Purification of an Insulin-like Growth Factor II Receptor from Rat Chondrosarcoma Cells*

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An insulin-like growth factor II (IGF-II) receptor was purified from rat chondrosarcoma cells by Triton X-100 solubilization of a 100,000 × g membrane preparation and affinity chromatography on a multiplicity-stimulating activity (MSA)-Sepharose column. Analysis of the purified receptor by sodium dodecyl sulfate-gel electrophoresis and silver staining showed a major band of Mr = 210,000 (Mr = 250,000 after reduction with dithiothreitol). When 125I-MSA was chemically cross-linked to the purified receptor and analyzed by sodium dodecyl sulfate-gel electrophoresis (with and without dithiothreitol) and autoradiography, the radioactive bands coincided with the Mr = 210,000 and 250,000 bands identified by silver staining. The purified receptor also appeared to contain an Mr = <68,000 species identified by silver staining in addition to the Mr = 250,000 binding component. IGF-I, IGF-II, and MSA-II inhibited binding of 125I-MSA to the purified receptor with the same relative potency as for binding to the intact chondrosarcoma cell (IGF-II > MSA-II > IGF-I), and insulin did not inhibit binding. The association constant (Kd) for MSA-II binding to the purified receptor was 2 × 10^9 M⁻¹. The purified receptor bound to concanavalin A-Sepharose and wheat germ lectin-Sepharose columns and was eluted with α-methyl-D-mannoside and N-acetyl-D-glucosamine, respectively, showing that the receptor is a glycoprotein.

The IGFs' or somatomedins are mitogens for cells in culture and have weak insulin-like metabolic activity (1, 2). Competitive binding experiments comparing the ability of IGFs and insulin to compete with radiolabeled IGFs for binding to intact cells and membrane preparations show that IGF receptors are heterogeneous (3). One receptor type has a higher affinity for IGF-I than for IGF-II or MSA (the rat homologue of human IGF-II (4)) and also interacts with insulin. A second type of IGF receptor binds IGF-II more tightly than it does IGF-I and does not recognize insulin. In recent studies, radiolabeled IGFs were chemically cross-linked to purified membranes or intact cells and the resultant 125I-IGF-receptor complexes were characterized by SDS-PAGE. The receptor which preferred IGF-I and was insulin sensitive (Type I) had a binding subunit of Mr = 190,000 (5–8), the same size as the α subunit of the insulin receptor (9, 10). By contrast, the receptor which preferred IGF-II and was insulin insensitive (Type II) had an Mr of 280,000 (5, 11).

Definitive characterization of IGF receptor structure and chemistry will require purification of the receptors. Although several reports have described solubilization of functional IGF-I and IGF-II receptors from membranes (7, 12, 13), preparation of highly purified IGF receptors has not been accomplished. We previously demonstrated that IGF-I, IGF-II, and MSA stimulated proteoglycan synthesis in chondrosarcoma chondrocytes (14, 15), and competitive binding studies showed that these cells have both IGF-I and IGF-II receptors, with predominance of the IGF-II receptor (15). In this paper, we describe the purification of the IGF-II receptor from these chondrosarcoma cells.

EXPERIMENTAL PROCEDURES

Materials—IGF-I (16SPII) and IGF-II (SEIV) were kindly provided by René Humbel, Zürich, Switzerland. The IGF-I preparation contains 10% IGF-II. Activated CH-Sepharose 4B was purchased from Pharmacia.

MSA Purification—MSA polypeptides were purified from serum-free media conditioned by the BRL-5A rat liver cell line as previously described (16). Alternatively, MSA-III-2 (Mr = 7100) was prepared according to the procedure of Marquardt et al. (4). MSA-II-1 (Mr = 8700), MSA-III-2, and IGF-1 were iodinated with Na125I to a specific activity of 50 to 200 μCi/μg, using a modification of the chloramine-T procedure (17). Radiolabeled MSA-III-1 and MSA-III-2 were used interchangeably for receptor binding studies.

Measurement of IGF Binding to Intact Cells and Purified Receptor—The procedure for measurement of 125I-MSA binding to intact chondrosarcoma cells was as previously described (15) except that a cell suspension was used rather than a monolayer culture. After the incubation, the cell suspension was aspirated, the tip of the tube cut off, and radioactivity measured. Nonspecific binding was determined by addition of 1 μg/ml of MSA-III-2 to the incubation.

The procedure for measuring 125I-MSA binding to purified receptor preparations was the same as for intact cells except that BSA-coated charcoal was used to separate bound from free tracer at the conclusion.

The abbreviations used are: IGF, insulin-like growth factor; MSA, multiplication-stimulating activity; SDS, sodium dodecyl sulfate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BES, 2-[bis(2-hydroxyethyl)amino]ethanesulfonic acid; TES, N-tris(hydroxymethyl)methyl-2-amino-ethanesulfonic acid; BSA, bovine serum albumin; PBS, Dulbecco's phosphate-buffered saline without Ca^2+ or Mg^2+; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis.

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of the incubation. The charcoal method was used previously in the measurement of MSA binding to serum proteins (16).

The binding capacity for MSA at various steps in the purification was determined by analysis of MSA-I-2 binding data (110 different MSA concentrations from 0.002-μg ml) according to the method of Scatchard (19). To estimate the amount of the receptor from the MSA binding capacity, an M, of 250,000 was used for the receptor, and it was assumed that the stoichiometry of binding was 1:1.

Cross-linking of 125I-MSA to Intact Cells and Purified Receptor—

Radioiodinated MSA-I-1 was chemically cross-linked to monolayer cultures of chondrosarcoma cells with diisocyanidyl substrate as previously described (5).

Cross-linking of the purified receptor, 5 × 10^4 cpd, of 125I-MSA-I-1 was added to 0.2 μg of purified receptor in 100 μl of 0.1 M sodium phosphate buffer, pH 7.4, and incubated for 2 h at room temperature. A 50-μl aliquot was used to measure 125I-MSA binding using the chemical method described above. Two μl of 2.5 mM disuccinimidyl suberate was added to the remaining 50 μl and incubated for 20 min at 15 °C. The cross-linking reaction was quenched by addition of 50 μl of 10 mM Tris-HCl, 1 mM EDTA. The 125I-MSA receptor complex was boiled in 2% SDS with or without 100 mM DTT and analyzed by SDS-PAGE as described previously (5).

Preparation of Membranes from Swarm Rat Chondrosarcoma Cells—Cells were isolated from Swarm rat chondrosarcomas by sequential treatment of a tumor mince with trypsin and collagenase (15) and maintained in suspension culture in Dulbecco-Vogt medium supplemented with HEPES, BES, and TES, overnight at 37 °C in an atmosphere of 90% air, 5% CO₂. Cells were harvested by centrifugation and stored at −70 °C in medium containing 10% glycerol. During the entire isolation procedure, cells were not exposed to serum in order to prevent possible contamination with serum IGF binding proteins. Cells (2–5 × 10⁶) accumulated from 15 to 20 large tumors were washed and suspended in 200 ml of cold 0.25 M sucrose containing 9.7 μg of phenylmethylsulfonyl fluoride and 250 μg of antipain. Homogenization and subcellular fractionation were performed at 4 °C. The cell suspension was homogenized with a Brinkmann Polytron for 2.5 min at a setting of 7. After centrifugation at 600 × g for 10 min, the supernatant was centrifuged at 12,000 × g (15,000 rpm) for 30 min in a Beckman 50.2TI rotor. The 15,000 × g supernatant was adjusted to 0.1 M NaCl, 0.2 mM MgSO₄, and centrifuged at 100,000 × g (37,000 rpm) for 90 min. The 100,000 × g pellet was suspended with the Polytron at a low setting in 200 ml of 0.05 M Tris-HCl, pH 7.4. The 100,000 × g centrifugation was repeated twice and the final pellet suspended in 40 ml of Tris-HCl.

Solubilization of the Receptor with Detergent—The receptor was solubilized from the 100,000 × g pellet suspended in 40 ml of Tris-HCl, pH 7.4, by addition of Triton X-100 to a final concentration of 1.5%, and after 30 min at room temperature the suspension was centrifuged at 100,000 × g for 90 min. The pellet was discarded and the supernatant was dialyzed overnight against 4 liters of 0.1 M sodium phosphate, pH 7.4, 0.1% Triton X-100.

MSA-Sepharose Affinity Chromatography—MSA-I-2 was coupled to the N-hydroxysuccinimide ester of CH-Sepharose 4B (activated CH-Sepharose 4B) under conditions described by Knaus et al. (20). MSA-I-2 (3.7 mg) was added to 4.5 g of activated Sepharose. The efficiency of coupling was 50% as estimated by recovery of radioactivity from 125I-MSA included in the reaction mixture.

Half of the Triton X-100 solubilized receptor (20 ml) was applied to 6 ml of MSA-Sepharose in a column (8 × 1.5 cm) at 22 °C. Application was in 2.5-ml aliquots, allowing 10-min equilibration between additions. The flow-through was collected and applied by the same stepwise procedure. The second flow-through was collected and the column was washed successively with the following buffers containing 0.1% Triton X-100: PBS, 30 ml of 0.05 M sodium phosphate, pH 6.0; and 30 ml of 0.05 M sodium acetate, pH 6.0. The fractions were analyzed by SDS-PAGE (5% acrylamide) using the discontinuous buffer system of Laemmli (23). The gel was stained for protein using a modification of a silver stain procedure developed by Switzer et al. (24).

RESULTS AND DISCUSSION

Purification of the IGF-II Type Receptor—The purification scheme was adapted from the procedure described by Cuatrecasas and Parikh (25) for purification of the insulin receptor from rat liver. Scatchard analysis of MSA binding to intact chondrosarcoma cells from nine different isolates demonstrated a range of 80,000 to 300,000 IGF-II type receptors/cell. Membranes (100,000 × g) were prepared from the chondrosarcoma cells as described under "Experimental Procedures." The Triton X-100 solubilized receptor preparation was applied to an MSA-Sepharose affinity column (Fig. 1). Most of the protein was found in the flow-through fractions, whereas the highest concentration of receptor was eluted with the buffer containing 4.5 M urea.

Table I summarizes the purification of receptor from 2.2 × 10⁹ cells. A relatively large amount of receptor was found in the 600-× g and 12,000-× g pellets. Presumably this represents receptor present on unbroken cells or on large membrane fragments remaining after the homogenization step. The receptor purified by affinity chromatography (Table I) was analyzed by SDS-PAGE (Fig. 2A). The major band identified by silver staining has an M, = 210,000. In addition, 4 faintly staining components are seen; one of M, > 250,000, 2 bands between M, = 68,000 and 94,000, and a fourth component of M, < 68,000. The band of M, > 250,000 is probably not an intrinsic component of the Triton solubilized receptor since, if it were present in a 1:1 stoichiometry with the M, = 220,000 component, the M, > 250,000 band would be expected to stain more intensely. Alternatively, the M, > 250,000 component was analyzed by SDS-PAGE (5% acrylamide) using the discontinuous buffer system of Laemmli (23). The gel was stained for protein using a modification of a silver stain procedure developed by Switzer et al. (24).
could represent a dimeric form of the $M_r = 210,000$ species. The two bands between $M_r = 68,000$ and 94,000 were not seen in receptor preparations further purified by Sepharose 6B gel filtration (Fig. 2B), so these bands probably represent contaminants. However, the $M_r < 68,000$ component is also seen in preparations that were further purified by gel filtration (Fig. 2B) suggesting that this low molecular weight species is an intrinsic component of the Triton solubilized receptor.

Based on specific MSA binding capacity values (Table I), the receptor preparation from the affinity column was purified 600-fold. The low recovery (3%) is explained by the low functional capacity of the MSA-Sepharose; additional receptor can be purified by reapplying the flow-through to the affinity column.

**Characterization of the Purified Receptor—Experiments in which $^{125}\text{I}-\text{IGF-II}$ or $^{125}\text{I}-\text{MSA}$ was chemically cross-linked to intact cells or purified membranes and the IGF-receptor complexes were analyzed by SDS-PAGE and autoradiography showed that the IGF-II type receptor of chondrosarcoma cells as well as other cells and membranes was characterized by an $M_r$ of 220,000 without reduction and 260,000 with reduction (5, 8, 11). Presumably the increase in molecular weight following reduction is due to further unfolding of the molecule after cleavage of intramolecular disulfide bonds. In agreement with these cross-linking experiments, the major band present in the purified receptor had an $M_r$ of 210,000 before reduction (Fig. 2A) and 250,000 after reduction (Fig. 2C).

The absence of the IGF-I receptor ($M_r = 130,000$ with reduction) in the purified receptor preparation presumably is because the IGF-I receptor is present in very low amounts relative to the IGF-II receptor on intact cells as indicated by competitive binding studies (15) and experiments in which $^{125}\text{I}-\text{IGF-I}$ was chemically cross-linked to intact cells and the $^{125}\text{I}-\text{IGF-I}$ receptor complexes examined by SDS-PAGE (data not shown).

The purified receptor eluted between thyroglobulin ($M_r = 669,000$; Stokes radius = 85 Å) and ferritin ($M_r = 440,000$; Stokes radius = 61 Å) on Sepharose 6B gel filtration (data not shown). Because the contribution of detergent micelles to the size of the receptor is not known, the Triton solubilized receptor could contain one or more $M_r = 250,000$ receptor units. The finding that the $M_r = 250,000$ component is the major protein band identified by silver staining after SDS-PAGE of the purified receptor (Fig. 2) suggests that other large molecular weight species are not intrinsic components of the Triton X-100 solubilized receptor. However, as discussed above, the $M_r < 68,000$ species (Fig. 2) may be an intrinsic component of the Triton solubilized receptor.

A direct demonstration of the ability of the purified receptor to specifically bind $^{125}\text{I}-\text{MSA}$ was provided by the cross-linking experiments shown in Fig. 3, A and B. The mobility on SDS-PAGE of the $^{125}\text{I}-\text{MSA}$-receptor complex produced by chemically cross-linking radioligand to intact chondrosarcoma cells (Fig. 3C) agrees with the mobility of the complex produced by cross-linking $^{125}\text{I}-\text{MSA}$ to the purified receptor (Fig. 3B); however, the band corresponding to the $^{125}\text{I}-\text{MSA}$-receptor complex is broad (Fig. 3) compared to the band identified by silver staining (Fig. 2). We have no explanation for this discrepancy other than the possibility of intrachain cross-linking with disuccinimidyl suberate leading to receptor molecules with different conformations. A caveat in the interpretation of the chemical cross-linking experiments performed with intact cells or purified membranes has been the hypothetical possibility that the cross-linking reagent may cross-link the radioligand to a neighboring membrane component rather than to the receptor. Thus, the possibility remains that the $M_r < 68,000$ component is the actual binding subunit with chemical cross-linking to the $M_r = 260,000$ species.

Competitive binding experiments with intact chondrosarcoma cells demonstrated that insulin did not compete for binding of $^{125}\text{I}-\text{MSA}$ and the rank order of competing IGFs was IGF > MSA-II > IGF-I (15). Identical competitive binding data were obtained with the purified receptor (Fig. 4A).

**Table I**

**Summary of IGF-II receptor purification from chondrosarcoma cells**

Table I lists the major classes of molecules that were purified by Sepharose 6B gel filtration (5, 8, 11). Presumably the increase in molecular weight following reduction is due to further unfolding of the molecule after cleavage of intramolecular disulfide bonds. In agreement with these cross-linking experiments, the major band present in the purified receptor had an $M_r$ of 210,000 before reduction (Fig. 2A) and 250,000 after reduction (Fig. 2C).

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cross-linking procedure is described under 'Experimental Procedures.' The 125I-MSA-receptor complexes were analyzed by SDS-PAGE without (A) or with (B) reduction with DTT. To demonstrate specific binding, unlabeled MSA-II (10 μg/ml) was included in a parallel incubation as indicated below the gel. The large amount of radioactivity at the bottom of the autoradiogram is unbound 125I-MSA. The Mₚ = 68,000 band in B is 125I-BSA present in the 125I-MSA preparation. Molecular weight markers were as described in Fig. 2A. C, autoradiogram showing 125I-MSA-receptor complexes from intact chondrosarcoma chondrocytes, chemically cross-linked to the cells, and analyzed by SDS-PAGE as described under "Experimental Procedures." Specificity of binding was demonstrated by addition of unlabeled polypeptides (MSA, 1 μg/ml; insulin (INS), 10 μg/ml) to parallel incubations as indicated below the autoradiogram. The Mₚ values were assigned based on the mobility of a series of protein standards (Mₚ = 68,000 to 250,000) as in Fig. 2A.

Since the IGF-I preparation used in this experiment and the previous experiment on whole cells (15) was contaminated with 10% IGF-II, most of the reactivity of IGF-I is accounted for by its IGF-II content.

Scatchard analysis of MSA II binding to the purified receptor (Fig. 4B) demonstrated high affinity binding (Kₛ = 2.0 × 10⁶ M⁻¹). The MSA binding capacity of the purified receptor was 13.5 μg/mg (Table I) compared to an expected value of 28.5 μg/mg for a binding stoichiometry of 1:1. Because of the limitations of Scatchard analysis of binding data for determining binding capacity (20), a more precise determination of the stoichiometry of MSA binding to the purified type II receptor will require a different method.

The purified receptor is a glycoprotein, since it bound to wheat germ lectin-Sepharose 6MB and Concanavalin A-Sepharose columns and was eluted with N-acetyl-D-glucosamine and α-methyl-D-mannoside, respectively (data not shown).

Our experiments demonstrate the feasibility of obtaining highly purified IGF-II type receptor from Swarm rat chondrosarcoma cells. The purified receptor shows binding characteristics that are indistinguishable from those of the receptor on intact cells, and is the same size as previously demonstrated by cross-linking studies with whole cells.

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Purification of an insulin-like growth factor II receptor from rat chondrosarcoma cells.

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