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Purification of the Insulin-like Growth Factor II (IGF-II) Receptor From an IGF-II-Producing Cell Line, and Generation of an Antibody Which Both Immunoprecipitates and Blocks the Type 2 IGF Receptor

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SUMMARY: 18,54-SF cells, which secrete rat insulin-like growth factor II (rIGF-II), have abundant type 2 IGF receptors. We have purified the type 2 receptor from these cells by solubilization of crude membranes in Triton X-100, followed by chromatography on agarose-immobilized rIGF-II. A partially purified receptor preparation, obtained by chromatography of solubilized membranes over wheat germ agglutinin, was used to immunize a rabbit. The antibody generated both immunoprecipitates the type 2 receptor, and specifically inhibits IGF-II binding to a variety of rat tissues, including 18,54-SF cells, BRL-3A cells and placenta. The presence of abundant type 2 receptors on an rIGF-II-secreting cell line is consistent with an autocrine role for IGF-II in select cells.

The somatomedins comprise a family of mitogenic peptides with major structural homology with proinsulin (1). In humans, two somatomedins have been identified, purified and sequenced: insulin-like growth factors (IGF) I and II (1,2). A rat IGF-II has also been identified, and found to have 94% sequence homology with human IGF-II (3). The metabolic and mitogenic actions of these growth factors are believed to be mediated by binding to specific receptors on target cell membranes (4). The insulin and IGF-I (type 1) receptors are structurally related, both migrating on electrophoretic gels with an apparent Mr >300,000 and consisting of disulfide-linked subunits (5). The IGF-II (type 2) receptor, on the other hand, migrates with an apparent Mr of 220-250,000, with no evidence of disulfide-linked subunits (5,6). This receptor also differs from the insulin and type 1 IGF receptor in its lack of affinity for insulin.

The biologic role of the type 2 receptor remains uncertain, despite extensive characterization by competitive binding studies, affinity cross-linking, and extraction and purification (5-8). Although the presence of abundant type 2 receptors in fetal and embryonic tissue, accompanied by high circulating levels of IGF-II in the fetus and infant, have suggested an important role for IGF-II and its receptor in fetal growth, this hypothesis remains unproven (9,10). Studies from Mottola and Czech (11) have indicated that the type 2 receptor is not involved in the stimulation of DNA synthesis in H-35 hepatoma cells, and recent studies from our laboratory demonstrate that the mitogenic action of IGF-II in human fibroblasts can be blocked by an antibody specifically directed at the IGF-I receptor. On the other hand, we have recently reported that two cell lines which secrete rIGF-II into conditioned media are also characterized by abundant type 2 receptors,

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suggesting a possible autocrine function for IGF-II and its receptor in the growth of these cells (12). In the process of characterizing this system, we have purified the type 2 receptor from one of these rIGF-II-secreting cell lines, and generated a rabbit polyclonal antibody which both immunoprecipitates the type 2 receptor and specifically blocks IGF-II binding.

MATERIALS AND METHODS

Peptides: Pure human IGF-II for iodination was kindly provided by Drs. G. Enberg and K. Hall (Stockholm, Sweden). For displacement studies, we employed synthetic IGF-II, generously provided by Dr. C. H. Li (San Francisco, CA). Pure biosynthetic (Thr-59)-IGF-I was purchased from Amgen Biologicals (Thousand Oaks, CA). Crystalline porcine insulin was obtained from Elanco Products Company (Indianapolis, IN). Iodination was performed by a modification of the chloramine-T technique, to specific activities of 250 uCi/ug for IGF-I and -II, and 100 uCi/ug for insulin. Prior to binding studies, iodinated peptides were further purified by Sephadex G-50 gel chromatography. For determination of nonspecific binding, we employed a partially purified, insulin-free IGF preparation, containing 10 ug/mg weight IGF-II and 20 ug/mg weight IGF-I.

Cells: 18,54-SF cells, initially isolated from a human pituitary adenoma, were cultured in Coon's modified Ham's F-12 medium without serum and passaged at 72-96 hour intervals after detachment with trypsin/EDTA. Subsequent studies have indicated that these cells are rat in origin, on the basis of 1) sequence analysis of the IGF peptide produced by these cells, which is characteristic of rIGF-II (J. Wyche, K. Nishikawa, personal communication); 2) chromosome analysis, which is consistent with rat; and 3) Southern blots, which are consistent with rat DNA (A. Ullrich, personal communication). While the origin of these rat cells remains uncertain, they clearly differ from BRL-3A cells in terms of serum requirements for cell attachment, growth characteristics, and morphology. BRL-3A cells (12) and IM-9 cells (13) were grown as previously described. The human fibroblast line N3652, derived from a 24 year old male donor, passage 11, was obtained from the Human Genetic Mutant Cell Repository (Camden, NJ).

Membrane Preparation: Cells were grown in 150x15 mm petri dishes, and confluent monolayers detached by the addition of 20 ml PBS, 1mM EDTA. Each plate was washed with an additional 5 ml of PBS, 1 mM EDTA, and the wash then added to the original cell pool. Cells were centrifuged at 500xg for 10 minutes at 4C, and pellets resuspended in 10mM sodium phosphate, pH 7.4, 1mM EDTA, 0.25M sucrose, 0.15M NaCl, 1mM phenylmethylsulfonyl fluoride (PMSF), 2mM iodoacetic acid. Cells were sonicated, and the lysate centrifuged at 12,000xg for 30 minutes at 4C. The pellet was discarded, and the supernatant centrifuged at 40,000xg for 1 hour at 4C. The resulting pellet was resuspended in 50mM HEPES, pH 7.4, 0.15M NaCl, 1mM PMSF, 2 mM magnesium sulfate and recentrifuged at 40,000xg for 1 hour at 4C. Membranes were resuspended in the above buffer, and frozen at -20C until used for assay or receptor purification.

Membranes from BRL-3A rat liver cells were prepared as previously described (12). Rat placentas were obtained during the final three days of pregnancy, and membranes prepared by the method of Daughaday et al (14), with minor modifications.

Purification of Receptors: Crude membrane preparations were thawed, and Triton X-100 added to a final concentration of 1% (v/v). After end-over-end mixing overnight at 4C, the mixture was centrifuged at 100,000xg for one hour at 4C. Further purification was by wheat germ agglutinin (WGA) chromatography to obtain partially purified receptor for immunization, or by chromatography on agarose-immobilized rIGF-II for final receptor purification. A 10 ml column, containing 7 mg/ml of WGA-agarose (Vector Laboratories, Burlingame, CA) was washed with 50mM HEPES, pH 7.6, 0.15M NaCl, 0.1% Triton X-100, 2mM PMSF, 4mM iodoacetic acid, after which 10-20 ml of solubilized membranes were applied (5-15 mg protein), allowed to sit for 1 hour at 4C, and recycled through the column three times. The column was then washed with 50 bed volumes of the above buffer, followed by elution of the bound material with the above buffer + 0.3M N-acetyl-D-glucosamine. Samples were concentrated in a Centricon-30 microconcentrator with a 30,000 MW cutoff (Amicon, Danvers, MA).

For preparation of the rIGF-II affinity column, rIGF-II was purified from the conditioned media of BRL-3A cells by the method of Moses et al (15). Cyanogen bromide-activated Sepharose 4B was purchased from Pharmacia (Piscataway, NJ) and the affinity column prepared as follows: 2g of gel were swollen with 15 ml of 1mM HCl for 15 minutes, and then washed over a sintered-glass filter with 500 ml of 1mM HCl applied in 50 ml aliquots. The gel was washed with 50 ml of coupling buffer containing 0.1M sodium bicarbonate, pH 8.0, 0.5M NaCl and was immediately added to coupling buffer containing 5.3 mg dry weight of pooled MSA II and III in a total volume of 12 ml. The mixture was then rotated end-over-end for 2 hours at room temperature. Coupling buffer was removed and the reaction quenched by end-over-end rotation in 15 ml of 0.2M glycine, pH 8.0 for 2 hours at

room temperature. The gel was transferred to a 10 x 0.7 cm Econocolumn (Bio-Rad Laboratories, Richmond, CA) and washed at room temperature with three cycles of coupling buffer alternating with 0.1M Na acetate, pH 4.0, 0.5M NaCl. Coupling efficiency, as determined by the addition of small amounts of iodinated IGF-II to the reaction mixture, was approximately 80%, thus giving a final concentration of approximately 0.5 mg MSA/ml gel. The gel was stored at 4C in 100 mM HEPES, pH 5.8, 0.05% sodium azide.

IGF-II receptors were purified using the rIGF-II-agarose gel, employing a modification of the method of Oppenheimer and Czech (8). Two ml of the gel were poured into a 10 x 0.7 cm column and washed with 50 ml of 50 mM HEPES, pH 7.4, 1% (v/v) Triton X-100. Five ml of solubilized cell membranes, containing 0.4 mg protein/ml, were made 0.5M in NaCl, introduced onto the column, and recirculated overnight at 4C at a flow rate of 7-8 ml/hr. The flow through was collected and the column washed with 40 ml of 10mM HEPES, pH 7.4, 0.5M NaCl, 0.5% Triton X-100, 1mM PMSF. The receptor was eluted with 40 ml of 10mM sodium acetate, pH 5.0, 1.5M NaCl, 0.2% Triton X-100, 1mM PMSF. Fractions (0.5 ml) were collected in 0.5 ml of 0.1M sodium phosphate, pH 7.4.

Protein Determination: Protein determinations were performed by the modified Bradford assay (Bio-Rad) and by the BCA protein assay (Pierce Chemical Co., Rockford IL).

Binding Assays: IGF-II, IGF-I and insulin binding to crude membranes was performed as previously described (12). For solubilized receptors, Triton extracts were incubated overnight at 4C in a 250 ul final volume of 50mM Tris HCl, pH 7.4, 0.5% BSA, containing 10,000 cpm of iodinated peptide, in the presence or absence of unlabeled peptide. Binding was terminated by the addition of 1 ml of ice-cold 50mM Tris HCl, pH 7.4, 0.1% Triton X-100, 0.25% BSA, 0.2% bovine gamma globulin (Sigma). The mixture was vortexed, followed by the addition of 1 ml of 20% ice-cold (w/v) polyethylene glycol. After standing for 30 minutes at 4C, the samples were centrifuged at 10,000xg, and the pellets counted in an automatic gamma counter. Nonspecific binding was defined as the binding observed in the presence of a partially purified somatomedin preparation,containing 1 ug/ml unlabeled IGF-II and 2 ug/ml unlabeled IGF-I.

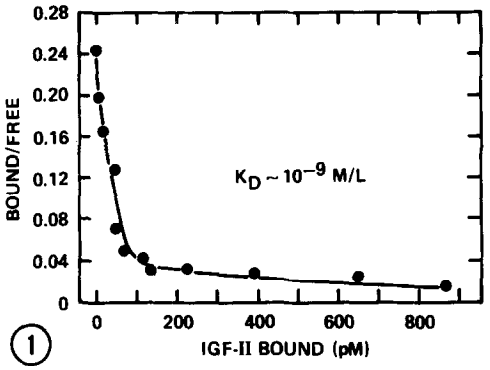
Affinity Labeling: Cross-linking of iodinated peptides to both crude and solubilized membranes and gel electrophoresis in sodium dodecyl sulfate were performed as previously described, using a 6% separating gel (5,12).

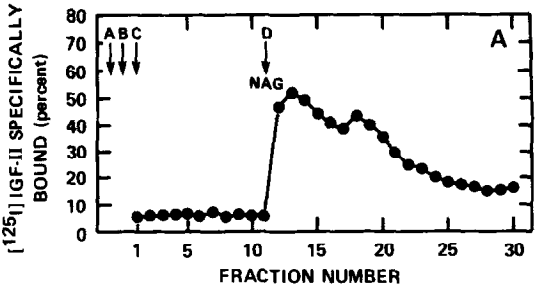
Anti-Receptor Antibodies: Fractions eluted from the WGA column were concentrated in a Centricon-30 microconcentrator. An albino New Zealand rabbit was injected at multiple subcutaneous sites in the scapular region with approximately 20 ug of partially purified receptor emulsified in an equal volume of Freund's complete adjuvant. The rabbit was boosted with 10 ug of partially purified receptor mixed with an equal volume of Freund's incomplete adjuvant at 2 weeks and at 5 weeks. Three weeks after the second boost, blood was drawn from the ear, and the serum was either frozen at -20C or precipitated in 45% saturated ammonium sulfate, resuspended in PBS, and dialyzed three times against PBS.

The effect of anti-receptor Ig on IGF binding was determined by incubating fibroblasts, IM-9 cells, or crude membranes from 18,54-SF cells, BRL-3A cells or rat placenta with serial dilutions of antiserum or ammonium sulfate-purified Ig in a 300 ul volume of 100 mM HEPES buffer, containing 120mM NaCl, 5 mM KCl, 1.2 mM magnesium sulfate, 10mM dextrose, 15 mM sodium actetate, 1mM EDTA, 0.5% BSA, pH 7.5 for 90 minutes at room temperature. At that point, 150 ul of iodinated peptide (10,000 cpm) in HEPES buffer, plus 50 ul of buffer or nonspecific concentrations of unlabeled peptide were added, and the mixture incubated at room temperature for an additional two hours. Incubation was terminated by the addition of 1 ml of ice cold HEPES buffer, followed by centrifugation, aspiration of the supernatant, and measurement of radioactivity in the remaining pellet.

For immunoprecipitation studies, iodinated IGF-II was cross-linked to solubilized 18,54-SF membranes, as described above. 50 mM Tris-HCl was added, yielding a final Triton X-100 concentration of 0.5% (v/v) in a final volume of 500ul. 50 ul of control or immune serum were added, followed by end-over-end mixing at 4C for seven hours. At the end of this incubation, 500 ul of goat anti-rabbit immunoglobulin covalently coupled to an agarose bead (Bio-Rad, Richmond, CA) and suspended in PBS, 0.1% BSA at a concentration of 4 mg/ml (final concentration 2 mg/ml) were added, followed by end-over-end mixing overnight at 4C. The mixture was centrifuged, the pellet removed, and the tube washed with 3 ml of 50mM Tris HCl, pH 7.6, 0.1% Triton X-100, 0.1% BSA, which were added to the pellet, followed by recentrifugation at 3000 rpm for 10 minutes. Pellets were counted for determination of immunoprecipitated radioactivity, followed by solubilization and SDS-PAGE, as described above.

RESULTS: As previously reported, specific binding of IGF-II to crude membrane preparations from 18,54-SF cells averaged 20% per 20 ug/ml protein (12). Under similar conditions, specific binding of IGF-I and insulin averaged 2.6% and 1.0%, respectively. Figure 1 shows a Scatchard plot of IGF-II binding to crude membranes.





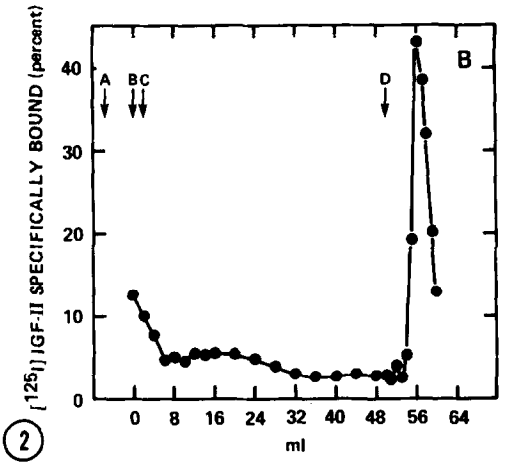


Figure 1. Scatchard plot of IGF-II binding to crude membranes prepared from 18,54-SF cells. The Kd of the high affinity component is shown.

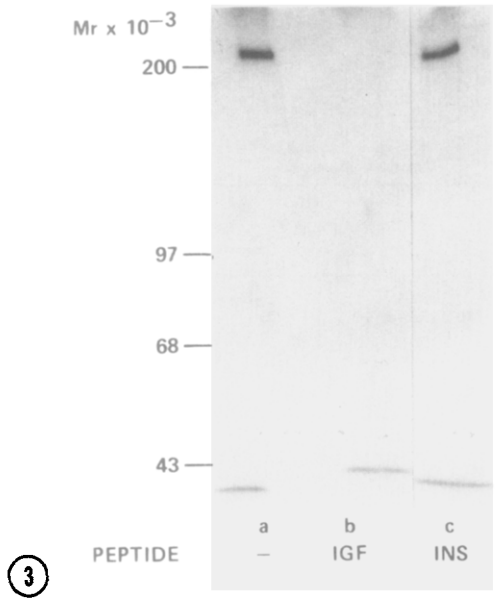
Figure 2. A. Wheat germ agglutinin chromatography of Triton-soluble receptor. 4.8 mg of solubilized membrane protein was applied, and 5 ml fractions collected. The designated points are A, starting buffer; B, sample; C, starting buffer; D, 0.3M N-acetyl-D-glucosamine (NAG) in starting buffer. 50 ul aliquots of eluate in a final volume of 300 ul were used for determination of specific binding.

B. rIGF-II-agarose chromatography of Triton-soluble receptor. 2.1 mg of solubilized membrane protein was applied, and 1 ml fractions collected. The designated points are A, sample; B, flow through; C, wash buffer; D, elution buffer. From point D on, 0.5 ml fractions were collected in 0.5 ml of 0.1M sodium phosphate buffer. For the first 50 ml collected, 50 ul aliquots of eluate in a final volume of 300 ul were used for determination of specific binding. For the final 10 ml collected, 20 ul aliquots of eluate were assayed; for these assays, specific binding was multiplied by 2.5, to correct for volume differences. Correction for the 1:2 dilution was not made.

The high affinity component of this curvilinear plot has a Kd of 1 nM, with a receptor concentration of 1 ug receptor/mg protein. In other membrane preparations, the estimated concentration of IGF-II receptors has been as high as 2.5 ug receptor/mg protein.

Solubilization of 18,54-SF membranes with Triton X-100 did not interfere with specific binding of IGF-II. Figure 2A depicts the elution of this receptor from a wheat germ agglutinin column with 0.3M N-acetyl-D-glucosamine. As can be seen, the overwhelming majority of the binding activity elutes in the glycoprotein fraction.

Purification of the type 2 receptor from the Triton X-100 extract is shown in Figure 2B. Elution of binding activity was successfully achieved with 10mM sodium acetate, pH 5.0, containing 1.5M NaCl, 0.2% (v/v)



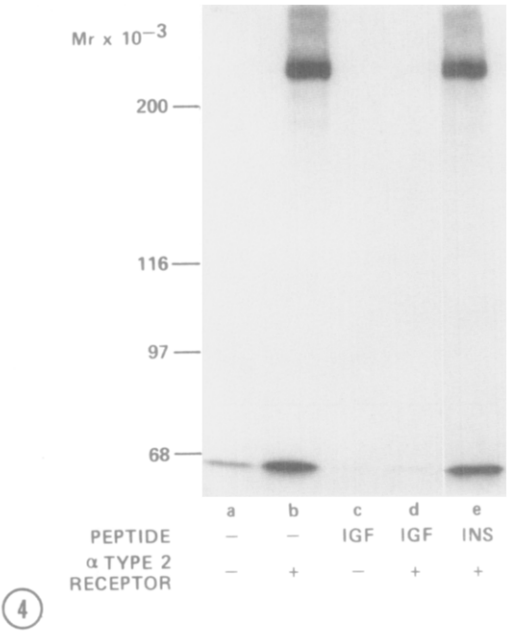
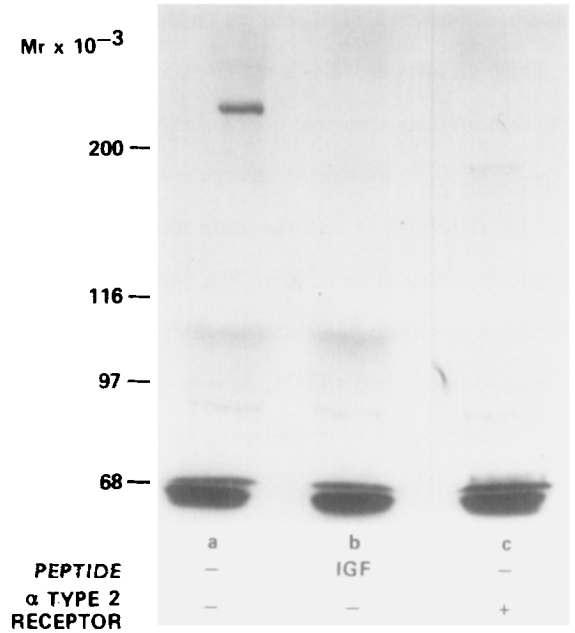


Figure 3. SDS-PAGE of rIGF-II affinity-purified receptor cross-linked to iodinated IGF-II and run under reducing conditions without unlabeled peptide (a), or in the presence of excess unlabeled IGF (b) or insulin (c).

Figure 4. SDS-PAGE of immunoprecipitated IGF-II receptor cross-linked to iodinated IGF-II under non-reducing conditions in the absence of unlabeled peptide (a,b) or in the presence of excess unlabeled IGF (c,d) or insulin (e). Cross-linked receptor was then incubated with a 1/100 dilution of pre-immune serum (a,c), or a 1:100 dilution of immune serum (b,d,e).

Triton X-100, and 1mM PMSF, as previously reported (8). Figure 3 demonstrates iodinated IGF-II cross-linked to the purified type 2 receptor eluted from the rIGF-II affinity column. A single band with an apparent Mr of 250K is visualized. This band is totally ablated by co-incubation with excess unlabeled IGF, but not altered by unlabeled insulin. Cross-linking of affinity-purified receptors with iodinated IGF-I or insulin failed to demonstrate specific bands (data not shown).

Immunoprecipitation experiments were performed with solubilized receptors cross-linked with iodinnated IGF-II and a 1:100 dilution of pre-immune and immunized rabbit sera (Figure 4). While no autoradiographic bands were observed in the lanes employing pre-immune serum (lanes a,c), immune serum resulted in precipitation of a doublet band with an apparent Mr of 245K and 250K (lane b). Interestingly, a similar doublet band was visualized on silver stain of the rIGF-II affinity-purified receptor. Immunoprecipitation of the doublet band was prevented by incubation of receptors with excess unlabeled IGF (lane d), but not by unlabeled insulin (lane e). Additionally, two less intense bands are visualized both above and below the 250K band. These bands are readily displaced by unlabeled IGF, but not by insulin.



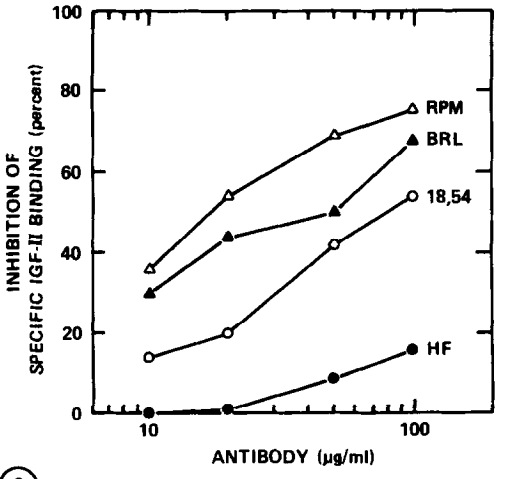


Figure 5. Unreduced SDS-PAGE demonstrating inhibition of IGF-II binding to crude membranes prepared from 18,54-SF cells. Membranes were incubated without (a,b) or with (c) a 1:100 dilution of immune serum for 90 minutes prior to the addition of iodinated IGF-II, in the absence (a,c) or presence of excess unlabeled IGF.

Figure 6. Inhibition of binding of IGF-II to membrane preparations from 18,54 cells (○), rat placental membranes (△), BRL-3A cells (▲), or human fibroblasts (●). Ammonium sulfate precipitated immunoglobulin concentrations are expressed as ug/ml.

Figure 5 shows the effect of a 1:100 dilution of immune rabbit serum upon IGF-II binding and cross-linking to rIGF-II affinity-purified receptors. In the absence of antibody, cross-linking revealed a 250K band (lane a), which was abolished by unlabeled IGF (lane b). Incubation with immune serum completely eliminated the 250K band, although a less intense band with an apparent Mr of 190K is now visualized (lane c).

Figure 6 demonstrates the specificity of this antibody for rat IGF-II receptors. At antibody concentrations of 100 ug/ml, inhibition of specific binding of IGF-II to 18,54-SF, BRL-3A and rat placental membranes ranged from 50-80%. At these antibody concentrations, inhibition of IGF-II binding to human fibroblasts or IM-9 cells was less than 20%. The antibody had no effect on insulin or IGF-I binding to either human or rat cells.

DISCUSSION: The rat type 2 receptor has been previously purified to homogeneity following extraction from chondrosarcoma cells (7) and placenta (8). We have now purified this receptor from 18,54-SF cells, a rIGF-II-secreting cell line, using a minor modification of the method of Oppenheimer and Czech (8). This approach depends upon elution of the type 2 receptor from agarose-immobilized rIGF-II by a high salt, low pH solution, rather than by urea. Under these conditions, the purified receptor appeared as a major band on both

silver stain and SDS-PAGE of affinity cross-linked receptors. Interestingly, on some gels a 245-250K doublet was observed. The eluted receptor continues to manifest a high affinity for IGF-II and no affinity for insulin, as is characteristic of the type 2 receptor. The high affinity component of the purified receptor has a Kd of 0.5×10^{-9} M/L, correlating approximately with the Kd of 10^{-9} M/L seen in the crude membrane preparation. It is of interest that Scatchard analysis of IGF-II binding to both the crude membranes and to the purified receptor demonstrated a curvilinear plot, while Scatchard plots of purified type 2 receptors from rat chondrosarcoma cells and rat placenta were linear. While it is conceivable that this discrepancy may be due to small amounts of IGF-I receptor eluting from the agarose-immobilized rIGF-II, we were unable to identify any type 1 receptors in our purified preparation following affinity cross-linking with iodinated IGF-I or insulin.

Immunization of a rabbit with a partially purified type 2 receptor generated an antibody capable of both immunoprecipitating the type 2 receptor and specifically blocking IGF-II binding to this receptor. Immunoprecipitation of solubilized 18,54-SF membranes cross-linked to iodinated IGF-II demonstrated a tight doublet band, with an apparent Mr of 245K and 250K, as well as several less intense bands of higher and lower apparent Mr. Since binding of IGF-II to these minor bands was ablated by excess unlabeled IGF, but not insulin, it is likely that these bands represent proteolytic products or alternative glycosylations of the type 2 receptor. We have previously reported similar autoradiographic bands following cross-linking of iodinated IGF-II to BRL 3A membranes (12).

This antibody also specifically inhibits IGF-II binding, not only to 18,54-SF cells, but also to rat placental membranes and BRL-3A membrane preparations. Inhibition of IGF-II binding to human fibroblasts and IM-9 cells was substantially less than that seen with rat tissues, further demonstrating that the 18,54-SF cells employed in these studies are rat in origin. Recent studies in our laboratory have indicated that these cells: 1) possess a single polyadenylated IGF-II mRNA, which hybridizes to a human IGF-II cDNA probe (16); and 2) secrete rIGF-II into conditioned media (12) and into the plasma of nude mice and rats transplanted with these cells. These observations, together with the demonstration of an abundance of classical type 2 receptors, are consistent with an autocrine role for IGF-II in select cell lines. Future studies employing this anti-type 2 receptor antibody will explore the role of the type 2 receptor in the metabolic activities and replication of such cells.

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