Characterization of Human Placental Insulin-like Growth Factor-I/Insulin Hybrid Receptors by Protein Microsequencing and Purification†

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ABSTRACT: Protein microsequencing of human placental IGF-I receptors purified by immunoaffinity chromatography using IGF-I receptor specific monoclonal antibody revealed amino acid sequences of both IGF-I and insulin receptors. Since this finding indicated the presence of IGF-I/insulin receptor hybrids, hybrid receptors were further purified by immunoaffinity chromatography using insulin receptor specific monoclonal antibody. The molecular size of the nonreduced hybrid receptor was ~350K, indicating that the IGF-I and insulin receptor $\alpha\beta$ halves were disulfide-linked. The ratio of IGF/insulin binding activity of purified hybrid receptors was ~25 when measured using tracer amounts of radioactive ligands. ^{125}I-IGF binding to these receptors was inhibited by IGF-I and insulin with IC_{50}s of ~2 and ~1000 nM, respectively. ^{125}I-Insulin binding to these receptors was similarly inhibited by IGF-I and insulin with IC_{50} of ~3 nM. Autophosphorylation and kinase activities of the hybrid receptor were stimulated by IGF-I more effectively than insulin in a dose-dependent manner. Thus, the present studies indicate that hybrid receptors purified from human placenta have the functional properties of an IGF-I receptor.

Insulin and insulin-like growth factor (IGF)[1] I receptors are structurally similar (Ullrich *et al.*, 1986). Both are composed of two α subunits with M_r = ~135K and two β subunits with M_r = ~90K, which are disulfide-linked in a β–α–α–β form. The α and β subunits are biosynthesized as an $\alpha\beta$ form of the precursor. Two $\alpha\beta$ forms are dimerized and processed into the matured $\alpha_2\beta_2$ form. There is evidence that IGF-I/insulin receptor hybrids exist (Moxham & Jacobs, 1992; Whittaker *et al.*, 1990). First, hybrid receptor tetramers can be constructed *in vitro* with $\alpha\beta$ dimeric forms of each receptor (Treadway *et al.*, 1989). Second, it has been reported that hybrid forms of insulin and IGF-I receptors exist, both in established cultured cell lines and in human placenta tissue (Moxham *et al.*, 1989; Soos & Siddle, 1989; Soos *et al.*, 1990). These hybrid receptors are believed to be composed of one IGF-I receptor $\alpha\beta$ half and one insulin receptor $\alpha'\beta'$ half in a β–α–α'–β' form. The studies suggesting the presence of hybrid receptors are based primarily on the observations that (1) antibodies to the insulin receptor can immunoprecipitate high-affinity IGF-I binding (Treadway *et al.*, 1989; Soos & Siddle, 1989; Soos *et al.*, 1990) and (2) in some tissues, the β subunit of the IGF-I/insulin receptor hybrid is a doublet with one species having a phosphopeptide map that is very similar to the IGF-I receptor and the other that is very similar to the insulin receptor (Moxham *et al.*, 1989).

In order to document that IGF-I/insulin receptor hybrids are present in target tissues, it is necessary to demonstrate that the amino acid sequences of both receptors are present in highly purified receptor preparations. In the present study we have immunopurified receptors from placenta with αIR-3, a monoclonal antibody that is highly specific for the IGF-I receptor (Kull *et al.*, 1983). Microsequencing of this receptor preparation demonstrates the presence of both insulin and IGF-I receptors. Characterization of the purified hybrid receptor demonstrates that the hybrid receptor has the functional properties of the IGF-I receptor.

EXPERIMENTAL PROCEDURES

Materials. αIR-3, a monoclonal antibody to the IGF-I receptor, was a gift from Dr. Steven Jacobs (Wellcome Research Laboratories, Research Triangle Park, NC). IGFR1-2, an anti-peptide (IGFIR 1323–1337) monoclonal antibody (Soos *et al.*, 1993), was a gift from Dr. Kenneth Siddle (University of Cambridge, England). An anti-insulin receptor α subunit peptide (657–670) (Rosenzweig *et al.*, 1990) was a gift from Dr. Steven A. Rosenzweig (Medical University of South Carolina). IGFIR α subunit specific monoclonal antibody, 1H7, has been prepared in our laboratory (Li *et al.*, 1993). Anti-IR C-terminal peptide (1327–1343) and MA51 antibodies have been described previously (Roth *et al.*, 1982; Kathuria *et al.*, 1986). Recombinant IGF-I was purchased from Mallinckrodt (Chesterfield, MI). Crystalline porcine insulin was supplied by Eli Lilly (Indianapolis, IN). PMSF, BAEE, BSA, DTT, leupeptin, aprotinin, pepstatin A, and poly(Glu–Tyr) (4:1) were purchased from Sigma Chemical Co. (St. Louis, MO). PVDF membranes were from Millipore (Bedford, MA). Molecular size markers for SDS–PAGE were obtained from Bio-Rad (Richmond, CA). ^{125}I-Labeled IGF-I was purchased from Amersham (Arlington Heights, IL).

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[1] Abbreviations: IGF, insulin-like growth factor; IR, insulin receptor; IGFIR, IGF-I receptor; HR, hybrid receptor; PMSF, phenylmethane sulfonyl fluoride; BAEE, *N,O*-benzoyl-L-arginine ethyl ester; BSA, bovine serum albumin; DTT, dithiothreitol; PVDF, poly(vinylidene difluoride); PTH, phenylthiohydantoin; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide electrophoresis; WGA, wheat germ agglutinin.

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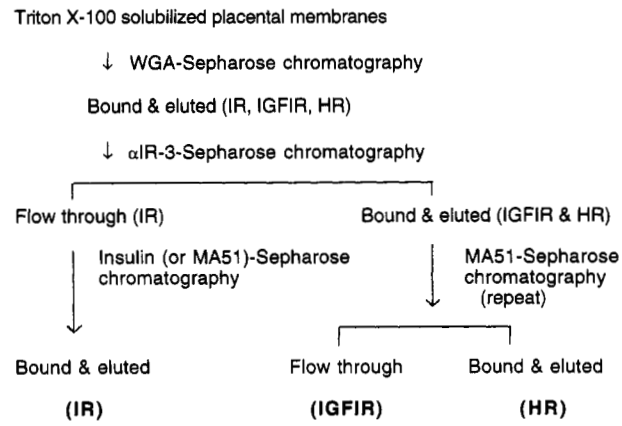

FIGURE 1: Schematic representation of purification of the insulin receptor (IR), IGF-I receptor (IGFIR), and hybrid receptor (HR). The insulin receptor is usually purified by insulin–Sepharose chromatography. However, for the fifth experiment, WGA–Sepharose eluates were passed through the αIR-3–Sepharose column three times to completely remove the hybrid receptor, and then the insulin receptor was purified by MA51–Sepharose chromatography from the flowthrough fractions of the αIR-3–Sepharose column. αIR-3–Sepharose eluates were passed through the MA51–Sepharose column three times to completely remove the hybrid receptor from the IGF-I receptor which did not bind to the column.

[γ-^{32}P]ATP and ^{125}I(A14)-labeled insulin were from DuPont–New England Nuclear (Wilmington, DE).

Purification of Insulin, IGF-I, and Hybrid Receptors. Receptors were purified to apparent homogeneity from human placentas as described (Fujita-Yamaguchi *et al.*, 1983; Fujita-Yamaguchi & LeBon, 1990; LeBon *et al.*, 1986). Briefly, membranes prepared from two fresh term placentas were solubilized in 50 mM Tris-HCl, pH 7.4, containing 2% Triton X-100, 1 mM PMSF, 2 mM BAEE, and 1 μg/mL each aprotinin, leupeptin, and pepstatin A, and the 100000*g* (90 min) supernatant was applied to a WGA–Sepharose column (2 × 8 cm) at 4 °C. The glycoprotein fractions that eluted from this column were applied to either an insulin–Sepharose column (2 × 8 cm) or an αIR-3–Sepharose column (2 × 6.4 cm). The fractions were eluted from these columns according to the published procedures and concentrated by pressure dialysis. To purify hybrid receptors, αIR-3–Sepharose eluates were applied to a MA51–Sepharose column (2 × 4 cm), and the flowthrough fractions were collected. The receptors bound to the column were eluted with 50 mM Tris-HCl buffer, pH 7.4, containing 1 M NaCl and 2.5 mM $MgCl_2$ and immediately applied to a small WGA–Sepharose column for concentration and removal of the salt. The receptors were eluted from the column with 50 mM Tris-HCl, pH 7.4, containing 0.3 M *N*-acetylglucosamine and 0.1% Triton X 100. The purification scheme is shown in Figure 1.

Binding Assays. IGF-I and insulin binding assays were performed as described (Fujita-Yamaguchi *et al.*, 1983; Fujita-Yamaguchi & LeBon, 1990; LeBon *et al.*, 1986). Briefly, samples were incubated at 4 °C for 16 h with ^{125}I-labeled IGF-I or insulin (20 000 cpm) in a final volume of 0.4 mL of 50 mM Tris-HCl buffer, pH 7.4, containing 0.1% Triton X-100 and 0.1% BSA. The receptor–^{125}I-IGF-I or –^{125}I-insulin complexes were separated from free ^{125}I-IGF-I or ^{125}I-insulin by adding 0.1 mL of 0.4% bovine γ-globulin and 0.5 mL of 20% poly(ethylene glycol) 6000 and allowing the mixture to precipitate for 20 min at 4 °C. The proteins were precipitated by centrifuging at 1500*g* for 20 min at 4 °C. The supernatants were aspirated, and the radioactivity in the pellets was counted with a γ counter. The radioactivity precipitated in the presence of excess unlabeled IGF-I (1 μg/mL) or insulin (20 μg/mL) was considered "nonspecific" binding. Competition binding studies were performed by varying the amount of unlabeled IGF-I or insulin in the assay tube. For heterologous ^{125}I-labeled ligand binding assays, *e.g.*, ^{125}I-insulin binding to the IGF-I receptor, an assay volume of 0.2 mL was used to reduce the amount of the receptors required.

Protein Microsequencing. The purified receptors were subjected to SDS–PAGE and then electroblotted to a PVDF membrane previously rinsed in 100% methanol and soaked in the transfer buffer for 20–30 min (Xu & Shively, 1988) using a Mini-Blot apparatus (Bio-Rad, Richmond, CA) essentially according to the method of Towbin *et al.* (1979). After proteins were electrotransferred for 135 min with a constant current of 0.3 A at 4 °C, the PVDF membrane was quickly washed in distilled water and stained with 0.2% Coomassie blue R-250 in 10% acetic acid–45% methanol for ~5 s. The membrane was then destained in 7% acetic acid–45% methanol for ~10 min, rinsed in distilled water for 1 min, air-dried, and stored at –20 °C. Coomassie-stained protein bands were cut out and subjected to microsequencing using a gas-phase microsequencer as described (Hawke *et al.*, 1983; Shively *et al.*, 1988). The yields of PTH-amino acids were 2–10 pmol.

Kinase and Autophosphorylation Assays. Kinase and autophosphorylation assays were performed as previously described (Xu *et al.*, 1991). Briefly, a phosphorylation reaction was performed in 15 or 30 μL of 50 mM Tris-HCl buffer, pH 7.4, containing 0.1% Triton X-100, 0.1% BSA, 2 mM $MnCl_2$, 15 mM $MgCl_2$, and 40 μM ATP (12 000 cpm/pmol) in the presence of 1 mM substrate (kinase assays) or in the absence of the substrate (autophosphorylation). For kinase assays, phosphorylation of an exogenous substrate was initiated by adding poly(Glu–Tyr) (4:1) into the enzyme reaction mixtures to final concentrations of 1 mM. The reaction was allowed to proceed for 40 min at 25 °C and terminated by adding $^1/_5$ volume of 20 mM ATP. Duplicate aliquots were spotted onto pieces of phosphocellulose paper (Whatman P81). The papers were extensively washed in 5% or 10% trichloroacetic acid solution as described (Sahal *et al.*, 1988). Incorporation of ^{32}P into the polymer was quantified by liquid scintillation counting. For autophosphorylation assays, the reaction mixtures were analyzed by SDS–PAGE (7.5% gel) under reducing conditions and autoradiography.

Western Immunoblotting. Purified receptors were electrophoresed in a SDS–polyacrylamide gel (7.5%), and blotted onto a nitrocellulose sheet as previously described (Kathuria *et al.*, 1986). After incubation with first antibodies and respective second antibodies, the bands were visualized using enhanced chemiluminescence (ECL) Western blotting detection reagent (Amersham, Arlington Heights, IL).

RESULTS AND DISCUSSION

N-Terminal Amino Acid Sequence Analysis of Purified Receptors. Protein microsequencing of the α and β subunits of the receptors purified by αIR-3–Sepharose chromatography revealed both IGF-I and insulin receptor sequences (Table I). On the basis of recovery, the ratio of IGF-I and insulin receptors in the preparations was estimated to be 2:1 from three independent sequence analyses.

Previously, when we determined the N-terminal amino acid sequence of the IGF-I receptor purified by αIR-3–Sepharose chromatography, we found a major PTH-amino acid corresponding to an unknown protein sequence and a minor PTH-amino acid derived from the insulin receptor after each Edman cycle (Fujita-Yamaguchi *et al.*, 1986). The former was later

Table I: N-Terminal Amino Acid Sequences of Purified Receptors

isolated from	subunit	N-terminal amino acid sequences[a]
αIR-3–Sepharose eluate	α	EI(C)GPGIDI(R)NDYQQ
		HLYPGEV(C)PGMDIR(N)
	β	DVMQVA(N)TTM
		SLGDVG(N)VTV
insulin receptor	α	HLYPGEV(C)PGM(D)I(R)N(N)LT
	β	SLG(D)VG(N)VTVAVP(T)VAAFP(N)

[a] Amino acid residues in parentheses indicate the residues that were not positively identified by these analyses.

confirmed to be the IGF-I receptor by molecular cloning (Ullrich *et al.*, 1986). The insulin receptor contaminating the IGF-I receptor preparation was less than 10% and was not as significant as the protein sequence results we obtained recently. The reason we did not previously recover the insulin receptor component after αIR-3–Sepharose chromatography is most likely because insulin receptors were first depleted in the glycoprotein fractions applied to the immunoaffinity column.

For comparison, purified insulin receptors were also subjected to protein microsequencing. The α and β subunits of this preparation contained only the insulin receptor sequence (Table I).

These results suggested two possibilities; either the αIR-3–Sepharose eluates contained significant amounts of both IGF-I and insulin receptors or they contained IGF-I/insulin hybrid receptors as well as IGF-I receptors. In contrast, the insulin receptor preparation contained essentially pure insulin receptors.

Purification of IGF-I/Insulin Hybrid Receptors on MA-51–Sepharose. Since antibody αIR-3 is specific for the IGF-I receptor α subunit, it was considered unlikely that ~30% of the receptors in the αIR-3–Sepharose eluates were insulin receptors. However, in order to further determine whether either insulin receptors or hybrid receptors were present, we used a second immunoaffinity chromatography step employing monoclonal antibody MA51, that has very high epitope specificity for the insulin receptor α subunit (Roth *et al.*, 1982; Forsayeth *et al.*, 1987). The αIR-3–Sepharose eluates were applied to MA51–Sepharose, and the fractions bound to MA51–Sepharose were eluted. Approximately half of the total IGF-I binding activity applied to the column was retained in the column. After elution and concentration by WGA–Sepharose chromatography, 8%, 8%, and 25% of the total IGF-I binding activity applied was recovered from three independent experiments, while ~50% of the activity was recovered in the flowthrough fractions. This low yield in the eluates may have been due either to inactivation of the receptor binding activity or to low recovery from the immunoaffinity column.

Since the receptor preparation passed through the column was found to contain significant amounts of insulin receptor components as determined by protein microsequencing, purification procedures were changed. For the fourth experiment, the αIR-3–Sepharose eluates were incubated with MA51–Sepharose for 16 h and the flowthrough fractions were reapplied to the MA51–Sepharose affinity column. The recovery of the hybrid receptor was 9.4% and 6.2% for the first and second affinity chromatographies, respectively. This experiment confirmed that insulin receptor components found in the flowthrough fractions were due to the low capacity of the column. For the fifth experiment, we repeated MA51–Sepharose chromatography three times in order to remove hybrid receptors from the flowthrough fractions in which IGF-I receptors should have remained.

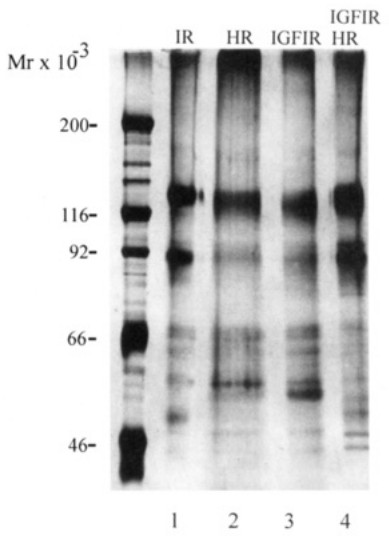

FIGURE 2: SDS–PAGE analysis of purified receptors. Purified receptors from the fifth experiment were analyzed by SDS–PAGE (7.5% gel) under reducing conditions and silver-stained. Shown are receptor preparations of MA51–Sepharose-purified insulin receptors (IR, lane 1), hybrid receptors (HR, lane 2), IGF-I receptors (IGFIR, lane 3), and αIR-3–Sepharose eluates (IGFIR/HR mixture, lane 4). Molecular weight markers used are myosin (200K), β-galactosidase (116K), phosphorylase *b* (92K), bovine serum albumin (66K), and ovalbumin (46K).

Purified receptor preparations corresponding to insulin receptor, hybrid receptor, IGF-I receptor, and αIR-3–Sepharose eluates containing IGF-I and hybrid receptors were analyzed by SDS–PAGE under reducing conditions (Figure 2, lanes 1, 2, 3, and 4, respectively). Receptor preparations containing IGF-I receptor components showed a broad α subunit band of 125–135 kDa and a broad β subunit band of 90–95 kDa (Figure 2, lanes 2–4), whereas purified insulin receptor preparations revealed a distinct α subunit of 135 kDa and a distinct β subunit of 90 kDa (Figure 2, lane 1; see below for more details). IGF-I/insulin hybrid receptors, however, revealed the presence of more distinct subunit bands as seen in the insulin receptor preparation (Figure 2, lane 2).

Western Immunoblotting of Purified Receptors. In order to verify whether each receptor preparation contained the respective receptor as summarized in Figure 1, the purified receptor subunits derived from the fifth experiment were separated by SDS–PAGE under reducing conditions, blotted onto a nitrocellulose membrane, and analyzed by either insulin receptor-specific antibodies [anti-IR(657–670) and anti-IR-(1327–1343)] or IGF-I receptor-specific antibodies [monoclonal antibody 1H7 and monoclonal antibody 1–2]. The results are shown in Figure 3. In panel A, comparable amounts of α subunits of insulin receptors (MA51–Sepharose-purified, Figure 1), hybrid receptors, IGF-I receptors, and αIR-3–Sepharose eluates containing hybrid/IGF-I receptor mixtures were analyzed for their reactivity with α subunit-specific antibodies. Anti-IR(657–670) reacted with insulin receptors, hybrid receptors, and hybrid/IGF-I receptor mixtures at least ~10 times more strongly than the IGF-I receptor as quantitated by densitometry, whereas anti-IGF-I receptor monoclonal antibody 1H7 immunostained hybrid receptors, IGF-I receptors, and hybrid/IGF-I receptor mixtures but did not react with the insulin receptor at all. In panel B, immunoreactivity of the β subunits with anti-insulin receptor β C-terminal peptide (1327–1343) was compared (lanes 1–4), which indicated that virtually no insulin receptor component was present in the IGF-I receptor preparation and that the hybrid receptor β subunit showed the signal (lane B2)

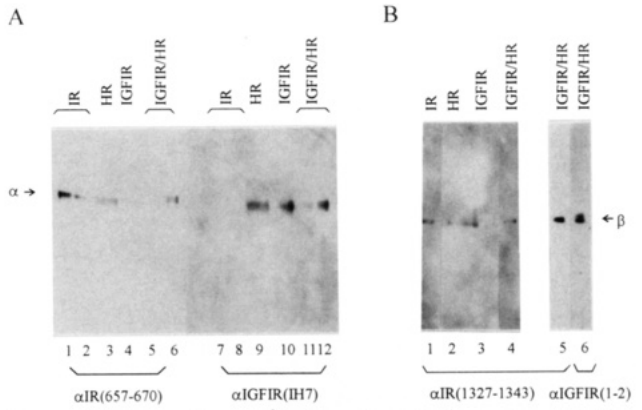

FIGURE 3: Western immunoblotting of purified receptors. Purified receptors from the fifth experiment shown in Figure 2 were used to examine their reactivity with anti-insulin receptor (αIR) or anti-IGF-I receptor (αIGFIR). In panel A, α subunits were assayed for their reactivity with rabbit αIR(657–670) antibody (lanes 1–6) or mouse monoclonal 1H7 antibody (lanes 7–12). In an attempt to adjust the protein amount of each preparation to the comparable level, various amounts of receptor preparations were applied to the SDS–polyacrylamide gel; relative to the silver-stained gel shown in Figure 2, 133% (lanes 1 and 7) and 40% (lanes 2 and 8) of the insulin receptor (IR), 500% of the hybrid receptor (HR, lanes 3 and 9), 200% of the IGF-I receptor (IGFIR, lanes 4 and 10), and 20% (lanes 5 and 11) and 67% (l anes 6 and 12) of αIR-3–Sepharose eluates (IGFIR/HR mixtures). Note: due to the irregular running, the insulin receptor subunits were condensed in smaller areas on the blot, which resulted in intensified signal (lanes 1 and 2). Thus, the intensity of the insulin receptor α subunit in lanes 2 and 3 is comparable. In panel B, comparable amounts of β subunits were applied to the SDS–polyacrylamide gel (lanes 1–4), and their reactivity with rabbit αIR-(1327–1343) was examined. In lanes 5 and 6, αIR-3–Sepharose eluates (IGFIR/HR mixtures), equivalent to approximately 4 times more than that applied to lane 4, were examined for their reactivity with rabbit αIR(1327–1343) antibody (lane 5) or mouse monoclonal αIGFIR 1–2 antibody (lane 6).

comparable to that of the insulin receptor (lane B1). Taken together, the insulin receptor preparation was free of the hybrid receptor as expected from the purification protocols shown in Figure 1, and the IGF-I receptor was apparently >90% pure. The hybrid receptor reacted with both anti-insulin receptor and anti-IGF-I receptor antibodies as expected.

These immunoblot experiments revealed the size differences in the α and β subunits of insulin and IGF-I receptors. As seen in Figure 3A, the insulin receptor α subunit was ~135 kDa (lanes 1–6), whereas the band corresponding to the IGF-I receptor α subunit was wider than the band for the insulin receptor α subunit, where showed molecular mass of ~125–135 kDa (lanes 9–12). In terms of the β subunit, the αIR-3–Sepharose eluates containing both receptor components were compared side by side after visualization by immunostaining with receptor-specific antibodies (Figure 3B, lanes 5 and 6). The insulin receptor β subunit was ~90 kDa whereas the IGF-I receptor β subunit was a doublet of ~90–95 kDa.

Thus, receptors isolated according to our immunoaffinity purification protocols as illustrated in Figure 1 were proven to be correct as judged by immunoblotting experiments of the resulting purified receptors using additional receptor-specific antibodies. In summary, we have shown that the hybrid receptor which was purified by αIR-3–Sepharose and MA51–Sepharose chromatography reacted with anti-insulin receptor α domain-specific (657–670) antibody, anti-insulin receptor β C-terminal peptide (1327–1343) antibody, and anti-IGF-I receptor α subunit-specific monoclonal antibody (1H7). We have not been able to confirm the sequences of α and β subunits of the hybrid receptor, mainly due to insufficient yield of the subunits for protein microsequencing. However, we did obtain

Table II: IC_{50} Values for Inhibition of ^{125}I-IGF-I or ^{125}I-Insulin Binding to Purified Receptors[a]

^{125}I-ligand	competing ligand	IC_{50} (nM) HR	IGFIR	IR
IGF-I	IGF-I	0.83,[b] 2.7[c]	0.83,[b] 2.9[c]	6.4, 9.0
	insulin	500,[b] 1600[c]	1700,[b] 1600[c]	4.2, 3.4
insulin	IGF-I	3.1, 3.4	3.0, 3.9	72, 200
	insulin	3.1, 3.4	3.0, 3.8	0.22, 0.28

[a] The concentration of unlabeled IGF-I or insulin required for half-maximal inhibition of ^{125}I-IGF-I or ^{125}I-insulin binding (IC_{50}) to purified hybrid receptors (HR), IGF-I receptors (IGFIR), and insulin receptors (IR) was determined from competition experiments shown in Figure 4. Two sets of representative data are shown. [b,c] For HR and IGFIR, the data marked with *b* or *c* were from competition experiments that were performed at the same time.

evidence that the relative content of insulin receptor-derived PTH-amino acids in the flowthrough fraction of the first-round MA51–Sepharose chromatography decreased by 30–50% as compared to those of the αIR-3–Sepharose eluates applied.

IGF-I and Insulin Binding Activities of Hybrid Receptors. IGF-I and insulin binding activities of the purified hybrid receptor were measured using tracer amounts of ^{125}I-labeled ligands. IGF-I binding activity was 24.0 ± 4.8 (n = 3) times higher than insulin binding activity.

Competition of unlabeled IGF-I and insulin for ^{125}I-IGF-I or ^{125}I-insulin binding to purified hybrid receptors, IGF-I receptors, and insulin receptors is shown in Figure 4. IC_{50} values from those shown in Figure 4 and other experiments are tabulated in Table II. ^{125}I-IGF-I binding to both hybrid receptors and IGF-I receptors was similarly inhibited by IGF-I and insulin with IC_{50} of ~2 and ~1000 nM, respectively (Figure 4A,C). ^{125}I-Insulin binding to both receptors was also similarly inhibited with IC_{50} of ~3 nM (Figure 4B,D). In contrast, ^{125}I-insulin binding to insulin receptors was inhibited by insulin >100 times more potently than IGF-I (Figure 4F), while ^{125}I-IGF-I binding to insulin receptors was inhibited by insulin slightly more potently than IGF-I, IC_{50} of 3.4 nM *vs* 9.0 nM (Figure 4E).

Thus, both homologous and heterologous ligand competition data provided evidence that the hybrid receptor has characteristics of the IGF-I receptor.

Autophosphorylation and SDS–PAGE Analysis of Hybrid Receptors. The subunit structure of purified hybrid receptors was analyzed by SDS–PAGE under either reducing (Figure 5A) or nonreducing (Figure 5B) conditions. Hybrid receptors were autophosphorylated in the absence or presence of 100 nM IGF-I or insulin, electrophoresed, silver-stained, and autoradiographed. IGF-I and insulin stimulated autophosphorylation of the hybrid receptors ~3-fold and ~2.5-fold, respectively. SDS–PAGE under nonreducing conditions demonstrated that the hybrid receptor was a disulfide-linked tetramer; *i.e.*, the IGF-I receptor $\alpha\beta$ half and the insulin receptor $\alpha'\beta'$ half are disulfide-linked in a β–α–α'–β' form.

Dose-Dependent Stimulation of Autophosphorylation and Kinase Activities of the Hybrid Receptor by IGF-I and Insulin. Dose-dependent stimulation of autophosphorylation of the hybrid receptor β subunit was measured three times in the presence of 0–1000 nM insulin or IGF-I. Radioactivity incorporated into the β subunit was quantitated by the AMBIS radioanalytic imaging system (AMBIS System, Inc., San Diego, CA). IGF-I stimulated autophosphorylation of the β subunit more significantly than insulin in a dose-dependent manner (Figure 6A). The dose dependency of ligand-stimulated kinase activity of hybrid receptors was measured

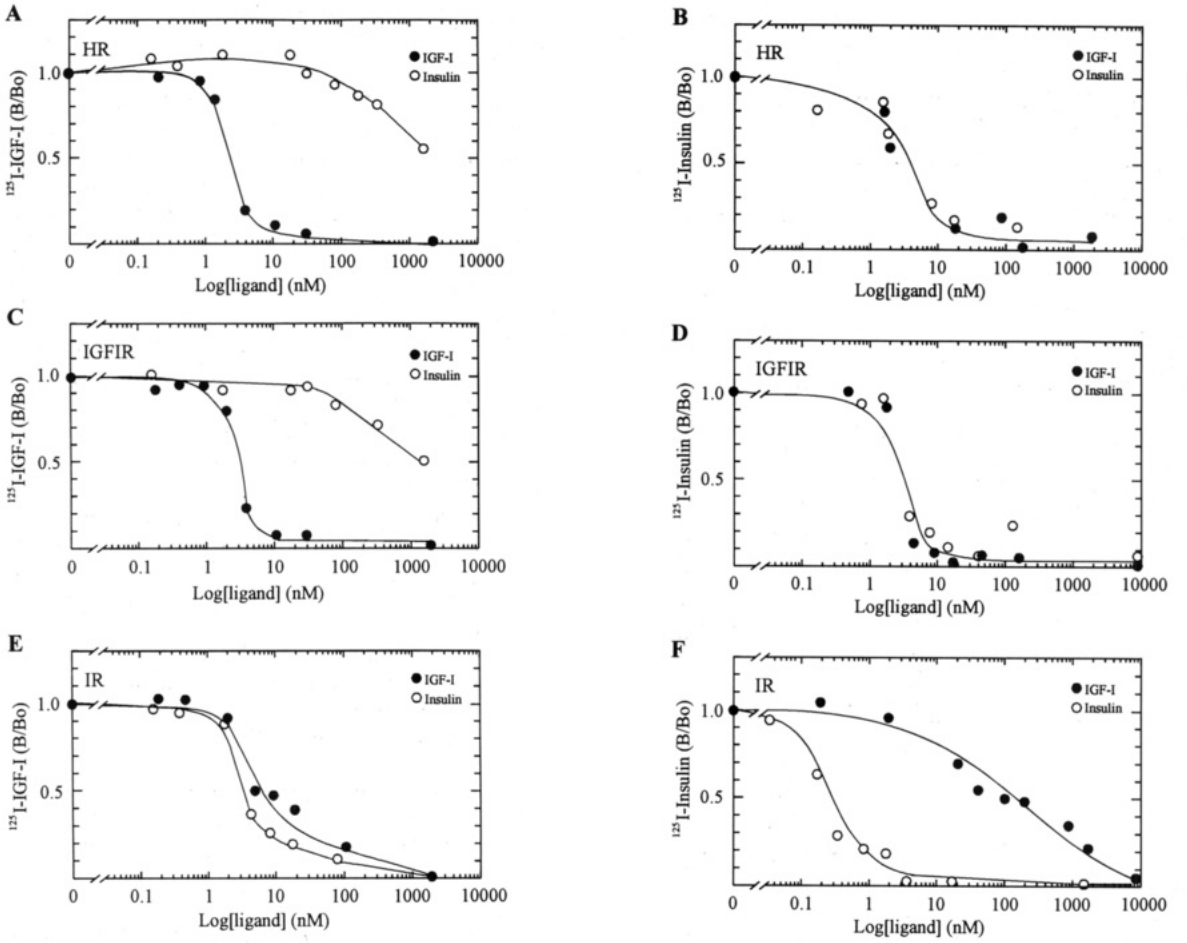

FIGURE 4: Competitive inhibition of ^{125}I-IGF-I (left side) or ^{125}I-insulin (right side) binding to purified hybrid receptors (panels A and B), IGF-I receptors (panels C and D), and insulin receptors (panels E and F) by IGF-I and insulin. Purified receptors from the fifth experiment shown in Figures 2 and 3 were used to compare IC_{50} values. Shown are one of two or three independent experiments. The ratios of the receptors used for heterologous ligand assays per homologous ligand assays were 18, 14, and 20 for HR, IGFIR, and IR, respectively.

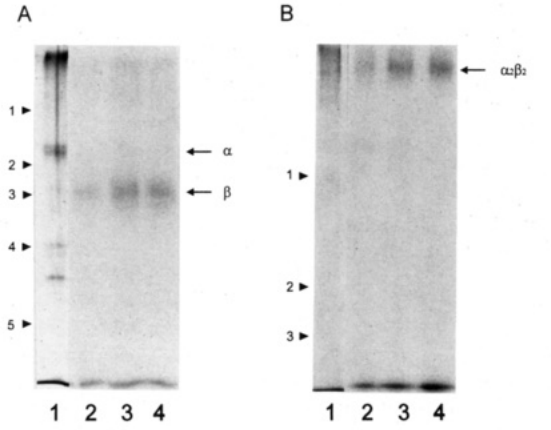

FIGURE 5: Autophosphorylation and SDS–PAGE analyses under reducing (A) and nonreducing (B) conditions of purified hybrid receptors. Purified hybrid receptors were autophosphorylated and subjected to SDS–PAGE under either reducing (A, 7.5% gel) or nonreducing conditions (B, 5% gel). The gels were stained with silver, dried, and autoradiographed. Lane 1 shows silver-stained hybrid receptors. Lanes 2, 3, and 4 show autoradiograms of basal and IGF-I- and insulin-stimulated autophosphorylation of the hybrid receptor, respectively. Molecular weight markers are indicated with (▲) arrowheads: (1) myosin, 200K; (2) β-galactosidase, 116K; (3) phosphorylase *b*, 92K; (4) bovine serum albumin, 66K; and (5) ovalbumin, 46K.

using poly(Glu–Tyr) (4:1) as an exogenous substrate. The results shown in Figure 6B indicated that kinase activation of hybrid receptors by IGF-I was more significant than that of insulin. Ligand stimulation of autophosphorylation and kinase activities in these experiments was lower than that observed with other autophosphorylation experiments (Figure 5). This result is most likely due to partial inactivation of the receptor kinase during extensive purification since the receptors from the fourth experiment were used in this experiment.

Since the initial submission of our manuscript, studies describing purification of the hybrid receptor have been published (Soos *et al.*, 1993). They purified the three receptors by batch methods using combinations of antibodies against the insulin receptor and IGF-I receptor and reported binding and immunological characteristics of those three receptors. Their conclusion that the hybrid receptor binds IGF-I, but not insulin, with high affinity is in good agreement with our conclusion. However, discrepancies are found in some of the relative IC_{50} values. They observed that the hybrid receptor binds insulin with ~10 times higher affinity than IGF-I and ~10 times lower affinity as compared to IC_{50} of the insulin receptor, whereas we find that IC_{50} of the hybrid receptor for insulin is nearly identical to that of the IGF-I receptor. They also reported that IGF-I inhibited ^{125}I-insulin binding to hybrid receptors or IGF-I receptors more effectively than insulin (~1 nM and 0.04 nM [IGF-I] *vs* 4 nM and 4 nM [insulin] for HR and IGFIR, respectively), whereas we observed that both IGF-I and insulin inhibited hybrid and IGF-I receptors at IC_{50} of ~3 nM. Furthermore, in the present studies, we have presented protein sequencing data of IGF-I receptor/hybrid receptor mixtures. We also showed that insulin receptor $\alpha\beta$ and IGF-I receptor $\alpha\beta$ are disulfide-linked and that

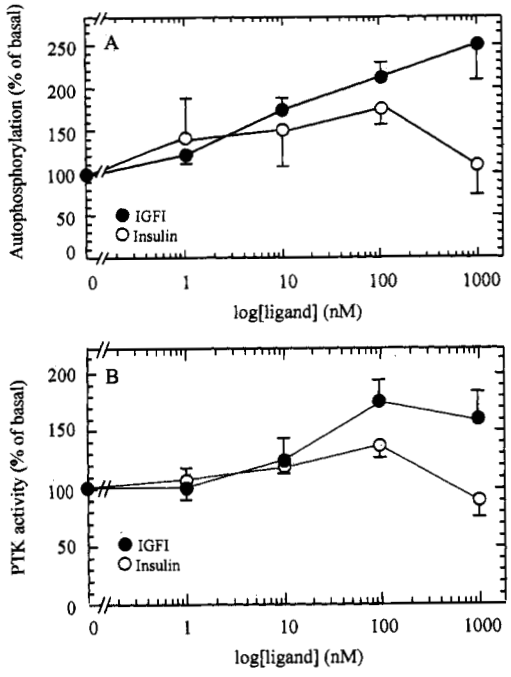

FIGURE 6: Dose dependency of ligand-stimulated autophosphorylation and kinase activity of purified hybrid receptors. Purified hybrid receptors preincubated with indicated concentrations of IGF-I (●) or insulin (○) were assayed for autophosphorylation (A) and kinase activity using poly(Glu–Tyr) (4:1) as an exogenous substrate (B). Three autophosphorylation experiments were performed, from which the averages ± SD were calculated. Two independent kinase assays were performed in duplicate. The average shown is the average ± SD of four data points.

autophosphorylation and kinase activity of the hybrid receptor were stimulated by IGF-I in a dose-dependent manner.

In summary, we have purified and characterized IGF-I/insulin receptor hybrids. Our primary information, that the hybrid receptor is a functional IGF-I receptor in terms of binding and kinase activity, should provide the basis for future investigations on postreceptor signal transduction and potential physiological roles of the hybrid receptor. Determination of binding sites for IGF-I and insulin in the hybrid receptor will be an important subsequent step since recent studies suggested that the primary domains responsible for the two ligands are different (Kjeldsen *et al.*, 1991; Zhang & Roth, 1991). The fact that the hybrid receptor has high affinity for IGF-I but low affinity for insulin is consistent with the possibility that the IGF-I binding site is located on the IGF-I receptor $\alpha\beta$ half whereas the insulin binding site requires two insulin receptor $\alpha\beta$ halves (Yip, 1992). Protein chemical analysis, however, will be required to determine how binding sites for IGF-I and insulin are modified in the hybrid receptor and how disulfide bonds are formed between the two heterologous α subunits.

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