Separation of the High Affinity Insulin-like Growth Factor I Receptor from Low Affinity Binding Sites by Affinity Chromatography*

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We have identified high and low affinity insulin-like growth factor I (IGF I)-binding sites with mean dissociation constants of 0.37 and 6.25 nM, respectively, in solubilized placental membranes. We have separated these sites and purified the high affinity IGF I receptor 1,300-fold, with an overall yield of 9.9%, using wheat germ agglutinin-Sepharose chromatography, insulin affinity chromatography, and IGF I affinity chromatography. The Scatchard plot of IGF I binding to the high affinity receptor is linear, suggesting the purification of a single homogeneous class of binding sites. Insulin is two orders of magnitude less effective than IGF I in competitively inhibiting IGF I binding to this receptor. The high affinity IGF I receptor is composed of two subunits with apparent molecular weights of 135,500 and 96,200, respectively. IGF I at concentrations of ≥50 ng/ml stimulates autophosphorylation of the β subunit of the purified high affinity receptor 4.6-fold. Low affinity IGF I-binding sites run through the IGF I affinity column or are eluted from the insulin affinity column. The separation of IGF I receptors with different binding affinities by sequential affinity chromatography will make it possible to examine directly the determinants of receptor affinity.

Insulin-like growth factor I or somatomedin C appears to play a key role in mediating the growth effects of pituitary growth hormone (1-3). IGF I interacts with a specific receptor in the plasma membrane of responsive cells. Analysis by affinity cross-linking and by immunoprecipitation with an anti-receptor antibody has demonstrated that the receptor is a disulfide-linked heterotetramer, composed of two α and two β subunits (4-9). The Mr of the α and β subunits, estimated from their electrophoretic mobility under reducing conditions, are 130,000–135,000 and 95,000, respectively. The IGF I receptor is structurally homologous to the insulin receptor.

The formation of a specific IGF I-receptor complex results in both rapid and more slowly developing responses. In intact cells (10, 11) and in partially purified receptor preparations (10, 12–14), IGF I stimulates a tyrosine-specific protein kinase activity which leads to β subunit phosphorylation (10–14) and to phosphorylation of exogenous tyrosine-containing substrates (13, 14). Studies with partially purified receptor preparations from BRL-3A2 rat liver cells (14) and from human placenta (15) have suggested that the substrate specificity, activation by phosphorylation, and nucleotide and cation preference of the IGF I receptor kinase closely resemble those of the insulin receptor kinase. By analogy to the insulin receptor, to receptors for a variety of growth factors including epidermal growth factor and platelet-derived growth factor (16), and to the protein kinases encoded by viral oncogenes (17), it has been argued that the tyrosine kinase activity of the IGF I receptor is intrinsic to the β subunit.

Recent studies have suggested that heterogeneity exists among IGF I receptors. Binding studies have suggested considerable variability in the affinity of the IGF I receptor for insulin and IGF II in different tissues (18). Hintz et al. (19) detected 125I-labeled IGF II binding activity which was displaced almost equipotently by insulin, IGF I, and IGF II in IM-9 human lymphoid cells and in human placental membranes. Jonas et al. (20) showed that an atypical receptor with high affinity for IGF I and II copurified with insulin receptors. The Kd for IGF I binding ranged from 2–4 nM, and insulin was 5–15 times more effective than IGF I in displacing 125I-labeled IGF I. Jonas and Harrison (21) also identified two forms of the IGF I receptor in human placental membranes on the basis of their immunoreactivity with a human autoantiserum (B-2) to the insulin receptor. The B-2 reactive IGF I receptor form bound with lower affinity to IGF I (Kd = 0.71 nM) than the B-2 nonreactive form (Kd = 0.21 nM). Insulin was somewhat more effective in displacing 125I-labeled IGF I from the B-2 reactive than from the B-2 nonreactive receptor form. Morgan and Roth (22) showed that a monoclonal antibody (6D9) could inhibit IGF I binding to its receptor in solubilized placental receptor preparations and in IM-9 human lymphoid cells but not in all tissues. Although their data suggested that different species of the IGF I receptor might exist in the same tissue, the affinities and relative amounts of the receptor species were not determined.

Massague and Czech (6) have shown by affinity cross-linking that the human placenta contains high amounts of IGF I and insulin receptors and low amounts of IGF II receptor. By careful analysis of binding data, we have identified IGF I receptors with different affinities in solubilized placental membranes. We have been able to separate these and to purify the high affinity IGF I receptor from human placental membranes using an IGF I affinity column. The development of IGF I affinity chromatography to purify the high affinity IGF I receptor and the characteristics of the receptor are described in this report.

*This work was supported in part by United States Public Health Service Grants AI20543 and HD28905 and by a grant from the Juvenile Diabetes 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.

Received for publication, April 30, 1987

Printed in U.S.A.

Vol. 262, No. 34, Issue of December 5, pp. 16461-16469, 1987

Published by The American Society for Biochemistry and Molecular Biology, Inc

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EXPERIMENTAL PROCEDURES

Materials—CNBr-activated Sepharose 4B was obtained from Pharmacia Fine Chemicals. Affi-Gel 10 and Bio-Gel P-2 were purchased from Bio-Rad. DEAE-cellulose (prewollen microgranular anion exchanger DE52) was a product of Whatman. Sephadex G-75-120, wheat germ agglutinin, lactoperoxidase (EC.11.1.1.7) from bovine milk, and ATP from equine muscle were obtained from Sigma. N-acetyl-b-glucosamine was obtained from Flonstiehl Laboratories, Inc. Crystalline porcine insulin was a gift of Dr. Ronald D. Glomar (Lilly). IGF I used in binding assays was purchased from AmGen Biotechnologies, and IGF I used to prepare the IGF I affinity adsorbent was the generous gift of Dr. B. D. Burleigh (International Minerals and Chemicals Corp., Northbrook, IL). [125I]-labeled insulin (monoiodeinated, specific activity 305-370 cpm/ng) was purified by high pressure liquid chromatography, was provided by Dr. Ronald L. Gingerich, Washington University. Na[I] (13-17 mCi/100 g) and [35S]PHTP (3000 Ci/mmol) were supplied by Amersham and Du Pont-New England Nuclear Research Products, respectively. Immunoglobulin fractions of normal rabbit serum and of rabbit antiserum to human albumin were produced by DAKO-immunoglobulins a/s. Pansorbin cells were purchased from Behring Diagnostics.

Preparation of Affinity Adsorbents—Wheat germ agglutinin was coupled to CNBr-activated Sepharose 4B, exactly as described by the manufacturer. Coupling efficiency, estimated by determining the amount of remaining uncoupled, was >99%, resulting in the coupling of 5.8 mg of WGA/ml of gel.

The insulin affinity adsorbent was prepared by mixing 25 ml of Affi-Gel 10 with an equal volume of 0.1 M HEPES buffer, pH 7.5, with 80 mM CaCl₂ and 6 M urea, containing 1 mg/ml insulin and a small amount of N-acetyl-b-glucosamine, as described by Pike et al. (25). Coupling efficiency, estimated by determining the [125I]-labeled insulin remaining uncoupled, was >97%.

To prepare the IGF I affinity adsorbent, ~3.5 ml of Affi-Gel 10 was washed as described by the manufacturer and added to an equal volume of 0.1 M HEPES buffer, pH 7.5, containing 1 mg/ml IGF I. A small amount of [125I]-labeled IGF I was added to the IGF I solution to monitor coupling efficiency. The suspension was gently agitated for 4 h at 4°C. After centrifugation, the gel was resuspended in an equal volume of 0.1 M glycine in 0.1 M HEPES buffer, pH 8.0, and agitated for an additional 1 h at 4°C to block unreacted groups. After centrifugation, the gel was resuspended in 0.1 M HEPES buffer, pH 7.5, transferred to a column, and washed until the A₂₈₀ of the column effluent fell to 0. The column was then washed with 3 cycles of alternating 0.1 M sodium acetate buffer, pH 4.0, with 0.5 M NaCl and 0.1 M NaHCO₃, pH 8.5, with 0.5 M NaCl. The column was equilibrated in 40 mM imidazole-HCl buffer, pH 7.4, 0.1% Triton X-100, and 10% glycerol, to a specific activity of 5.8 mg of WGA/ml of gel.

The WGA-Sepharose eluate was mixed with the insulin affinity adsorbent and rotated end-over-end for 2 h at 4°C. The adsorbent was then poured into a column and washed with Buffer A with 0.5 M NaCl until the A₂₈₀ of the column effluent fell to <0.015. Adsorbed protein was eluted with 0.1 M sodium acetate buffer, pH 5.0, with 1.5 M NaCl, 0.1% Triton X-100, and 10% glycerol. Fractions of 1 ml were collected into tubes containing 1 M Tris-HCl, pH 7.4, for immediate neutralization and were placed on ice. Fractions containing insulin binding activity were pooled and dialyzed against 40 mM imidazole-HCl buffer, pH 7.4, with 10% glycerol. To concentrate the receptor activity, the fractions were diluted with an equal volume of Buffer A and applied to a DEAE-cellulose column equilibrated in Buffer A. Bound protein was eluted with 0.25 M NaCl in Buffer A and stored at -70°C. In most preparations, the run-through/first column wash from the insulin affinity column was mixed with the insulin affinity adsorbent again, and this step was repeated to deplete it further of insulin binding activity.

The run-through/first column wash from the insulin affinity column was pooled and applied to the IGF I affinity column. In early preparations, the run-through/wash was recycled through the column for 18-24 h at 4°C at a flow rate of 20 ml/h. In more recent preparations, the run-through/wash was concentrated to 10 ml by ultrafiltration using a YM-10 Diaflo membrane (Amicon), mixed with the IGF I affinity adsorbent, and rotated end-over-end overnight at 4°C. The adsorbent was then poured into a column and was washed until the A₂₈₀ of the column effluent fell to <0.015. Adsorbed protein was eluted with 10 mM sodium acetate buffer, pH 5.0, with 1.5 M NaCl, 0.1% Triton X-100, and 10% glycerol. Fractions of 1 ml were collected into tubes containing 1 M Tris-HCl, pH 7.4, for immediate neutralization and were placed on ice. Fractions containing IGF I binding activity were pooled and dialyzed against 40 mM imidazole-HCl buffer, pH 7.4, with 10% glycerol. To concentrate the receptor activity, the fractions were diluted with an equal volume of Buffer A and applied to a DEAE-cellulose column equilibrated in Buffer A. Bound protein was eluted with 0.25 M NaCl in Buffer A and stored at -70°C. The run-through/first column wash from the IGF I affinity column was mixed with the IGF I affinity adsorbent, and this step was repeated at least twice.

Binding Studies—IGF I was radioiodinated using lactoperoxidase, essentially as described by Tait et al. (26). [125I]-Labeled IGF I was purified from the reaction mixture by gel filtration on a Sephadex G-75 column (1.5 x 25 cm) equilibrated in 0.1 M acetic acid. The specific activity ranged from 200 to 240 cpm/100 pg. [125I]-Labeled IGF I was stored in 0.1 M acetic acid containing 10 mg/ml BSA at -70°C and was routinely repurified by gel filtration before use.

Binding assays were performed in 1.6-m1 polystyrene microtiter plates. Reactants in a final volume of 0.2 ml were (1) [125I]-labeled IGF I or [35S]PHTP (0.25-1.0 pg); (2) 125I-labeled IGF I or [35S]PHTP (0.25-1.0 pg); (3) 125I-labeled IGF I or [35S]PHTP (0.25-1.0 pg); and (4) solubilized membrane fraction, 10-75 g/l. Tris-HCl buffer, pH 7.4, with 10% acetic acid. The addition of 0.05 M NaCl was the generous gift of Dr. B. D. Burleigh (International Minerals and Chemicals Corp., Northbrook, IL). The values reported for binding capacities and dissociation constants were obtained from a fit based on one of several classes of sites that exhibited in the fit by adding another class of sites. Reactant in binding buffer, 0 or 50 & (26), 5 mg/ml BSA (binding buffer), 150-265 & (26), 2) unlabeled ligand in binding buffer, 0 or 50 & (26), and 3) [125I]-labeled IGF I or [35S]PHTP in binding buffer, 0 or 50 & (26). Protein concentration on 0.1 M acetic acid was calculated. Binding data were analyzed by Ligand, a computer program developed by Munson and Rodbard (27). The values reported for binding capacities and dissociation constants were obtained from a fit based on a model that included the additional complexity.

Radioiodination of Purified IGF I Receptor Preparations—Purified IGF I receptor or solubilized placental membrane protein was dialyzed against PBS on a Millipore VMWP filter and was radioiodinated using chloramine T (28). The [125I]-labeled protein was purified from the reaction mixture by gel filtration on a Sephadex G-75 column (1.5 x 25 cm) equilibrated in PBS with 0.1% Triton X-100, 0.2 mM phenylmethylsulfonyl fluoride, and 1 & (28). Fractions in the void volume of this column were pooled and stored at -20°C until use.
High Affinity IGF I Receptor

Immunoprecipitation of Contaminating Albumin from Purified IGF I Receptor Preparations—125I-Labeled IGF I receptor in 40 nM imidazole-HCl buffer, pH 7.4, with 0.125 M NaCl, 0.05% Triton X-100, 5% glycerol, 0.2 mM phenylmethylsulfonyl fluoride, and 1 μM leupeptin (final volume 200 μl) was incubated with 10 μl of rabbit anti-human albumin immunoglobulins or with 10 μl of normal rabbit immunoglobulins for 2 h on ice. 200 μl of a 10% suspension of Pansorbin in the above buffer was added to the incubation for an additional 30 min to precipitate immune complexes. After centrifugation, the supernatants were removed, and the pellets were washed, resuspended in 100 μl of SDS sample buffer, and heated for 5 min at 100 °C. The supernatants and dissociated immune complexes were analyzed by SDS-PAGE, as described below. Immunoprecipitation of contaminating albumin from unlabelled IGF I receptor was performed in exactly the same manner, but the supernatants were used in competition binding studies.

Polyacrylamide Gel Electrophoresis and Autoradiography—SDS-PAGE was performed according to the method of Laemmli (29) with 7.5% acrylamide resolving gels. Samples were precipitated with trichloroacetic acid and washed with acetone to extract the Triton X-100 before SDS-PAGE. Precipitated protein was suspended in sample buffer containing 2% SDS and 5% β-mercaptoethanol and heated for 5 min at 100 °C. Molecular weight standards (Bio-Rad) included myosin (200,000), β-galactosidase (116,250), phosphorylase b (92,500), BSA (66,200), and ovalbumin (45,000). Gels were fixed, stained, and destained, as previously described (30) and were then dried and exposed to Kodak XAR-5 x-ray film at -70 °C. Autoradiographs were scanned in an LKB Ultrascan XL laser densitometer.

Phosphorylation Assay—Purified IGF I receptor was incubated in the absence or presence of IGF I in 40 nM imidazole-HCl buffer, pH 7.4, with 0.125 M NaCl, 0.05% Triton X-100, and 5% glycerol. After 20 min at room temperature, MnCl2 (final concentration 5 mM) was added, and phosphorylation was initiated by the addition of ATP (final concentration 5 μM) and 40 μCi of [γ-32P]ATP. The final reaction volume was 100 μl. After 10 min at room temperature, the reaction was terminated by the addition of 200 μl of 30% trichloroacetic acid and washed with acetone to extract the Triton X-100. IGF I binding activity is efficiently solubilized from human placental membranes with 0.5% Triton X-100. Recovery of insulin binding activity that can be extracted is extracted with 0.5% Triton X-100, and 10% glycerol at 30 °C. High affinity insulin binding activity recovered per placenta has ranged from 68.5 to 123.7 pmol of insulin bound. Similar yields have been reported by others (23, 24, 33–35).

RESULTS

IGF I Binding to Solubilized Placental Membranes—Binding of IGF I to solubilized membranes at 4 °C is saturable, as shown in Fig. 1A for membrane protein solubilized with 0.5% Triton X-100. Analysis of IGF I binding to solubilized membranes was complicated in that the Scatchard plots are curvilinear. In order to obtain optimal estimates of the binding parameters, the curves have been fit by a two-site binding model, as described by Munson and Rodbard (27). The Scatchard plot of IGF I binding to solubilized protein, analyzed by LIGAND, is shown in Fig. 1B. The mean ± S.D. for the binding parameters derived from analysis of IGF I binding to solubilized protein and to the WGA-Sepharose eluate in 7 placental preparations has been calculated. The dissociation constants of the high affinity and low affinity sites are 0.37 ± 0.19 and 6.25 ± 2.94 nM, respectively. Although there is some variation in the dissociation constants among the different preparations, the dissociation constant of the high affinity site is always at least an order of magnitude lower than that of the low affinity site in each preparation. The IGF I binding capacity of the low affinity site ranges from 3.5 to 8.5 times greater than that of the high affinity site in the preparations.

The dissociation constants of IGF I binding are similar to those derived from analysis of insulin binding to solubilized membranes. In seven placental preparations, the mean ± S.D. for the dissociation constant of the high affinity insulin binding site is 0.38 ± 0.08 nM. High affinity binding is about half of total insulin binding activity. Placental membranes solubilized with 0.5% Triton X-100 contain at least 4 times more high affinity insulin than high affinity IGF I-binding sites.

Purification of the IGF I Receptor from Human Placental

Membranes—We have purified the IGF I receptor from human placental membranes by the procedure described under “Experimental Procedures.” Competition binding studies are performed at each step in the purification procedure, and the binding capacity of the high affinity IGF I-binding site is used to determine the yield and fold purification. Table I summarizes a typical receptor purification from three human placentas.

Several features of the purification procedure are noteworthy. IGF I binding activity is efficiently solubilized from human placental membranes with 0.5% Triton X-100. Re-extraction of the residual membranous pellets with 2% Triton X-100 demonstrated that over 80% of the IGF I binding activity that can be extracted is extracted with 0.5% Triton X-100. Similarly, approximately 90% of the insulin binding activity that can be extracted is extracted with 0.5% Triton X-100. IGF I and insulin binding activities are adsorbed by WGA-Sepharose and are eluted together with 0.4 M N-acetylglucosamine. This step results in a 5.4- to 13.3-fold purification of the IGF I receptor.

In order to separate the IGF I and insulin binding activities, the WGA-Sepharose eluate is batch-adsorbed to the insulin affinity adsorbent. Adsorbed insulin binding activity is eluted with 50 mM sodium acetate buffer, pH 5.0, with 0.5 M MgCl2, 0.1% Triton X-100, and 10% glycerol at 30 °C. High affinity insulin binding activity recovered per placenta has ranged from 68.5 to 123.7 pmol of insulin bound. Similar yields have been reported by others (23, 24, 33–35). More importantly,
this step results in \( \geq 85\% \) depletion of total insulin binding activity from the insulin affinity column run-through/wash. In the preparation summarized in Table I, 43.6\% of the high affinity IGF I-binding sites are recovered at this step. As discussed below, IGF I binding activity is also eluted from the insulin affinity column.

To purify the IGF I receptor further, the insulin affinity column run-through/wash is concentrated and batch-adSORBED to the IGF I affinity adsorbent. Adsorbed IGF I binding activity is eluted with 10 mM sodium acetate buffer, pH 5.0, with 1.5 mM NaCl, 0.1\% Triton X-100, and 10\% glycerol. A typical elution profile is shown in Fig. 2A. The binding capacity of the high affinity IGF I-binding site recovered per placenta has ranged from 6.7 to 9.2 pmol of IGF I bound, an overall yield of 6.8–9.9\%. The specific activity of the purified receptor is 690 pmol/mg of protein, resulting in a purification of 1300-fold. The difference between the observed specific activity of the purified receptor and the theoretical specific activity, i.e. \( \sim 2800 \) pmol of IGF I bound/mg of protein,\(^5\) may result from the presence of contaminating protein (see below) and/or partial denaturation of the receptor during the purification procedure.

To assess the purity of the IGF I receptor, the IGF I affinity column eluate was radioabeled with \(^{125}\)I and analyzed by SDS-PAGE under reducing conditions and autoradiography. SDS-PAGE of the purified IGF I receptor, shown in Fig. 2B, resolves two protein bands with estimated \( M_r = 130,000 \) and 95,000.\(^6\) These agree well with the molecular weights of the \( \alpha \) and \( \beta \) subunits of the IGF I receptor, previously determined by affinity cross-linking and by immunoprecipitation with an anti-receptor antibody (4–9). A protein band with estimated \( M_r = 65,000 \) is also observed (see below).

**IGF I Binding Studies**—IGF I binding to the insulin affinity column run-through/wash, to the purified IGF I receptor eluted from the IGF I affinity column, and to the final IGF I affinity column run-through/wash is shown in Fig. 3. The dissociation constants and the relative IGF I binding capacities of the low affinity and high affinity sites do not differ significantly in the solubilized membrane protein (Fig. 1B), in the WGA-Sepharose eluate (not shown), and in the insulin affinity column run-through/wash (Fig. 3A), but the sites are effectively separated by IGF I affinity column chromatography. The Scatchard plot of IGF I binding to the combined IGF I affinity column eluates (Fig. 3B) is linear, and the dissociation constant derived from this analysis is 0.26 nM. Hence, IGF I receptor with high affinity is preferentially purified. The Scatchard plot of IGF I binding to the final IGF I affinity column run-through/wash (Fig. 3C) is also linear, and the dissociation constant derived from this analysis is 4.0 nM, an order of magnitude higher. The binding capacity of the low affinity IGF I-binding site recovered in the final IGF I affinity column run-through/wash per placenta is 32.3 pmol of IGF I bound, an overall yield of \( \sim 10\% \) (data not shown). The similar yields of high affinity (9.9\%, as shown in Table I) and low affinity binding sites suggest that the loss of IGF I binding activity that occurs during the purification procedure is not selective.

**TABLE I**

| Volume | Total protein | IGF I binding specific activity | Yield | Purification |
|--------|---------------|---------------------------------|-------|-------------|
| ml     | mg            | pmol bound/mg protein           | %     | -fold       |
| Solubilized protein | 93.5          | 590                             | 0.53  | 100         | 1           |
| WGA-Sepharose eluate | 70           | 44.9                            | 7.1   | 100        | 13.3        |
| Insulin affinity column |            |                                  |       |             |             |
| Run-through/wash 1 | 126          | 30.2                            | 4.8   | 48.6        | 9           |
| Run-through/wash 2 | 171          | 29.2                            | 4.9   | 43.6        | 9.2         |
| IGF I affinity column eluates (1–3) | 1.8         | 0.04*                           | 690   | 9.9         | 1300        |

* Determined by amino acid analysis after postcolumn ninhydrin-based derivatization, employing a Beckman 6300 autoanalyzer.

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\(^{5}\) A molecular weight of 350,000 for the IGF I receptor has been used for this calculation. Based on preliminary data in our laboratory, a stoichiometry of 1 mol of IGF I bound per mol of receptor has been assumed.

\(^{6}\) The mean \( \pm S.D. \) apparent \( M_r \) of the \( \alpha \) and \( \beta \) subunits of the purified IGF I receptor in four placental preparations have been calculated and are \( 135,500 \pm 2,200 \) and 96,200 \( \pm 3,100 \), respectively. The variation among preparations does not differ from that obtained by repeated analysis of the same preparation, i.e. 133,800 \( \pm 2,100 \) and 95,900 \( \pm 3,000 \), for the \( \alpha \) and \( \beta \) subunits, respectively.

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High Affinity IGF I Receptor

Purification of the high affinity IGF I receptor from human placental membranes

IGF I receptor was solubilized from membranes from three human placentas and purified as described under "Experimental Procedures." The insulin affinity column step was repeated twice, and aliquots from the three eluates were combined for assay. Competition binding studies were performed at each step in the purification procedure, as shown in Fig. 1. The binding capacity of the high affinity IGF I-binding site was used to determine the yield and -fold purification. Protein was measured by the method of Lowry et al. (31), as modified by Peterson (32), using BSA as the standard.
**High Affinity IGF I Receptor**

A. elution profile from the IGF I affinity column. The IGF I affinity adsorbent was poured into a column and washed with Buffer A with 1.0 M NaCl at a flow rate of 40 ml/h. Adsorbed protein was eluted with 10 mM sodium acetate buffer, pH 5.0, with 1.5 M NaCl, 0.1% Triton X-100, and 10% glycerol at 30 °C, as indicated by the arrow. IGF I binding activity was monitored by incubating 20 μl of every other fraction with 19,000 cpm (0.0076 × 10^9 nmol) of 125I-labeled IGF I for 100 min at 15 °C and precipitating receptor-bound 125I-labeled IGF I with polyethylene glycol, as described under “Experimental Procedures.”

B. autoradiograph of 125I-labeled solubilized placental membrane protein (S) and of 125I-labeled IGF I receptor eluted from the IGF I affinity column (R), analyzed by SDS-PAGE under reducing conditions. The migration of molecular weight standards is indicated.

**IGF I Receptor**—The major contaminant in the purified IGF I receptor preparations has been a protein with $M_r \approx 65,000$. Because this protein migrated with the BSA standard on SDS-PAGE and varied in amount among the preparations, its identity was suspected to be albumin. To confirm this, the 125I-labeled IGF I receptor was incubated with the immunoglobulin fraction of rabbit antiserum to human albumin or of normal rabbit serum, and immune complexes were precipitated with Pansorbin cells. The supernatants and immune complexes precipitated were analyzed by SDS-PAGE under reducing conditions and autoradiography, as shown in Fig. 5A. The protein with $M_r \approx 65,000$ is precipitated with the rabbit anti-human albumin (lane 2) but not the normal rabbit (lane 4) immunoglobulins. The supernatant remaining after immunoprecipitation with the rabbit anti-human albumin immunoglobulins (lane 1) contains only the $\alpha$ and $\beta$ subunits of the IGF I receptor.

To examine the possibility that the protein with $M_r =$
Fig. 4. Competition curves of IGF I binding. The specificity of IGF I binding to the high affinity IGF I receptor in the IGF I affinity column eluates and to the low affinity IGF I-binding site in the final IGF I affinity column run-through/wash was analyzed in competition binding studies. Nonspecific binding has not been subtracted. Data are expressed as B/Bo, the fraction of maximum bound. The solid curves are the computer-generated best fit for a one-site binding model. A, inhibition of ¹²⁵I-labeled IGF I binding to the high affinity IGF I receptor by unlabeled IGF I (■) or unlabeled insulin (□). In this study, 24.1% of the ¹²⁵I-labeled IGF I was bound in the absence of unlabeled ligand. B, inhibition of ¹²⁵I-labeled IGF I binding to the low affinity IGF I-binding site by unlabeled IGF I (■) or unlabeled insulin (□). In this study, 22.3% of the ¹²⁵I-labeled IGF I was bound in the absence of unlabeled ligand.

~65,000 affects IGF I binding to the purified IGF I receptor, unlabeled IGF I receptor was incubated with the immunoglobulin fraction of rabbit antiserum to human albumin or of normal rabbit serum, and immune complexes were precipitated with Pansorbin cells. The supernatants remaining after immunoprecipitation were then used in competition binding studies. The inhibition of ¹²⁵I-labeled IGF I binding by unlabeled IGF I in these studies is shown in Fig. 5A. The dissociation constants derived from analysis of IGF I binding to these supernatants are 0.21 and 0.22 nM, indicating that the presence or absence of albumin in the IGF I receptor preparation does not affect IGF I binding. To verify that albumin had been completely precipitated from the unlabeled IGF I receptor preparation, immunoprecipitation of a separate sample of unlabeled IGF I receptor to which ¹²⁵I-labeled IGF I receptor had been added was performed. The supernatant remaining after immunoprecipitation with rabbit anti-human albumin immunoglobulins contained only the α and β subunits of the IGF I receptor (data not shown).

Kinase Activity of the Purified IGF I Receptor—IGF I has previously been reported to stimulate the phosphorylation of the β subunit of the IGF I receptor in partially purified receptor preparations from IM-9 human lymphoid cells (10), from placenta (12), and BRL-3A2 rat liver cells (13, 14). The possibility that the kinase was a distinct protein closely associated with the receptor or that the presence of insulin receptor in the partially purified preparations was responsible

Fig. 5. Immunoprecipitation of albumin from the purified IGF I receptor preparation. IGF I receptor was purified as described under “Experimental Procedures.” ¹²⁵I-Labeled or unlabeled IGF I receptor was incubated with the immunoglobulin fraction of rabbit antiserum to human albumin or of normal rabbit serum, and immune complexes were precipitated with Pansorbin cells, as described under “Experimental Procedures.” A, autoradiograph of the ¹²⁵I-labeled IGF I receptor preparation, analyzed by SDS-PAGE under reducing conditions. Lane 1, the supernatant remaining after immunoprecipitation with rabbit anti-human albumin immunoglobulins, and lane 2, the immune complex precipitated. Lane 3, the supernatant remaining after immunoprecipitation with normal rabbit immunoglobulins, and lane 4, the immune complex precipitated. The migration of molecular weight standards is indicated. B, inhibition of ¹²⁵I-labeled IGF I binding by unlabeled IGF I. The supernatants remaining after immunoprecipitation of unlabeled IGF I receptor with rabbit anti-human albumin immunoglobulins (□) or with normal rabbit immunoglobulins (■) were diluted with binding buffer, and IGF I binding studies were performed, as described under “Experimental Procedures.” Nonspecific binding has not been subtracted. The solid curves are the computer-generated best fit for a one-site binding model.
for the observed kinase activity could not be rigorously excluded in these studies. To demonstrate directly that kinase activity is intrinsic to the IGF I receptor, hormone-dependent autophosphorylation of the highly purified IGF I receptor was investigated. The high affinity IGF I receptor was incubated in the absence or presence of increasing concentrations of IGF I for 20 min, and phosphorylation was initiated by the addition of [γ-32P]ATP, as described under "Experimental Procedures." A, autoradiogram of the phosphorylated receptor, analyzed by SDS-PAGE under reducing conditions. IGF I was present at concentrations of 5-500 ng/ml, as noted. The migration of molecular weight standards is indicated. β, 32P incorporated into the β subunit, determined by densitometric scanning. The area in the absence of IGF I (not shown) was 0.9.

**DISCUSSION**

We report here the identification of IGF I receptors with different binding affinities in solubilized placental membranes. Binding data have been analyzed by LIGAND, a computer program developed by Munson and Rodbard (27). This program facilitates a systematic, objective data analysis by providing estimates of the binding parameters, evaluating the adequacy of fit of the data, and allowing convenient formulation and comparison of a variety of models. The best model among these may then be chosen on a statistically meaningful basis. IGF I binding to solubilized protein, to the WGA-Sepharose eluate, and to the insulin affinity column run-through/wash is best fit by a two-sites binding model. Although there is some variation in the dissociation constants among the different preparations, the dissociation constant of the high affinity site is always at least an order of magnitude lower than that of the low affinity site in each preparation. Curvilinear Scatchard plots have been obtained by others for IGF I binding to particulate placental membranes (36, 37), and the association constants agree well with those obtained by us for IGF I binding to solubilized placental membranes.

We have used an IGF I affinity column to purify the high affinity IGF I receptor from human placental membranes. The affinity adsorbent contains ~1 mg/ml IGF I, and repeated adsorption of the run-through/wash is performed to improve the yield at this step in the purification procedure. The Scatchard plot of IGF I binding to the combined IGF I affinity column eluates is linear, suggesting the purification of a single homogeneous class of binding sites. The dissociation constant derived from this analysis is 0.26 nM. The dissociation constant derived from analysis of IGF I binding to the final IGF I affinity column run-through/wash is 4.0 nM. Hence, IGF I receptors with different affinities are effectively separated by this purification step. While this work was in progress, LeBon et al. (38) described the purification of the IGF I receptor from human placental membranes by immunoaffinity chromatography using αIR-3, a monoclonal antibody directed against the IGF I receptor. Although this antibody preferentially immunoprecipitates IGF I receptors, it also cross-reacts weakly with insulin receptors (9) and may not distinguish IGF I receptors with different IGF I binding affinities. The Scatchard plot of IGF I binding to receptor purified by immunoaffinity chromatography using αIR-3 is curvilinear (38), suggesting that, in fact, IGF I receptors with different IGF I binding affinities are copurified. The utility of immunochemical methods which require monoclonal antibodies may also be restricted by cell type or species specificity.

The high affinity IGF I receptor purified by IGF I affinity chromatography has intrinsic kinase activity, and IGF I stimulates autophosphorylation of the β subunit of the receptor 4.6-fold. IGF I-dependent stimulation of phosphorylation, with maximal 3-4-fold stimulation at ≈25 nM (200 ng/ml), has been reported in partially purified receptor preparations from placenta (12) and from BRL-3A2 rat liver cells (13, 14). Morgan et al. (39) have also reported recently that the IGF I receptor from BRL-3A rat liver cells, which was highly purified by chromatography using a monoclonal antibody to the cytoplasmic domain of the insulin receptor, possesses intrinsic kinase activity. In contrast, there is no detectable stimulation of phosphorylation of the β subunit by IGF I in experiments with IGF I receptor isolated by immunoprecipitation with αIR-3 (10), and IGF I-stimulated kinase activity of the receptor purified by immunoaffinity chromatography has been reported to be <2-fold at 1 μM (7.65 μg/ml) IGF I (40, Fig. 6). These results suggest that the interaction of the IGF I receptor with αIR-3 itself or the elution conditions required to break this interaction alter receptor activity. The use of IGF I affinity column chromatography makes possible the purifi-
cation of high affinity IGF I receptor which retains IGF I-dependent kinase activity.

In the course of our studies, we have also noted that IGF I binding activity is eluted from the insulin affinity column. The dissociation constant derived from our analysis of IGF I binding to the insulin affinity column eluates is 1.0-3.2 nM, and insulin is about 5 times more effective than IGF I in competitively inhibiting $^{125}$I-labeled IGF I binding to this receptor (data not shown). Our data suggest that this binding activity is distinct from the insulin receptor but that it represents about 30% of the total insulin binding activity present in the insulin affinity column eluates. It is likely that the binding activity we have noted is similar to that described by Hintz et al. (19) and Jonas et al. (20).

The heterogeneity in IGF I receptor affinities is not readily explained. The complete amino acid sequence of the IGF I receptor has recently been deduced from human placental cDNA clones (41). The deduced sequence predicts a 1367-amino acid receptor precursor, including a 30-amino acid signal peptide. There are close similarities in the overall organization of the IGF I and insulin receptor precursors, and amino acid homology in the domains of the two precursors ranges from 41-84%, except in the signal peptide and transmembrane domains. cDNA encoding the human insulin receptor precursor hybridizes with four major mRNAs of 10.3, 9.6, 8.5, and 6.7 kilobases. Interestingly, two insulin receptor species which differ by a 12-amino acid insertion at the C terminus of the $\alpha$ subunit have been described by Ullrich et al. (42) and Ehina et al. (43). A different pattern of Northern blot hybridization is detected with human IGF I receptor cDNA, which hybridizes to two major mRNAs of 11.0 and 7.0 kilobases. It is not known whether both mRNAs code for the same IGF I receptor precursor sequence or whether structural variants exist. Jonas and Harrison (44) presented evidence that the lower affinity B-2 immunoreactive form of the IGF I receptor was converted into the higher affinity nonimmuno-reactive form by reduction of receptor disulfide bonds. However, the change in affinity after disulfide bond reduction was minimal ($K_d = 0.71$ nM to $K_d = 0.5$ nM). In addition, they could not detect the reduced $\alpha\beta$ dimeric form in placental membranes by affinity cross-linking of $^{125}$I-labeled IGF I. Our analysis of the purified high affinity IGF I receptor by SDS-PAGE under nonreducing conditions has not demonstrated the $\alpha\beta$ dimer (data not shown). Because IGF I affinity column chromatography allows a significant separation of IGF I receptors with different IGF I binding affinities, it will now be possible to examine the determinants of receptor affinity directly.

Steady state binding to many membrane receptors has been demonstrated distinct species of receptors differing in their affinity for ligand. Although curvilinear Scatchard plots may be explained by negative cooperativity (46), alternative mechanisms have been proposed. The interleukin 2 receptor appears to associate with a protein with an apparent $M_1 = 70,000$, which converts it from a low affinity to a high affinity form (46, 47). We have noted a protein with $M_1 = 65,000$ in our purified IGF I receptor preparations, and a protein with a similar $M_1$ has been noted by others in purified insulin receptor preparations (15, 23, 40). As shown here, the protein in our preparations can be precipitated with rabbit anti-human albumin immunoglobulins, suggesting that albumin can interact nonspecifically with the affinity adsorbents used in the purification procedure. The presence or absence of albumin does not affect IGF I binding to the purified high affinity IGF I receptor. Hence, because no other major proteins were detected in the purified IGF I receptor preparations, it is unlikely that the IGF I receptor requires a "converter" protein for high affinity binding. Covalent modifications of plasma membrane receptors by phosphorylation may also regulate receptor function and distribution (for review, see 48). Studies to examine the possibility that post-translational modifications may alter the affinity of the receptor for IGF I are underway.

Acknowledgments—We wish to thank Dr. Linda J. Pike for advice in preparing placental membranes and in using the LIGAND program and Dr. Ned Seigel at Monsanto Company, St. Louis, MO, for amino acid analysis of the purified IGF I receptor.

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