-I are required to induce this response. BAYK8644 induced  $Ca^{++}$  influx in primed competent cells resulted in a similar increase in  $^3H$ -thymidine incorporation (Nishimoto *et al.*, 1987a and b). IGF-II appears to act through a G-protein as it attenuated pertussis toxin induced ADP ribosylation of a 40 kDa G-protein in these cells (Nishimoto *et al.*, 1989). Finally, an antibody to the IGF-II/Man 6-P receptor increases thymidine uptake and  $Ca^{++}$  influx in this system (Kojima *et al.*, 1988).

There are a number of pieces of evidence which indicate that the IGF-II/Man 6-P receptor plays no role in mediating the responses of IGF-II. The IGF-I receptor has been shown to mediate the IGF-II induced increase in  $\alpha$ -aminoisobutyric acid uptake in rat soleus muscle (Yu and Czech, 1984a) and in DNA synthesis in human fibroblasts (Furlanetto, DiCarlo, and Wisehart, 1987) and rat granulosa cells (Adashi, Resnick, and Rosenfeld, 1990). Using anti-IGF-II/Man 6-P receptor antibodies, it has been demonstrated that the IGF-II/Man 6-P receptor plays no role in the IGF-II induced increase in DNA synthesis in H-35 Hepatoma cells (Mottola and Czech, 1984) and in  $\alpha$ -aminoisobutyric acid uptake, glucose transport, and protein synthesis by L6 myoblasts, although the anti-receptor antibody was able to block IGF-II binding to its receptor and decrease the rate of degradation of IGF-II (Kiess *et al.*, 1987). These pieces of evidence have disproven the proposed role of the IGF-II/Man 6-P receptor in mediating these particular responses.

Other types of experiments also question the role of IGF-II/Man 6-P receptor in signalling responses to IGF-II in general, but in more indirect ways. A disruption of the IGF-II gene in mice results only in a decrease in growth (DeChiara, Efstratiadis, and Robertson, 1990). Such an effect argues against a critical role for the IGF-II binding capability of the IGF-II/Man 6-P receptor. More evidence comes from chick myotubes, which lack IGF-II/Man 6-P receptors, but show an increase in amino acid uptake, protein synthesis, and decrease in protein degradation at 5 nM IGF-II (Janeczko and Etlinger, 1984; Conover *et al.*, 1986), similar to responses seen in cells possessing IGF-II/Man 6-P receptors. The presence of a second binding site on the IGF-I receptor which preferentially binds IGF-II and is not blocked by  $\alpha$ IR-3, the anti-IGF-I receptor antibody used in the studies discussed above (Casella *et al.*, 1986) calls into question experiments implicating

the IGF-II/Man 6-P receptor as the signalling receptor by blocking IGF-I receptor with this antibody. IGF-II responses could also be mediated by a recently discovered receptor which has been cloned and sequenced and which resembles the insulin and IGF-I receptors (Shier and Watt, 1989). This may be a new IGF-II receptor. Finally, some pieces of data provide alternative explanations for some of the observed phenomenon. The ability of insulin to inhibit pertussis toxin catalyzed ADP-ribosylation of G-proteins through its own receptor (Rothenburg and Kahn, 1988; Ciaraldi and Maisel, 1989) brings up the possibility that the effect in primed competent Balb/c 3T3 cells, discussed above, could be mediated by the insulin or IGF-I receptor. There is also evidence that the normal secretory pathway of the IGF-II/Man 6-P receptor may involve G-proteins and  $Ca^{++}$  based on the ability of alterations of these components to alter lysosomal enzyme release (Goda and Pfeffer, 1988; Warren, 1989) and affinity for ligand (Bryson and Baxter, 1987). It would not be unusual if ligands for the receptor altered its intracellular movement, and thereby these parameters. The fact that IGF-II/Man 6-P receptor phosphorylation is altered by binding of lysosomal enzymes or mannose 6-phosphate (Panneerselvam and Balasubramanian, 1989) further suggests that receptor conformation may be altered upon ligand binding and transmitted across the membrane; however, there is no indication that this phosphorylation plays any further role in signalling.

## N. Receptor Forms in Serum

The initial "soluble receptors" were generated by special treatments of lymphocytes or liver membranes to induce the release of these proteins. Insulin receptors (Gavin, Buell, and Roth, 1972) and growth hormone receptors (McGuffin et al., 1976; Herington, Elson, and Ymer, 1981) were generated in this manner. Recently, however, true serum receptors have been identified. Recent publications have proposed the use of serum receptors in both the diagnoses and treatment of certain diseases, as described below..

### 1. The Transferrin Receptor

The transferrin receptor has been demonstrated in human serum as ~90 kDa band in immunoblots. It is present at approximately 255 ng/ml in healthy adults. Alterations in the levels of the serum transferrin receptor have been observed in a number of disease states: acute leukemia (368 ng/ml), myeloma (422 ng/ml), and malignant lymphoma (478 ng/ml; Kongo *et al.*, 1986). Recently an immunoassay has been devised to measure serum transferrin receptor levels (Flowers *et al.*, 1989; Trowbridge, 1989). Using this assay, the normal transferrin receptor level in serum is 5 mg/l, while in aplastic anemia the level is decreased to ~2.5 mg/l (Flowers *et al.*, 1989; Trowbridge, 1989). Iron deficiency anemia or sickle cell anemia result in significant increases in serum transferrin receptor, to 18 mg/l and 33 mg/l, respectively (Flowers *et al.*, 1989; Trowbridge, 1989).

## 2. The Growth Hormone Receptor

The growth hormone receptor is the best characterized of the serum receptors. The serum receptor is capable of binding growth hormone (Ymer and Herington, 1985; Baumann *et al.*, 1986), is immunologically similar to the membrane receptor (Barnard and Waters, 1986; Baumann and Shaw, 1988), and is similarly regulated by growth hormone (Bick *et al.*, 1990). The serum receptor is derived from a soluble cytosolic receptor found in liver (Herington, Elson, and Ymer, 1981; Ymer, Stevenson, and Herington, 1984; Ymer and Herington, 1984; Herington, Ymer, and Stevenson, 1986; Barnard and Waters, 1986; Spencer *et al.*, 1988) and is produced from an alternatively spliced message from the membrane receptor (Leung *et al.*, 1987; Baumbach, Horner, and Logan, 1989). While this protein is normally present in human serum (Herington, Ymer, and Stevenson, 1986), it is absent in patients with Laron dwarfism (Daughaday and Trivedi, 1987).

## 3. The Nerve Growth Factor Receptor

A truncated form of the nerve growth factor receptor having an Mr ~50 kDa as opposed to the cellular nerve growth factor receptor with an Mr ~80 kDa has been detected in the medium conditioned by schwann cells, schwannoma, PC12 cells, and superior cervical ganglionic neurons (DiStefano and Johnson, 1988). This protein can also be found in neonatal rat urine, amniotic fluid, and in the stomach of the fetal rat (DiStefano and Johnson, 1988).

#### 4. Neural Cell Adhesion Molecule (NCAM)

NCAM, a neural adhesion molecule, exists as transmembrane forms, in forms lacking a transmembrane region but possessing a lipid membrane anchor, and as a soluble form released from cells, all of which are generated by alternate splicing of a single gene (Gower *et al.*, 1988).

#### 5. The Class I Antigens

Soluble forms of the class I, or major histocompatibility, antigens have been detected in serum complexed with  $\beta_2$ -microglobulin (Gussow and Ploegh, 1987). At present there is evidence that such proteins could be generated from different genes, different alternatively spliced messages, or be proteolytic products of the normal cell associated class I antigens (Gussow and Ploegh, 1987).

#### 6. The Interleukin Receptors

Unlike the serum receptors described above in which most of the information relates to the structure and synthesis of the receptor, the serum interleukin receptors have been studied predominantly for their clinical importance. The serum interleukin 2 receptor is released by cytotoxic T-cells upon activation, apparently by a proteolytic mechanism (Baran, Korner, and Theze, 1988). The amount of receptor detected in serum varies by the detection method used, but all results show significant increases with various types of malignancies: malignant lymphoma (Harrington *et al.*, 1988), lung cancer (1.66 fold;

Marino *et al.*, 1990), Kawasaki disease (4.2-5.2 fold, depending on disease activity; Lang *et al.*, 1990), and multiple sclerosis (2.3 fold; Greenberg *et al.*, 1988; Capra *et al.*, 1990; Rudnick and Barna, 1990).

Recent experiments have also suggested a possible clinical application for the serum interleukin 1 receptor. Injection of mice with serum interleukin 1 receptor significantly extended the survival of heart transplants and prevented lymph node hyperplasia in response to injection of allogeneic cells (Fanslow *et al.*, 1990). In either case, injection of interleukin 1 reversed the effects of the serum interleukin 1 receptor (Fanslow *et al.*, 1990). This suggests that serum receptors can be used to bind and/or remove their ligands, a process that could have clinical implications for a number of the serum receptors discovered, and could promote the synthesis of serum receptors not found in nature.

## 7. The IGF-II/Man 6-P Receptor

The serum form of the IGF-II/Man 6-P receptor was initially discovered in rat serum (Kiess *et al.*, 1987), and later in sheep (Gelato *et al.*, 1989), monkeys (Gelato *et al.*, 1988), and man (Causin *et al.*, 1988). It is identical to the cellular receptor in its ability to bind ligand, although it is ~10 kDa smaller than the cellular receptor (Kiess *et al.*, 1987; Causin *et al.*, 1988). This difference in molecular weight cannot be removed by endoglycosidase treatment (Kiess, Gelato, and Nissley, 1989). The serum receptor is high in fetal and neonatal serum and decreases with age (Kiess *et al.*, 1987), similar to the developmental regulation observed for the cellular receptor in various tissues (Alexandrides, Moses, and Smith, 1989; Sklar *et al.*, 1989; Valentino, Ocrant, and Rosenfeld, 1990). The serum IGF-II/Man 6-P receptor is not likely to play a significant

role as it can account for only a small percentage of the serum IGF binding proteins: 3-7% in adult rats and sheep (Kiess *et al.*, 1987; Gelato *et al.*, 1989), 20% in monkey (Gelato *et al.*, 1988), but up to 50% in fetal sheep (Gelato *et al.*, 1989). The apparent lack of effect of an IGF-II deletion on development further calls into question the importance of IGF-II binding by the IGF-II/Man 6-P receptor (DeChiara, Efstratiadis, and Robertson, 1990). The presence of both IGF-II/Man 6-P receptor (Causin *et al.*, 1988) and lysosomal enzymes (Belfiore *et al.*, 1974) in urine suggests that the serum form of the IGF-II/Man 6-P receptor may play a role in binding lysosomal enzymes in serum and removing them through the urine



## Chapter II

### Introduction

The studies described in the enclosed manuscripts are designed to increase our understanding of the structure, regulation, and function of the IGF-II/Man 6-P receptor by studying forms of this protein that have not been studied previously: non-mamalian receptors and the serum form of the receptor. While only a few specific questions have been addressed, a number of interesting implications are suggested by the results as are several new areas of research.

The first question addressed is whether Man 6-P receptors from species other than those previously studied (a number of mammals) possess the capacity to bind IGF-II. The rationale for these experiments arises from two different pieces of evidence. The first is that a number of studies to address the presence of IGF receptors in chickens have identified only IGF-I type receptors (Kasuga *et al.*, 1982b; Bassas *et al.*, 1988). Second, given the general and critical role of the Man 6-P receptor in sorting lysosomal enzymes, it is unlikely that this protein would be absent in any species. The approach taken to answer this question is to affinity purify Man 6-P receptors, then to use  $^{125}\text{I}$ -IGF-II in affinity crosslinking and binding experiments. Receptors were isolated from rat (as a positive control), chicken, and *Xenopus* (to address the general pattern of binding in evolution) and both rat and chicken  $^{125}\text{I}$ -IGF-II were used in the binding studies.

The remainder of the manuscripts involve a number of studies to address the question of the relationship between the serum and cellular IGF-II/Man 6-P receptors and to further characterize the serum receptor. The first studies addressed the structure and binding functions of the serum IGF-II/Man 6-P receptor. In order to compare the

structures of the cellular and serum IGF-II/Man 6-P receptors, antibodies were raised against two peptides: one in the extracellular region of the receptor, and a second in the cytoplasmic tail of the receptor. These antibodies were then used in immunoblots to detect possible truncations of the serum receptor as compared to its cellular counterpart. In order to assess the binding functions of the serum receptor, receptor immobilized on anti-IGF-II/Man 6-P receptor Affigel or PMP-Sepharose was affinity crosslinked to  $^{125}\text{I}$ -IGF-II. In this way its ability to bind mannose 6-phosphate, as shown by its specific binding to PMP-Sepharose, and to bind IGF-II, as assessed by crosslinking to iodinated ligand, could be demonstrated.

The next set of studies continued studies of the receptor in the whole animal to further elucidate the relationship between the cellular and serum receptors. As has been discussed in an earlier section, the IGF-II/Man 6-P receptor in cells, most notably adipocytes, is translocated from an intracellular compartment to the cell surface in response to insulin. If the serum receptor is a proteolytic product of the cellular receptor produced by proteolysis from the cell surface, we would expect the serum levels of the IGF-II/Man 6-P receptor to increase in response to insulin. This effect has been monitored using the immunoadsorption and immunoblotting technique described above.

Finally, the question of a possible precursor/product relationship between the cellular and serum IGF-II/Man 6-P receptor was addressed. A system was developed where cultured cells were briefly labeled with  $^{35}\text{S}$  amino acids, then IGF-II/Man 6-P receptor immunoadsorbed from cell membranes or the medium at several times thereafter. Additionally, the effects of a number of agents, including insulin, protease inhibitors, and ligands for the receptor, on the process of cellular receptor degradation/serum receptor

production was studied. These experiments are discussed in the final manuscript in the Materials, Methods and Results section.

**Chapter III**  
**Materials, Methods and Results**

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**Chicken and *Xenopus* Mannose-6-Phosphate Receptors Fail to Bind**

**Insulin-like Growth Factor II**

Running Title: Non-mammalian Man-6-P Receptors Fail to Bind IGF-II

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## SUMMARY

The recent demonstration that a single mammalian receptor protein binds both mannose-6-phosphate (Man-6-P) and insulin-like growth factor II (IGF-II) with high affinity has suggested a multifunctional physiological role for this receptor, possibly including signal transduction. In order to better understand the functions of this receptor, we have investigated the properties of Man-6-P receptors from non-mammalian species. Receptors were affinity purified from Triton X-100 extracts of total membranes from *Xenopus* and chicken liver as well as rat placenta using pentamannosyl-6-phosphate (PMP)-Sepharose. The Man-6-P receptor was adsorbed to the PMP-Sepharose and specifically eluted by Man-6-P in all three species, as evaluated by sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by silver staining. When the purified receptors from these three species were crosslinked to  $^{125}\text{I}$ -IGF-II with disuccinimidyl suberate (DSS), only receptors isolated from rat membranes were affinity labelled. To further evaluate the properties of these Man-6-P receptors, binding of  $^{125}\text{I}$ -rat-IGF-II and  $^{125}\text{I}$ -chicken YGTA-IGF-II to purified receptors from *Xenopus*, chicken, and rat was evaluated by polyethylene glycol (PEG) precipitation. Only the rat Man-6 P receptor exhibited detectable binding of  $^{125}\text{I}$ -IGF-II. These data suggest that the emergence of a high affinity IGF-II binding site on the Man-6-P receptor occurred in evolution after the divergence of mammals from other vertebrates. Thus, the biological actions of IGF-II in chickens and frogs appears to be initiated by the type I IGF receptor.

The insulin-like growth factors I and II (IGF-I and II) are widespread among animals, yet the physiologic roles and mechanisms of action of these hormones is incompletely understood. The synthesis and release of IGF-I into the circulation has been demonstrated to be regulated by growth hormone (1), and IGF-I induces growth when administered directly (1). Similarly, IGF-II has been implicated in a number of physiologic effects, including stimulation of thymidine incorporation (2-5), RNA and protein synthesis (6),  $\text{Ca}^{2+}$  influx (2), glucose (6-7) and amino acid (6) uptake, glucose incorporation into glycogen (8), glucose oxidation (4), protein phosphorylation (9), and cell proliferation (3) and differentiation (10). IGF-II has also been reported to inhibit protein degradation (6) and change intracellular pH (11). Biological responses of IGF-I and IGF-II have been shown to be mediated by a type I IGF receptor which is structurally very similar to the insulin receptor, a heterotetrameric tyrosine kinase composed of two  $\alpha$  and two  $\beta$  subunits (1). Another receptor for IGF-II, the type II IGF receptor, bears no resemblance to these signalling receptors. It consists of a single polypeptide chain with one transmembrane segment, a short cytoplasmic domain, and no protein kinase activity (12-18). Furthermore, this IGF-II receptor has been shown to be identical to the cation independent Man-6-P receptor as demonstrated by sequence analysis (12-16) and binding studies (12).

A number of experiments have been designed to elucidate through which receptor or receptors the biological activities of IGF-II are mediated. Some of these studies indicate that IGF-II effects are mediated through the insulin or IGF-I receptor, either by analysis of dose response relationships or by the use of antibodies which block binding of peptide ligands to insulin (4) and IGF-I (6) receptors. Other results have been interpreted to suggest that some biological effects may be mediated through the IGF-II/Man-6-P receptor (7-9, 11). Some of these effects of IGF-II are mimicked by anti-IGF-II/Man-6-P receptor antiserum (2, 8). However, two preparations of anti-receptor antibodies which block ligand binding to the IGF-II/Man-6-P receptor did not block biological responses (19, 20). The signalling potential of the IGF-II/Man-6-P receptor thus remains controversial.

The presence of IGF's has been reported in chickens (21), reptiles, amphibians, and fish (22) where their presence has been assayed by radioimmunoassay or the ability to compete with the mammalian peptide for binding to mammalian IGF-II/Man-6-P receptor. Furthermore, mammalian IGF's have been shown to stimulate DNA synthesis, nutrient transport and cell multiplication in chick embryo fibroblasts (3, 23) and in cultured chick

myotubes (6). These peptides also act to initiate differentiation in chick myoblasts (9). Other processes affected by IGF's include DNA, RNA and protein synthesis in chick chondrocyte cultures (24) and glycogen, RNA, and protein synthesis in chick liver cells (25). These data strongly support the hypothesis that the avian and mammalian IGF's have been highly conserved during evolution in both function and structure. The partial amino acid sequences of the chicken IGF's further support this conclusion (21). However, studies on binding and affinity crosslinking of  $^{125}\text{I}$ -IGF-II to cell membranes have indicated that chickens possess an IGF receptor which resembles the mammalian IGF-I receptor and binds both ligands with near equal affinity (3, 26). No labelling of a type II IGF receptor was detected in such experiments. In this study, we have addressed this issue directly by assessing the ability of affinity purified Man-6-P receptors from rat, chicken, and *Xenopus* to bind with rat and chicken  $^{125}\text{I}$ -IGF-II.



## EXPERIMENTAL PROCEDURES

**Materials.** Rat IGF-II was purified from BRL-3A cell conditioned medium as described (27). Chicken YGTA variant form of IGF-II was a gift from Dr. F.J. Ballard, CSIRO, Adelaide, South Australia. Purified IGF-II was radioiodinated using Enzymobeads (BioRad) to a specific activity of 40-250 Ci/g. Pentamannosyl-6-phosphate (PMP), prepared from the native Y-2448 Q-phosphomannan from *H. holstii*, was a gift of Dr. M.E. Slodki, Midwest Area Northern Regional Research Center, Peoria, IL. PMP-Sepharose was prepared as described (28). Nitrocellulose filters with a 45  $\mu$ m pore size were obtained from the Millipore Corporation. All other chemicals were reagent grade or better.

**Preparation of Membranes from Rat Placenta, Chicken Liver, and Frog Liver.** A microsomal membrane fraction was isolated from rat placentas taken at day 19 of gestation as described previously (29), and chicken and *Xenopus* liver membranes were prepared by the same technique. Membrane extracts (5 mg protein/ml) were prepared by incubation with 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), 0.15 M NaCl, 1% Triton X-100, 5 mM  $\beta$ -glycerophosphate, pH 7.4, plus protease inhibitors by gentle mixing for 1 h at 30°C. The protease inhibitors were leupeptin, antipain and benzamidine at concentrations of 10  $\mu$ g/ml each, 20  $\mu$ g/ml aprotinin, 12.5  $\mu$ g/ml chymostatin, and 1 mM PMSF. The mixture was centrifuged for 10 min at 15000 x g in a microcentrifuge and the supernatant fraction was used as the extract. Protein was assayed by the Bradford method (30).

**Purification of Man-6-P Receptors with PMP-Sepharose.** Triton X-100 extracts of microsomal membranes containing 1 mg membrane protein from rat placenta, chicken liver, or *Xenopus* liver were adsorbed with PMP-Sepharose in 50 mM Hepes, 0.15 M NaCl, 0.05% Triton X-100, 5 mM  $\beta$ -glycerophosphate, pH 7.4, with antiproteases. After an overnight incubation at 4°C, the resin was loaded into a column and, for the rat and chicken membranes, prewashed in 50mM Hepes, 0.5 M NaCl, 0.1% Triton X-100, 5 mM  $\beta$ -glycerophosphate, pH 7.4. Material was then eluted sequentially in buffer alone, buffer containing 5 mM Man-1-P, containing 5 mM glucose-6-phosphate, and finally 5 mM Man-6-P.

**Affinity Crosslinking of  $^{125}\text{I}$ -IGF-II to Purified Receptors.** Following purification with PMP-Sepharose, receptors from 100  $\mu\text{g}$  membrane protein were suspended to a volume of 0.2 ml with KRP buffer containing 1% BSA plus 4 nM  $^{125}\text{I}$ -IGF-II with or without 400 nM unlabelled IGF-II. These mixtures were then incubated for 4 h at 23°C. The samples were subjected to affinity cross-linking with DSS as described before (31).

**Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE).** Electrophoresis was carried out under reducing conditions, samples were heated to 100°C for 10 min in SDS sample buffer with dithiothreitol. Electrophoresis was performed as described (32) on 6% polyacrylamide gels. Silver staining was performed using a BioRad silver stain kit as described (33).

**Quantitation of Ligand Binding Using the Polyethylene Glycol (PEG) Precipitation Method.** The studies of ligand binding to receptor were performed as previously described (29). Briefly, 0.1 ml of a stock solution of 4 nM  $^{125}\text{I}$ -IGF-II and unlabelled IGF-II at 0, 10, 40, 100, 400, and 1000 nM was combined with an equal volume of a solution of purified receptors at 4  $\mu\text{g}$  of protein. Following incubation for 1 h at 3°C, 0.5 ml of 0.9 mg/ml bovine  $\gamma$ -globulins in 0.1 M sodium phosphate buffer, pH 7.4 and 0.5% PEG were added to each sample, the sample mixed, and incubated at 0°C for 15 min. This mixture was then filtered through nitrocellulose filters which were blocked by incubation in KRP containing 1% bovine serum albumin, the filters washed 3 times with 8% PEG, and the  $\gamma$ -radiation from the filters measured.

## RESULTS AND DISCUSSION

In the first experimental protocol (Fig. 15), PMP-Sepharose was used to affinity purify Man-6-P receptors from membrane extracts of rat placenta (lanes 1, 2, 3), chicken liver (lanes 4, 5, 6), and *Xenopus laevis* liver (lanes 7, 8, 9). A Triton X-100 extract of whole membranes was incubated with PMP-Sepharose overnight, then this material was loaded onto a column and the fractions eluted by buffer alone (not shown), mannose-1-phosphate (lanes 1, 4, and 7), glucose-6-phosphate (lanes 2, 5, and 8), and Man-6-P (lanes 3, 6, and 9). From each fraction one aliquot was electrophoresed (SDS-PAGE) then silver stained (panel B). A duplicate sample was incubated with  $^{125}\text{I}$ -IGF-II and the crosslinking agent DSS and subjected to SDS-PAGE, and the gel autoradiographed (panel A). Although most of the protein fails to bind to the column and elutes in the first washes with buffer, the Man-6-P receptor binds the column strongly and is mostly eluted by Man-6-P as demonstrated by a silver stained Mr~250K band (lanes 3, 6, 9).

Affinity crosslinking of the purified rat receptor preparations yielded a labelled band at Mr~260K, corresponding to the ligand-receptor complex (lanes 1, 2, and 3). Very low but significant amounts of labelled rat receptor were also visualized in the mannose-1-phosphate and glucose-6-phosphate elutions (lanes 1A and 2A) even though silver stained receptor was not evident. In contrast, no labelled receptor bands were detected when either chicken or *Xenopus* receptor preparations were crosslinked with  $^{125}\text{I}$ -IGF-II (lanes 4-9). Increasing the exposure times of the autoradiograms of the chicken and frog preparations five-fold also failed to reveal labelled bands (not shown). Using this technique, the presence of receptors with up to a 10 fold lower affinity than mammalian IGF-II/Man-6-P receptors should be easily detected.

The data in Figure 15 strongly support the idea that Man-6-P receptors from *Xenopus* and chicken fail to bind IGF-II, yet it is possible that IGF-II and its receptor have diverged in evolution such that ligand-receptor interactions are species specific. Direct binding studies using both rat and chicken IGF-II using the purified Man-6-P receptors were performed to definitively test this hypothesis. In Figure 16 (panel A) is shown the competition by excess unlabelled rat IGF-II of  $^{125}\text{I}$ -rat IGF-II binding to Man-6-P receptors purified from the three species described in Figure 15. While rat  $^{125}\text{I}$ -IGF-II binds significantly to rat receptor by a saturable process, no binding to the chicken or

*Xenopus* receptor by this ligand is observed. This experiment was repeated (Fig. 2, panel B) using  $^{125}\text{I}$ -chicken IGF-II as ligand with similar results: neither chicken nor *Xenopus* Man-6-P receptors bind chicken  $^{125}\text{I}$ -IGF-II significantly, while the rat receptors bind this ligand with high affinity.

The data presented above demonstrate that chicken and *Xenopus* liver cells contain Man-6-P receptors, but that they lack the ability to bind IGF-II with measurable affinity under the experimental conditions described here. In these species, the presence of IGF's has been demonstrated, and, in the chicken, IGF-II has been shown to cause a number of physiologic responses, many similar to those documented in mammals. Importantly, the chicken Man-6-P receptor fails to bind chicken  $^{125}\text{I}$ -IGF-II with high affinity, while the rat receptor binds this ligand with high affinity. It has previously been demonstrated that chickens possess a type I IGF receptor tyrosine kinase which binds IGF-I and II with near equal affinity (3). It thus seems likely that both IGF-I and IGF-II mediate biological actions by interacting with this type I IGF receptor in chickens.

**FOOTNOTE**

The abbreviations used are: IGF I and II, insulin like growth factor I and II; Man-6-P, mannose-6-phosphate; PMP, pentamannosyl-6-phosphate; DSS, disuccinimidyl suberate; KRP, Krebs Ringer phosphate buffer; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BSA, bovine serum albumin; PEG, polyethylene glycol.

### ACKNOWLEDGEMENTS

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### Note Added in Proof

While this manuscript was undergoing review, a paper was published (Canfield, W.M. and Kornfeld, S. (1989) *The Journal of Biological Chemistry* **264**, 7100-7103) which also reported that the chicken Man 6-P receptor failed to bind IGF-II with high affinity.

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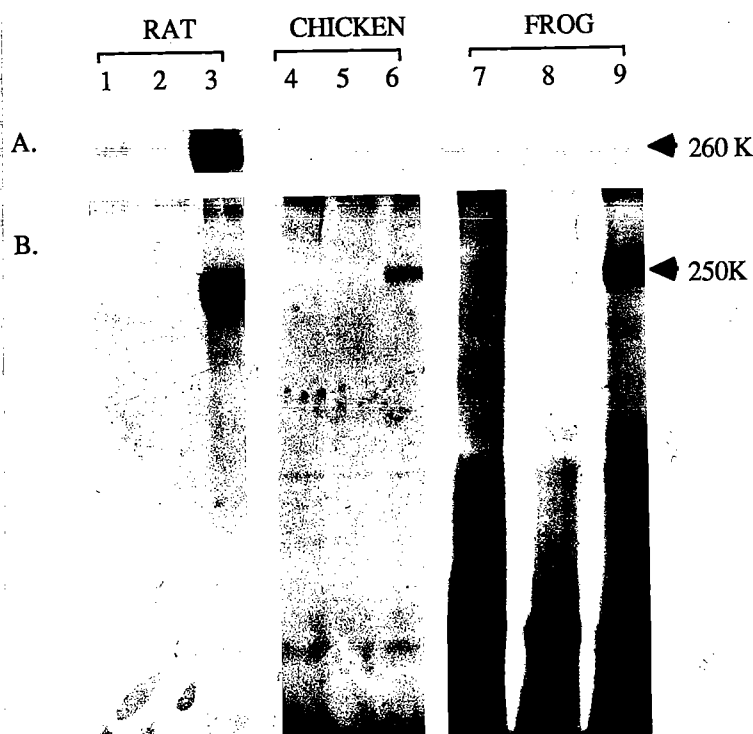
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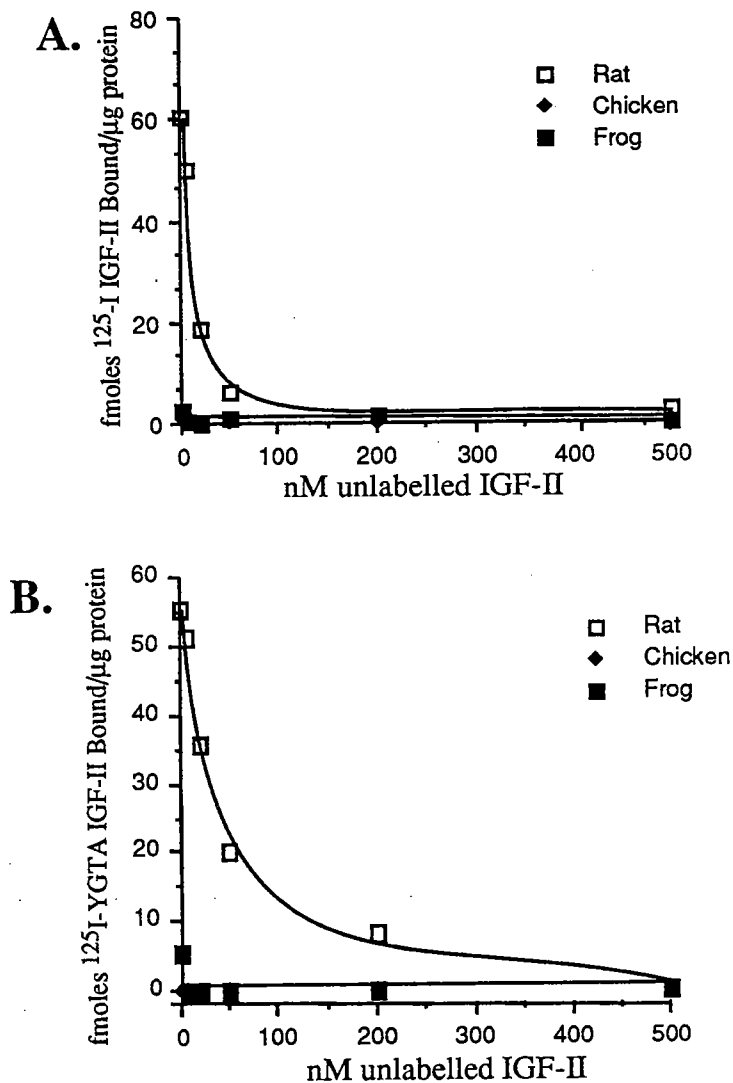
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**Figure 15.** Analysis of fractions eluted from a PMP-Sepharose column by affinity crosslinking. Triton X-100 extracts were prepared of 1 mg of protein from rat placenta (lanes 1, 2, 3), chicken liver (lanes 4, 5, 6), and frog liver (lanes 7, 8, 9). These extracts were incubated in a total volume of 2 ml with 1 ml of PMP Sepharose overnight at 4°C. Following the incubation, the rat and chicken membranes were prewashed in a high salt buffer as described in the Experimental Procedures. Fractions were eluted with 1 ml of buffer alone (not shown), buffer containing 5 mM mannose-1-phosphate (lanes 1, 4, 7), buffer containing 5 mM glucose-6-phosphate (lanes 2, 5, 8), or buffer containing 5 mM Man-6-P (lanes 3, 6, 9). One aliquot of each fraction (0.1 ml) was then analysed by SDS-PAGE followed by silver staining (panel B), while another aliquot (50  $\mu$ l) was analysed by crosslinking with DSS to 4 nM  $^{125}$ I-IGF-II, followed by SDS-PAGE and autoradiography (panel A). The apparent Mr of the receptor band in the crosslinking study is increased to 260K due to the added molecular mass of the crosslinked IGF-II.



**Figure 16.** Analysis of the binding of  $^{125}\text{I}$ -rat and chicken IGF-II to purified Man-6-P receptors. Receptors purified on PMP-Sepharose ( $4\ \mu\text{g}$  protein) were incubated in a total volume of  $0.2\ \text{ml}$  with  $2\ \text{nM}$   $^{125}\text{I}$ -IGF-II from rat (panel A) or chicken (panel B) containing  $0, 5, 20, 50, 200,$  or  $500\ \text{nM}$  unlabelled rat IGF-II. Following a  $1\ \text{h}$  incubation at  $3^\circ\text{C}$ , samples were PEG precipitated as described (29) and the  $\gamma$ -radiation from the filters measured.

**Serum Form of the Rat Insulin-like Growth Factor II/Mannose-6-phosphate  
Receptor is Truncated in the Carboxyl-terminal Domain\***

Running Title: Serum IGF-II/Man-6-P receptor is C-terminally Truncated

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Medical Center Diabetes and Endocrinology Research Center (NIH grant DK 32520).

Some of the work in the following paper was performed by Dr. Richard MacDonald. For the sake of ensuring a proper distribution of credit for this manuscript the following information is provided for readers of this thesis:

Contributions of Dr. R.G. MacDonald:

Purification of peptides 13D and 22C.

Generation and characterization of anti-13D antisera.

Contributions of K.B. Clairmont:

Generation and characterization of anti-22C antisera.

Development and performance of solid-phase crosslinking assays.

Efforts in which both authors contributed significantly:

Purification of IGF-II/Man 6-P receptor for antibody generation and characterization.

Performance of immunoblots used in the manuscript.

Preparation of the manuscript

### Summary

The insulin-like growth factor (IGF)-II/mannose-6-phosphate (Man-6-P) receptor present in mammalian tissues as an apparent Mr = 250 K glycoprotein has recently been detected in fetal rat serum in a lower Mr form (240K). In the present studies the serum receptor was affinity labelled with <sup>125</sup>I-IGF-II after its adsorption onto pentamannosyl-6-phosphate-Sepharose, demonstrating that it can also bind both ligands simultaneously. The receptors in both serum and fresh plasma exhibited the lower Mr compared to tissue receptors, indicating this form circulates *in vivo*. In order to probe the structural basis of the serum receptor's lower Mr, we raised antipeptide antibodies against cytoplasmic and extracellular domains of the tissue form of the rat receptor deduced from complementary DNA clones (MacDonald, R.G., *et al* Science 239, 1134-1137, 1988). Peptide 22C, EEETDENETE WLMEEIQVPAPR, located in the cytoplasmic domain 32 residues C-terminal to the transmembrane region, and peptide 13D, YYLNVCRPLNPVPGCN, located 1476 residues N-terminal to the transmembrane domain were synthesized and used as immunogens in rabbits. IGF-II/Man-6-P receptors were first immunoprecipitated from either rat serum or a Triton X-100 extract of rat placental plasma membranes using a polyclonal antireceptor antibody. The immunoadsorbed receptors were then reduced, alkylated, electrophoresed on SDS-PAGE, blotted onto nitrocellulose, and probed with antipeptide antibodies. Anti-13D revealed the major receptor band in all the membrane and serum samples tested as well as several minor species of lower apparent Mr in serum. Fetal and neonatal rat sera contained 3-4 times as much of the receptor as adult serum. In contrast, anti-22C recognized the membrane IGF-II/Man-6-P receptor, but failed to recognize any of the serum receptor species. These results indicate that the serum IGF-II/Man-6-P receptor is truncated or altered in its cytoplasmic domain, consistent with the hypothesis that it is derived from cells by proteolytic cleavage.

The type II insulin-like growth factor (IGF)<sup>1</sup> receptor is one of a group of receptors that bind the insulin-like growth factors (IGFs) with high affinity (1-3). It consists of a single polypeptide chain of approximate Mr =250 K (4-8) which has no apparent tyrosine kinase activity *in vitro* (9). These properties contrast with the type I insulin and IGF-I receptors which share similar heterotetrameric structures (1-3, 10, 11) containing intrinsic tyrosine kinase activities apparently required to produce biological effects in response to ligand binding (12-15). Although several recent studies have reported biological effects potentially mediated through the IGF-II receptor (16-18), there is no definitive evidence that this receptor has a signalling function (19-22).

Recently, Morgan *et al.* (23) and we (24) have reported isolation of complementary DNA clones for the type II IGF receptor from the human and rat, respectively. The deduced amino acid sequences were found to be greater than 80 % identical to that of the bovine cation-independent mannose-6-phosphate (Man-6-P) receptor reported by Lobel *et al.* (25). Subsequent isolation of the full-length complementary DNA (cDNA) for the bovine receptor (26) and the recent independent cloning of the human cation-independent Man-6-P receptor cDNA (27) confirm this high sequence identity among these species. Direct biochemical and immunological experiments have demonstrated that a single receptor protein does indeed contain distinct binding sites for both IGF-II and Man-6-P-containing ligands (23, 24, 28-30). The IGF-II/Man-6-P receptor from all three species contains a single stretch of 22-23 hydrophobic amino acids comprising the transmembrane domain. This region is bounded by a short (163-167 residues) C-terminal cytoplasmic domain and a very large (>2000 residues) N-terminal extracellular domain characterized by fifteen repeat sequences of ~150 residues in length (23-27). Binding of IGF-II and Man-6-P to either the rat (24) or human (28), but not bovine (30), receptors exhibited positive cooperativity.

Kiess *et al.* (31) have recently reported the existence of immunoreactive IGF-II/Man-6-P receptor in rat serum. Affinity cross-linking studies using  $^{125}\text{I}$ -IGF-II revealed a developmental pattern of expression of the circulating form of the receptor, with high levels found during late gestation and early neonatal life (31). Moreover, the serum IGF-II/Man-6-P receptor species exhibits a slightly lower apparent  $M_r$  than the cellular receptor upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). In this study, we report structural analysis of the serum form of the rat IGF-II/Man-6-P receptor using antibodies directed against peptide sequences present in the cytoplasmic and extracellular domains of the receptor. The results demonstrate that the serum IGF-II/Man-6-P receptor binds both IGF-II and Man-6-P-containing ligands but is incapable of binding anti-cytoplasmic peptide antibodies. The data are consistent with the hypothesis that the serum IGF-II/Man-6-P receptor is truncated at its C-terminal domain and is devoid of its transmembrane anchor.



## Experimental Procedures

**Materials.** Rat IGF-II was purified from BRL-3A cell conditioned medium as described (32). Purified IGF-II was radioiodinated using Enzymobeads (BioRad) to a specific activity of 40-250 Ci/g. Pentamannosyl-6-phosphate (PMP), prepared from the native Y-2448 Q-phosphomannan from *H. holstii*, was a gift of Dr. M.E. Slodki, Midwest Area Northern Regional Research Center, Peoria, IL. PMP-Sepharose was prepared as described (33). Dithiothreitol (DTT) was 99% pure from Chemical Dynamics Corporation, and sodium iodoacetate (Fisher Biotech) was recrystallized twice from methanol. All other chemicals were reagent grade or better.

**Preparation of Serum and Plasma.** Male Sprague-Dawley rats obtained from Taconic Farms, Germantown, NY, were used at ages 14 d (~25 g), 35 d (~75 g), 70 d (~200 g), and 200 d (300-350 g). Blood was obtained by decapitation. Serum was prepared by clotting blood at 3°C for 20 h followed by centrifugation at 2000 x g for 10 min. Sera were then filtered through 0.45 µm sterile filters and stored at -20°C until use. For the preparation of plasma, blood was collected directly into 3 volumes of buffered saline containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 3 mM EDTA, and 80 U/ml heparin. Cells were removed from the dilute plasma by centrifugation.

**Preparation of Plasma Membranes from Rat Placenta.** Plasma membranes were isolated from rat placentas taken at day 19 of gestation as described previously (4). Membrane extracts (5 mg protein/ml) were prepared by incubation with 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), 1% Triton X-100, pH 7.4, plus antiproteases by gentle mixing for 1 h at 3°C. The antiproteases were leupeptin, antipain and benzamidine at concentrations of 10 µg/ml each, 20 µg/ml aprotinin, 12.5 µg/ml chymostatin, and 1 mM PMSF. The mixture was centrifuged for 10 min at 15000 x

g in a microcentrifuge and the supernatant fraction was used as the extract. Protein was assayed by the Bradford method (34).

**Antisera Against the IGF-II/Man-6-P Receptor.** IGF-II/Man-6-P receptors were purified from rat placental plasma membranes by IGF-II-Sepharose chromatography as previously described (4). The primary structure of the rat IGF-II/Man-6-P receptor deduced from cDNA clones (24) was the basis for design of synthetic peptides. Peptides were synthesized on an Applied Biosystems Model 430A solid phase peptide synthesizer (University of Massachusetts Medical Center Peptide Synthesis Facility). Each of the peptides was then purified by reverse-phase high pressure liquid chromatography and analyzed for amino acid composition by the PICO-TAG method (University of Massachusetts Medical Center Protein Chemistry Facility, supported by the Diabetes and Endocrinology Research Center). Peptides were coupled to keyhole limpet hemocyanin (KLH) as described (35). Rabbits were immunized with 1 mg of peptide-KLH emulsified in Freund's complete adjuvant, boosted 4 weeks later with 1 mg peptide-KLH in Freund's incomplete adjuvant (East Acres Biologicals, Southbridge, MA). Sera were assayed by enzyme-linked immunosorbent assay and dot immunoblot against either peptide 13D or 1 mg: 5 mg peptide 22C-bovine serum albumin (BSA) conjugate (Figure 17). The polyclonal antibody raised against the native purified receptor has been described previously (19,36). Crude immunoglobulin fractions were obtained by ammonium sulfate precipitation (36), and were further purified by chromatography on diethylaminoethyl-cellulose in 5 mM phosphate buffer, pH 6.5.

**Immunoabsorption of the IGF-II/Man-6-P Receptor and Adsorption with Pentamannosyl-6-Phosphate-Sepharose.** IGF-II/Man-6-P receptors were immunoabsorbed from serum, plasma, or Triton X-100 extracts of rat placental plasma membranes as described before (6,37). For adsorption with PMP-Sepharose, serum (50

$\mu\text{l}$ ) or membrane extracts (0.1 mg protein in 50  $\mu\text{l}$ ) were added to 25  $\mu\text{l}$  of the resin in 0.5 ml total volume of 50 mM Hepes, 0.15 M NaCl, 0.05% Triton X-100, 5 mM  $\beta$ -glycerophosphate, pH 7.4, with antiproteases. After incubation for 2 h at 4°C, the resin was washed twice with the same buffer.

#### **Affinity Crosslinking of $^{125}\text{I}$ -IGF-II to Immobilized Receptors.**

Following immunoadsorption or adsorption with PMP-Sepharose, the washed resin pellets were resuspended to a volume of 0.2 ml with Krebs-Ringer phosphate buffer containing 1% BSA plus 4 nM  $^{125}\text{I}$ -IGF-II with or without 400 nM unlabelled IGF-II. These mixtures were then incubated for 4 h at 23°C. The samples were subjected to affinity cross-linking with disuccinimidyl suberate as described before (1). Briefly, 50  $\mu\text{l}$  of resin (antibody Affigel or PMP-Sepharose) with material bound was incubated in KRP + 1% BSA for 2-4 h at 23°C. To this was added 25 nanomoles of disuccinimidyl suberate, then the sample was placed on ice for 1 h. The sample was then washed in 1 ml of 100 mM Tris-HCl, pH 7.4 before electrophoresis.

**Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis.** For the antipeptide antibodies to recognize the IGF-II/Man-6-P receptor, the receptor had to be reduced and alkylated under denaturing conditions (Figure 17). For reductive alkylation, washed resin or membrane pellets were heated for 15 min at 100°C in 0.1 ml of 10% SDS, 0.25 M Tris, 10 mM DTT, pH 8.3. The samples were cooled, 5  $\mu\text{l}$  of 0.5 M sodium iodoacetate was added, followed by incubation in the dark at 23°C for 30 min. For electrophoresis under non-reducing conditions, samples were heated to 100°C for 10 min in SDS sample buffer without DTT. Electrophoresis was performed as described (38) on 6% polyacrylamide gels.

**Western Immunoblotting.** IGF-II/Man-6-P receptors were transferred from polyacrylamide gels to nitrocellulose as described (37). The immunoblots were blocked for 30-60 min at 23°C in 0.22  $\mu\text{m}$ -filtered 0.5% nonfat dry milk dissolved in 15 mM Tris, 0.15 M NaCl, pH 7.4. Incubations with the anti-receptor, anti-13D, or anti-22C antibodies

were done at 30°C for 16-20 h using 1:800, 1:500, or 1:200 dilutions, respectively, of purified immunoglobulin fractions in blocking buffer. The blots were then washed, incubated with  $^{125}\text{I}$ -protein A, and washed again as described (37).

## Results

**Characterization of antireceptor and antipeptide antisera.** Peptides 13D and 22C were synthesized on the basis of sequences in the extracellular and cytoplasmic domains, respectively, of the deduced amino acid sequence of the rat IGF-II/Man-6-P receptor (24, Fig. 17A). Antisera raised in rabbits against each of these peptides exhibited immunoreactivity against the corresponding peptide in dot blots and enzyme-linked immunoassays (data not shown). In order to test the structural requirements for immunoreactivity of each antiserum against the IGF-II/Man-6-P receptor, rat placental plasma membrane proteins were electrophoresed under either non-reducing conditions, after reduction or reductive alkylation. Western immunoblots of these gels are shown in Fig. 17B. The polyclonal antireceptor antiserum recognized the Mr ~230 K receptor species under non-reducing conditions, but did not react with either the reduced or reductively alkylated receptor (Fig. 17B). In contrast, anti-13D immunoglobulin failed to recognize the non-reduced receptor, but reacted strongly with the receptor after reductive alkylation under denaturing conditions. The receptor migrates at a higher apparent Mr (~280 K) under these conditions. Anti-22C immunoglobulin specifically recognized the high Mr IGF-II/Man-6-P receptor under all three conditions, but the degree of reactivity improved progressively as a function of increasing disruption of disulfide bonds in the receptor (Fig. 17B). In addition, anti-22C cross reacted with an unknown low Mr species derived from placental membranes which migrated just above the dye front in SDS-PAGE on 6% gels (Fig. 17B, lanes 7-9). Unlike the receptor, optimal recognition of this low Mr protein by anti-22C apparently does not depend on reductive alkylation. Based on these results the antiserum against the native receptor was subsequently used to immunoadsorb IGF-II/Man-6-P receptors from sera or Triton X-100 extracts of placental plasma

membranes, whereas the antipeptide antibodies have been employed as Western blot probes following SDS-PAGE of the immunoadsorbed receptors.

**Structural analysis of the cellular and serum forms of the receptor using domain-specific antisera.** Fig. 18A shows a Western immunoblot of IGF-II/Man-6-P receptors immunoadsorbed from a Triton X-100 extract of rat placental plasma membranes and rat sera and probed with the polyclonal antireceptor antiserum. A single high molecular weight receptor species of Mr ~230 K is specifically immuno-adsorbed from the plasma membrane extract, whereas serum samples from rats aged 14 d through 200 d contained a single immunoreactive species of slightly lower apparent Mr than the cellular receptor. These results obtained by direct immunoadsorption of the IGF-II/Man-6-P receptor from serum samples agree with the findings of Kiess *et al.* (31), who partially purified the serum receptor using gel filtration chromatography. In addition, the concentration of receptors in rat serum is developmentally regulated, with maximal levels found in early neonatal life (31, Fig. 18A). Quantitative analysis of the data in Fig. 18A demonstrated that neonatal rat serum contained approximately 3-4 times as much receptor as found in serum from adult (200 d) rats. We observed that the concentration of receptors in fetal rat serum was approximately the same as that found in serum from day 14 neonatal rats (data not illustrated).

Analysis of immunoblots containing reduced and alkylated IGF-II/Man-6-P receptors using the anti-peptide antibodies revealed striking differences between the structures of the cellular and serum receptors (Fig. 18B and 18C). In addition to recognizing a major 260 K species, the antiserum raised against the extracellular domain peptide 13D reacted with at least three discrete species of lower apparent Mr, the smallest of which had an Mr of ~160 K (Fig. 18B). All forms of the serum IGF-II/Man-6-P receptor had reduced apparent Mr relative to the 280 K cellular receptor under these conditions. We

conclude from these data that, despite the apparent heterogeneity of the IGF-II/Man-6-P receptors in rat serum, the region in the extracellular domain of the receptor that is recognized by the anti-13D antiserum is essentially intact.

Immunoblots of IGF-II/Man-6-P receptors probed with the cytoplasmic domain-specific anti-22C antiserum revealed another important structural difference between the serum and cellular receptors (Fig. 18C). Whereas the cellular receptor (Fig 18C, *lane 1*) was recognized by this antiserum under identical conditions to those used in Fig 18B, none of the serum receptor species reacted significantly with anti-22C (Fig 18C, *lanes 3-10*). The autoradiogram in Fig 18C (*lanes 5-10*) also reveals reaction of anti-22C with several bands of Mr~130-140K, the serum concentration of which appears to increase with the age of the rats. However, these species are not derived from the IGF-II/Man-6-P receptor because they react with both anti-receptor and control non-immune immunoglobulin affinity resins. These data demonstrate that the cytoplasmic domain is either absent or altered in the serum IGF-II/Man-6-P receptor.

**Comparison of IGF-II/Man-6-P receptors present in rat serum and plasma.** One possible explanation for the observed heterogeneity of the serum IGF-II/Man-6-P receptor as depicted in Figure 18 is proteolytic cleavage during the process of clotting blood to obtain serum. In order to address this hypothesis, we prepared plasma from day 14 neonatal rats and directly compared the structures of IGF-II/Man-6-P receptors in plasma and serum using the antipeptide antibodies described above (Figure 19). Neither the receptors isolated from serum nor dilute plasma reacted with anti-22C, but both exhibited an identical set of polypeptide bands which reacted with anti-13D. These results demonstrate that the structural properties of the serum receptor are representative of the IGF-II/Man-6-P receptor that circulates in the animal.

### Ligand binding characteristics of the serum IGF-II/Man-6-P receptor.

The data in Fig. 20A demonstrate that  $^{125}\text{I}$ -IGF-II may be specifically cross-linked to the serum receptor immobilized on antibody-Affigel. This experiment shows that a single ~230 K receptor band is observed under non-reducing conditions (Fig. 20A, *lane 7*). Reductive alkylation of the cross-linked serum receptor results in an increase in the apparent Mr of the major affinity-labeled species to ~260 K, plus the appearance of several species of lower apparent Mr (Fig. 20A, *lane 9*). In contrast, cross-linking of  $^{125}\text{I}$ -IGF-II to the plasma membrane receptor revealed only a single labeled species regardless of treatment (Fig. 20A, *lanes 1-5*). These findings indicate that the serum form of the IGF-II/Man-6-P receptor specifically binds  $^{125}\text{I}$ -IGF-II. Furthermore, in conjunction with the data illustrated in Figure 18, these observations suggest that the receptor circulates in blood as a mixture of species having identical apparent Mr, some of which exist as proteolytically cleaved complexes held together by intramolecular disulfide bonds.

It was not previously known whether the serum form of the IGF-II/Man-6-P receptor retains the capacity to bind Man-6-P-containing ligands. In order to address this question, we developed a method for  $^{125}\text{I}$ -IGF-II affinity labeling of receptors adsorbed to pentamannosyl-6-phosphate (PMP)-Sepharose (Fig. 20B). Both the plasma membrane receptor (Fig. 20B, *lane 1*) and the receptor from day 14 neonatal rat serum (Fig. 20B, *lane 5*) were adsorbed by PMP-Sepharose. Washing the affinity resin with 5 mM Man-6-P readily eluted both receptor species (Fig. 20B, *lanes 3 and 7*). These data demonstrate that despite the apparent structural differences between the cellular and serum forms of the IGF-II/Man-6-P receptor, each is capable of binding both IGF-II and Man-6-P.



## Discussion

The objective of the present studies was to begin a detailed structural characterization of the serum form of the rat IGF-II/Man-6-P receptor. For this purpose, we have acquired and characterized three different antisera specific for the rat IGF-II/Man-6-P receptor. A polyclonal antiserum raised against the native receptor was used to immunoadsorb receptors from serum under mild and relatively rapid conditions. The immunoadsorbed receptors were subsequently immunoblotted with two antisera raised against peptides derived from deduced amino acid sequence within the extracellular versus cytoplasmic domains of the rat IGF-II/Man-6-P receptor. These studies demonstrate that the serum receptor fails to react with an antiserum specific for a peptide sequence located in the cytoplasmic domain of the receptor, 32 residues C-terminal to the transmembrane domain (Figs. 17 and 18). These data strongly suggest that the cytoplasmic domain is either missing or substantially altered in the serum receptor.

Taken together, the present results and previous observations are consistent with the hypothesis that the serum form of the IGF-II/Man-6-P receptor lacks both the C-terminal intracellular and transmembrane domains: 1) The rat IGF-II/Man-6-P receptor is an intrinsic membrane glycoprotein which is normally anchored in the membrane by a single 22-residue hydrophobic sequence (24,31). Thus, the presence of this receptor in serum implies changes in the membrane-spanning region of the protein. 2) The 10-15 K reduction in apparent  $M_r$  of the serum receptor relative to the cellular receptor suggests the removal of a small portion of the protein upon formation of the serum receptor (31, this study). Examination of the amino acid sequence of the rat IGF-II/Man-6-P receptor predicted from cDNA clones (24) indicates that truncation of the receptor by deletion of both the transmembrane and cytoplasmic domains would involve removal of 189 amino

acids producing a reduction in  $M_r$  of 21,134. This value is reasonably consistent with the observed differences in electrophoretic migration of tissue versus serum forms of the receptor upon SDS-PAGE analysis. 3) Failure of the serum receptor to react with the anti-22C antiserum demonstrates that the peptide sequence constituting the epitope(s) for this immunologic probe are significantly different or missing from the serum receptor. The possibility still remains that the peptide sequence is merely altered rather than deleted from the serum receptor. Further studies will be required to determine the actual C-terminal sequence of the serum receptor.

It appears that a minor fraction of circulating IGF-II/Man-6-P receptor is proteolytically nicked. The antiserum specific for peptide 13D located in the extracellular domain of the receptor recognized at least three distinct immunoreactive species lower than  $M_r = 260$  K in serum isolated from rats aged 14 d through 200 d (Figs. 18 and 19). This discovery was an unexpected consequence of reductive alkylation of the receptor under denaturing conditions in order to permit reaction with the antiserum. The multiple receptor species observed ranged in apparent  $M_r$  between 160 K and 260 K, indicating that although all circulating forms of the receptor had an altered cytoplasmic domain, several species were severely truncated. Does the serum IGF-II/Man-6-P receptor circulate as multiple, independent species of different apparent  $M_r$  or as a mixture of proteolytically nicked forms of intact  $M_r \approx 220$  K receptor? Our experiments favor the latter conclusion. Western blots of non-reduced receptors using polyclonal anti-receptor antisera revealed only a single 220 K serum receptor (Fig. 18A). Results of affinity cross-linking experiments using  $^{125}\text{I}$ -IGF-II also support this concept. In agreement with the data of Kiess et al. (31), we found only a single IGF-II binding species in rat sera, which exhibited an apparent  $M_r$  of  $\sim 230$  K under non-reducing conditions (Figure 20). However, when the affinity cross-linked

receptors were subjected to reductive alkylation, the receptor migrated as a family of bands dominated by a 260 K major species (Figure 20).

The structural features of the serum receptor revealed in our studies suggest a proteolytic process by which cells release IGF-II/Man-6-P receptors into the circulation. The pathways by which the IGF-II/Man-6-P receptor recycles between intracellular and cell-surface membrane compartments in order to transport lysosomal enzymes and IGF-II to the lysosomes are well known (39,40). An important stage in each of these processes is the release of receptor-bound IGF-II or lysosomal enzymes upon acidification of endosomes. Previous studies have demonstrated that, after releasing these ligands, the IGF-II/Man-6-P receptors return to the Golgi apparatus or the cell surface, thus escaping degradation in lysosomes (39-42). However, a significant percentage of the receptors within the endosomal lumen may undergo partial proteolysis at discrete, susceptible sites near the transmembrane domain. Liberated from its anchorage, the receptor extracellular domain could subsequently be released. Alternatively, the receptor may be proteolytically cleaved at the cell surface by unknown proteases. It is possible that the serum IGF-II/Man-6-P receptor is synthesized from an alternatively spliced mRNA to produce a truncated protein which is subsequently secreted. However, this explanation is inconsistent with the observation of a single ~9 kb IGF-II/Man-6-P receptor transcript in RNA isolated from several species (23). The serum receptor is clearly not derived from cellular receptors by proteolysis during the process of collecting or clotting the blood. Thus, the data in Figure 19 demonstrate that the receptor is present in plasma at levels comparable to those in serum, and that the receptors in serum and plasma have similar structures. These data suggest that the receptor species we have studied is the normal circulating form of the receptor. In addition, we have found that amniotic fluid taken at days 19-20 of gestation also contains

high levels of IGF-II/Man-6-P receptors which are structurally similar to those in serum (data not illustrated).

The existence of several other soluble receptors has also been documented. Among these are the transferrin receptor (43), the EGF receptor (44), the growth hormone receptor (45-47) and the insulin receptor (48). Of these, the soluble insulin and EGF receptors have not been demonstrated in circulation (44, 48), and the precise structure and physiologic roles, if any, of the transferrin and growth hormone receptors in serum have not been shown (43, 45-47). The source and function of IGF-II/Man-6-P receptors in blood is also unknown. Kiess et al. (31) detected receptors in the blood of rats up to one year old. Indeed our studies indicate that the concentration of receptors in the circulation declines by only a factor of 3- to 4-fold from day 19 of gestation to day 200 postpartum (Figure 18). During this period, levels of the receptor in many rat tissues, including brain, lung, liver and intestine, decline dramatically near birth by factors of 5 to 10 (49).<sup>2</sup> The relatively high level of receptors in blood of adult rats suggests that receptors may be released into the circulation as a normal consequence of receptor function rather than as a means of shedding unneeded receptors from the tissues following completion of fetal development. Acting as a carrier of circulating IGF-II appears to be ruled out in view of the much higher levels of carrier proteins (31), but the receptor may serve to scavenge lysosomal enzymes which have been released from cells. Our data (Fig. 20B) demonstrate that the serum receptor retains the ability to bind Man-6-P containing ligands. In this way, the receptor may inactivate the enzymes or transport them to an unknown destination for internalization or degradation, in a manner similar to the action of  $\alpha_2$ -macroglobulin (50,51). This hypothesis predicts the existence of cell-surface receptors which bind the IGF-II/Man-6-P receptor and internalize the ligand receptor complex, a possibility that could be tested directly.

### Footnotes

<sup>1</sup> The abbreviations used are: IGF-I and -II, insulin-like growth factor-I and II; Man-6-P, mannose-6-phosphate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PMP, pentamannosyl-6-phosphate; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; KLH, keyhole limpet hemocyanin.; BSA: bovine serum albumin.

<sup>2</sup>Tepper, M.A., MacDonald, R.G., Brocklebank, C.M., Ullrich, A., and Czech, M.P., manuscript in preparation.

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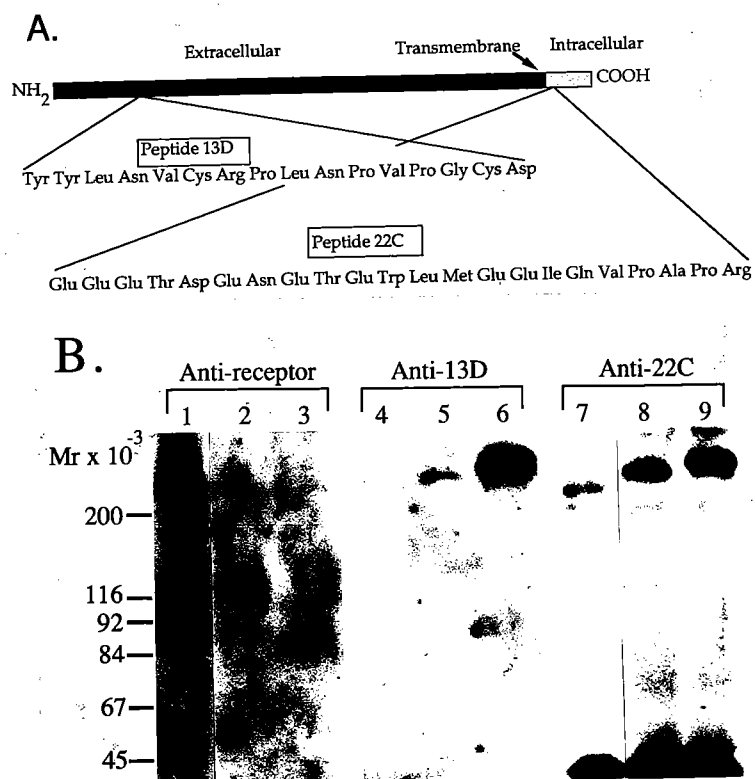
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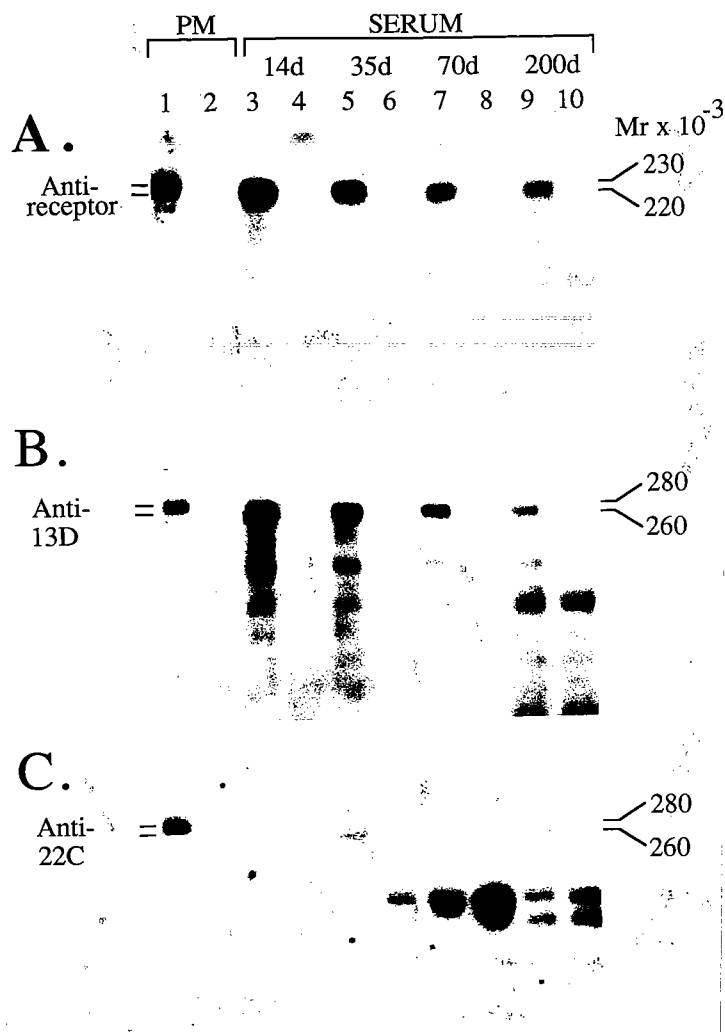


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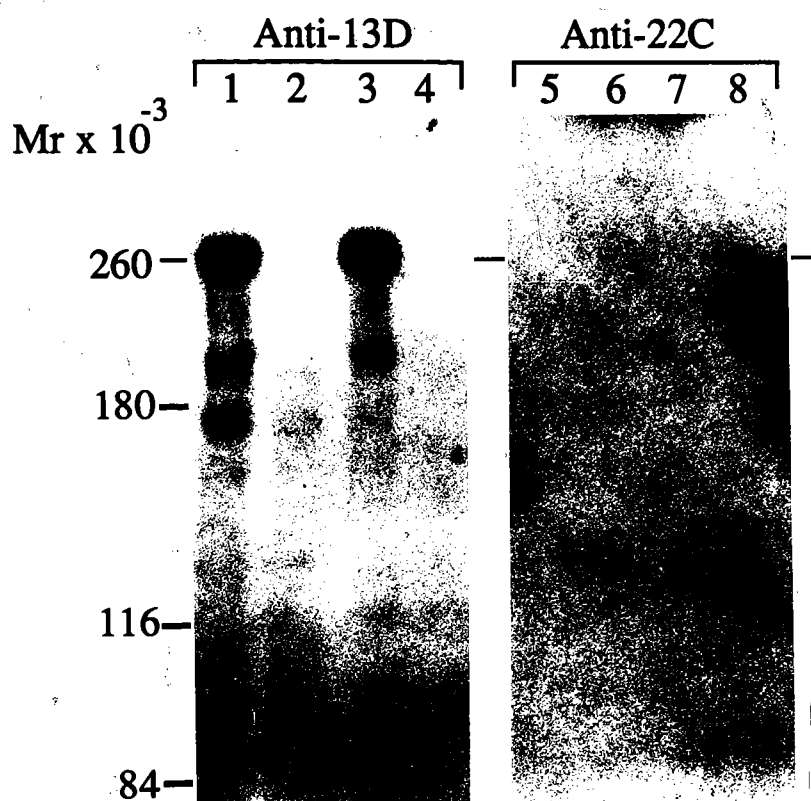
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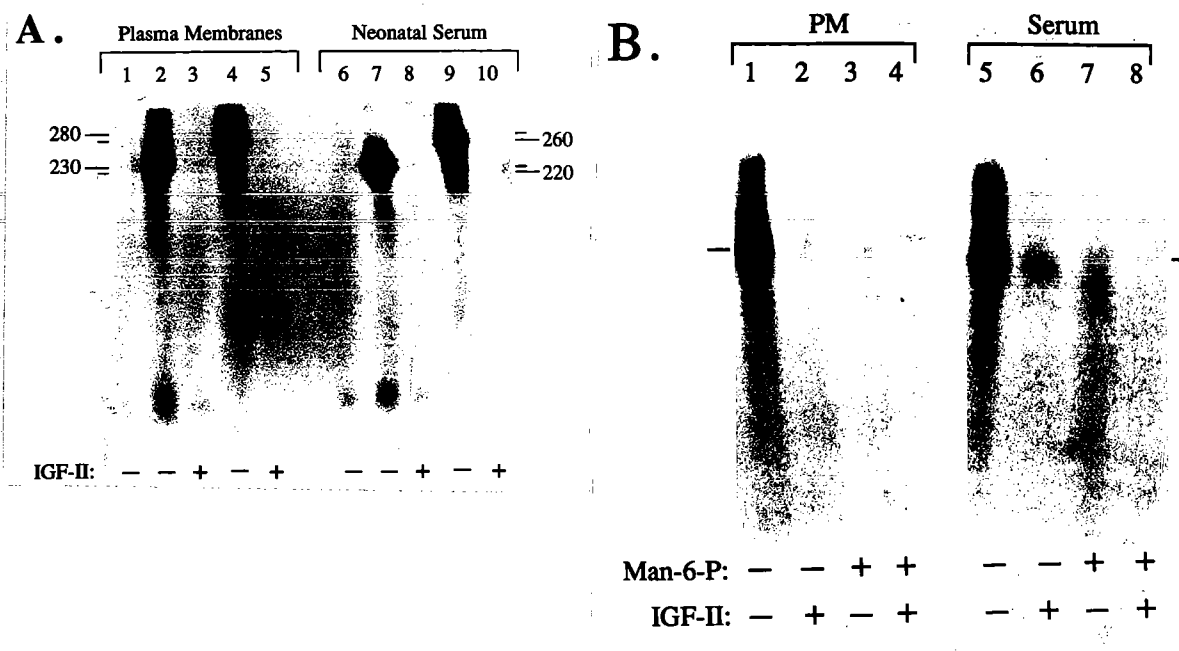
**Fig 17. Antipeptide antibodies specific for the cytoplasmic and extracellular domains of the IGF-II/Man-6-P receptor.** A. Schematic diagram representing the coding region of the rat IGF-II/Man-6-P receptor cDNA (24). The transmembrane domain is indicated by the *solid bar*. Peptide 13D is a 16-amino acid sequence located 1476 residues N-terminal to the transmembrane domain. Peptide 22C is a 22-amino acid peptide located 32 residues C-terminal to the transmembrane domain. B. Structural requirements for recognition of the IGF-II/Man-6-P receptor by antireceptor and antipeptide antibodies. Aliquots (0.4 mg protein) of rat placental plasma membranes were electrophoresed under non-reducing conditions (*Lanes 1,4,7*), after reduction with 10 mM DTT (*2,5,8*), or following reductive alkylation (*3,6,9*). Electrophoresed proteins were transferred to nitrocellulose, and probed with antireceptor (*1-3*), anti-13D (*4-6*), or anti-22C (*7-9*) immunoglobulins as indicated. Values for molecular weights of pre-labelled standard proteins are indicated.



**Fig. 18. Characterization of cellular and serum forms of the IGF-II/Man-6-P receptor using antipeptide antibodies.** Triton X-100 extract of rat placental plasma membranes (0.4 mg protein, *Lanes 1,2*) or serum (1.0 ml) isolated from rats aged 14 d (*3,4*), 35 d (*5,6*), 70 d (*7,8*), or 200 d (*9,10*) were immunoadsorbed with antireceptor immunoglobulin-Affigel (*odd numbered lanes*) or nonimmune immunoglobulin-Affigel (*even numbered lanes*). Prior to electrophoresis, washed resin pellets were either heated for 15 min at 100°C in SDS sample buffer without DTT (*Panel A*) or reduced and alkylated as described (*B and C*). The parallel immunoblots were probed with antireceptor (*A*), anti-13D (*B*), or anti-22C (*C*) antibodies as indicated. Autoradiograms of the immunoblots are shown.



**Fig. 19.** The plasma IGF-II/Man-6-P receptor has the same structure as the serum receptor. Neonatal rat serum (0.5 ml, *Lanes 1,2,5,6*) and dilute neonatal plasma (2 ml, *3,4,7,8*) were immunoadsorbed with anti-receptor (*odd*) or nonimmune (*even*) immunoglobulin-Affigel resins. The washed resins were reduced and alkylated, electrophoresed on SDS-PAGE, and then the gel was immunoblotted with anti-13D (*1-4*) or anti-22C (*5-8*) antibodies as noted.



**Fig 20.** Affinity crosslinking of  $^{125}\text{I}$ -IGF-II to serum and cellular IGF-II/Man-6-P receptors. **A.** Triton X-100 extracts of plasma membranes (0.1 mg protein, *Lanes 1-5*) or neonatal serum (0.1 ml, *6-10*) were immunoadsorbed with anti-receptor immunoglobulin-Affigel (*2-5, 7-10*) or nonimmune immunoglobulin-Affigel (*1, 6*). The washed resin pellets were then incubated with 4 nM  $^{125}\text{I}$ -IGF-II in the absence (*1, 2, 4, 6, 7, 9*) or presence (*3, 5, 8, 10*) of 400 nM unlabelled IGF-II. After incubation for 4 h at 23°C, the samples were further incubated for 1 h at 3°C with 0.125 mM disuccinimidyl suberate, and electrophoresed on SDS-PAGE. **B.** Triton X-100 extracts of plasma membranes (0.1 mg protein, *Lanes 1-4*) or neonatal serum (0.1 ml, *5-8*) were adsorbed with 25  $\mu\text{l}$  of PMP-Sepharose. The resin pellets were washed in the absence (*1,2,5,6*) or presence (*3,4,7,8*) of 5 mM Man-6-P as indicated. The samples were then incubated with 4 nM  $^{125}\text{I}$ -IGF-II with (*even*) or without (*odd*) 400 nM unlabelled IGF-II followed by cross-linking with disuccinimidyl suberate and electrophoresis on SDS-PAGE. Autoradiograms of the dried gels are shown.

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STRUCTURE AND REGULATION OF THE SERUM INSULIN-LIKE GROWTH  
FACTOR II/MANNOSE-6-PHOSPHATE RECEPTOR

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INTRODUCTION

A significant and rapid effect of insulin administration is the translocation of a number of proteins from an intracellular compartment to the cell surface. Among these proteins are glucose transporters (1-4) and receptors for IGF-II/Man-6-P (5, 6), transferrin (7, 8), and  $\alpha_2$  macroglobulin (9). Translocation of the glucose transporter appears to play a role in increasing cellular uptake of glucose, while the receptors may increase the uptake of nutrients and other factors required for cell growth. The increase in the number of cell surface receptors correlates with an increased binding of ligand with concomitant increase in uptake of ligand, and a decrease in the number of intracellular receptors which fractionate in low density microsomes (5-9). However, while the number of cell surface glucose transporters is only increased approximately two- to five-fold, the amount of glucose transport may be increased by as much as 20-fold (1-4), indicating that intrinsic activity of transport is also increased by insulin.

Of the proteins translocated to the cell surface in response to insulin, the IGF-II/Man-6-P receptor (10-12) and the transferrin receptor (13) have been detected as having serum forms. We have previously shown that the serum IGF-II/Man-6-P receptor is C-

terminally truncated, and that its levels decrease over time beyond 14 days after birth (12 and Figure 22). We hypothesized that the serum receptor was most likely a proteolytic product of the cellular receptor. The serum receptor could be a product of the action of proteases on the cell surface. In this case, it would be expected that insulin could induce an increase in the release of IGF-II/Man 6-P receptor into serum. In this paper we investigate the acute regulation of serum receptor levels by insulin.



## MATERIALS AND METHODS

Materials. IGF-II/Man-6-P receptors were purified from rat placental plasma membranes by IGF-II-Sepharose chromatography as previously described (13). The primary structure of the rat IGF-II/Man-6-P receptor deduced from cDNA clones (14) was the basis for design of synthetic peptides. Dithiothreitol (DTT) was 99% pure from Chemical Dynamics Corporation, and sodium iodoacetate (Fisher Biotech) was recrystallized twice from methanol. All other chemicals were at least reagent grade.

Preparation of Serum. Male Sprague-Dawley rats at 150 g, obtained from Taconic Farms, Germantown, NY, were injected subcutaneously with 0.2ml PBS alone or with 2U insulin in 0.2 ml PBS. Thirty minutes following injection, blood was obtained by decapitation. Serum was prepared by clotting blood at 3°C for 6 h followed by centrifugation at 2000 x g for 10 min. Sera were then filtered through 0.45 mm sterile filters and used immediately. Insulin treatment resulted in a decrease of serum glucose levels from 110 to 30. Enzyme activities (creatine kinase and lactate dehydrogenase) measured in serum were not significantly altered by this treatment.

Preparation of Plasma Membranes from Rat Placenta. A plasma membrane fraction was isolated from rat placentas taken at day 19 of gestation as described previously (13). Membrane extracts (5 mg protein/ml) were prepared by incubation with 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), 1% Triton X-100, pH 7.4, plus antiproteases by gentle mixing for 1 h at 3°C. The antiproteases were leupeptin, antipain and benzamidine at concentrations of 10 mg/ml each, 20 mg/ml aprotinin, 12.5 mg/ml chymostatin, and 1 mM PMSF. The mixture was centrifuged for 10 min at 15000 x g in a microcentrifuge and the supernatant fraction was used as the extract. Protein was assayed by the Bradford method (15).

Antisera and Antibodies. The anti-IGF-II/Man-6-P receptor antisera used are as described by MacDonald et al (12). Briefly, the antireceptor antiserum was raised in rabbits against intact purified rat cellular receptor. Anti-22C was raised against the peptide EEETDENETEWLMEEIQVPAPR which is located 32 residues C terminal to the transmembrane domain, and anti-13D was raised against the peptide YYLNVCRPLNPVPGCD located 1476 residues N-terminal to the cytoplasmic domain (Figure 21A). In the experiments described, the antireceptor antiserum was immobilized on affigel as described (12) to immunoadsorb the receptors from serum or membrane extracts. The antipeptide antisera were used to immunoblot reduced and alkylated receptors. For the transferrin receptor experiments, a single monoclonal antibody obtained from Dr. Roger Davis was used for the immunoadsorption and the immunoblotting (16).

Immunoadsorption of the IGF-II/Man-6-P Receptor and the Transferrin Receptor. IGF-II/Man-6-P receptors were immunoadsorbed from Triton X-100 extracts of rat placental plasma membranes as described before (17, 18). For immunoadsorption from serum, serum was diluted 1:1 with HBS containing protease inhibitors, then immunoadsorbed in the same manner as a membrane extract. The same procedure was used for immunoadsorption of the transferrin receptor.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis. For the antipeptide antibodies to recognize the IGF-II/Man-6-P receptor, the receptor had to be reduced and alkylated under denaturing conditions, as shown (12 and Figure 2 1B). For reductive alkylation, washed resin or membrane pellets were heated for 15 min at 100°C in 0.1 ml of 10% SDS, 0.25 M Tris, 10 mM DTT, pH 8.3. The samples were cooled, 5 ml of 0.5 M sodium iodoacetate was added, followed by incubation in the dark at 23°C for 30 min or more. Reduction alone was adequate to immunoblot the transferrin receptor, so it was

simply heated to 100°C for 5 min in sample buffer containing 10 mM DTT. Electrophoresis was performed as described (19) on 6% polyacrylamide gels.

Western Immunoblotting. IGF-II/Man-6-P receptors were transferred from polyacrylamide gels to nitrocellulose as described (18). The immunoblots were blocked for 30 min at 23°C in 0.22 mm-filtered 0.5% nonfat dry milk dissolved in 15 mM Tris, 0.15 M NaCl, pH 7.4. Incubations with the anti-13D, anti-22C, or anti-transferrin antibodies were done at 3°C for 16-20 h using 1:500, 1:200, or 1:1000 dilutions, respectively, of purified immunoglobulin fractions or ascites fluid in blocking buffer. The blots were then washed, incubated with <sup>125</sup>I-protein A (anti-13D and anti 22C) or <sup>125</sup>I-goat anti-mouse IgG (anti-transferrin receptor), and washed again as described (18). The autoradiograms are shown. Quantitation was performed by scanning the appropriate lanes on the autoradiograph on a Beckman DU-8 Spectrophotometer, cutting out the appropriate peaks, and weighing them. Thus, areas are measured in mg corresponding to the weight of the paper containing the peak.

## RESULTS AND DISCUSSION

While the presence of receptors in serum has been known for many years, little is known about how the receptors are released into the serum or what regulates this process. In a previous paper (12), we addressed the structure and developmental regulation of the serum IGF-II/Man 6-P receptor. The three anti-IGF-II/Man 6-P receptor antibodies used differed greatly in their ability to recognize receptor in immunoblots. The anti-receptor antibody recognized receptor only under nonreduced conditions, anti-13D only following reductive alkylation, while anti-22C was able to recognize receptor under all conditions tested although it, too, preferred reductive alkylation (Figure 21B). Using these antibodies we showed that the serum receptor lacked a cytoplasmic domain (Figure 22). This experiment further demonstrates that the levels of IGF-II/Man-6-P receptor in serum is developmentally regulated (Figure 22). This regulation is similar to the developmental regulation of the cellular receptor which peaks near the time of birth, then falls in a number of tissues (19).

To further investigate the relationship of the cellular and serum receptors, we have looked at a more acute regulator of cell surface levels of IGF-II/Man-6-P receptor. Insulin addition to insulin sensitive cells causes a rapid redistribution of IGF-II/Man-6-P receptors from an intracellular compartment to the cell surface (6). If serum receptor is generated from receptors at the cell surface by a proteolytic mechanism, this process might lead to an increase in the levels of receptor in serum. To measure the levels of receptor in serum, IGF-II/Man 6-P receptor was immunoabsorbed to antireceptor antibody affigel, washed thoroughly as described in the methods, then immunoblotted with anti-22C, anti-13D, or silver stained. As can be seen in the silver stained gel (Figure 23) a single band, corresponding to the IGF-II/Man-6-P receptor, is seen to be increased in the immunoprecipitates. That this effect is not due to differences in the amount of antibody

used in the immunoadsorption or to gross differences in blood proteins is demonstrated by the amount of protein in the antibody and contaminating bands being very similar in both the control and insulin treated animals. While, as previously demonstrated (Figure 22), anti-22C recognizes only cellular receptor (Figure 24), the anti-13D antibody recognizes a protein in cell extracts as well as a protein of slightly lower molecular weight in serum (Figure 24). The serum receptor from insulin treated animals is present at approximately 2.5 fold the level seen in control animals.

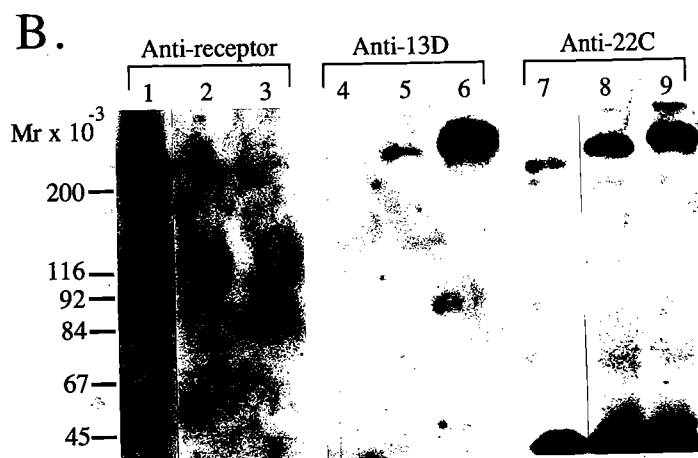
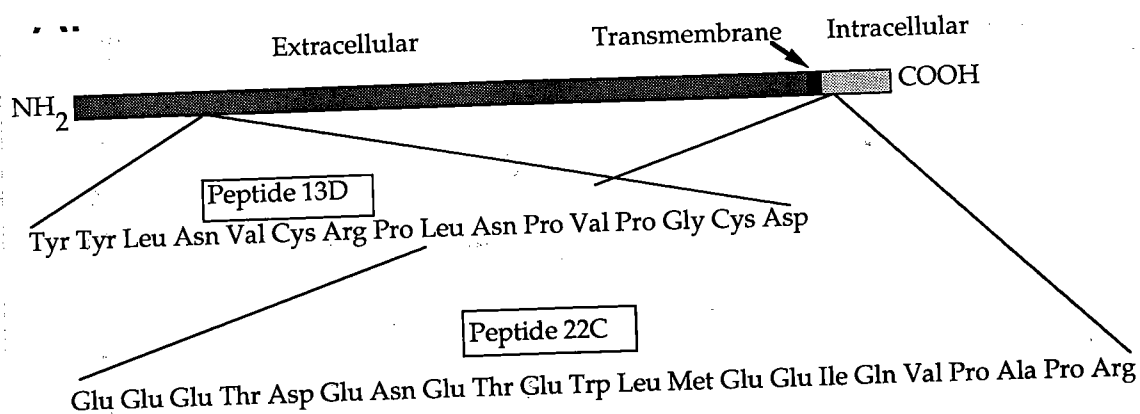
To determine if this acute regulation of serum receptor levels was specific for the IGF-II/Man-6-P receptor, we used the same procedure to measure levels of transferrin receptor in serum from the same animals. Transferrin receptor levels in serum are regulated similarly to the IGF-II/Man-6-P receptor, being increased twofold following insulin treatment (data not shown).

The acute regulation of serum receptor levels has several interesting implications. First, such acute regulation argues against the possibility of serum receptor arising from a message distinct from the cellular receptor: the time necessary to synthesize the IGF-II/Man-6-P receptor (17) is very much greater than the period of insulin treatment in these animals. Second, the same process that generates the serum IGF-II/Man-6-P receptor may generate the serum transferrin receptor. It has recently been shown that these two receptors coexist in intracellular vesicles which also contain the HepG2 type glucose transporter, and that these vesicles move to the surface in response to insulin (20). A likely explanation for the results presented is that proteolysed receptors exist in these vesicles and that insulin stimulation causes these vesicles to migrate to the cell surface, releasing the serum receptor fragments. Another possibility is that receptors are cleaved from the cell surface directly.

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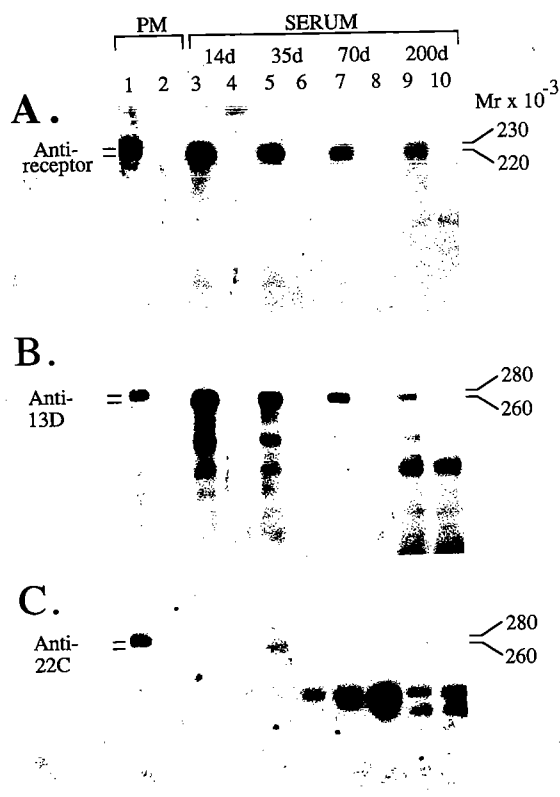
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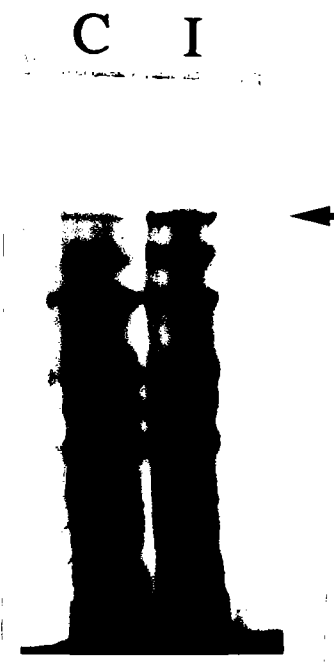


**Figure 21. Antibodies which recognize the IGF-II/Man 6-P receptor. A.** A schematic of the IGF-II/Man 6-P receptor indicating the locations of the peptides against which antibodies were raised. **B.** The structural requirements for each antibody to recognize receptor in an immunoblot differ. Aliquots (0.4 mg of protein) were electrophoresed under nonreducing conditions (lanes 1, 4, and 7), following reduction with 10 mM DTT (lanes 2, 5, and 8), or after reductive alkylation with 10 mM DTT followed by 2.5  $\mu$ M IAA (lanes 3, 6, and 9). Gels were then transferred to nitrocellulose and immunoblotted as described. The molecular weights of prestained molecular weight standards are indicated. Figure used with permission of the Journal of Biological Chemistry.

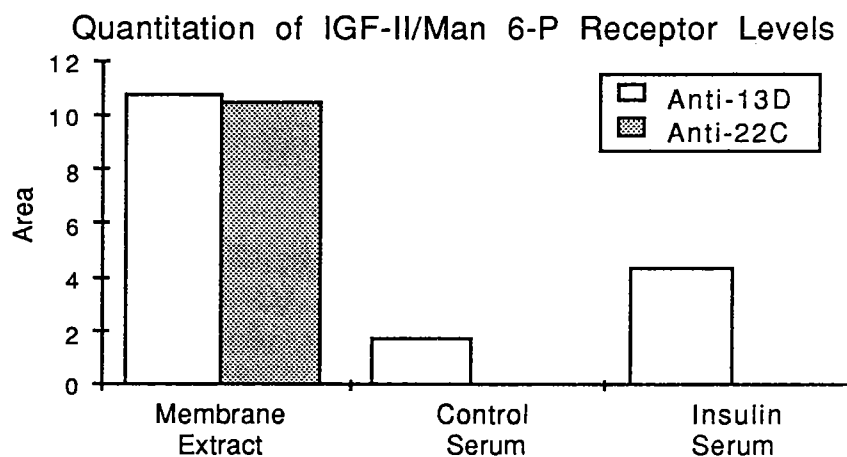




**Figure. 22. Structure and developmental regulation of the serum IGF-II/Man-6-P receptor.** Triton X-100 extracts of rat placental plasma membrane (0.4 mg protein, lanes 1 and 2), or 1 ml of serum from rats aged 14 days (lanes 3 and 4), 35 days (lanes 5 and 6), 70 days (lanes 7 and 8), or 200 days (lanes 9 and 10) were immunoadsorbed to anti-IGF-II/Man 6-P receptor antibody affigel (odd lanes) or a preimmune antibody affigel (evenlanes). Samples were electro-phoresed under nonreducing conditions (A) or following reductive alkylation (B and C). Following transfer the nitrocellulose filters were immunoblotted with the indicated antibodies. An autoradiogram of these immunoblots is shown. Figure used with permission of the Journal of Biological Chemistry.



**Figure 23.** Silver stain of immunoprecipitates of IGF-II/Man-6-P receptor from serum. IGF-II/Man-6-P receptors were immunoabsorbed from serum as described in Figure 21, then subjected to SDS-PAGE under reducing conditions on a 6% polyacrylamide gel, followed by silver staining. This silver stain demonstrates that the levels of immunoglobulin used in the immunoabsorption and the amount of serum used for the immunoabsorption are constant while the IGF-II/Man-6-P receptor level is increased approximately twofold (band indicated by arrow).



**Figure 24.** The regulation of receptor forms in serum by insulin. Receptors were immunoadsorbed from an extract of 0.5 mg plasma membrane protein (E), or 5 ml serum from rats injected with insulin (I) or carrier (C) alone 30 minutes before sacrifice, using polyclonal antisera raised against IGF-II/Man-6-P receptors. Immunoadsorbed receptors were then reduced and alkylated, then subjected to SDS-PAGE under reducing conditions on 6% polyacrylamide gels, and transferred to nitrocellulose. The nitrocellulose filters were then immunoblotted with anti-22C antiserum or an anti-13D antiserum followed by  $^{125}\text{I}$ -protein A. Each immunoblot was then quantitated as described in the methods.

Insulin Injection Increases the Levels of Serum Receptors for Transferrin and Insulin-like  
Growth Factor II/Mannose 6-Phosphate in Intact Rats

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Running Title: Insulin Increases Serum Receptor Levels

### Abstract

Previous work indicates a serum form of the insulin-like growth factor II/mannose 6-phosphate (IGF-II/Man 6-P) receptor that circulates in mammals is a truncated form of the cellular receptor which lacks its cytoplasmic domain (MacDonald, R.G. et al (1989) *J. Biol. Chem.* 264, 3256-3261). In this study, the effects of insulin administration on the levels of the serum forms of IGF-II/Man 6-P and transferrin receptors were measured. Following subcutaneous injection of insulin into rats fasted overnight, the amount of IGF-II/Man 6-P receptor in serum increases to approximately twice that observed in untreated animals within 30 minutes, then decreases to a level lower than the initial level by 60 minutes before returning to control values by 90 minutes. The time course of the initial increase in serum receptor levels in response to insulin is similar to that observed for the decrease in blood glucose concentrations. Measurements of serum enzyme activities (creatine kinase and lactate dehydrogenase) during this time show no significant increase in response to insulin treatment. Furthermore, the increase in serum of IGF-II/Man 6-P receptors is proportional to the amount of insulin injected over the range tested. In diabetic rats the serum IGF-II/Man 6-P receptor concentration is decreased to approximately 80% as much serum receptor as normal, age matched rats. The acute response of serum IGF-II/Man 6-P receptor levels to insulin administration is similar in both time course and extent of change to the increase in isolated fat cells of cell surface receptor levels due to insulin action. These results suggest the hypothesis that insulin stimulates the movement of cellular IGF-II/Man 6-P receptors to the cell surface where they are proteolytically cleaved and released into serum.

## Introduction

A significant and rapid effect of insulin administration is the translocation of a number of proteins from an intracellular compartment to the cell surface. Among the proteins for which this has been demonstrated in rat adipocytes are glucose transporters (1-4) and receptors for IGF-II/Man-6-P (5, 6), transferrin (7, 8), and  $\alpha_2$  macroglobulin (9). Translocation of the glucose transporter appears to play a role in increasing cellular uptake of glucose. The increase in the number of these cell surface receptors correlates with increased cellular binding of ligands with concomitant increases in uptake of ligands. This phenomenon is associated with a concomitant decrease in the number of intracellular receptors which fractionate in low density microsomes (5-9).

Of the proteins translocated to the cell surface in response to insulin, the IGF-II/Man-6-P receptor (10-12) and the transferrin receptor (13) have been detected as having serum forms. We have previously shown that the serum IGF-II/Man-6-P receptor is truncated at the C-terminus, and that its levels in rats decrease over time beyond 14 days after birth (12). We hypothesized that the serum receptor is most likely a proteolytic product of the cellular receptor, produced by the action of proteases on the cell surface. In this case, it would be expected that insulin might induce an increase in the release of IGF-II/Man 6-P receptor into serum, secondary to its effect to increase cell surface receptor numbers.

In this paper we investigate the regulation of serum IGF-II/Man 6-P and transferrin receptor levels by insulin. Serum receptors assayed in rats 30 minutes following insulin injection showed an approximately two-fold increase in the levels of both receptors in serum as compared to untreated animals. The increased amount of IGF-II/Man 6-P receptor over time following insulin injection demonstrated similarity to the effect on blood glucose levels. Similarly, as has been shown for the decrease in blood glucose levels, the increase in IGF-II/Man 6-P receptor in circulation was dependent upon the amount of insulin injected. Serum IGF-II/Man 6-P receptors were also assayed in RT6 depleted diabetic rats and found to be decreased compared to controls. The evidence presented demonstrates that the level of IGF-II/Man 6-P receptors in serum is regulated by insulin.

## Materials and Methods

Materials. IGF-II/Man-6-P receptors for antibody generation were purified from rat placental plasma membranes by IGF-II-Sepharose chromatography as previously described (13). The primary structure of the rat IGF-II/Man-6-P receptor deduced from cDNA clones (14) was the basis for design of synthetic peptides used in the generation of anti-peptide antibodies. Dithiothreitol (DTT) was 99% pure from Chemical Dynamics Corporation, and sodium iodoacetate (Fisher Biotech) was recrystallized twice from methanol. All other chemicals were at least reagent grade.

Preparation of Serum. Male Sprague-Dawley rats at 100 to 150 g, obtained from Taconic Farms, Germantown, NY, were injected subcutaneously with 0.2 ml phosphate buffered saline (0.01 M sodium phosphate, 0.15M NaCl, pH 7.4) alone or with 0.5U insulin (or the indicated dosage) in 0.2 ml phosphate buffered saline. Thirty minutes following injection (or at the times indicated) blood was obtained by decapitation. Serum was prepared by clotting blood at 4°C for 6 h followed by centrifugation at 2000 x g for 10 min. Sera were then used immediately. Insulin treatment resulted in a decrease of serum glucose levels (measured in the clinical chemistry facility at the University of Massachusetts Medical Center) from 110 to 30 mg/dl (Fig. 25C and 26). Enzyme activities (creatine kinase and lactate dehydrogenase) measured in serum were not significantly altered by this treatment (2708±628 to 2950±991 and 607±183 to 853±125, respectively).

Diabetic rats, generously provided by Dr. Aldo Rossini, were of the BB-DR/Wor strain and were either spontaneous diabetics (1 animal) or RT6 depleted (3 animals). Diabetes was confirmed by glycosuria and a blood glucose >250 mg/dl on the day of sacrifice and animals were never treated with insulin. These animals and nondiabetic littermates, or age matched rats of the same strain, were sacrificed at approximately two weeks of age and blood obtained by heart puncture. Blood glucose levels were 317±172 for the diabetic rats and 150±15 for the matched controls.

Preparation of Plasma Membranes from Rat Placenta. A plasma membrane fraction was isolated from rat placentas taken at day 19 of gestation as described previously (13). Membrane extracts (5 mg protein/ml) were prepared by incubation with 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), 1% Triton X-100, pH 7.4, plus antiproteases by gentle mixing for 1 h at 3°C. The antiproteases were leupeptin, antipain and benzamidine at concentrations of 10 mg/ml each, 20 mg/ml aprotinin, 12.5 mg/ml



chymostatin, and 1 mM PMSF. The mixture was centrifuged for 10 min at 15000 x g in a microcentrifuge and the supernatant fraction was used as the extract. Protein was assayed by the Bradford method (15). The cellular form of the IGF-II/Man 6-P receptor was isolated from this preparation.

Immunoabsorption of the IGF-II/Man-6-P Receptor and the Transferrin Receptor. IGF-II/Man-6-P receptors were immunoabsorbed from Triton X-100 extracts of rat placental plasma membranes as described before (16, 17). For immunoabsorption from serum, serum was diluted 1:1 with Hepes buffered saline (50 mM Hepes, 0.15M NaCl, pH 7.4) containing protease inhibitors, then immunoabsorbed in the same manner as a membrane extract. The same procedure was used for immunoabsorption of the transferrin receptor.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis. For the antipeptide antibodies to recognize the IGF-II/Man-6-P receptor in western immunoblotting, the receptor had to be reduced and alkylated under denaturing conditions, as previously described (12). For reductive alkylation, washed anti-receptor antibody affigel or membrane pellets were heated for 15 min at 100°C in 0.1 ml of 10% SDS, 0.25 M Tris, 10 mM DTT, pH 8.3. The samples were cooled, 5 ml of 0.5 M sodium iodoacetate was added, followed by incubation in the dark at 23°C for 30 min or more. Reduction alone was adequate to immunoblot the transferrin receptor, so it was simply heated to 100°C for 5 min in sample buffer containing 10 mM DTT. Electrophoresis was performed as described (18) on 6% polyacrylamide gels.

Antisera and Antibodies. The anti-IGF-II/Man-6-P receptor antisera used were those described by MacDonald et al (12). Briefly, the antireceptor antiserum was raised in rabbits against intact purified rat cellular receptor. Anti-22C was raised against the peptide EEETDENETEWLMEEIQVPAPR which is located 32 residues C terminal to the transmembrane domain, and anti-13D was raised against the peptide YYLNVC RPLNPVPGCD located 1476 residues N-terminal to the cytoplasmic domain (12). In the experiments described, the antireceptor antiserum was immobilized on affigel as described (12) to immunoabsorb the receptors from serum or membrane extracts. The antipeptide antisera were used to immunoblot reduced and alkylated receptors, or a polyclonal anti-receptor antibody to immunoblot nonreduced receptors. For the detection of transferrin receptors a monoclonal antibody obtained from Dr. Roger Davis was used for the immunoabsorption and the immunoblotting (19).

**Western Immunoblotting.** IGF-II/Man-6-P receptors were transferred from polyacrylamide gels to nitrocellulose as described (17). The immunoblots were blocked for 30 min at 23°C in 0.22  $\mu$ m-filtered 0.5% nonfat dry milk dissolved in 15 mM Tris, 0.15 M NaCl, pH 7.4. Incubations with the anti-13D, anti-22C, or anti-transferrin antibodies were done at 3°C for 16-20 h using 1:500, 1:200, or 1:1000 dilutions, respectively, of purified immunoglobulin fractions or ascites fluid in blocking buffer. The blots were then washed, incubated with  $^{125}$ I-protein A (anti-13D and anti 22C) or  $^{125}$ I-goat anti-mouse IgG (anti-transferrin receptor), and washed again as described (17). The autoradiograms are shown. Quantitation was performed by scanning of autoradiograms on an LKB Ultrosan XL densitometer and integrating peak areas; or by cutting out the bands of interest and counting them directly. Thus, amounts of receptors are expressed in areas determined by the densitometer or in counts per minute.

## Results

In order to measure the effect of insulin on levels of receptors in serum, rats were injected with 0.5 U of insulin in phosphate buffered saline or with saline alone. At various times following this injection, the animals were sacrificed and serum obtained as described in Material and Methods. From this serum, IGF-II/Man 6-P receptors were isolated by immunoabsorption, electrophoresed on SDS-PAGE, and transferred to nitrocellulose. As shown in Figure 25, serum receptor levels increase rapidly to reach approximately twice the control level 20 minutes following insulin injection. Following this peak at 20 minutes, levels decrease rapidly in the next 40 minutes to a level approximately 50% of control levels, before returning to the initial concentration at 90 minutes. The serum glucose levels were also measured at these times (Fig. 25C). In general, the initial increase in serum receptor levels correlated with the decrease in glucose levels (Fig. 25B and 25C), although the degree of change differed. After this initial change, however, the glucose levels remained low while the serum receptor concentrations rapidly decreased below the initial level by 60 minutes, then returned to resting levels by 90 minutes.

We next studied the sensitivity of serum IGF-II/Man 6-P receptor levels to insulin by injecting the rats with different amounts of insulin. The levels of receptor in serum increased with increasing doses of insulin injected (Fig. 26) up to very high levels (10 Units/rat). The gradual increase in serum receptor levels over the range of 0.0001 Unit to 10 Units in response to increasing amounts of insulin injected contrasts sharply with the acute dose dependence of glucose levels in which no effect is seen below 0.01 Unit and a

maximal effect is obtained by 1 Unit. It is of interest that a significant change in serum receptor levels is observed at 0.01 Units while no change in blood glucose is seen at this dosage.

If insulin induces an increase in the levels of IGF-II/Man 6-P receptor in circulation, it might be expected that decreases in circulating insulin should result in a decrease in serum receptor levels. In order to test this hypothesis, rats were injected with an anti-RT6 antibody to induce diabetes (20) and their serum IGF-II/Man 6-P receptor levels measured as described above, and compared to matched controls as described in the methods. Diabetic rats had circulating IGF-II/Man 6-P receptor levels that were slightly but significantly decreased ( $83.1 \pm 1.1$  %; mean  $\pm$  S.D. 3 matched sets) as compared to nondiabetic controls. Furthermore, the rats of this genetic strain are prone to diabetes, and the nondiabetic littermates had blood glucose levels that were markedly elevated as compared to the Sprague-Dawley control rats used for the other experiments (150 as compared to 110 mg/dl). A comparison of these diabetic rats to the Sprague-Dawley control animals showed a 50% decrease in serum receptor levels in the diabetics (not illustrated).

In order to verify the identity of the protein studied as the serum IGF-II/Man 6-P receptor and to determine if other serum receptor proteins might be similarly regulated, additional antibodies were used. Animals were injected with 0.5 U insulin, then sacrificed 30 minutes later. IGF-II/Man 6-P receptors were immunoadsorbed from placental plasma membranes or the serum samples described above, electrophoresed on SDS-PAGE, and transferred to nitrocellulose as described in Materials and Methods. These filters were then probed with an antipeptide antibody as described in the legend of Figure 27. While, as previously demonstrated (12), anti-22C recognizes only cellular receptor, the anti-13D antibody recognizes a protein in cell extracts as well as a protein of slightly lower molecular weight in serum (Fig. 27). The serum IGF-II/Man 6-P receptor from insulin treated animals is present at approximately 2.5 fold the level seen in control animals. The lower Mr forms of the serum form of the IGF-II/Man 6-P receptor previously described by MacDonald *et al.* (12) are present in much lower amounts than the major form thus not contributing greatly to the total amount of serum IGF-II/Man 6-P receptor. They do, nevertheless, appear to be regulated in a manner similar to the major species shown. Analysis of similar preparations of immunoadsorbed receptor by silver staining showed that a single band, corresponding to the known migration of the IGF-II/Man-6-P receptor, is increased in the immunoprecipitates. Similarly, an approximately two-fold increase in

transferrin receptor levels in serum are observed following insulin treatment (from  $525 \pm 291$  to  $1100 \pm 91$  cpm, mean  $\pm$ S.D. N=3; Fig. 27). The measurement of other enzyme levels in serum, as described in Materials and Methods, demonstrates that these changes are not due to a general increase in serum protein levels.

## Discussion

The results presented support a model whereby the serum IGF-II/Man 6-P and transferrin receptors are degradation products of their cellular counterparts, produced either by proteolysis from the cell surface or by intracellular proteolysis within vesicles followed by release of vesicle contents. In these models, the initial increase in serum receptor levels would reflect the insulin induced increase in receptors at the cell surface. The decrease in serum receptor at 60 minutes would then reflect the recovery of the cells from the insulin induced increase causing a decrease in the exocytosis rate and the number of cell surface receptors. Finally, by 90 minutes the level of exocytosis would return to control levels as would the number of receptors available at the cell surface. The determination of which of these models (or others) correctly describes the release of receptors from cells will require the development of a tissue culture system which produces serum receptor forms.

A third possible explanation for the insulin induced increase in serum receptor levels is that the hormone causes a decrease in the rate at which receptors are cleared from circulation. It seems unlikely that insulin would induce a specific decrease in the clearance rate of these proteins, as circulating enzyme levels were not appreciably changed by this treatment. Furthermore, the time course of this response would require a decrease in the clearance rate initially, followed by an increased rate by 60 minutes, then another decrease to the control values by 90 minutes. However, such a possibility cannot be ruled out at this time. Such a hypothesis could be tested by injecting rats with radiolabelled serum receptor and measuring the rate of clearance in insulin treated and untreated animals. However, the low concentration of receptors in serum and their apparent occupancy state make this truncated protein difficult to purify.

The acute regulation of serum receptor levels has several interesting implications. First, such acute regulation argues against the possibility of serum receptor arising from RNA messages distinct from the cellular receptors: the time necessary to synthesize the IGF-II/Man-6-P receptor (16) is much greater than the period of insulin treatment in these animals. Second, the same process that generates the serum IGF-II/Man-6-P receptor may generate the serum transferrin receptor. It has recently been shown that these two receptors coexist in intracellular vesicles which also contain the HepG2 type glucose transporter, and that these vesicles move to the surface in response to insulin (21). It is possible, if not likely, that other proteins regulated by insulin, such as the  $\alpha_2$ -macroglobulin receptor, may also share this degradative pathway.

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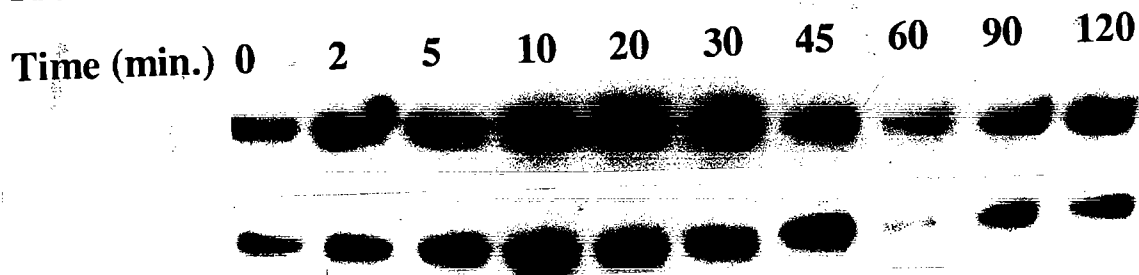
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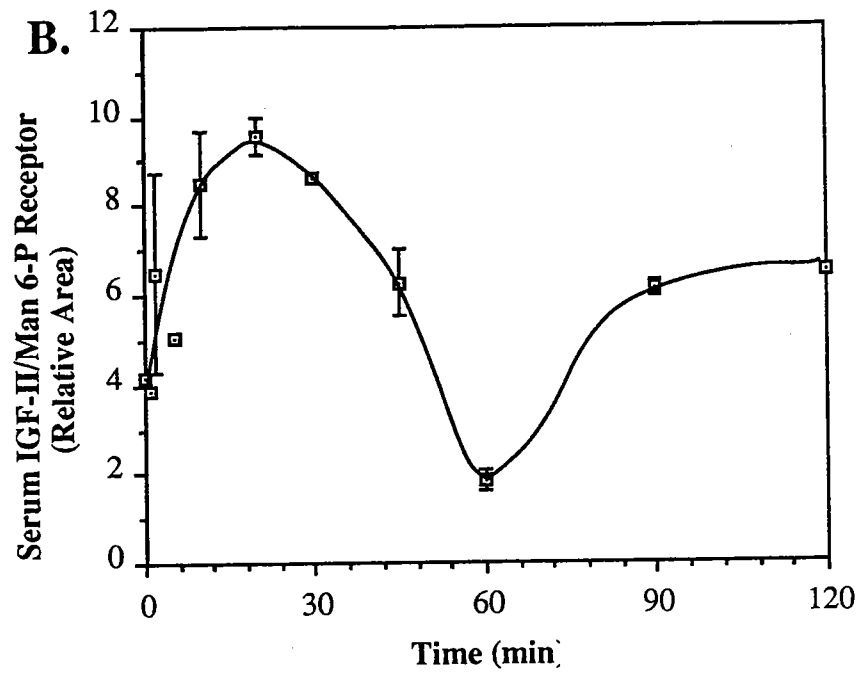


**Fig. 25.** Serum IGF-II/Man 6-P receptor levels at several times following insulin treatment. Male Sprague Dawley rats at 100-125 g were injected with 0.5U of insulin, then sacrificed at 0, 2, 5, 10, 20, 30, 45, 60, 90, or 120 minutes following injection and their blood collected. Serum was obtained, and IGF-II/Man 6-P receptors immunoadsorbed as described in materials and methods. **A.** Following immunoadsorption, serum receptors were electrophoresed following reductive alkylation on SDS-PAGE, transferred to nitrocellulose, and immunoblotted using anti-13D antibody followed by  $^{125}\text{I}$  protein A. Each point corresponds to serum pooled from two animals. The IGF-II/Man 6-P receptor bands excised from autoradiograms from two independent time courses are shown. **B.** The receptor levels from the immunoblots were quantitated by densitometry as described in the methods and averaged. Results are measured as means  $\pm$  S.D. (N=3), normalized by mean peak area over each time course. **C.** Serum glucose levels obtained over the same time course. Levels were determined as described and the results are shown as means  $\pm$  S.D. (N=3) normalized by the averages for each of the three time courses.

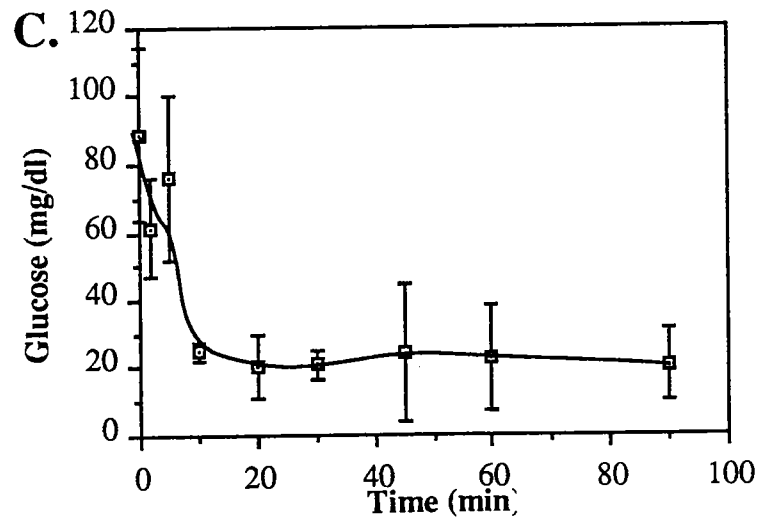
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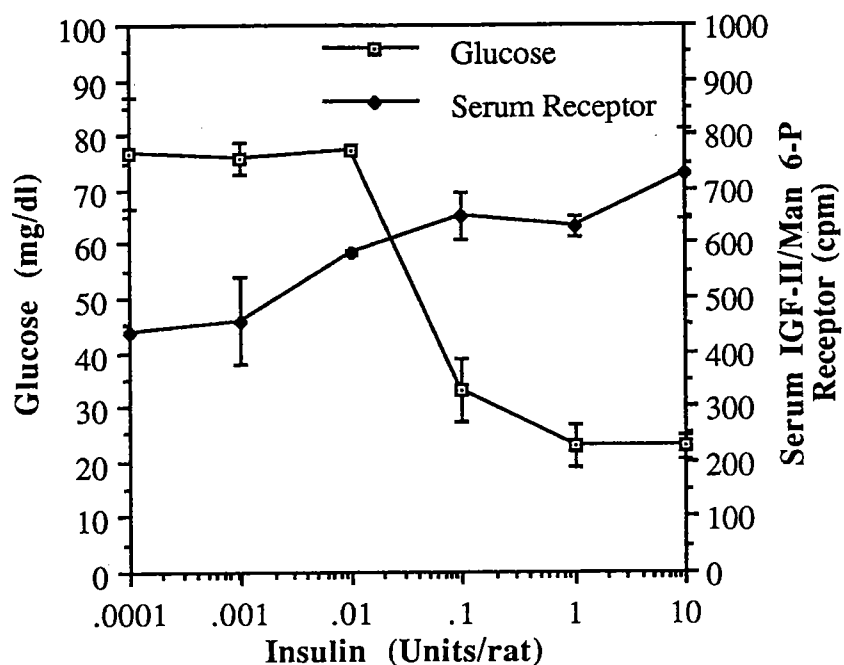


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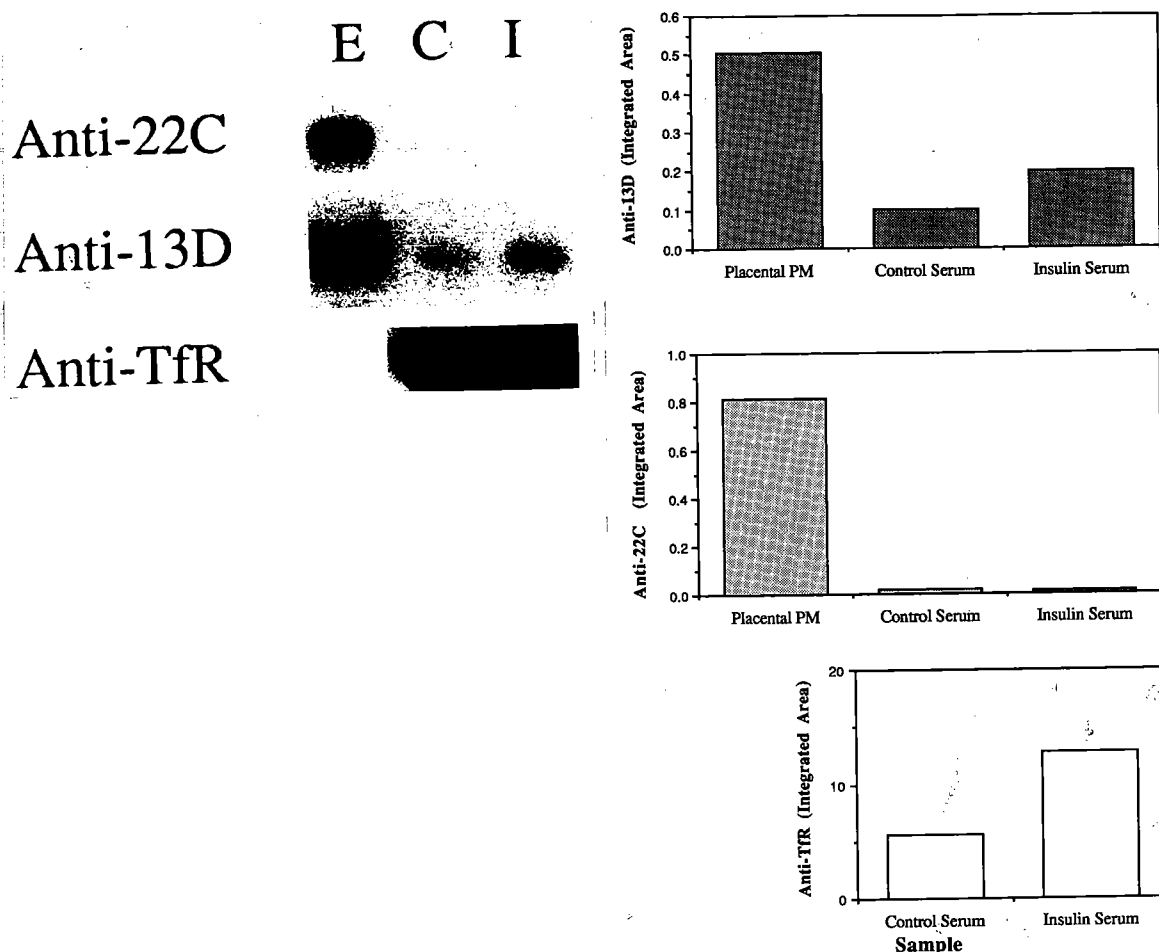


C.





**Fig. 26.** The serum IGF-II/Man 6-P receptor levels following treatment with various amounts of insulin. Male Sprague Dawley rats at 100-125g were injected with 0.0001, 0.001, 0.01, 0.1, 1.0, or 10.0 Units of insulin per rat. Animals were sacrificed 20 minutes following injection and serum obtained. Blood glucose and serum receptor levels were determined as described except that a polyclonal anti-IGF-II/Man 6-P receptor antibody was used to probe immunoblots in place of the anti-peptide antibody. Each data point represents the pooled serum from 2 animals. Blood glucose levels shown are means $\pm$ S.D. (N=3) and receptor levels are means $\pm$ S.D. (N=2).



**Fig. 27.** The levels of transferrin and IGF-II/Man 6-P receptors in serum of control and insulin treated rats. Receptors were immunoadsorbed from an extract of 0.5 mg plasma membrane protein (E), or 5 ml serum from pooled from 4 rats injected with insulin (I) or carrier (C) alone 30 minutes before sacrifice, using polyclonal antisera raised against IGF-II/Man-6-P receptors or a monoclonal specific for the transferrin receptor. Immunoadsorbed receptors were then subjected to SDS-PAGE as described in materials and methods and transferred to nitrocellulose. The nitrocellulose filters were then immunoblotted with anti-22C antiserum or anti-13D antiserum followed by  $^{125}\text{I}$ -protein A or anti-transferrin receptor antibody followed by  $^{125}\text{I}$ -goat anti-mouse Ig. A. The bands of interest excised from an autoradiogram are shown. B. The autoradiographs depicted in A were quantitated by densitometry and the areas under the receptor peaks plotted.

*Submitted for Publication*

Extracellular Release as the Major Degradative Pathway of the Insulin-like Growth Factor  
II/Mannose 6-phosphate (IGF-II/Man 6-P) Receptor

Running Title: IGF-II/Man 6-P Receptor Degrades into a Serum Form

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### Summary

The IGF-II/Man 6-P receptor directs lysosomal proteins to a prelysosomal compartment from which they go on to form lysosomes while the receptor returns to the Golgi apparatus. The degradation of the IGF-II/Man 6-P receptor thus must be extralysosomal. By pulse-chase labelling of cultured BRL-3A cells, we directly demonstrate that the loss of cellular IGF-II/Man 6-P receptor correlates with the appearance of a truncated form of the IGF-II/Man 6-P receptor which is found in the medium. The ability of serum and conditioned medium to increase the rate of appearance of receptor in the medium in a phosphate dependent manner suggests that the degradation of the cellular IGF-II/Man 6-P receptor occurs by lysosomal enzyme mediated proteolysis of the receptor from the cell surface to produce a truncated receptor form.

A number of published reports describe experiments using pulse-chase protocols to address the synthesis, processing, and degradation of the IGF-II/Man 6-P receptor (Goldberg, Gabel, and Kornfeld, 1983; Creek and Sly, 1983; Sahagian and Neufeld, 1983; Sahagian, 1984; MacDonald and Czech, 1985). These reports demonstrated that the receptor required a significant length of time to reach maturity (4-8 h), that it was glycosylated, that some processing was necessary for ligand binding, and that the receptor had a long half life (24-48 h). Through the use of a number of agents which disrupt lysosomes, these reports also demonstrated that the degradation of the receptor was not lysosomal (Creek and Sly, 1983; Sahagian, 1984). The mechanism of receptor degradation has remained an open question.

In 1987, the first report of a serum form of the IGF-II/Man 6-P receptor in circulation was reported (Kiess *et al.*, 1987). Subsequent reports demonstrated that the serum receptor could bind IGF-II and Man 6-P containing ligands simultaneously, that it was cytoplasmically altered or truncated as compared to the cellular receptor, and that in circulation the receptor was proteolysed into smaller fragments (MacDonald *et al.*, 1989). It has also been shown that the IGF-II/Man 6-P receptor can be found in urine (Causin *et al.*, 1988). This suggested that the serum IGF-II/Man 6-P receptor might be an intermediate form in the degradation of the protein.

In order to address the degradative pathway of the IGF-II/Man 6-P receptor and the possible role of the serum IGF-II/Man 6-P receptor in this process, we have performed pulse-chase protocols on cultured cells and isolated IGF-II/Man 6-P receptor forms from cell membranes and medium. In all cells studied the appearance of receptor in the medium correlates with the disappearance of receptor from the cells. In BRL-3A cells quantitation of cellular receptor loss and the appearance of receptor in the medium demonstrates that this

pathway accounts for essentially all receptor degradation. In studies in which differentiated 3T3-L1 adipocytes were fractionated, receptor appearance in the medium followed appearance at the cell surface. Cyclohexamide, a protein synthesis inhibitor, showed little or no effect on the production of the serum IGF-II/Man 6-P receptor when added after a 1 hour pulse. Results from all cell lines studied are consistent with the model that the serum IGF-II/Man 6-P receptor is the major, if not sole, degradative product of the cellular receptor generated by proteolysis of the cellular receptor at the cell surface.



## Results

### Radiolabelled IGF-II/Man 6-P Receptors Appear in the Medium of Cultured Cells

In order to develop a system in which to study the synthesis of the serum IGF-II/Man 6-P receptor and its relationship to the cellular receptor, cellular and medium receptors were isolated following a pulse-chase protocol on a number of different cell lines: BRL-3A (rat liver), L6 (rat myoblast), CHO-K1 (Chinese hamster ovary), and SL-29 (chick embryo fibroblasts). In each cell line, the cells were labelled with Tran<sup>35</sup>S label for 1 hour, then incubated in unlabelled medium for the times indicated (Figure 28). In each case receptor accumulated in the cells at 0 and about 3.5 hours following a 1 hour pulse label, then decreased to lower levels by about 20 hours. Simultaneously, serum receptor was absent from the medium at the start of the chase, then accumulated slightly by about 3.5 hours, with similar levels seen by about 20 hours. This is consistent with the loss of IGF-II/Man 6-P receptor from the cells being accounted for by the appearance of receptor in the medium and previous information on the specificity of the antibody used (MacDonald and Czech, 1985). The primary translation product (232 kDa) is not recognized by the antibody until it has been glycosylated to ~245 kDa. This post-translational modification accounts for the additional receptor appearing between 0 and about 3.5 hours following the pulse. During this time, receptor is also appearing in the medium and, as will be discussed further in the following section, serum receptor in the medium is gradually lost, possibly by further degradation or reuptake. Taking these factors into account, it appears that a significant portion of the receptor lost from the cells appears in the medium. This is the case with all cell lines studied regardless of the tissue or species from which receptor is obtained.

The Appearance of Receptors in the Medium Quantitatively Accounts for the Loss of Radiolabelled IGF-II/Man 6-P Receptors from Cell Membranes

As described above, the quantitation of the amount of receptor lost from cells and appearing in the medium is difficult due to the dynamics of this system: cellular receptor is synthesized and processed slowly making the 0 time point following the 1 hour pulse label an inappropriate starting value to determine the total amount of labelled receptor in the experiment. On a similar scale of time, cellular receptor is degraded in to medium receptor then, after some lag, serum receptor is further degraded to a form not identified in our assays. In order to address this issue better a representative cell line, BRL-3A cells, were labelled for 1 hour with EXPRE<sup>35</sup>S<sup>35</sup>S label then incubated with unlabelled medium for the times indicated (Figure 29). At the start of the experiment, there is no receptor in the medium, and only the ~245 kDa precursor is seen in cells. Over the next several hours the ~245 kDa precursor is converted into the ~250 kDa mature receptor and a truncated receptor is first seen in the medium. At later times, cellular receptor is gradually converted into the truncated form of the receptor found in the medium. Quantitation of the results shows that  $109 \pm 39\%$  of the receptor lost from the cells appears in the medium. Protein synthesis is not necessary for the synthesis of the serum receptor as it occurs in the presence of the protein synthesis inhibitor cyclohexamide (Figure 30, lanes 11 and 12). Furthermore, neither form of the IGF-II/Man 6-P receptor was detected in the supernatant following the pelleting of total membranes (data not shown), demonstrating that no detectable amount of the serum receptor is stored in the cytoplasm or in the lumens of disrupted vesicles prior to appearance in the medium. These data further support the idea of proteolysis from the cell surface, and rule out any possibility that the serum receptor is translated from a message distinct from that of the cellular receptor.

### The Effect of Ligands, Serum, and Conditioned Medium on the Production of IGF-II/Man 6-P Receptor in the Medium

It would be expected that agents which cause a redistribution of IGF-II/Man 6-P receptors or bind to it might alter the rate at which the cellular IGF-II/Man 6-P receptor is proteolysed into a truncated form of the receptor. In order to test this hypothesis, BRL-3A cells were pulse-labelled for 1 hour, then chased in serum free medium with or without insulin, fetal bovine serum, conditioned medium, or ligand. Insulin (Figure 30, lane 2), IGF-II (Figure 30, lane 3), and Man 6-P (Figure 30, lane 5) cause little or no significant change in the rate at which serum receptor is released into the medium as compared to cells grown in control medium (Figure 30, lane 1). The lysosomal enzyme  $\beta$ -galactosidase (Figure 30, lanes 6-8) likewise causes no significant change in the rate at which this process occurs. Addition of fetal bovine serum (Figure 30, lane 9) or conditioned medium (Figure 30, lane 11) increases the rate at which serum receptor is produced, with the effect being reduced or negated by alkaline phosphatase treatment (Figure 30, lanes 10 and 12). The results suggest that serum and conditioned medium may contain a Man 6-P containing protease which binds to the receptor and causes the indicated cleavage. The ineffectiveness of other ligands in negating this process, however, is unclear. Another possibility is that the dephosphorylation inactivates the protease which is involved in this process.

### The Effect of Protease Inhibitors on the Production of the Medium Receptor

The predicted proteolytic release of IGF-II/Man 6-P receptors from the cell surface would be expected to be hindered by the addition of protease inhibitors to the chase medium of pulse labelled BRL-3A cells. Addition of a combination of the protease inhibitors leupeptin, antipain, aprotinin, and benzamidin (Figure 31) or chymostatin and

PMSF inhibit the proteolytic cleavage which produces the serum receptor. Individually, the protease inhibitors aprotinin, chymostatin, or PMSF completely inhibit the production of the receptor in medium from the cellular receptor, while benzamidine inhibits the process to a lesser extent. Leupeptin and antipain have no effect on the production of serum receptor in the medium as compared to cells incubated in the absence of protease inhibitors for the chase period. Taken together the data are suggestive of a chymostatin-like protease which are present in serum or released from cells into the medium, and can then bind and cleave the IGF-II/Man 6-P receptor at any of a number of sites in the juxtamembrane region. The results do not, however, the inhibition of this process by protease inhibitors through another mechanism.

#### Subcellular Fractionation of 3T3-L1 Adipocytes

In order to address the site from which the serum IGF-II/Man 6-P receptor is released into the medium, a cell line for which membranes can easily be fractionated, the 3T3-L1 adipocyte, was used. The adipocytes were labelled with  $^{35}\text{S}$ -cysteine for 1 hour, then the medium replaced with nonradioactive medium. At several times thereafter cells were placed on ice, medium removed, and cells homogenized. Over the time course studied the receptor was lost from the total membranes and quantitatively appeared in the medium (Figure 32). Next, in order to address the site of proteolysis of the cellular receptor to produce the serum receptor, cells were labelled for 30 minutes, then placed in unlabelled medium for 0, 10, 20, 30, 40, and 50 minutes (Figure 33). The homogenates from each of these times was then fractionated into plasma membranes and low density microsomes (panel A). A precursor form (~245 kDa) is seen at the very short times only in the low density microsomes, and is converted over time into the mature form of the receptor (~250 kDa). Following maturation, the mature form of the receptor is found at the

plasma membrane (panel B). At yet later times, serum receptor is detected in the medium (panel C). This time course suggests that plasma membrane receptors are a prerequisite to generating serum receptors. Taken together this evidence supports proteolysis of the cellular IGF-II/Man 6-P receptor from the plasma membrane to produce the serum form of the receptor.

## Discussion

### The Synthesis of Medium Receptors

While proteolysis from the cell surface would seem to be the most straightforward way to produce a serum form of a receptor when a cellular form already exists, this mechanism has not previously been identified as a significant route whereby serum receptors are produced. The most studied serum receptor, the serum growth hormone receptor, has been definitively demonstrated to arise from an independently produced protein product from the cellular receptor which is always in a soluble form (Herington, Ymer, and Stevenson, 1986; Leung *et al.*, 1987). Additionally, there is evidence of multiple messages including some lacking transmembrane domains for the neural cell adhesion molecule (Gower *et al.*, 1988) and the class I antigens (Gussow and Pleogh, 1987). In those cases in which the serum receptor forms are independently produced, a serum receptor function independent of the cellular receptor is indicated. Most likely, these functions are similar to those indicated for the serum IGF-binding proteins: inactivation and removal of ligand and/or an increase in the half-life of ligand in circulation.

The demonstration that the serum form of the IGF-II/Man 6-P receptor is a product of the cellular receptor brings up an additional possibility: perhaps the serum IGF-II/Man 6-P receptor is simply a degradative intermediate in the breakdown of the cellular receptor. This idea will be discussed further below. It is also worth considering again at this time that evidence regarding serum binding proteins has indicated that the serum receptor is not a major binding protein of IGF's in circulation (Kiess *et al.*, 1987). The other type of ligand for this receptor, mannose 6-phosphate containing lysosomal enzymes, have also been demonstrated in serum (Belfiore *et al.*, 1974; Lombardo *et al.*, 1980). However, there is

little evidence addressing the role of the serum form of the IGF-II/Man 6-P receptor in binding these proteins in serum. The low concentration of serum IGF-II/Man 6-P receptor in serum argues against an important role in this function.

#### The Synthesis and Degradation of the IGF-II/Man 6-P Receptor

The IGF-II/Man 6-P receptor is synthesized as a 2491 amino acid preprotein, from which 50 amino acids are removed to give rise to the mature receptor (Morgan *et al.*, 1987). The mature peptide has an  $M_r \sim 232$  kDa in the absence of glycosylation (MacDonald and Czech, 1985; Figure 34). A partially processed protein containing some glycosylation is produced fairly rapidly which migrates with an  $M_r \sim 245$  kDa and is the earliest receptor form recognized by the antibody used in these studies. Upon further processing this protein is converted into a mature form of  $M_r \sim 250$  kDa. The conversion of the 245 kDa form to the 250 kDa form occurs with a halftime of approximately 2 hours in H35 cells. Data presented in this manuscript (Figure 33) demonstrates that maturation precedes the arrival of IGF-II/Man 6-P receptor at the plasma membrane. Such processing is not necessary, however, for the acquisition of the ability to bind mannose 6-phosphate containing ligands (Sahagian and Neufeld, 1983). The observed very long processing time is consistent with experiments which have suggested that the IGF-II/Man 6-P receptor may bind lysosomal enzymes in the cis Golgi, and carry them from there to the lysosomes (Brown and Farquhar, 1984; Brown, Goodhouse, and Farquhar, 1986). The IGF-II/Man 6-P receptor may cycle from the cis region of the Golgi apparatus to the prelysosomal compartment, then back to the same region of the Golgi apparatus. During each cycle, a small percentage of the receptors escape this pathway, traverse the remainder of the Golgi apparatus where they may be further glycosylated, then arrive at the plasma membrane. Thus, the receptor could undergo a number of cycles of delivery of lysosomal enzymes to

the prelysosomal compartment while still at the immature Mr of 245 kDa. A small percentage of receptors would escape this pathway during each cycle, get processed to the 250 kDa mature form, and reach the plasma membrane.

Those receptors that reach the plasma membrane would also be available to be proteolytically released and degraded. Data presented in this manuscript suggests that such a protease may be released from cells and accumulate in the medium or in serum. The protease would then bind to the receptor at a Man 6-P binding site and cause the proteolysis which produces the serum receptor form. This process is generally unaffected by the binding of mannose 6-phosphate, IGF-II, or  $\beta$ -galactosidase. The proteolysis appears to be dependent on Man 6-P as pretreatment with alkaline phosphatase eliminates this activity, although effects of alkaline phosphatase on proteolytic activity cannot be ruled out. The lack of an effect of insulin on this process may be due to a lack of sensitivity of the BRL-3A cells to insulin or a lack of receptor redistribution in these cells caused by insulin. In other experiments (not shown) insulin did increase the initial rate at which serum receptor was produced in 3T3-L1 cells, a cell line in which IGF-II/Man 6-P receptors are redistributed in response to insulin. Taken together the data suggest proteolysis from the cell surface by a previously released lysosomal protease (Figure 34). The released receptor fragment would then appear in the medium, arriving in serum in vivo where it may be further degraded or removed through endocytosis.

### Conclusion

For no other protein has proteolytic cleavage from the cell been demonstrated as a significant mechanism of protein degradation, although a proteolytic mechanism may be involved in the transcytosis of IgA. The IGF-II/Man 6-P receptor, however, appears to use this route as the major, if not sole, mechanism of degradation. This process is



dependent upon the presence of IGF-II/Man 6-P receptors at the cell surface which can then be proteolytically cleaved by a lysosomal protease which was previously released from the cell. Such a degradative mechanism may have evolved as a necessary part of the sorting of lysosomal enzymes by this receptor. The efficiency with which receptor is sorted from lysosomal enzymes in the prelysosomal compartment has been demonstrated by the failure of numerous ultrastructural studies to find this protein in lysosomes. However, the other sorting steps, lysosomal enzyme binding to the receptor in the Golgi apparatus and sorting of receptor (and receptor lysosomal enzyme complexes) in coated vesicles destined for the prelysosomal compartment, are not as efficient. Lysosomal enzymes fail to bind to receptor, they then arrive at the cell surface. Receptors also escape sorting to reach the cell surface, where they can capture the released lysosomal enzymes. One model is that a lysosomal protease which has escaped the normal sorting pathway can cleave the cellular receptor from the cell surface to release the serum form of the IGF-II/Man 6-P receptor. This degradative mechanism may, therefore, be required in order for a protein to be efficiently sorted away from the lysosome, the major degradative compartment of the cell.

## Experimental Procedures

Materials. IGF-II/Man-6-P receptors for antibody generation were purified from rat placental plasma membranes by IGF-II-Sepharose chromatography as previously described (Oppenheimer and Czech, 1983). The anti-IGF-II/Man-6-P receptor antisera used were those previously described (MacDonald *et al* , 1989). Cell lines were purchased from the American Type Culture Collection. Cell cultured reagents are from Gibco.. Sodium iodoacetate (Fisher Biotech) was recrystallized twice from methanol. All other chemicals were at least reagent grade.

Radioactive Labelling of Cells. Prior to labelling, cells were grown to 90% confluence (all cell lines other than 3T3-L1) or grown to confluence and differentiated as described (for 3T3-L1; Frost and Lane, 1985). Medium was then removed and replaced by serum free modified Eagles medium buffered with 15 mM HEPES and lacking the labelled amino acid(s). To this was added 0.15-1.0 mCi of  $^{35}\text{S}$ -cysteine, Tran $^{35}\text{S}$ -label (ICN, Costa Mesa, CA), or EXPRE $^{35}\text{S}$  Label (New England Nucleur, Boston, MA) and cells were incubated for 30-60 minutes at 37°C. Following this labelling period, this medium was removed and replaced by serum free Dulbeccos modified Eagles medium. Incubation was continued for the indicated times in serum free DME or DME supplemented as indicated prior to homogenization.

Preparation of Membranes from Cultured Cell Lines. Cells were scraped into a buffer consisting of 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 0.25 M sucrose, and 1 mM (ethylenedinitrilo)tetraacetic acid (EDTA) pH 7.4, plus protease inhibitors at 0°C. The protease inhibitors were leupeptin, antipain and benzamidine at concentrations of 10 mg/ml each, 20 mg/ml aprotinin, 12.5 mg/ml chymostatin, and 1 mM

PMSF. Following homogenization, total membranes were obtained by a single centrifugation at 200,000 x g.

Fractionation of 3T3 L1 adipocytes into plasma membranes and low density microsomes was carried out by differential centrifugation as described (Simpson *et al.*, 1983). Briefly, cells homogenized as described above were centrifuged at 16000 x g for 20 minutes. The pellet from this spin was then resuspended in 20 mM Hepes, 1 mM EDTA pH 7.4 and layered atop a sucrose cushion of this buffer containing 1.12 M sucrose. This was centrifuged at 100,000 x g for one hour and the material that remained above the sucrose cushion diluted 10 fold in 20 mM Hepes, 1 mM EDTA, pH 7.4 and centrifuged at 30,000 x g. This final pellet is plasma membranes. The supernatant from the 16000 x g spin was centrifuged at 30,000 x g for 30 minutes. The pellet from this centrifugation is high density microsomes. A low density microsome pellet was obtained by a 200,000 x g centrifugation of the supernatant from the previous step. Results from our laboratory (Clancy and Czech, 1990) show an 8-fold enrichment in 5' nucleotidase in the plasma membranes and no enrichment of activity in microsomes as compared to a cellular extract. Furthermore, the lack of a doublet at short time points in the plasma membranes (Figure 33) argues against gross contamination of the plasma membranes by microsomes. All membranes were resuspended in 20 mM Hepes, 1 mM EDTA, pH 7.4 and frozen at -80°C until use. Membrane protein was determined by the BCA method (Pierce, Rockford, IL; Smith *et al.*, 1985).

To obtain a membrane extract the membranes obtained as described above were solubilized as follows. A volume of the membrane suspension obtain above equivalent to 0.2 mg of protein or the total membrane protein from one well was pelleted by centrifugation for 5 minutes at 15,000 x g in a microfuge. The supernatant was removed

and the pellet resuspended in 50  $\mu$ l of 15 mM Tris, .15 M NaCl, pH 7.4 containing 1% Triton X-100, 1% deoxycholate, and 0.1% sodium dodecylsulfate with the protease inhibitors described above. Following a 1 hour incubation at 4°C on an end-over-end mixer, the mixture was centrifuged for 10 min at 15000 x g in a microcentrifuge and the supernatant fraction was used as the extract. The cellular form of the IGF-II/Man 6-P receptor was isolated from this preparation.

Preparation of Medium for Immunoabsorption. Prior to homogenization of the cells, medium was removed into centrifuge tubes and protease inhibitors added as described above. The medium was then centrifuged at 35,000 x g or 200,000 x g to remove cellular or membrane contaminants. Both techniques produced identical results (data not shown). Medium was frozen at -80°C until needed. Prior to immunoabsorption, a fraction of the medium corresponding to 0.2 mg membranes or the total medium from one well was concentrated using a Centricon 30 (Amicon, Danvers, MA) or a Centrifugal UltraFree with a 30,000 N.M.W.L. (Millipore, Bedford, MA.) to a volume of 0.1-0.5 ml.

Immunoabsorption of the IGF-II/Man-6-P Receptor. IGF-II/Man-6-P receptors were immunoabsorbed essentially as described (MacDonald *et al.*, 1989). For immunoabsorption from a membrane extract the 50  $\mu$ l of extract was diluted to 0.9 ml with 50 mM HEPES, pH 7.4 and to a final concentration of 0.15 M NaCl and 5 mM mannose 6-phosphate. For medium, 50  $\mu$ l of extraction buffer was added to the medium and it was diluted in the same manner as the membrane extracts. Finally, to each was added 0.1 ml of a 50% slurry of Anti-receptor antibody Affigel (BioRad, Richmond, CA) in 50 mM HEPES, pH 7.4 containing 0.1% Triton X-100. These mixtures were incubated overnight at 4°C on an end-over-end mixer. Unbound material was removed by withdrawing the supernatant following a 1 minute centrifugation in a microfuge at 15,000 x g. This material

was then washed in this manner four times in 50 mM Hepes, 0.5 M NaCl, pH 7.4 containing 0.1% Triton X-100, then once in each 50 mM Hepes, 0.15 M NaCl, pH 7.4 with 0.05% Triton X-100 and 50 mM Hepes, pH 7.4.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis. Samples labelled with  $^{35}\text{S}$  were reduced by incubation in electrophoresis sample buffer contain 100 mM dithiothreitol. Electrophoresis was performed as described (Laemmli, 1970) on 6% polyacrylamide gels.

Autoradiography of  $^{35}\text{S}$ -labelled Gels and Quantitation of Results. Gels containing  $^{35}\text{S}$  labelled material were stained with Coomassie brilliant blue and destained. They were then treated with EN<sup>3</sup>HANCE (New England Nucleur, Boston, MA) according to the manufacturers instructions. The gels were then dried and subjected to autoradiography. Quantitation was performed using an LKB Ultrosan XL densitometer (LKB, Rockville, MD) to scan the autoradiographs or by cutting out the bands and counting them in a scintillation counter. Quantitated results shown are means  $\pm$  standard deviations for three repeats of each experiment except for Figure 5B where the results are the quantitation of a single experiment.

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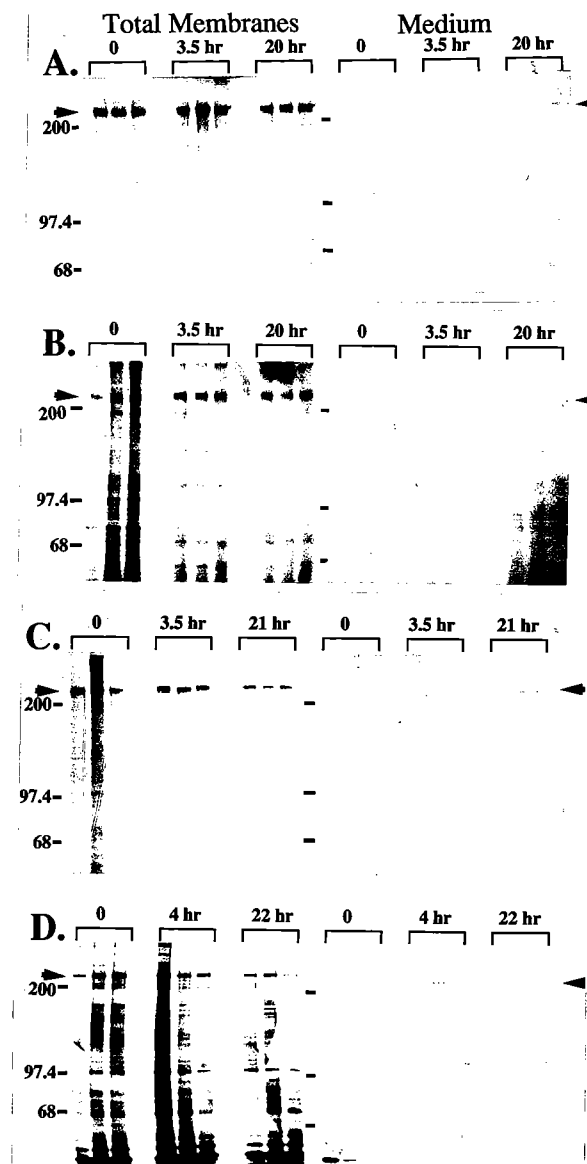
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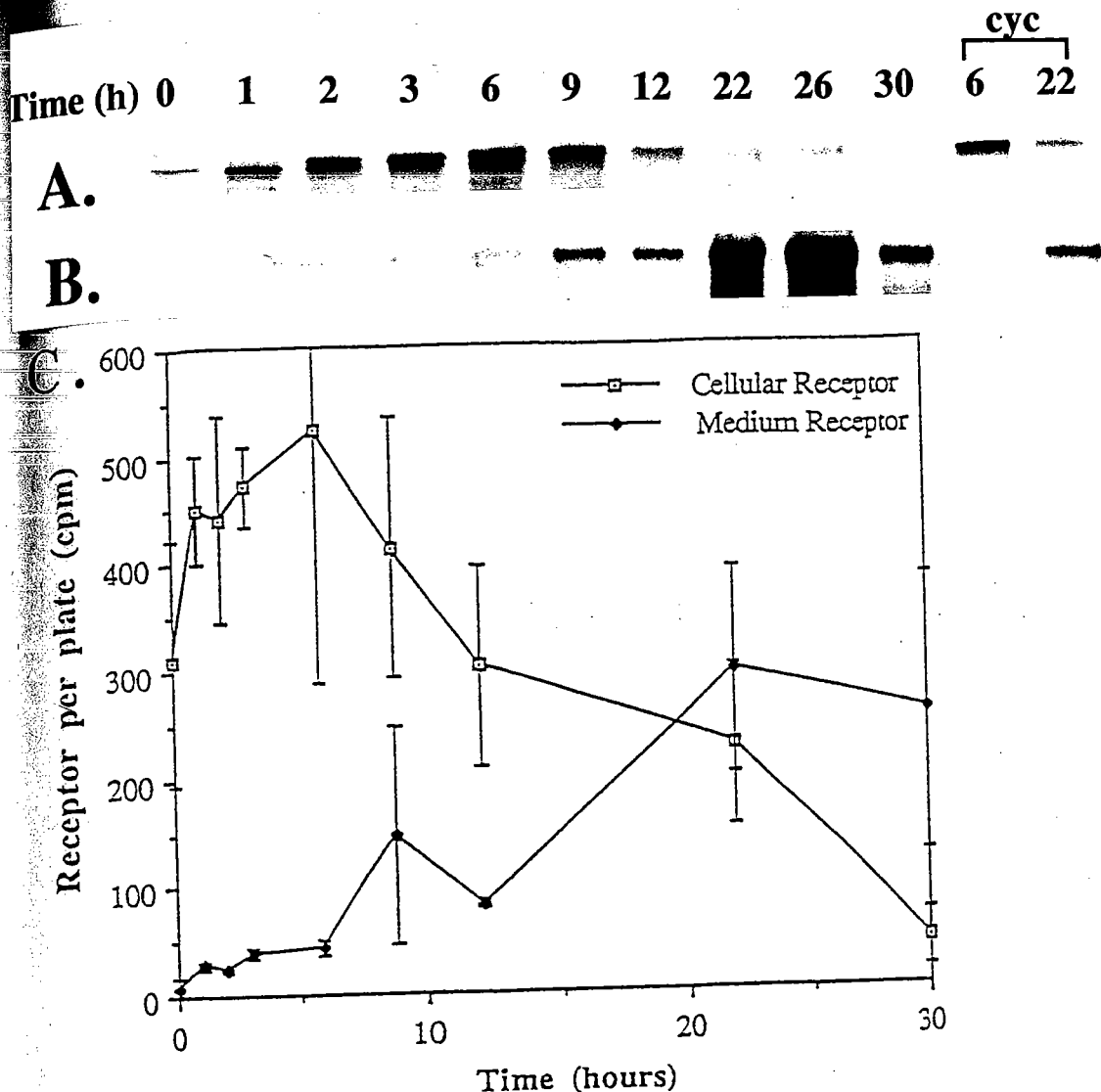
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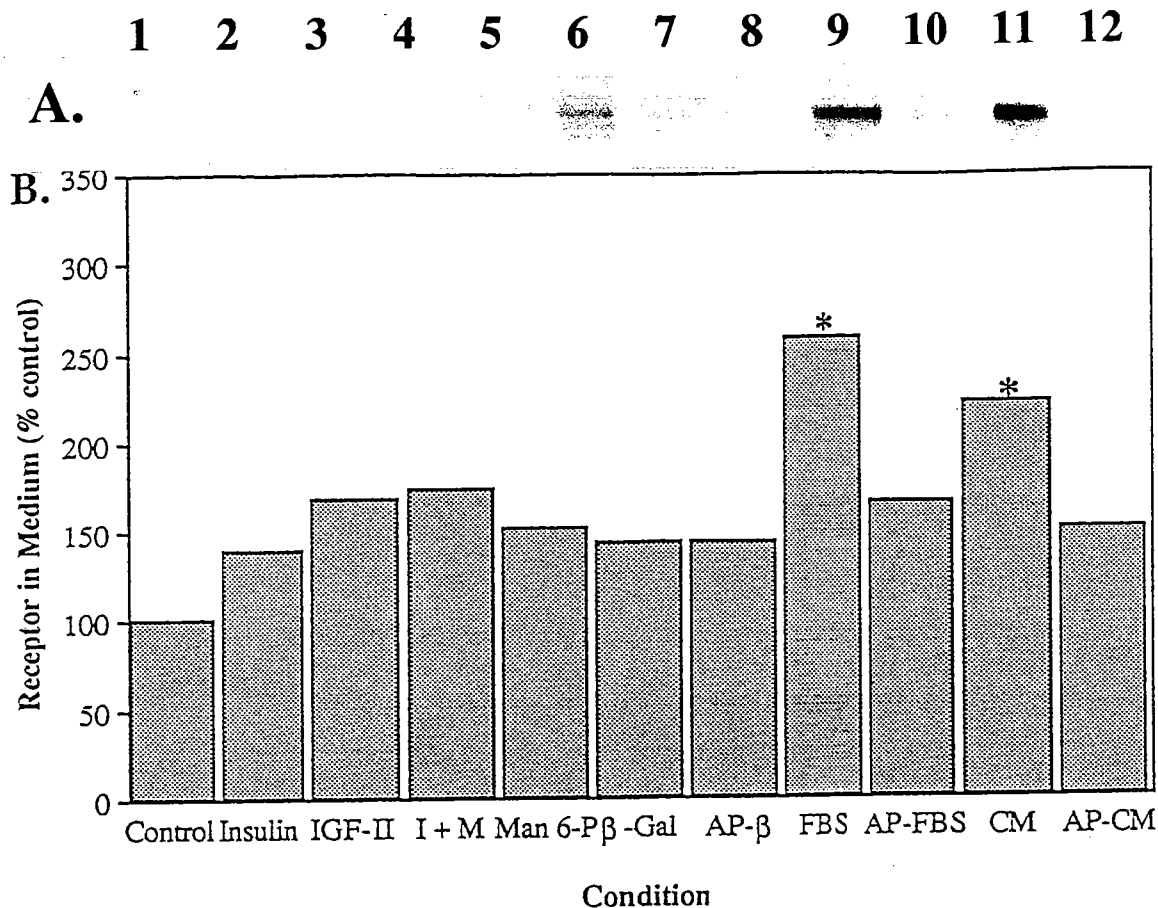




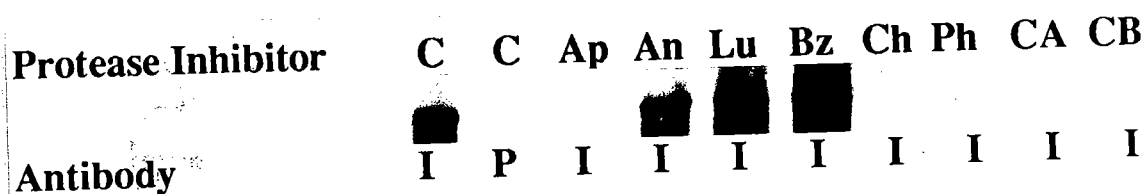
**Figure 28:** The Medium Receptor Form Appears as Cellular Receptor is Lost in BRL-3A, CHO-K1, L6, and SL-29 Cells. Cells were grown to 90% confluence, then labelled with 0.3 mCi Tran<sup>35</sup>S Label per 100 mm plate for one hour followed by the indicated period in serum free medium. Cells were then placed on ice, medium removed, cells homogenized and total membranes obtained. These samples were then electrophoresed on SDS-PAGE, stained, destained, and EN<sup>3</sup>HANCED. A. BRL-3A rat liver cells (0, 3.5, and 20 hours). B. CHO-K1 chinese hamster ovary cells (0, 3.5, and 20 hours). C. L6 rat myoblasts (0, 3.5, and 21 hours). D. SL-29 chick embryo fibroblasts (0, 4, and 22 hours).



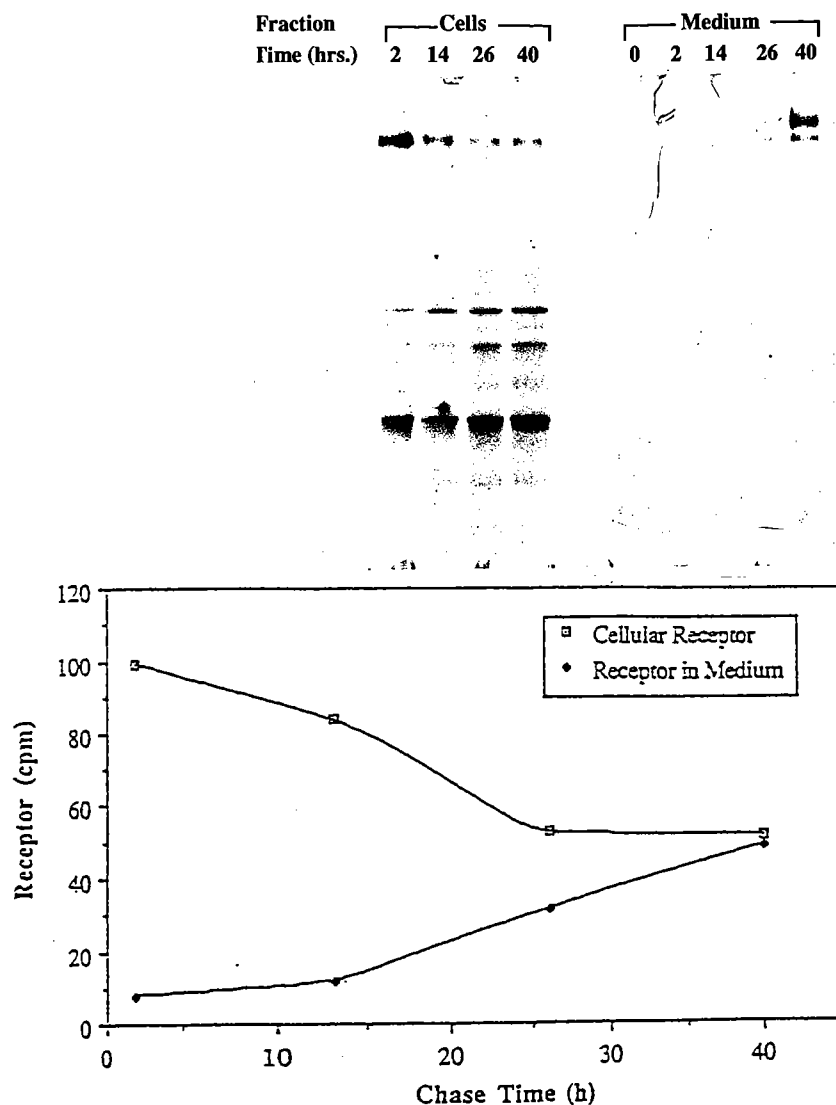
**Figure 29:** In BRL-3A Cells the Serum IGF-II/Man 6-P Receptor Quantitatively Accounts for the Loss of Cellular Receptor. BRL-3A rat liver cells were grown to 90% confluence in 35 mm plates, then labelled with 0.4 mCi EXPRE<sup>35</sup>S<sup>35</sup>S Label for one hour. Radioactive medium was then replaced with serum free DME with or without 0.2 mM cyclohexamide for the indicated times. Cells and medium were then separated and receptors purified as described in the methods. A. Total membrane samples from 0.2 mg membrane protein were electrophoresed on SDS-PAGE, stained, destained, and EN<sup>3</sup>HANCED. A representative autoradiogram is shown. B. Medium receptor from one plate was electrophoresed on SDS-PAGE, stained, destained, and EN<sup>3</sup>HANCED. A representative autoradiogram is shown. C. Quantitation of the results depicted in panels A and B by cutting of receptor regions from gels and measuring radioactivity in a scintillation counter.



**Figure 30: Certain Agents Can Alter the Rate of Serum Receptor Production.** Cells grown in 6 well multiwell plates were labelled with 150  $\mu$ Ci of EXPRE<sup>35</sup>S<sup>35</sup>S Label per well for one hour, after which this medium was removed and replaced with serum free DME (1) or DME containing 1  $\mu$ M insulin (2), 5 nM IGF-II (3), 5 mM Man 6-P and 5 nM IGF-II (4), 5 mM Man 6-P (5), 10  $\mu$ g  $\beta$ -galactosidase (6), alkaline phosphatase treated  $\beta$ -galactosidase (7), mock treated  $\beta$ -galactosidase (8), 10% fetal bovine serum (9), alkaline phosphatase treated fetal bovine serum (10), medium conditioned by BRL-3A cells for 24 hours (11), or alkaline phosphatase treated conditioned medium (12). **A.** Following a 42 hr incubation, cells were placed on ice and medium and membranes obtained. Receptors were adsorbed to anti-IGF-II/Man 6-P receptor antibody, electrophoresed on SDS-PAGE, stained, destained and EN<sup>3</sup>HANCED. **B.** The receptor bands were cut out from the gels and  $\beta$ -emission measured in a scintillation counter. The bars represent the averages of three experiments with those conditions which are consistently different than the control indicated by an asterisk.

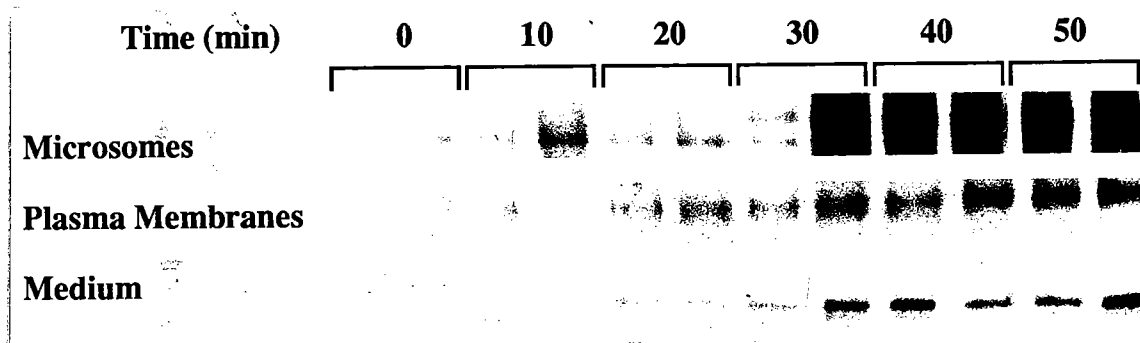


**Figure 31: The Effect of Protease Inhibitors on the Production of the Serum IGF-II/Man 6-P Receptor.** Cells were labelled with 0.5 mCi EXPRE<sup>35</sup>S<sup>35</sup>S Label for 1 hour followed by incubation in serum free DME (C) or DME containing the following: Ap-aprotinin, An-antipain, Lu-leupeptin, Bz-benzamidine, Ch-chymostatin, Ph-PMSF, CA-leupeptin, antipain, aprotinin, and benzamidine, CB-PMSF and chymostatin at the concentrations indicated in the methods for 20 h each following labelling period. The control samples were then immunadsorbed to immune or preimmune Affigel 10 while all other samples were immunoadsorbed only to the immune Affigel 10.

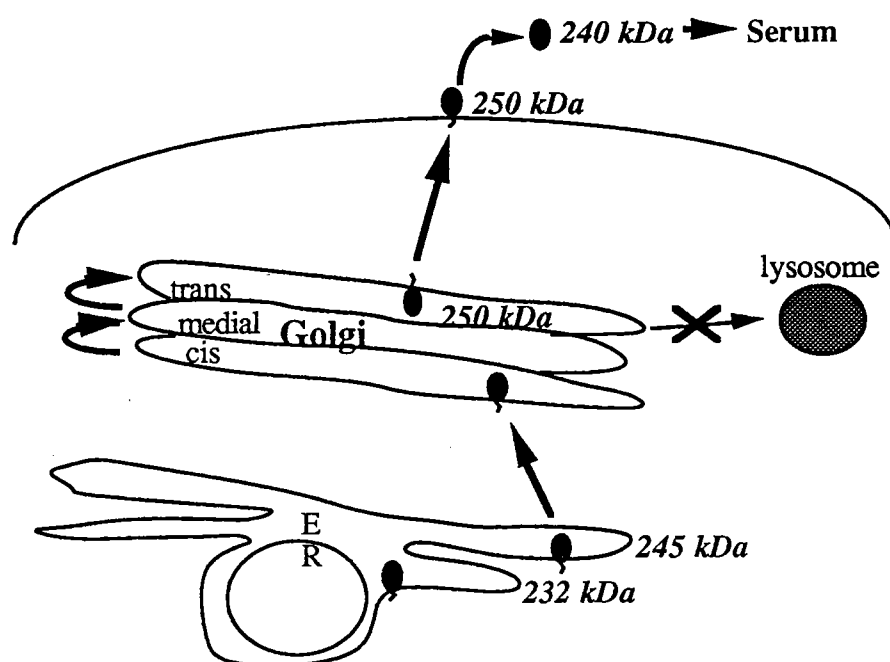


**Figure 32:** The Serum Form of the IGF-II/Man 6-P Receptor Appears in the Medium from 3T3-L1 Cells as it is Lost from the Total Membranes.

Differentiated 3T3-L1 adipocytes were labelled with 1 mCi  $^{35}\text{S}$  cysteine/150 mm plate for 1 hour. Labelling medium was then removed and the cells grown in serum free DME for the indicated times. Cells were then placed on ice, the medium removed, and the cells homogenized and total membranes obtained. Receptors were isolated from membranes or medium as indicated in the experimental procedures. A. Immunoabsorbed receptors were electrophoresed on SDS-PAGE, stained with Coomassie Brilliant Blue, and treated with EN<sup>3</sup>HANCE. B. The receptor regions indicated were cut out of the gel and counted in a scintillation counter.



**Figure 33:** The Appearance of the Receptor in the Medium Follows its Appearance at the Cell Surface. Differentiated 3T3-L1 adipocytes were labelled with 0.3 mCi  $^{35}\text{S}$ -cysteine for 30 minutes, then placed in unlabelled medium for the time indicated. Cells were then placed on ice, medium removed, and the cells homogenized and fractionated into plasma membrane and low density microsomes as described in the methods. The receptors were immunoadsorbed, electrophoresed, and EN<sup>3</sup>HANCED as described in experimental procedures.



**Figure 34: The Life Cycle of the IGF-II/Man 6-P Receptor.** The IGF-II/Man 6-P receptor is synthesized as a 2491 amino acid pre-receptor from which a 50 amino acid signal sequence is removed to give the mature peptide. The mature deglycosylated peptide migrates with an Mr~232 kDa. Glycosylation of the peptide produces a receptor with an Mr~245 kDa which is fully capable of binding ligand (MacDonald and Czech, 1985) and which is further glycosylated to the mature 250 kDa receptor form with a half time of several hours. The immature 250 kDa receptor may actively transport lysosomal enzymes from the cis Golgi to the prelysosomal compartment (Brown and Farquhar, 1984; Brown, Goodhouse, and Farquhar, 1986) or may be retained in the endoplasmic reticulum or Golgi apparatus until fully glycosylated. The fully glycosylated form may then escape from the Golgi apparatus and reach the cell surface. At the cell surface some of the receptor is proteolytically cleaved to release the serum form of the IGF-II/Man 6-P receptor while the majority is re-internalized intact where it may direct more rounds of lysosomal enzyme sorting. The serum receptor that is released may be further degraded in serum, taken up by cells, or carried in the serum to the kidneys and released in urine (Causin *et al.*, 1988).

## Chapter IV

### Discussion

#### A. Non-mammalian Man 6-P Receptors Fail to Bind IGF-II

The identification in chicken and *Xenopus* of a protein of the same molecular weight as the mammalian IGF-II/Man 6-P receptor which has high affinity for PMP-Sepharose and can be specifically eluted by mannose 6-phosphate has a number of interesting implications. These will be discussed in the following order: 1. Implications Relating to the Function of the IGF-II/Man 6-P Receptor; 2. Possible Applications of This Finding to Future Research; and 3. An Evolutionary Perspective.

##### 1. Implications Relating to the Function of the IGF-II/Man 6-P Receptor

The presence of a 250 kDa Man 6-P receptor in non-mammalian species provides a new aspect to the ongoing debate as to whether IGF-II signals through the IGF-II/Man 6-P receptor. This problem was initially discussed as **Background (I. L.)**, and will now be discussed and a conclusion reached (Table 2). That data which relies simply on the ability of IGF-II to cause a response (Hill and Milner, 1984) or upon dose response data (Beguinet *et al.*, 1985; Mellas, Gavin, and Hammerman, 1986; Tally, Li, and Hall, 1987; van Dijk, Tanswell, and Challis, 1988) as proof of signalling through the IGF-II/Man 6-P receptor provide essentially no evidence as this relationship can be explained by the modulation of effects through the insulin and IGF-I receptor by IGF binding proteins or signalling through the second site on the IGF-I receptor which preferentially binds IGF-II. The presence of such an IGF-II preferring site on the IGF-I receptor also explains the lack of inhibition of effects by the antibody  $\alpha$ IR-3 (Hari *et al.*, 1987), which blocks only binding to the first, IGF-I preferring, site (Casella *et al.*, 1986). Biological effects



**Table 2: Evidence for the IGF-II/Man 6-P Receptor Signalling.** There have been numerous publications supporting or refuting the role of the IGF-II/Man 6-P in mediating the responses of cells to IGF-II. The list that follows shows that evidence and any arguments to refute the published demonstrations of such a role of the IGF-II/Man 6-P receptor.

<u>System</u>	<u>Effect(s)</u>	<u>Evidence</u>	<u>Weakness(es)</u>	<u>Reference</u>
Costal cartilage	Increases thymidine and Sulfate incorporation	IGF-II causes response	Could be through insulin or IGF-I receptor	Hill and Milner, 1984
L6 myoblasts	Increases glucose and amino acid uptake	Ligand specificity	Effect could be through Insulin or IGF-I receptor, with specificity altered by serum IGF binding proteins.	Beguinet <i>et al.</i> , 1985
L6 myoblasts	Increases glucose transport amino acid uptake and protein synthesis	IGF-II receptor antibody blocks IGF-II binding but has no effect on IGF-II stimulated response	Single system	Kiess <i>et al.</i> , 1987
Soleus muscle	Increases amino acid uptake	IGF-I receptor antibody blocks effect.	Single system	Yu and Czech, 1984a
K562 erythroblast	Growth	Ligand specificity	(as above)	Tally, Li, and Hall, 1987
Proximal tubules	Increases Na <sup>+</sup> /H <sup>+</sup> exchange	Ligand specificity	(as above)	Mellas, Gavin, and Hammerman, 1986
Proximal tubules	Increases IP <sub>3</sub> production	Potentiation by Man 6-P	Effects of Man 6-P on other growth factor not determined.	Rogers and Hammerman, 1989
Adrenal cells	Growth	Ligand specificity	(as above)	van Dijk, Tanswell, and Challis, 1988
Fibroblasts	Increases thymidine incorporation	Antibody blocks insulin receptor	Could be through IGF-I Receptor	King <i>et al.</i> , 1980

<u>System</u>	<u>Effect(s)</u>	<u>Evidence</u>	<u>Weakness(es)</u>	<u>Reference</u>
Fibroblasts	Increases DNA synthesis	IGF-I receptor antibody blocks effect	Single system	Furlanetto, DiCarlo, and Wischart, 1987
Basolateral membranes	Phosphorylation of insulin receptor $\alpha$ subunit	Antibody blocks insulin receptor	(as above)	Hammerman and Gavin, 1984
hepatoma	Increases glycogen synthesis	Ligand specificity, not blocked by antibodies to Insulin or IGF-I receptors and mimicked by IGF-II receptor antibody.	Could be IRR or IGF-II preferring site of the IGF-I receptor. Specificity of IGF-II receptor antibody not demonstrated.	Hari <i>et al.</i> , 1987
H-35 hepatoma	Increases DNA synthesis	IGF-II receptor antibody blocks IGF-II binding but not IGF-II response.	Single system	Mottola and Czech, 1984
Balb/c 3T3	Increases $Ca^{++}$ influx and thymidine incorporation	Ligand specificity and mimicked by IGF-II receptor antibody	As above and antibody specificity not demonstrated	Kojima <i>et al.</i> , 1988 Nishimoto <i>et al.</i> , 1987a, b, and 1989.
Chick myotubes	Increases amino acid and	Cells lack IGF-II receptor	Indirect	Janeczko and Etlinger, 1984 Conover <i>et al.</i> , 1986
IGF-I receptor		$\alpha$ IR-3 fails to block IGF-II binding to IGF-I receptor.	Indirect	Casella <i>et al.</i> , 1986
IRR (Insulin receptor-like receptor)		Member of insulin receptor with unknown specificity	May or may not be relevant	Shier and Watt, 1989
Mannose 6-phosphate receptor		Non-mammalian Man 6-P receptors don't bind IGF-II	Indirect, possible that IGF binding and signalling co-evolved	Canfield and Kornfeld 1989; Clairmont and Czech, 1989
Rats		Disruption of IGF-II gene only reduces size.	limits role of IGF-II, relates indirectly to receptor	DeChiara, Efstratiadis, and Robertson, 1990

mimicked by antibodies to the IGF-II/Man 6-P receptor (Hari *et al.*, 1987; Kojima *et al.*, 1988) may be caused by contaminating antibodies to the IGF-I receptor, as both receptors can co-purify on IGF-II Sepharose.

The responses observed in Balb/c 3T3 cells have used the types of evidence refuted above to show that the effect is mediated by the IGF-II/Man 6-P receptor. This evidence is inconclusive. The responses themselves also have several possible alternative explanations. The first, most obvious explanation is that the responses are occurring through the insulin or IGF-I receptor. Such an explanation is suggested by the recent observation that insulin, like IGF-II, can inhibit the pertussis toxin catalyzed ADP-ribosylation of G-proteins (Rothenburg and Kahn, 1988; Ciaraldi and Maisel, 1989). Additionally, ligands which bind to the IGF-II/Man 6-P receptor have been shown to alter the intracellular movement of this protein (Braulke *et al.*, 1989). It is possible that such alterations could effect other receptors or the machinery involved in receptor cycling. G-proteins and calcium have been suggested to play a role in this process (Bryson and Baxter, 1987; Warren, 1989); thus an alteration in the process may also alter these parameters.

Therefore, there is no conclusive evidence that IGF-II signals through the IGF-II/Man 6-P receptor. There is considerable evidence that the IGF-II receptor isn't responsible for signalling the effects of IGF-II. First, as discussed above (I. L.) a number of responses have been directly demonstrated not to be mediated by the IGF-II/Man 6-P receptor (Mottola and Czech, 1984; Yu and Czech, 1984; Furlanetto, DiCarlo, and Wisheart, 1987; Kiess *et al.*, 1987). Second, IGF-II exists in birds and signals certain responses, although birds lack an IGF-II/Man 6-P receptor (Kasuga *et al.*, 1982b; Janeczko and Etlinger, 1984; Conover *et al.*, 1986; Bassas *et al.*, 1988). We (Clairmont and Czech, 1989a) and others (Canfield and Kornfeld, 1989) have recently demonstrated

that chickens possess a Man 6-P receptor incapable of binding IGF-II. This demonstrates that the receptor did not evolve primarily to bind IGF-II or to signal, and is not likely to serve such a purpose even in species with IGF-II/Man 6-P receptors. IGF-II most likely signals through the insulin, IGF-I, or the new, recently discovered member of the insulin receptor family (Shier and Watt, 1989). The IGF-II/Man 6-P receptor primarily serves to sort lysosomal enzymes to the lysosomes. At the cell surface it serves to scavenge lysosomal enzymes, thereby removing them from circulation and the IGF-II binding site may serve to allow the removal of IGF-II from circulation and lead to its degradation.

## 2. Possible Applications of This Finding to Future Research

An interesting line of research is suggested as a direct extension of the demonstration that mammalian Man 6-P receptors possess a binding site for IGF-II, while non-mammalian receptors do not. The cloning and sequencing of the cDNA of a nonmammalian Man 6-P receptor may suggest that a domain lacking from the nonmammalian receptor but present in the mammalian receptor as the binding site for IGF-II. Such a putative binding domain could be tested directly by mutagenesis in this interesting region of the receptor. Whether or not such an obvious sequence difference exists, a non-mammalian receptor will be a useful tool as chimeras can be made between mammalian and non-mammalian Man 6-P receptors to confer IGF-II binding to a non-mammalian Man 6-P receptor. Such a system will allow the identification of the IGF-II binding site. A similar system has recently been used to localize the epidermal growth factor binding site on its receptor (Lax *et al.*, 1988; Lax *et al.*, 1989).

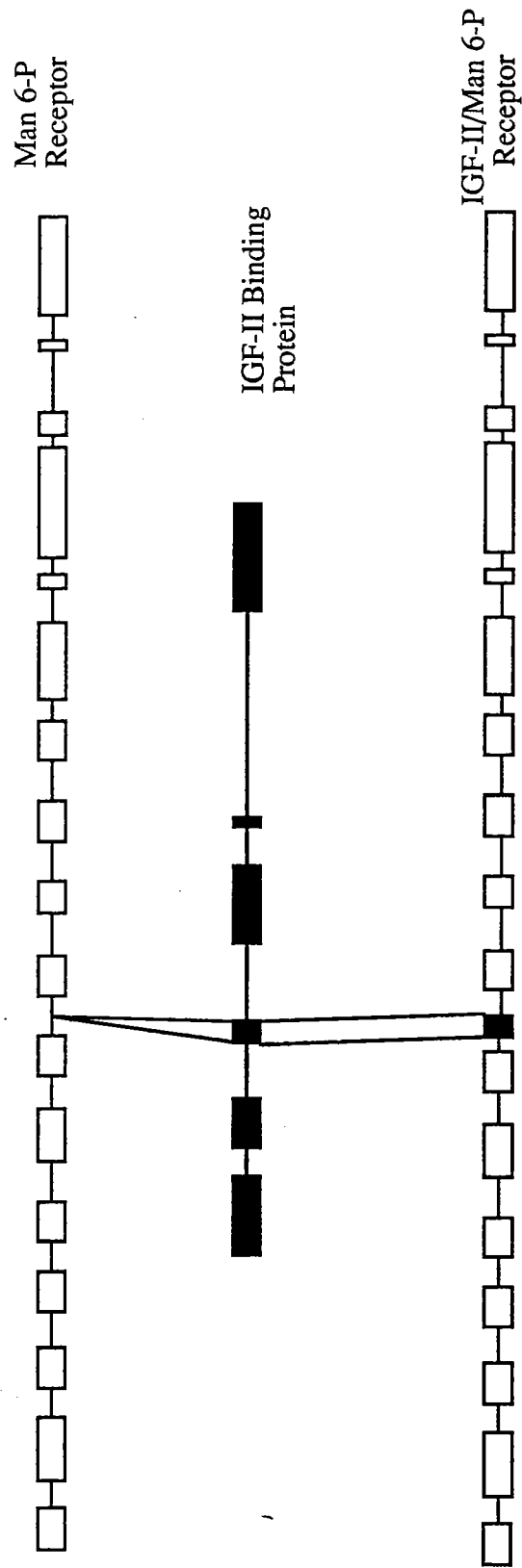
Subsequent cloning and sequencing of the gene for the mammalian and non-mammalian Man 6-P receptor in the region of the binding site can give insight into the evolution of function. Specifically, a popular theory holds that exons contain the

information to code for structural or functional regions, and that these may be moved and copied among genes for proteins to confer such a structural or functional domain on a new protein. Introns are simply regions of noncoding information which separate exons and facilitate such movement by allowing exons to move freely without interrupting other coding regions. There has been, however, no direct proof for this theory. If the Man 6-P receptor gained the ability to bind IGF-II by gaining an exon from an IGF-II binding protein, this would provide the first direct evidence for such an occurrence (Figure 35).

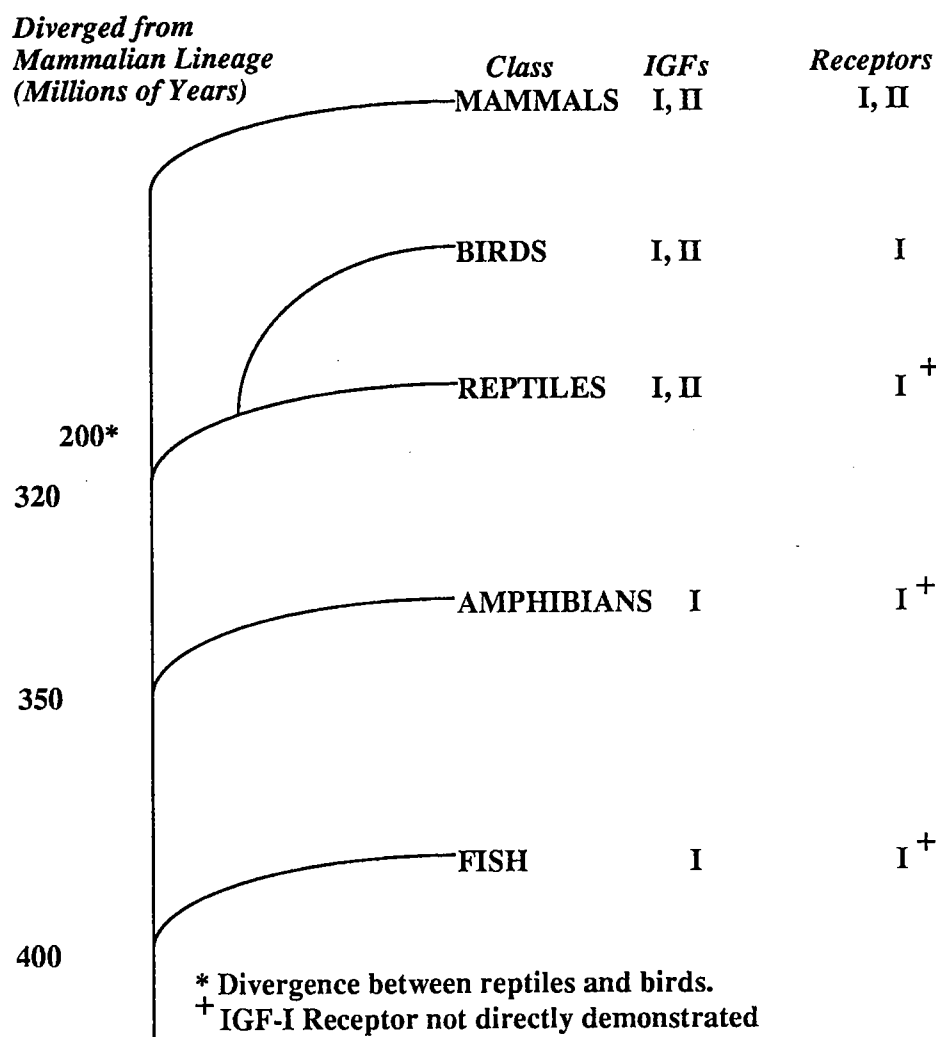
### 3. An Evolutionary Perspective.

According to Darwin's theory of evolution (Darwin, 1859), natural selection will serve to retain favorable traits and eliminate unfavorable ones. For this reason we generally assume that a conserved function or sequence is important to the organism. By this consideration, we would expect the IGF-II binding capacity to play an important role in mammals. What could this function be?

One possibility, mentioned above, is that the primary purpose of the binding of IGF-II by the IGF-II/Man 6-P receptor is to degrade IGF-II. Under what circumstance would this be so important of a function that it would be retained in all mammals? In vertebrates, there is considerable variation in the IGFs observed and the receptors that exist for them. Specifically, fish and amphibians possess only IGF-I, higher vertebrates both IGF-I and IGF-II (Daughaday *et al.*, 1985; Engstrom, Bell, and Schofield, 1987; Dawe *et al.*, 1988). Mammals possess both IGF-I and IGF-II/Man 6-P receptors, while all lower vertebrates possess only IGF-I receptors (Canfield and Kornfeld, 1989; Clairmont and Czech, 1989). This leaves us with two phyla, reptiles and birds, which possess IGF-II but no specific receptor for this molecule (Figure 36).



**Figure 35: Addition of an IGF-II Binding Domain by Exon Shuffling.** As discussed in the text, it has been hypothesized that proteins gain structural and functional regions by exchange of exons (Gilbert, 1978). Shown is a cartoon depicting such a mechanism for the acquisition of IGF-II binding capacity by the Man 6-P receptor. Another possibility not depicted is random mutations of the Man 6-P receptor to acquire an IGF-II binding site. These possibilities could be differentiated based on a comparison of the cDNA and genomic sequences surrounding the binding site in mammals and non-mammals.



**Figure 36: The Evolution of IGFs and IGF Receptors.** IGF-I has been found in all vertebrate species examined, while IGF-II exists only in reptiles, birds, and mammals (Daughaday *et al.*, 1985; Engstrom, Bell, and Schofield, 1987; Dawe *et al.*, 1988). The IGF-I receptor, likewise, is present in all vertebrates which have been tested (Janeczco and Etlinger, 1984; Conover *et al.*, 1986), while the IGF-II/Man 6-P receptor exists only in mammals (Canfield and Kornfeld, 1989; Clairmont and Czech, 1989). This information suggests a duplication of the IGF gene upon the divergence of reptiles from amphibians, and the acquisition of IGF-II binding capacity by the Man 6-P receptor upon the divergence of mammals from reptiles and birds.

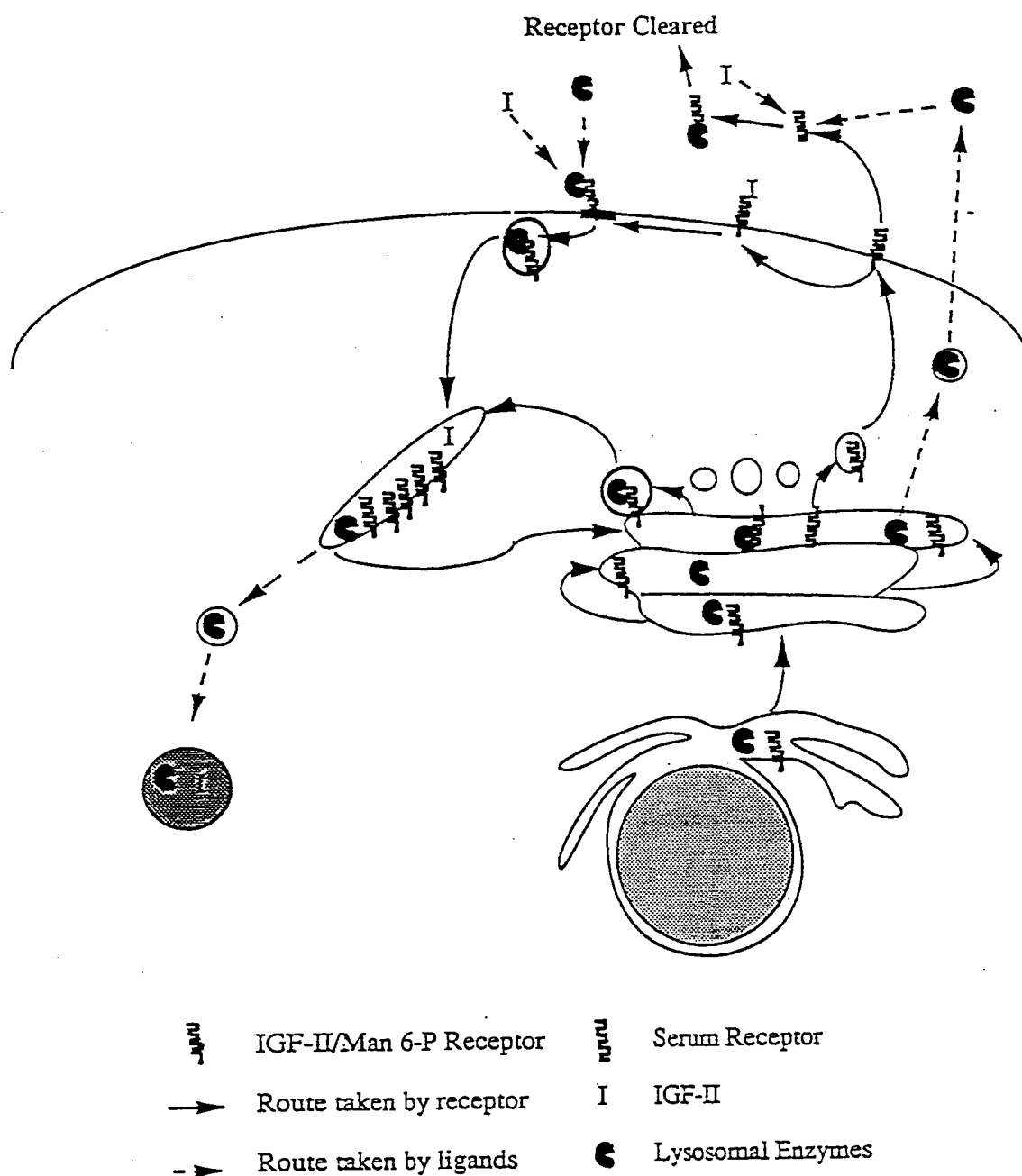
This restricts the role of the IGF-II binding capacity of the Man 6-P receptor to a secondary role: not only was this function added to a functional Man 6-P receptor, but it evolved after IGF-II. The question remains as to why the possession of an IGF-II molecule and its receptor which serves only as a degradative route has been conserved. One possibility is suggested by the recent disruption of the IGF-II gene in mice (DeChiara, Efstratiadis, and Robertson, 1990) which induces reduced size. Perhaps when IGF-II initially evolved it caused a significant increase in the size of the animals possessing it, most likely early reptiles. Perhaps after some time, there was a niche formed for small, highly evolved animals. Such a selective pressure would be expected to give rise to mechanisms to remove IGFs from circulation. One solution would be the evolution of a receptor to degrade these growth factors. While such an idea would explain the pattern of IGFs and receptors we see in animals today, there is no strong evidence in support of this idea, and a number of other hypothesis could be imagined.



## B. The Serum Form of the IGF-II/Man 6-P Receptor

The majority of the studies discussed in this manuscript have dealt with the serum form of the IGF-II/Man 6-P receptor. These studies have directly addressed its origin in culture, and its structure, regulation, and functionality in circulation. These studies have demonstrated that the serum receptor is a product of the cellular receptor in a number of diverse cultured cell lines. This protein released from cultured cells, like the serum receptor in circulation, is truncated as compared to the cellular receptor as determined by binding of an antibody directed against a cytoplasmic peptide to these proteins. The serum receptor also retains the ability to bind both types of ligand: IGF-II and lysosomal enzymes possessing the mannose 6-phosphate recognition marker. Finally, insulin is able to acutely increase the levels of receptor in serum and to increase the rate of release of receptor from cultured cells. In cultured cells this increase in release correlates with an increase in the cell surface concentration of receptors.

A possible problem with the data demonstrating that the serum form of the IGF-II/Man 6-P receptor is regulated by insulin involves a lack of knowledge of the occupancy state of the receptors in serum before and after insulin treatment. In the cultured cell system, immunoadsorption showed some dependence upon the occupancy state as mannose 6-phosphate increased the proportion of IGF-II/Man 6-P receptors immunoadsorbed. However, while in the presence of Man 6-P the immunoadsorption was essentially quantitative, approximately 90% of receptors were immunoadsorbed even in its absence. It is therefore unlikely that changes in ligand occupancy of the IGF-II/Man 6-P receptor could account for the significantly larger changes observed in the amount of receptors immunoadsorbed from the serum of diabetic, control, and insulin treated rats.



**Figure 37: The Serum IGF-II/Man 6-P Receptor.** The serum receptor is generated by proteolytic cleavage from the cellular receptor at the plasma membrane and leaves behind an ~10 kDa cytoplasmic fragment. The receptor in serum can then bind IGF-II and lysosomal enzymes in serum and remove them. The cell surface IGF-II/Man 6-P receptor may also play a role in the clearance of these ligands from circulation.

From this data, a model can be proposed to explain the generation of the serum receptor from the cellular IGF-II/Man 6-P receptor (Figure 37). The IGF-II/Man 6-P receptor is located primarily in intracellular locations. At any time, however, approximately 5-10% of receptor is at the cell surface. This population is available to a plasma membrane protease which cleaves the receptor to release the extracellular domain of the receptor as the serum receptor. The sorting of the remaining cytoplasmic piece is then altered such that it is sorted to lysosomes for degradation. Such sorting of this isolated cytoplasmic domain would be consistent with the observation that the IGF-II/Man 6-P receptor cytoplasmic domain is not capable of directing the proper sorting of an EGF receptor extracellular domain in a chimera of these proteins (Dintzis and Pfeffer, 1990).

The cleavage of the receptor from the cell surface is consistent with all of the available data: the degradation rate is increased by insulin which increases the cell surface concentration of receptors and by small ligands for the receptor which would be expected to alter the conformation of the receptor. Larger ligands, however, inhibit the degradative process as do certain protease inhibitors. Finally, in the shorter pulse chase studies it is possible to follow receptor from inside the cell, to the plasma membrane, and then into the medium. This lag is consistent with the proposed model, but not with serum and plasma membrane receptors travelling in the same vesicles to the cell surface. It is possible that the serum receptor reaches the cell surface through an alternate route, but, given that it is a soluble protein, it would be expected to move by bulk flow. If this is the case, the membrane bound receptor could reach the cell surface no sooner than the medium receptor.

Once released, the serum receptor circulates as a functional binding protein capable of binding both IGF-II and mannose 6-phosphate containing ligands. The role of the

receptor in binding IGF-II in circulation has been assessed to be minimal in most species, being significant only in sheep fetus (Gelato *et al.*, 1989) or monkey (Gelato *et al.*, 1988).

On the other hand, the role of the serum receptor in binding lysosomal enzymes has not been well evaluated. Lysosomal enzymes have been shown to exist in circulation (Belfiore *et al.*, 1974; Lombardo *et al.*, 1980). It is of interest that their levels are significantly increased in diabetes (Belfiore *et al.*, 1974; Poon *et al.*, 1979; Kohler, Sheth, and Good, 1981; Miralles *et al.*, 1982; Perdichizzi *et al.*, 1983) and that their levels have been correlated with complications of this disease (Pitkanen *et al.*, 1980; Goi *et al.*, 1986; Goi *et al.*, 1987)). It is certainly possible, if not likely, that circulating lysosomal enzymes could play a significant role in causing such complications as retinopathy and other types of vascular and kidney degeneration. From this information and data presented in this thesis it is possible to propose a hypothesis to explain the elevated lysosomal enzyme levels in diabetes. The sorting of lysosomal enzymes by the IGF-II/Man 6-P receptor is an imperfect process. Some lysosomal enzymes fail to bind to the IGF-II/Man 6-P receptor and escape from the cell. The sorting of the IGF-II/Man 6-P receptor is also imperfect and some receptors reach the cell surface. Receptors at the cell surface can then serve to bind and re-internalize escaped lysosomal enzymes.

Insulin increases the concentration of IGF-II/Man 6-P receptors at the cell surface and in circulation, and will therefore be expected to increase the rate of clearance of lysosomal enzymes. In diabetes, the serum receptor level, and likely the cell surface concentration as well, is decreased. This would be expected to result in a decrease in the rate of clearance of lysosomal enzymes and an increase in their circulating level, as has been observed. Such a model could be tested by measuring the effects of mannose 6-

phosphate and anti-IGF-II/Man 6-P receptor antibodies on the levels of circulating lysosomal enzymes in control and insulin treated animals.

The lysosomal enzyme level has been proposed as an acute measure of insulin in diabetics to be used in measuring metabolic control (Goi *et al.*, 1986; Goi *et al.*, 1987). The serum IGF-II/Man 6-P receptor levels are much more acutely regulated than blood glucose or lysosomal enzymes. This provides a possible explanation for the lysosomal enzyme levels and could provide a more sensitive and more mechanistically understood measure of metabolic control in diabetics. Another serum receptor, the serum form of the transferrin receptor, has recently proven very useful in the diagnosis of anemias. Such a role could develop for serum IGF-II/Man 6-P receptor levels in diabetes, especially as an extremely acute measurement to fine tune the insulin treatment in diabetics.

### C. Conclusions

The Man 6-P receptor evolved primarily as a protein to sort lysosomal enzymes to a prelysosomal compartment and to repeat this cycle many times without reaching the lysosome where it, too, would be degraded. As a consequence of such an efficient sorting away from the lysosome, this receptor cannot be degraded by that pathway. Instead some of the receptor is improperly sorted in the Golgi apparatus and reaches the cell surface instead of the prelysosomal compartment. IGF-II/Man 6-P receptor at the cell surface can then be proteolysed to release the serum receptor. The cell surface IGF-II/Man 6-P receptors also serve to remove lysosomal enzymes from circulation. This serves both to protect the body from these proteins and to scavenge lysosomal enzymes needed by the cell. This scavenging has been demonstrated in female carriers of Hunter disease, an X-linked loss of iduronate sulfatase. Those cells unable to produce this enzyme scavenge enzyme released from producing cells into circulation.

The released serum receptor is eventually cleared from circulation and has been observed in urine. While in circulation the receptor binds lysosomal enzymes. The serum and cell surface receptors may also play a role in removing IGF-II from circulation in mammals, as has been discussed in a previously. The mammalian IGF-II/Man 6-P receptor is unlikely to signal responses to IGF-II, but may modulate responses to IGF-II through the alteration of circulating levels by binding and removing ligand from the blood.

In conclusion, the IGF-II/Man 6-P receptor serves primarily to sort and scavenge lysosomal enzymes. The receptor's ability to bind IGF-II serves only in a clearance and degradative route for this protein. The IGF-II/Man 6-P receptor binding of IGF-II may,

however, modulate signalling responses mediated by the insulin, IGF-I, or other unidentified IGF receptor.

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