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Receptors for insulin and insulin-like growth factor-I can form hybrid dimers

Characterisation of hybrid receptors in transfected cells

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We have demonstrated the formation of hybrid insulin/insulin-like growth factor-I(IGF-I) receptors in transfected rodent fibroblasts, which overexpress human receptors, by examining reactivity with species- and receptor-specific monoclonal antibodies. In NIH 3T3 and Rat 1 fibroblasts, endogenous IGF-I receptors were unreactive with anti-(human insulin receptor)monoclonal antibodies (47–9, 25–49, 83–14, 83–7, 18–44). However, in transfected cells expressing high levels of insulin receptors, 60–80% of high-affinity IGF-I receptors reacted with these antibodies, as assessed either by inhibition of ligand binding in intact cells or by precipitation of solubilized receptors. Conversely, endogenous insulin receptors in NIH 3T3 cells were unreactive with anti-(IGF-I receptor) antibodies αIR-3 and 16–13. However, approx. 50% of high-affinity insulin receptors reacted with these antibodies in cells expressing high levels of human IGF-I receptors. The hybrid receptors in transfected cells bound insulin or IGF-I with high affinity. However, responses to these ligands were asymmetrical, in that binding of IGF-I inhibited subsequent binding of insulin, but prior binding of insulin did not affect the affinity for IGF-I. The existence of hybrid receptors in normal tissues could have important implications for metabolic regulation by insulin and IGF-I.

INTRODUCTION

Insulin and insulin-like growth factor-I (IGF-I) exert their biological effects by binding to specific plasma membrane receptors which show many similarities of structure and function. Each receptor is synthesized initially as a proreceptor polypeptide which is processed by glycosylation and proteolytic cleavage to give α- and β-subunits, the mature and functional receptors being symmetrical structures of two disulphide-linked (αβ) units (reviewed in Czech, 1985; Rechler & Nissley, 1985; Duronio & Jacobs, 1988; Yarden & Ullrich, 1988). The α-subunit is extracellular and contains the ligand binding site. The transmembrane β-subunit possesses ligand-stimulated tyrosine kinase activity which appears to be an essential component of the signalling pathways mediating hormone action (reviewed in Rosen, 1987; Zick, 1989). The cloning and sequencing of cDNAs coding for the respective proreceptors has confirmed that receptors for insulin and IGF-I are the products of separate genes, with substantial similarity of amino acid sequence (Ullrich *et al.*, 1985,1986).

There has been debate as to the basis of the overlapping but apparently distinct biological effects of insulin and IGF-I (Froesch *et al.*, 1985). These might be a consequence of inherently different signalling capacities of the respective receptors, or of differences in receptor distribution among tissues with different capacities for metabolic response to a given signal. Insulin and IGF-I induce the phosphorylation of common endogenous substrates for the receptor tyrosine protein kinase (Izumi *et al.*, 1987; Kadowaki *et al.*, 1987). No differences in inherent signalling capacity were apparent when human insulin and IGF-I receptors were expressed in Chinese hamster ovary cells following cDNA transfection (Steele-Perkins *et al.*, 1988). However, expression of normal and chimaeric receptors in NIH 3T3 fibroblasts revealed differences in signalling potential of the insulin- and IGF-I-receptor cytoplasmic domains, at least for stimulation of DNA synthesis (Lammers *et al.*, 1989).

It has recently been reported that in human tissues and cell lines expressing both insulin and IGF-I receptors, a proportion of receptors exists as hybrid ($\alpha\beta\alpha'\beta'$) structures (Soos & Siddle, 1989; Moxham *et al.*, 1989). The existence of hybrid receptors could have important functional consequences if such structures are capable of binding and responding to both insulin and IGF-I. However, other work has suggested the existence of two distinct IGF-I receptor polypeptides as a possible basis for signalling by insulin via the IGF-I receptor (Garofalo & Rosen, 1989; Alexandrides & Smith, 1989). The availability of rodent cell lines transfected with human insulin- or IGF-I-receptor cDNA (Whittaker *et al.*, 1987; McClain *et al.*, 1987; Lammers *et al.*, 1989) has allowed us to examine the consequences for receptor assembly when one type of receptor is overexpressed relative to the other. We have demonstrated conclusively the ability of insulin- and IGF-I-receptor subunits to assemble as interspecies hybrids in these cells, by using species- and receptor-specific monoclonal antibodies. We have also been able to

Abbreviations used: IGF-I, insulin-like growth factor-I; WGA, wheat-germ agglutin; PEG, poly(ethylene glycol); DMEM, Dubecco's modified Eagle's medium; PBS, phosphate-buffered saline; k.i.u., kallikrein-inactivating unit; rIR/hIGFR and hIR/rIGFR, complexes between rodent insulin receptor and human IGF-I receptor, and human insulin receptor and rodent IGF-I receptor, respectively.

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examine some of the ligand-binding properties of these hybrid receptors, and to show that both insulin and IGF-I are bound with high affinity.

EXPERIMENTAL

Materials

Bovine insulin (for displacement studies) was from Sigma Chemical Co., Poole, Dorset, U.K., and highly purified desamido-free bovine insulin (for iodination) was a gift from Dr. D. Brandenburg, Deutsches Wollforschungsinstitut, Aachen, Germany. Recombinant human IGF-I was generously provided by Ciba–Geigy, Basle, Switzerland. Proteinase inhibitors, BSA, methotrexate, Sephadex G-50 and wheat-germ-agglutinin–Sepharose (WGA–Sepharose) 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, Watford, Herts., U.K. All tissue culture reagents and Geneticin (G418 sulphate) were from Gibco Ltd., Paisley, Scotland, U.K. Sheep anti-(mouse IgG) antibodies were coupled to aminocellulose to obtain immunoadsorbents as described previously (Soos *et al.*, 1986).

Radioiodinations

Mono-^{125}I-insulin with a specific radioactivity of 100–200 μCi/μg was prepared from highly purified bovine insulin as described by Linde *et al.* (1981). IGF-I was iodinated to a specific activity of 50–150 μCi/μg using a stoichiometric chloramine-T method (Roth, 1975) and was purified by gel filtration on Sephadex G-50 to separate ^{125}I-IGF-I from free [^{125}I]iodide.

Antibodies

Monoclonal antibodies specific for human insulin receptors (Soos *et al.*, 1986) were purified from ascites fluids by precipitation with $(NH_4)_2SO_4$ followed by chromatography on hydroxyapatite (Stanker *et al.*, 1985). Monoclonal antibody 16–13, specific for human IGF-I receptors, was obtained following fusion of NSO myeloma cells with spleen cells from a mouse immunized with mouse fibroblasts overexpressing human IGF-I receptors (IGF-I-R/3T3 cells) using standard techniques (Galfre & Milstein, 1981). Monoclonal antibody αIR-3 (also specific for human IGF-I receptors) was kindly donated by Dr. Steven Jacobs (Wellcome Research Laboratories, Research Triangle Park, NC, U.S.A.).

Cell culture

All the cell lines were grown in medium with 10% (v/v) foetal calf serum, penicillin (100 units/ml) and streptomycin (100 μg/ml). Rat 1 cells (untransfected) (McClain *et al.*, 1987) were grown in Dulbecco's modified Eagle's medium (DMEM). The cell lines NIH 3T3 NEO (mock-transfected), NIH 3T3 HIR3.5 (Whittaker *et al.*, 1987) and IGF-I-R/3T3 (Lammers *et al.*, 1989) were routinely grown in DMEM plus 0.4 mg of Geneticin/ml. HIRc-B cells (McClain *et al.*, 1987) were grown in F12/DMEM (1:1, v/v) plus 500 nM-methotrexate and 0.4 mg of Geneticin/ml. Cultures were maintained in a humidified atmosphere of air/CO_2 (19:1) at 37 °C. For the ^{125}I-hormone binding studies, cells were seeded on to 24-well tissue culture plates at 2×10^4 cells per well. They were cultured for 2 days to approximately half confluence for ^{125}I-insulin binding to NIH 3T3 HIR3.5 and HIRc-B cells and for ^{125}I-IGF-I binding to IGF-I-R/3T3 cells, and for 3 days to confluence for all other binding studies.

Receptor preparations

Receptors from NIH 3T3 NEO, NIH 3T3 HIR3.5 and IGF-I-R/3T3 cell lines were partially purified from approx. 3×10^8 cells. After washing with phosphate-buffered saline (PBS; 150 mM-NaCl/10 mM-sodium phosphate, pH 7.4), cells were removed from flasks by scraping into PBS containing proteinase inhibitors [0.2 mM-phenylmethanesulphonyl fluoride, 0.4 mg of benzamidine/ml, 1 μg of leupeptin/ml, 1 μg of pepstatin/ml, 1 μg of antipain/ml, 200 kallikrein-inactivating units (k.i.u.) of aprotinin/ml and 0.2 mg of bacitracin/ml]. The cell pellet after centrifugation (150 *g*, 7 min) was solubilized for 1 h at 4 °C in 20 ml of 0.05 M-Hepes, pH 7.4, containing the proteinase inhibitors and 1% (v/v) Triton X-100. The supernatant after centrifugation (50000 *g* for 30 min, 4 °C) was diluted 1:1.5 with 0.05 M-Hepes, pH 7.4, containing 0.15 M-NaCl, 0.025 M-$MgCl_2$ and proteinase inhibitors, and mixed for 16–20 h at 4 °C with 1 ml of WGA–Sepharose. The WGA–Sepharose was then washed with 10–20 ml of 0.05 M-Hepes, pH 7.4, containing 0.15 M-NaCl and 0.1% Triton X-100 before elution with 0.5 M-*N*-acetylglucosamine in the same buffer. The single protein peak was pooled for use in assays. Solubilized Rat 1 and HIRc-B receptors were prepared by solubilizing approx. 10^7 cells in 1.5 ml of 0.05 M-Hepes, pH 7.4, containing the proteinase inhibitors and 1% Triton X-100 as described above. This Triton X-100 extract was used without further purification.

^{125}I-Hormone binding assays

Cells were washed twice with PBS before addition of insulin, IGF-I or antibody in 0.25 ml of modified DMEM (containing 25 mM-Hepes, pH 7.8, and 7 mM-sodium bicarbonate) supplemented with 1 mg of BSA/ml and 250 k.i.u. of aprotinin/ml. After 30 min at 4 °C, 0.05 ml of ^{125}I-insulin or ^{125}I-IGF-I (approx. 30000 d.p.m.) was added. After a further 4 h, cells were washed twice with ice-cold PBS and solubilized with 0.03% SDS for determination of radioactivity. Non-specific binding was determined in the presence of 1 μM-insulin or 0.1 μM-IGF-I as appropriate.

Binding to solubilized receptors was performed as described previously (Soos & Siddle, 1989), except that receptors were incubated with ^{125}I-insulin or ^{125}I-IGF-I (approx. 30000 d.p.m. in a total volume of 0.25 ml), together with unlabelled hormone, for 18 h at 4 °C. Receptor-bound radioactivity was determined by precipitation with PEG 6000 (Baron & Sonksen, 1982). Non-specific binding was determined in the presence of 1 μM-insulin or 0.1 μM-IGF-I as appropriate. The concentration of Triton X-100 in all soluble binding assays was maintained at 0.05%.

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

Assays were performed as described in Soos *et al.* (1986) by preincubating solubilized receptors with ^{125}I-insulin or ^{125}I-IGF-I (approx. 30000 d.p.m. in a total volume of 0.1 ml) for 18 h at 4 °C before addition of 0.1 ml of antibody for a further 6 h. To establish the specificity of binding of labelled hormones, unlabelled hormones were included in the first incubation. Antibody-bound radioactivity was determined using a sheep anti-(mouse IgG) adsorbent as previously described (Soos *et al.*, 1986). Total receptor-bound radioactivity was measured by precipitation with PEG 6000 (Baron & Sonksen, 1982).

RESULTS

Relative levels of insulin and IGF-I receptors in cell lines

Estimates of numbers of receptors in the cell lines studied are summarized in Table 1. These values take no account of whether ligand binding is to homomeric or hybrid receptors. The NIH

Table 1. Numbers of insulin and IGF-I- receptors in transfected cell lines

Values indicate receptor numbers per cell. Data are taken from *Hoffman *et al.* (1989), †McClain *et al.* (1987), Maegawa *et al.* (1988) and ‡Lammers et al. (1989). Rodent receptors were determined in corresponding untransfected cells, but levels were very similar in transfected cells where measured.

	10^{-3} × Receptor no.			
	Rodent receptors (untransfected cells)		Human receptors (transfected cells)	
Cell line	Insulin	IGF-I	Insulin	IGF-I
NIH 3T3 HIR3.5*	< 3	180	3000	–
HIRc-B†	1.7	120	1250	–
IGF-I-R/3T3‡	5.4	3.4	–	1311

3T3 and Rat 1 fibroblasts transfected with insulin receptor cDNA possess a moderately high number of endogenous rodent IGF-I receptors but relatively few insulin receptors. The NIH 3T3 subline used for transfection with IGF-I receptor cDNA clearly differs from that used for insulin receptor transfection in having low levels of endogenous receptors for both insulin and IGF-I. The levels of human receptors in transfected cells greatly exceeded those of endogenous receptors in all cases. The NIH 3T3 HIR3.5 cell line (Ebina *et al.*, 1985; Whittaker *et al.*, 1987) was transfected with a cDNA sequence encoding a receptor with an additional 12-amino-acid segment compared with that expressed in the Rat 1 HIRc-B cells (Ullrich *et al.*, 1985; McClain *et al.*, 1987). There is no evidence in any of these cells that type II IGF receptors (Froesch *et al.*, 1985) contribute significantly to ^{125}I-IGF-I binding. Thus ^{125}I-IGF-I binding was effectively displaced by insulin, albeit at high concentrations, as expected for reactivity with 'classical' IGF-I receptors. Further, type II receptors, because of their totally different structure compared with insulin- and IGF-I-receptors, would not be expected to react with any of the antibodies used in this study.

Binding studies with intact cells overexpressing human insulin receptors

Monoclonal anti-(insulin receptor) antibodies 47-9 and 25-49 were tested for effects on ligand binding to normal fibroblasts (NIH 3T3, Rat 1) and to cells transfected with human insulin receptor cDNA. These antibodies recognize distinct epitopes on the human insulin receptor and inhibit insulin binding (Soos *et al.*, 1986). They do not react with rodent insulin receptors (Soos *et al.*, 1986) nor with human IGF-I receptors in IGF-I-R/3T3 cells (results not shown). As expected, therefore, these antibodies had no effect on the binding of IGF-I (Fig. 1) or insulin (results not shown) to mock-transfected NIH 3T3 NEO or untransfected Rat 1 cells. The IGF-I binding sites behaved as a homogeneous population, in showing inhibition of tracer binding by low concentrations of unlabelled IGF-I [concn. giving 50% inhibition of binding (IC_{50}) 0.5–0.6 nM], but only by high concentrations of insulin (IC_{50} 400–1000 nM, Fig. 1).

The level of IGF-I binding to NIH 3T3 HIR3.5 and HIRc-B cells was similar to that in the corresponding untransfected cell lines. However, in the cells expressing human insulin receptors, antibodies 47–9 and 25–49 inhibited not only the binding of insulin (Figs. 2*c* and 3*c*) but also the binding of IGF-I (Figs. 2*d* and 3*d*). Maximum inhibition of insulin binding was 95–98% and of IGF-I binding it was 70–80%. The antibody concentration-dependence of binding inhibition was similar for both ligands with antibody 25–49, but antibody 47–9 was consistently approximately 2-fold less potent with IGF-I than

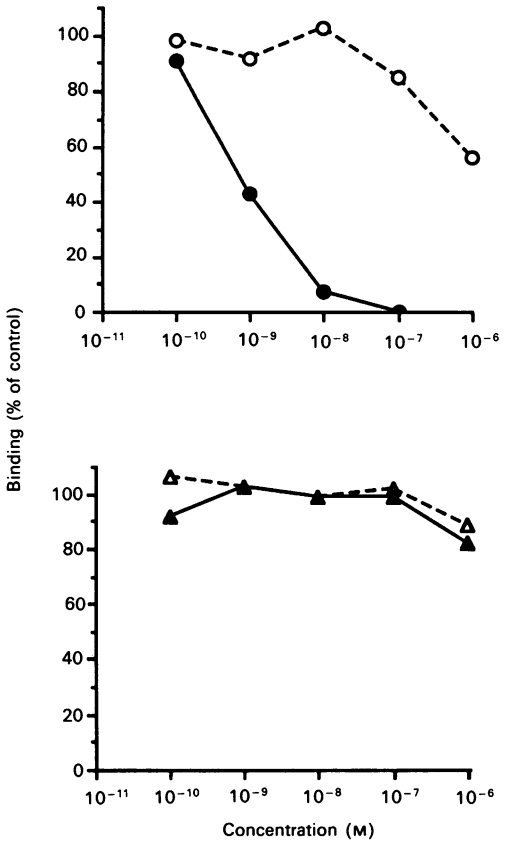

Fig. 1. Inhibition of ^{125}I-IGF-I binding to NIH 3T3 NEO cells

Binding of labelled IGF-I to cells was measured in the presence of the indicated concentrations of unlabelled insulin (○), IGF-I (●), 47-9 (△) or 25-49 (▲) as described in the Experimental 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. Total cell-bound ^{125}I-IGF-I was 2.7% and non-specific binding was 0.2% of the total radioactivity. Very similar results were obtained with Rat 1 fibroblasts. ^{125}I-IGF-I binding was inhibited by low concentrations of unlabelled IGF-I (IC_{50} 0.5 nM) and high concentrations of insulin (IC_{50} 400 nM), whereas antibodies 47-9 and 25-49 were without effect.

with insulin. Monovalent Fab fragments of antibodies 47–9 and 25–49 (10–100 nM) inhibited binding of both IGF-I and insulin to the same maximum extent as bivalent antibodies. Anti-(insulin receptor) antibodies which did not inhibit insulin binding (e.g. 83–7) were without effect on IGF-I binding (results not shown).

In both transfected cell types the binding of ^{125}I-IGF-I appeared to be heterogeneous in terms of inhibition by unlabelled insulin (Figs. 2*b* and 3*b*). Approx. 75% of IGF-I binding was inhibited by low concentrations of IGF-I (IC_{50} 0.5–1 nM) but only by high concentrations of insulin (IC_{50} approx. 800 nM). Thus most of the IGF-I binding sites still showed the properties expected of high-affinity IGF-I receptors, as in the untransfected cells. However, a fraction (approx. 25%) of IGF-I binding was inhibited by low insulin concentrations (IC_{50} approx. 0.2 nM) and was assumed to reflect low-affinity binding of IGF-I to the large excess of insulin receptors.

There was a significant difference in the reactivity of IGF-I with insulin receptors in the two different transfected cell lines. Under the conditions of these experiments, unlabelled IGF-I was considerably more potent at inhibiting tracer binding in HIRc-B cells (IC_{50} approx. 20 nM, Fig. 3*a*) than in NIH 3T3 HIR3.5 cells ($IC_{50} \geqslant 100$ nM, Fig. 2*a*), although displacement by unlabelled

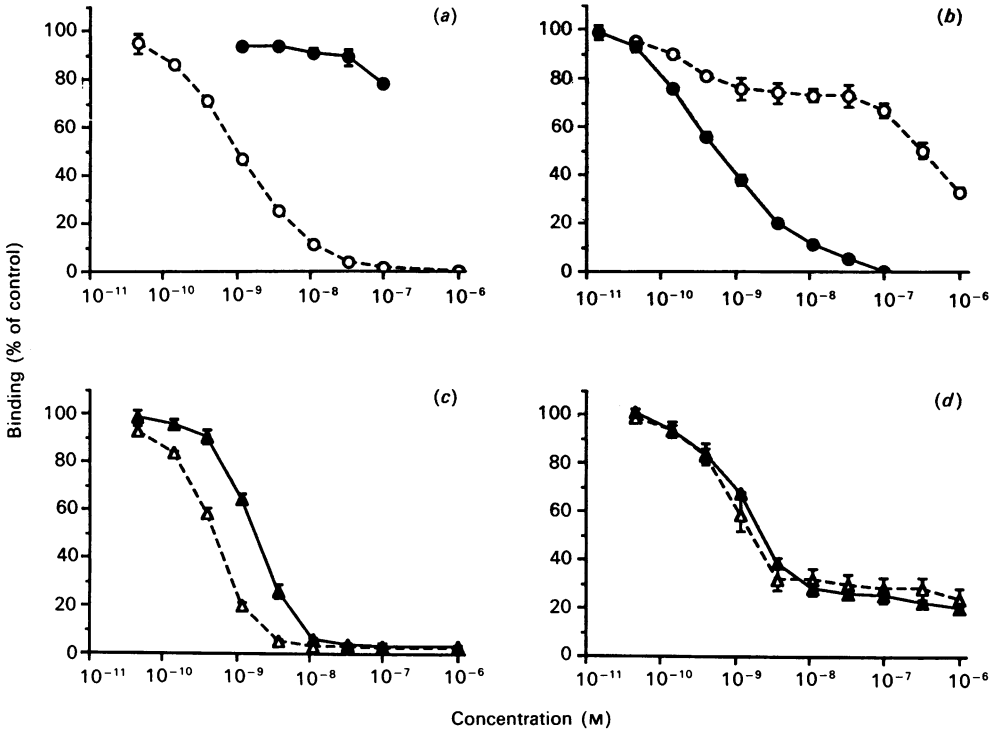

Fig. 2. Inhibition of ^{125}I-hormone binding to NIH 3T3 HIR 3.5 cells

Binding of labelled insulin (*a*,*c*) and IGF-I (*b*,*d*) to cells was measured in the presence of the indicated concentrations of unlabelled insulin (○), IGF-I (●), 47–9 (△) or 25–49 (▲) as described in the Experimental section. The means ± S.E.M. of three independent experiments, performed in duplicate, are shown. Specific binding is expressed as a percentage of that in the absence of unlabelled ligand. Total cell-bound radioactivity was 36 ± 2 % (^{125}I-insulin) and 4.1 ± 0.4 % (^{125}I-IGF-I), and non-specific binding was 0.23 ± 0.13 % (^{125}I-insulin) and 0.33 ± 0.04 % (^{125}I-IGF-I) of the total radioactivity.

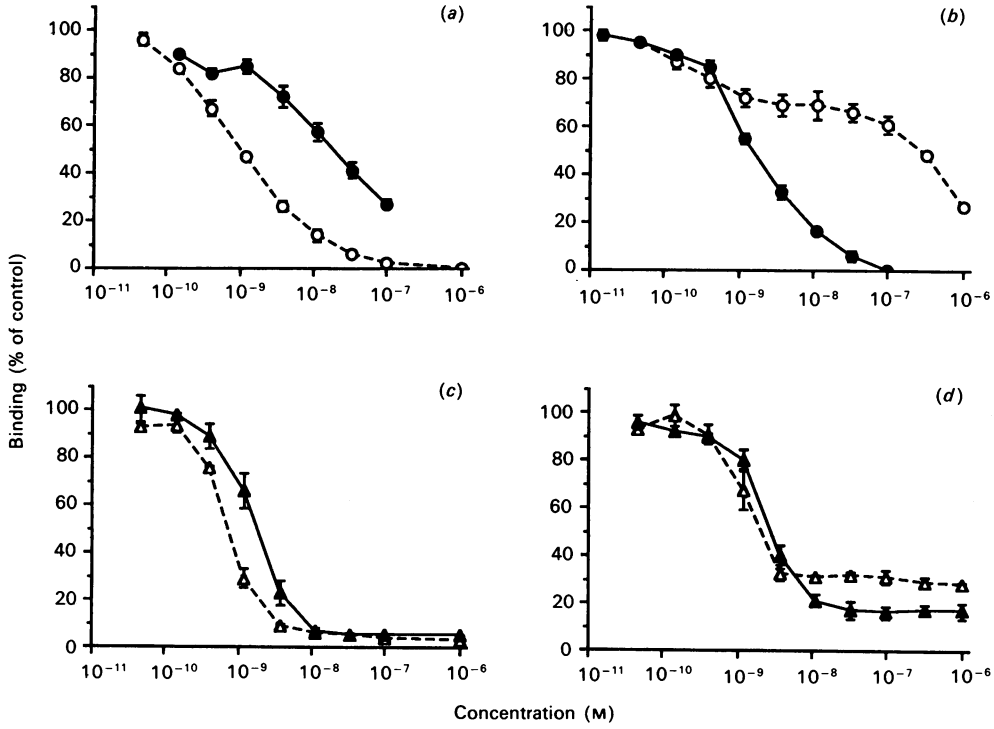

Fig. 3. Inhibition of ^{125}I-hormone binding to HIRc-B cells

Binding of labelled insulin (*a*,*c*) and IGF-I (*b*,*d*) to cells was measured in the presence of the indicated concentrations of unlabelled insulin (○), IGF-I (●), 47–9 (△) or 25–49 (▲) as described in the Experimental section. The means ± S.E.M. of three independent experiments performed in duplicate are shown. Specific binding is expressed as a percentage of that in the absence of unlabelled ligand. Total cell-bound radioactivity was 34 ± 7.9 % (^{125}I-insulin) and 10.9 ± 2.1 % (^{125}I-IGF-I), and non-specific binding was 0.16 ± 0.07 % (^{125}I-insulin) and 0.45 ± 0.0 % (^{125}I-IGF-I) of the total radioactivity.

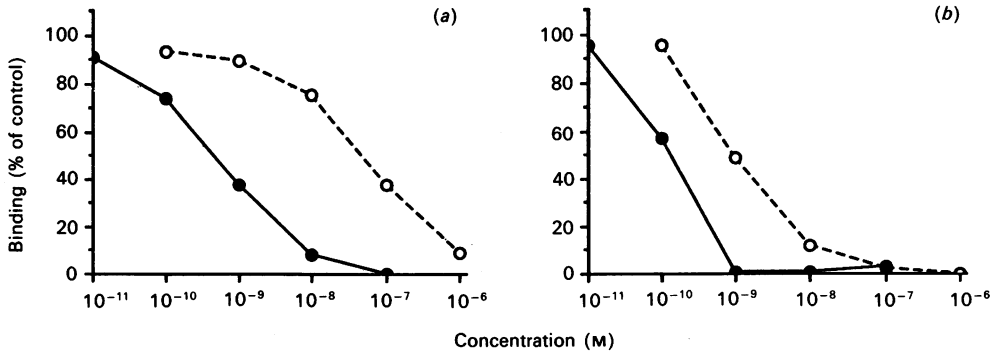

Fig. 4. Inhibition of ^{125}I-hormone binding to partially purified receptors

Binding of ^{125}I-IGF-I to WGA–Sepharose-purified NIH 3T3 HIR 3.5 receptors (*a*) and of ^{125}I-insulin to WGA–Sepharose-purified IGF-I-R/3T3 receptors (*b*) was measured in the presence of the indicated concentrations of unlabelled insulin (○) or IGF-I (●) as described in the Experimental 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. Total receptor-bound radioactivity was 9.4% (*a*) and 7.1% (*b*), and non-specific binding was 4% (*a*) and 0.9% (*b*).

insulin was very similar in both cell types. The basis of the apparently higher cross-reaction of IGF-I with human insulin receptors expressed in Rat 1 compared with NIH 3T3 HIR3.. cells is unknown. This might reflect the differences in recepto sequence or differences in glycosylation which influence binding affinity for IGF-I but not insulin.

The conclusion from these experiments is that approx. 60–70% of endogenous rodent IGF-I receptors became reactive with anti-(insulin receptor) specific antibodies when a 10–20-fold excess of human insulin receptors was co-expressed. This is consistent with the idea that a large fraction of IGF-I binding sites were in hybrid receptors ($\alpha\beta\alpha'\beta'$) in which binding of ligand to the α'-subunit was inhibited by binding of antibody or Fab to particular epitopes on α. In other respects, including affinity for IGF-I and insulin, these IGF-I binding sites were very similar to IGF-I receptors in untransfected cells.

Binding studies with solubilized receptors from cells overexpressing human insulin receptors

Receptors solubilized and partially purified from transfected cells were studied in order to permit the investigation of reactivity with antibodies which did not necessarily inhibit ligand binding. In these experiments, ^{125}I-IGF-I or ^{125}I-insulin was preincubated with receptor before determination of the fraction of receptor–ligand complexes which was immunoprecipitable.

Solubilized receptors from NIH 3T3 HIR3.5 and HIRc-B cells behaved similarly to those from the intact cells in terms of inhibition of ^{125}I-ligand binding by unlabelled insulin and IGF-I. The different potency of cross-reaction of IGF-I with insulin receptors from the two cell lines was still apparent after solubilization. However, the minor component of ^{125}I-IGF-I binding which was inhibitable by low concentrations of insulin in intact cells was not apparent with solubilized receptors (Fig. 4*a*). This probably reflects the fact that conditions for separation of bound and free ligand in the soluble receptor assays (PEG precipitation) do not detect the low-affinity component of IGF-I binding because of the dissociation during precipitation and washing. Binding of ^{125}I-IGF-I in this assay therefore reflects only high-affinity IGF-I receptors (IC_{50} 0.4 nM for IGF-I and 40–150 nM for insulin; Fig. 4*a*).

Antibodies for three different insulin receptor epitopes (83–7 and 83–14, α-subunit; 18–44, β-subunit) precipitated not only receptor–^{125}I-insulin complexes but also the bulk of receptor–^{125}I-IGF-I complexes from solubilized NIH 3T3 HIR3.5 and HIRc-B cells (Table 2). None of the antibodies reacted at all with

Table 2. Reaction of antibodies with receptor–^{125}I-hormone complexes

WGA–Sepharose-purified receptors (NIH 3T3 HIR3.5 and IGF-I-R/3T3 cells) or Triton X-100-solubilized receptors (HIRc-B) were preincubated with ^{125}I-insulin or ^{125}I-IGF-I before addition of 10 nM-antibody. Total and immunoreactive receptor-bound radioactivity was determined as described in the Experimental section. In the absence of antibody, total specific receptor-bound ^{125}I-insulin was 2483 c.p.m. (NIH 3T3 HIR3.5), 2521 c.p.m. (HIRc-B) and 1564 c.p.m. (IGF-I-R/3T3), and total receptor-bound ^{125}I-IGF-I was 1492 c.p.m. (NIH 3T3 HIR3.5), 1673 c.p.m. (HIRc-B) and 2370 c.p.m. (IGF-I-R/3T3). Values are the means of duplicate incubations within representative experiments. None of the antibodies precipitated receptors from control cells (NIH 3T3, Rat 1) which do not express human insulin or IGF-I receptors. N.D., not determined.

	Immunoreactive receptors (%)					
	NIH 3T3 HIR3.5		HIRc-B		IGF-I-R/3T3	
Antibody	Insulin	IGF-I	Insulin	IGF-I	Insulin	IGF-I
83–7	96	82	70	67	0	0
83–14	94	100	86	76	0	1
18–44	102	83	93	71	0	1
αIR-3	0	0	0	2	44	85
16–13	0	N.D.	0	2	50	77

receptors from untransfected cells or with human IGF-I receptors in IGF-1-R/3T3 cells. As expected, antibodies αIR-3 and 16–13, specific for distinct epitopes on the human IGF-I receptor, did not react with rodent IGF-I receptors in either untransfected or transfected cells (Table 2).

It was concluded that a substantial fraction of rodent IGF-I receptors in transfected cells was incorporated into hybrid structures which were then reactive with the whole panel of antibodies specific for human insulin receptors.

Binding studies with solubilized receptors from cells overexpressing human IGF-I receptors

The level of specific ^{125}I-insulin binding to intact IGF-I-R/3T3 cells was too low to permit quantitative studies. Moreover, a significant fraction of this binding appeared to reflect sites of low affinity, in terms of competition by unlabelled insulin. Solubilized receptor preparations from IGF-I-R/3T3 cells could be studied

more readily than receptors in intact cells because it was possible to achieve greater specific binding of insulin, both in absolute terms and relative to non-specific background. Binding of ^{125}I-insulin to solubilized receptors was inhibited by low concentrations of unlabelled insulin (IC_{50} approx. 1 nM; Fig. 4*b*), whereas binding of ^{125}I-IGF-I was inhibited only at much higher concentrations (IC_{50} approx. 500 nM; results not shown). It was concluded that binding of ^{125}I-insulin reflected a high-affinity interaction with rodent insulin receptors, and not low-affinity binding to human IGF-I receptors. Nevertheless, binding of ^{125}I-insulin was strikingly inhibited by low concentrations of IGF-I, almost identical with those which inhibited ^{125}I-IGF-I binding (IC_{50} 0.1–0.2 nM, Fig. 4*b*). High-affinity insulin binding in extracts of mock-transfected NIH 3T3 NEO cells was inhibited only by high concentrations of IGF-1 (IC_{50} > 100 nM; results not shown).

The immunoreactivity of receptors solubilized from IGF-I-R/3T3 cells was investigated with two antibodies specific for human IGF-I receptors: αIR-3, which inhibits insulin binding (Kull *et al.*, 1983), and 16–13, which does not inhibit binding. Both antibodies precipitated the bulk of IGF-I receptors in IGF-1-R/3T3 extracts, as expected. However, these antibodies also precipitated approx. 50% of the rodent insulin receptors in these extracts (Table 2), although they were unreactive with insulin receptors in untransfected cells. Total (PEG-precipitable) ^{125}I-insulin binding under the conditions of these experiments was decreased by antibody αIR-3 and increased by antibody 16–13 (700 c.p.m. and 2339 c.p.m. respectively compared with the control value of 1564 c.p.m.). This suggests that αIR-3 accelerates the dissociation of receptor-bound insulin, whereas 16–13 increases the binding affinity.

It is concluded that rodent insulin receptors in IGF-I-R/3T3 cells were substantially incorporated into hybrid structures with human IGF-I receptors. These hybrids differed from normal insulin receptors in that binding of ^{125}I-insulin was inhibited by low concentrations of both insulin and IGF-I.

DISCUSSION

Receptors for insulin ($\beta\alpha\alpha\beta$) and IGF-I ($\beta'\alpha'\alpha'\beta'$) have generally been considered to be distinct but structurally similar symmetrical heterotetramers (Czech, 1985; Rechler & Nissley, 1985; Yarden & Ullrich, 1988). Various observations have indicated that there may be subtypes of both receptors (Jonas, 1988), but the structural basis and functional significance of this heterogeneity is unclear. Two distinct hypotheses have been advanced. We have proposed that a significant fraction of IGF-I receptors in human placenta occurs as hybrids with insulin receptors ($\beta\alpha\alpha'\beta'$), based on reactivity with a panel of anti-receptor antibodies (Soos & Siddle, 1989). The existence of hybrid structures was suggested independently as a result of studies of IGF-I-induced receptor phosphorylation in Hep G2 cells (Moxham *et al.*, 1989). However, similar autophosphorylation work in brain (Garofalo & Rosen, 1989) and muscle (Alexandrides & Smith, 1989) was interpreted as evidence for two distinct IGF-I receptor polypeptides differing in primary sequence and/or glycosylation (and therefore in immunological recognition), as well in developmental regulation. We now provide conclusive evidence for the formation in intact cells of hybrids which incorporate subunits of receptors for both insulin and IGF-I. When human insulin receptors (hIR) were overexpressed in rat or mouse fibroblasts by transfection with cloned cDNA, rodent IGF-I receptors (rIGFR) were largely incorporated into hybrid structures which were detected by their reaction with multiple anti-(human insulin receptor) specific monoclonal antibodies (Figs. 2 and 3; Table 2). Conversely, when human IGF-I receptors (hIGFR) were overexpressed in similar cells, rodent insulin receptors were incorporated into hybrids which then reacted with human IGF-I-receptor-specific antibodies (Table 2).

In NIH 3T3 HIR3.5 and HIRc-B cells, hybrids were clearly demonstrated in intact as well as solubilized cells by the criterion of inhibition of high-affinity IGF-I binding by monoclonal antibodies specific for insulin receptors (Figs. 2 and 3). In IGF-I-R/3T3 cells, hybrids were demonstrated by immunoprecipitation of solubilized preparations (Table 2), although little high-affinity insulin binding was detected in intact cells. Hybrid receptors clearly pre-exist in intact cells but the possibility cannot be ruled out that the binding specificity, or even the formation, of hybrids is influenced by solubilization. In previous studies with placental microsomal membranes, the proportion of IGF-I receptors appearing as hybrids appeared higher in solubilized than in particulate preparations (Soos & Siddle, 1989). It has recently been demonstrated that hybrid insulin/IGF-I receptors can be assembled *in vitro* from the respective $\alpha\beta$ receptor halves, although this required the presence of the respective ligands or of Mg ATP (Treadway *et al.*, 1989).

Hybrid receptors displayed a high affinity for both insulin and IGF-I, as studied with the rIR/hIGFR and rIGFR/hIR hybrids respectively (Fig. 4). However, comparison of ligand binding to these two hybrids revealed a striking asymmetry in properties. The rIGFR/hIR hybrids behaved similarly to homomeric IGF-I receptors, in that binding of ^{125}I-IGF-I was inhibited by low concentrations of IGF-I but only by high concentrations of insulin. In contrast, binding of ^{125}I-insulin to rIR/hIGFR hybrids was inhibited by low concentrations of both insulin and IGF-I (Fig. 4). It is unknown in either case whether the unlabelled ligands remain bound together with ^{125}I-labelled ligand and therefore whether hybrid receptors can bind both insulin and IGF-I simultaneously. It is possible that the different properties of rIR/hIGFR compared with hIR/rIGFR reflects the species asymmetry. However, it is tempting to speculate that the differences in the properties of the two hybrids are a consequence of asymmetry of ligand–receptor interactions common to both structures. This would imply that the insulin–IR/IGFR complex retained a high affinity for IGF-I, whereas the IR/IGFR–IGF-I complex no longer had a high affinity for insulin. It has been shown that the homomeric insulin receptor binds only one molecule of insulin with high affinity, this interaction converting the unoccupied site to a low-affinity state (Pang & Shafer, 1984; Boni-Schnetzler *et al.*, 1987; Sweet *et al.*, 1987). The homomeric IGF-I receptor may behave differently in binding two molecules of ligand with high affinity (Feltz *et al.*, 1988), although this is not certain (Tollefsen & Thompson, 1988). A model can be proposed in which the unoccupied half of a hybrid receptor displays the same kinetic properties as in the corresponding homomeric receptor (Fig. 5). It therefore appears that the interaction between heterologous receptor halves is sufficient not only to allow their covalent association but also to permit inter-subunit conformational transitions.

Further evidence for inter-subunit interactions is provided by the observation that the anti-(IGF-I receptor) antibody αIR-3 accelerated dissociation of insulin from hybrid receptors, whereas the anti-(insulin receptor) antibody 25–49 accelerated dissociation of IGF-I (M. A. Soos & K. Siddle, unpublished work). Antibody 47–9 consistently showed a lower affinity for hybrid receptors than for homomeric receptors in transfected cells (Figs. 2 and 3), as in placenta (Soos & Siddle, 1989). This suggests that the conformation of the insulin receptor α-subunit may differ somewhat in the two types of receptor. It is perhaps surprising that three distinct insulin receptor antibodies 47–9, 25–49 and (results not shown) 83–14 all inhibited binding of IGF-I to hybrids not only as bivalent antibodies but also as Fab

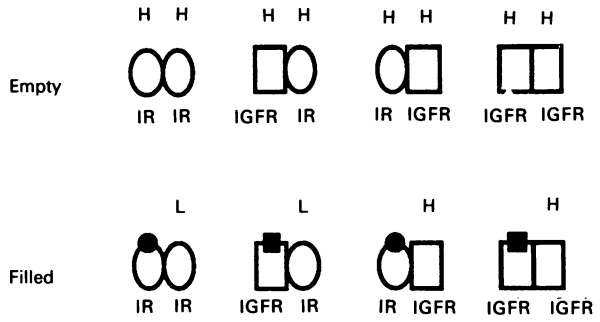

Fig. 5. Schematic representation of the affinity of ligand binding sites

Homomeric (IR.IR, IGFR.IGFR) and hybrid (IGFR.IR, IR.IGFR) receptors are represented in the empty state and with one site occupied by insulin (●) or IGF-I (■). Affinities of the empty sites are represented as high (H) or low (L).

fragments. It is unclear whether steric factors alone could account for this inhibition, as the relationships of epitopes and potential ligand binding sites in the heterotetrameric receptor is unknown. However, this observation might also reflect an ability of antibodies bound at one α-subunit to influence the ligand affinity for the other α-subunit within a hybrid molecule by conformational effects.

Several reports have previously described receptors with anomalous properties, which might now be attributed to the presence of hybrids. In the rat L6 myocyte line (Burant *et al.*, 1987) and in bovine neural retina (Waldbillig & Chader, 1988) binding of ^{125}I-insulin was observed which was competed by IGF-I with greater potency than by insulin itself. This could be explained if most of the insulin receptors exist as hybrids like the rIR/hIGFR species of the IGF-I-R/3T3 cells. The converse situation of ^{125}I-IGF-II binding which is competed by insulin at unexpectedly high potency has been described in IM-9 cells and placenta (Hintz *et al.*, 1984; Misra *et al.*, 1986; Jonas *et al.*, 1986). These properties have been attributed to 'atypical' insulin receptors. However, we did not observe such behaviour when using ^{125}I-IGF-I as tracer either in previous studies with IM-9 cells and placenta (Soos & Siddle, 1989) or with rIGFR/hIR hybrids in the present study (Figs. 2 and 3). It is possible, though unlikely, that binding of insulin to hybrid receptors differentially affects their affinity for IGF-I and IGF-II. However 'atypical' receptors in placenta reacted with anti-(insulin receptor) antibodies but not with the anti-(IGF-I receptor) antibody αIR-3, which would not be consistent with a hybrid species (Jonas *et al.*, 1989). The presence of hybrid receptors in some tissues does not of course preclude the existence of receptor heterogeneity, dependent perhaps on differences in primary sequence (reflecting products of distinct genes or alternative RNA splicing) and/or differential glycosylation.

It appears that hybrids as well as homomeric receptors occur in normal human tissues in which insulin and IGF-I receptors are co-expressed (Soos & Siddle, 1989), and this could have important implications for metabolic regulation by the respective ligands. The tyrosine kinase activity of these receptors appears to be an essential component of their signalling mechanism (Rosen, 1987; Zick, 1989). Ligand-induced activation of the insulin receptor kinase occurs through an intramolecular autophosphorylation reaction within intact heterotetramers (O'Hare & Pilch, 1988; Morrison *et al.*, 1988; Wilden *et al.*, 1989). The observation that both insulin and IGF-I bind to hybrid receptors with high affinity raises the question of whether both ligands can activate both receptor kinases within hybrids. This is difficult to answer in transfected cells because of the very large excess of homomeric human receptors. However, in Hep G2 and NIH 3T3 cells it appeared that low concentrations of IGF-I stimulated autophosphorylation of both β-subunits within putative hybrids, although insulin was less potent (Moxham *et al.*, 1989). Interestingly, NIH 3T3 HIR3.5 cells show enhanced responses not only to insulin but also to IGF-I, concomitant with overexpression of insulin receptors (Hofmann *et al.*, 1989). This suggests that some effects of IGF-I might be signalled more efficiently via hybrid receptors than via homomeric IGF-I receptors. However, the full physiological implications of the finding that insulin and IGF-I receptors behave as isoenzymes which can combine to form hybrid as well as homomeric structures require further investigation. Studies with cells co-transfected with cDNAs for both insulin and IGF-I receptors may shed light on this issue, and on the relative efficiency of formation of hybrid and homomeric structures.

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REFERENCES

Alexandrides, T. K. & Smith, R. J. (1989) J. Biol. Chem. **264**, 12922–12930
Baron, M. D. & Sonksen, P. H. (1982) Biosci. Rep. **2**, 785–793
Boni-Schnetzler, M., Scott, W., Waugh, S. M., DiBella, E. & Pilch, P. (1987) J. Biol. Chem. **262**, 8395–8401
Burant, C. F., Treutelaar, M. K., Allen, K. D., Sens, D. A. & Buse, M. G. (1987) Biochem. Biophys. Res. Commun. **147**, 100–107
Czech, M. P. (1985) Annu. Rev. Physiol. **47**, 357–381
Duronio, V. & Jacobs, S. (1988) Recept. Biochem. Methodol. **12B**, 19–36
Ebina, Y., Ellis, L., Jarnagin, K., Edery, M., Graf, L., Clauser, E., Ou, J.-H., Masiarz, F., Kan, Y. W., Goldfine, I. D., Roth, R. A. & Rutter, W. J. (1985) Cell **40**, 747–758
Feltz, S. M., Swanson, M. L., Wemmie, J. A. & Pessin, J. E. (1988) Biochemistry **27**, 3234–3242
Froesch, E. R., Schmid, C., Schwander, J. & Zapf, J. (1985) Annu. Rev. Physiol. **47**, 443–467
Galfre, G. & Milstein, C. (1981) Methods Enzymol. **73**, 3–46
Garofalo, R. S. & Rosen, O. M. (1989) Mol. Cell. Biol. **9**, 2806–2817
Hintz, R. L., Thorsson, A. V., Enberg, G. & Hall, K. (1984) Biochem. Biophys. Res. Commun. **118**, 774–782
Hofmann, C., Goldfine, I. D. & Whittaker, J. (1989) J. Biol. Chem. **264**, 8606–8611
Izumi, T., White, M. F., Kadowaki, T., Takaku, F., Akanuma, Y. & Kasuga, M. (1987) J. Biol. Chem. **262**, 1282–1287
Jonas, H. A. (1988) Recept. Biochem. Methodol. **12B**, 19–36
Jonas, H. A., Newman, J. D. & Harrison, L. C. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 4124–4128
Jonas, H. A., Cox, A. J. & Harrison, L. C. (1989) Biochem. J. **257**, 101–107
Kadowaki, T., Koyasu, S., Nishida, E., Tobe, K., Izumi, T., Takaku, F., Sakai, H., Yahara, I. & Kasuga, M. (1987) J. Biol. Chem. **262**, 7342–7350
Kull, F. C., Jacobs, S., Su, Y.-F., Svoboda, M. E., Van Wyk, J. J. & Cuatrecasas, P. (1983) J. Biol. Chem. **258**, 6561–6566
Lammers, R., Gray, A., Schlessinger, J. & Ullrich, A. (1989) EMBO J. **8**, 1369–1375
Linde, S., Hansen, B., Sonne, O., Holst, J. J. & Glieman, J. (1981) Diabetes **30**, 1–8
Maegawa, H., Olefsky, J. M., Thies, S., Boyd, D., Ullrich, A. & McClain, D. (1988) J. Biol. Chem. **265**, 12629–12737
McClain, D. A., Maegawa, H., Lee, J., Dull, T. J., Ullrich, A. & Olefsky, J. M. (1987) J. Biol. Chem. **262**, 14663–14671
Misra, P., Hintz, R. L. & Rosenfeld, R. G. (1986) J. Clin. Endocrinol. Metab. **63**, 1400–1405
Morrison, B. D., Swanson, M. L., Sweet, L. J. & Pessin, J. E. (1988) J. Biol. Chem. **263**, 7806–7813
Moxham, C. P., Duronio, V. & Jacobs, S. (1989) J. Biol. Chem. **264**, 13238–13244

O'Hare, T. & Pilch, P. (1988) Biochemistry **27**, 5693–5700
Pang, D. T. & Shafer, J. A. (1984) J. Biol. Chem. **259**, 8589–8596
Rechler, M. M. & Nissley, S. P. (1985) Annu. Rev. Physiol. **47**, 425–442
Rosen, O. M. (1987) Science **237**, 1452–1458
Roth, J. (1975) Methods Enzymol. **37**, 223–233
Soos, M. A. & Siddle, K. (1989) Biochem. J. **263**, 553–563
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
Stanker, L. H., Vanderlaan, H. & Juarez-Salinas, H. (1985) J. Immunol. Methods **76**, 157–169
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
Sweet, L. J., Morrison, B. D. & Pessin, J. E. (1987) J. Biol. Chem. **262**, 6939–6942
Treadway, J. L., Morrison, B. D., Goldfine, I. D. & Pessin, J. E. (1989) J. Biol. Chem. **264**, 21450–21453
Tollefsen, S. E. & Thompson, K (1988) J. Biol. Chem. **263**, 16267–16273
Ullrich, A., Bell, J. R., Chen, E. Y., Herrera, R., Petruzzelli, L. M., Dull, T. J., Gray, A., Coussens, L., Liao, Y.-C., Tsubokawa, M., Mason, A., Seeburg, P. H., Grunfield, C., Rosen, O. M. & Ramachandran, J. (1985) Nature (London) **313**, 756–761
Ullrich, A., Gray, A., Tam, A. W., Yang-Fen, T., Tsubokawa, M., Collins, C., Henzel, W., Le-Bon, T., Kathuria, S., Chen, E., Jacobs, S., Francke, U., Ramachndran, J. & Fujita-Yamaguchi, Y. (1986) EMBO J. **5**, 2503–2512
Waldbillig, R. J. & Chader, G. J. (1988) Biochem. Biophys. Res. Commun. **151**, 1105–1112
Whittaker, J., Okamoto, A. K., Thys, R., Bell, G. I., Steiner, D. F. & Hofmann, C. A. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 5237–5241
Wilden, P. A., Morrison, B. D. & Pessin, J. E. (1989) Biochemistry **28**, 785–792
Yarden, Y & Ullrich, A. (1988) Annu. Rev. Biochem. **57**, 443–478
Zick, Y. (1989) Crit. Rev. Biochem. Mol. Biol. **24**, 217–269

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