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The Rat Liver Insulin Receptor†

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ABSTRACT: Using insulin affinity chromatography, we have isolated highly purified insulin receptor from rat liver. When evaluated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis under reducing conditions, the rat liver receptor contained the M_r 125 000 α-subunit, the M_r 90 000 β-subunit, and varying proportions of the M_r 45 000 β'-subunit. The specific insulin binding of the purified receptor was 25–30 μg of ^{125}I-insulin/mg of protein, and the receptor underwent insulin-dependent autophosphorylation. Rat liver and human placental receptors differ from each other in several functional aspects: (1) the adsorption–desorption behavior from four insulin affinity columns indicated that the rat liver receptor binds less firmly to immobilized ligands; (2) the ^{125}I-insulin binding affinity of the rat liver receptor is lower than that of the placental receptor; (3) partial reduction of the rat liver receptor with dithiothreitol increases its insulin binding affinity whereas the binding affinity of the placental receptor is unchanged; (4) at optimal insulin concentration, rat liver receptor autophosphorylation is stimulated 25–50-fold whereas the placental receptor is stimulated only 4–6-fold. Conversion of the β-subunit to β' by proteolysis is a major problem that occurs during exposure of the receptor to the pH 5.0 buffer used to elute the insulin affinity column. The rat receptor is particularly subject to destruction. Frequently, we have obtained receptor preparations that did not contain intact β-subunit. These preparations failed to undergo autophosphorylation, but their insulin binding capacity and binding isotherms were identical with those of receptor containing β-subunit. Proteolytic destruction and the accompanying loss of insulin-dependent autophosphorylation can be substantially reduced by proteolysis inhibitors. In summary, rat liver and human placental receptors differ functionally in both α- and β-subunits. Insulin binding to the α-subunit of the purified rat liver receptor communicates a signal that activates the β-subunit; however, major proteolytic destruction of the β-subunit does not affect insulin binding to the α-subunit. This suggests that communication does not occur in the reverse direction, i.e., $\beta \rightarrow \alpha$.

Jacobs et al. (1977) reported the first isolation of an insulin receptor preparation, i.e., that of rat liver. Their material exhibited a specific activity of 2.4 μg of ^{125}I-insulin bound/mg of protein. The scheme employed in this pioneering investigation, which provided the blueprint for all subsequent isolations of insulin receptors, involved (1) preparation of a crude membrane fraction, (2) solubilization of the receptor with Triton X-100, (3) chromatography on DEAE-cellulose, and (4) affinity chromatography on insulin–agarose resins. Although a number of studies characterizing the rat liver insulin receptor have appeared, we are not aware of studies aimed at isolation and characterization of homogeneous rat liver receptor. This paper deals with this subject.

In a previous paper (Finn et al., 1984) we have described an affinity resin for the routine isolation of human placental insulin receptor that was prepared by noncovalently attaching ligand II (Figure 1) to succinoylavidin–Sepharose. Having available a reliable affinity resin for the isolation of insulin receptors from human placenta, we reasoned that it should be a simple matter to isolate insulin receptor from rat liver by the same procedure. This, however, proved not to be the case. We were surprised to find that wheat germ lectin purified rat insulin receptor failed to bind to the above-mentioned affinity resin under conditions that were employed for the isolation of the human placental receptor. This observation suggested that placenta and rat liver receptors were different and prompted a systematic comparison of the adsorption–desorption characteristics of the two receptors with a series of affinity resins containing different ligands. In connection with these studies, we have employed two new ligands (compounds III and IV, Figure 1), whose syntheses are described.

Using an affinity resin based on ligand IV, we succeeded in isolating purified rat liver insulin receptor in good yield essentially by the procedure described for the isolation of the human placental receptor. Unfortunately, many of our

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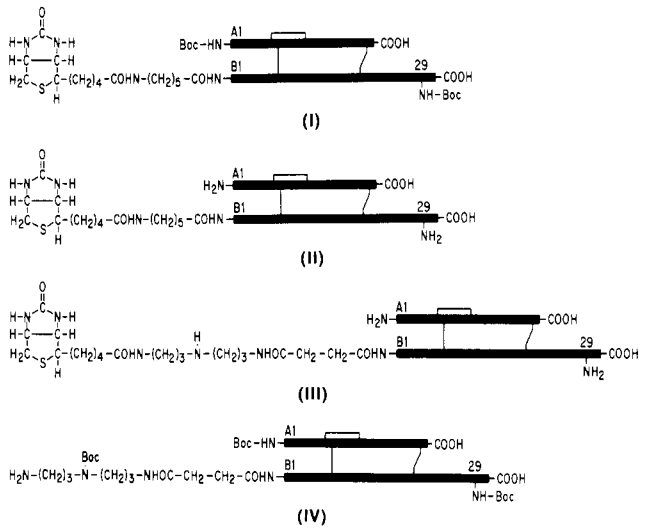

FIGURE 1: Simplified structures of ligands.

preparations failed to undergo insulin-dependent autophosphorylation, and separation of ^{125}I-labeled receptor subunits by SDS–PAGE[1] under reducing conditions revealed that the preparations contained no intact β-subunit. The addition of a "cocktail" containing six protease inhibitors to the elution buffer of the affinity column afforded receptor preparations exhibiting a high level of insulin-dependent autophosphorylation and containing intact β-subunit. A comparison of highly purified human placental and rat liver receptors provided compelling evidence that the two receptors are not identical.

Materials and Methods

Biotin was a gift from Dr. W. Scott of Hoffmann-La Roche Inc., Nutley, NJ, and $(Boc)_2$-insulin was obtained from Professor R. Geiger of Hoechst, A.G., Frankfurt am Main, West Germany. Msc-OSu was obtained from Dr. C. Schwabe, Department of Biochemistry, Medical University of South Carolina, Charleston, SC, and Dr. R. Andreatta, Ciba-Geigy Inc., Basel, Switzerland. Wheat germ lectin was from E. Y. Laboratories, San Mateo, CA. The proteinase inhibitors were obtained from the following sources: benzamidine from Aldrich; TLCK, BAEE, antipain, and soybean trypsin inhibitor from Sigma; leupeptin, aprotinin, and pepstatin from Boehringer Mannheim; bacitracin from Upjohn. The AG 1X2 resin was from Bio-Rad, and the IRA-400 was from Mallinckrodt. The solvent systems for ascending TLC on silica gel (E. Merck and Co., Darmstadt, West Germany) were (I) 1-butanol–glacial acetic acid–water (60:20:20) and (II) chloroform–methanol–water (8:3:1). Compounds were visualized on thin-layer plates by fluorescamine (Udenfriend et al., 1972), the chlorine reagent (exposure to hypochlorite followed by spraying with a 1:1 mixture of 0.4% KI and 1% starch solution in water), and the biotin reagent *p*-(dimethylamino)cinnamaldehyde (McCormick & Roth, 1970). The elemental analysis was by Schwarzkopf Microanalytical Laboratory, Woodside, NY. The pH 8.0 buffer is 0.1 M $NaHCO_3$/1 M NaCl; the pH 4.0 buffer is 0.1 M sodium acetate/1 M NaCl; the pH 7.6 buffer is 50 mM Hepes containing 1 M NaCl and 0.1% Triton X-100. Sepharose 4B immobilized wheat germ lectin and succinoylavidin–Sepharose 4B were prepared as described previously (Finn et al., 1984). The ^{125}I-insulin binding capacity of receptor at various stages of purification was determined by PEG assay (Cuatrecasas, 1972) at an insulin concentration 0.16–33 nM. Wheat germ agglutinin column eluates were assayed at concentrations of 10–20 μg of protein; eluates from the affinity resins were assayed at 1–2 μg. Charcoal assays were performed according to Williams and Turtle (1979) at an insulin concentration of 0.33 nM. Protein was determined with fluorescamine (Udenfriend et al., 1972). Receptor labeling was performed as described previously (Finn et al., 1984).

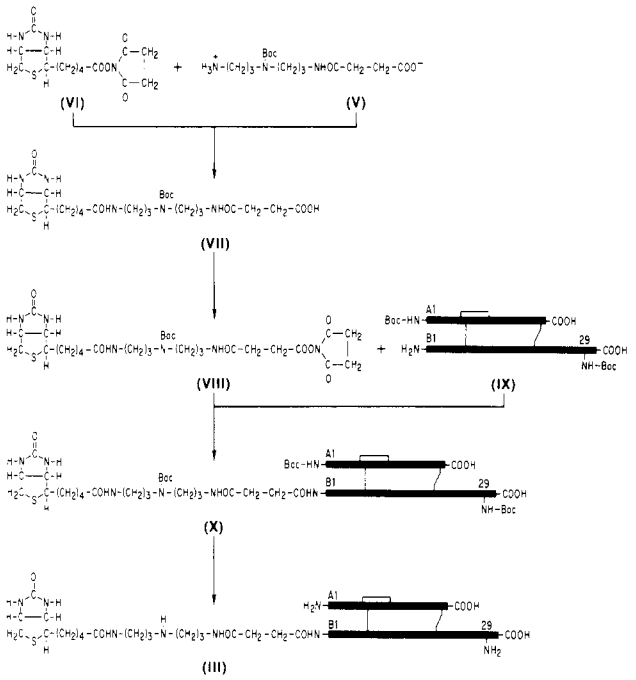

FIGURE 2: Synthetic route to ligand III.

Autophosphorylation Assays. Autophosphorylation was initiated by addition of [^{32}P]ATP (4.25 Ci/mmol) (final concentration 6.7 μM) to 2.2 pmol of insulin binding activity in the presence or absence of 100 nM insulin plus $MnCl_2$ (final concentration 4.5 mM) in a total volume of 55 μL. The reaction proceeded for 30 min at 4 °C and was terminated by the addition of stopping buffer (10 μL) containing SDS (10% w/v), bromophenol blue (0.05% w/v), glycerol (50% v/v), and DTT (0.5 M) in 50 mM NaH_2PO_4, pH 7.0. The mixture was boiled for 2 min, and samples were analyzed by SDS–PAGE as described by Laemmli (1970) with 4% stacking and 7.5% resolving gels. Autoradiograms were obtained with Kodak XAR-5 film and a Cronex intensifying screen at −70 °C. The autoradiograms were scanned with an LKB 2202 Ultrascan laser densitometer. Autophosphorylation units correspond to the areas of the peaks.

Synthesis of Ligand III (Figure 2). TEA (0.153 mL, 1.1 mmol) was added to a suspension of *N*-hydroxysuccinimido biotinate (VI) (341 mg, 1.0 mmol) and *N*-[3-[[(3-aminopropyl)carboxy]amino]propyl]succinamic acid *N*-*tert*-butyl ester (V) (Hofmann et al., 1984) (364 mg, 1.1 mmol) in DMF

[1] Abbreviations: BAEE, benzoylarginine ethyl ester; Boc, *tert*-butoxycarbonyl; DCC, *N*,*N*′-dicyclohexylcarbodiimide; DCU, *N*,*N*′-dicyclohexylurea; DIPEA, diisopropylethylamine; DMF, dimethylformamide; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HPLC, high-performance liquid chromatography; Me_2SO, dimethyl sulfoxide; Msc, [[(methylsulfonyl)ethyl]oxy]carbonyl; Msc-OSu, [(methylsulfonyl)ethyl]succinimido carbonate; NEM, *N*-ethylmaleimide; OSu, *N*-hydroxysuccinimide ester; PMSF, phenylmethanesulfonyl fluoride; PEG, poly(ethylene glycol); SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TLC, thin-layer chromatography; TEA, triethylamine; THF, tetrahydrofuran; Tris, tris(hydroxymethyl)aminomethane; TFA, trifluoroacetic acid; TLCK, (N^{α}-*p*-tosyl-L-lysyl)chloromethane; $(Boc)_2$-insulin, N^{α,A^1},$N^{\epsilon,B^{29}}$-$(Boc)_2$-insulin; GlcNAc, *N*-acetyl-D-glucosamine.

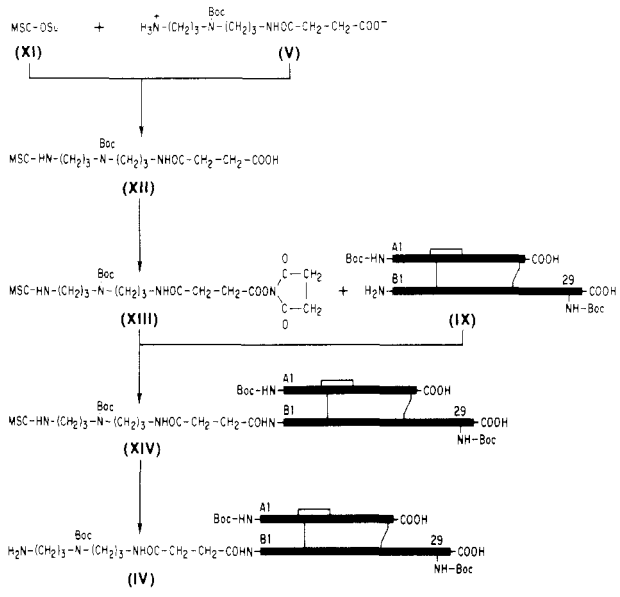

FIGURE 3: Synthetic route to ligand IV.

(15 mL), and the mixture was stirred at room temperature for 22 h. The DMF was evaporated, and the residue dissolved in water (20 mL) was applied to a column of AG 1X2 (acetate cycle) (1.9 × 10 cm), which was eluted with water until the effluent was fluorescamine negative. The column was then eluted with 5% acetic acid until the biotin test became negative (approximately 120 mL required). The eluates were pooled and evaporated, and the residue was dried in vacuo. The material was dissolved in methanol and precipitated by addition of ethyl ether: yield of VII 472 mg (77%); R_f^I 0.45, R_f^{II} 0.2; single biotin positive spot. A sample for analysis was reprecipitated from methanol with ether and dried. Anal. Calcd for $C_{25}H_{42}N_5O_7S \cdot {}^1/_2H_2O$: C, 53.08; H, 7.66; N, 12.38. Found: C, 53.02; H, 7.69; N, 12.31.

For conversion to the OSu ester (VIII), DCC (78 mg, 0.38 mmol) was added at room temperature to a solution of VII (142 mg, 0.25 mmol) and *N*-hydroxysuccinimide (44 mg, 0.38 mmol) in DMF (3 mL), and the solution was stirred at room temperature for 24 h. The suspension was filtered, and the filtrate was evaporated to dryness. The residue dissolved in 90% aqueous 2-propanol (20 mL) was percolated through a column (1.5 × 10 cm) of acetate-cycle AG 1X2 equilibrated with the same solvent. Biotin-positive fractions were pooled, evaporated to dryness, and triturated with ether to give a solid, yield 170 mg. On TLC, this material showed the presence of two spots with R_f^{II} values of 0.5 and 0.6. The material having the higher R_f value was tentatively identified as the acylurea derivative of VIII since, in contrast to the higher running spot, it failed to regenerate the acid VII on exposure to alkali. The crude active ester was used to acylate $(Boc)_2$-insulin as follows: A solution of 62 mg (10 μmol) of $(Boc)_2$-insulin (IX), 115 mg of VIII, and imidazole (28 mg) in Me_2SO (4 mL) was stirred for 14 h at room temperature. The solution was diluted with 0.05 M ammonium bicarbonate and desalted on a column of Sephadex G-25 (4 × 55 cm) with 0.05 M ammonium bicarbonate as the solvent. The compound was deprotected in 1 mL of TFA for 40 min at room temperature, and TFA ions were exchanged for acetate ions on a column of acetate-cycle IRA-400 (0.9 × 11 cm) with 10% acetic acid as the solvent. Fractions containing the desired material (III) were pooled and lyophilized, yield 56 mg.

Synthesis of Ligand IV (Figure 3). To a stirred solution of *N*-[3-[[(3-aminopropyl)carboxy]amino]propyl]succinamic acid *N-tert*-butyl ester (V) (Hofmann et al., 1984) (166 mg, 0.5 mmol) in DMF (5 mL) and water (2 mL) containing 10% DIPEA/DMF (0.85 mL, 0.5 mmol) was added solid Msc-OSu (XI) (159 mg, 0.60 mmol), and the solution was stirred at room temperature for 24 h. Solvents were removed in vacuo, and the oily residue (R_f^I 0.6, chlorine positive, fluorescamine negative) was dried. The residue was dissolved in THF (10 mL), *N*-hydroxysuccinimide (86 mg, 0.75 mmol) followed by solid DCC (155 mg, 0.75 mmol) was added, and the reaction mixture was stirred at room temperature for 24 h. DCU was removed by filtration, and the filtrate was evaporated to dryness. The residue was dissolved in a small volume of acetone, and additional DCU was removed by filtration and the filtrate was evaporated. The residue dissolved in 90% aqueous 2-propanol (20 mL) was percolated through a column of acetate-cycle AG 1X2 (1.5 × 11 cm) equilibrated with the same solvent. Chlorine test positive fractions were pooled and evaporated to dryness: yield of oily XIII 276 mg (52%); R_f^{II} 0.75. This crude active ester was used to acylate $(Boc)_2$-insulin.

$(Boc)_2$-insulin (IX) (62 mg, 10 mmol), the active ester (XIII) (72 mg, 125 mmol), and imidazole (28 mg, 412 mmol) were dissolved in Me_2SO (4 mL), and the solution was stirred at room temperature for 12 h; the cooled reaction mixture was diluted with ice-cold 0.05 M ammonium bicarbonate and desalted on a column of Sephadex G-25 (3.5 × 55 cm) in 0.05 M ammonium bicarbonate. Fractions containing the desired material (XIV) were pooled and lyophilized, yield 61 mg.

For removal of the Msc group, this material was dissolved in 24 mL of an ice-cold solution containing dioxane (9 mL), methanol (1.5 mL), 2 N NaOH (3 mL), and water (10.5 mL). After 2 min at 0 °C, glacial acetic acid (6 mL) was added, and the solution was applied to a Sephadex G-25 column (4.5 × 53 cm) that was developed with 1 M acetic acid. Fractions containing the desired material were pooled and lyophilized, yield of IV 48 mg.

Preparation of Affinity Resins. Ligands I, II, and III were attached to Sepharose 4B immobilized succinoylavidin (140–160 nmol/mL of settled resin) in the manner described previously (Finn et al., 1984). Ligand loading was approximately 112 μg/mL of settled resin. For attachment of ligand IV to the solid support, CNBr-activated Sepharose 4B (2 g) was washed with 1 mM HCl (400 mL), and the washed resin was rotated for 16 h at 4 °C with a solution of ligand IV (20 mg) in 5 mL of 0.1 M $NaHCO_3$, pH 8.0, containing 0.5 M NaCl and 10^6 cpm of ^{125}I-labeled IV as a tracer. The resin was collected and rotated at 4 °C for 3 h with 1 M ethanolammonium chloride, pH 8.0 (5 mL). The resin was collected, washed with three cycles each of 100 mL of pH 8.0 and 4.0 buffers, and then washed with 1 L of pH 7.6 buffer and stored in the Hepes buffer containing 0.05% sodium azide. Ligand loading was 1.25 mg/mL of settled resin (high loading). Resin loaded with approximately 140 μg of ligand IV/mL of settled resin was also prepared (low loading).

Preparation of Wheat Germ Eluate. Livers from 38 rats (392 g) were collected into ice-cold 0.25 M sucrose, pH 7.5. The sucrose was decanted, and the tissue was homogenized in a Polytron (setting 5) for 1 min at a concentration of 2 mL/g of tissue in 0.25 M sucrose/5 mM Tris, pH 7.4/0.1 mM PMSF. The suspension was centrifuged at 12000*g* for 10 min, and the supernatant was collected. Sodium chloride and $MgSO_4$ were added to a concentration of 0.1 M and 0.2 mM, respectively, and the suspension was centrifuged at 40000*g* for 40 min. Prior to solubilization, the crude membrane fraction was washed by suspension and centrifugation at 40000*g* with 50 mM Tris, pH 7.4/0.1 mM PMSF/2 mM EDTA (800 mL) and twice with 50 mM Hepes, pH 7.6/0.1 mM PMSF/2 mM

EGTA/25 mM benzamidine hydrochloride (800 mL). The pellet was suspended (15 mg of protein/mL) in the preceding buffer, and Triton X-100, in the same buffer, was added to a final concentration of 1%. The mixture was stirred at 4 °C for 30 min and centrifuged at 100000*g* for 2 h, and the supernatant was collected and frozen at −70 °C. This material, in three equal batches, was made 10 mM in $MgCl_2$ and added to 40 mL of immobilized wheat germ lectin; the suspensions were rotated at 4 °C for 16 h and poured into a chromatography column, which was washed with 3 L of 50 mM Hepes, pH 7.6/10 mM $MgCl_2$/0.1% Triton X-100/0.1 mM PMSF. The column was then eluted with 0.3 M GlcNAc in 50 mM Hepes/0.1% Triton X-100/0.1 mM PMSF.

Fractions were assayed (charcoal assay) (Williams & Turtle, 1979) for insulin binding at a level of 0.1 pmol of ^{125}I-insulin per tube. Fractions containing ^{125}I-insulin binding activity were pooled and frozen at −70 °C.

Affinity Chromatography. Columns containing 5 mL of the desired settled resin (2 mL for resin derived from ligand IV, high loading) were washed with 1 L of pH 7.6 buffer. Wheat germ eluate (corresponding to 800 pmol of receptor) was adjusted to 1 M with NaCl and circulated through the affinity column for 16 h at 4 °C. The flow-through was retained for assay and recycling, and the resin was washed with the pH 7.6 buffer (1 L) and eluted in the manner previously described (Fujita-Yamaguchi et al., 1984; Finn et al., 1984). To minimize destruction of insulin-dependent autophosphorylation activity, it is of critical importance to neutralize the pH 5.0 eluates immediately. Elutions in the presence of protease inhibitors were performed as follows: The receptor-loaded and washed columns were equilibrated with 20 mL of pH 7.6 buffer containing per milliliter aprotinin, pepstatin, antipain, and leupeptin (1.5 μg each), benzamidine (15 μg), and bacitracin (2 mg) ("cocktail") or BAEE (2 mM) or TLCK (1 mM) or no additions. PMSF (0.1 mM) was also present in the latter three buffer solutions. After the columns were equilibrated, they were eluted with pH 5.0 buffer containing the same additives. Fractions containing ^{125}I-insulin binding activity (charcoal assay) were pooled and subjected to Scatchard (1949) analysis. The solution was then concentrated in an Amicon stirred cell (PM-10 filter) to a receptor concentration of approximately 0.1 mg/mL, and aliquots of this solution were employed for autophosphorylation experiments.

Results

The syntheses of ligands III and IV (Figures 2 and 3) are straightforward and establish the assigned structures. *N*-[3-[[(3-Aminopropyl)carboxy]amino]propyl]succinamic acid *N*-*tert*-butyl ester (V) was the key intermediate for the synthesis of both ligands. For the preparation of III, compound V was acylated with the *N*-hydroxysuccinimido ester of biotin (VI) to give VII, which was converted to the *N*-hydroxysuccinimide ester (VIII). Unchanged VII was removed from the active ester by ion-exchange chromatography on the resin AG 1X2 in the acetate cycle. On TLC, the amorphous purified ester was found to be contaminated with a higher moving spot, which appeared to represent the acylurea corresponding to VIII since it failed to regenerate VII on exposure to alkali. $(Boc)_2$-insulin (IX) was acylated with VIII in the manner previously described (Hofmann et al., 1982) except that imidazole served as the base instead of *N*-methylmorpholine. Compound X was deprotected with TFA in the usual manner.

For the synthesis of IV, the amino carboxylic acid (V) was acylated with Msc-OSu (XI) to give XII, which was converted to the *N*-hydroxysuccinimide ester (XIII). Unchanged acid (XII) was removed by ion-exchange chromatography on AG 1X2 in the acetate cycle. The oily active ester served to acylate $(Boc)_2$-insulin (IX). The final products were purified by HPLC. (Profiles not shown).

Table I: Adsorption–Desorption Behavior of Rat Liver and Human Placental Insulin Receptors[a]

ligand	receptor	bound (pmol)	eluted (pmol)
I	liver		
I	placenta	438	285
II	liver		
II	placenta	367	131
III	liver	410	41
III	placenta	550	328
IV	liver	293	
IV	placenta	543	178
IV[b]	liver	577	351
IV[b]	placenta	710	168

[a] See Materials and Methods for preparation and operation of the affinity columns. The values are averages of several experiments. [b] High loading.

The results of the adsorption–desorption studies with affinity resins prepared from ligands I–IV are summarized in Table I. It is apparent that columns prepared from ligands I and II bound the placental receptor very well and the bound material could be eluted. Both affinity resins failed to bind the rat receptor, which was recovered quantitatively in the column effluent. To examine the possibility that a longer spacer was necessary for binding of the rat liver receptor, we prepared ligand III and attached it to the immobilized succinoylavidin. The results with this column were inconclusive. The column bound 410 pmol of the rat receptor, but only 41 pmol of the bound material could be eluted. Conversely, this column bound the placental receptor very well and 60% of the bound material could be recovered. The effect that succinoylavidin may have on receptor binding was explored by the use of a column in which ligand IV was attached directly to CNBr-activated Sepharose 4B. Again, the two receptors exhibited significantly different adsorption–desorption behavior on this column. Rat liver receptor yield was poor when either ligand III or ligand IV (low loading) columns were used. Therefore we concluded that poor binding of the rat liver receptor is not due solely to the presence of succinoylavidin. It would appear that because of its low affinity for resins prepared from ligands III and IV, the bound rat receptor was lost during the extensive washing step preceding elution.

Regardless of the ligand used, rat liver receptor bound less firmly than did placenta receptor, and it seemed reasonable to predict that higher binding might be achieved if the ligand density were increased. Thus, columns containing ligand IV at a density of 1.25 mg/mL of resin were prepared and tested. The behavior of the two receptors on these columns confirmed this hypothesis. The high-density columns bound 577 pmol of rat receptor, and 351 pmol (61%) could be eluted. A higher amount of placental receptor bound (710 pmol), but only 24% of the bound receptor could be eluted. These columns were selected for routine purification of the rat liver receptor.

Scatchard (1949) analyses of ^{125}I-insulin binding to the rat liver and human placental receptors (Figure 4) indicate that the rat liver receptor binds insulin less firmly than does the placental material, an observation that supports the adsorption–desorption findings. Binding to insulin imbedded in the affinity resin matrix appears to provide a more discriminating system for recognizing the differences between the two receptors than does insulin binding in solution.

The specific insulin binding of various preparations of rat liver receptor is 25–30 μg of ^{125}I-insulin bound/mg of protein,

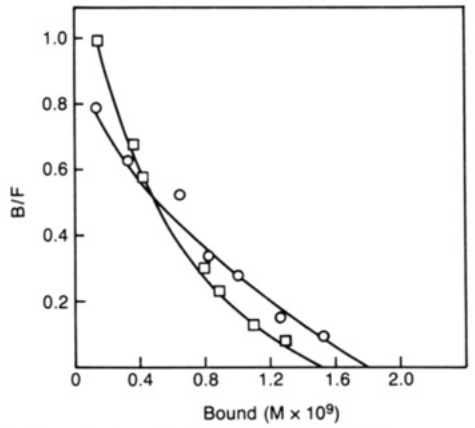

FIGURE 4: Scatchard analysis of ^{125}I-insulin binding to rat liver (○) and human placental (□) insulin receptors.

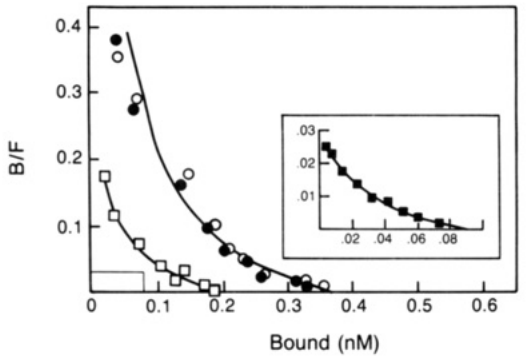

FIGURE 5: Scatchard analysis of ^{125}I-insulin binding by purified human placental receptor following exposure to DTT. Purified receptor (8.75 pmol of binding activity) was incubated for 16 h at 4 °C without DTT (○) or with the addition of DTT: 0.1 (●), 0.5 (□), or 2.0 mM (■). NEM (2 mol/mol of DTT) was added, and incubation was continued for 24 h. Aliquots (100 μL) were assayed for ^{125}I-insulin binding by the PEG assay. Curves were generated by computer analysis of the binding data.

a value in agreement with the binding capacity of purified human placental receptor (Fujita-Yamaguchi et al., 1984; Finn et al., 1984). The possibility that the purified receptor may be contaminated by small amounts of IGF_1 receptor cannot be ruled out.

We have examined the effect of DTT on the insulin binding activity of highly purified receptor from human placenta and rat liver. To prevent insulin destruction, the reaction mixture was alkylated with NEM (2:1 ratio of NEM to DTT) prior to conducting the binding assays. The binding capacity of receptors from both sources is virtually eliminated when they are incubated with 5 mM DTT (data not shown).

Although DTT destroys the insulin binding of both receptors, Scatchard (1949) analyses of the effects of several concentrations of DTT reveal that the reducing agent affects the two receptors differently. With the placental receptor (Figure 5) DTT, at a concentration of 0.1 mM, exerts no measurable effect either on the binding affinity or on the number of receptor sites available. Higher concentrations of DTT (0.5 and 2.0 mM) reduce the number of insulin binding sites to 50% and 31%, respectively. At 2 mM DTT, the affinity of the receptor for insulin is decreased.

The binding affinity of the rat liver receptor (Figure 6) is increased by 0.1 mM DTT. Increasing the DTT concentration to 0.5 mM reduces the number of binding sites, but higher binding affinity is still evident.

A major problem during purification of insulin receptors is the conversion, presumably by proteolysis, of the β-subunit to the β′ species. This process is accompanied by loss of

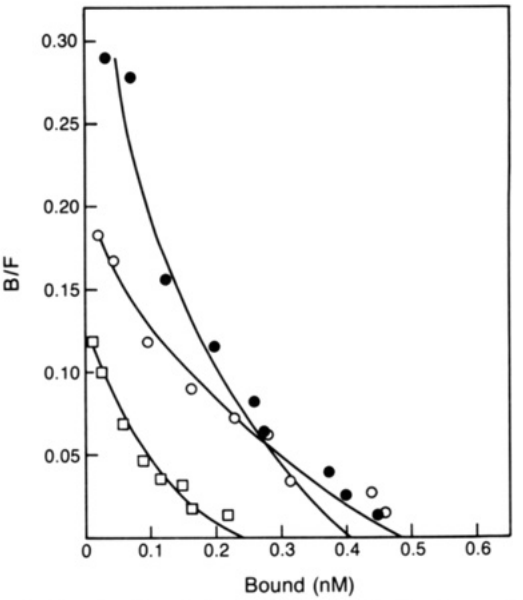

FIGURE 6: Scatchard analysis of ^{125}I-insulin binding by purified rat liver receptor following exposure to DTT. Purified receptor (8.25 pmol of binding activity) was incubated for 16 h at 4 °C without DTT (○) or with the addition of DTT: 0.1 (●) and 0.5 mM (□). NEM (2 mol/mol of DTT) was added, and incubation was continued for 24 h. Aliquots (100 μL) were assayed for insulin binding by the PEG assay. Curves were generated by computer analysis of the binding data.

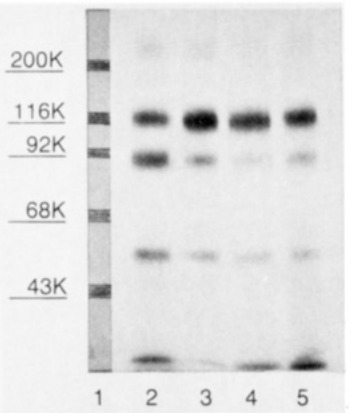

FIGURE 7: SDS–PAGE of ^{125}I-labeled highly purified rat liver insulin receptor (reducing conditions) eluted with buffer containing various additivies. See Materials and Methods for concentration of additives. (Lane 1) standards; (lane 2) cocktail, β/α ratio 1.03; (lane 3) BAEE, β/α ratio 0.21; (lane 4) TLCK, β/α ratio 0.11; (lane 5) no additions, β/α ratio 0.21.

insulin-dependent autophosphorylation. In the case of the isolation of the placental receptor, destruction of the β-subunit can be minimized by rapidly eluting the affinity column and by immediately neutralizing the eluates. β-Subunit destruction is a more serious problem with the rat liver receptor. Destruction is very rapid, and while rapid elution and neutralization are helpful, most preparations contained very little intact β-subunit. We have now observed that a "cocktail" of protease inhibitors added to the pH 5.0 elution buffer affords receptor preparations in which the β-subunit is largely preserved. However, we have never obtained a preparation free of β′-subunit (Figure 7).

We have also tested the ability of the trypsin substrate BAEE and the trypsin inhibitor TLCK to protect the β-subunit during elution of the affinity column. Both compounds were ineffective. When the autoradiograms of ^{125}I-labeled receptor were scanned and the ratios of the areas corresponding to the α- and β-subunits were compared, the ratio of the β- to α-

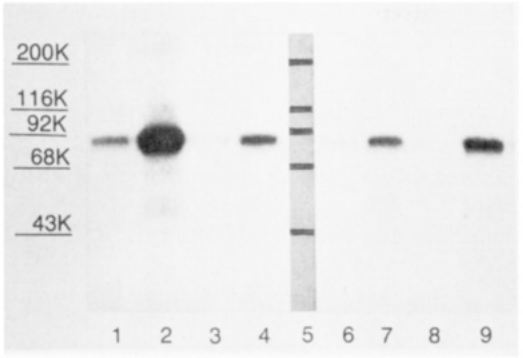

FIGURE 8: Insulin-dependent autophosphorylation of rat liver insulin receptor eluted from the affinity column with buffer containing various additives. See Materials and Methods for concentrations of additives. (Lane 1) Cocktail minus insulin; (lane 2) cocktail plus insulin; (lane 3) TLCK minus insulin; (lane 4) TLCK plus insulin; (lane 5) standards; (lane 6) BAEE minus insulin; (lane 7) BAEE plus insulin; (lane 8) PMSF minus insulin; (lane 9) PMSF plus insulin.

subunit of the preparation eluted in the presence of the cocktail was unity. Receptor eluted in the presence of BAEE or TLCK or with no additions contained significantly less β- and α-subunit. β'-Subunit was present in all the eluates.

Rat liver receptor preparations eluted in the presence of the cocktail showed a marked enhancement in autophosphorylating capacity (Figure 8). Both basal and insulin-stimulated activities were significantly increased. The insulin-stimulated autophosphorylation of the receptor eluted in the presence of the cocktail was 145 units as compared with 26 units for the BAEE sample, 20 units for the TLCK sample, and 35 units for the sample eluted with no additions. Maximum stimulation occurs at an insulin concentration of approximately 1 μM and is 25–50-fold above basal. Autophosphorylation of the placental receptor is stimulated 4–6-fold by insulin, and the optimal insulin concentration is 0.1 μM (data not shown). Addition of cocktail to samples that had been eluted in its absence did not result in reactivation, and the cocktail did not contain components that migrate at 90K. The autophosphorylation results are based on multiple experiments.

Discussion

In this paper we describe the isolation of highly purified insulin receptor preparations from rat liver that exhibit insulin-dependent autophosphorylation and contain the well established α- and β-subunits (Figures 7 and 8). Significant quantities of the β'-subunit are also present. Using *three* criteria, i.e., (1) adsorption–desorption behavior from four insulin affinity columns, (2) ^{125}I-insulin binding, and (3) behavior toward reduction with DTT, we demonstrate that the α-subunits of the rat liver and human placental receptors are not identical.

Morgan et al. (1986) found that an antibody directed to the α-subunit of the human placental insulin receptor failed to cross-react with the α-subunit of the rat receptor, indicating that the α-subunits are structurally different. The data presented here indicate that the two α-subunits are functionally different as well.

Scatchard analysis of the binding of placental receptor exposed to 0.1 mM DTT indicates that this concentration of reducing agent has no effect on binding affinity or the number of binding sites. At 0.5 mM DTT, the concentration at which the majority of receptor molecules are reduced to $\alpha\beta$ halves (Massaguē & Czech, 1982), binding affinity is unchanged although the number of functioning binding sites is markedly reduced (Figure 5). This is consistent with the hypothesis that DTT destruction of binding is due to an all or none modification at the binding site; i.e., binding is either present or absent. At higher concentrations of DTT (2.0 mM) presumably receptor conformation undergoes major modifications resulting in a change in binding affinity as well as further decreases in binding site number.

In contrast, rat liver receptor affinity is increased by 0.1 mM DTT (Figure 6). Higher concentrations of the reducing agent (0.5 mM) reduce the number of insulin binding sites, but increased affinity is still evident. By analogy, this would mean that reduction is at least a two-step phenomenon involving first an increase in affinity followed by a total loss of binding capacity.

Our results are qualitatively similar to those of Massaguē and Czech (1982), who evaluated the changes in insulin binding of rat liver and human placental receptors in crude membrane fractions after exposure to a high concentration of DTT (20 mM). Dithiothreitol increased the affinity of the rat liver membranes for insulin while no change in affinity was noted with the placental receptor. In both instances, the number of binding sites was decreased.

Pike et al. (1986) have exposed purified placental receptor to 2.0 mM DTT and have reported that 40% of the binding sites were lost but binding affinity remained unchanged. We have observed that at this concentration of DTT both the binding site number and binding affinity are decreased.

Substantial proteolysis occurs on the β-subunit during receptor purification. Although the degree of β-subunit destruction can be reduced by shortening the exposure of the receptor to pH 5.0 during elution from the insulin affinity column, proteolysis cannot be totally inhibited. In a recent publication, Kathuria et al. (1986) reported that the β-subunit of human placental insulin receptor is gradually converted from a protein of M_r 90 000 to a species of M_r 88 000 and, finally, to β', M_r 45 000, during prolonged storage at 4 °C. They conclude that this destruction is the result of proteolysis by an enzyme exhibiting trypsin-like specificity. They were able to suppress this destruction by addition of the trypsin substrate BAEE. As is evident from the results presented in Figure 7, BAEE, included in the buffer employed to elute the rat liver receptor from the insulin affinity resin, did not suppress β-subunit cleavage. Addition of the specific trypsin inhibitor TLCK was also ineffective (Figure 7).

We have had considerable success in suppressing β-subunit degradation by adding a cocktail of protease inhibitors to the pH 5.0 buffer used to elute the receptor from the insulin affinity resin (Figure 7). There is significant increase in the ratio of β- to α-subunit in lane 2 where the cocktail was included. The finding that BAEE (lane 3) and the very potent trypsin inhibitor TLCK (lane 4) fail to inhibit proteolysis of the rat liver β-subunit does not support the involvement of a trypsin-like enzyme in the degradation process. BAEE and TLCK are also ineffective in preserving autophosphorylation (compare lane 9, no inhibitors, with lanes 4 and 7, Figure 8).

A striking enhancement of autophosphorylation was observed when the receptor was eluted in the presence of the cocktail (Figure 8, lane 2). The effect on autophosphorylation of the various additions to the eluting medium has been evaluated quantitatively by scanning the autoradiographs (see Results). The concentration of insulin receptor is the same in each lane and is based on ^{125}I-insulin binding activity. Both basal and insulin-stimulated autophosphorylation levels were dramatically increased by adding the cocktail to the elution buffer.

The rat liver receptor autophosphorylation is remarkably stimulated by insulin. A 25–50-fold increase over basal

phosphorylation was elicited with 1 μM insulin in preparations eluted in the presence of cocktail. The same was true for receptor preparations eluted with BAEE or TLCK or with no additions. Our finding that autophosphorylation of the human placental receptor (at 0.1 μM insulin) is stimulated only 4–6-fold by insulin is in good agreement with the results of Kasuga et al. (1983). The higher insulin concentrations necessary for optimal stimulation of autophosphorylation with the rat receptor are consistent with the observation that rat liver receptor has lower affinity for insulin than does the placental receptor.

The mechanism of subunit destruction remains to be elucidated. Whether the putative protease(s) involved has (have) a pH maximum (maxima) at pH 5 or whether exposure to pH 5 causes a conformational change in the β-subunit rendering it more susceptible to proteolytic attack is yet to be established. Kathuria et al. (1986) have reported that proteolysis of the human placental β-subunit is a stepwise process leading first to the creation of an M_r 88 000 species. Clearly, further studies will be necessary to define the events that lead to inactivation of autophosphorylation.

Although it is clear that proteolysis of the β-subunit is accompanied by loss of autophosphorylation, receptor preparations (rat liver or placenta) containing no intact β-subunit are able to bind insulin. The specific binding activities of such preparations are 25–30 μg of ^{125}I-insulin bound/mg of receptor, and the binding isotherms are curvilinear (data not shown). Roth et al. (1983) digested human placental insulin receptor with a collagenase preparation and found that while insulin binding activity did not decrease, the insulin-dependent autophosphorylation was rapidly destroyed. Thus, although occupation of the insulin binding site on the receptor activates the tyrosine kinase, destruction of the β-subunit has no measurable effect on insulin binding by the α-subunit in purified rat liver and human placental insulin receptors. Communication occurs from the α- to the β-subunit but apparently not in the opposite direction.

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