Biochem. J. (1989) 263, 553–563 (Printed in Great Britain)

Immunological relationships between receptors for insulin and insulin-like growth factor I

Evidence for structural heterogeneity of insulin-like growth factor I receptors involving hybrids with insulin receptors

Maria A. SOOS and Kenneth SIDDLE*
Department of Clinical Biochemistry, University of Cambridge, Addenbrooke's Hospital, Hills Road, Cambridge CB2 2QR, U.K.

The receptors for insulin and insulin-like growth factor-I (IGF-I) are closely related in primary sequence and overall structure. We have examined the immunological relationships between these receptors by testing the reactivity of anti-(insulin receptor) monoclonal antibodies with IGF-I receptors in various tissues and cell lines. Antibodies for six distinct epitopes reacted with a subfraction of IGF-I receptors, as shown by inhibition of ^{125}I-IGF-I binding, precipitation of ^{125}I-IGF-I–receptor complexes or immunodepletion of receptor from tissue extracts before binding assays. Both immunoreactive and non-immunoreactive subfractions displayed the expected properties of 'classical' IGF-I receptors, in terms of relative affinities for IGF-I and insulin. The proportion of total IGF-I receptors which was immunoreactive varied in different cell types, being approx. 40% in Hep G2 cells, 35–40% in placental membranes and 75–85% in IM-9 cells. The immunoreactive fraction was somewhat higher in solubilized receptors than in the corresponding intact cells or membranes. A previously described monoclonal antibody, α-IR-3, specific for IGF-I receptors, inhibited IGF-I binding by more than 80% in all preparations. When solubilized placental receptors were pretreated with dithiothreitol (DTT) under conditions reported to reduce intramolecular (class I) disulphide bonds, the immunoreactivity of IGF-I receptors was abolished although total IGF-I binding was little affected. Under the same conditions insulin receptors remained fully immunoreactive. When solubilized receptor preparations were fractionated by gel filtration, both IGF-I and insulin receptors ran as symmetrical peaks of identical mobility. After DTT treatment, the IGF-I receptor was partially converted to a lower molecular mass form which was not immunoreactive. The insulin receptor peak showed a much less pronounced skewing and remained fully immunoreactive in all fractions. It is concluded that the anti-(insulin receptor) antibodies do not react directly with IGF-I receptor polypeptide, and that the apparent immunoreactivity of a subfraction of IGF-I receptors reflects their physical association with insulin receptors, both in cell extracts and in intact cells. The most likely basis for this association appears to be a 'hybrid' receptor containing one half ($\alpha\beta$) of insulin receptor polypeptide and the other ($\alpha'\beta'$) of IGF-I receptor polypeptide within the native ($\alpha\beta\beta'\alpha'$) heterotetrameric structure.

INTRODUCTION

Two very different cell surface receptors have been described for insulin-like growth factors (IGFs) [1–4]. The IGF-I receptor (sometimes referred to as the Type I IGF receptor) shows considerable structural and functional similarity to the insulin receptor. Both are heterotetrameric molecules of structure $\beta\alpha\alpha\beta$ which possess intrinsic tyrosine-specific protein kinase activity and are subject to autophosphorylation [2,5]. The tyrosine kinase is essential to the signalling function of the insulin receptor [6,7]. The cloning of cDNAs coding for the respective precursors has revealed a high degree of sequence similarity, especially in the intracellular tyrosine kinase domains of the β-subunits [3,8]. There is evidence for heterogeneity of both insulin receptors and IGF-I receptors [9], although only a single gene has been identified for each [3,8]. In the case of the insulin receptor this heterogeneity is reflected in different binding affinities of receptors from different tissues [10] and in the separation of two forms of receptor with distinct binding specificities [11]. The IGF-II receptor (Type II IGF receptor) is structurally unrelated to the IGF-I receptor and also functions as a mannose 6-phosphate receptor [4,12]. It is unclear whether or not this receptor has any signalling role.

The primary physiological role of IGF-I is generally considered to be in regulating cell proliferation, although in some cells acute metabolic effects similar to those of insulin are observed [13,14]. It is not clear whether the different but overlapping effects of IGF-I and insulin are a reflection of differences in the signalling capacity of the two receptors, or due simply to differences in the distribution of receptors with essentially similar activities but distinct target pathways in different cell types. This issue is further complicated by the cross-reaction of

Abbreviations used: IGF-I, insulin-like growth factor I, DTT, dithiothreitol; NEM, *N*-ethylmaleimide; PEG, poly(ethylene glycol).

* To whom correspondence should be addressed.

IGF-I with the insulin receptor and vice versa [1,13,14], and by the reported heterogeneity in both types of receptor.

We have used monoclonal antibodies to multiple epitopes on the insulin receptor as probes of structure and function [15]. These antibodies are potentially useful as insulin receptor agonists [16] or antagonists [17]. Such studies require however that the antibodies are specific for the insulin receptor, although the sequence similarity between IGF-I receptors and insulin receptors suggest that both common and specific epitopes are to be expected. We report here an investigation of the reactivity of the anti-(insulin receptor) antibodies with IGF-I receptors, demonstrating a subpopulation of IGF-I binding sites which are immunologically very similar to insulin receptors. It is suggested that this subpopulation consists of 'hybrid' receptors containing both insulin receptor ($\alpha\beta$) and IGF-I receptor ($\alpha'\beta'$) polypeptides in a $\beta\alpha\alpha'\beta'$ configuration.

EXPERIMENTAL

Materials

Bovine insulin (for displacement studies) was from Sigma (London) Chemical Co., Poole, Dorset, U.K., and highly purified desamido-free bovine insulin (for iodination) was a gift from Dr. D. Brandenburg, University of Aachen, Aachen, Germany. Recombinant human IGF-I was generously given by Ciba–Geigy Ltd., Basle, Switzerland. Antibodies MC51 [18] and α-IR-3 [19] were kindly provided by Dr. R. Roth and Dr. S. Jacobs respectively. Protease inhibitors, dithiothreitol (DTT), *N*-ethylmaleimide (NEM) and bovine serum albumin were from Sigma, poly(ethylene glycol) (PEG) 6000 was from BDH Chemicals, Dagenham, Essex, U.K., and Na^{125}I (IMS 30) was from Amersham International, Aylesbury, Bucks, U.K. Hydroxyapatite was purchased from Bio-Rad Laboratories, St Albans, Herts., U.K., and Sephadex G-50 and Sephacryl S300 HR were from Sigma. IM-9 lymphocytes were obtained from Flow Laboratories, Irvine, Scotland, and Hep-G2 hepatoma cells from the European Collection of Animal Cell Cultures, Porton Down, Salisbury, U.K. Sheep anti-(mouse IgG) antibodies were coupled to aminocellulose to obtain immunoadsorbents as described previously [20]. Normal human placenta was freshly obtained at delivery.

Radioiodinations

Mono-^{125}I-insulin with a specific activity of 100–200 μCi/μg was prepared from highly purified bovine insulin as described [21]. IGF-I was iodinated to a specific activity of 50–100 μCi/μg using a stoichiometric chloramine-T method [22] and purified by gel filtration on Sephadex G-50 to separate ^{125}I-IGF-I from free [^{125}I]-iodide.

Antibodies

Monoclonal antibodies were as previously reported [15]. Antibodies were partially purified from ascites fluid by precipitation with 40% saturated (v/v) $(NH_4)_2SO_4$ followed by reconstitution to the original ascites volume in phosphate-buffered saline (150 mM-NaCl/10 mM-sodium phosphate, pH 7.4). The purity (50–70%) and concentration (2–10 mg/ml) of antibodies was estimated as previously described [16]. For some experiments, antibody was further purified (> 90%) by hydroxyapatite chromatography [23].

Receptor preparations

Human placental membranes were prepared as described [24]. Membranes (20–40 mg of protein/ml) were solubilized in 50 mM-Tris/HCl (pH 7.4 at 4 °C), containing 2% (v/v) Triton X-100 and protease inhibitors (1 mM-phenylmethanesulphonyl fluoride, 0.4 mg of benzamidine/ml, 1 μg of pepstatin/ml, 1 μg of antipain/ml and 1 μg of leupeptin/ml) for 1 h at 4 °C with stirring. The clear supernatant obtained after centrifugation at 150000 *g* for 1 h at 4 °C was used for assays. Membranes were prepared from 10^9 IM-9 lymphocytes as described [25] and solubilized (10 mg/ml) with 1% (v/v) Triton X-100.

^{125}I-hormone binding assays

In all assays the concentration of ^{125}I-labelled hormone was 50–100 pM (approx. 20000 c.p.m., counting efficiency 70%). Non-specific binding was determined by incubation with 1 μM-insulin or 0.1 μM-IGF-I as appropriate.

Binding to IM-9 lymphocytes was determined as described [26] by preincubating cells (5×10^6 and 6×10^5 for IGF-I and insulin binding assays respectively) for 30 min at 15 °C with antibody or unlabelled hormone (total volume 200 μl) before addition of 50 μl of labelled hormone for a further 90 min. Total binding was typically approx. 9% (^{125}I-IGF-I) and 20% (^{125}I-insulin) in the absence of unlabelled ligand, while non-specific binding was 3% (^{125}I-IGF-I) and 2% (^{125}I-insulin).

Binding to Hep G2 cells (75% confluent on 10 cm^2 dishes) was carried out as described [27] by preincubating cells with antibody or unlabelled hormone (total volume 900 μl) for 30 min at 4 °C before addition of 100 μl of labelled hormone for a further 6 h. Total binding was typically approx. 7% (^{125}I-IGF-I) and 13% (^{125}I-insulin), while non-specific binding was 0.5% (^{125}I-IGF-I) and 0.3% (^{125}I-insulin).

Binding to particulate placental membranes (40–80 μg of protein) was performed as previously described [15] by preincubating membranes with antibody or unlabelled hormone (total volume 200 μl) for 3–4 h at 4 °C before addition of 50 μl of labelled hormone for a further 18 h. Total binding was 7–10% (^{125}I-IGF-I) and 15–30% (^{125}I-insulin), while non-specific binding was 0.7% for both hormones.

Binding to solubilized placental membranes (20–40 μg) or IM-9 membranes (5–10 μg) was performed as described for particulate membranes, except that 0.05% Triton X-100 was included in the buffer and receptor-bound radioactivity was determined by precipitation with PEG 6000 [28]. Total binding was 10–20% (^{125}I-IGF-I) and 25–30% (^{125}I-insulin) for placental receptors and 5–10% (^{125}I-IGF-I) and 20–30% (^{125}I-insulin) for IM-9 receptors. Non-specific binding was approx. 3% (^{125}I-IGF-I) and 1% (^{125}I-insulin) for both placental and IM-9 receptors.

Co-precipitation of receptor–^{125}I-hormone complexes

Assays were performed as described in [15] by preincubating receptors solubilized from placental membranes (20–40 μg of protein) or IM-9 membranes (5–10 μg of protein) with labelled hormone (50–100 pM in 100 μl) for 20 h at 4 °C before addition of antibody

(100 μl) for a further 20 h. To establish the specificity of binding of labelled hormones, unlabelled hormones were sometimes included in the first incubation, as specified for individual experiments. Antibody-bound radioactivity was determined using a sheep anti-(mouse IgG) adsorbent as previously described [15]. Non-specific binding was approx. 1% and 0.5% for ^{125}I-IGF-I and ^{125}I-insulin respectively. Total receptor-bound radioactivity was measured by precipitation with PEG 6000 [28].

Immunodepletion

Solubilized placental receptors (approx. 100 μg of protein/ml) were incubated with or without antibody (10^{-8} M) for 18 h at 4 °C. Sheep anti-(mouse IgG) adsorbent was then added for a further 2 h followed by centrifugation (1700 *g*, 10 min, 4 °C) to remove antibody-bound receptor. Supernatants were assayed for binding activity as described above.

DTT treatment

Solubilized placental membranes (0.2–0.4 mg of protein/ml in 0.075 M-Tris/HCl, pH 7.3/0.03 M-NaCl) were incubated with DTT (2–10 mM) for 20 min at room temperature. The reaction was stopped by the addition of a 2.5-fold higher concentration of NEM (15 min at room temperature). In control incubations, DTT and NEM were premixed before addition. Samples were then assayed in the co-precipitation assay as described above.

Gel-filtration chromatography

Solubilized placental membranes (approx. 3 mg of protein/ml) were incubated with or without 5 mM-DTT as described above. Samples (1 ml) were then applied to a Sephacryl S300 HR gel filtration column (1.6 cm × 60 cm) equilibrated with 30 mM-Hepes, pH 7.6/150 mM-NaCl/0.02% NaN_3/0.05% Triton X-100, and 40 ml was voided before collection of 0.6 ml fractions at a flow rate of 10 ml/h. Aliquots (50 μl) of fractions were removed for determination of total and immunoreactive (antibody 83-14) receptors as described for the co-precipitation assay.

RESULTS

Antibody inhibition of IGF-I binding

As an initial test of specificity we compared the effects of selected antibodies on binding of ^{125}I-IGF-I and ^{125}I-insulin in different human cell types. Antibodies 47-9 and 25-49, which recognize distinct epitopes on the insulin receptor α-subunit [15], both inhibited ^{125}I-IGF-I binding, by approx. 30% in placental membranes, 40% in Hep G2 hepatoma cells, and 60–70% in IM-9 lymphocytes (Table 1). The same concentration of antibody inhibited binding of ^{125}I-insulin by approx. 90% in all cases, as previously reported [15,16]. In contrast, α-IR-3, a monoclonal antibody specific for IGF-I receptors [19], inhibited ^{125}I-IGF-I binding by 80–90% and

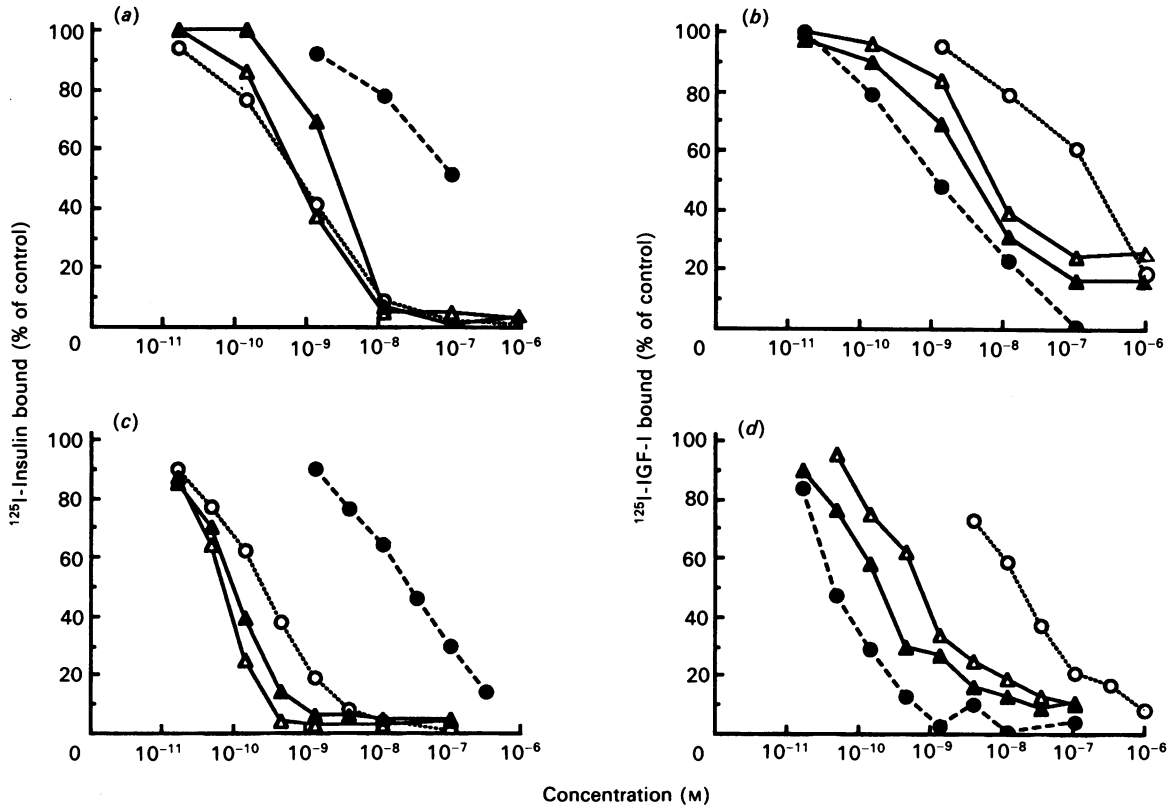

Fig. 1. Inhibition of ^{125}I-hormone binding to IM-9 receptors

Binding of labelled insulin (*a*,*c*) and IGF-I (*b*,*d*) to cells (*a*,*b*) or solubilized membranes (*c*,*d*) was measured in the presence of the indicated concentrations of unlabelled insulin (○), IGF-I (●), 47-9 (△) or 25-49 (▲). 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. Irrelevant monoclonal antibodies had no effect at concentrations up to 10^{-7} M and gave only slight inhibition (< 10%) at 10^{-6} M.

Table 1. Effect of antibodies on IGF-I binding

Binding assays were carried out as described in the Experimental section with and without preincubation with 10^{-8} M-insulin (Ins), IGF-I, 47-9 and 25-49. The exact concentrations of α-IR-3 and MC51 were unknown but were probably of similar magnitude. Data points are the means of two separate experiments (IM-9 cells and placental membranes) or of duplicate determinations within a single experiment (Hep G2 cells). nd, Not determined.

	% Inhibition of specific binding of labelled hormone to:					
	IM-9 cells		Hep G2 cells		Placental membranes	
Unlabelled ligand	IGF-I	Ins	IGF-I	Ins	IGF-I	Ins
Insulin	21	91	12	90	15	92
IGF-I	77	22	98	16	99	25
47-9	61	95	37	88	29	92
25-49	69	93	38	87	28	87
α-IR-3	83	2	96	nd	82	10
MC51	nd	nd	nd	nd	11	81

^{125}I-insulin binding by less than 10%. Antibody MC51, described as relatively specific for insulin receptors [18], inhibited ^{125}I-IGF-I binding to placental membranes by only 11% at the one concentration tested, but was also less effective than other antibodies at inhibiting insulin binding.

To test whether the different effects of antibodies on binding of IGF-I and insulin reflected differences in maximum inhibition or in antibody concentration dependence, binding was examined in more detail in IM-9 cells (Fig. 1) and placenta (Fig. 2), using solubilized receptors as well as intact cells or membranes. In all cases, antibodies 47-9 and 25-49 failed to inhibit IGF-I binding to the same extent as insulin binding, although maximum effects, which were the same for both antibodies, were clearly obtained within the concentration range used. The antibody-inhibitable fraction of IGF-I binding was greater for IM-9 cells (approx. 80%) than for placental membranes (approx. 40%), and also greater for solubilized receptors than for the corresponding cells or membranes (approx. 90 and 70% for IM-9 cells and placenta respectively). The concentrations of antibody 25-49 producing half-maximal effects on IGF-I and insulin binding were very similar for a given receptor preparation. In contrast, the concentration of antibody 47-9 required for a half-maximal effect on IGF-I

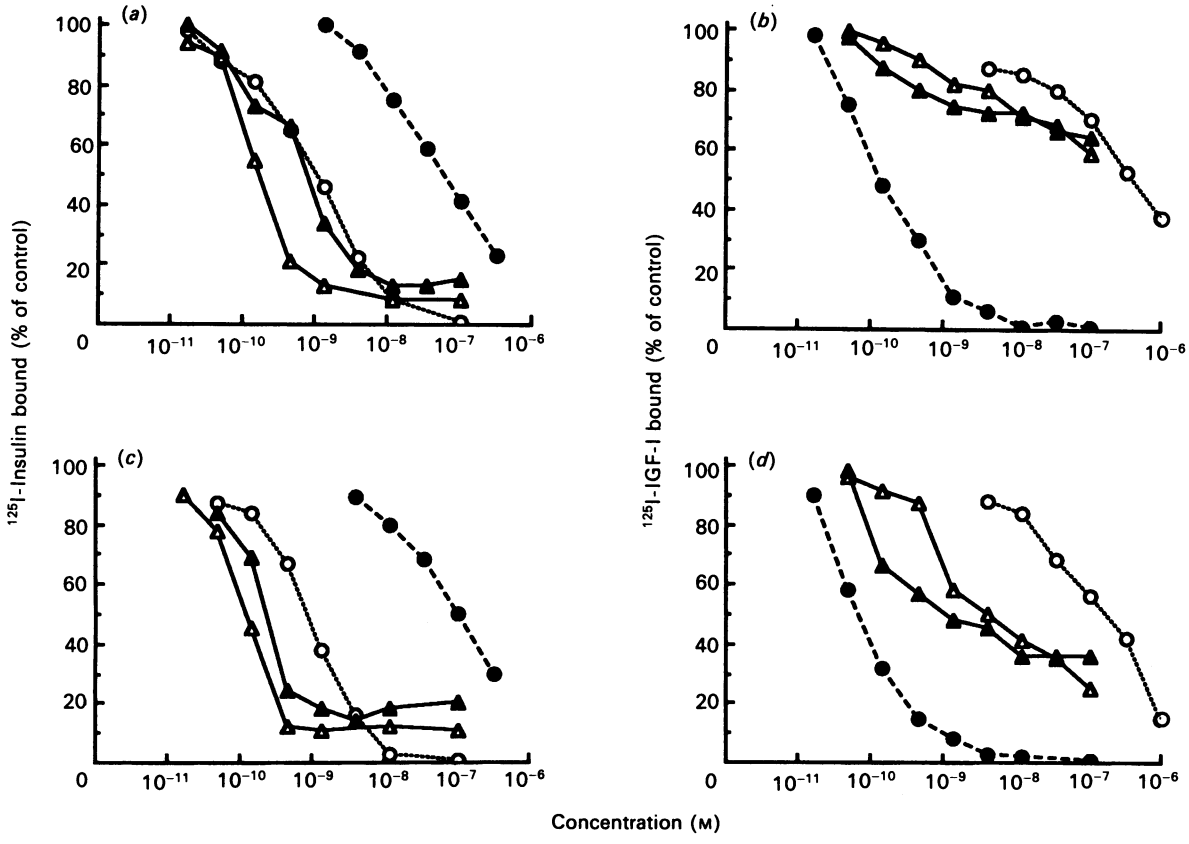

Fig. 2. Inhibition of ^{125}I-hormone binding to placental receptors

Binding of labelled insulin (*a*,*c*) and IGF-I (*b*,*d*) to particulate membranes (*a*,*b*) or solubilized membranes (*c*,*d*) was measured in the presence of the indicated concentrations of unlabelled insulin (○), IGF-I (●), 47-9 (△) or 25-49 (▲). 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. Irrelevant monoclonal antibodies had no effect at concentrations up to 10^{-7} M and gave only slight inhibition (< 10%) at 10^{-6} M.

binding was consistently 5–10-fold higher than that for inhibition of insulin binding in the same preparation. Thus 47-9 was more potent than 25-49 for inhibition of insulin binding, but less potent for inhibition of IGF-I binding. Both antibodies displayed an affinity for insulin receptors similar to (at 15 °C) or slightly higher than (at 4 °C) that of insulin itself.

It was confirmed that binding of labelled IGF-I and insulin displayed properties appropriate for each specific receptor [1]. Thus ^{125}I-IGF-I binding was half-maximal in the presence of 1 nM-IGF-I (intact cells at 15 °C) or 0.05–0.2 nM-IGF-I (membranes or solubilized receptors at 4 °C), while 200–2000-fold higher concentrations of insulin were required to achieve similar inhibition (Figs. 1 and 2). The converse pattern was seen for ^{125}I-insulin binding, in that inhibition by insulin was 60–100-fold more potent than by IGF-I. There was no evidence that a significant fraction of ^{125}I-IGF-I binding was inhibited by low concentrations of insulin. A detailed analysis of the relative numbers of insulin and IGF-I receptors in each of the preparations was not performed. Placental membranes (intact or solubilized) bound 2–3 times as much insulin as IGF-I at tracer concentrations of ligand. Scatchard analysis of binding data suggested however that the number of high-affinity insulin-binding sites (K_d 0.4–0.9 nM) was approx. 7–10 times greater than the number of IGF-I binding sites (K_d 0.1–0.2 nM). Given the difference in binding affinities, the relative binding of tracers overestimates the level of IGF-I receptors compared with insulin receptors, at least in placenta. IM-9 cells under various conditions specifically bound 7–30 times more insulin than IGF-I, and Hep G2 cells bound approx. 2-fold more insulin that IGF-I, at tracer concentrations of ligands. Thus in all cases it is likely that insulin receptors were in considerable excess over IGF-I receptors.

It appeared from these experiments that IGF-I receptors are immunologically heterogeneous, such that one subfraction contains epitopes in common with or closely related to those on insulin receptors, while another subfraction lacks these epitopes. This immunological heterogeneity was not reflected in any gross differences between the two subfractions in affinity for either IGF-I or insulin.

Reaction of antibodies with receptor–IGF-I complexes

To assess whether other insulin receptor epitopes were present on IGF-I receptors, we tested the ability of antibodies to precipitate receptor–^{125}I-hormone complexes. Results for four antibodies to different epitopes, tested with solubilized placental receptor, are shown in Fig. 3. Antibodies 83-14 and 83-7 (reacting

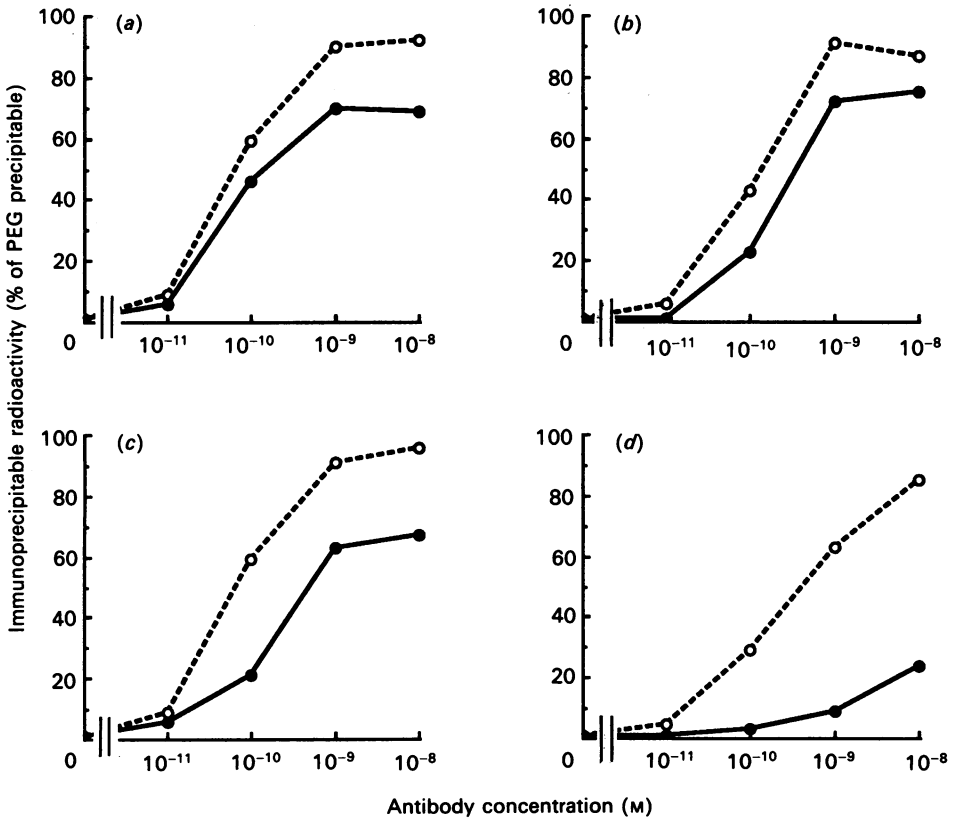

Fig. 3. Reaction of antibodies with receptor–hormone complexes

Solubilized placental membranes were incubated with ^{125}I-IGF-I (●) or ^{125}I-insulin (○) before precipitation with varying concentrations of antibody 83-14 (*a*), 83-7 (*b*), 18-44 (*c*) and 18-34 (*d*) according to the co-precipitation protocol. Data points are the means of duplicate incubations within separate representative experiments. Results are expressed as a percentage of the total (PEG-precipitable) receptor-bound radioactivity: (*a*) 3100 c.p.m. (IGF-I), 5400 c.p.m. (insulin); (*b*) 2400 c.p.m. (IGF-I), 5540 c.p.m. (insulin); (*c*) 3460 c.p.m. (IGF-I), 5270 c.p.m. (insulin); (*d*) 2100 c.p.m. (IGF-I), 5400 c.p.m. (insulin). Irrelevant monoclonal antibodies did not precipitate either receptor–hormone complex.

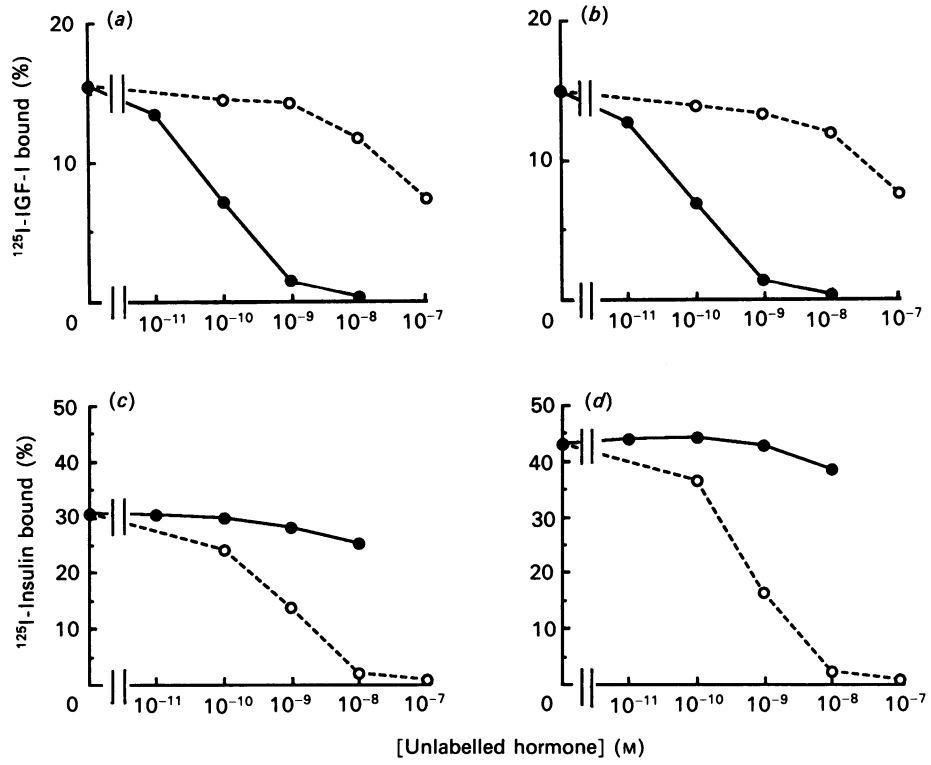

Fig. 4. Specificity of immunoprecipitable receptors

Solubilized placental membranes were incubated with ^{125}I-IGF-I (*a*,*b*) or ^{125}I-insulin (*c*,*d*) together with unlabelled IGF-I (●) or insulin (○) before precipitation with antibody 83-14 (*a*,*c*) or 18-44 (*b*,*d*) according to the co-precipitation protocol. Data points are the means of duplicate incubations. Radioactivity specifically precipitated is expressed as a percentage of the total added. Total specific receptor-bound radioactivity (PEG precipitable) in the absence of unlabelled hormone was: (*a*) 22.6%, (*b*) 23.6%, (*c*) 33.1%, (*d*) 48.1%.

with α-subunit) and 18-44 (reacting with β-subunit) maximally precipitated approx. 70% of receptor-bound ^{125}I-IGF-I, but more than 90% of receptor-bound ^{125}I-insulin. Maximal precipitation was not achieved with the lower affinity antibody 18-34, but nevertheless there was significant reaction of this antibody with IGF-I receptors. The affinity of antibodies 83-14 and 83-7 for IGF-I receptors was similar to that for insulin receptors, as judged by the concentrations for half-maximal precipitation. Antibody 18-44 had a slightly lower affinity for IGF-I receptors than for insulin receptors, and 18-34 reacted relatively poorly with IGF-I receptors. Comparable results were obtained with solubilized receptors from IM-9 cells, except that the immunoprecipitable fraction of IGF-I receptors was greater than for placenta (approx. 85%; results not shown).

The magnitude of immunoprecipitable IGF-I binding compared with insulin binding made it very unlikely that this could be accounted for by binding of IGF-I to insulin receptors. However, to investigate this possibility further we measured the effect of addition of unlabelled insulin and IGF-I on the immunoprecipitation (Fig. 4). It was confirmed that the immunoprecipitable ^{125}I-IGF-I binding was displaced only by very high concentrations of insulin compared with IGF-I, as expected for classical IGF-I receptors. It was concluded that antibodies which do not inhibit hormone binding, as well as binding inhibitory antibodies, react with a subfraction of IGF-I receptors.

Immunodepletion of IGF-I receptors

The reactivity of both binding inhibitory and non-inhibitory antibodies was further assessed by immunodepletion studies (Table 2). Solubilized placental receptors were preincubated with antibodies and the immune complexes were removed by using an immobilized second antibody. The binding capacity for IGF-I and insulin

Table 2. Immunodepletion of IGF-I receptors

Immunodepletion of receptors and binding assays were carried out as described in the Experimental section using 10^{-8} M antibody. Identical results were obtained with 10^{-9} M antibody, indicating that maximal depletion had been obtained. Data points are the means of duplicate incubations. Irrelevant monoclonal antibodies did not deplete either receptor.

	Depletion of receptor (%)	
Antibody	^{125}I-Insulin	^{125}I-IGF-I
47-9	96	68
25-49	99	74
83-14	99	70
83-7	99	67
18-44	91	67
α-IR-3	2	88

was measured before and after the immunodepletion. By this method all of the anti-insulin receptor antibodies removed the same fraction (approx. 70%) of IGF-I receptors but almost all of the insulin receptors. In contrast, antibody α-IR-3 removed 88% of IGF-I receptors and a negligible quantity of insulin receptors. Thus in the solubilized placental preparation the fraction of IGF-I receptors reacting with anti-(insulin receptor) antibodies was the same for all antibodies, whether assessed by inhibition of IGF-I binding, co-precipitation of receptor–IGF-I complexes or immunodepletion. These results again indicated that a subfraction of IGF-I receptors possessed all the epitopes of the insulin receptor while the remaining subfraction lacked these epitopes. However, both subfractions reacted with antibody α-IR-3.

Effect of DTT on immunoreactivity of IGF-I receptors

It has previously been reported that the reactivity of placental IGF-I receptors with a polyclonal human autoantibody B2 was lost following treatment of receptors with DTT, and that this was accompanied by an apparent increase of binding affinity [29]. We therefore examined the effects of DTT treatment on receptor reactivity with monoclonal antibodies and on ligand binding (Fig. 5). After incubation with 10 mM-DTT for 20 min at pH 7.3, placental IGF-I receptors were completely unreactive with antibodies 83-14, 18-44 and (results not shown) 83-7, although binding of ^{125}I-IGF-I was slightly increased. Under the same conditions, immunoreactivity of insulin receptors was not affected but total insulin binding decreased. In control experiments it was shown that the simultaneous addition of DTT and NEM had no effect on the immunoreactivity or ligand-binding properties of either receptor (results not shown). As previously observed [15], antibody 18-44 increased binding of insulin to solubilized receptors, an effect which was lost after DTT treatment. However 18-44 did not show any comparable stimulation of IGF-I binding (Fig. 5).

In separate experiments it was found that the ability of antibodies 47-9 and 25-49 to inhibit binding of ^{125}I-IGF-I to solubilized placental receptors was also lost following DTT treatment, although binding of ^{125}I-insulin was still inhibited (results not shown).

The complete loss of reactivity of IGF-I receptors with anti-(insulin receptor) antibodies, under conditions in which binding of IGF-I was maintained and immunoreactivity of occupied insulin receptors was unaffected, strongly suggested that the antibodies were not reacting directly with IGF-I receptor polypeptide. The possibility that the immunoreactivity of IGF-I receptors reflected a

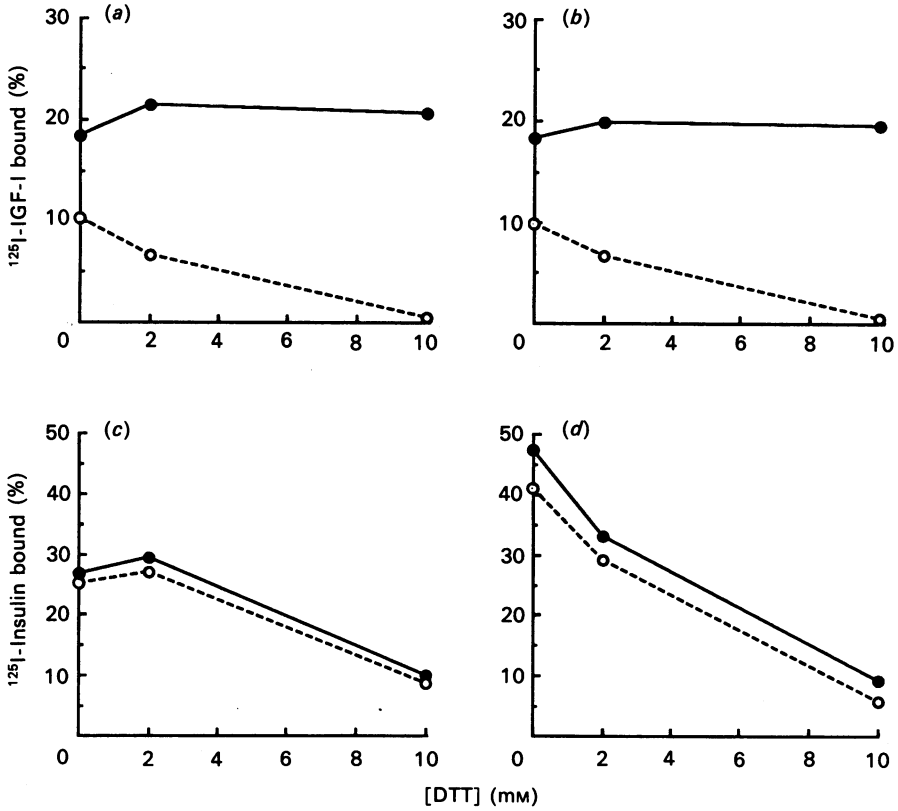

Fig. 5. Immunoreactivity of DTT-treated receptors

Solubilized placental membranes treated with DTT as described in the Experimental section were incubated with ^{125}I-IGF-I (*a*,*b*) or ^{125}I-insulin (*c*,*d*) before addition of antibody 83-14 (*a*,*c*) or 18-44 (*b*,*d*) and precipitation with sheep anti-(mouse IgG) immunoadsorbent (○) or PEG (●) to determine immunoreactive and total binding respectively, according to the co-precipitation protocol. Data points are the means of duplicate incubations within a representative experiment. Radioactivity specifically precipitated is expressed as a percentage of the total added. Total specific receptor-bound radioactivity (PEG-precipitable) in the absence of antibody was 18% (^{125}I-IGF-I) and 28% (^{125}I-insulin).

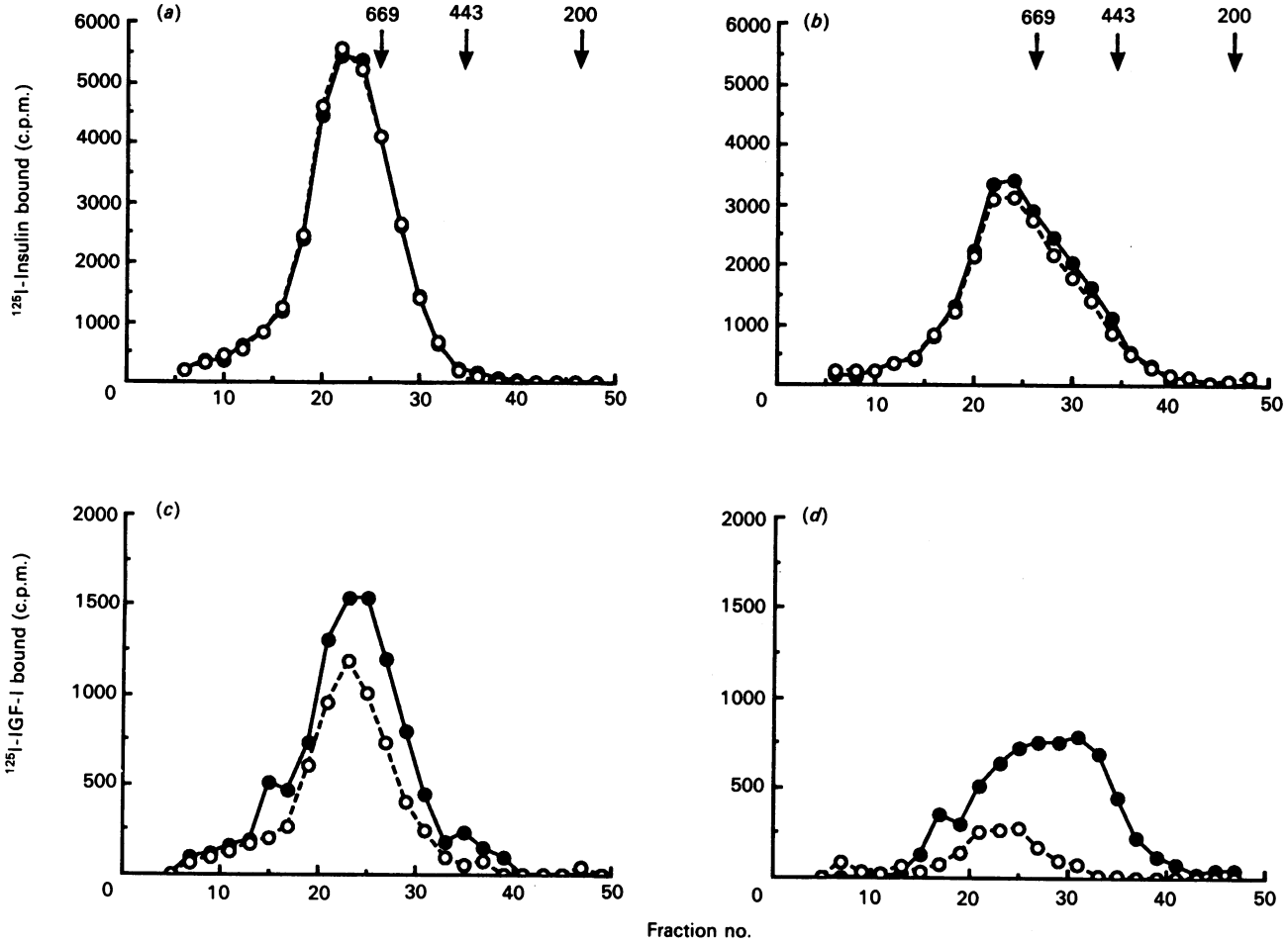

Fig. 6. Gel filtration of DTT-treated receptors

Solubilized placental membranes were fractionated on a Sephacryl S300 HR column before (*a,c*) or after (*b,d*) DTT treatment as described in the Experimental section. Aliquots of fractions were incubated with ^{125}I-insulin (*a,b*) or ^{125}I-IGF-I (*c,d*) before addition of antibody 83-14 and precipitation with sheep anti-(mouse IgG) immunoadsorbent (○) or PEG (●) to determine immunoreactive and total binding respectively. The immunoprecipitable radioactivity of samples before gel filtration was: (*a*) 100%, (*b*) 90%, (*c*) 68%, (*d*) 26% of the total receptor-bound radioactivity. The column was calibrated with thyroglobulin (669 kDa), apoferritin (443 kDa) and β-amylase (200 kDa) which are indicated on the Figure.

DTT-sensitive association with insulin receptors was investigated by gel filtration of solubilized placental membranes before and after DTT treatment (Fig. 6). Preparations which had not been DTT-treated displayed a single symmetrical peak of IGF-I binding which co-migrated precisely with the peak of insulin binding (apparent molecular mass 800–900 kDa). Antibody 83-14 precipitated approx. 70% of the IGF-I receptors and more than 95% of the insulin receptors in all fractions. Following a DTT treatment calculated to abolish most but not all of the immunoreactivity of IGF-I receptors, the gel-filtration profiles for the binding of IGF-I and insulin were different. The peak of IGF-I binding broadened with the appearance of a peak of binding activity of a lower molecular size (400–500 kDa) which was not immunoreactive. The residual immunoreactive material ran in the same position as receptor before DTT treatment, as did a significant amount of non-immunoreactive receptor. The peak of insulin binding remained in the same position after DTT treatment but the overall distribution now showed significant skewing towards a lower molecular size, although all fractions remained fully immunoreactive. This experiment was repeated using Ultrogel AcA 22 rather than Sephacryl S300 HR as gel-filtration medium with qualitatively identical results.

These observations suggest that a subfraction of IGF-I receptors exists which is reactive with anti-(insulin receptor) antibodies by virtue of an association with insulin receptors. This hybrid species is of the same molecular size as classical $(\alpha\beta)_2$ insulin and IGF-I receptors, but unlike these molecules dissociates into its component $\alpha\beta$ halves following reduction with DTT under the conditions used. The reduction therefore generates both insulin-binding and IGF-I-binding species of lower molecular mass, of which only the former is now immunoreactive. The low molecular mass form appears as a much greater fraction of IGF-I receptors than of insulin receptors because of the difference in overall receptor concentrations.

DISCUSSION

Several lines of evidence (reviewed in [9]) have suggested that both insulin receptors and IGF-I receptors

are heterogeneous with respect to their ligand-binding properties and immunoreactivity. We observed that a subfraction of IGF-I receptors reacted with each of six distinct monoclonal anti-(insulin receptor) antibodies, while the remaining receptor population was unreactive with all of these antibodies. The size of the immunoreactive subfraction as a proportion of total IGF-I binding was variable for different receptor preparations, being greatest in IM-9 cells and less in Hep G2 cells and placenta. The immunoreactive fraction in a given receptor preparation was similar whether assessed in binding inhibition (Figs. 1 and 2), co-precipitation (Fig. 3) or immunodepletion (Table 2) experiments. Under the same conditions, insulin receptors from all sources were fully immunoreactive. In contrast, the monoclonal antibody α-IR-3 reacted with essentially all IGF-I receptors but not with insulin receptors in all tissues studied, as reported previously [19]. The partial immunoreactivity of IGF-I receptors with insulin receptor antibodies was first described for a human polyclonal auto-antiserum B2 [30], and subsequently with a single monoclonal antibody 5D9 [31]. Inspection of data for other 'cross-reacting' auto-antisera suggests that although these reacted with the majority of IGF-I receptors in IM-9 cells, only a subfraction of receptors was reactive in human placenta [32]. The extent of cross-reaction of a rabbit anti-(insulin receptor) antiserum A410 is unclear [32,33]. Although antibodies have been described which clearly do cross-react fully with both insulin receptors and IGF-I receptors, these are directed against the highly conserved intracellular domain [34].

Both the immunoreactive and non-immunoreactive IGF-I binding sites displayed the properties expected of 'classical' IGF-I receptors in terms of their relative affinities for insulin and IGF-I (Figs. 1, 2 and 4). Thus the immunoreactive subfraction was not attributable to binding of IGF-I to receptors for insulin or IGF-II [1] and was distinct from the previously described 'atypical' insulin receptors [11,35]. Partial immunoreactivity of IGF-I receptors with insulin receptor antibodies has been ascribed to heterogeneity in glycosylation, disulphide formation or redox state [29,31]. Structural heterogeneity might also arise as a result of alternative splicing of a primary RNA transcript to produce different receptor sequences from a single gene as apparently occurs for the insulin receptor [36].

Several observations indicated that interaction of antibodies with the IGF-I receptor was not directly with the receptor peptide, but rather was indirect through an association with insulin receptors. The human IGF-I receptor expressed in rodent cells by transfection with cloned cDNA is not reactive with the antibody 5D9 [37] nor with any of the antibodies used in the present study (M. A. Soos & K. Siddle, unpublished work). It appears extremely unlikely, given only 52% overall sequence identity between the extracellular domains [3], that all insulin receptor epitopes would be conserved in any variant form of the IGF-I receptor. Strikingly, the immunoreactivity of occupied insulin receptors was unaffected and total IGF-I binding was if anything increased immunoreactivity of insulin receptors was unaffected and total IGF-I binding was if anything increased (Fig. 5). A similar observation was made previously with the auto-antiserum B2, and in this case it was further shown that the B2-reactive IGF-I receptors were of somewhat lower affinity than the non-immunoreactive fraction, and that DTT increased the binding affinity concomitant with the loss of immunoreactivity [29]. In the present experiments DTT treatment resulted in the conversion of the immunoreactive receptor subfraction to a lower molecular mass non-immunoreactive form (Fig. 6).

We propose that a hybrid or chimaeric receptor, having one $\alpha\beta$ half of the insulin receptor and the other of the IGF-I receptor in a heteromeric $\beta\alpha\alpha'\beta'$ structure, is responsible for the immunoreactive IGF-I binding sites, rather than a higher order association of homomeric insulin and IGF-I receptors. The fact that several antibodies inhibit IGF-I binding is most easily explained by assuming intramolecular steric hindrance within hybrid receptors. Moreover, the DTT treatment which abolishes IGF-I receptor immunoreactivity has previously been shown to reduce class I (α–α) disulphide bonds [38,39], although reduction at the neutral pH used here does not lead to dissociation of $\alpha\beta$ halves of the insulin receptor under non-denaturing conditions [40]. However, it appears that the $\alpha\beta$ halves of hybrid insulin/IGF-I receptors do dissociate following reduction producing roughly equivalent amounts of insulin and IGF-I binding activity of approx. half the original molecular mass (Fig. 6). We observed no change after DTT treatment in the size of the majority of insulin receptors, nor of the presumed homomeric IGF-I receptors which were initially unreactive with antibodies (Fig. 6). The apparent molecular masses of the homomeric receptors and $\alpha\beta$ halves (800–900 and 400–500 kDa respectively) were somewhat higher than those expected for these structures (600 and 300 kDa respectively, assuming that each transmembrane domain is associated with a Triton micelle of 70 kDa). Such anomalous behaviour on gel filtration has been noted previously by other workers [41,42].

The association between insulin and IGF-I receptors pre-exists in intact cells as indicated by inhibitory effects of antibodies on binding of IGF-I to Hep G2 and IM-9 cells. The size of the immunoreactive fraction in placental membranes increased somewhat after solubilization (Fig. 2) possibly due to differential recovery of receptors. The possibility cannot be completely ruled out that a certain amount of subunit interchange occurs on solubilization, although the addition of NEM to block any association reaction dependent on disulphide exchange did not influence the immunoreactivity of IGF-I receptors (results not shown). Moreover, when two transfected rodent cell lines separately expressing human IGF-I receptors and insulin receptors were solubilized together, the IGF-I receptors remained unreactive with insulin receptor antibodies (M. A. Soos & K. Siddle, unpublished work).

The immunoreactive (hybrid) insulin/IGF-I receptors are generally present in amounts comparable with non-immunoreactive (classical) IGF-I receptors, and in IM-9 cells appear to be the major form. Although the presence of hybrid receptors cannot be presumed in all tissues, the question arises as to why they have not been detected previously. Some insulin receptor antibodies have been described which apparently do not react with IGF-I receptors, including human autoantibodies B9 and B10 [32,43] and some anti-peptide antibodies [44], while monoclonal antibodies MC51 [18], α-IR-1 [19] and in this work 47-9 (Figs. 1 and 2) and 18-34 (Fig. 3) display a lower affinity for IGF-I receptors than insulin receptors.

It may be that these antibodies recognize epitopes which are substantially modified in hybrid receptors compared with classical insulin receptors by conformational differences or dependence on peptide elements from more than one subunit. Alternatively, affinity differences might reflect in part the potential for bivalent versus monovalent binding of antibody. There are also obviously tissue differences in the abundance of hybrid receptors. The cross-reaction of 47-9 with a subfraction of IGF-I receptors is not inconsistent with its use for selective blockade of insulin receptors in studies designed to investigate the biological role of IGF-I receptors [17]. It is likely that in this and similar cases [27] there exists a sufficient number of homomeric, classical IGF-I receptors to permit a maximum response to IGF-I even if hybrid receptors are present which are blocked by antibodies. Purified preparations of IGF-I receptors have been reported to be very low in insulin-binding activity [45,46], indicating that hybrid receptors if present initially were not co-purified, or did not significantly bind insulin at tracer concentrations. Interestingly, two of only three monoclonal antibodies obtained after immunizing mice with purified placental insulin receptors were in fact specific for IGF-I receptors [19]. It would not be expected that an antibody such as α-IR-3 would easily detect hybrid receptors as an apparent cross-reaction with a subpopulation of insulin receptors, as in most of the tissues commonly studied insulin binding is in excess over IGF-I binding so that hybrid receptors would contribute a much smaller proportion of insulin binding than of IGF-I binding. We estimated that the solubilized placental receptor preparations contained up to 10 times as many high-affinity binding sites for insulin as for IGF-I. Even so, we might have expected to detect the presence of a significant (5–10%) fraction of hybrid insulin receptors reacting with α-IR-3 in immunodepletion studies (Table 2). However, it cannot be assumed that hybrid and homomeric receptors would have the same binding affinities for insulin, so that relative tracer binding might not reflect relative numbers of different forms of receptor. Indeed it has previously been shown that homomeric ($\alpha_2\beta_2$) insulin receptors possess only one high-affinity binding site for insulin, and that the binding affinity is less in dissociated ($\alpha\beta$) receptor halves [47–49]. Thus the affinity of insulin is clearly dependent on the associations between α-subunits and on their occupancy, although it is not clear that the same is true for IGF-1 [39]. The possibility cannot at this stage be ruled out that, compared with their corresponding homomeric receptors, hybrid receptors bind IGF-I quite well but insulin only relatively poorly.

The biological role of hybrid receptors is unclear but certainly merits investigation. It is not certain at present whether insulin and IGF-I receptors have intrinsically different activities when expressed within the same cell type [37], so it is difficult to speculate on whether hybrids would be functional or whether they would have a similar or different activity to homomeric receptors. The reported affinity differences [29] between what we now interpret as hybrid and homomeric receptors might be significant in terms of cellular sensitivity to IGF-I and insulin. Evidence that hybrid receptors can undergo conformational changes typical of classical insulin receptors is provided by the observation that antibodies 47-9 and 25-49 accelerate the dissociation of bound IGF-I as they do for insulin (M. A. Soos & K. Siddle, unpublished work), a phenomenon analogous to the negatively co-operative effect of insulin itself [10,50]. It may well be that hybrid receptors are functional in metabolic regulation, at least in response to IGF-I. It has been noted by several workers that IGF-I-induced autophosphorylation of receptors in IM-9 and Hep G2 cells appears as a doublet of presumed β-subunits, one of which co-migrates with insulin receptor β-subunit, following SDS/polyacrylamide-gel electrophoresis [5]. This observation has recently been interpreted as reflecting the existence and activation of hybrid receptors, based on peptide mapping and on immunoprecipitability of the two phosphorylated bands before and after DTT treatment [51].

The factors which govern the combination of insulin receptor and IGF-I receptor $\alpha\beta$ units to form homomeric or hybrid receptors are unknown, and it is possible that in cells expressing both receptor types this combination might be random, non-random or susceptible to regulation. Significantly, the proportion of hybrid receptors was greater in IM-9 cells, where total insulin binding was in considerable excess over IGF-I binding, than in Hep G2 cells, where the amounts of insulin and IGF-I binding were more comparable. It has been proposed that inter-species hybrid insulin receptors may occur when the human insulin receptor gene is expressed in rodent cells [6], although in one case the proportion of receptors occurring as such hybrids was shown to be quite small and there was no evidence that overexpression of mutant insulin receptors interfered with the functioning of endogenous insulin or IGF-I receptors [52]. Thus the formation of inter-species or inter-receptor hybrids may not occur readily under all circumstances. The availability of cDNA clones for both insulin and IGF-I receptors will permit co-transfection studies to confirm the existence of hybrid receptors, to investigate factors controlling their assembly and to elucidate their biological activity.

We are very grateful to Dr. Steven Jacobs and Dr. Richard Roth for the gift of antibodies, to Dr. A. Ullrich and Dr. R. Lammers for the gift of cells transfected with human IGF-I receptor cDNA, and to Dr. K. Scheibli for the gift of IGF-I. We also thank the Wellcome Trust, the Medical Research Council and Serono Diagnostics Ltd. for their financial support, and Jill Stigter for her technical assistance. K. S. is a Wellcome Trust Senior Lecturer.

REFERENCES

1. Rechler, M. M. & Nissley, S. P. (1985) Annu. Rev. Physiol. **47**, 425–442
2. Duronio, V. & Jacobs, S. (1988) in Insulin Receptors, Part B: Clinical Assessment, Biological Responses, and Comparison to the IGF-I Receptor (Kahn, C. R. & Harrison, L. C., eds.), pp. 3–18. Alan R. Liss Inc., New York
3. Ullrich, A., Gray, A., Tam, A. W., Yang-Feng, T., Tsubokawa, M., Collins, C., Henzel, W., Le Bon, T., Kathuria, S., Chen, E., Jacobs, S., Francke, U., Ramachandran, J. & Fujita-Yamaguchi, Y. (1986) EMBO J. **5**, 2503–2512
4. Morgan, D. O., Edman, J. C., Standring, D. N., Fried, V. A., Smith, M. C., Roth, R. A. & Rutter, W. J. (1987) Nature (London) **329**, 301–307
5. Jacobs, S. & Cuatrecasas, P. (1986) J. Biol. Chem. **261**, 934–939
6. Ebina, Y., Araki, E., Taira, M., Shimada, F., Mori, M., Craik, C. S., Siddle, K., Pierce, S. B., Roth, R. A. & Rutter, W. J. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 704–708

7. Chou, C. K., Dull, T. J., Russell, D. S., Gherzi, R., Lebwohl, D., Ullrich, A. & Rosen, O. M. (1987) J. Biol. Chem. **262**, 1842–1847
8. Ebina, Y., Ellis, L., Jarnagin, K., Edery, M., Graf, L., Clauser, E., Ou, J., Masiarz, F., Kan, Y. W., Goldfine, I. D., Roth, R. A. & Rutter, W. J. (1985). Cell **40**, 747–758.
9. Jonas, H. A. (1988) in Insulin Receptors, Part B: Clinical Assessment, Biological Responses, and Comparison to the IGF-I Receptor (Kahn, C. R. & Harrison, L. C., eds.), pp. 19–36, Alan R. Liss Inc., New York
10. Gammeltoft, S. (1984) Physiol. Rev. **64**, 1321–1378.
11. Jonas, H. A., Cox, A. J. & Harrison, L. C. (1989) Biochem. J. **257**, 101–107.
12. Kiess, W., Blickenstaff, G. D., Sklar, M. M., Thomas, C. L., Nissley, S. P. & Sahagian, G. G. (1988) J. Biol. Chem. **263**, 9339–9344.
13. Froesch, E. R., Schmid, C., Schwander, J. & Zapf, J. (1985) Annu. Rev. Physiol. **47**, 443–467.
14. Kahn, C. R. (1985) Annu. Rev. Med. **36**, 429–451.
15. Soos, M. A., Siddle, K., Baron, M. D., Heward, J. M., Luzio, J. P., Bellatin, J. & Lennox, E. S. (1986) Biochem. J. **235**, 199–208.
16. Taylor, R., Soos, M. A., Wells, A., Argyraki, M. & Siddle, K. (1987) Biochem. J. **242**, 123–129
17. Bar, R. S., Siddle, K., Dolash, S., Boes, M. & Dake, B. (1988) Metabolism **37**, 714–720
18. Roth, R. A., Maddux, B., Wong, K. Y., Styne, D. M., Van Vliet, G., Humbel, R. E. & Goldfine, I. D. (1983) Endocrinology (Baltimore) **112**, 1865–1867
19. Kull, F. C., Jacobs, S., Su, Y-F., Svoboda, M. E., Van Wyk, J. J. & Cuatrecasas, P. (1983) J. Biol. Chem. **258**, 6561–6566
20. Hales, C. N. & Woodhead, J. S. (1980) Methods Enzymol. **70**, 334–355
21. Linde, S., Hansen, B., Sonne, O., Holst, J. J. & Gliemann, J. (1981) Diabetes **30**, 1–8
22. Roth, J. (1975) Methods Enzymol. **37**, 223–233
23. Stanker, L. H., Vanderlaan, M. & Juarez-Salinas, H. (1985) J. Immunol. Methods **76**, 157–169
24. Fujita-Yamaguchi, Y., Choi, S., Sakamoto, Y. & Itakura, K. (1983) J. Biol. Chem. **258**, 5045–5049
25. Lang, U., Kahn, C. R. & Harrison, L. C. (1980) Biochemistry **19**, 64–70
26. Roth, R. A., Cassell, D. J., Wong, K. W., Maddux, B. A. & Goldfine, I. D. (1982) Proc. Natl. Acad. Sci. U.S.A. **79**, 7312–7316
27. Verspohl, E. J., Roth, R. A., Vigneri, R. & Goldfine, I. D. (1984) J. Clin. Invest. **74**, 1436–1443
28. Baron, M. D. & Sonksen, P. H. (1982) Biosci. Rep. **2**, 785–793
29. Jonas, H. A. & Harrison, L. C. (1986) Biochem. J. **236**, 417–423
30. Jonas, H. A. & Harrison, L. C. (1985) J. Biol. Chem. **260**, 2288–2294
31. Morgan, D. O. & Roth, R. A. (1986) Biochem. Biophys. Res. Commun. **138**, 1341–1347
32. Kasuga, M., Sasaki, N., Kahn, C. R., Nissley, S. P. & Rechler, M. M. (1983) J. Clin. Invest. **72**,1459–1469
33. Jacobs, S., Cook, S., Svoboda, M. E. & Van Wyk, J. J. (1986) Endocrinology (Baltimore) **118**, 223–226
34. Morgan, D. O. & Roth, R. A. (1986) Biochemistry **25**, 1364–1371
35. Jonas, H. A., Newman, J. D. & Harrison, L. C. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 4124–4128
36. Seino, S. & Bell, G, I. (1989) Biochem. Biophys. Res. Commun. **159**, 312–316
37. Steele-Perkins, G., Turner, J., Edman, J. C., Hari, J., Pierce, S. B., Stover, C., Rutter, W. J. & Roth, R. A. (1988) J. Biol. Chem. **263**, 11486–11492
38. Czech, M. P. (1985) Annu. Rev. Physiol. **47**. 357–381
39. Feltz, S. M., Swanson, M. L., Wemmie, J. A. & Pessin, J. E. (1988) Biochemistry **27**, 3234–3242
40. Sweet, L. J., Morrison, B. D., Wilden, P. A. & Pessin, J. E. (1987) J. Biol. Chem. **262**, 16730–16738
41. Pollet, R. J., Haase, B. A. & Standaert, M. L. (1981) J. Biol. Chem. **256**, 12118–12126
42. Johnson, J. D., Wong, M. L. & Rutter, W. J. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 7516–7520
43. Jonas, H. A., Baxter, R. C. & Harrison, L. C. (1982) Biochem. Biophys. Res. Commun. **109**, 463–470
44. Herrera, R., Petruzzelli, L. M. & Rosen, O. M. (1986) J. Biol. Chem. **261**, 2489–2491.
45. Le Bon, T. R., Jacobs, S., Cuatrecasas, P., Kathuria, S. & Fujita-Yamaguchi, Y. (1986) J. Biol. Chem. **261**, 7685–7689
46. Maly, P. & Luthi, C. (1986) Biochem. Biophys. Res. Commun. **137**, 695–701
47. Pang, D. T. & Shafer, J. A. (1984) J. Biol. Chem. **259**, 8589–8596
48. Boni-Schnetzler, M., Scott, W., Waugh, S. M., DiBella, E. & Pilch, P. F. (1987) J. Biol. Chem. **262**, 8395–8401
49. Sweet, L. J., Morrison, B. & Pessin, J. E. (1987) J. Biol. Chem. **262**, 6939–6942
50. De Meyts, P., Bianco, A. R. & Roth, J. (1975). Biochem. Biophys. Res. Commun. **66**, 1118–1126
51. Moxham, C. P., Duronio, V. & Jacobs, S. (1989) J. Biol. Chem. in the press
52. Maegawa, H., Olefsky, J. M., Thies, S., Boyd, D., Ullrich, A. & McClain, D. A. (1988) J. Biol. Chem. **263**, 12629–12637

Received 14 April 1989/30 May 1989; accepted 7 June 1989