Purified hybrid insulin/insulin-like growth factor-I receptors bind insulin-like growth factor-I, but not insulin, with high affinity

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Hybrid insulin/insulin-like growth factor-I (IGF-I) receptors have previously been described in human placenta, but it has not been possible to study their properties in the presence of classical insulin receptors and type I IGF receptors. To facilitate the purification of hybrids, we produced an anti-peptide monoclonal antibody IGFR 1-2, directed against the C-terminal peptide of the type I IGF receptor β-subunit. The antibody bound native human and rat type I IGF receptors, and reacted specifically with the β-subunit on immunoblots. Solubilized placental microsomal membranes were depleted of classical type I IGF receptors by incubation with an immobilized monoclonal antibody IGFR 24-55, which reacts well with type I receptors but very poorly with hybrid receptors. Residual hybrid receptors were then isolated by incubation with immobilized antibody IGFR 1-2, and recovered by elution with excess of synthetic peptide antigen. Binding properties of hybrids were compared with those of immunoaffinity-purified insulin receptors and type I IGF receptors, by using the radioligands ^{125}I-IGF-I and ^{125}I-insulin. Hybrids bound approx. 20 times as much ^{125}I-IGF-I as ^{125}I-insulin at tracer concentrations (approx. 0.1 nM). The binding of ^{125}I-insulin, but not ^{125}I-IGF-I, to hybrids increased after treatment with dithiothreitol to reduce disulphide bonds between the α-subunits. Hybrids behaved very similarly to type I receptors with respect to the inhibition of ^{125}I-IGF-I binding by unlabelled IGF-I and insulin. By contrast, the affinity of hybrids for insulin was approx. 10-fold lower than that of classical insulin receptors, as assessed by inhibition of ^{125}I-insulin binding by unlabelled hormone. It is concluded that the properties of insulin receptors, but not IGF receptors, are markedly affected by assembly as hybrid compared with classical structures, and that hybrids are more likely to be responsive to IGF-I than insulin under physiological conditions.

INTRODUCTION

The insulin receptor and type I IGF receptor share many similarities of structure and function (reviewed by Czech, 1985; Rechler and Nissley, 1985; Humbel, 1990), reflecting similarities in primary sequence (Ullrich et al., 1985, 1986). Each receptor is synthesized as a single polypeptide and is proteolytically cleaved to give α- and β-subunits, which are disulphide-linked in a β-α-α-β configuration. The ligand-binding site is contained within the extracellular α-subunit (Yip et al., 1988; Wedekind et al., 1989; Kjeldsen et al., 1991; Schumacher et al., 1991), and binding specificity is such that in spite of some cross-reaction each receptor should significantly bind only its own ligand at physiological concentrations (Humbel, 1990; Adamo et al., 1992). The tyrosine kinase activity of the transmembrane β-subunit, which is stimulated by ligand binding, is essential for intracellular signalling (reviewed by Zick, 1989; Olefsky, 1990). The protein pp185/IRS-1 is an endogenous substrate for both receptor tyrosine kinases (White et al., 1985; Izumi et al., 1987; Sun et al., 1991), although the role of this protein and of other substrates in intracellular signalling remains unclear. The insulin receptor and type I IGF receptor mediate a similar range of biological effects in a given cell background (Shimizu et al., 1986; Steele-Perkins et al., 1988; Weiland et al., 1991), although there is evidence that they differ in relative efficiency for initiating growth-promoting and acute metabolic actions (Lammers et al., 1989; Takata et al., 1991).

In cells expressing both insulin receptors and type I IGF receptors, a proportion of the receptor $\alpha\beta$ halves assemble as hybrid structures (Soos and Siddle, 1989; Moxham et al., 1989; Soos et al., 1990). Hybrids can also be generated *in vitro* from isolated $\alpha\beta$ receptor halves (Treadway et al., 1989, 1991). Analogous hybrids have been described for other tyrosine kinase receptor families, including α and β PDGF receptors (Seifert et al., 1989; Heidaran et al., 1991) and EGF receptor/p185^{neu} (Wada et al., 1990; Qian et al., 1992). There is as yet little information about the distribution and properties of insulin/IGF-I hybrid receptors, and it is therefore difficult to judge their functional significance. To facilitate further characterization, we have devised an immunoaffinity protocol for isolation of hybrid receptors from human placenta. We previously used free peptide to elute functionally active human and rat insulin receptors from an insulin-receptor-specific anti-peptide monoclonal antibody (Ganderton et al., 1992). We also recently reported the production of a panel of monoclonal antibodies to type I IGF receptors (Soos et al., 1992). We describe here the production and characterization of an additional anti-peptide monoclonal antibody for the type I IGF receptor, and its use in the purification of type I and hybrid IGF receptors. This has allowed us for the first time to study the binding of insulin and IGF-I to hybrids without interference from classical receptors.

MATERIALS AND METHODS

Materials

Rat 1 fibroblasts (McClain et al., 1987) were a gift from Dr. J. Olefsky (Department of Medicine, University of California,

Abbreviations used: IGF, insulin-like growth factor; IRS-1, insulin-receptor substrate-1; PDGF, platelet derived growth factor; EGF, epidermal growth factor; Fmoc, 9-fluorenylmethoxycarbonyl; Boc, butoxycarbonyl; KLH, keyhole-limpet haemocyanin; WGA, wheat-germ agglutinin; PEG, poly(ethylene glycol).

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San Diego, CA, U.S.A.). IGF-1R/3T3 mouse fibroblasts transfected with human type I IGF receptor cDNA (Lammers et al., 1989) were a gift from Dr. A. Ullrich (Max-Planck Institut für Biochemie, Martinsried, Germany). NIH 3T3 HIR3.5 fibroblasts transfected with human insulin receptor cDNA (Whittaker et al., 1987) were a gift from Dr. J. Whittaker (Department of Medicine, SUNY, Stony Brook, NY, U.S.A.). Recombinant IGF-I was a gift from Ciba–Geigy, Basle, Switzerland. Peptides were synthesized by the Fmoc solid-phase method on a Milligan 9050 peptide synthesizer, using butyl and Boc side-chain protection on poly(dimethylacrylamide)–kieselguhr resin. Peptide was cleaved with trifluoroacetic acid in the presence of appropriate scavengers, analysed by h.p.l.c., and the sequence checked.

Antibodies and immunoadsorbents

The hexadecapeptide YRKNERALPLPQSSTC [corresponding to the C-terminal 15 amino acids of the human type I IGF receptor (Ullrich et al., 1986) together with an N-terminal tyrosine], was coupled to keyhole-limpet haemocyanin (KLH) at a weight ratio of 1:4 by using bis-diazotolidine (Basiri and Utiger, 1972; Briand et al., 1985). Balb/c mice were injected subcutaneously with 0.1 mg of peptide–KLH conjugate at 6-weekly intervals (the first injection in Freund's complete adjuvant, and two subsequent injections in Freund's incomplete adjuvant). After a final intravenous boost of peptide–KLH conjugate together with WGA–Sepharose-purified type I IGF receptors from IGF-1R/3T3 cells (approx. 2 μg of receptor), spleen cells were fused with NSO/1 myeloma cells (Galfre and Milstein, 1981; Soos et al., 1986). Antibodies were detected by reaction with receptor/^{125}I-IGF-I complexes (Soos and Siddle, 1989; Soos et al., 1992). Antibody-secreting cells were cloned at limiting dilution and amplified as ascites. Isotyping was performed by a haemagglutination assay (Serotec), using rabbit antisera specific for mouse IgG1, IgG2a, IgG2b, IgG3, IgA and IgM.

Other monoclonal antibodies specific for the insulin receptor or type I IGF receptor were as described previously (Soos et al., 1986, 1992; Ganderton et al, 1992). Antibodies were partially purified from ascites fluid by precipitation with 40% satd. $(NH_4)_2SO_4$. Immunoadsorbents were prepared by covalent coupling of partially purified antibody to finely divided aminocellulose via a diazotization reaction (Hales and Woodhead, 1980). For some experiments antibodies were further purified by hydroxyapatite chromatography (Stanker et al., 1985) and radioiodinated as previously described (Soos et al., 1984).

Receptor preparations and ^{125}I-ligand-binding assays

Human placental microsomal membranes were prepared and solubilized as described (Soos et al., 1986; Soos and Siddle, 1989) and used in binding assays without further purification. IGF-1R/3T3 cells were solubilized and receptors partially purified by chromatography on WGA–Sepharose as described (Le Bon et al., 1986; Soos et al., 1990). Binding of ^{125}I-IGF-I or ^{125}I-insulin (20000 c.p.m., counting efficiacy 75%, approx. 50–100 pM) to solubilized receptors was measured after incubation for 16 h at 4 °C as described (Soos et al., 1990). Receptor-bound radioactivity was determined by precipitation with PEG 6000. Non-specific binding was determined in the presence of 1 μM insulin or 0.1 μM IGF-I.

For immunodepletion before ligand-binding assays, receptors were incubated for 1 h at 4 °C with anti-receptor antibody covalently coupled to cellulose. After centrifugation to remove antibody-bound receptor (5 min at 4 °C in a microcentrifuge), supernatants were assayed for binding activity as described above. Co-precipitation of receptor/^{125}I-ligand complexes was performed as described (Soos et al., 1986, 1990). Receptors were preincubated with ^{125}I-IGF-I or ^{125}I-insulin for 16 h before addition of antibody for a further 1 h, and antibody-bound radioactivity was then determined by using a sheep anti-(mouse IgG) adsorbent.

Immunoblotting

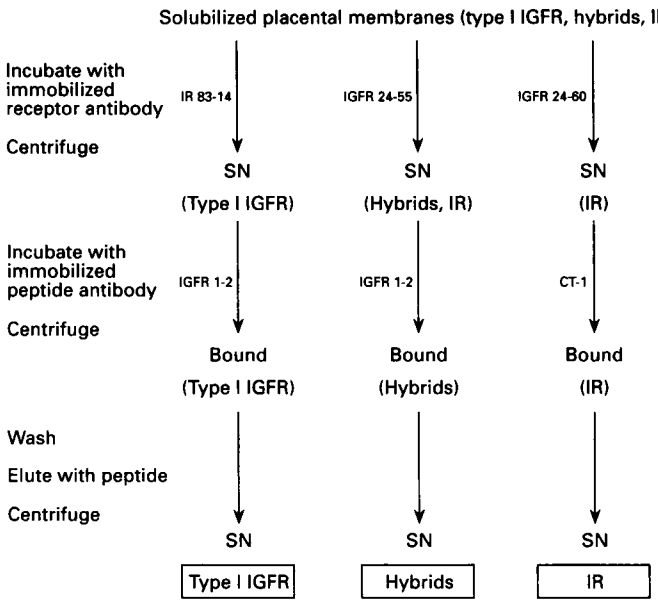
Receptors isolated from detergent-solubilized IGF-1R/3T3 and NIH 3T3 HIR3.5 cells by WGA–Sepharose chromatography were subjected to SDS/PAGE, and proteins were blotted onto poly(vinylidene difluoride) membranes. After blocking in 50 mM Tris/HCl (pH 7.4)/100 mM NaCl (TBS) containing 5% milk powder and 0.1% Tween 20 for 16 h at 4 °C, blots were incubated with anti-receptor antibody [1/200 dilution of 40%-$(NH_4)_2SO_4$-precipitated ascites fluid in TBS/5% milk powder/0.02% Tween 20] for 1.5 h at 22 °C. After washing, the blots were then incubated with horseradish-peroxidase-coupled second antibody (1/2000 dilution) for 1 h, and developed by using the Amersham ECL system according to the manufacturer's instructions. An image was obtained by using the developed blot to expose Fuji RX NIF X-ray film for 15 s.

Two-site immunoassay

Receptors were incubated with labelled anti-(insulin-receptor) antibody 83-14 (20000 c.p.m., approx. 25–50 pM) for 16 h at 4 °C in 100 μl of assay buffer (0.075 M Tris/HCl, pH 7.3 at room temperature, 0.03 M NaCl, 0.01 M glucose, 0.5 mM EDTA, 0.1% BSA), before addition of 50 μl of immobilized antibody IGFR 17-69 or IR 83-7 for a further 1 h. The immunoadsorbents were then washed three times with ice-cold 0.1 M sodium phosphate buffer, pH 7.4, containing 0.1% BSA and 0.1% Triton X-100, and bound radioactivity was measured in a γ-radiation counter. Non-specific binding was determined by omitting the receptor preparation.

Immunoaffinity purification of receptors

Insulin receptors, type I IGF receptors and hybrid receptors were separated from each other by immunoaffinity purification using receptor-specific monoclonal antibodies (Scheme 1). Immunoadsorbents were prepared by coupling partially purified antibody to aminocellulose (100 μg of antibody/mg of cellulose) as described previously (Hales and Woodhead, 1980; Soos et al., 1986). Human placental membranes were prepared and solubilized with 2% (w/v) Triton X-100 as described by Fujita-Yamaguchi et al. (1983). Solubilized membranes (5 ml) were diluted with an equal volume of buffer containing protease inhibitors (0.075 M Tris/HCl, pH 7.3, 0.03 M NaCl, 0.5 mM EDTA, 0.1% BSA, 0.02 mM phenylmethanesulphonyl fluoride, 0.4 mg/ml benzamidine, 1 μg/ml pepstatin, 1 μg/ml antipain, 1 μg/ml leupeptin, 200 kallikrein-inhibitor units/ml aproptinin, 0.2 mM bacitracin), and mixed for 1 h at 4 °C with 5 mg of appropriate immunoadsorbent (Scheme 1). Immunoadsorbents were then pelleted by centrifugation (5 min in a microcentrifuge, 4 °C), and the supernatant was taken for further purification of residual receptors using 0.5 mg of the appropriate immobilized anti-peptide antibody (Scheme 1). After batchwise incubation for 1 h at 4 °C, immunoadsorbents were pelleted as above and washed five times with phosphate-buffered saline. Receptors were eluted by incubating the immunoadsorbents with peptide corresponding to the antibody epitope (5 ml, 50 μg/ml in assay



Scheme 1 Immunoaffinity purification of receptors

Type I IGF receptors (IGFR), hybrids and insulin receptors (IR) were purified from solubilized placental membranes by sequential use of two receptor-specific monoclonal antibodies as described in the Materials and methods section.

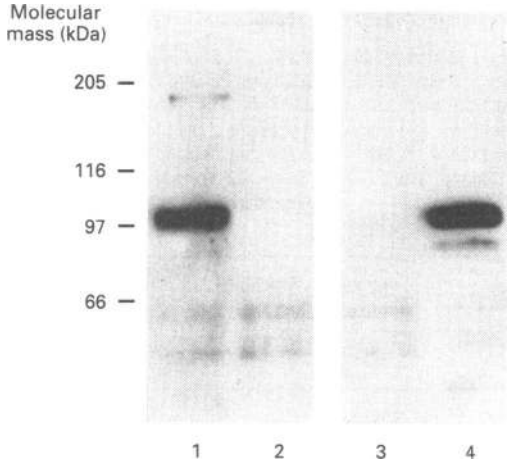


Figure 1 Reactivity of antibody 1-2 with denatured receptor on immunoblots

Receptors, partially purified by WGA–Sepharose chromatography from detergent-solubilized IGF-1R/3T3 cells (type I IGF receptors, tracks 1 and 3) and NIH 3T3 HIR3.5 cells (insulin receptors, tracks 2 and 4), were subjected to electrophoresis under reducing conditions on SDS/7.5%-polyacrylamide minigels (receptors from approx. 3×10^5 cells per track), and blotted on to poly(vinylidene difluoride) membranes. Blots were probed with 1:200 dilutions of partially purified monoclonal antibodies IGFR 1-2 (tracks 1 and 2) and CT-1 (tracks 3 and 4) as described in the Materials and methods section. Bound antibodies were detected by using horseradish-peroxidase-coupled second antibody (1/2000 dilution) and the Amersham ECL system.

Table 1 Immunodepletion of IGF-I receptors

Receptors, solubilized from human placental membranes or partially purified from IGF-1R/3T3 cells by using WGA–Sepharose, were incubated with 10 nM antibody for 1 h at 4 °C before addition of sheep anti-(mouse IgG) antibody covalently coupled to cellulose for 30 min. After centrifugation to remove immunoreactive receptors, residual ^{125}I-IGF-I binding to the supernatant was determined as described in the Materials and methods section. Specific IGF-I binding before depletion was 2340 c.p.m. (IGF-1R/3T3) and 3470 c.p.m. (placenta). ^{125}I-IGF-I binding after depletion by antibody is expressed as a percentage of that of control incubations with cellulose. Non-specific binding (460 c.p.m.) has been subtracted, and values are the means of duplicate determinations within a representative experiment. Irrelevant monoclonal antibodies were without effect.

	Depletion (%)	
Antibody	IGF-1R/3T3	Placenta
IR 83-14	0	77
IR 47-9	0	76
IGFR 1-2	78	64
IGFR 24-31	> 98	89
IGFR 17-69	98	91
IGFR 24-57	96	64
IGFR 24-55	96	53
IGFR 24-60	> 98	97
IGFR 26-3	76	39
IGFR 16-13	90	71

buffer) for 5 h at 4 °C. After centrifugation to remove the immunoadsorbent, eluted receptors were stored in liquid N_2.

RESULTS

Production and characterization of antibody 1-2

To produce an antibody for use in purification of type I and hybrid IGF receptors, mice were immunized with a peptide corresponding to the C-terminal sequence of the IGF receptor β-subunit. Five fusions were performed, two of which yielded six positive wells on initial screening. A single hybridoma was cloned, secreting an IgG1 monoclonal antibody designated IGFR 1-2. The screening assay employing receptor/^{125}I-IGF-I complexes ensured that only antibodies reacting with native receptor were detected, and it was confirmed that antibody IGFR 1-2 also bound unoccupied receptors (Table 1). The efficiency of reaction of antibody 1-2 was less than that of previously obtained monoclonal antibodies to IGF receptors suggesting that 1-2 was of relatively low affinity. In spite of this low affinity, antibody 1-2 reacted well with denatured receptor β-subunit on immunoblots (Figure 1).

The reaction of antibody 1-2 with the type I IGF receptor was highly specific. There was no detectable reaction with human insulin receptor from NIH 3T3 HIR3.5 cells on immunoblots (Figure 1) or in a ^{125}I-insulin/receptor co-precipitation assay (results not shown). The anti-peptide antibody (CT-1) (Ganderton et al., 1992) was specific for the insulin receptor under the same conditions (Figure 1). Antibody 1-2 precipitated just three polypeptides, corresponding to the α- and β-subunits of the receptor and unprocessed proreceptor, from metabolically labelled IGF-1R/3T3 cells (Soos et al., 1992). Species cross-reaction was investigated using co-precipitation assays with ^{125}I-IGF-I bound to receptors from Rat 1 fibroblasts. In contrast with all monoclonal antibodies to IGF receptor previously described (Kull et al., 1983; Soos et al., 1992), antibody 1-2 reacted equally with rat and human receptors (results not shown).

Differential reactivity of antibodies with hybrid and type I IGF receptors

An immunoaffinity approach for purification of hybrid receptors necessitates first removing classical receptors which would also react with a chosen antibody. Selective removal of insulin receptors was not possible, as all available anti-insulin-receptor

Table 2 Sequential depletion of receptors

Placental receptors which were unreactive with IGFR 24-55 or IR 83-14 (see Table 1) were subjected to a second depletion by mixing with the indicated immobilized antibodies for 1 h at 4 °C, followed by centrifugation to remove immunoreactive receptors. Residual ^{125}I-IGF-I binding in the supernatants was determined as described in the Materials and methods section, by using 23000 c.p.m. of ^{125}I-IGF-I. Values are the means of two experiments, and non-specific binding (750 c.p.m.) has been subtracted. Specific IGF-I binding is expressed as a percentage of that remaining after the first depletion, which was 2120–2850 c.p.m. (24-55) and 870–1100 c.p.m. (83-14).

First depletion with antibody	Depletion of ^{125}I-IGF-I binding (%) by a second incubation with:		
	IR 83-14	IGFR 24-60	IGFR 24-55
IGFR 24-55	> 90	88	8
IR 83-14	4	> 90	> 90

Table 3 Immunodepletion of purified receptors

Purified hybrids, type I IGF receptors (IGFR) and insulin receptors (IR) were incubated with cellulose (no antibody) or immobilized antibody IGFR 24-60 or IR 83-14 for 1 h at 4 °C. After centrifugation to remove immunoreactive receptors, residual ^{125}I-ligand binding to the supernatant was determined as described in the Materials and methods section. Values are the means of duplicate determinations within a representative experiment. Results are expressed as specific ^{125}I-ligand bound by 10 μl of receptor. The actual volumes of receptor used in the experiment were 5 μl (hybrids and type I IGF receptors) for ^{125}I-IGF-I binding, and 50 μl (hybrids) and 2 μl (insulin receptors) for ^{125}I-insulin binding. The percentage of ^{125}I-ligand binding activity depleted by antibody is shown in parentheses.

Receptor	^{125}I-ligand	Residual ^{125}I-ligand bound (c.p.m.) after depletion with:		
		No antibody	IGFR 24-60	IR 83-14
Hybrids	IGF-I	2965	232 (93)	62 (98)
	Insulin	132	14 (90)	13 (90)
IGFR	IGF-I	1760	56 (97)	1840 (0)
IR	Insulin	7490	7780 (0)	15 (100)

antibodies react with the hybrid subfraction of IGF receptors in human placenta (Soos and Siddle, 1989). We therefore further investigated antibodies for various epitopes on the type I IGF receptor (Soos et al., 1992). Some of these antibodies (e.g. 24-55, 26-3) bound only 40–50 % of placental IGF-I receptors, although binding essentially all the classical type I receptors in extracts of IGF-1R/3T3 cells (Table 1). Other antibodies (e.g. 17-69, 24-60) bound > 90 % of receptors from both sources. As previously described (Soos and Siddle, 1989), anti-insulin-receptor antibodies (e.g. 83-14) reacted with hybrid receptors representing approx. 75 % of IGF-I binding in placental extracts, but were unreactive with type I IGF receptors in IGF-1R/3T3 cells.

The subfractions of receptor which were unreactive with the anti-IGF-receptor antibody 24-55 or the anti-insulin-receptor antibody 83-14 were subjected to a second depletion. Receptor which was not depleted by a first incubation with antibody 24-55 was almost fully removed by subsequent incubation with either the anti-insulin-receptor antibody 83-14 or the anti-IGF-receptor antibody 24-60 (Table 2), and therefore represents hybrids. Only a small fraction of these receptors was removed by a second incubation with antibody 24-55 itself. Conversely, material not depleted by a first incubation with anti-insulin-receptor antibody 83-14 was efficiently removed by subsequent incubation with both the anti-IGF-receptor antibodies 24-55 and 24-60, but not by a second incubation with antibody 83-14 itself (Table 2), as expected for classical type I IGF receptors. Our interpretation of these results is that antibody 24-55 reacts well with classical receptors but poorly with hybrids, whereas antibody 24-60 reacts with all IGF-I receptors.

Purification of receptors

Two-step immunoaffinity purification schemes were developed to isolate the different receptors present in solubilized placental membranes (Scheme 1). In each case, preparations were first depleted of unwanted receptors by using an appropriate immobilized antibody. Required receptors remaining in the supernatant were then purified by binding to a second (anti-peptide) antibody, followed by specific elution with free peptide.

To obtain hybrid receptors, classical type I IGF receptors were first removed by using immobilized antibody IGFR 24-55, under conditions which left insulin receptors and most of the hybrids in solution. Hybrids were then isolated by using immobilized antibody IGFR 1-2. This gave a 14 % yield of IGF-I binding activity, which was estimated to be a 22 % yield of hybrid receptors. Classical type I IGF receptors were also isolated by using immobilized IGFR 1-2, but after first removing hybrid and insulin receptors with immobilized IR 83-14. This gave an 11 % yield of IGF-I binding activity, which was estimated to be 30 % of the type I IGF receptors. Insulin receptors were isolated by using immobilized antibody CT-1 (Ganderton et al., 1992), after removal of hybrid and classical type I IGF receptors with IGFR 24-60. The yield of insulin receptors was 42 %.

It was confirmed that purified hybrid receptors were essentially free of classical insulin receptors and type I IGF receptors. Thus the binding activity of the hybrid preparation for both ^{125}I-IGF-I and ^{125}I-insulin was removed (> 90 %) by immunodepletion with either immobilized antibody IGFR 24-60 or antibody IR 83-14 (Table 3). These antibodies are known to be specific for the respective receptors (Soos et al., 1990, 1992), although both epitopes must also be accessible in hybrids. In contrast, ligand-binding activity of purified type I receptors was significantly depleted only by antibody IGFR 24-60, and that of insulin receptors only by antibody IR 83-14. This was the case not only for binding of each ligand to its own receptor (Table 3), but also for the very low level of binding of each radioligand to the heterologous receptor. Thus, for the insulin-receptor preparation, 90 % of the ^{125}I-IGF-I binding activity was removed by IR 83-14 but only 20 % by IGFR 24-60, whereas for the type I receptor preparation 98 % of the ^{125}I-insulin-binding activity was removed by IGFR 24-60, but only 15 % by IR 83-14 (results not shown).

Ligand-binding studies

Purified hybrid receptors readily bound ^{125}I-IGF-I, as previously demonstrated with intact cells and crude cell lysates (Soos and Siddle, 1989; Soos et al., 1990). The concentration of unlabelled IGF-I for half-maximal inhibition of ^{125}I-IGF-I binding was 0.1–0.2 nM for hybrids and 0.05–0.1 nM for type I receptors (Figure 2a, Table 4). The corresponding values for IGF-II were 2 nM and 1 nM respectively (results not shown). Thus hybrids and type I receptors have very similar affinities for both IGF-I and IGF-II. Purified hybrids bound only approx. 1/20 as much ^{125}I-insulin as ^{125}I-IGF-I at the low ligand concentrations used in routine assays (Table 3), indicating that the affinity of hybrids for insulin was substantially less than for IGF-I. The concentration

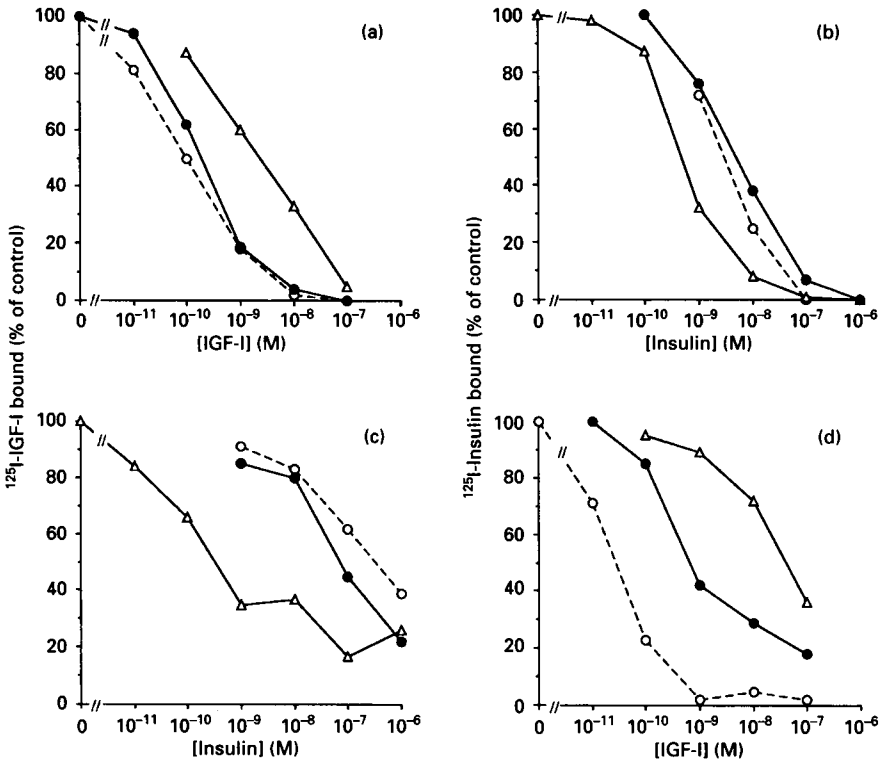


Figure 2 Inhibition of ^{125}I-ligand binding to purified receptors

Binding of ^{125}I-IGF-I (**a, c**) and ^{125}I-insulin (**b, d**) to purified hybrids (●), type I IGF receptors (○) and insulin receptors (△) was measured in the presence of the indicated concentrations of unlabelled IGF-I (**a, d**) or insulin (**b, c**) as described in the Materials and methods section. Data points are the means of duplicate incubations within a representative experiment. Specific binding is expressed as a percentage of that in the absence of unlabelled ligand. Non-specific binding in the presence of 1 μM insulin (215 c.p.m.) or 100 nM IGF-I (400 c.p.m.) has been subtracted.

Table 4 IC_{50} values for inhibition of ^{125}I-ligand binding to purified receptors

The concentration of unlabelled IGF-I or insulin required for half-maximal inhibition of ^{125}I-ligand binding (IC_{50}) to purified hybrids, type I IGF receptors (IGFR) and insulin receptors (IR) was determined from competition experiments described in Figure 2. Values shown are for a single experiment (^{125}I-IGF-1, insulin receptors; ^{125}I-insulin, type I IGF receptors) or the range for two experiments (^{125}I-IGF-I, hybrids and type I IGF receptors) or three experiments (^{125}I-insulin, hybrids and insulin receptors).

		IC_{50} (nM)		
^{125}I-ligand	Competing ligand	Hybrids	IGFR	IR
IGF-I	IGF-I	0.1–0.2	0.05–0.1	3
	Insulin	70–100	300–400	0.2
Insulin	IGF-I	0.7–1.5	0.04	40–50
	Insulin	3–5	4	0.3–0.5

of unlabelled insulin required for half-maximal inhibition of ^{125}I-insulin binding was 3–5 nM for hybrids and 0.3–0.5 nM for insulin receptors (Figure 2b, Table 4), confirming the relatively low affinity of hybrids for insulin. Scatchard analysis (not shown) of the data from Figures 2(a) and 2(b) indicated very similar numbers of binding sites for insulin and IGF-I in hybrid receptors, as would be expected.

It can be shown that the IC_{50} value from a competitive displacement curve approximates to the dissociation constant K_d for a ligand–receptor interaction, provided that concentrations of both the labelled ligand and the receptor are substantially less than the K_d (Jacobs et al., 1975; Chang et al., 1975). In the present experiments, this condition was satisfied for insulin binding (concentrations of both ^{125}I-insulin and receptor $\leqslant IC_{50}/5$, receptor concentrations being estimated by Scatchard analysis). Therefore the IC_{50} values for insulin binding to classical and hybrid receptors over-estimate the corresponding average K_d values by a factor of only 1.1–1.2. However, IC_{50} values for IGF binding to type I and hybrid receptors will significantly over-estimate corresponding K_d values by a factor of 2–3, because of the very high affinity of IGF binding (concentration of ^{125}I-IGF-I and receptor $\approx IC_{50}$).

Inhibition of radioligand binding to hybrid receptors by heterologous unlabelled ligand was also examined. In this case, inhibition could in principle result from a '*trans*' effect involving co-operative interaction between receptor halves, as well as by direct '*cis*' competition for a given binding site. In fact, binding of ^{125}I-IGF-I to hybrids was inhibited only by relatively high concentrations of insulin (IC_{50} 70–100 nM), similar to those observed with type I receptors (IC_{50} 300–400 nM) (Figure 2c, Table 4). There was thus no evidence for an effect of insulin on IGF-I binding at concentrations appropriate to occupancy of the insulin half-receptor within a hybrid (IC_{50} 3–5 nM). Binding of ^{125}I-insulin to hybrids was inhibited by IGF-I at concentrations (IC_{50} 0.7–1.5 nM) which were intermediate between those required to inhibit binding of ^{125}I-insulin to insulin receptors (IC_{50} 40–50 nM) and binding of ^{125}I-insulin or ^{125}I-IGF-I to type I receptors (IC_{50} 0.04–0.1 nM) (Figure 2a and 2d, Table 4). Moreover, the IGF-I/^{125}I-insulin displacement curve for hybrids

Table 5 Effect of dithrothreitol (DTT) on ligand binding to purified receptors

Purified insulin receptors (IR), hybrids and type I IGF receptors (IGFR) were treated with 1.25 mM DTT as described in the Materials and methods section. Receptors were then incubated with 20000 c.p.m. of ^{125}I-insulin (with 2 μl of insulin receptors or 50 μl of hybrids) or ^{125}I-IGF-I (with 10 μl of type I IGF receptors or 10 μl of hybrids) in a final volume of 250 μl. After 16 h at 4 °C, specific receptor-bound ^{125}I-ligand was determined as described in the Materials and methods section. Values are the means of duplicate determinations within a representative experiment. Non-specific binding in the presence of 1 μM insulin (200 c.p.m.) or 100 nM IGF-I (550 c.p.m.) has been subtracted.

		Receptor-bound ^{125}I-ligand (c.p.m.)	
^{125}I-ligand	Receptor	No DTT	+ DTT
Insulin	IR	3092	825
	Hybrids	769	1395
IGF-I	IGFR	1424	1708
	Hybrids	1755	1288

Table 6 Reactivity of purified receptors in a two-site immunoassay

A two-site immunometric assay for receptors, purified from solubilized placental membranes, was carried out as described in the Materials and methods section. Receptors were incubated with labelled anti-insulin-receptor antibody 83-14 (19300 c.p.m.) for 16 h at 4 °C before addition of immobilized antibody IGFR 17-69 or IR 83-7 for 1 h. Results are expressed as radioactivity bound to the immunoadsorbent after subtraction of non-specific binding (50 c.p.m.), determined in the absence of receptor. Values are the means of duplicate determinations within a representative experiment.

	^{125}I-antibody IR 83-14 bound (c.p.m.)			
Immobilized antibody	PE	Type 1	Hybrids	IR
IGFR 17-69	428	0	695	0
IR 83-7	4305	26	757	4103

was more shallow than other displacement curves, suggesting that IGF-I may have been binding to two sites with different affinities. The possibility that high-affinity binding of IGF-I to the IGF half-receptor indirectly influences the affinity of the insulin half-receptor therefore cannot be ruled out.

Effect of disulphide reduction on ligand binding to hybrid receptors

Isolated insulin receptor $\alpha\beta$ halves display decreased affinity for insulin compared with native heterotetrameric receptors (Böni-Schnetzler et al., 1987; Sweet et al., 1987). The question therefore arises whether the insulin-binding affinity of hybrids is comparable with that of isolated half receptors. To test this, ^{125}I-ligand binding to the purified receptors was determined before and after treatment with dithiothreitol, under conditions known to reduce α-α disulphides (Table 5). As previously reported, binding of ^{125}I-insulin to insulin receptors was substantially decreased after disulphide reduction (to 27 ± 2% of control values; mean$\pm$S.D. for three independent experiments). However, binding of ^{125}I-insulin to hybrids actually increased after reduction (to 177 ± 18% of controls; $n = 3$), indicating that hybrids have an even lower affinity for insulin than isolated half-receptors. By contrast, binding of ^{125}I-IGF-I to type I receptors was little affected by reduction (105 ± 33% of controls; $n = 3$), whereas binding to hybrids was slightly decreased (73 ± 6% of controls; $n = 3$). Thus the state of assembly of type I receptors has only a minor influence on their affinity for IGF-I.

Two-site immunoassay of hybrid receptors

Hybrid receptors were originally detected as a fraction of ^{125}I-IGF-I binding sites reactive with insulin-receptor-specific antibodies (Soos and Siddle, 1989; Soos et al., 1990). This is applicable as an assay for hybrids under conditions where insulin receptors are in excess, and therefore hybrids account for a significant fraction of total IGF binding. However, it would be difficult to detect hybrids by this means when type I IGF receptors were in large excess and the fraction of IGF-binding sites contributed by hybrids was small. Because hybrids bind insulin with relatively low affinity, they cannot be quantified as a fraction of ^{125}I-insulin binding reactive with anti-IGF-I receptor antibodies. We therefore tested whether ^{125}I-labelled anti-insulin-receptor antibody could be used in place of ^{125}I-insulin in this kind of assay. Hybrid receptors were incubated with ^{125}I-antibody 83-14 and then with immobilized anti-IGF-receptor antibody 17-69 or anti-insulin receptor antibody 83-7. Both immobilized antibodies bound similar amounts of ^{125}I-antibody 83-14, as expected (Table 6). This result confirms that insulin-receptor-specific and IGF-receptor-specific antibodies can bind simultaneously to hybrid receptors, a property which could form the basis for a quantitative assay of hybrids in tissue extracts.

When insulin-binding assays were carried out using the same receptor concentrations and assay format as in Table 6, the insulin-receptor preparation bound 1140 c.p.m. of ^{125}I-insulin (compared with 4103 c.p.m. of ^{125}I-83-14), whereas hybrids bound < 20 c.p.m. of ^{125}I-insulin (compared with 757 c.p.m. of ^{125}I-83-14). It is uncertain whether antibody 83-14 binds with the same affinity to hybrid and classical receptors, and whether classical receptors have the potential to bind one or two molecules of 83-14. However, if receptor concentrations are normalized for equal binding of 83-14, insulin binding to hybrids is < 10% of that to classical receptors, consistent with the calculated difference in relative affinity for insulin.

When crude placental extracts were incubated with ^{125}I-antibody 83-14, the radioactivity bound by immobilized anti-IGF-receptor antibody 17-69 was approx. 10% of that bound by anti-insulin-receptor antibody 83-7 (Table 6). Allowing for uncertainty as to whether classical receptors bind one or two molecules of 83-14, these data suggest that hybrids contain 10–20% of the tissue insulin receptor halves. On the other hand, based on the reactivity of IGF-I binding sites with anti-insulin-receptor antibodies, hybrids appeared to account for 50–70% of the placental type I receptor halves (Soos and Siddle, 1989). It has been estimated that the total concentration of IGF-I receptors in human placenta is approx. 10% of the insulin receptor concentration (Le Bon et al., 1986), although by Scatchard analysis we find up to 50% as many high-affinity binding sites for IGF-I as for insulin in solubilized membranes.

DISCUSSION

The physiological role of hybrid insulin/IGF-I receptors remains unclear. Although it might be expected that hybrids would bind both insulin and IGF-I, there are indications that they respond more readily to IGF-I than to insulin, at least in terms of autophosphorylation (Moxham et al., 1989; Moxham and Jacobs, 1992). We therefore devised a method for immunoaffinity purification of hybrid receptors in order to study their properties *in vitro* without interference from other receptor types.

Several anti-IGF-receptor antibodies were identified which reacted much less efficiently with hybrid receptors from placenta than with type I receptors from transfected fibroblasts (Table 1). Some of these antibodies recognized epitopes in the region 440–586 of the type I receptor α-subunit (Soos et al., 1992), and these epitopes must depend on local conformations or inter-subunit contacts which differ between hybrid and classical IGF receptors. By using one of these antibodies, IGFR 24-55, it was possible selectively to deplete solubilized placental membranes of classical type I receptors without removing the majority of hybrid receptors. This permitted the purification of residual hybrids by using the IGF-receptor-specific anti-peptide monoclonal antibody, IGFR 1-2. This antibody was directed against the C-terminus of the receptor β-subunit, analogously to the previously described anti-insulin-receptor antibody CT-1 (Ganderton et al., 1992). Antibody 1-2 appeared to be of relatively low avidity, a property which made it particularly suitable for use in immunoaffinity purification because of the ease of recovering dissociated receptor in the presence of excess free peptide antigen. Insulin receptors and type I IGF receptors were also purified from placenta by immunoaffinity techniques and peptide elution under mild conditions (Scheme 1). Previous methods for purifying type I IGF receptors, using IGF-I–Sepharose (Maly and Luthi, 1986) or specific monoclonal antibodies (Le Bon et al., 1986), involved potentially denaturing conditions for elution of bound receptor.

It was clearly shown that purified hybrids bound IGF-I, but not insulin, with high affinity. The affinity of hybrids for IGF-I, as indicated by the IC_{50} for displacement of ^{125}I-IGF-I, was similar to that of classical type I receptors. However, the affinity of hybrids for insulin was approx. 10-fold lower than that of classical insulin receptors, and 20-fold lower than the affinity of hybrids for IGF-I. The concentration of insulin required to inhibit binding of ^{125}I-insulin to hybrids (IC_{50} approx. 4 nM) was considerably lower than that required to inhibit binding of ^{125}I-IGF-I (IC_{50} approx. 100 nM), confirming that insulin is indeed binding to the insulin half-receptor within a hybrid, albeit with relatively low affinity. Hybrids reacted well with all available anti-insulin-receptor antibodies, but not all anti-IGF-receptor antibodies, implying that conformational changes responsible for altered ligand binding and immunoreactivity of hybrid compared with classical receptors are distinct and relatively localized within the receptor structure.

These observations on hybrid receptors extend previous studies indicating that ligand-binding affinity of the insulin half-receptor is dramatically dependent on its state of assembly. Classical insulin-receptor heterotetramers bind only a single molecule of insulin with high affinity (Pang and Shafer, 1983, 1984), and isolated insulin half-receptors have 10-fold lower affinity for insulin that do intact heterotetramers (Böni-Schnetzler et al., 1987; Sweet et al., 1987). When hybrids were subjected to disulphide reduction to release the individual half-receptors, ^{125}I-insulin binding increased 1.8-fold (Table 5), indicating that the affinity for insulin of intact hybrids is even less than that of isolated half-receptors. Previous data suggesting that hybrids reconstituted *in vitro* from isolated receptor halves bound comparable amounts of ^{125}I-insulin to classical insulin receptors (Treadway et al., 1989) may in part reflect the use of higher ligand concentrations, which would diminish the effects of affinity differences. It may also be that hybrids assembled *in vitro* differ in subtle respects from naturally occurring hybrids, perhaps in terms of incomplete formation of inter-subunit disulphides.

It is generally assumed that receptor-binding interactions of insulin are entirely within a single α-subunit. On this model, binding affinity depends on conformation as influenced by an adjacent α-subunit: combination of two unoccupied insulin receptor α-subunits is essential for high-affinity binding, and single occupancy within a classical heterotetramer is proposed to decrease the affinity of the unoccupied α-subunit by negatively co-operative interactions (De Meyts et al., 1976; Pang and Shafer, 1983). Combination of an IGF half-receptor with the insulin half-receptor in a hybrid may similarly impose a conformation with low affinity for insulin. It will be interesting to test whether hybrids display negative co-operativity in terms of effects of IGF on dissociation of insulin, and vice versa. Equilibrium binding studies did not clearly show whether one ligand can affect binding of the other by '*trans*' co-operative interactions, as opposed to direct '*cis*' competition, although there was some indication that insulin binding was decreased by low concentrations of IGF (Figure 2d).

An alternative model for different affinity states of the insulin receptor is suggested by recent work on the growth-hormone receptor, in which it has been shown that a single growth-hormone molecule forms a high-affinity complex by asymmetric interaction with both halves of a receptor dimer (Cunningham et al., 1991). In a similar way, '*trans*' interactions with both α-subunits of a heterotetrameric insulin receptor may contribute to high-affinity ligand binding and conformational changes involved in receptor activation. This proposal would be consistent with studies of photoaffinity cross-linking of insulin, which have shown that different parts of the insulin molecule contact distinct regions of the receptor (Yip et al., 1988; Wedekind et al., 1989; Fabry et al., 1992). On this model, the low affinity of hybrids for insulin might reflect the absence of appropriate '*trans*' interactions necessary to increase the affinity above that observed with isolated half-receptors. It should be possible to use hybrid receptors, together with photo-activatable insulin analogues, to probe the relative proximity of different parts of the bound ligand to the two receptor halves.

Whatever the molecular basis for the various affinity states of insulin receptors, it is clear that IGF receptors behave differently. Hybrid and classical type I receptors displayed similar affinity for IGF-I (Table 4), and, in agreement with a previous study (Feltz et al., 1988) this was little affected by reduction of inter-subunit disulphides (Table 5). Most workers report linear Scatchard plots for IGF-I binding to type 1 receptors from diverse sources, suggesting there is no co-operativity, although there are conflicting reports as to whether the receptors bind one or two molecules of IGF with high affinity (Feltz et al., 1988; Tollefsen and Thompson, 1988).

Other investigators have described 'atypical' receptors which bind both IGF-I and insulin with relatively high affinity (Jonas, 1988). Atypical receptors reportedly display specificity in their reaction with anti-receptor antibodies, in contrast with hybrids, which react with both anti-insulin-receptor and anti-IGF-receptor antibodies. To confirm that hybrid and atypical receptors are distinct entities, we looked for 'atypical' properties in immunoaffinity-purified type I IGF receptors and insulin receptors which were free of hybrids. Purified type I receptors bound IGF-I with high affinity as expected, but there was a considerable discrepancy between the concentrations of insulin required to inhibit binding of ^{125}I-IGF-I and ^{125}I-insulin (IC_{50} 300 nM and 4 nM respectively, Table 5). This indicates the presence of an atypical subfraction of IGF receptors with anomalously high affinity for insulin, as described previously in several cell types (Hintz et al., 1984; Misra et al., 1986; Burant et al., 1987; Waldbillig and Chader, 1988; Milazzo et al., 1992). With purified insulin receptors, IGF-I was more effective in competing binding of ^{125}I-IGF-I than that of ^{125}I-insulin (IC_{50} 3 nM and 50 mM respectively; Table 5). Atypical insulin recep-

tors defined by similar criteria have been described previously in placenta (Jonas et al., 1989), and in IM-9 lymphocytes (Jonas and Cox, 1990). The structural basis of atypical receptors is unclear, although a subfraction of insulin receptors displaying atypical binding kinetics was seen in transfected cells expressing cloned receptor cDNA (Jonas et al., 1990). It may be that IGF-receptor cDNA can similarly give rise to both normal and atypical receptors in transfected cells, and we now believe that most of the relatively high-affinity insulin binding to solubilized receptors from transfected IGF-1R/3T3 cells (Soos et al., 1990) is to atypical rather than hybrid IGF receptors.

In summary, we have purified hybrid insulin/IGF receptors which are distinct from previously described atypical receptors, and demonstrated that the properties of insulin receptors, but not IGF receptors, are markedly affected by assembly as hybrid compared with classical structures. It is concluded that hybrids are more likely to be responsive to IGF-I than to insulin under physiological conditions, but a number of questions regarding the biological importance of hybrid receptors remain to be answered. The purification procedures described here will allow examination of hybrid receptor activation by insulin and IGF-I, and the availability of a specific two-site immunoassay will permit investigation of the tissue distribution of hybrids.

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