Purification of the Type II Insulin-like Growth Factor Receptor from Rat Placenta*

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The membrane receptor for insulin-like growth factor II (IGF II) has been purified to near homogeneity from rat placenta by chromatography of crude plasma membranes solubilized in Triton X-100 on agarose-immobilized IGF II. Elution of the IGF II receptor from the matrix at pH 5.0 in the presence of 1.5 M NaCl resulted in a receptor purification of 1100-fold from isolated plasma membranes, or 340-fold from the Triton extract with an average yield of about 50% in five separate purifications. Analysis of 125I-IGF II binding to the solubilized receptor in the Triton extract and in purified form by the method of Scatchard demonstrated no change in receptor affinity (K_a = 0.72 nM). Sodium dodecyl sulfate electrophoresis of the purified receptor showed one major band at M_r = 250,000 with only minor contamination. Affinity labeling of the receptor in isolated placenta membranes and in purified form using 125I-IGF II and the cross-linking agent di-succinimidyl suberate resulted in covalent labeling of only the M_r = 250,000 band. Such labeling was abolished by unlabeled IGF II but was unaffected by insulin, consistent with the previously reported specificity of IGF II receptor (Massague, J., and Czech, M. P. (1982) J. Biol. Chem. 257, 5038–5045). These results establish a one step affinity method for the purification of the type II IGF receptor that is rapid and highly efficient.

IGF II is a small polypeptide which closely resembles insulin in sequence, structure, and biological effects (1–3). The potency of IGF II in mediating short term metabolic effects in adipose tissue is 60-fold less than that of insulin, while its capacity to stimulate cell multiplication, receptor for IGF II, distinct from the insulin and insulin-like growth factor I receptors, has been demonstrated in a variety of tissues (4) and has been shown by affinity labeling to be a single polypeptide of M_r = 250,000 (5–7). This receptor, also denoted as the type II IGF receptor, has high affinity for IGF II, moderate affinity for IGF I, and no affinity for insulin (7). Recent studies on H-35 rat hepatoma cells suggest, but do not prove, that the IGF II receptor may mediate the growth stimulatory effects of IGF II on these cells (8).

In rat adipocytes and H-35 rat hepatoma cells, binding of IGF II to the IGF II receptor is rapidly increased by physiological concentrations of insulin, apparently due to an insulin-induced increase in IGF II receptor affinity (3, 8–11). In the course of studies investigating the molecular mechanism of this receptor-receptor interaction, it was necessary to develop a rapid method to obtain microgram quantities of purified receptor. We report here the rapid, one step purification of the IGF II receptor from rat placenta to near homogeneity using affinity chromatography on IGF II-agarose.

MATERIALS AND METHODS

Preparation of IGF II and IGF II-Agarose—IGF II was purified from the conditioned media of BRL-3A rat liver cells as previously described (11, 12). Purified hormone was iodinated by the chloramine T method (13) to a specific activity of 100 Ci/g. IGF I was a generous gift of Professor R. E. Humbel (Biochemisches Institut der Universität, Zürich, Switzerland).

IGF II was covalently coupled to CNBr-activated Sepharose 4B (Pharmacia) according to directions supplied by the manufacturer. One mg of hormone containing tracer 125I-IGF II was incubated with 1 ml of activated gel for 16 h at 4 °C in 0.1 M NaHCO_3, pH 8.0, 0.5 M NaCl. Unreacted sites were quenched by incubation with 0.2 M glycine, pH 8.3, for 2 h at 24 °C. Coupled gel was extensively washed on a sintered glass filter with alternating cycles of 0.1 M sodium acetate, pH 4.0, 0.5 M NaCl and 0.1 M sodium bicarbonate, pH 9.0, 0.5 M NaCl, and was stored in 50 mM Hepes, 0.05% sodium azide. After use, the gel was recycled using the same washing procedure. It could be successfully reused without change in performance at least five times.

Hormone Binding—IGF II binding to membranes was carried out as previously described (11). Triton extracts were incubated in 200 μl of Krebs-Ringer phosphate buffer, pH 7.4, containing 10 mg/ml of bovine serum albumin and 0.1% (w/v) Triton X-100, 125I-IGF II (1 nM), and unlabeled IGF II for 30 min at 24 °C. Binding was terminated by the additon of 0.55 ml of ice-cold 0.1 M sodium phosphate, pH 7.4, containing 1 mg/ml of bovine γ globulin (Sigma), and 0.5 ml of ice-cold 25% (w/v) polyethylene glycol. The mixture was vortexed, allowed to stand on ice for 15 min, and filtered under vacuum on an Amicon Microporous Filter, 0.45 micron size, which was pre-soaked in Krebs-Ringer phosphate buffer containing 10 mg/ml of bovine serum albumin. The assay tube was washed with 2.5 ml of ice-cold 0.1 M Tris-Cl, pH 7.4, containing 8% (w/v) polyethylene glycol, which was also applied to the filter. Filters were counted in a γ counter to quantitate binding. Nonspecific binding, determined at a 250-fold excess of unlabeled IGF II, was subtracted and represented 10–20% of maximum binding. Binding was analyzed by the method of Scatchard (14). Preparation of Membranes—Rat placental were obtained from Sprague-Dawley rats (Taconic Farms) in the seventeenth day of pregnancy. The tissue (50 g) was immediately homogenized (2 × 30 s) in 250 ml of ice-cold 10 mM sodium phosphate, pH 7.4, 1 mM EDTA, 0.25 M sucrose (PES) containing 1 mM PMSF using a Brinkmann Polytron. All subsequent steps were carried out at 4 °C. The homogenate was filtered through 2 layers of cheesecloth and centrifuged at 600 × g for 10 min. The pellets were discarded and the supernatant centrifuged at 3,000 × g for 10 min. The supernatant was then centrifuged at 30,000 × g for 40 min. The pellets were resuspended in 10 mM Tris Cl, pH 7.4, 1 mM EDTA, 1 mM PMSF, and centrifuged at 30,000 × g for 30 min. The pellets were resuspended in a small volume of the same buffer and stored at −20 °C. They were supported by Grant AM 30648 from the National Institutes of Health and by a grant from the Kroc Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: IGF, insulin-like growth factor; PMSF, phenylmethylsulfonyl fluoride; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DSS, diisuccinimidyl suberate.

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stable for at least a month with little or no change in IGF II binding activity.

**Purification of the IGF II Receptor**—Membranes (20 mg) were resuspended in 4 ml of 50 mM Hepes, pH 7.4, 1% (w/v) Triton X-100, containing 1 mM PMSF, 10 μg/ml of leupeptin, and 20 μg/ml of aprotinin and incubated with end-over-end mixing for 1 h at 4°C. The suspension was then centrifuged at 100,000 x g for 1 h. The supernatant was made 0.5 M in NaCl, then incubated with 0.8 ml of IGF II-agarose which had been washed with 50 mM Hepes, 1% (w/v) Triton X-100. The suspension was incubated on a Fisher Hemotest mixer for 1 h at 4°C, then poured into a column (0.7 x 2.0 cm) and the flowthrough collected. The column was washed with 10 ml of 50 mM Hepes, pH 7.4, 0.5 M NaCl, 0.5% (w/v) Triton X-100 containing 1 mM PMSF, 10 μg/ml of leupeptin and 20 μg/ml of aprotinin. The receptor was eluted with 4 ml of 10 mM sodium acetate, pH 5.0, 1.5 M NaCl, 0.2% (w/v) Triton X-100 containing 1 mM PMSF, 10 μg/ml of leupeptin, and 20 μg/ml of aprotinin. Fractions (0.5 ml) were collected directly in 0.5 ml of 0.1 M sodium phosphate, pH 7.4, for immediate neutralization. After assay, fractions containing active receptor were pooled, made 20% (v/v) in glycerol, and stored at −26°C.

**Protein Assay**—Membrane protein concentration was determined by the method of Lowry (16), and in Triton extracts by the method of Markovic et al. (17). Protein concentration of the purified receptor was determined by amino acid analysis using precolumn derivatization with o-phthalaldehyde in the presence of 0-mercaptoethanol followed by reverse phase high pressure liquid chromatography by a modification of the method of Jones et al. (17).

**Gel Electrophoresis and Autoradiography**—Samples were boiled in the presence of Laemmli sample buffer (18) containing 1% sodium dodecyl sulfate and 50 mM dithiothreitol, then subjected to sodium dodecyl sulfate electrophoresis on 5% polyacrylamide gels or 5 to 12% gradient polyacrylamide gels according to the method of Laemmli (19). Gels were silver stained according to a method supplied by Bio-Rad or stained with Coomassie blue, dried, and subjected to autoradiography using Kodak X-OMAT film with enhancing screen (19).

**Affinity Labeling**—Affinity labeling of the receptor in membranes using DSS was carried out as previously described (11). Membranes (200 μg) were incubated in 250 μl of Krebs-Ringer phosphate buffer, pH 7.4, containing 10 mg/ml of bovine serum albumin and 5 nm 125I-IGF II for 90 min at 10°C. The membranes were centrifuged for 3 min at 13,000 x g in a Fisher microcentrifuge and resuspended in 300 μl of Krebs-Ringer phosphate buffer. DSS was added with vortexing at a final concentration of 0.2 mM and the samples were incubated for 15 min on ice. The reaction was quenched with 1 ml of 10 mM Tris-Cl, 1 mM EDTA, and the samples centrifuged as before. The pellets were retained for electrophoresis.

Purified receptor solubilized in Triton X-100 was incubated in 300 μl of Krebs-Ringer phosphate containing 5 nM 125I-IGF II, 10 mg/ml of bovine serum albumin, and 0.1% (w/v) Triton X-100 for 90 min at 10°C. The reaction was then added with vortexing to a final volume of 0.2 mM and the samples incubated for 15 min on ice. The reaction was quenched by the addition of 0.1 ml of 0.1 M Tris Cl, pH 7.4. In order to remove the unbound hormone, samples were gel filtered on columns (1.0 x 6.0 cm) of Sephadex G-75 equilibrated in Krebs-Ringer phosphate buffer, pH 7.4, containing 1 mg/ml of bovine serum albumin, 0.1% (w/v) Triton X-100. The peak of radioactivity at this void volume was pooled and subjected to electrophoresis and autoradiography.

**RESULTS**

Placenta was chosen as a source of IGF II receptor for our studies because affinity labeling studies carried out as previously described (7) demonstrated that this tissue is rich in IGF II receptors but lacks IGF I receptors (data not shown). Because IGF I receptors in some tissues have moderately high affinity for IGF II as well as IGF I (7), such receptors might be expected to copurify with the IGF II receptor on IGF II-agarose. When crude plasma membranes prepared from pregnant rat plasma membranes were solubilized with 1% (w/v) Triton X-100 and 1% (w/v) octylglucoside, an increase in binding receptor activity of approximately 2-fold is observed. This may be due to the presence of receptor in inside-out vesicles which become available for binding in the presence of detergent. Increasing detergent concentration to 2% did not improve solubilization of activity. Triton X-100 was used for solubilization of activity.

| Step | Protein | Specific activity a | Purification from membranes | Purification from Triton extract | Yield of receptor from Triton extract a |
|------|---------|-------------------|-------------------------------|----------------------------------|--------------------------------------|
| Triton extract | 1.2 | 6.2 | 3.3 | 1 | 100 |
| Triton extract after treatment with IGF II-agarose | 8.4 | 2.6 | 1.4 | 0 | 0 | 29 |
| NaCl wash | 5.3 | 2.6 | 1.4 | 0 | 0 | 19 |
| Eluate | 0.018 | 2100 | 1100 | 340 | 37 |

*Binding analyzed by the method of Scatchard (14).

Yield is expressed relative to Triton extract since detergent solubilization gave a 2-fold increase in total binding activity.

Final protein concentration was 3.8 μg/ml.

**TABLE I**

Purification of the IGF II receptor from Triton extract of rat placenta

Triton extract was prepared from 20 mg of crude rat placenta plasma membranes (approximately 10 g of placental tissue) as described under “Materials and Methods.” The data presented are taken from a representative experiment. The average yield from five separate preparations was 48%.

Elution of the receptor from the immobilized IGF II was achieved with 10 mM sodium acetate, pH 5.0, containing 1.5 M NaCl and 0.2% Triton X-100. Attempts to elute IGF II binding activity at pH 7.0 in the presence of 2 M NaCl, at pH 5.0 in the absence of 1.5 M NaCl, or at pH 9.0 or above in the presence or absence of 2 M NaCl were unsuccessful. Similarly, elution at pH 4.0 in the presence of 2 M NaCl resulted in a much lower yield of binding activity, presumably due to denaturation of the receptor at the lower pH. In order to minimize such acid-induced denaturation, eluting fractions were neutralized immediately by direct collection in phosphate buffer at neutral pH. Elution was normally complete in two bed volumes, so that further concentration of the pooled receptor was not necessary. Purified receptor was stored at −26°C in the presence of 20% glycerol. Under these conditions, receptor binding activity was stable for months.

Fig. 1 shows analysis by the method of Scatchard (14) of 125I-IGF II binding to the IGF II receptor in Triton X-100 extracts of plasma membrane and in the purified receptor preparation. No significant difference in affinity is seen with an average Kd = 0.72 nM. No evidence of curvilinearity of the Scatchard plots was observed. Assuming 1 mol of 125I-IGF II
bound per mol of receptor, one can calculate from Scatchard analysis that the affinity-purified fraction in the experiment presented in Table I should contain 7 pg of active receptor. Amino acid analyses indicated that 13 µg of total protein was present. Thus, in this preparation, the IGF II receptor attained at least 55% of theoretical purity by Scatchard analysis, indicating that it is close to homogeneity. Electrophoretic analysis of the purified receptor (Fig. 2) indicated that the actual purity is much higher (see below). Overall purification of 1100-fold from membranes or 340-fold from the Triton X-100 extract was achieved with a final yield (relative to the Triton X-100 extract) of 37%. In five separate experiments, the yield averaged 48% with a range of 31–63%. The receptor could be recycled over IGF-II-agarose for an additional one or two elutions. However, this gave no further purification with a substantial decrease in overall yield.

Fig. 2 shows the sodium dodecyl sulfate-gel electrophoresis pattern obtained for crude plasma membranes, Triton extracts, and purified receptor when electrophoresed in the presence of dithiothreitol. The purified receptor preparation contained a major species at M, = 250,000 as well as a minor contaminant at M, = 67,000 which is probably bovine serum albumin (lane C). When the purified receptor was examined on a 5–12% gradient gel (lane D), no other contaminant bands of molecular weight 25,000 or higher were observed.

Fig. 3 shows autoradiographs of affinity labeled IGF II receptor in rat placenta plasma membranes and in the purified preparation, using 125I-IGF II (5 nM) and disuccinimidyl suberate (0.2 mM). In both cases, a single band at M, = 250,000 was observed, which co-migrated with the major silver-stained species in the purified preparations. No lower molecular weight bands were seen in the purified preparation even on overexposure of the autoradiograph (Fig. 3, lanes G–I). Electrophoresis of the affinity labeled receptor on a 5–12% gradient polyacrylamide gel to examine lower molecular weight proteins showed that no other band, including the M, = 67,000 band, was specifically labeled by 125I-IGF II (data not shown). This demonstrated the absence of proteolytic fragments of the IGF II receptor and lack of contamination with the IGF I receptor, whose major affinity labeled subunit migrates at M, = 130,000 under reducing conditions. Labeling of the M, = 250,000 band was completely abolished by the presence of 600 nM unlabeled IGF II but was unaffected by the presence of 3 µM unlabeled insulin. This is consistent with the results of binding assays in which binding of 125I-IGF II (1 nM) to the purified receptor was abolished by the addition of 500 nM unlabeled IGF II but was unaffected by the presence of 5 µM insulin or 5 µM proinsulin. The addition of 10 nM unlabeled IGF I inhibited binding of 125I-IGF II (1 nM) to the receptor by approximtely 60% (data not shown).
DISCUSSION

We report here the purification of the IGF II receptor to near homogeneity by a single step procedure of affinity chromatography on IGF II-agarose. Such immobilized ligand chromatography is the only feasible means of purifying the receptor in the native state since it represents only 0.1% of the protein in crude plasma membranes. The technique has been widely applied to other growth factor receptors including insulin (20), epidermal growth factor (21), and nerve growth factor (22). Preliminary steps such as ion exchange or lectin chromatography prior to the affinity column proved unnecessary in our present studies, increasing the speed and yield of the purification.

The conditions reported here for eluting the IGF II receptor from the immobilized IGF II (pH 5.0 and high salt) resulted in elution of the receptor in small volume and high yield (Table I). The conditions also avoided the use of denaturing agents such as urea, which has been used for elution of the insulin receptor from insulin-agarose (20). Dissociation of the ligand-receptor complex below pH 5.5 has been reported for several ligands including epidermal growth factor (23), insulin (24), lysosomal enzymes (25), and asialoglycoproteins (26). This may represent the physiological mechanism for removal of internalized ligand, as suggested by recent findings that endocytotic vesicles containing receptors for α2-macroglobulin (27) and transferrin (28) are rapidly acidified to pH 5.0 before fusion with lysosomes.

The affinity of the purified IGF II receptor from rat placenta (Kd = 0.72 nM, Fig. 1) is somewhat higher than that reported for the high affinity form of this receptor previously observed in plasma membranes and low density microsomes of control and insulin-treated adipocytes (11) is present in rat placenta, it does not survive homogenization and preparation of membranes. Affinity labeling of the purified receptor shows that the M, = 250,000 species, the only band observed upon silver staining except for a minor contaminant of bovine serum albumin (Fig. 2), is the only band covalently labeled by 125I-IGF II and the cross-linking agent DSS (Fig. 3). The fact that unlabeled insulin does not compete for binding or affinity labeling of the receptor, even at a concentration of 3 μM, is consistent with previous finding that the IGF II receptor has no affinity for insulin (7). IGF I, however, shows significant affinity for this receptor in competition binding assays, again consistent with previous results (7).

The purification reported here is sufficiently rapid and quantitative to permit studies on possible physiological covariant modifications of the IGF II receptor and in particular to investigate the molecular mechanism of its rapid modulation by insulin. The method should be applicable to purification of the receptor from many other tissues. In addition, the method can easily be scaled up to obtain large amounts of purified material suitable for antibody production and protein chemistry studies.

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