laser techniques to at least 100 GPa [C. S. Zha *et al.*, *Eos* **67**, 1216 (1986)].
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# A Single Receptor Binds Both Insulin-Like Growth Factor II and Mannose-6-Phosphate

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**Amino acid sequences deduced from rat complementary DNA clones encoding the insulin-like growth factor II (IGF-II) receptor closely resemble those of the bovine cation-independent mannose-6-phosphate receptor (Man-6-P receptor$^{CI}$), suggesting they are identical structures. It is also shown that IGF-II receptors are adsorbed by immobilized pentamannosyl-6-phosphate and are specifically eluted with Man-6-P. Furthermore, Man-6-P specifically increases by about two times the apparent affinity of the purified rat placental receptor for $^{125}$I-labeled IGF-II. These results indicate that the type II IGF receptor contains cooperative, high-affinity binding sites for both IGF-II and Man-6-P–containing proteins.**

THE INSULIN-LIKE GROWTH FACTORS (IGF) I and II exhibit substantial sequence homology to the hormone insulin, which regulates important metabolic effects in humans and other animals (*1*). The IGF peptides mimic insulin action on cellular transport and metabolic pathways and, in many cell types, enhance cell proliferation more potently than insulin (*1*). Three major cell surface receptors bind these peptides with overlapping specificities (*2*). Insulin and IGF-I receptors, denoted as type I, bind their respective ligands with high affinity (dissociation constant, $K_d = 1$ n*M*), but also bind both heterologous peptides with lower affinity. The type I receptors possess heterotetrameric $\alpha_2\beta_2$ subunit structures (*3*) and intrinsic ligand-activated tyrosine kinase activities (*4*) that are essential for their biological functions (*5*). In contrast, the type II IGF receptor preferentially binds IGF-II ($K_d = 1$ n*M*) with higher affinity than IGF-I, but has no affinity for insulin. This receptor consists of a single polypeptide chain (*2, 6*) lacking detectable kinase activity (*7*), and its physiological role is unclear.

In this study of IGF-II receptor function, we obtained amino acid sequences of 14 tryptic peptides derived from the affinity-purified rat placental receptor and synthesized oligonucleotide probes based on two of these sequences (*8*). Several overlapping clones encoding the receptor were isolated from a complementary DNA (cDNA) library prepared from total polyadenylated rat placental RNA (*9*). The composite sequence of 7470 bp contains a 6180-bp open reading frame coding for 2060 amino acids followed by a 1290-bp 3′ noncoding region. The cDNA sequence encodes 11 of the 14 chemically identified receptor peptides, but is still incomplete because the reading frame extends to the extreme 5′ end. Comparison of our deduced rat IGF-II receptor sequence with other proteins contained within a laboratory protein data bank revealed a remarkably high degree of identity (79%) with the recently described sequence of the bovine cation-independent mannose-6-phosphate receptor (Man-6-P receptor$^{CI}$) (*10*).

Several interesting structural features are revealed upon comparison of the deduced amino acid sequences of the putative cytoplasmic domains of the rat IGF-II receptor, the human IGF-II receptor (*11*), and the bovine Man-6-P receptor$^{CI}$ (*10*). These sequences are 167, 164, and 163 amino acids long, respectively, with an overall 58% sequence identity (Fig. 1). Five regions (Fig. 1, boxed and numbered 1 to 5) show a high degree of sequence conservation among the three species (91% identity compared with 38% for the remaining segments). The five highly conserved regions contain sequences that suggest important functional domains. Region 1, adjacent to the transmembrane sequence, is highly charged as expected for a cytoplasmic stop transfer sequence. Region 2 contains a sequence with the potential to be a substrate for protein kinase C or adenosine 3′,5′-monophosphate (cAMP)–dependent protein kinase, or both. Region 3 is a highly acidic domain analogous to those of several other receptors [for example, the low density lipoprotein (LDL), transferrin, and asialoglycoprotein receptors] that occur at about the same distance from the transmembrane domain (*12*). This region also contains potential tyrosine and serine phospho-

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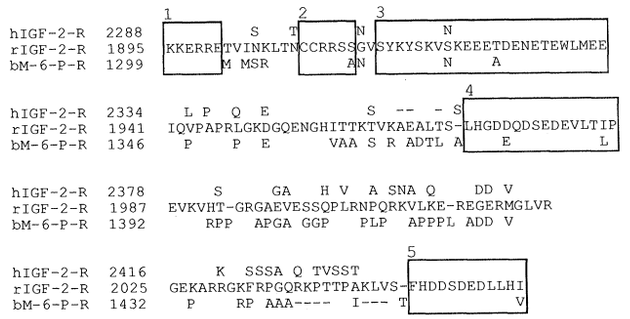


**Fig. 1.** Alignment of the cytoplasmic domains of the rat, human, and bovine IGF-II/Man-6-P receptors. The deduced amino acid sequences corresponding to the putative cytoplasmic domains of the rat (this report), human [Morgan *et al.* (*11*)], and bovine [Lobel *et al.* (*10*)] receptors have been aligned for optimal sequence identity. Only differences with the rat sequence are shown. Regions of highly conserved sequence are boxed and numbered 1 to 5 (see text for details).

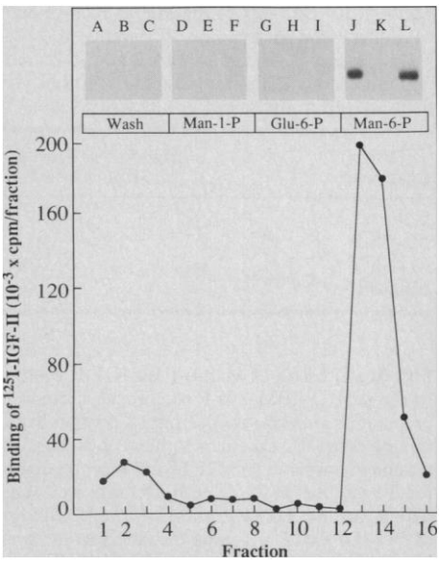


**Fig. 2.** Chromatography of the type II IGF receptor on immobilized pentamannosyl-6-phosphate. Rat placental plasma membranes were solubilized in 50 m*M* Hepes, *p*H 7.4, 0.15*M* NaCl, 5 m*M* β-glycerophosphate, 0.05% Triton X-100 (1.5 mg of protein in 3 ml). This extract was incubated for 1 hour at 3°C with 1 ml of pentamannosyl-6-phosphate–Sepharose (*26*). After collecting the flow-through material, the column was washed with 1 ml of buffer and then eluted sequentially with four 1-ml portions each of buffer containing 5 m*M* mannose-1-phosphate (Man-1-P), glucose-6-phosphate (Glu-6-P), or Man-6-P (see bar). Fractions (1 ml) were collected, and duplicate 0.1-ml portions were assayed for $^{125}$I-IGF-II binding activity (*8*). (Top) Affinity–cross-linking of $^{125}$I-IGF-II to material eluted from the column. Portions (50 μl) of pooled fractions 1 to 4 (wash, lanes A to C), fractions 5 to 8 (Man-1-P, lanes D to F), fractions 9 to 12 (Glu-6-P, lanes G to I), or fractions 13 to 16 (Man-6-P, lanes J to L) were incubated for 2 hours at 3°C with 3 n*M* $^{125}$I-IGF-II alone (lanes A, D, G, and J) or with 300 n*M* unlabeled IGF-II (lanes B, E, H, and K) or 300 n*M* insulin (lanes C, F, I, and L). The samples were treated with 0.25 m*M* disuccinimidyl suberate for 30 minutes at 3°C and then subjected to electrophoresis on SDS-PAGE. An autoradiogram of the dried gel shows the $M_r$ 250,000 band specifically eluted by Man-6-P. Labeling of this band is displaced by excess IGF-II but not insulin, consistent with the properties of the high-affinity type II IGF receptor (*2*).

rylation sites and one conserved threonine that might serve as a casein kinase I substrate.

Regions 4 and 5 (Fig. 1) contain multiple acidic residues surrounding single serines that should be excellent substrates for casein kinases I and II (*13*). These conserved phosphorylation sites may relate to the mechanism whereby rapid redistribution of IGF-II receptors from intracellular membranes to cell-surface membranes occurs in response to insulin (*14, 15*). Associated with this redistribution, insulin causes a rapid decrease in the $^{32}$P specific activity of the receptor present in plasma membranes derived from $^{32}$P-labeled adipocytes (*7, 16*). Although indirect evidence suggests that the insulin-regulated phosphorylation sites on the IGF-II receptor are specific for casein kinase II or a similar kinase (*17*), it is not known whether these phosphorylation sites are critical for regulation of IGF-II receptor sorting by insulin.

The high degree of sequence identity between the rat and human IGF-II receptor and bovine Man-6-P receptor$^{CI}$ observed by us and Morgan *et al.* (*11*) suggests that the same receptor binds both IGF-II– and Man-6-P–containing proteins. We therefore subjected IGF-II receptors in a Triton X-100 extract of rat placental plasma membranes to chromatography on immobilized pentamannosyl-6-phosphate (Fig. 2). Over 70% of the $^{125}$I-IGF-II binding activity was retained by the affinity resin and was specifically eluted by 5 m*M* Man-6-P but not by the related sugar phosphates Man-1-P or Glu-6-P. Analysis of the Man-6-P–eluted material in fractions 13 to 16 by affinity–cross-linking with $^{125}$I-IGF-II confirmed the presence of the $M_r$ 250,000 type II IGF receptor (Fig. 2, top). Thus, binding sites for both IGF-II and Man-6-P oligosaccharides coexist in a single receptor.

The presence of binding sites for different ligands on the IGF-II/Man-6-P receptor suggests the possibility that occupancy by one of the ligands alters binding of the other ligand. We therefore measured $^{125}$I-IGF-II binding to receptor in plasma membranes, microsomes, or Triton X-100 membrane extracts, in the presence or absence of Man-6-P or related sugars (Fig. 3A). Man-6-P (2 m*M*) almost doubled the binding of a subsaturating concentration of $^{125}$I-IGF-II to the receptor, whereas several other sugar derivatives had no such effect.

One possible explanation for these results is that Man-6-P displaces endogenously bound lysosomal enzymes that might sterically block the IGF-II binding site. To test this hypothesis, we incubated plasma membranes with Man-6-P, then washed them three times and assayed them for $^{125}$I-IGF-II binding (Table 1). Washing the membranes to remove Man-6-P and possible endogenous Man-6-P–containing proteins readily reversed the effect of Man-6-P on $^{125}$I-IGF-II binding. Addition of Man-6-P to the washed membranes again stimulated $^{125}$I-IGF-II binding. Thus, enhancement of $^{125}$I-IGF-II binding depends on the presence of Man-6-P.

To determine whether Man-6-P stimulates IGF-II binding by direct interaction with the receptor, we examined its effect on rat placental IGF-II/Man-6-P receptors that had been affinity-purified on immobilized IGF-II. Figure 3B depicts $^{125}$I-IGF-II binding isotherms obtained by incubating the partially purified receptor with increasing concentrations of $^{125}$I-IGF-II (2 to 500 n*M*). The data were analyzed by the method of Scatchard (*18*). Man-6-P at a concentration of 4 m*M* reduced the apparent equilibrium dissociation constant for $^{125}$I-IGF-II binding to the receptor without changing the apparent number of available IGF-II binding sites. Dissociation constants calculated from three experiments were 2.4 ± 0.2 (± SEM) n*M* for control as opposed to 1.2 ± 0.2 n*M* for Man-6-P–treated receptor. These results provide further evidence that the two ligands bind to independent sites on the same receptor and suggest positive cooperativity between these sites.

The conclusion that the authentic IGF-II receptor and Man-6-P receptor$^{CI}$ are the same glycosylated polypeptide is consistent with biochemical analyses of these receptors in many laboratories that have yielded similar results in terms of molecular size, electrophoretic mobility, disulfide bridge content, primarily intracellular location, and widespread occurrence in many cell types (*6, 19–21*). Furthermore, antibody-induced down-regulation of the Man-6-P receptor$^{CI}$ has been shown to lead to secretion of lysosomal hydrolases (*22*), consistent with its presumed biological function. Similarly, we showed that enhanced cellular uptake and degradation of IGF-II is associated with modulation of IGF-II receptor recycling by insulin (*14*). Morgan *et al.* (*11*) and we have also observed that antibodies raised against

**Table 1.** Reversibility of the Man-6-P effect on $^{125}$I-IGF-II binding to plasma membranes. Membranes were incubated initially for 30 minutes at 22°C with or without 5 m*M* Man-6-P as described in the legend to Fig. 3. The membranes were collected by centrifugation, resuspended in wash buffer with or without Man-6-P, then incubated for 1 hour. This step was repeated twice. Binding of $^{125}$I-IGF-II was then assayed in the samples as described (*14*). Values are mean ± SEM, $n$ = 3.

| Initial treatment | Man-6-P in wash | Man-6-P in assay | $^{125}$I-IGF-II binding (cpm) |
|---|---|---|---|
| Control | − | − | 6,940 ± 200 |
| Man-6-P | + | + | 10,470 ± 390 |
| Man-6-P | − | − | 7,570 ± 300 |
| Man-6-P | − | + | 10,300 ± 110 |

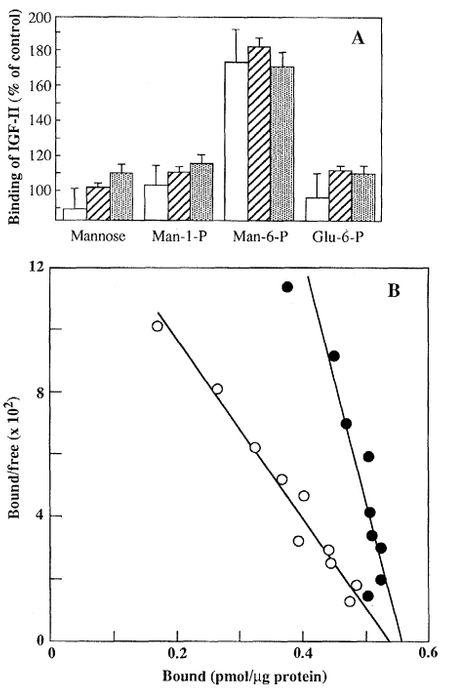


**Fig. 3.** (**A**) Effect of Man-6-P on IGF-II binding to the rat IGF-II/Man-6-P receptor in membrane preparations. Portions (0.2 mg of protein in 0.2 ml) of rat placental microsomes (open bars) or plasma membranes (hatched bars) were incubated for 30 minutes at 22°C with the indicated sugar derivatives at a concentration of 2 m*M*. Binding of $^{125}$I-IGF-II (2 n*M*) was measured in the presence and absence of 200 n*M* unlabeled IGF-II for an additional 30 minutes at 22°C as described previously (*14*). Portions (0.15 mg of protein in 0.2 ml) of a Triton X-100 extract of plasma membranes (stippled bars) were incubated with the sugar derivatives (2 m*M*) for 1 hour at 3°C, and then assayed for $^{125}$I-IGF-II binding (*8*). Values for $^{125}$I-IGF-II specifically bound relative to controls are expressed as mean ± SEM where $n$ = 9 for microsomes or plasma membranes, and $n$ = 6 for Triton X-100 extracts. (**B**) Scatchard analysis of $^{125}$I-IGF-II binding to affinity-purified IGF-II/Man-6-P receptor. Rat placental IGF-II receptor was purified by IGF-II–Sepharose chromatography as described (*8*). Portions (0.2 μg of protein in 0.2 ml) were incubated with 2 n*M* $^{125}$I-IGF-II with increasing concentrations of unlabeled IGF-II (0 to 500 n*M*) for 16 hours at 3°C in the presence (●) or absence (○) of 4 m*M* Man-6-P and assayed for $^{125}$I-IGF-II binding as described (*8*). Equilibrium binding data were analyzed by the method of Scatchard (*18*).

the respective affinity-purified receptors cross-react. However, it should be noted that the data we present here do not rigorously exclude the possibility that the IGF-II/Man-6-P receptor is a multimeric complex of two or more very similar but nonidentical, noncovalently associated subunits.

What might be the biological significance of a single receptor binding IGF-I–, IGF-II–, and Man-6-P–containing proteins? The general structure of the IGF-II/Man-6-P receptor is similar to that of other cell surface receptors having transport rather than signaling functions, such as the LDL receptor, the transferrin receptor, and the asialoglycoprotein receptor (*12*). All four receptor species recycle rapidly between intracellular compartments and the cell surface (*21, 23*). The primary intracellular destination of these receptors is an acidic endosome (*23*). Ligands segregate from their respective receptors in this organelle and are ultimately directed to lysosomes for storage (lysosomal enzymes) or degradation (LDL, asialoglycoproteins). These observations are consistent with our hypothesis that a major function of the IGF-II/Man-6-P receptor is to mediate internalization and processing of IGF-I and IGF-II (*14*). It is likely that the routing of the IGF-II/Man-6-P receptor among cellular compartments during recycling must fulfill the biological requirements of both IGFs and lysosomal enzymes. It will be important to test whether this receptor binds still other ligands.

Could a multifunctional receptor play a major role in targeted sorting of IGFs and lysosomal hydrolases as well as transduce signals across the plasma membrane? The IGF-II receptor fails to mediate certain biological responses in H-35 hepatoma cells and L6 myoblasts (*24*). However, other investigators claim modulation of cellular ion flux and glycogen synthesis by IGF-II via the type II receptor (*25*). Thus, the issue of whether the type II IGF receptor mediates the biological effects of IGF-II through a signal transduction mechanism is unresolved. The availability of IGF-II/Man-6-P receptor cDNA clones reported here should facilitate new experimental approaches toward clarifying the biological roles of this multiligand binding receptor.

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# Estrogen-Dependent in Vitro Transcription from the Vitellogenin Promoter in Liver Nuclear Extracts

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**One approach to analyzing the molecular mechanisms of gene expression in vivo is to reconstitute these events in cell-free systems in vitro. Although there is some evidence for tissue-specific transcription in vitro, transcriptionally active extracts that mimic a steroid hormone–dependent enhancement of transcription have not been described. In the study reported here, nuclear extracts of liver from the frog *Xenopus laevis* were capable of estrogen-dependent induction of a homologous vitellogenin promoter that contained the estrogen-responsive element.**

IN FEMALE FROGS OF THE SPECIES *Xenopus laevis*, the vitellogenin genes that encode the precursor of the yolk proteins are activated in the liver by estrogen, whereas in the males the vitellogenin genes are normally silent (*1*). However, hormone treatment of males leads to both an increase in estrogen receptor and a strong de novo activation of vitellogenin transcription (*2*). This activation can be mimicked in a variety of cell lines and in *Xenopus* oocytes, provided that both the estrogen receptor and the *Xenopus* target sequences, the estrogen-responsive element (ERE), are present (*3, 4*). Since these earlier studies did not address the regulation of the genes in their natural context, we prepared extracts of nuclei isolated from *Xenopus* liver and assayed their ability in vitro to sustain hormone-dependent transcription from the vitellogenin B1 cloned promoter (*5*).

We first tested a nuclear extract (*6*) prepared from adult female *Xenopus* livers, which express the vitellogenin genes and thus are most likely to yield a transcriptionally active extract. The template consisted of the vitellogenin B1 gene 5′ flanking sequences from −596 to +8 fused to the chloramphenicol acetyltransferase (CAT) reporter gene. This construct is inducible by estrogen after transfer into a hormone-responsive human cell line (*3*). We assayed its transcription by primer extension. The amount of extract and the amount of DNA in the transcription assays were optimized in the presence of 17β-estradiol (Fig. 1). The vitellogenin-specific extension product appeared at a range of concentrations but the optimum was at 1.4 mg of protein per milliliter with three extracts tested (Fig. 1A). Furthermore, optimal transcription was at 1000 ng of specific template (Fig. 1B). Thus the ratio of protein to the DNA template in the reaction must be carefully maintained to obtain efficient transcription, a requirement reported previously for other in vitro transcription systems (*6, 7*). Vitellogenin transcription was inhibited by α-amanitin (0.5 μg/ml) and was thus due to RNA polymerase II.

The crucial question was whether the observed transcription from the vitellogenin promoter was dependent on the estradiol added to the assay. We therefore tested the transcription of the same template as above in the absence or presence of hormone. We also analyzed a second template in which the ERE had been deleted (*3*) (Fig. 2B). As an estrogen-independent internal control, we included a chimeric gene in which the SV40 early promoter was linked to the CAT gene (*8*) (Fig. 2B). Very little transcription of either vitellogenin template occurred in the

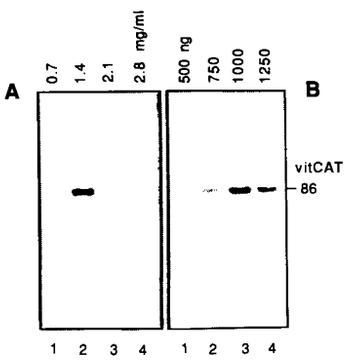


**Fig. 1.** Optimization of transcription conditions. (**A**) Effect of nuclear extract concentrations on transcription in vitro. Transcriptions were performed in a total reaction volume of 20 μl containing increasing amounts of the extract. Numbers at the top of the figure indicate the final protein concentration in each reaction. The amount of specific DNA template was 750 ng per reaction. The template was the pB1(5′/−596+8)CAT8+ construct (Fig. 2B). The size (86 nucleotides) of the correctly initiated transcripts, measured by primer extension, is indicated. (**B**) Effect of specific template concentrations on transcription in vitro. Assays were performed with increasing amounts of pB1(5′/−596+8) CAT8+ DNA brought to a constant total amount of 1250 ng per reaction with plasmid DNA. The nuclear extract concentration gave a final protein concentration of 1.4 mg/ml in each reaction. The numbers above the lanes indicate the amount of specific template. Transcription was assayed by primer extension. *Xenopus laevis* adults were induced by two injections of 1 mg of 17β-estradiol into the dorsal lymph sac with an interval of 3 days between the two injections, or they were left unstimulated. They were killed 1 day after the final injection. Extracts of liver nuclei were prepared essentially as described (*6*), except that the livers were perfused with 75% phosphate-buffered saline containing 0.5 mg of heparin per milliliter, and the homogenization buffer contained 2.4*M* sucrose and 15 μg of aprotinin per milliliter. Possible residual estradiol in the extracts was eliminated during the dialysis in the final step of the preparation. For in vitro transcription the standard reaction consisted of the following components (final volume, 20 μl): 33 m*M* Hepes (*p*H 7.9), 60 m*M* KCl, 6 m*M* $MgCl_2$, 5 m*M* creatine phosphate, 0.6 m*M* nucleoside triphosphates, 10% glycerol, 0.6 m*M* dithiothreitol, 0.06 m*M* EDTA, 1 μl RNAsin (Promega), and 2.5 n*M* 17β-estradiol when not indicated otherwise. After 45 minutes at 30°C, the reaction was terminated by the addition of 180 μl of STOP-mix [1% SDS, 200 m*M* NaCl, 25 m*M* tris-HCl (*p*H 7.5), 5 m*M* EDTA, and transfer RNA (20 μg/ml)], and nucleic acids were purified by extraction in phenol-chloroform and recovered by precipitation with ethanol. Primer extension analysis was performed as described (*12*), with 80,000 cpm of $^{32}P$ end-labeled CAT primer per reaction. Half of the reaction product was denatured and analyzed on a 6% polyacrylamide 7.5*M* urea sequencing gel.

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