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Binding of insulin-like growth factor II (IGF-II) by human cation-independent mannose 6-phosphate receptor/IGF-II receptor expressed in receptor-deficient mouse L cells

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Mouse L cells deficient in expression of the murine cation-independent mannose 6-phosphate receptor/insulin-like growth factor II receptor (CI-MPR/IGF-IIR) were stably transfected with a plasmid containing the cDNA for the human receptor. Transfected cells expressed high levels of the human receptor which functioned in the transport of lysosomal enzymes and was capable of binding ^{125}I-IGF-II, both at the cell surface and intracellularly. Cell surface binding of ^{125}I-IGF-II by the receptor could be inhibited by pretreatment of cells with antibodies to the receptor or by coincubation with the lysosomal enzyme, β-glucuronidase. Expression of the receptor conferred on transfected cells the ability to internalize and degrade ^{125}I-IGF-II. Cells transfected with the parental vector and those expressing the human CI-MPR/IGF-IIR were found to express an atypical binding site for IGF-II that was distinct from the CI-MPR/IGF-IIR and the type I IGF-receptor. The availability of two cell lines, one of which overexpresses the human CI-MPR/IGF-IIR and one deficient in expression of the murine receptor, may help in the analysis of the role of the receptor in mediating the biological effects of IGF-II. They should also be useful in examining the significance of binding of ligands, such as transforming growth factor-β1 precursor and proliferin to this receptor.

Introduction

The cation-independent mannose 6-phosphate receptor* (CI-MPR) and the insulin-like growth factor II (IGF-II) receptor (IGF-IIR) constitute a single bifunctional receptor (CI-MPR/IGF-IIR). This was originally inferred from the sequence homology of the independently cloned cDNAs for these receptors (Lobel *et al.*, 1987, 1988; Morgan *et al.*, 1987; MacDonald *et al.*, 1988; Oshima *et al.*, 1988). Several laboratories have confirmed the identity of these two receptors by showing that receptor purified as either the CI-MPR or as the IGF-IIR binds both lysosomal enzymes and IGF-II (Braulke *et al.*, 1988; Kiess *et al.*, 1988; MacDonald *et al.*, 1988; Tong *et al.*, 1988).

While the role of the CI-MPR/IGF-IIR in targeting of lysosomal enzymes is well documented (for review, see von Figura and Hasilik, 1986; Dahms *et al.*, 1989), its involvement in mediating the biological actions of IGF-II is unclear. One of the reasons for this is that IGF-II, in addition to binding to the CI-MPR/IGF-IIR, also binds to the type I IGF receptor and to the insulin receptor with varying degrees of affinity (Froesch *et al.*, 1985). Another complication is that most cells express more than one of these three receptors (Rechler and Nissley, 1985). It has been difficult, therefore, to decide which, if any, of the biological effects of IGF-II are mediated through interaction with the CI-MPR/IGF-IIR. In fact, it had been concluded that most, if not all, of these effects could be interpreted as due to interaction of the growth factor with insulin or type I IGF receptors (Massague *et al.*, 1982; Mottola and Czech, 1984; Ewton *et al.*, 1987; Kiess *et al.*, 1987; DiCicco-Bloom and Black, 1988). Recently, however, there have been several reports of biological effects of IGF-

* Abbreviations: CI-MPR, cation-independent mannose 6-phosphate receptor; DMEM, Dulbecco's modified Eagle medium; DSS, disuccinimidyl suberate; FBS, fetal bovine serum; Hepes, 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid; HSA, human serum albumin; IGF, insulin-like growth factor; IGF-IIR, insulin-like growth factor II receptor; Man 6-P, mannose 6-phosphate; PBS, phosphate buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

II that are apparently elicited through the CI-MPR/IGF-IIR (Schimizu *et al.*, 1986; Hari *et al.*, 1987; Nishimoto *et al.*, 1987; Tally *et al.*, 1987; Blanchard *et al.*, 1988; Lewis *et al.*, 1988; Rogers and Hammerman, 1988, 1989). The mechanism by which signal transmission through the CI-MPR/IGF-IIR occurs may be novel, since the sequence of the receptor does not encode a tyrosine kinase activity such as found in many other growth factor receptors (Hunter and Cooper, 1985). There is some evidence that the CI-MPR/IGF-IIR is directly coupled to a G-protein (Nishimoto *et al.*, 1989).

The availability of several well characterized mouse cell lines deficient in the expression of the CI-MPR/IGF-IIR prompted us to see if transfection of these cells with the cDNA for the human receptor would provide a useful model system in which to study the potential role of this receptor in IGF-II function. We (Kyle *et al.*, 1988) and others (Lobel *et al.*, 1989) have previously demonstrated the usefulness of such an approach in studying the role of this receptor in transport of lysosomal enzymes.

Here we report the transfection of receptor-deficient mouse L cells with the cDNA for the human CI-MPR/IGF-IIR. We show that the expressed receptor binds ^{125}I-IGF-II and that this binding is inhibited by treatment of cells with antibody to the receptor, or by coincubation with a lysosomal enzyme, β-glucuronidase. Receptor-expressing cells are capable of mediating the degradation of IGF-II, in contrast to the control, CI-MPR/IGF-IIR-deficient cells. In addition, we show that CI-MPR/IGF-IIR-deficient cells possess an IGF-II-binding site that is distinct from the type I receptor and from the CI-MPR/IGF-IIR and that does not appear to mediate endocytosis and degradation of IGF-II.

Results

Transfected mouse L cells express the human CI-MPR/IGF-IIR

Mouse L cells that are deficient in expression of the CI-MPR/IGF-IIR (Gabel *et al.*, 1983) were transfected with the plasmid pMSXND or with this plasmid containing a 9 kb insert encoding the CI-MPR/IGF-IIR (pMSXND-hMPR-9) (Kyle *et al.*, 1988). Stably transfected cells were selected by growth of cells in the antibiotic, G418. The pMSXND plasmid also contains the gene for mouse dihydrofolate reductase, which allows for amplification of the dihydrofolate reductase sequence and adjacent gene sequences by growth of cells in an inhibitor of this enzyme, methotrexate. Cells transfected with pMSXND-hMPR-9, but not those transfected with the parental plasmid, and selected for growth in 3.2 μM methotrexate, synthesize and accumulate large amounts of receptor. This is shown by immunofluorescence analysis of fixed permeabilized cells with antiserum to the human CI-MPR/IGF-IIR (Figure 1). Cells transfected with vector containing the cDNA for the human CI-MPR/IGF-IIR display a bright perinuclear fluorescence (Figure 1A). Cells transfected with the parental vector (Figure 1B) show only a background level of fluorescence similar to that seen when cells were stained with non-immune serum (not shown).

Expressed human CI-MPR/IGF-IIR participates in intracellular sorting of lysosomal enzymes and mediates mannose 6-phosphate-dependent endocytosis of ligand

The receptor-deficient mouse L cells used in this study have been previously shown to be less efficient in the targeting of newly synthesized lysosomal enzymes than CI-MPR/IGF-IIR-expressing cells. Instead of being transported to lysosomes, the majority of the lysosomal enzymes are secreted into the extracellular medium of these cells (Gabel *et al.*, 1983). Table 1 shows that cells expressing the human CI-MPR/IGF-IIR secrete substantially lower amounts of two lysosomal enzymes, β-glucuronidase and β-hexosaminidase, than cells transfected with the parental plasmid.

CI-MPR/IGF-IIR-deficient L cells do not possess the ability to specifically endocytose Man 6-P-containing ligands (Gabel *et al.*, 1983). Table 2 shows the Man 6-P-dependent association of human spleen β-glucuronidase with transfected cells at either 4°C (representing cell surface binding) or at 37°C (representing cell surface binding and internalization). Transfected L cells expressing the receptor are capable of binding and internalizing this ligand in a Man 6-P-specific manner. Cells transfected with pMSXND show no such detectable binding or endocytosis.

These experiments indicate that the human CI-MPR/IGF-IIR expressed in receptor-deficient mouse L cells is fully functional in terms of transporting lysosomal enzymes. We have previously reported similar results with another receptor-deficient mouse cell line, the macrophage line, P388D$_1$, (Kyle *et al.*, 1988). Lobel *et*

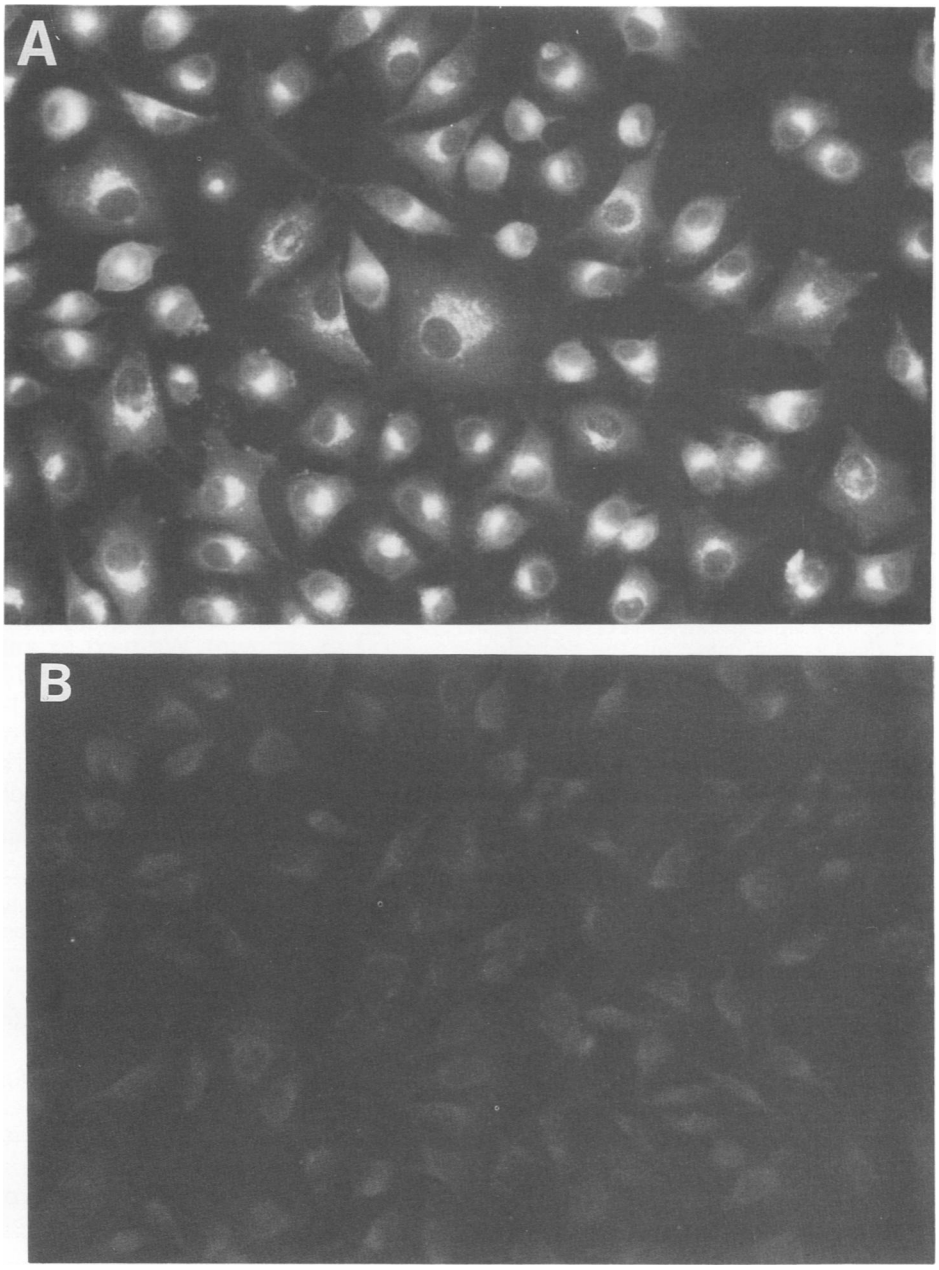

Figure 1. **Immunofluorescent labeling of transfected mouse L cells.** Mouse L cells transfected with pMSXND vector containing the cDNA for the human CI-MPR/IGF-IIR (B) or with the parental pMSXND vector (A) and selected for growth in 3.2 μM methotrexate, were grown on glass coverslips, fixed and permeabilized with methanol, and stained with rabbit antiserum to the human receptor followed by fluorescein-conjugated $F(ab')_2$ fragment goat anti-rabbit IgG, as described in Methods.

al. (1989) have recently shown that the bovine CI-MPR/IGF-IIR is functional in this respect in transfected mouse L cells.

Human CI-MPR/IGF-IIR expressed in receptor-deficient mouse L cells is capable of binding ^{125}I-IGF-II

To determine whether these cells might be a useful model system in which to study the role of the CI-MPR/IGF-IIR in mediating the biological effects of IGF-II, it was important to demonstrate that the expressed receptor was capable of interacting with the growth factor. To address this question, we performed affinity cross-linking experiments using the noncleavable cross-linking reagent, DSS. In these experiments, cells transfected with either pMSXND-hMPR-9 or with the parental plasmid pMSXND were incubated at 4°C with ^{125}I-IGF-II in the absence or presence of excess unlabelled IGF-I, IGF-II, or insulin. Subsequently, the

Table 1. Secretion of newly synthesized acid hydrolases by transfected mouse L cells

	Acid hydrolase activity in medium (% of newly synthesized)	
Vector insert	β-Glucuronidase	β-Hexosaminidase
No cDNA insert (control cells)	70	70
hCI-MPR/IGF-IIR cDNA	16	42

Subconfluent mouse L cells transfected with the pMSXND vector containing no insert (control cells) or with this vector containing the cDNA for the human CI-MPR/IGF-IIR were washed once with DMEM and incubated at 37°C with 2 ml fresh medium containing heat-inactivated FBS and 5 mM Man 6-P. After 48 h, the conditioned medium was removed and cells solubilized. Cells and media were assayed for enzyme activity as described in Methods. Knowing the amount of both enzymes present in the cells at zero time, the level of newly synthesized enzyme activity could be estimated and the fraction present in the medium determined. Three 35-mm dishes of cells, the enzyme activity levels of which differed by <10%, were used for each data point. This experiment is typical of the results obtained in three independent experiments.

cells were exposed to DSS, solubilized and subjected to SDS-PAGE under reducing conditions (Laemmli, 1970). The results of one such experiment are shown in Figure 2. Cells transfected with pMSXND-hMPR-9 show a very prominent labeled band that migrates at the expected position for the CI-MPR/IGF-IIR (Figure 2B). The intensity of the band decreases greatly when labeling is performed in the presence of excess IGF-II but only slightly in the presence of excess IGF-I and not at all in the presence of excess insulin. This is consistent with the previously reported specificity of the CI-MPR/IGF-IIR (Massague *et al.*, 1981; Rechler and Nissley, 1985).

Under these exposure conditions, no labeled species were seen in the cells transfected with the parental pMSXND (Figure 2A). With a very long exposure of the portion of the gel shown in panel 2A, a faint band (which does not photograph well) was seen at a position corresponding to a molecular weight of 130 kDa. This probably represents binding of the ligand to a type I IGF receptor, since this is the molecular weight of the binding subunit of this receptor (Kasuga *et al.*, 1981). Furthermore, the 130 kDa band was not present in the lanes corresponding to incubation in the presence of excess IGF-II, IGF-I, and insulin, consistent with the reported specificity of the type I IGF receptor. These data suggest the presence of relatively low levels of this receptor in these cells. The presence of low levels of the type I IGF receptor was confirmed by direct binding studies using ^{125}I-IGF-I (see Table 3).

Pretreatment of human CI-MPR/IGF-IIR-expressing cells with antibody to the receptor inhibits formation of ^{125}I-IGF-II-receptor complexes

We have previously reported that treatment of human fibroblasts with antibodies raised to the bovine CI-MPR/IGF-IIR receptor interfered with the ability of the receptor to participate in sorting of lysosomal enzymes and in endocytosis of exogenous lysosomal enzymes (Nolan *et al.*, 1987). Similarly, preincubation of receptor-expressing transfected mouse L cells at 37°C for 3 hr with antibodies to the receptor inhibited by 80% the subsequent ability of these cells to bind β-glucuronidase at the cell surface (data not shown). We next examined the effect of such pretreatment of transfected cells on the subsequent binding of ^{125}I-IGF-II. Figure 3 shows the affinity labeling of hCI-MPR/IGF-IIR-expressing cells following exposure to nonimmune serum or to antireceptor serum. There is clearly

Table 2. Cell surface binding and endocytosis of human spleen β-glucuronidase by transfected mouse L cells

	Man 6-P-specific cell-associated human β-glucuronidase activity* (U/mg protein)	
Vector insert	4°C	37°C
No cDNA insert (control cells)	—	1
hCI-MPR/IGF-IIR cDNA	327	1554

Mouse L cells transformed with the pMSXND vector containing no insert (control cells) or with this vector containing the cDNA for the human CI-MPR/IGF-IIR were incubated for 2 h at 4°C or at 37°C, with human spleen β-glucuronidase (4000 U/ml) (in the absence or presence of 5 mM Man 6-P), as described in Methods. At the end of the incubation, cells were washed with PBS, solubilized in 0.5% sodium deoxycholate and assayed for β-glucuronidase activity. Values shown represent the average of assays performed on duplicate 35-mm dishes of cells. Duplicate values did not differ by >10%. The results presented here are typical of those obtained in three independent experiments.

* The amount of human spleen β-glucuronidase associated with the cells in Man 6-P inhibitable manner was determined by subtracting the activity associated with the cells in the presence of Man 6-P from the total cell-associated activity.

a large inhibitory effect of the antiserum on the affinity labeling as would be expected if the labeled species is in fact the expressed CI-MPR/IGF-IIR. By densitometric scanning of five autoradiograms similar to that shown in Figure 3, the decrease in labeling of the receptor was estimated to be 73 ± 6% (±SD).

Binding of Man 6-P-containing ligand inhibits formation of ^{125}I-IGF-II-receptor complex

It has been reported that a macromolecular Man 6-P-containing ligand, the lysosomal enzyme β-galactosidase, can partially inhibit the binding of ^{125}I-IGF-II to the CI-MPR/IGF-IIR in C6 and BRL 3A2 cells in culture (Kiess *et al.*, 1988). It was suggested that such interaction of distinct ligands may be an important means of regulating both functions of this receptor (Roth, 1988; Dahms *et al.*, 1989; Kiess *et al.*, 1989). We examined this interaction by studying the effect of β-glucuronidase on the affinity cross-linking of ^{125}I-IGF-II to cells expressing the human CI-MPR/IGF-IIR. As shown in Figure 4A, coincu-

Table 3. Binding of ^{125}I-IGF-I and ^{125}I-IGF-II to CI-MPR/IGF-IIR-deficient mouse L cells transformed with the pMSXND vector

Radioactive ligand	Competing ligand	^{125}I-IGF bound* (cpm/well)
^{125}I-IGF-I	—	
	IGF-II	3935 ± 37
	IGF-I	612 ± 24
	Insulin	905 ± 81
^{125}I-IGF-II	—	
	IGF-II	16 574 ± 1002
	IGF-I	4743 ± 190
	Insulin	12 629 ± 841

Mouse L cells transformed with the pMSXND vector and selected for growth in 3.2 μM methotrexate were incubated with ^{125}I-IGF-I or with ^{125}I-IGF-II (5 ng/ml) at 4°C for 2 h, in the absence of a competing ligand or in the presence of excess unlabeled IGF-I (500 ng/ml), IGF-II (500 ng/ml) or insulin (50 μg/ml), as described in Methods. The cells were then washed, solubilized, and counted for radioactivity.

* Values are means ± SD.

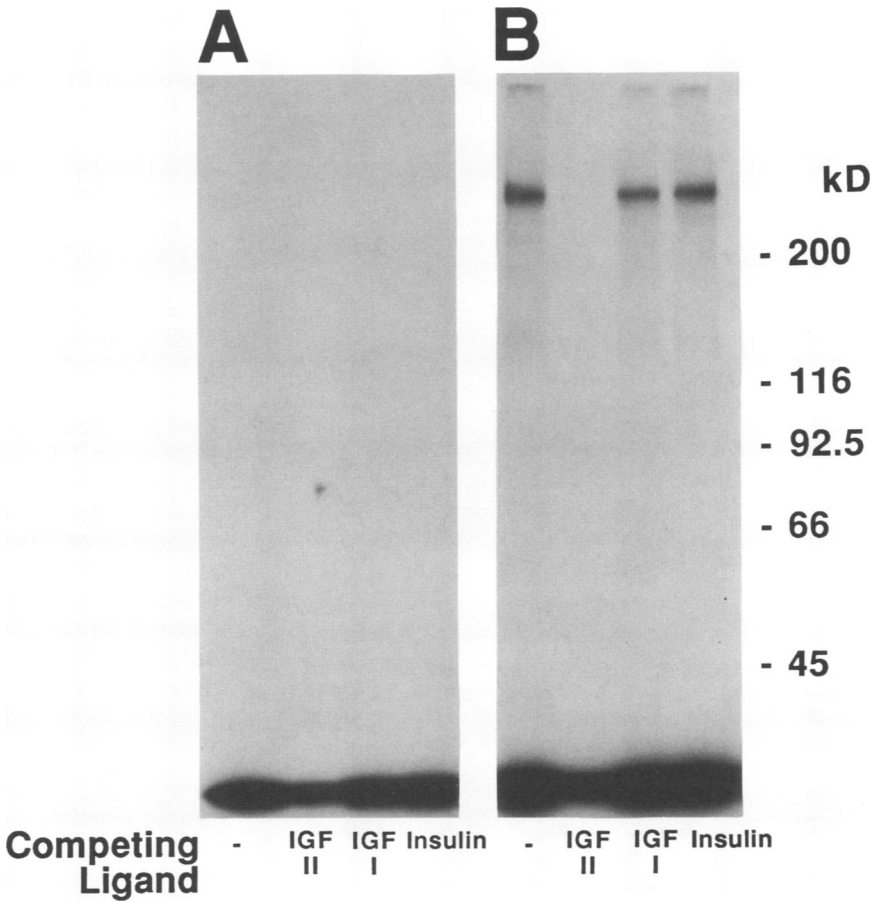

***Figure 2.* Affinity labeling of cell surfaces of transfected mouse L cells with ^{125}I-IGF-II.** Mouse L cells transfected with parental vector (A) or with vector containing the cDNA for the human CI-MPR/IGF-IIR (B), and selected for growth in 3.2 μM methotrexate, were incubated at 4°C with 10 ng/ml ^{125}I-IGF-II, in the absence or presence of a 100-fold excess of unlabeled IGF-II or IGF-I or of a 10 000-fold excess of insulin, as described in Methods. Cells were then incubated with 0.5 mM DSS for 15 min at 4°C. Following quenching of the reaction, cells were solubilized in Laemmli sample buffer (Laemmli, 1970) and analyzed by SDS-PAGE and autoradiography.

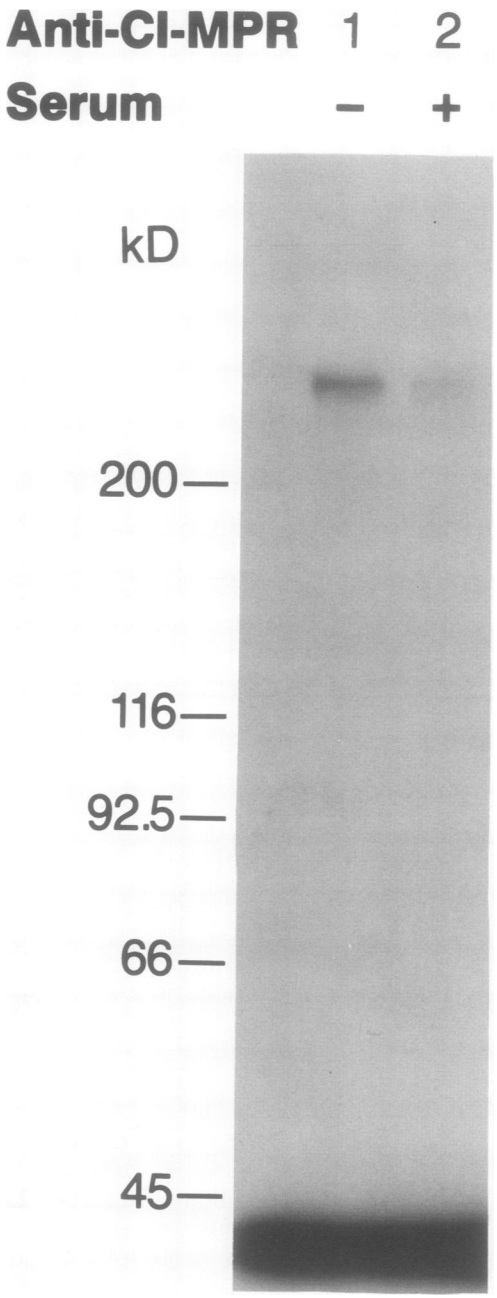

Figure 3. **Effect of antibodies to the CI-MPR on affinity-labeling of mouse L cells expressing the human CI-MPR/IGF-IIR.** Mouse L cells transformed with vector containing the cDNA for the human CI-MPR were incubated, for 3 h at 37°C, with a 1:50 dilution of either nonimmune rabbit serum (lane 1) or rabbit anti-human CI-MPR serum (lane 2). Binding of ^{125}I-IGF-II for 2 h at 4°C and cross-linking by DSS were then performed, as described in Methods. Following solubilization of the cells, the samples were analyzed by SDS-PAGE and autoradiography of the dried gel.

bation with β-glucuronidase significantly inhibits the formation of ^{125}I-IGF-II-receptor complex (lane 2). By densitometric scanning of five independent autoradiograms, we estimated that the β-glucuronidase-induced decrease in receptor labeling was 51 ± 11%. Coincubation with Man 6-P alone did not have a significant inhibitory effect (lane 3). The inhibition induced by β-glucuronidase, however, was prevented by coincubation with Man 6-P (Figure 4B, lanes 2, 3). These results indicate that binding of IGF-II to the human CI-MPR/IGF-IIR expressed in mouse L cells can be modulated by interaction of the receptor with ligand at the Man 6-P-ligand binding site.

Intracellular human CI-MPR/IGF-IIR in transfected mouse L cells is capable of binding ^{125}I-IGF-II

In most cells expressing the CI-MPR/IGF-IIR, only about 10–20% of the total cellular receptors are present on the cell surface. The remainder are located in an intracellular compartment (Fischer *et al.*, 1980a; Geuze *et al.*, 1985; Griffiths *et al.*, 1988). It has recently been recognized that the intracellular interaction of growth factors with their receptors may be important in generating the biological effects of these growth factors (Huang and Huang, 1988). For this reason we looked at the ability of the intracellular pool of CI-MPR/IGF-IIR to bind ^{125}I-IGF-II following permeabilization of cells expressing the receptor with saponin. As shown in Figure 5, the labeling intensity of the band corresponding to the receptor is much greater in permeabilized cells (lanes 3 and 4) than in those not exposed to saponin (lanes 1 and 2). Binding of ligand to the intracellular receptor was inhibited by excess unlabeled IGF-II (data not shown). Furthermore, this band was not seen when control cells were permeabilized and exposed to ^{125}I-IGF-II and DSS (data not shown). These results indicate that the intracellular receptor, in addition to the cell surface receptor, is capable of binding IGF-II. Washing the cells with Man 6-P prior to exposure to ^{125}I-IGF-II (lanes 2 and 4) did not appear to affect the subsequent binding of the ligand. This observation suggests that in these cells that express very high levels of the CI-MPR/IGF-IIR, most of the Man 6-P binding sites are unoccupied, i.e., the receptors exceed the supply of acid hydrolases.

Binding of ^{125}I-IGF-II by transfected mouse L cells

We next quantitated the binding of the ligand to the cell surfaces of transfected cells. Figure

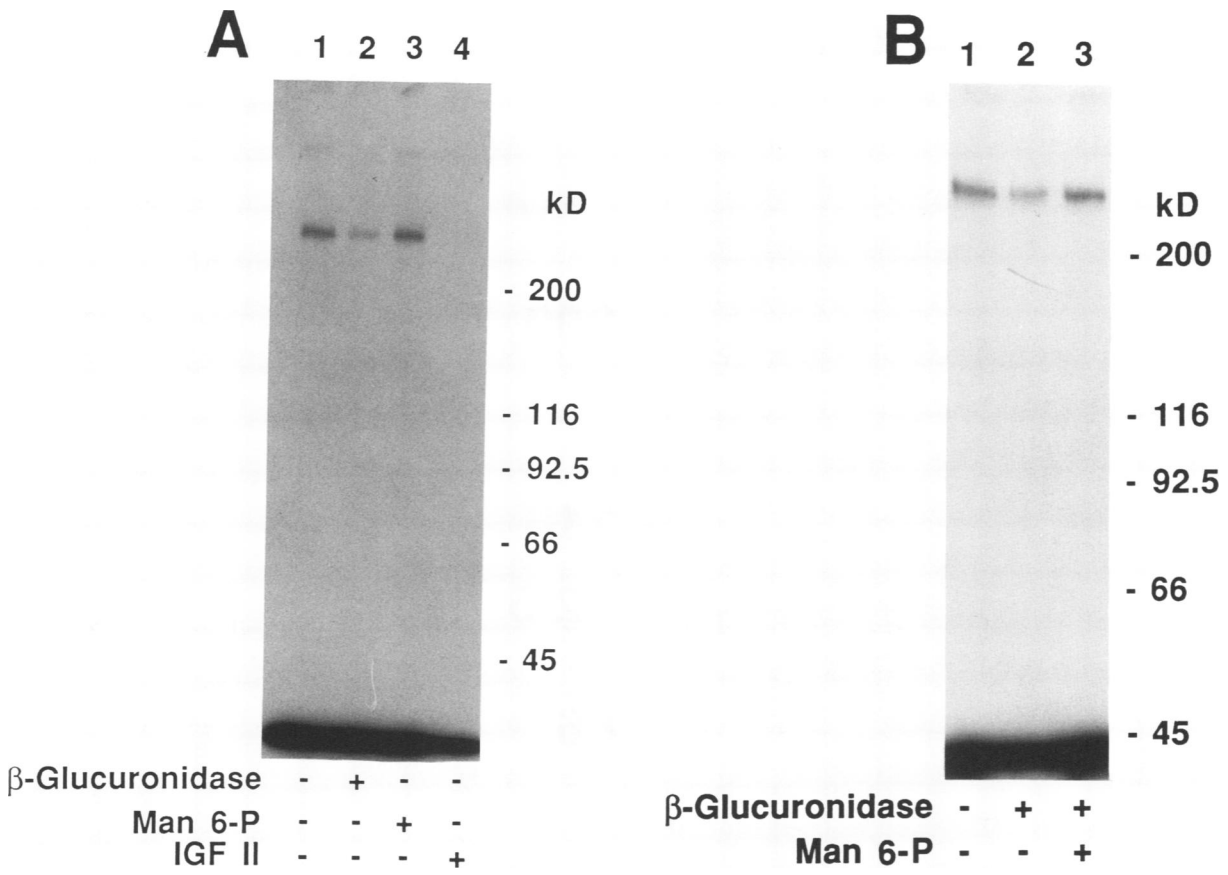

***Figure 4.* β-Glucuronidase inhibits binding of ^{125}I-IGF-II to the expressed human CI-MPR/IGF-IIR.** (A) Mouse L cells expressing the human CI-MPR/IGF-IIR were incubated with 10 mM Man 6-P at 4°C for 30 min. The cells were then washed with PBS and incubated with ^{125}I-IGF-II in the absence of any competing ligand (lane 1) or in the presence of 10 000 U/ml human recombinant β-glucuronidase (lane 2), 10 mM Man 6-P (lane 3) or of excess unlabeled IGF-II (lane 4). (B) Cells expressing the human CI-MPR/IGF-IIR were incubated with ^{125}I-IGF-II in the absence of a competing ligand (lane 1) or in the presence of 10 000 U/ml β-glucuronidase with (lane 3) or without (lane 2) 10 mM Man 6-P.

6 shows the specific binding (that which is inhibitable by excess unlabeled ligand) of increasing concentrations of ^{125}I-IGF-II to the cell surfaces of human CI-MPR/IGF-IIR-expressing cells or to control cells. Both CI-MPR/IGF-IIR-expressing cells and cells not expressing the receptor (control cells) show a significant level of binding of ^{125}I-IGF-II. Binding to the control cells is due in large part to binding to an atypical IGF-II-specific cell surface binding site (see below and Table 3), in addition to binding of the ligand to the type I IGF receptor described above. The presence of the large amount of ligand binding to the control cells prevented us from performing Scatchard analysis of the binding data (Scatchard, 1949), as it was difficult to determine the fraction of the binding that was due to the expressed human CI-MPR/IGF-IIR. Receptor-expressing cells pretreated with antihuman CI-MPR/IGF-IIR serum at 37°C as described in Figure 3 showed a 33% decrease in subsequent ^{125}I-IGF-II binding activity compared with cells treated with nonimmune serum. Since the efficiency of the antiserum appears to be 73–80%, based on the inhibition of β-glucuronidase binding and on the densitometric scanning of gels such as that shown in Figure 3, the binding of ligand to the expressed receptor can be roughly estimated to be ~45% of the total cell surface binding.

Mouse L cells possess an atypical IGF binding site

As shown in Figure 6, control mouse L cells that have been stably transfected with the parental plasmid (without insert) and that do not express the CI-MPR/IGF-IIR, show a significant level of

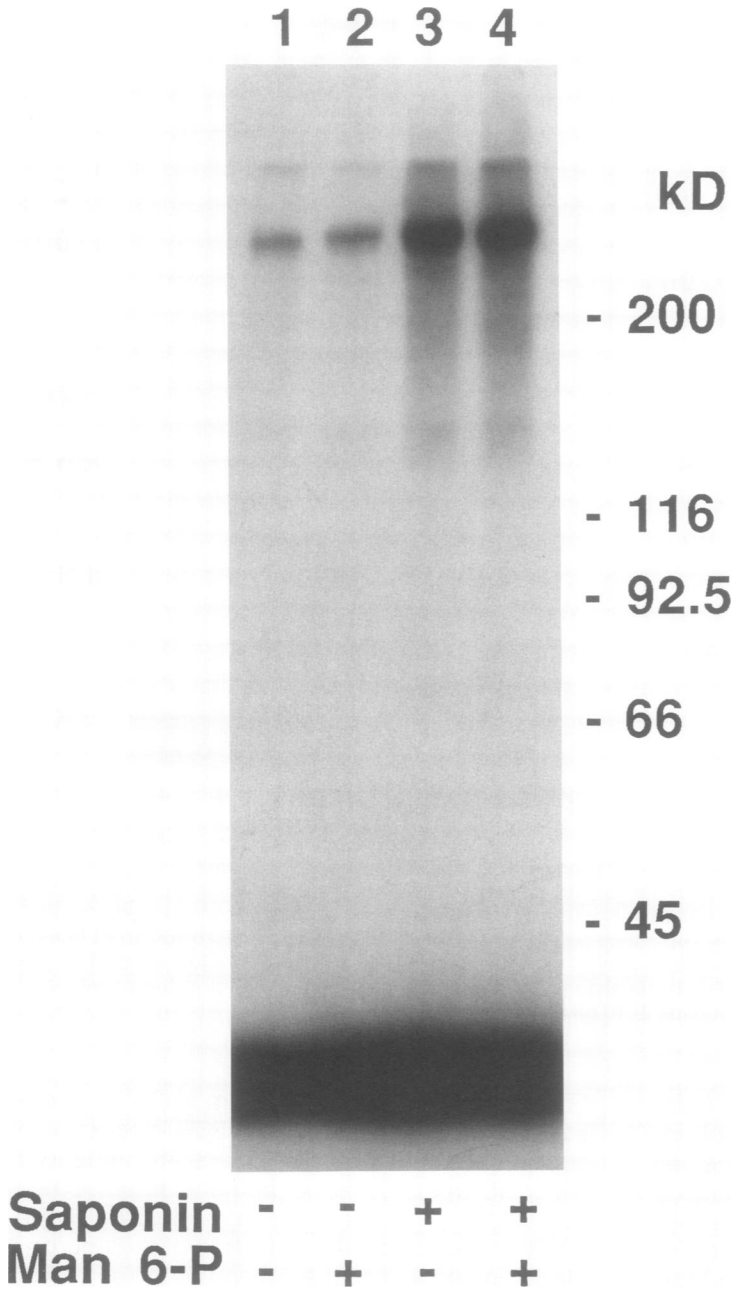

Figure 5. **Intracellular CI-MPR/IGF-IIR binds ^{125}I-IGF-II.** Mouse L cells transfected with vector containing the cDNA for the human CI-MPR/IGF-IIR were incubated with PBS with or without 0.25% saponin, in the presence or absence of 10 mM Man 6-P, as indicated, for 1 h at 4°C. The cells were then washed with PBS and incubated with ^{125}I-IGF-II (150 ng/ml) at 4°C for 2 h. Cross-linking and SDS-PAGE were performed as described in Methods.

^{125}I-IGF-II binding. This may be partially explained by binding of the ligand to a type I IGF receptor. In fact, the affinity cross-linking experiments suggested that these cells do express very low levels of the type I IGF receptor. Table 3 shows the binding of ^{125}I-IGF-I and ^{125}I-IGF-II to cells transfected with the parental plasmid, pMSXND, in the presence of excess unlabeled IGF-I, IGF-II, or insulin. The binding of ^{125}I-IGF-I to these cells is inhibited equally well by excess IGF-I and IGF-II and is significantly inhibited by excess insulin. This specificity is typical of that of the type I IGF receptor (Rechler and Nissley, 1985) and suggests that ^{125}I-IGF-I is binding to a type I IGF receptor. The binding of ^{125}I-IGF-II to these cells is inhibited by excess IGF-II but is only partially inhibited by IGF-I and is not significantly affected by excess insulin. These data indicate that the binding of IGF-II to these cells cannot be interpreted com-

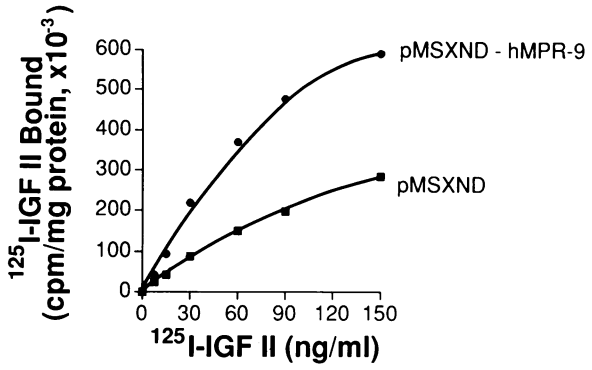

Figure 6. **Binding of ^{125}I-IGF-II by transfected mouse L cells.** Cells transfected with parental pMSXND vector (■ —— ■) or with vector containing the cDNA for the human CI-MPR/IGF-IIR (● —— ●) were incubated for 2 h at 4°C with increasing concentrations of ^{125}I-IGF-II. At the end of the incubation, the cells were washed, solubilized, and counted for radioactivity. Non-specific binding was measured by incubating cells with radioactive ligand in the presence of a 100-fold excess of unlabeled IGF-II and has been subtracted from all values.

pletely as binding to a classical type I IGF receptor and suggest the presence in these cells of an atypical IGF binding site which preferentially recognizes IGF-II.

Mouse L cells secrete IGF binding proteins

Many cells in culture synthesize and secrete proteins that bind the insulin-like growth factors (Baxter and Martin, 1989). It seemed likely that the atypical binding site observed in the L cells used in these experiments might represent such IGF-binding proteins associated with the cell surface. To test this hypothesis, we performed affinity cross-linking experiments on control cells with ^{125}I-IGF-I and ^{125}I-IGF-II and analyzed the results on SDS-PAGE, under conditions which allowed the resolution of proteins from 14–400 kDa in molecular weight. We were not able to identify a cross-linked species that accounted for the binding to the control cells. We also collected conditioned media from control cells and from receptor-expressing cells and performed affinity cross-linking experiments with ^{125}I-IGF-I and ^{125}I-IGF-II. The results of one such experiment are shown in Figure 7.

When ^{125}I-IGF-I is the ligand (Figure 7A), three cross-linked species are seen, with molecular weights in the range 40–50 kDa. The appearance of these bands is inhibited by the presence of excess unlabeled IGF-I or IGF-II but not insulin. When ^{125}I-IGF-II is the ligand (Figure 7B) two cross-linked species are seen at molecular weights of 40–45 kDa. The appearance of these bands is also inhibited by excess unlabeled IGF-I or IGF-II but not by insulin. Although it is not seen on this autoradiogram, longer exposures indicated that ^{125}I-IGF-II also formed a cross-linked species of the same molecular weight and with the same ligand specificity as the higher molecular weight species seen in Panel A.

These binding proteins were stable to acid treatment, as shown by their ability to bind ligand following gel filtration in 0.1 N acetic acid (data not shown). The binding specificity and the acid stability of these proteins are similar to those of previously reported binding proteins (Baxter and Martin, 1989). Conditioned medium from L cells inhibited the binding of ^{125}I-IGF-II to control cells and receptor-expressing cells, as did the binding protein-containing fractions obtained following gel filtration of acidified conditioned medium (data not shown).

Mouse L cells expressing the human CI-MPR/IGF-IIR receptor are capable of degrading bound ^{125}I-IGF-II

One well-characterized response of cells to exposure to IGF-II is internalization and degradation of the bound ligand. It has been suggested that this response is mediated by the CI-MPR/IGF-IIR (Oka *et al.*, 1985; Kiess *et al.*, 1987). To examine this in control mouse cells and in mouse cells overexpressing the human CI-MPR/IGF-IIR, we incubated the cells with ^{125}I-IGF-II at 4°C, to allow binding of the ligand to cell surface receptors. Unbound ligand was then washed off, fresh medium was added, and the cells were incubated at 37°C for various times.

Figure 8 shows the results in control pMSXND-transfected cells. With these cells there is a rapid loss of 75% of the cell-associated ligand into the medium as TCA-precipitable material. This loss is essentially complete within 30 min and most likely represents dissociation of intact ligand from the atypical IGF-II binding site discussed above. In contrast, cells expressing the CI-MPR/IGF-IIR receptor show an initial loss of radioactivity of ~25% of the total cell-associated and thereafter a slower loss of radioactivity into the medium (Figure 9). This slowly appearing radioactivity is predominantly TCA-soluble material. By the end of the incubation period, the trichloroacetic acid-soluble material in the medium represents more than 50% of the initial cell-associated radioactivity, indicating significant degradation of the ^{125}I-IGF-II in CI-MPR/IGF-IIR-expressing cells. This deg-

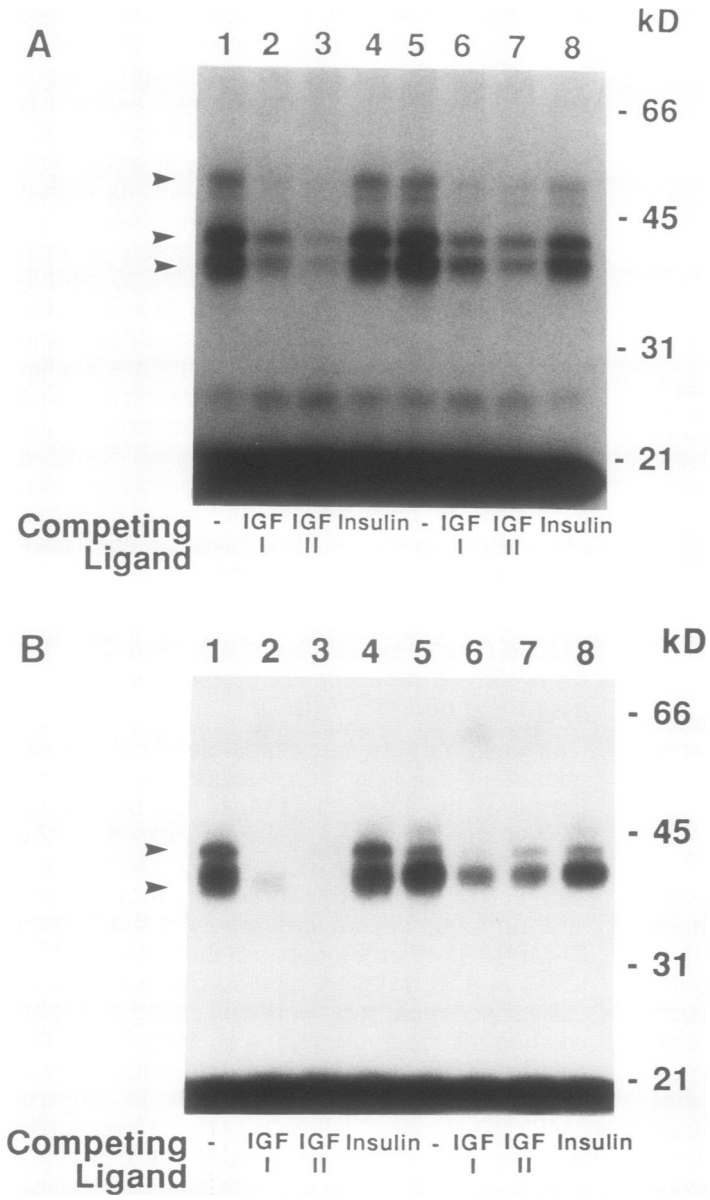

Figure 7. **Transfected L cells secrete IGF-binding proteins.** Conditioned medium of mouse L cells transfected with the parental vector (lanes 1–4) or with this vector containing the cDNA for the human CI-MPR/IGF-IIR (lanes 5–8) was incubated with ^{125}I-IGF-I (A) or with ^{125}I-IGF-II (B), in the presence or absence of excess unlabeled IGF-I, IGF-II, or insulin, and exposed to the cross-linking reagent, DSS, as described in Methods. Aliquots of the cross-linked media were then run, under reducing conditions, on 8% SDS-PAGE, and the dried gels were analyzed by autoradiography.

radation appears to occur at least partly in an acidic compartment, since it could be inhibited by 56% by performing the 37°C incubation in medium containing 10 mM NH_4Cl, a lysosomotropic agent that is known to neutralize intracellular acidic compartments (Okhuma and Poole, 1978; Gonzalez-Noriega *et al.,* 1980; Merion and Sly, 1983).

Discussion

Our aim in the study reported here was to develop a system that might be of use in clarifying the role of the CI-MPR/IGF-IIR in mediating the biological responses of cells to IGF-II. We wished to take advantage of the availability of murine cells deficient in the expression of this receptor (Gabel *et al.,* 1983) and of the ability to express high levels of the human receptor in transformed cells (Oshima *et al.,* 1988). The generation of a pair of cell lines that differ only in the presence or absence of CI-MPR/IGF-IIR expression should be very helpful in attempting to decide which of the biological effects of IGF-II are elicited through interaction with the CI-MPR/IGF-IIR. This is an important question in

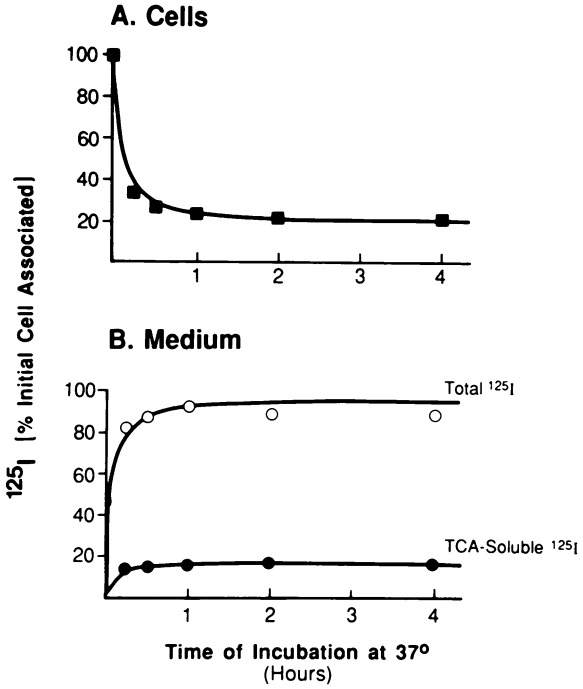

Figure 8. **Dissociation of ^{125}I-IGF-II from CI-MPR/IGF-IIR-deficient L cells at 37°C.** CI-MPR/IGF-IIR deficient mouse L cells transfected with the pMSXND vector were incubated for 2 h at 4°C with ^{125}I-IGF-II, as described in Methods. Cells were then washed and were incubated with fresh medium at 37°C. At the indicated times, the cells and media were harvested and counted for ^{125}I-IGF-II. The medium was also analyzed for trichloroacetic acid (TCA)-soluble ^{125}I-IGF-II. (A) Cell-associated radioactivity; (B) radioactivity present in the medium. Radioactivity is expressed as a percentage of that present at the start of the 37°C incubation.

view of the documented role of this receptor in the transport of lysosomal enzymes, the uncertainty concerning the in vivo function of IGF-II and the role played by the CI-MPR/IGF-IIR in this function, and the lack of understanding of the method by which signal transmission occurs through the receptor.

In this report we describe the transformation of mouse L cells that do not normally express the CI-MPR/IGF-IIR (Gabel *et al.*, 1983) with the cDNA for the human receptor (Oshima *et al.*, 1988) and amplification of the cDNA so that the cells express very high levels of the receptor. The expressed receptor is clearly functional in terms of its ability to participate in sorting of newly synthesized lysosomal enzymes and in mediating the endocytosis of lysosomal enzymes from the extracellular medium. We were able to demonstrate very clearly that the expressed receptor was capable of binding IGF-II by an affinity cross-linking procedure. Complex formation was inhibited by coincubation of radioactive ligand with excess unlabeled IGF-II but not by IGF-I or insulin (Figure 2). This is the specificity expected for binding of IGF-II to the CI-MPR/IGF-IIR (Rechler and Nissley, 1985). Formation of receptor-ligand complex was also inhibited by incubation of receptor-expressing cells with antiserum to the receptor (Figure 3).

The realization that the CI-MPR and the IGF-IIR constitute a single bifunctional receptor has generated much speculation as to the in vivo significance of a single receptor binding such apparently different ligands. It has been suggested that this receptor may serve to integrate different signals. In this regard, several groups have examined the effects of binding of a Man 6-P-containing ligand on the binding of IGF-II to the receptor. The results range from a lack of effect (Braulke *et al.*, 1988; Tong *et al.*, 1988), to enhanced binding affinity for IGF-II (Roth *et al.*, 1987; MacDonald *et al.*, 1988), to inhibition of IGF-II binding (Kiess *et al.*, 1988). It has also

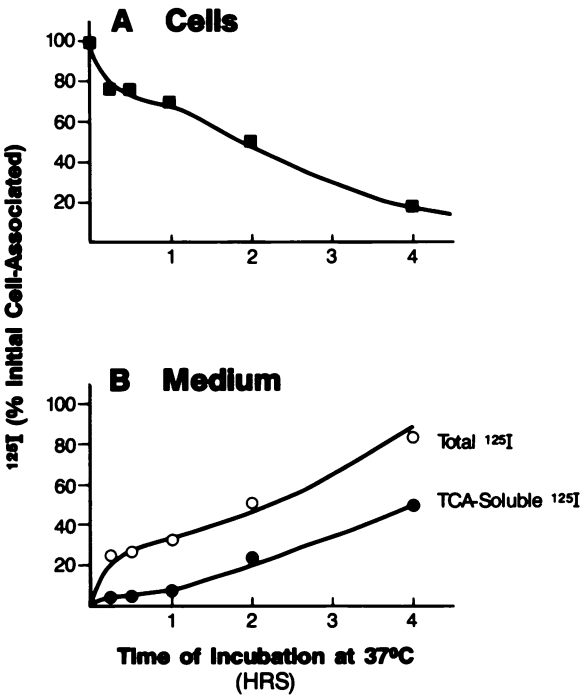

Figure 9. **Degradation of cell-associated ^{125}I-IGF-II by mouse L cells expressing the human CI-MPR/IGF-IIR.** Mouse L cells transfected with the pMSXND vector containing the cDNA for the CI-MPR/IGF-IIR were incubated with ^{125}I-IGF-II at 4°C, followed by addition of fresh medium and incubation at 37°C, as described in the legend to Figure 7. (A) Cell-associated radioactivity; (B) radioactivity present in the medium. Radioactivity is expressed as a percentage of that present at the start of the 37°C incubation.

been reported that Man 6-P potentiates IGF-II-induced phosphotidylinositol turnover in kidney-derived membranes (Rogers and Hammerman, 1989).

It is clear that while the receptor appears to possess distinct binding sites for these two classes of ligands, binding of ligand at one site is capable of influencing binding at the other site. Binding of a lysosomal enzyme, β-galactosidase, inhibited the binding of IGF-II to purified receptor, whereas the reverse was also true in cultured rat cells (Kiess *et al.,* 1988, 1989). In our studies, β-glucuronidase clearly inhibited the binding of IGF-II to the human receptor expressed in mouse cells. This inhibitory effect of β-glucuronidase was prevented by coincubation of cells with β-glucuronidase and Man 6-P. Whether the observed inhibitory effect of β-glucuronidase is due to steric effects or to conformational changes induced in the receptor by binding of the lysosomal enzyme is not yet clear. However, the results clearly support the suggestion that one macromolecular ligand can modulate the binding activity of the other (Roth, 1988; Dahms *et al.,* 1989; Kiess *et al.,* 1989).

The demonstration that the intracellular CI-MPR/IGF-IIR, as well as the cell surface receptor, is capable of binding IGF-II indicates that interaction of this ligand with the receptor in an intracellular location may be important for the biological activity of the growth factor in cells that express both IGF-II and the CI-MPR/IGF-IIR, such as the differentiating muscle cell line described by Tollefson and co-workers (1989). It has been suggested that such intracellular interaction of growth factor and receptor occurs in the case of *v-sis,* the transforming protein of simian sarcoma virus, and the PDGF receptor (Huang and Huang, 1988).

An interesting and unexpected observation made during the course of these experiments was that cells that do not express the CI-MPR/IGF-IIR are nevertheless capable of binding a significant level of IGF-II. This binding is mediated in small part by the type I IGF receptor, but it appears that the majority of the IGF-II binding to these cells is through an atypical binding site that has a greater affinity for IGF-II than for IGF-I. Binding of IGF-II to this atypical binding site does not appear to result in internalization and degradation of ligand, in contrast to binding to the CI-MPR/IGF-IIR.

The nature of the atypical IGF-II-specific binding activity in CI-MPR/IGF-IIR-deficient cells is not yet clear. One possibility is that it represents binding of the ligand to a cell surface binding protein. Many cells produce IGF binding proteins that are generally unrelated to the IGF receptors (for review see Baxter and Martin, 1989). Most of these binding proteins are secreted proteins found in the medium of cultured cells, but some have been shown to interact with cell surfaces (Clemmons *et al.,* 1986; Bar *et al.,* 1987; DeLeon *et al.,* 1989). We have found that the mouse L cells used in these experiments do in fact secrete IGF-binding proteins that can readily be identified in the conditioned medium of the cells (Figure 7). It appears unlikely that these represent the atypical ^{125}I-IGF-II binding site for two reasons: 1) while we have been readily able to cross-link both ^{125}I-IGF-I and ^{125}I-IGF-II to these proteins in conditioned medium and in extracts of L cells, we have not seen these species when cell-associated ligand was exposed to the cross-linking reagent, even when high percentage SDS-PAGE gels were run that allowed resolution of proteins of such molecular weights; 2) unlike the atypical cell surface IGF-II binding site, which preferentially binds IGF-II, the secreted binding proteins appear to bind IGF-I to a similar extent as IGF-II. The radioactivity that dissociated from the control cells at 37°C (Figure 8) did not appear to represent ligand associated with a binding protein, as evidenced by the fact that it co-migrated with ^{125}I-IGF-II that had not been exposed to cells when both samples were run on non-reducing SDS-PAGE. Nor were we able to show by affinity cross-linking experiments that the dissociated ligand was associated with any binding protein (Nolan, unpublished results).

It may be that these cells produce a binding protein capable of associating with the cell surface but that does not cross-link with ^{125}I-IGF-II under the conditions that are effective with the CI-MPR/IGF-IIR and with the secreted binding proteins. Alternatively, the association of IGF-II with the cell surface may represent binding of the growth factor to a component of the extracellular matrix. Such interaction has been shown to occur in the case of basic fibroblast growth factor (Vlodavsky *et al.,* 1987; Folkman *et al.,* 1988), granulocyte/macrophage colony stimulating factor, and interleukin 3 (Roberts *et al.,* 1988). The association of growth factors with cell surface binding sites other than their receptors may be a mechanism for increasing the local concentration of such ligands at the cell surface and perhaps improving the efficiency of their interaction with their receptors. The functional significance of the IGF-II binding site seen in CI-MPR/IGF-IIR-deficient L cells or the nature

of its interaction, if any, with the CI-MPR/IGF-IIR in receptor-expressing cells is at present unclear and warrants further investigation.

It has recently been demonstrated that transforming growth factor-β1 precursor, a protein with a wide range of biological activities, and proliferin, a prolactin-related protein produced by many murine cells in culture, can bind to the CI-MPR/IGF-IIR through interaction of Man 6-P moieties in their oligosaccharide side chains with the receptor (Lee and Nathans, 1988; Purchio *et al.*, 1988; Kovacina *et al.*, 1989). It has been suggested that the interaction of the transforming growth factor-β1 precursor with the CI-MPR/IGF-IIR may be important in the activation of the precursor (Miyazono *et al.*, 1989). These ligands may play an important role in processes such as embryonic development, bone and tissue remodeling, and tumor invasion. The availability of the cells described in this report, one of which expresses high levels of the receptor, the other demonstrating undetectable levels, may be useful in analyzing the biological significance of the binding of these ligands as well as that of IGF-II to the CI-MPR/IGF-IIR. They may also be useful in furthering our understanding of the role of this multifunctional receptor in signal transduction.

Methods

Materials

The eukaryotic expression vector, pMSXND, was kindly provided by Se-jin Lee and Dan Nathans (Johns Hopkins University, Baltimore, MD). The cloning of the cDNA for the human CI-MPR into this vector has been previously reported (Kyle *et al.*, 1988). Methotrexate was from Aldrich Chemical Co., Milwaukee, WI. G418 was obtained from Gibco. ^{35}S-methionine (Tran^{35}S-label) was from ICN. IgG-Sorb was purchased from The Enzyme Center. Na^{125}I and En^{3}Hance were obtained from New England Nuclear. Disuccinimidyl suberate (DSS) was from Pierce Chemical Co., Rockford, IL. Sephadex G-75 was from Pharmacia, Piscataway, NJ. Lactoperoxidase, hydrogen peroxide, and porcine insulin were from Sigma Chemical Co., St. Louis, MO. Recombinant human IGF-II was a kind gift of Michele Smith, Eli Lilly and Co., Indianapolis, IN. Recombinant human IGF-I was purchased from Bachem Inc., CA. Human spleen β-glucuronidase was purified as described previously (Natowicz *et al.*, 1979). In some experiments, human recombinant β-glucuronidase was purified by immunoaffinity chromatography from the conditioned medium of CI-MPR/IGF-IIR-deficient mouse L cells (Gabel *et al.*, 1983) stably transfected with a plasmid containing the cDNA for the human enzyme (Oshima *et al.*, 1987; Kyle and Grubb, unpublished results). Antibodies to the CI-MPR were raised in rabbits by immunization with purified human liver CI-MPR, and have been described previously (Nolan *et al.*, 1987). Fluorescein-conjugated F(ab^1)$_2$ fragment goat anti-rabbit IgG (heavy and light chain specific) was purchased from Cappel Laboratories, Malvern, PA.

Miscellaneous methods

Protein concentrations were determined by the method of Lowry *et al.* (1951), using bovine serum albumin as standard. β-Glucuronidase and β-hexosaminidase activities were determined fluorimetrically, as described previously (Fischer *et al.*, 1980a,b). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (1970).

Cell culture

Mouse L cells that are deficient in expression of the murine CI-MPR/IGF-IIR (Gabel *et al.*, 1983) were a generous gift of Stuart Kornfeld, Washington University, St. Louis, MO. They were maintained as monolayers in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% fetal bovine serum (FBS). Cells were grown at 37°C in 5% CO_2 and were passaged frequently.

Cell transfection and selection of transfectants

The construction of the plasmid, pMSXND-hMPR-9, containing the cDNA for the human CI-MPR/IGF-IIR has been reported previously (Kyle *et al.*, 1988). This plasmid also contains the genes for G418 resistance and mouse dihydrofolate reductase (Lee and Nathans, 1988). Mouse L cells deficient in the CI-MPR/IGF-IIR were transfected with pMSXND-hMPR-9 or with the parental vector, pMSXND, by a calcium phosphate procedure, and selected for G418 resistance as previously described (Kyle *et al.*, 1988). G418-resistant cells were further selected by growth in methotrexate-containing medium as described by Lee and Nathans (1988). This medium consisted of DMEM supplemented with 5% FBS, which had been dialyzed against sterile phosphate buffered saline (PBS), and 0.2 μM methotrexate. A second methotrexate selection step was performed by growing cells in medium containing 3.2 μM methotrexate. All cells used in the experiments described in this report have been selected by growth in medium containing 5% dialyzed FBS and 3.2 μM methotrexate and are routinely passaged in this medium.

Immunofluorescent labeling of transfected mouse L cells

Transfected mouse L cells were grown on glass coverslips. Subconfluent cells were washed with PBS and fixed and permeabilized in 100% methanol at −20°C for 10 min. They were incubated for 1 h at room temperature with rabbit antiserum to the human liver CI-MPR/IGF-IIR (diluted 1:50 in PBS containing 1% normal goat serum and 0.2% gelatin) followed by fluorescein-conjugated goat F(ab^1)$_2$ antirabbit IgG (diluted 1:20 in the same buffer) and examined and photographed with a fluorescent microscope equipped with a filter suitable for green fluorescence.

Binding and endocytosis of human spleen β-glucuronidase by transfected cells

Transfected cells, in 35 mm petri dishes, were incubated at 4°C for 15 min with DMEM containing 5 mM mannose 6-phosphate (Man 6-P) to remove endogenous cell surface ligands. The cells were then washed three times with DMEM and incubated with DMEM containing 5 mg/ml human serum albumin (HSA) and 4000 U/ml human spleen β-glucuronidase, at 4°C or at 37°C, for 2 h. Replicate dishes were in-

cubated with enzyme in the presence of 5 mM Man 6-P. The cells were then washed four times with PBS, solubilized in 0.5% sodium deoxycholate, and assayed for β-glucuronidase activity. The endogenous mouse β-glucuronidase activity was measured by incubating cells in medium containing no added human β-glucuronidase. One unit of enzyme activity is defined as the release of 1 nmol of β-glucuronic acid/h at 37°C.

Secretion of newly synthesized lysosomal enzymes by transfected cells

Subconfluent cells, in 35 mm petri dishes, were washed once with DMEM. Replicate dishes were frozen at −20°C. The remainder of the dishes were incubated with 2 ml of DMEM, 3.2 μM methotrexate, containing 5% dialyzed FBS that had been heat inactivated for 10 min at 70°C (to inactivate serum β-glucuronidase and β-hexosaminidase) and 5 mM Man 6-P (to prevent reinternalization of any secreted lysosomal enzymes by receptor expressed on the cell surface). After 48 h of incubation, the conditioned media were harvested and frozen at −20°C. The cells were washed three times with PBS and frozen at −20°C.

At the end of the experiment, all samples were thawed and β-glucuronidase and β-hexosaminidase activities measured. For measurement of β-glucuronidase activity, cells were solubilized in 0.5% sodium deoxycholate. For measurement of β-hexosaminidase activity, cells were solubilized by freeze/thaw in distilled water. From measurement of the initial enzyme activities present intracellularly, the amount of newly synthesized enzyme could be calculated and the proportion of the newly synthesized enzyme that was secreted into the medium was determined.

Radioiodination of recombinant human IGF-II and IGF-I

Radioiodination was performed using the lactoperoxidase method (Tait *et al.*, 1981). Briefly, 5 μg of IGF-II or of IGF-I in 0.2 M sodium phosphate, pH 7.6, was incubated with $Na^{125}I$ (1.0 mCi) and lactoperoxidase (0.8 μg) in a total volume of 55 μl. Hydrogen peroxide (500 μM in H_2O) was added in two 4 μl aliquots over the 8-minute reaction period. Following quenching of the reaction by addition of 150 μl of 1 M NaCl, 100 mM NaI, 50 mM sodium phosphate, 1 mM sodium azide, 1 mg/ml protamine sulfate, 2 mg/ml HSA, pH 7.5, the reaction products were separated on a Sephadex G-75 gel-filtration column equilibrated with 0.1 N ammonium acetate containing HSA (2 mg/ml). The specific radioactivity of the radiolabeled ligands was estimated by assuming a 70% recovery of the ligand and was approximately 100,000 cpm/ng.

Cell surface binding of ^{125}I-IGF-II and of ^{125}I-IGF-I by transfected cells

Cell surface binding measurements were performed essentially as described by Rechler *et al.* (1977). Cells, in 24-well cluster plates, were grown to confluence in DMEM, 3.2 μM methotrexate, 5% dialyzed FBS. Forty-eight hours before the binding assay, the medium was changed to DMEM, 3.2 μM methotrexate, 1 mg/ml HSA. Cells were chilled on ice for 15 min, washed twice with cold PBS and incubated, at 4°C for 2 h, with iodinated IGF-II or IGF-I (5–150 ng/ml) in binding buffer (15 mM 4-[2-Hydroxyethyl]-1-piperazine-ethanesulfonic acid [Hepes], 150 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 8 mM glucose, 5 mg/ml HSA, pH 7.6). At the end of the incubation, cells were washed three times with 15 mM Hepes, 150 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 1 mg/ml HSA, pH 7.6, solubilized in 0.4 ml of 0.4 N NaOH and counted for radioactivity. Nonspecific binding was determined by measuring the cell-associated radioactivity when binding was performed in the presence of 100-fold excess of the appropriate unlabeled IGF, and was subtracted from the total binding to determine specific IGF binding. Nonspecific binding routinely constituted <20% of the total binding.

Affinity cross-linking of ^{125}I-IGF-II to transfected cells

Cells in 24-well cluster plates were incubated for 48 h prior to binding experiments in serum-free medium, as described above. Binding of ^{125}I-IGF-II was performed as described above, using 10 ng/ml of ^{125}I-IGF-II, for 2 hr at 4°C. The cells were then washed twice with ice-cold PBS and incubated with 0.2 ml of 15 mM Hepes, 150 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, pH 7.6 to which the cross-linking agent DSS (freshly dissolved in dimethyl sulfoxide) had been added, to a final concentration of 0.5 mM. After 15 min on ice, the reaction was quenched by the addition of 1.0 ml of 0.25 M sucrose, 10 mM Tris, 1.0 mM EDTA, pH 7.2. The cells were washed once with this buffer, solubilized in SDS-PAGE sample buffer (Laemmli, 1970) and boiled for 5 min. Following SDS-PAGE on 6% gels, molecular weights markers were visualized by staining with Coomassie Blue, and the gels were dried and exposed to x-ray films at −70°C.

The specificity of the reaction was determined by performing the binding in the presence of 100-fold excess unlabeled IGF-II or IGF-I or in the presence of a 10 000-fold excess of insulin. To study the effect of antibodies to the human CI-MPR on the cross-linking of ^{125}I-IGF-II to transfected cells, cells were preincubated at 37°C with a 1:50 dilution of either non-immune rabbit serum or of rabbit antiserum to the purified human liver CI-MPR, for three hours prior to the ^{125}I-IGF II binding reaction. To determine the effect of β-glucuronidase on cross-linking of ^{125}I-IGF-II, cells were pre-incubated with DMEM containing 5 mg/ml HSA and 10 mM Man 6-P for 30 min at 4°C. The cells were then washed three times with PBS and incubated with ^{125}I-IGF-II (10 ng/ml) in the presence of a saturating concentration (10 000 U/ml) of human recombinant β-glucuronidase or of 10 mM Man 6-P or of both ligands. After 2 h at 4°C, cross-linking was performed as described above.

Cross-linking of ^{125}I-IGF-I and ^{125}I-IGF-II to conditioned medium of L cells

Confluent dishes of transfected mouse L cells were washed three times with DMEM and then incubated at 37°C for five days with DMEM containing 3.2 μM methotrexate and 1 mg/ml HSA. At the end of the incubation, the medium was collected and centrifuged at 1000 × *g* for 15 min. For cross-linking to IGF-binding proteins, 0.2 ml of conditioned medium was incubated with 5 ng ^{125}I-IGF-I or ^{125}I-IGF-II for 2 h at 4°C, in the presence or absence of a 100-fold excess of unlabeled IGF-I or IGF-II or of a 10 000-fold excess of insulin, at which time 4.0 μl of 50 mM DSS freshly dissolved in dimethyl sulfoxide was added. After 20 min at room temperature, 40 μl of 50 mM tris, pH 7.2, was added. Aliquots of the mixture were analyzed by SDS-PAGE under reducing conditions followed by autoradiography.

Degradation of ^{125}I-IGF-II by transfected cells

Cells were allowed to bind ^{125}I-IGF-II for 2 h at 4°C, as described above. At the end of the incubation, cells were

washed three times with Hepes binding buffer containing 1 mg/ml HSA, and 0.5 ml of DMEM containing 5 mg/ml HSA was added. The cells were then incubated at 37°C for various times up to 4 h. At each time point, the conditioned medium was harvested, counted for radioactivity, and incubated with ice-cold trichloroacetic acid to determine the amount of trichloroacetic acid-soluble and trichloroacetic acid-insoluble material. Cells were solubilized in 0.4 N NaOH and counted for radioactivity.

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