Insulin receptor/IGF-I receptor hybrids are widely distributed in mammalian tissues: quantification of individual receptor species by selective immunoprecipitation and immunoblotting

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The insulin receptor (IR) and type I insulin-like growth factor (IGF-I) receptor (IGFR) are both widely expressed in mammalian tissues, and are known to be capable of heteromeric assembly as insulin/IGF hybrid receptors, in addition to the classically described receptors. By selective immunoadsorption of radioligand/receptor complexes and by immunoblotting we have determined the fraction of insulin receptors and IGF receptors occurring as hybrids in different tissues. Microsomal membranes were isolated from tissue homogenates and solubilized with Triton X-100. Solubilized receptors were incubated with ¹²⁵I-IGF-I, and radioligand/receptor complexes bound by IR-specific and IGFR-specific monoclonal antibodies were quantified. The fraction of IGF-I binding sites behaving as hybrids (anti-IR-bound/anti-IGFR-bound) was approx. 40% in liver and spleen, 70% in placenta, and 85–90% in skeletal muscle and heart, similar results being obtained in rabbit and human tissues. There was no correlation between the proportion of hybrids and the ratio of ¹²⁵I-insulin/¹²⁵I-IGF-I binding in different tissues. The fraction of ¹²⁵I-insulin bound to hybrids was too low for accurate quantification, because of the relatively low affinity

of hybrids for insulin. The fraction of insulin receptors present in hybrids was therefore determined by immunoblotting. Receptors in solubilized human placental microsomal membranes were precipitated with IR-specific or IGFR-specific monoclonal antibodies, and after SDS/PAGE, blots were prepared and probed with IR-specific and IGFR-specific antisera. It was found that 15% of IR and 80% of IGFR were present in hybrids. Consistent with these figures, the overall level of IR was estimated, by blotting with the respective antibodies at concentrations shown to give equal signals with equal amounts of receptor, to be 4-fold greater than IGFR. Overall it was concluded that a significant fraction of both IR and IGFR occurs as hybrids in most mammalian tissues, including those that are recognized targets of insulin and IGF action. The fraction of hybrids in different tissues was not a simple function of the relative levels of IR and IGFR, possibly because of heterogeneity of receptor expression in different cell types. However, in placenta the proportions of IR, IGFR and hybrids were consistent with a process of random assembly reflecting the molar ratio of IR and IGFR half-receptors.

INTRODUCTION

The insulin receptor (IR) and type I insulin-like growth factor (IGF-I) receptor (IGFR) are structurally related transmembrane glycoproteins that are widely expressed in mammalian tissues. Each receptor is the product of a single gene, and the IR and IGFR proreceptors display approx. 50% amino acid sequence identity, as do their ligands insulin and IGF-I [1,2]. Post-translational processing in the endoplasmic reticulum and Golgi apparatus results in dimerization and disulphide linkage of proreceptors followed by proteolytic cleavage, which generates α and β subunits [3]. Mature and functional receptors thus have the structure ($\alpha\beta$)₂. The extracellular α subunit contains the ligand-binding site and the transmembrane β subunit possesses tyrosine kinase activity. The native ($\alpha\beta$)₂ structure is essential for ligand-induced activation of the tyrosine kinase, by a trans-autophosphorylation reaction between β subunits [4,5]. However, $\alpha\beta$ 'half receptors' that retain high-affinity ligand-binding and ligand-independent tyrosine kinase activities can be generated experimentally by mild reduction of holoreceptors [6–8].

The distinct physiological functions of insulin and IGFs depend on differences in the distribution and/or signalling potential of their respective receptors. Despite this, it has been shown in human placenta and certain cultured cell lines that a proportion of IR and IGFR assemble as hybrid structures containing an ($\alpha\beta$) half of the IR disulphide-linked to an ($\alpha'\beta'$)

half of the IGFR [9–12]. These hybrid receptors are functional, in that they bind IGF-I with high affinity and insulin with somewhat lower affinity [13,14], and display IGF-I-induced autophosphorylation both *in vitro* and *in situ* [11,14]. Most mammalian cells express both IR and IGFR, albeit at different levels in different cell types, and therefore have the potential to form functional hybrids. However, the significance of hybrid receptors *in vivo* remains unclear. In particular, the occurrence of hybrids in tissues that are known to be physiologically important targets of insulin or IGF-I has not been demonstrated, and the relative abundances of IR, IGFR and hybrids in such tissues have not been quantified. Simple radioligand binding measurements do not provide a reliable means of comparing levels of IR, IGFR and hybrids because of differences in ligand affinity between receptors. The aim of the present work was to determine the fraction of hybrid receptors in a variety of tissues from different mammalian species, and to devise methods for quantitative assessment of the relative levels of different receptor types.

EXPERIMENTAL

Materials

Bovine insulin was obtained from Sigma, and recombinant human IGF-I was a gift from Dr. W. Märki (Ciba-Geigy, Basel,

Abbreviations used: IGF, insulin-like growth factor; IGFR, type I IGF receptor; IR, insulin receptor; PEG, polyethylene glycol; TBST buffer, Tris-buffered saline/Triton X-100 buffer.

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Switzerland). Mono-[A14]- 125 I-insulin (specific radioactivity approx. 1 μ Ci/pmol) was prepared as described previously [8] and 125 I-IGF-I (specific radioactivity 1.88 μ Ci/pmol) was a gift from Dr. L. Schaffer (Novo-Nordisk, Bagsvaerd, Denmark). Wheatgerm lectin coupled to beaded agarose and proteinase inhibitors were from Sigma, and Na 125 I was from Amersham International.

Monoclonal antibodies IR 83-14 and IGFR 24-60, directed against epitopes in the α -subunits of the human IR and IGFR respectively, were as described previously [15–17]. Rabbit polyclonal antisera IR.CT and IGFR.CT were generated against glutathione S-transferase fusion proteins incorporating the C-terminal 100 residues of the human IR or IGFR β -subunits respectively, as described previously [18].

Preparation of membrane fractions

Tissues were removed from male Dutch rabbits immediately after exsanguination, cut into small pieces and rapidly frozen in liquid nitrogen. Human tissues obtained at operation were similarly treated, and only non-diseased portions of tissue were retained for study. Membrane preparations were obtained by modification of the method of Alexandrides et al. [19]. Frozen tissue samples were crushed with a hammer, transferred to 4 vol. (w/v) of ice-cold buffer [50 mM Hepes (pH 7.6)/150 mM NaCl/1 mM bacitracin/1 mM PMSF/200 k-i.u./ml aprotinin], and homogenized for 1 min at 4 °C in a Polytron homogenizer (1 cm diameter probe) on setting 7. The homogenate was centrifuged at 10000 *g* for 10 min at 4 °C on a Sorvall RC-5B centrifuge; the pellet was discarded. The supernatant was centrifuged at 200000 *g* for 50 min at 4 °C in a Beckman OTD65B ultracentrifuge, and the resulting pellets were resuspended in homogenizing buffer containing 1% (w/v) Triton X-100 (1 ml of buffer per g of original wet weight of tissue), and stirred slowly for 90 min at 4 °C. The solubilized membranes were again centrifuged at 200000 *g* for 50 min and the supernatants were stored frozen until assay. The protocol was modified for fat tissue, which was initially disrupted in a motor-driven Potter–Elvehjem homogenizer. The crude homogenate was centrifuged at 300 *g* for 10 min and the supernatant was centrifuged at 200000 *g* as for other tissues. The protein content of receptor preparations was determined with a Bio-Rad assay kit in accordance with the manufacturer's instructions.

For some experiments, soluble receptors were partly purified on a column of wheatgerm lectin immobilized on cross-linked beaded agarose (WGA). A 1 ml column bed of WGA was equilibrated with wash buffer [50 mM Hepes (pH 7.6)/150 mM NaCl/0.1% Triton X-100]. Solubilized receptor preparations from 1 g of tissue were passed through the column four times; the column was then washed with 50 vol. of wash buffer. Receptors were eluted with 3 vol. of wash buffer containing 0.45 M *N*-acetylglucosamine, 0.2 mM PMSF and 0.2 mM bacitracin. Fractions of 1 ml were collected and frozen until assay.

Solubilization of insulin and IGF-I receptors from placenta

Microsomal membranes were prepared from fresh normal human placenta [15] and stored in aliquots at –70 °C at a protein concentration of 11 mg/ml protein. Membranes were solubilized by the addition of an equal volume of ice-cold buffer containing 100 mM Hepes, pH 7.4, 4% (w/v) Triton X-100, 4 μ M pepstatin A, 2 μ g/ml antipain, 2 μ g/ml leupeptin, 400 μ M 4-(2-aminoethyl)benzenesulphonyl fluoride, HCl [Calbiochem–Novobiochem (U.K.) Ltd] and 5 mM benzamidine, for 2 h on ice. After centrifugation at 48000 *g* for 1 h, the supernatant was collected and stored in 100 μ l aliquots at –70 °C.

Radioligand binding assays

The binding of 125 I-ligands to soluble receptors was determined essentially as previously described [8,15,16]. Solubilized microsomal membranes were diluted in assay buffer [75 mM Tris (pH 7.8 at 4 °C)/30 mM NaCl/0.5 mM EDTA/0.1 mM PMSF/1 mM *N*-ethylmaleimide/200 k-i.u./ml aprotinin/0.1% BSA/0.1% Triton X-100]. The receptor preparations were incubated for 18 h at 4 °C with 125 I-insulin (20000 c.p.m.) or 125 I-IGF-I (10000 c.p.m.), with or without unlabelled ligand, in a total volume of 250 μ l of assay buffer. Receptor-bound radioactivity was quantified by γ -counting after specific immunoprecipitation with antibodies 83-7 (IR-specific) or 17-69 (IGFR-specific) in conjunction with sheep anti-(mouse IgG) immunoadsorbent, or after non-specific precipitation with polyethylene glycol (PEG) 6000 [8,15,16]. Non-specific binding was determined in the presence of 1 μ M insulin or 100 nM IGF-I as appropriate. Counting efficiency was approx. 70%.

Immunoprecipitation of insulin and IGF-1 receptors

Immunoabsorbents were prepared by coupling antibodies IR 83-14 and IGFR 24-60 to aminocellulose, as described [15]. The immunoabsorbents (100 μ l of 5 mg/ml cellulose) were washed extensively with Tris-buffered saline/Triton X-100 (TBST) buffer containing 50 mM Tris, 44 mM HCl, 150 mM NaCl and 0.1% Triton X-100, and pelleted by centrifugation at 10000 *g* for 5 min. Solubilized placental membranes (5–30 μ l, 2.7 mg/ml protein) were added to the pellet, which was then resuspended. After incubation on ice for 4 h, 100 μ l of TBST buffer was added and the immunoabsorbent was pelleted by centrifugation. The supernatant was retained, and the immunoabsorbent pellet was washed five times with 1 ml of TBST buffer at 4 °C. The washed pellets were incubated with 100 μ l of Laemmli SDS/PAGE sample buffer containing 100 mM dithiothreitol at 100 °C for 5 min and centrifuged again; 40 μ l of supernatant was applied to an SDS/7.5% (w/v) polyacrylamide gel.

Western blotting

After SDS/PAGE, proteins were transferred to PVDF membranes (Immobilon-P, Millipore Corporation) by the semi-dry method of Western blotting [20]. The blots were incubated in PBS containing 5% (w/v) dried milk powder and 0.05% Tween-20 for 2 h at room temperature or overnight at 4 °C. The blots were developed with anti-IR C-terminal serum (at 1:1000 dilution) and anti-IGFR C-terminal serum (at 1:2000 dilution) used either at room temperature for 2 h or at 4 °C overnight, followed by 125 I-protein A (0.75 μ Ci per 10 ml in each 10-lane blot). The immunoreactive material was quantified on a Fuji BAS 2000 phosphorimager.

RESULTS

Determination of fraction of hybrid receptors by radioligand binding

Hybrid receptors were originally detected in detergent-solubilized microsomal membranes by the ability of IR-specific antibodies to precipitate a significant proportion of receptor-bound IGF-I. This approach was applied to the detection of hybrid receptors in extracts of various human and rabbit tissues (Table 1). Advantage was taken of the fact that the IR-specific antibody 83-7 and IGFR-specific antibody 17-69, both raised originally against human receptors, were found to cross-react fully with the corresponding rabbit receptors. Previous experience with human

Table 1 Determination of hybrid receptors in rabbit and human tissues by radioligand binding

Microsomal membranes were prepared and solubilized, and binding assays were performed with ^{125}I -insulin and ^{125}I -IGF-I, as described in the Experimental section. Receptor-bound radioactivity was determined by immunoprecipitation (IP) with IR-specific antibody 83-7 or IGFR-specific antibody 17-69, as indicated. Results for rabbit tissues are expressed as radioligand bound (c.p.m.) per mg of protein. The quantities of protein in binding assays were in the range 210–760 μg , except for fat (17–30 μg). Data shown are means $\pm$ range ($n = 2$) for insulin binding, and means $\pm$ S.D. ($n = 3$ independent determinations) for IGF-I binding. Hybrid receptors (83-7-precipitable IGF-I binding) were determined as a percentage of total IGFR (17-69-precipitable IGF-I binding), and mean values were calculated from three separate assays. Hybrid fractions in human tissues were determined in the same way as for rabbit tissues. Abbreviation: n.d., not determined.

Tissue	Radioligand binding to rabbit tissues (c.p.m./mg of protein)			Hybrids (% of total IGFR)	
	Insulin (IP IR 83-7)	IGF-I (IP IGFR 17-69)	IGF-I (IP IR 83-7)	Rabbit tissues	Human tissues
Muscle	930 $\pm$ 187	1866 $\pm$ 357	1410 $\pm$ 114	86 $\pm$ 12	74 $\pm$ 9
Heart	2925 $\pm$ 16	6169 $\pm$ 558	5496 $\pm$ 289	90 $\pm$ 5	87 $\pm$ 6
Kidney	1225 $\pm$ 115	6630 $\pm$ 684	4112 $\pm$ 210	63 $\pm$ 8	70 $\pm$ 2
Liver	3179 $\pm$ 330	1457 $\pm$ 208	560 $\pm$ 10	40 $\pm$ 10	n.d.
Fat	10088 $\pm$ 1823	20382 $\pm$ 2235	13911 $\pm$ 1382	69 $\pm$ 9	68 $\pm$ 7
Brain	1486 $\pm$ 301	10352 $\pm$ 764	5634 $\pm$ 764	55 $\pm$ 11	n.d.
Lung	3782 $\pm$ 270	6943 $\pm$ 1844	3504 $\pm$ 592	50 $\pm$ 8	n.d.
Spleen	1478 $\pm$ 241	7565 $\pm$ 1537	2822 $\pm$ 302	40 $\pm$ 10	53 $\pm$ 14
Placenta	n.d.	n.d.	n.d.	n.d.	72 $\pm$ 4

placenta had shown that it was difficult to detect insulin binding to hybrids because insulin receptors were present in substantial excess and possessed a higher affinity for insulin, so that the fraction of total insulin binding accounted for by hybrids was very small. Experiments were therefore restricted to the use of ^{125}I -IGF-I as ligand.

It was shown that in all tissues except skeletal muscle and heart the levels of PEG-precipitable and antibody 17-69-precipitable ^{125}I -IGF-I binding were very similar. This confirmed that type I IGFR (classical or hybrid) accounted for most (more than 85 %) of IGF binding in tissue extracts. In skeletal muscle and heart, antibody-precipitable ^{125}I -IGF-I binding was only approx. 30 % and 60 % respectively of PEG-precipitable binding. Moreover, competition experiments with unlabelled insulin showed that although antibody-precipitable binding was inhibited by insulin with an IC_{50} of approx. 300 nM, as expected for type I IGFR, insulin did not affect the non-immunoprecipitable component of ^{125}I -IGF-I binding (results not shown). Thus it seems likely that type II IGFR or IGF-binding proteins, which do not bind insulin, contribute substantially to the total (PEG-precipitable) ^{125}I -IGF-I binding observed in extracts of heart and skeletal muscle, but relatively little in other tissues.

The fraction of type I IGFR present as hybrids was assessed by determining the amount of ^{125}I -IGF-I binding that was precipitable by IR-specific antibody 83-7, relative to that precipitated by IGFR-specific antibody 17-69 (Table 1). In all tissues, hybrids accounted for a substantial fraction of IGFR, varying from approx. 40 % in liver and spleen to approx. 90 % in skeletal muscle and heart. The proportions of hybrids were very similar in corresponding human and rabbit tissues. It was confirmed with representative extracts that no additional IGF binding was precipitated by a second incubation with IR-specific antibody (results not shown). Moreover, the precipitability of IGF binding by IR-specific antibody was substantially decreased (by 60–90 % in different tissues) after treatment with dithiothreitol to reduce disulphide links between receptor α -subunits (results not shown). Thus, in terms of immunoreactivity, hybrid receptors detected in rabbit tissues behaved like those originally described in human placenta [8].

The absolute levels of ^{125}I -IGF-I and ^{125}I -insulin binding varied considerably between tissues. Because solubilized IGF receptors bind IGF-I with higher affinity than soluble insulin receptors bind insulin (M. Soos and K. Siddle, unpublished

Figure 1 Relationship between hybrid receptor fraction and insulin-to-IGF binding ratio in rabbit tissues

The fraction of total IGFR occurring as hybrids is plotted against the ratio of ^{125}I -insulin binding to ^{125}I -IGF-I binding in different rabbit tissues. Data are taken from Table 1. Abbreviations: H, heart muscle; M, skeletal muscle; F, fat; K, kidney; B, brain; S, spleen; Lu, lung; Li, liver.

work), the relative bindings of ^{125}I -insulin and ^{125}I -IGF-I in a tissue will not be a direct measure of the relative numbers of respective receptors. However, it can be assumed that the higher this binding ratio, the greater must be the excess of IR over IGFR. There was no correlation between the fraction of IGFR present as hybrids and the ratio of ^{125}I -insulin binding to ^{125}I -IGF-I binding in different tissues (Figure 1), suggesting that the formation of hybrids is not a simple function of the relative levels of IR and IGFR.

Determination of fraction of hybrid receptors by immunoblotting

The fraction of IR present as hybrids cannot readily be assessed by selective immunoprecipitation of receptor-bound ^{125}I -insulin with IGFR-specific antibody because of the relatively low affinity of hybrids for insulin [13]. Instead, total and hybrid insulin receptors were compared by immunoblotting with IR-specific polyclonal antiserum, after precipitation with IR-specific and IGFR-specific monoclonal antibodies respectively. In a similar

Figure 2 Measurement of IR/IGFR hybrids by immunoprecipitation and Western blotting

Duplicate samples of solubilized placental membrane proteins (10 μ l) were immunoprecipitated with anti-IR and anti-IGFR immunoadsorbents. The immunoprecipitates were eluted with 100 μ l of SDS/PAGE sample buffer and two 40 μ l samples of each eluate were subjected to electrophoresis on a 7.5% (w/v) gel. Proteins were transferred to nitrocellulose and the blot was cut in half. One portion was developed with IR-CT antiserum (**A**) and the other with IGFR-CT antiserum (**B**). Lanes 1 and 2, IR immunoprecipitates; lanes 3 and 4, IGFR immunoprecipitates; lanes 5 and 6, unprecipitated material from the IR immunoprecipitations (equivalent to 25% of material loaded in lanes 1 and 2); lanes 7 and 8, unprecipitated material from the IGFR immunoprecipitations (equivalent to 25% of material loaded in lanes 3 and 4); lane 9, 5 μ l of starting material (equivalent to 125% of material loaded in lanes 1–4). The positions of 97 and 66 kDa molecular mass markers are indicated.

way, the fraction of IGFR in hybrids was estimated by blotting with IGFR-specific polyclonal antiserum, after precipitation with IGFR-specific and IR-specific monoclonal antibodies.

In the crude microsomal membrane extract the anti-IR antiserum detected a diffuse component of approx. 96 kDa (Figure 2A, lane 9) that co-migrated with the IR β -subunit purified from NIH3T3 fibroblasts overexpressing the human IR (results not shown) and was precipitable by both IR-specific and IGFR-specific antibodies (Figure 2A, lanes 1–4). Additional proteins of approx. 88 and 84 kDa were also detected and were assumed to represent non-specific reactions. The supernatant from the anti-IR precipitation contained a well-defined component of slightly greater electrophoretic mobility than the bulk of the IR (Figure 2A, lanes 5 and 6). This component comprised 10% of the total immunoreactive material of molecular mass 90–98 kDa, and a 6-fold variation in the ratio of immunoadsorbent to placental extract did not affect its appearance (results not shown). It was therefore concluded that this protein was not related to the IR but represented a further non-specific reaction. The proportion of IR in hybrids was determined by quantifying the anti-IR- and anti-IGFR-precipitable β -subunits by phosphorimaging. It was estimated that $15 \pm 2\%$ of the IR was present as hybrid (mean $\pm$ S.D., for three independent determinations on one membrane extract).

The anti-IGFR antiserum detected two diffuse components of similar intensities in the placental extract, of approx. 97 and 93 kDa (Figure 2B, lane 9), of which the former co-migrated with IGFR purified from NIH3T3 fibroblasts overexpressing the human IGFR (results not shown). Both components were immunoprecipitated by the anti-IR and anti-IGFR antibodies. Similar heterogeneity of the IGFR has been noted previously in placenta, muscle, brain and fibroblasts [14,21–26] and it was suggested that this might be due in part to differential glycosylation [21]. It has also been proposed that there exist two distinct forms of IGFR, characteristic of fetal and adult tissues [22,24], but no second gene or alternative splicing mechanism has been identified to account for this. Alternatively the heterogeneity might be an experimental artifact arising from partial proteolysis. This would have to involve the extracellular N-terminal portion of the β -subunit, which has been shown to be relatively susceptible to tryptic cleavage [27], as size heterogeneity is still detected with antibodies directed against the extreme C-terminal sequence [14]. Both forms of the placental IGFR were present in hybrids, in

contrast with previous work that suggested that only the more slowly migrating component of the IGFR β -subunit doublet formed hybrids with IR in rat 1 fibroblasts [26].

The supernatant from the anti-IGFR precipitation contained a well-defined component of approx. 93 kDa (Figure 2B, lanes 5–8), which comprised approx. 25% of the total immunoreactivity present in the 90–98 kDa region. It was assumed that this protein was not related to the IGFR but reflected a non-specific reaction. The proportion of IGFR in hybrids was estimated by quantifying the anti-IGFR- and anti-IR-precipitable β -subunits by phosphorimaging. By this method, $80 \pm 8\%$ of the IGFR was present as hybrid (mean $\pm$ S.D., for three independent determinations on one membrane extract), in reasonable agreement with the results of ligand/receptor co-precipitation experiments on the same extract, which indicated that 72% of 125 I-IGF-I binding was to hybrids.

Determination of relative concentrations of IR and IGFR by immunoblotting

In our hands it has not proved possible to obtain reliable estimates of the relative concentrations of IR and IGFR polypeptide in a receptor preparation from measurements of radio-ligand binding. The amount of ligand bound at tracer concentrations will depend on the binding affinity, so that the relative bindings of 125 I-insulin and 125 I-IGF-I will not be a simple measure of relative receptor concentrations. In principle, the receptor concentration can be calculated by Scatchard analysis of binding data but this involves imprecise extrapolations and subjective judgements as to the significance of high-affinity and low-affinity sites. Therefore we sought to compare receptor concentrations by immunoblotting with specific antisera.

We first defined conditions under which anti-IR and anti-IGFR antisera would react equally with their respective antigens. IR and IGFR were isolated from transfected NIH3T3 fibroblasts overexpressing the receptors, by immunoprecipitation with specific monoclonal antibodies. The receptor subunits were resolved by SDS/PAGE under reducing conditions and quantified by densitometric scanning of the silver-stained gels. The ratio of staining of α and β subunits was the same for both receptors, allowing the calculation of the relative receptor concentrations in the respective cell extracts (results not shown). Equal amounts of the receptors were subjected to electrophoresis and blots were

Figure 3 Equalization of Western blotting signals of anti-IR and anti-IGFR sera

Equal amounts of immunopurified IR and IGFR, over a 30-fold concentration range, were electrophoresed, Western blotted and developed with IR.CT antiserum at 1:1000 dilution and IGFR.CT antiserum at 1:2000 dilution. The intensity of immunostaining of the β -subunits of each receptor was determined by phosphorimaging and plotted as a function of the amount of receptor loaded.

probed with the respective specific antisera at a range of dilutions (1:1000 to 1:10000) to determine the conditions under which equal signals were obtained in subsequent phosphorimaging. It was found that equal signals were obtained by using anti-IR serum at twice the concentration of anti-IGFR serum, for the batches of serum used throughout these experiments. When IR blots were probed with 1:1000 anti-IR antiserum, and IGFR blots with 1:2000 anti-IGFR antiserum, equal amounts of the receptors gave equal signals with their respective antibodies across a 30-fold range of receptor concentration (Figure 3).

The antibodies were used at the same dilutions to probe blots of solubilized placental membranes (Figure 4), and phosphorimager signals from anti-IR and anti-IGFR reactions were quantified over a range of concentrations of placental extract. These signals were corrected for the non-specifically reacting material co-migrating with receptor β -subunits (10% of anti-IR reaction; 25% of anti-IGFR), and the corrected signals were plotted as a function of protein concentration (Figure 5). Both receptors gave linear plots over a substantial range of extract concentrations, from which it was deduced that the concentration of IR β -subunit in the extract was 4-fold that of IGFR. It had

Figure 5 Quantification of placenta blots

The blots in Figure 4 were quantified by phosphorimaging and the data were corrected for the presence of non-specific reactions. The immunoblot signal for IR was taken to be 90%, and that for IGFR to be 75%, of the total immunoreactive material in the β -subunit regions (approx. 90–100 kDa) of the respective blots (see text for details).

been calculated previously for the same batch of extract that 15% of insulin receptors and 80% of IGF receptors were present as hybrids, which must necessarily contain equal amounts of IR and IGFR. Within experimental error, the data are therefore internally consistent, as 15% of 4 approximately equals 80% of 1.

DISCUSSION

Previous work has documented the presence of hybrid insulin/IGF receptors in placenta, a tissue in which the metabolic significance of insulin and IGF receptors is unclear, and in several cultured cell lines including transfected cells overexpressing insulin receptors [8–12]. The present study has shown for the first time that hybrid insulin/IGF receptors occur widely in mammalian tissues, including physiologically important targets for insulin and IGF. Indeed, hybrids represent the major form of IGF-I receptors in heart and skeletal muscle from both rabbits and humans.

The factors regulating the assembly of hybrid receptors, as opposed to classical insulin and IGF receptors, are unknown. Ligand-induced recombination of isolated half-receptors into

Figure 4 Blots of placental IR and IGFR with normalized antibodies

Solubilized placental membrane proteins were subjected to electrophoresis, Western blotted and developed with IR.CT antiserum at 1:1000 dilution (A) or IGFR.CT antiserum at 1:2000 dilution (B). Lanes 1–8 contained 54, 27, 13.5, 6.8, 3.4, 1.35, 0.68 and 0.27 μ g of solubilized proteins respectively. The molecular masses (in kDa) of the major immunoreactive components are indicated, as calculated from mobility relative to standard markers.

homo- or heterodimers *in vitro* occurred randomly and in proportion to the relative concentrations of each receptor [8]. The extent of hybrid receptor formation was also proportional to the molar ratio of insulin and IGF receptors when receptors were transiently co-expressed at high levels in transfected fibroblasts [28]. These data suggest that the structural features essential for dimerization are highly conserved between insulin and IGF receptors, such that preferential homodimerization to form classical receptors does not occur. It might then be expected that the relative levels of classical and hybrid receptors in a tissue should be predictable from the relative expression of IR and IGF polypeptides, and that the proportion of IGF receptors occurring as hybrids would increase as the ratio of IR to IGF increased. However, assembly of hybrids *in vitro* or in transfected cells might bypass regulatory mechanisms that could operate in normal tissues *in vivo*. In the present study there was no correlation between the fraction of hybrid receptors and the relative levels of insulin and IGF binding in tissue extracts. Cellular heterogeneity of tissues might contribute substantially to this lack of correlation: for instance, within liver it is likely that insulin receptors are expressed predominantly on hepatocytes, and IGF receptors on endothelial cells; the overall tissue levels will therefore not predict the hybrid fraction. A more detailed analysis of relative receptor levels was performed in term human placenta, where IR and IGF are localized primarily to cells of the fetal endothelium [29,30]. Co-precipitation of receptor/IGF complexes suggested that approx. 72 % of IGF-I binding was to hybrids in these cells, consistent with previous data [9]. Assuming equal affinity of classical and hybrid receptors for IGF-I, and allowing for the possibility that classical IGF have the capacity to bind either one or two molecules of IGF with high affinity [31,32], this would indicate that 56 % or 72 % respectively of IGF polypeptide is in hybrids. Selective inhibition of IGF binding to intact membranes suggested a somewhat smaller fraction of hybrids [8], possibly because of different affinities of receptors in membranes and in soluble form. Data from other laboratories indicated that the combined classical IGF plus hybrids from human placenta contained IGF and IR polypeptides in a ratio 2:1 [14], consistent with 50 % of the IGF polypeptide's being in hybrids. Similar approaches have not been fruitful in revealing the fraction of IR present as hybrids, because this fraction is probably quite small when IR polypeptides are in excess over IGF, and because hybrids have a lower affinity for insulin than classical IR [13].

The relatively abundant receptor expression in placenta facilitates detection by immunoblotting, in conjunction with specific immunoprecipitation, allowing the quantitative analysis of different receptor types without concern for ligand affinity. It was found that 15 ± 2 % of IR and 80 ± 8 % of IGF were in hybrids. If the total amounts of IR and IGF polypeptides are I and G , and the fractions of each in hybrids are f_{IR} and f_{IGF} respectively, then the level of hybrids is given by $H = I \cdot f_{IR} = G \cdot f_{IGF}$, and the relative levels of IR and IGF polypeptide are given by $r = I/G = f_{IGF}/f_{IR}$. Thus it can be deduced from the hybrid fractions that $I/G = 5.3 \pm 0.9$. The relative levels of IR and IGF polypeptide were also measured directly by using receptor-specific antisera under conditions adjusted to allow the quantitative comparison of signals from immunoblots. This analysis produced a value for I/G of 4, which is consistent within experimental error with the value deduced from the hybrid fractions.

Assuming that the residual IR and IGF polypeptides not in heterodimeric hybrids exist as homodimeric classical receptors, it can be calculated that the relative concentrations of IR homodimers, hybrids and IGF homodimers will be approx. 12:4:1 (0.70:0.24:0.06). Random dimerization of IR and IGF poly-

Figure 6 Expected fractions of hybrid receptors for a process of random assembly

If the mole fraction of IR polypeptide is f , and that of IGF polypeptide is $(1-f)$, then the expected fractions of IR homodimers, IGF homodimers and hybrid heterodimers will be respectively f^2 , $(1-f)^2$ and $2f(1-f)$. In (A) these predicted fractions are plotted as a function of the mole fraction of IR polypeptide. If the ratio of IR polypeptide to IGF polypeptide is r , then the expected fractions in hybrids will be respectively $1/(r+1)$ and $r/(r+1)$. In (B) these predicted fractions are plotted as a function of the ratio of IR to IGF.

peptides, at a relative expression level of $I/G = r$, would generate IR homodimers, hybrids and IGF homodimers in a ratio $r^2:2r:1$. Under these conditions, the fraction f_{IR} of IR in hybrids is $2r/(2r^2+2r)$ or $1/(r+1)$, and the fraction f_{IGF} of IGF in hybrids is $2r/(2r^2+2r)$ or $r/(r+1)$ (Figure 6). Random assembly from a pool where $I/G = 4$ would generate native IR homodimers, hybrids and IGF homodimers in a ratio 16:8:1 (0.64:0.32:0.04), with 20 % of IR polypeptide and 80 % of IGF polypeptide in hybrids. Corresponding ratios for $I/G = 5.3$ would be 23:9:1 (0.70:0.27:0.03), with 16 % of IR polypeptide and 84 % of IGF polypeptide in hybrids. In general, $f_{IR} + f_{IGF} = 1$ for a process of random assembly, whereas $f_{IR} + f_{IGF} < 1$ if classical receptors (homodimers) form more readily than hybrids (heterodimers), and $f_{IR} + f_{IGF} > 1$ if hybrid formation occurs preferentially.

The data for placental receptors ($f_{IR} + f_{IGF} = 0.95$) are therefore consistent, within experimental error, with a process of random assembly in which hybrids and classical receptors are formed with equal efficiency and in proportions determined by the molar ratio of IR and IGF polypeptides. This interpretation assumes that the tissue is homogeneous with regard to levels of

IR and IGFR expression in all cells, and that the relative levels of mature receptor protein (presumed to be largely in the plasma membrane) accurately reflect the relative pools in the subcellular compartment where initial assembly takes place. Most probably, assembly (dimerization and disulphide linkage) occurs only in the endoplasmic reticulum [3], and there is no evidence for further disulphide exchange or rearrangement of receptor subunits during the subsequent life history of the receptors. The possibility cannot be ruled out that homodimerization might be facilitated by the spatial or temporal separation of the synthesis of IR and IGFR. Moreover, the relative levels of cell-surface receptor expression might not reflect the proportions initially assembled if there are differences in turnover of different receptor types [26]. Thus it is difficult to draw definitive conclusions regarding hybrid assembly from receptor levels in whole cells, but the present data are consistent with other studies [8,28] suggesting that the features on which dimerization depends are highly conserved between IR and IGFR. Further studies to examine a wider range of tissues and conditions will be necessary to determine whether receptor assembly is random under all circumstances. There is only limited information available about the transcriptional or translational controls of IR and IGFR expression, which determine basal receptor levels in different cell types or modulate levels within a given cell type. It is not known whether there are additional controls on the post-translational processing and assembly of receptors. Thus the possibility that hybrid assembly can be specifically promoted or inhibited under certain pathophysiological conditions cannot yet be ruled out.

It remains to be determined whether hybrid receptors have unique signalling properties, distinct from those of IR and IGFR, that would necessitate their specific regulation. Indeed it is still a matter of debate whether there are intrinsic differences in signalling capacity of the IR and IGFR [33–36]. These issues are surprisingly difficult to address experimentally because IR and IGFR, and therefore also hybrid receptors, are expressed in almost all cell types and completely specific ligands are not available. Thus it is not possible to examine unambiguously the consequences of selective activation of hybrid or classical receptors in a given cell type. The alternative approach of comparing responses in multiple cell types that express different fractions of receptors as hybrids suffers from the problem that any differences in response cannot be simply ascribed to properties of the receptors alone. However, the availability of cell lines derived from mice with targeted disruption of the IR or IGFR genes [37,38] might allow the consequences of hybrid formation to be examined after transfection with the appropriate receptor cDNA. It is clear that both insulin and IGF-I are able to stimulate autophosphorylation of both β -subunits within hybrid receptors ([14], and K. Dib and K. Siddle, unpublished work), although IGF-I is bound with higher affinity than insulin [13,14]. Thus the potential exists for hybrids to possess signalling properties that are at least the sum of those of IR and IGFR; the possibility that they have specific properties arising from synergy between separate IR and IGFR signals cannot be ruled out.

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