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## **Characterisation of insulin/IGF hybrid receptors: contributions of the insulin receptor L2 and Fn1 domains and the alternatively spliced exon 11 sequence to ligand binding and receptor activation**

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Abbreviations: IR, insulin receptor; IGFR, type I insulin-like growth factor receptor; IGF-I, insulin-like growth factor 1, IGF-II, insulin-like growth factor 2; Rluc, Renilla luciferase; R-luc, luciferase-tagged receptor; GFP, green fluorescent protein; YFP, yellow fluorescent protein; CHO, Chinese hamster ovary; BRET, bioluminescence resonance energy transfer; RLIC, radioligand immunocapture.

## Synopsis

The insulin receptor (IR) and type I IGF receptor (IGFR) are homo-dimers but the respective pro-receptors can also hetero-dimerise to form insulin/IGF hybrid receptors. There are conflicting data on the ligand affinity of hybrids, and especially on the influence of different IR isoforms. To investigate further the contribution of individual ligand binding epitopes to affinity and specificity in the IR/IGFR family, we generated hybrids incorporating both IR isoforms and IR/IGFR domain-swap chimeras, by ectopic co-expression of receptor constructs in CHO cells, and studied ligand binding using both radioligand competition and bioluminescence resonance energy transfer assays. We found that IR-A/IGFR and IR-B/IGFR hybrids bound insulin with similar and relatively low affinity, intermediate between that of homodimeric IR and homodimeric IGFR. However, both IR-A/IGFR and IR-B/IGFR hybrids bound IGF-I and IGF-II with high affinity comparable to homodimeric IGFR. Incorporation of a significant fraction of either IR-A or IR-B into hybrids resulted in abrogation of insulin- but not IGF-I-stimulated autophosphorylation. We conclude that the sequence of 12 amino acids encoded by IR exon 11 has little or no effect on ligand binding and activation of IR/IGFR hybrids, and that hybrids bind IGFs but not insulin at physiological concentrations regardless of the IR isoform they contain. To reconstitute high affinity insulin binding within a hybrid receptor, chimaeras in which the IGFR L1 or L2 domains had been replaced by equivalent IR domains were co-expressed with full length IR-A or IR-B. In the context of an IR-A/IGFR hybrid, substitution of IR residues 325-524 (L2 domain + part of first fibronectin domain) for the corresponding IGFR sequence increased the affinity for insulin by 20 fold. We conclude that the L2 and/or first fibronectin domains of IR contribute in *trans* with the L1 domain to create a high affinity insulin binding site within a dimeric receptor.

## Introduction

The insulin receptor (IR) and type I IGF receptor (IGFR) are highly homologous members of subclass II of the tyrosine kinase receptor super-family [1, 2]. Unlike other receptor tyrosine kinases that are activated by ligand-induced dimerisation, IR and IGFR exist at the cell surface as pre-formed dimers composed of two  $\alpha\beta$  halves disulphide linked in a  $\beta$ - $\alpha$ - $\alpha$ - $\beta$  structure. Dimerisation of pro-receptors occurs post-translationally in the endoplasmic reticulum, prior to cleavage to generate  $\alpha$  and  $\beta$  subunits [3]. Where co-expressed IR and IGFR pro-receptors also heterodimerise to create insulin/IGF hybrid receptors [4-6]. Heterodimerisation occurs with similar efficiency to homodimerisation, so that the proportion of hybrids is a function of the mole fractions of the individual receptors. The less abundant of the receptors (IR and IGFR) in any given cell type is thus found predominantly in hybrids rather than classical homodimers [7, 8].

The extracellular portions of IR and IGFR contain six structural domains, as initially revealed by molecular modelling and confirmed by crystallographic studies [9, 10]. The N-terminal half consists of homologous L1 and L2 domains flanking a cysteine rich domain, while the C-terminal half consists of three fibronectin type III (FnIII) domains. The central FnIII domain has an unstructured insert region, within which is the site of cleavage between  $\alpha$  and  $\beta$  subunits. Ligand binding determinants have been localised to the L1, cysteine rich and L2 domains and to the carboxyl-terminal (CT) peptide sequence in the  $\alpha$  subunit (reviewed in [11]). Surprisingly, in view of its symmetrical dimeric structure, the IR binds only a single molecule of insulin with high affinity, and binding displays negative co-operativity. These properties were accounted for in the models of De Meyts [12] and Schäffer [13] which proposed that high affinity ligand binding requires contacts with sites on both  $\alpha$ -subunits, and that receptor activation is a consequence of cross-linking of  $\alpha$ -subunits by ligand. In the absence of a crystal structure of receptor in complex with ligand, it remains unclear which of the several binding epitopes act in *cis* and which in *trans*, to achieve high affinity binding.

Alternative splicing gives rise to two isoforms of IR, IR-A (exon 11-) and IR-B (exon 11+), while there is only a single isoform of IGFR (effectively exon 11-). IR exon 11 encodes 12 amino acids at the carboxyl-terminus of the  $\alpha$ -subunit, immediately downstream of the CT peptide that is essential for ligand binding [14, 15]. The IR isoforms have very similar affinity for insulin [16, 17], but IR-A has approx 10 fold higher affinity for IGF-I and IGF-II than IR-B [18]. IR-A has been reported to bind IGF-II with high affinity approaching that of insulin [19] and has been shown to mediate growth effects of IGF-II during embryonic development [20]. It has also been suggested that IGF-II acts through IR-A to elicit predominantly

mitogenic rather than metabolic effects, and as a more potent mitogenic agonist than insulin [21]. Thus IR-A has been implicated along with IGFR in malignant transformation [22].

Early studies of IR/IGFR hybrids assembled from half-receptors *in vitro* suggested that hybrids bound both insulin and IGF-I with high affinity [6]. Subsequent studies of receptors purified from human placenta indicated that hybrids bound IGF-I with higher affinity than insulin, and had lower affinity for insulin than classical IR [23, 24]. However these studies did not take account of IR isoforms. More recent work has suggested that IR-A/IGFR hybrids have significantly higher affinity for insulin, IGF-I and IGF-II than IR-B/IGFR hybrids, such that IR-A/IGFR hybrids might be responsive to all these ligands at near physiological concentrations [25]. Thus the influence of the IR exon 11 sequence on ligand binding may depend not only on which ligand is considered (insulin or IGF) but also on the receptor context (IR homodimer or hybrid). The fact that hybrid receptors (especially those incorporating the IR-B isoform) bind insulin with lower affinity than classical IR also suggests that interactions in *trans* from an IR half-receptor profoundly influence insulin binding. The aims of this study were to determine the influence of IR exon 11 on ligand binding, and to identify which IR domains are required in *trans* from the IR L1 domain to support high affinity insulin binding, within the context of hybrid receptors.

## Experimental

### Materials

Bovine insulin was from Sigma, recombinant human IGF-I was a gift from Genentech Inc (South San Francisco, CA, USA) and recombinant human IGF-II was from Calbiochem (Darmstadt, Germany). <sup>125</sup>I-insulin and <sup>125</sup>I-IGF-I were from Amersham Biosciences (Bucks, UK). Anti-phosphotyrosine antibody 4G10 was from Upstate Biotechnology Inc. Anti-insulin receptor monoclonal antibodies 83-7 and 83-14 and anti-IGFR monoclonal antibody 17-69 were as previously described [26, 27]. Rabbit polyclonal anti-IR antibody used for blotting was raised in house against a 16-mer peptide corresponding to the carboxyl-terminal sequence of the human IR  $\beta$ -subunit. IR-GFP and IGF-1R-GFP constructs, a generous gift from Dr Rosemary O'Connor (Biosciences Institute, National University of Ireland, Cork), were as previously described [28]. CHO cells overexpressing IR-A [17] were a gift from Dr David Moller, CHO cells overexpressing IR-B [29] were a gift from Dr Leland Ellis and NIH3T3 cells overexpressing IGFR [30] were a gift from Dr Axel Ullrich. Cell culture reagents (DMEM, FBS) were from Gibco BRL. Antibiotics (G418, puromycin) were from Sigma, and Coelenterazine was from Assay Designs (MI, USA).

### ***Construction and expression of cDNA encoding tagged receptors***

Full length IGFR cDNA in which the stop codon had been mutated was excised from the plasmid pIGF-1R-GFP [28] using EcoRI and ligated in frame to the yellow fluorescent protein in Eco-RI digested pEYFP-N1 (Clontech) to generate the construct pIGFR-YFP. Renilla luciferase coding sequence was amplified, and flanking BamHI and XbaI restriction sites (in bold) introduced, by PCR using pRL-TK (Promega) as a template with the primers LucFw: 5'-CACTATAGGCT**GGATCCA**TGACTTCG-3', LucRev: 5'-GCGGCCGCT**CTAGA**ATTATTGTTC-3'. The product was digested using BamHI and XbaI and inserted in similarly digested pIGFR-YFP in place of YFP and in frame to the stop-mutated IGFR to generate pIGFR-Luc. Full length IR cDNA in which the stop and adjacent codons had been mutated to a HindIII site was inserted in frame into HindIII digested pEYFP-N1 to generate pIR-YFP. Finally pIR-Luc was generated by excising the IGFR from pIGFR-Luc using EcoRI, religating the resultant RLuc plasmid and digesting this with HindIII prior to insertion of the full-length stop-mutated IR sequence. Restriction sites used to generate tagged receptors together with linkers from the expression plasmids added a sequence of 20 amino acids between IGFR and YFP in IGFR-YFP (GSSRILQSTVPRARDPPVAT), 16 amino acids between IGFR and luciferase in IGFR-Luc (GSSRILQSTVPRARDP), 19 amino acids between IR and YFP in IR-YFP (KLRILQSTVPRARDPPVAT), and 15 amino acids between IR and luciferase in IR-Luc (KLRILQSTVPRARDP).

### ***Cell culture and transfection of tagged receptors***

CHO cells were maintained in Hams F12 medium supplemented with 10% newborn calf serum. To generate cells stably expressing IR-A/IGFR and IR-B/IGFR for analysis by radioligand immunocapture (RLIC) assays, CHO cells previously transfected with IR-A or IR-B [17, 29] were seeded at  $1.2 \times 10^6$  cells in a 10 cm dish. Transfections were performed 24 h later, when cells were 60-75 % confluent, using Lipofectamine (Gibco BRL) with 8 µg of pCDNA3.1 vector encoding IGFR and a puromycin resistance gene. Following transfection, cells were grown in normal medium for 48 h before adding puromycin (15 µg/ml). After a further 5 days cells were transferred to 75cm<sup>2</sup> flasks and the populations expanded. For transient expression of receptors for BRET and autophosphorylation assays, CHO cells were seeded at  $5 \times 10^5$  cells per 10 cm dish and transfected 24 h later using Lipofectamine (Gibco BRL). For BRET experiments, cells were co-transfected with 0.5 µg R-luc cDNA and 0.5 µg empty vector or with 0.5 µg of R-luc and 0.5 µg of R-YFP or R-GFP (R being either IR or IGFR). For autophosphorylation experiments amounts of DNA used for transfection were as detailed in figure legends. Cells were harvested for receptor analysis 24 h after transfection.

### ***Radio-Ligand Immuno-Capture (RLIC) binding assays***

Ligand binding studies were conducted as described previously [15] using Immulon 4 HBX plates from Dynex Technologies, coated with monoclonal antibodies IR 83-7 or IGFR 17-69 which are highly specific for their respective receptors. The epitope of antibody 83-7 has been mapped to the cysteine rich domain of the IR and this antibody has been shown to have no effect on insulin binding to solubilised receptors from placental membranes (M.A. Soos & K. Siddle unpublished). The epitope of antibody 17-69 has been mapped to the first FnIII domain of IGFR, and this antibody similarly has no effect on ligand binding [26].

Triton X-100 lysates from cells expressing IR-A, IR-B, IR-A+IGFR or IR-B+IGFR were diluted in binding buffer so that, following immunocapture, 15-20% of added  $^{125}\text{I}$ -insulin or  $^{125}\text{I}$ -IGF-I tracer was bound in the absence of unlabelled ligand. Immunocaptured receptors were incubated with 50 pM  $^{125}\text{I}$ -insulin or 25 pM  $^{125}\text{I}$ -IGF-I for 16 h at 4°C, together with unlabelled insulin or IGF-I as specified for individual experiments. Plates were then washed with cold phosphate-buffered saline, and bound radioactivity was determined in a  $\gamma$ -counter. IC<sub>50</sub> values were objectively determined using GraphPad Prism by curve-fitting with a one-site competition model.

### ***Bioluminescence resonance energy transfer measurements***

BRET measurements were made essentially as previously described [31, 32]. Transfected CHO cells were cultured in Hams F12 medium containing 50  $\mu\text{g}/\text{ml}$  streptomycin, 50 units/ml penicillin and 10% newborn calf serum and lysed at 4°C in 50 mM HEPES pH7.4, 150 mM NaCl, 10 mM EDTA, 30 mM NaF, 10 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 1% Triton X-100, 2.5 mM benzamidine and 1  $\mu\text{g}/\text{ml}$  each of pepstatin, antipain, and leupeptin. Lysate (approx 10  $\mu\text{g}$  protein) was preincubated with insulin or IGF in 96-well microplates for 45 min at 20°C in a total volume of 60  $\mu\text{l}$  containing 30 mM MOPS, and 1 mM Na<sub>3</sub>VO<sub>4</sub>. Coelenterazine (10  $\mu\text{l}$ , final concentration 15  $\mu\text{M}$ ) was added and light emission acquisition at 410 nm (filter window 70 nm) and 530 nm (filter window 35 nm) was started immediately using a Fusion Microplate Analyser (Packard). The BRET ratio (BR) was defined in terms of emissions (E) at 530 and 410 nm as

$$\text{BR} = [\text{E}530/\text{E}410 (\text{R-luc} + \text{R-YFP})] - [\text{E}530'/\text{E}410' (\text{R-luc alone})]$$

### ***Autophosphorylation of receptors***

Receptor phosphorylation in intact cells was assessed by stimulating with insulin or IGF-I at 37°C. Following stimulation, cells were placed on ice, lysed as above, centrifuged and the supernatants were immunoprecipitated then analysed by SDS-PAGE and immunoblotting.

## Results

### *Radioligand competition analysis of ligand binding to IR, IGFR and IR/IGFR hybrids*

Before investigating ligand binding to hybrid receptors, we confirmed that we could replicate previously described characteristics of IR and IGFR homodimers using radioligand immunocapture assays with lysates of cells overexpressing IR-A, IR-B or IGFR (Figures 1 and 2). Concentrations of unlabelled ligand producing 50% inhibition of radioligand binding ( $IC_{50}$ ) were determined as a measure of affinity (Table 1). Both IR isoforms bound insulin with high affinity, approx two fold higher for IR-A than IR-B. There was a more marked difference between the isoforms with respect to IGF binding, IR-A having approx 10-fold higher affinity for IGF-I and 5-fold higher affinity for IGF-II. Both IR isoforms had higher affinity for IGF-II than IGF-I, but even IR-A had approx 10-fold lower affinity for IGF-II than for insulin. IGFR displayed high affinity for IGF-I but >1000-fold lower affinity for insulin. These results are broadly consistent with published data [18, 19, 25, 33].

Extracts of cells expressing IR-A+IGFR or IR-B+IGFR were used as a source of hybrid receptors. The IR-B/IGFR cells expressed rather more IR than the IR-A/IGFR cells, but expression of IGFR was similar in both cells and greater than that of IR (data not shown). Receptor concentrations in binding assays were adjusted to give similar binding of radioligand (~ 20% of total) in all cases. The percentage of hybrids was estimated as the fraction of  $^{125}I$ -IGF-I binding that was captured with IR-specific antibody 83-7 compared to IGFR-specific antibody 17-69. Hybrids accounted for approx 20% of  $^{125}I$ -IGF-I binding in IR-A/IGFR cells, and 15% of binding in IR-B/IGFR cells, consistent with the expression of the individual receptors. It would be expected that most IR would be incorporated into hybrids given that IGFR was expressed at a higher level than IR. The assay conditions used allowed ligand binding to hybrids to be specifically determined in crude cell lysates, as IGFR will not be captured by IR-specific antibody and  $^{125}I$ -IGF-I tracer at 25 pM will not bind significantly to immunocaptured IR. These assumptions were verified by control experiments with extracts of cells expressing only IR or IGFR (data not shown). It was not possible to perform reciprocal assays using IGF-specific capture antibody and  $^{125}I$ -insulin tracer because of the low affinity of hybrids for insulin.

Competition curves using unlabelled insulin, IGF-I and IGF-II are shown in Figure 3, and  $IC_{50}$  values are summarised in Table 1. IR-A/IGFR and IR-B/IGFR hybrids behaved similarly, with all ligands. The affinity of hybrids for insulin was relatively low ( $IC_{50} = 70 \pm 12$  nM for IR-A/IGFR and  $76 \pm 12$  nM for IR-B/IGFR), and intermediate between that of homodimeric IR ( $IC_{50} = 0.25$ - $0.51$  nM) and homodimeric IGFR ( $IC_{50} > 1\mu M$ ). Hybrids



bound both IGF-I and IGF-II with high affinity similar to that of homodimeric IGFR, again regardless of IR isoform. These findings differ from previous data suggesting that IR-A/IGFR hybrids had significantly higher affinity than IR-B/IGFR hybrids for all ligands [25] ( $IC_{50}$  values (nM) for Hybrid-R<sup>A</sup> and Hybrid-R<sup>B</sup> respectively of 3.7 and >100 for insulin; 0.3 and 2.5 for IGF-I; 0.6 and 15 for IGF-II).

### ***BRET analysis of ligand binding to IR, IGFR and IR/IGFR hybrids***

Because of the unexpected discordance between our own and previous data, we elected also to investigate ligand binding to hybrid receptors by bioluminescence resonance energy transfer (BRET). This technique, which has been validated previously as a measure of ligand binding to homodimeric IR and IGFR [31, 32], requires co-expression of receptor constructs tagged with luciferase (as donor) and fluorescent protein (as acceptor). Luciferase and YFP/GFP fused to the carboxyl-termini of the receptor  $\beta$ -subunits can engage in BRET, and the efficiency of energy transfer is influenced by changes in the conformation or proximity of  $\beta$  subunits induced by ligand binding. A 3-4-fold increase in BRET is observed upon ligand binding to IR or IGFR [31, 32], although the overall efficiency of BRET is low compared to what can be achieved by direct fusion of luciferase to GFP. It was previously established that energy transfer, and a resulting spectral shift, is only observed as an intramolecular event between two halves of the same receptor and not as an intermolecular event between dimeric receptors [32].

We created constructs in which IR and IGFR were tagged with luciferase, YFP or GFP, expressed the fusion proteins in CHO cells and stimulated with insulin and IGF-I. Western blotting of cell lysates with anti-IR, anti-IGFR or anti-phosphotyrosine antibodies (as appropriate) revealed proteins of apparent  $M_r$  125-135 kDa (data not shown) corresponding to the expected size of receptor  $\beta$  subunits fused to luciferase or YFP/GFP. It was concluded that the tagged receptors were efficiently expressed at the cell surface and retained their ability to undergo ligand-stimulated autophosphorylation *in situ*.

We next established that we could observe BRET from tagged IR expressed in CHO cells, and compared BRET signals from IR-B homodimers and IR-A/IR-B heterodimers. IR-B-luc was co-expressed with either IR-B-YFP or IR-A-GFP and cell lysates were incubated with insulin. Although IR-B-luc/IR-A-GFP co-transfected cells will contain both IR-B-luc and IR-A-GFP homodimers, resonance energy transfer can only occur within IR-B-luc/IR-A-GFP heterodimers. BRET signals comparable in magnitude to those previously reported [31, 32] were observed for both IR-B homodimers and IR-A/B heterodimers, and the BRET ratios were increased 3-4-fold by insulin (Figure 4). Under all conditions the BRET ratio was a

small difference between two relatively large numbers (the 530/410 ratio for luciferase alone was approx 0.8) and the precision with which ratios could be determined was limited by levels of emission, which in turn were limited by measurement times and the amount of cell extract that could be contained in a well. Nevertheless by objective curve-fitting for replicate independent experiments, the concentrations of ligand producing half-maximal increases in BRET ratio ( $EC_{50}$ ) could be determined with reasonable precision.  $EC_{50}$  values for insulin stimulation of IR-B homodimers and IR-A/B heterodimers were not significantly different (Table 2).

To investigate the activation of hybrid receptors, and to compare hybrids with IGFR homodimers, IGFR-luc was co-transfected with IR-A, IR-B or IGFR tagged with GFP or YFP. BRET ratios were again determined as a function of ligand concentration (Figure 5), and  $EC_{50}$  values calculated (Table 2). IR-A/IGFR and IR-B/IGFR hybrids displayed very similar properties. Both had moderately high affinity for IGF-I and relatively low affinity for insulin. Affinity for IGF-I was slightly lower than that of homodimeric IGFR, while affinity for insulin was very much lower than that of homodimeric IR and comparable to homodimeric IGFR. The results of the BRET assays were therefore in broad agreement with those of RLIC assays, in showing that hybrid receptors bound IGF-I with affinity comparable to homodimeric IGFR, but had low affinity for insulin compared to homodimeric IR, regardless of IR isoform.

#### ***Ligand-induced autophosphorylation of IR and IR/IGFR hybrids***

We next compared properties of hybrid receptors and homodimeric IR in intact cells, using autophosphorylation as a measure of activation. CHO cells were transiently transfected with IR-A or IR-B alone or with a 5-fold excess of IGFR which is sufficient to drive most of the IR into hybrids. The concentrations of cDNA used were adjusted to give similar expression of total IR protein (as assessed by anti-IR immunoblotting) under all conditions. Following stimulation with 10 nM insulin or IGF-I (a concentration chosen to give near maximal activation of cognate homodimeric receptor with minimal activation of the heterologous receptor) cells were lysed and immunoprecipitated with either IR-specific or IGFR-specific antibody. Hybrid receptors should be precipitated by both anti-IR and anti-IGFR antibodies. The anti-IR immunoprecipitates will additionally contain homodimeric IR (though it is expected that approx 80% of IR will be in hybrids), while the anti-IGFR immunoprecipitates will also contain homodimeric IGFR (approximately 4-fold excess over hybrids). Immunoprecipitates were resolved on SDS-polyacrylamide gels, and blots were probed with an anti-phosphotyrosine antibody then stripped and reprobed with anti-IR antibody. Under the

conditions used the  $\beta$ -subunits of IR and IGFR were not resolved and the phospho-signal is therefore a composite value for both IR and IGFR protein. Assuming reciprocal trans-phosphorylation [34], it would be expected that both  $\beta$ -subunits are phosphorylated equally within activated hybrids. Analysis of IR-A/IGFR hybrids is shown in Figure 6 and of IR-B/IGFR hybrids in Figure 7.

Addition of insulin (10 nM) to cells expressing IR alone markedly stimulated receptor phosphorylation (lanes 1 and 4 in Figures 6 and 7). However, when the same level of IR protein was co-expressed with an excess of IGFR, and therefore was mostly in hybrids, phosphorylation in response to 10 nM insulin was reduced to near basal levels (lanes 5 and 6 in Figures 6 and 7). The differences phosphorylation of IR alone compared to co-expression with IGFR were highly significant ( $P < 0.02$ ) for both IR-A and IR-B. The slightly higher level of phosphorylation in anti-IR compared to anti-IGFR immunoprecipitates (lane 5 vs lane 6) is attributable to the presence of some IR homodimers in the former but not the latter. It is concluded that insulin induces little or no stimulation of either IR-A/IGFR or IR-B/IGFR hybrids at a concentration (10 nM) sufficient maximally to activate homodimeric IR. In contrast, 10 nM IGF-I stimulated greater phosphorylation of hybrid receptors than homodimeric IR (lane 8 vs lane 7). Indeed, IGF-I-stimulated phosphorylation of hybrids approached that of insulin-stimulated IR homodimers (lane 8 vs lane 4). There was no detectable phosphorylation of IR-B homodimers in response to IGF-I, but some phosphorylation of IR-A homodimers (lane 7, Figure 6 vs Figure 7), consistent with their 10-fold higher affinity for IGF-I. It is concluded that in intact cells both IR-A/IGFR and IR-B/IGFR hybrids respond well to IGF-I but very poorly to insulin.

#### ***Reconstitution of high affinity insulin binding in a hybrid receptor***

To investigate the domains required to support high affinity insulin binding, IR was co-expressed with previously described chimeric IGFR constructs in which segments 1-137 (most of L1 domain) or 325-524 (L2 + part of first FnIII domain) of the IR  $\alpha$ -subunit had been substituted for equivalent segments of the IGFR [35]. We confirmed by anti-IGFR immunoblotting that both chimeras were processed and expressed with similar efficiency to wild-type IGFR when transfected into CHO cells (data not shown). The IGFR.1RL1 (IGF-1R/IR C1) and IGFR.1RL2/Fn (IGF-1R/IR C3') chimeras were then co-expressed with IR-A or IR-B so as to generate corresponding hybrid receptors, and binding properties of were assessed by RLIC assay. The IGFR cysteine-rich domain is a critical determinant of IGF-I binding [36] and as this domain is retained in both the chimeras it was possible still to employ

$^{125}$ I-IGF-I as tracer. The use of IR-specific antibody 83-7 for immunocapture ensured that observed binding was attributable to hybrid receptors rather than IGFR homodimers.

Binding data for the hybrid chimeras are shown in Figure 8 and Table 3. Substitution of the IR-L2/Fn1 domains within the IGFR dramatically increased insulin binding affinity of hybrids with IR-A ( $IC_{50}$  4 nM for IR-A/IGFR.IRL2/Fn compared to 70 nM for IR-A/IGFRwt) but had only a marginal effect on affinity of hybrids with IR-B ( $IC_{50}$  33 nM for IR-B/IGFR.IRL2/Fn compared to 76 nM for IR-B/IGFRwt). Replacement of the IGFR L1 domain with the equivalent IR domain produced only small increases in insulin affinity, within both IR-A/IGFR and IR-B/IGFR hybrids (approx 1.4-fold for IR-A/IGFR and 2.3-fold for IR-B/IGFR) which were not statistically significant. It is concluded that lack of IR L2 and/or FnIII-1 domains *in trans* with other IR domains is in large part responsible for the low affinity of hybrid receptors for insulin, although the presence or absence of IR exon 11 modulates the contribution of an L2/Fn domain in *trans*.

## Discussion

It is still unclear whether insulin/IGF hybrid receptors have a specific physiological role, and uncertainties remain even with regard to their affinity for and activation by potential ligands. We previously reported that hybrids bind insulin poorly compared to IR homodimers [24] although other studies have since produced conflicting data [25]. The first aim of this work was to re-evaluate the affinity of hybrid receptors for insulin and IGFs with particular regard to dependence on IR isoform. A second aim was to determine, within the context of hybrid receptors, which binding epitopes on individual  $\alpha$ -subunits are required to create a high affinity insulin binding site.

Radioligand competition binding assays are commonly used to study interactions between ligands and receptors, while autophosphorylation provides a convenient measure of insulin/IGF receptor activation in intact cells. We used both assays in conjunction with specific immunocapture or immunoprecipitation to characterise ligand interactions with hybrid receptors. We additionally employed a BRET technique that has been previously developed to study activation of IR and IGFR [31, 32] and very recently hybrid receptors [37]. An advantage of the BRET approach is that it allows activation of hybrids to be studied without purification or immunoprecipitation to remove homodimers.

A very clear conclusion from our studies was that heterodimeric hybrid receptors possess considerably higher affinity for IGF-I than for insulin. The affinity of hybrids for IGF-I and IGF-II is similar to (perhaps slightly lower than) that of homodimeric IGFR,

whereas their affinity for insulin is very substantially less than that of homodimeric IR. This conclusion was supported by RLIC assays (Table 1), ligand-dependent BRET (Table 2) and autophosphorylation in intact cells (Figures 6 and 7), and is consistent with previous studies of ligand binding to immunoaffinity-purified hybrid receptors [23, 24] and hybrid receptor activation in vascular endothelial or smooth muscle cells [38, 39].

Recent years have seen renewed interest in IR isoforms generated by alternative splicing of exon 11, particularly in relation to the role of IR-A in mediating actions of IGFs and in malignant transformation [22, 40] and in terms of isoform-specific signalling [41]. The sequence of 12 amino acids encoded by exon 11 lies immediately downstream of a sequence of 16 amino acids that is well conserved between IR and IGFR and makes a critical contribution to ligand binding in both receptors [14, 42]. The exon 11-encoded sequence has an inhibitory influence on ligand binding. We found that IR-B (exon 11+) had approx 2-fold lower affinity for insulin than IR-A (exon 11-) (Table 4), in agreement with most previous reports [16, 17] although one study found IR-B to have higher affinity than IR-A [33]. We also found that IR-B had approx 10-fold lower affinity for IGF-I and 5-fold lower affinity for IGF-II compared to IR-A, again broadly in line with previous work [18, 25, 33]. For both IR-A and IR-B the rank order of affinities was insulin>IGF-II>IGF-I (Table 4).

A previous study by Pandini and colleagues reported that IR-A/IGFR hybrids have significantly higher affinity for insulin, IGF-I and IGF-II than IR-B/IGFR hybrids [25]. They concluded that IR-B acts as a highly specific receptor for insulin, while IR-A expression up-regulates the IGF system both by increasing affinity of hybrids for IGFs and by allowing insulin to activate the IGFR in hybrids, and proposed that regulated expression of IR isoforms thus constitutes a molecular switch. While our manuscript was in preparation a report was published which questioned these findings [43]. We found no significant dependence of the properties of hybrids on IR isoform, whether assessed by RLIC, BRET or autophosphorylation. In RLIC assays, both IR-A and IR-B hybrids displayed low affinity for insulin, closer to that of IGFR than IR. We also found that both IR-A and IR-B hybrids had high affinity for IGFs, similar to that of IGFR. Our data therefore differ significantly from those of Pandini et al in several important respects (Table 4) and indicate that hybrids are responsive to IGFs and unresponsive to insulin regardless of the IR isoform they contain.

We can offer no explanation for these discordant findings. Both our study and that of Pandini et al involved human receptors and RLIC assays with the same tracer (<sup>125</sup>I-IGF-I) and antibody (anti-IR 83-7). We extracted receptors from populations of transfected CHO cells and performed binding assays for 16 h at 4° C, whereas Pandini and colleagues used receptors

from clones of transfected murine R- fibroblasts and measured binding after 2 h at room temperature, but it is difficult to see why such technical differences would differentially influence the properties of IR-A and IR-B hybrids. The very recent study of Slaaby and colleagues [43] also failed to find any dependence of ligand affinities on IR isoform and also concluded that signalling of insulin through hybrid receptors is unlikely to be physiologically relevant. Indeed, given that IR-A and IR-B homodimers bind insulin with similar affinity, there is no a priori reason to expect that the affinity of hybrid receptors for insulin would depend on IR isoform. Moreover, we found that IR-A/IR-B heterodimers had high affinity for insulin similar to homodimeric IR (Figure 4), indicating that asymmetry of hybrids with respect to exon 11 is not intrinsically inhibitory towards ligand binding.

Heterodimerisation between IR-A and IR-B has previously been investigated in a pancreatic  $\beta$  cell line [41]. It was reported that the isoforms localised to different regions of the plasma membrane and did not form heterodimers. It was thus concluded that the peptide sequence encoded by exon 11 acted as a targeting signal directing IR-A and IR-B to distinct lipid raft microdomains, and that this resulted in differential signalling by the two isoforms. However, several studies have shown that in fibroblasts both IR isoforms heterodimerise efficiently with IGFR [18, 25] and our work confirms that in CHO cells they also readily form hybrids with each other. Moreover, dimerisation of pro-receptors, and disulphide bond formation between  $\alpha$ -subunits, takes place as an immediate post-translational event in the endoplasmic reticulum [3]. Segregation of isoforms to prevent heterodimerisation would have to occur in this compartment, there being no evidence for disulphide exchange at subsequent stages of receptor maturation and trafficking. We conclude that, if mechanisms do exist to prevent heterodimerisation of IR isoforms and to target them to different regions of the plasma membrane, these must be highly specific for certain cell types and are unlikely to be of general importance.

Determination of affinities for ligand-receptor interactions strictly requires analysis of binding data by Scatchard or similar plots. In the case of IR and IGFR such plots are curvilinear, indicating negative co-operativity and/or heterogeneity of binding sites [12], so that no single value can be assigned for ligand affinity. We took  $IC_{50}$  (RLIC assays) or  $EC_{50}$  (BRET assays) as measures of relative affinity which should approximate to mean dissociation constants under the conditions used. The rank order of  $IC_{50}$  values for insulin binding in RLIC assays performed over 16 h at 4°C (Table 1) was the same as corresponding  $EC_{50}$  values for BRET assays involving incubations for 45 min at 20°C (Table 2), namely homodimeric IR < IR/IGFR hybrids < homodimeric IGFR. There were however some

significant differences in absolute values. For instance, the EC<sub>50</sub> for activation of IGFR by insulin in BRET assays was 70 nM, whereas IC<sub>50</sub> for insulin binding in RLIC assays was >1000 nM. This may in part reflect the high affinity of IGF-I for its own receptor and the need for very high concentrations of insulin to displace IGF-I bound to the receptor.

The IC<sub>50</sub> for insulin competition with <sup>125</sup>I-IGF-I tracer binding to IR-A/IGFR and IR-B/IGFR hybrids in RLIC assays in the present study (approx 70 nM) was very similar to that reported previously for immuno-affinity purified receptors from human placenta, which should contain both forms of hybrid [24]. In the present study the quantities of hybrid receptors immunocaptured on microtitre plates were not sufficient to bind a significant fraction of <sup>125</sup>I-insulin at tracer concentrations, but in the previous study the different assay format and greater quantities of material available enabled competition studies to be carried out also with <sup>125</sup>I-insulin as tracer. These homogeneous binding assays produced a significantly lower IC<sub>50</sub> value for insulin binding to hybrids (3-5 nM), similar to what has been reported for isolated half-receptors [44, 45], although still some 10-fold higher than the value for homodimeric IR.

The dependence of IC<sub>50</sub> on the radioligand may to some extent be a function of the effectiveness of homologous versus heterologous competition between unlabelled ligand and radiolabelled tracer. However, it is probable that the different tracers bind to different sites on hybrid receptors, <sup>125</sup>I-insulin predominantly to the IR half and <sup>125</sup>I-IGF-I to the IGFR half, so that unlabelled insulin competes directly with <sup>125</sup>I-insulin but indirectly with <sup>125</sup>I-IGF-I. The question then arises as to which IC<sub>50</sub> value is more relevant to activation of hybrid receptors by insulin. In the present, study the EC<sub>50</sub> for activation of hybrids by insulin in BRET assays was similar to the IC<sub>50</sub> for heterologous competition in RLIC assays, suggesting that the latter is a good measure of productive ligand binding leading to receptor activation. Although there may be a site on hybrid receptors with higher affinity for insulin, as revealed in homogeneous radioligand competition assays [24], binding at this site appears not to be productive of receptor activation as assessed by BRET.

A framework for interpreting these anomalies is provided by the binding model of DeMeyts [11], which proposes that high affinity insulin binding to IR results from a single insulin molecule cross-linking two distinct binding sites, one on each  $\alpha$ -subunit. Site 1 on one  $\alpha$ -subunit contributes the greater part of the binding energy by interacting with the classical receptor-binding surface of insulin, while site 2 on the other  $\alpha$ -subunit has lower intrinsic affinity and interacts with a distinct and more recently defined binding surface of insulin. The model predicts two possible modes of insulin binding to a hybrid receptor, in which the

classical binding surface of insulin interacts with either the IR half or the IGFR half (Figure 9). The former mode would be expected to have the higher intrinsic affinity, but our data suggest that the latter may lead more readily to receptor activation. However, the fact that homodimeric IGFR can be activated by insulin, albeit at high concentrations, indicates that insulin can interact to some degree with both sites 1 and 2 of IGFR. In contrast to insulin, IGFs bind to hybrid and homodimeric IGFRs with similar affinity as measured by  $IC_{50}$  in RLIC assays, although there was a small difference in the respective  $EC_{50}$  values as determined in BRET assays. Thus it would appear either that effective site 2 interactions for IGF-I can be provided as effectively by IR sequences as by those in IGFR, as also suggested by studies of chimeric receptors [46], or that such interactions contribute relatively little to IGF binding. In fact it remains uncertain whether the binding mechanisms of insulin and IGF are completely analogous. For instance IR and IGFR exhibit subtle differences in the effect of disulphide reduction on ligand binding [44, 45, 47, 48] and in the concentration dependence of ligand dissociation kinetics [46].

The crystal structures of monomeric amino-terminal fragments of IR and IGFR revealed a putative ligand binding cavity flanked by the L1, Cys-rich and L2 domains [49, 50]. The L1 domain of IR has been identified as a key region conferring affinity and specificity for binding of insulin compared to IGFs [11], while the Cys-rich domain of IGFR makes contributions to binding of IGF-I but not IGF-II [36, 51]. However, IR or IGFR constructs consisting only of these three domains do not bind ligand, and a sequence of 16 amino acids from the  $\alpha$ -subunit carboxyl terminus (CT domain) is essential for ligand binding [14, 52]. The recently reported crystal structure of the disulphide-linked IR ectodomain dimer reveals a folded over conformation that places the ligand binding regions of both  $\alpha$ -subunits in juxtaposition [10], and allows descriptions of two ligand contact sites to be made that are fully consistent with the binding model of De Meyts [11]. Site 1, corresponding to the low affinity binding site of half-receptors, involves the L1 domain (and probably the CT domain, though this is not revealed in the crystal structure) and is believed to contact the classical binding surface of insulin. Site 2 involves one or more of the AB, CC' and EF loops between  $\beta$ -strands of the first FnIII domain (for historical reasons this is variously referred to by different authors as FnIII-0 or FnIII-1) and is believed to contact the hexamerisation surface of insulin. In fact the FnIII-1 domain together with the adjacent L2 domain has recently been characterised experimentally as a second insulin binding site [53]. The structure of the dimeric IR ectodomain thus supports a model of ligand binding involving interaction of insulin with site 1 of one  $\alpha$ -subunit and site 2' of the other, providing an explanation for high



affinity, negative co-operativity, and conformational changes leading to receptor activation. While the two L1 domains are too far apart to allow insulin to contact both simultaneously, the two L2 domains are much closer together [10]. In the absence of detailed structural information for receptor-ligand complexes, it remains unclear whether the L2 domain contributes to site 1 or site 2, i.e. in *cis* or in *trans* relative to the L1 domain.

We addressed this question by studying the properties of hybrid receptors comprised of wild-type IR together with chimeric IGFR in which either the L1 or L2/Fn domain had been replaced with the equivalent portion of IR. We found that, within the context of an IR-A/IGFR hybrid receptor, replacing the L2/Fn domain of the IGFR with the equivalent domain from the IR (residues 325-524, including almost the whole L2 domain and  $\beta$ -strands A, B and C of the FnIII-1 domain) increased the affinity for insulin approx 20-fold (Table 3). However, replacement of the same domain within IR-B/IGFR resulted in a much smaller increase in insulin binding affinity of approx 2-fold. We conclude that in dimeric receptors the IR L2/Fn1 domains contribute to insulin binding in *trans* from the major insulin contact site in the L1 domain. There is asymmetry between the IR and IGFR in that high affinity insulin binding requires specifically the IR L2/Fn1 region, while both the IGFR and IR L2/Fn1 regions support high affinity binding of IGF-I, in the context of chimeric receptors [46] as well as hybrids. It remains to be determined whether the critical contribution to insulin binding involves residues within the C-terminal portion of the L2 domain or N-terminal portion of the FnIII-1 domain. Moreover, the extent of this contribution is evidently influenced by the presence of the IR exon 11 sequence. Alanine scanning of the insulin binding site has revealed differences between the IR isoforms in the relative energetic contributions of common receptor side chains to insulin binding, suggesting that insulin employs different modes of interaction with the two isoforms to achieve similar binding and that there is significant accommodation for structural changes induced by the presence of the extra 12 amino acids in IR-B [54]. In conclusion, our data support a model in which bound insulin contacts both  $\alpha$ -subunits within dimeric receptors and suggest that effective cross-linking of  $\alpha$ -subunits is essential for receptor activation. Further studies of hybrid receptors incorporating IR/IGFR domain exchanges should allow the contributions of individual binding epitopes, in *cis* or in *trans*, to be defined more precisely.

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Receptor	Radioligand	IC <sub>50</sub> (nM)		
		Insulin	IGF-I	IGF-II
IR-A	<sup>125</sup> I-insulin	0.25 ± 0.11	9.0 ± 2.6	2.2 ± 0.1
IR-B	<sup>125</sup> I-insulin	0.51 ± 0.12	90 ± 6	10 ± 1
IR-A/IGFR	<sup>125</sup> I-IGF-I	70 ± 12	0.5 ± 0.2	0.7 ± 0.1
IR-B/IGFR	<sup>125</sup> I-IGF-I	76 ± 12	0.3 ± 0.1	0.3 ± 0.1
IGFR	<sup>125</sup> I-IGF-I	> 1μM	0.5 ± 0.1	-

**Table 1: IC<sub>50</sub> values of IR, IGFR and hybrids as measured by ligand competition assays**

Radioligand immunocapture assays were performed using <sup>125</sup>I-insulin or <sup>125</sup>I-IGF-I as tracer as shown in Figures 1, 2 and 3. IC<sub>50</sub> values were determined using GraphPad Prism by curve-fitting with a one-site competition model. Values are the means ± SEM of three independent experiments each conducted with triplicate incubations.

Receptor	Tags	EC <sub>50</sub> (nM)	
		Insulin	IGF-I
IR-A	-	-	-
IR-B	IR-luc/IR-YFP	0.1 ± 0.1	-
IR-A/IR-B	IR-luc/IR-GFP	0.06 ± 0.02	-
IR-A/IGFR	IGFR-luc/IR-GFP	60 ± 10	4.0 ± 0.5
IR-B/IGFR	IGFR-luc/IR-YFP	40 ± 5.0	4.0 ± 1.0
IGFR	IGFR-luc/IGFR-YFP	70 ± 12	1.0 ± 0.5

**Table 2: EC<sub>50</sub> values for IR, IGFR and IR/IGFR hybrids as measured by BRET assay**

BRET assays were performed as shown in Figures 4 and 5. EC<sub>50</sub> values were determined using GraphPad Prism by curve-fitting with a one-site competition model. The values are means ± range of two independent experiments each conducted in duplicate.

Receptor	Insulin IC <sub>50</sub> (nM)
IR-A/IGFR	70 ± 12
IR-B/IGFR	76 ± 12
IRA-IGFR.IRL1	50 ± 15
IRA-IGFR.IRL2Fn	4 ± 2
IRB-IGFR.IRL1	33 ± 18
IRB-IGFR.IRL2Fn	33 ± 13

**Table 3. IC<sub>50</sub> values of the domain swap IR/IGFR chimaeric hybrids as measured by ligand competition assays**

Radioligand immunocapture assays were performed using <sup>125</sup>I-IGF-I as tracer, as shown in Figure 8. IC<sub>50</sub> values were determined using GraphPad Prism by curve-fitting with a one-site competition model. Values are the means ± SE of three independent experiments each conducted with triplicate incubations.

		IC <sub>50</sub> (nM)		
		Insulin	IGF-I	IGF-II
IR-A/IR-A	This work	0.3	9	2.2
	Pandini et al	0.2	>30	0.9
	Yamaguchi et al	0.9	41	-
	Denley et al	2.8	120	18
IR-B/IR-B	This work	0.5	90	10
	Pandini et al	0.3	>30	11
	Yamaguchi et al	1.6	390	-
	Denley et al	1.4	366	68
IR-A/IGFR	This work	70	0.5	0.7
	Pandini et al	3.7	0.3	0.6
IR-B/IGFR	This work	76	0.3	0.3
	Pandini et al	>100	2.5	15

**Table 4. IC<sub>50</sub> values for ligand binding to IR isoforms and hybrids**

Data are from the present study, Pandini et al [25], Yamaguchi et al [18] or Denley et al [33]. See original publications for experimental details, but note that different studies used different conditions (intact cells or solubilised receptors, incubation at 4° or room temperature, <sup>125</sup>I-insulin, Eu-labelled insulin or <sup>125</sup>I-IGF-I tracer).

## Legends to Figures

### Figure 1: Ligand binding to IR-A and IR-B homodimers

IR-A and IR-B expressed in CHO cells were immunocaptured using anti-IR antibody 83-7 and binding of  $^{125}\text{I}$ -insulin (50 pM) was measured in the presence of increasing concentrations of unlabelled insulin (A), unlabelled IGF-I (B), or unlabelled IGF-II (C). Binding to IR-A (open symbols) and IR-B (filled symbols) is expressed as a percentage of the value in the absence of unlabelled ligand. Data points are the means  $\pm$  SEM of three independent experiments in (A) and triplicate samples within a representative experiment in (B) and (C).

### Figure 2: Ligand binding to IGFR homodimers

IGF-I receptors expressed in NIH3T3 cells were immunocaptured using anti-IGFR antibody 17-69 and binding of  $^{125}\text{I}$ -IGF-I (25 pM) was measured in the presence of increasing concentrations of unlabelled insulin (open symbols) or IGF-I (filled symbols). Binding is expressed as a percentage of the value in the absence of unlabelled ligand. Data points are the means  $\pm$  SEM of triplicate samples within a representative experiment.

### Figure 3: Ligand binding to IR-A/IGFR and IR-B/IGFR hybrids

Hybrid receptors IR-A/IGFR and IR-B/IGFR were immunocaptured using IR-specific antibody 83-7 and the binding of  $^{125}\text{I}$ -IGF-I (25 pM) was determined in the presence of unlabelled insulin (A), unlabelled IGF-I (B) or unlabelled IGF-II (C). Binding to IR-A/IGFR (open symbols) and IR-B/IGFR (filled symbols) is expressed as a percentage of the value in the absence of unlabelled ligand. Data points are the means  $\pm$  SEM of triplicate samples within a representative experiment in (A) and (C) and three independent experiments in (B).

### Figure 4: Effect of insulin on BRET signal of IR-B homodimers and IR-A/IR-B heterodimers

Lysates of cells co-expressing IR-B-luc with either IR-B-YFP (A) or IR-A-GFP (B) were incubated with insulin and the BRET ratio was measured as described in Methods. Results shown are means  $\pm$  range of two independent experiments.

### Figure 5: Effects of insulin and IGF-I on BRET signal of IGFR and IR/IGFR hybrids

Lysates of cells co-expressing IGFR-luc with either IGFR-YFP, IR-A-GFP or IR-B-YFP were incubated with insulin (A) or IGF-I (B) and the BRET ratio was measured as described in

Methods. Results are mean  $\pm$  range of two independent experiments each conducted in duplicate.

### **Figure 6: Phosphorylation of IR-A in homodimers and in hybrids**

CHO cells were transfected with 40 ng IR-A cDNA, or 1  $\mu$ g IGFR plus 0.2  $\mu$ g IR-A cDNAs. The cells were stimulated with 10 nM insulin or IGF-I and lysates were immunoprecipitated with either anti-IR 83-14 ( $\alpha$ IR) or anti-IGFR 17-69 ( $\alpha$ IGFR) and resolved on SDS-PAGE. A) Immunoblots were probed with anti-phosphotyrosine antibody (4G10). The membrane was then stripped and reprobed with anti-IR antibody (rabbit polyclonal recognising carboxyl-terminal domain). The blots shown are representative of three independent experiments. B) Anti-phosphotyrosine blots (as in A) were scanned and quantified using MacBAS V2.2, and values were normalised for receptor expression as determined by scanning the anti-IR blots. The results are mean  $\pm$  SEM for three independent experiments. Insulin-stimulated phosphorylation was significantly less (\* $P$  < 0.01) in presence of IGFR (lanes 5 and 6) compared to IR alone (lane 4) as assessed by two-tailed paired Student's T-test.

### **Figure 7: Phosphorylation of IR-B in homodimers and in hybrids**

CHO cells were transfected with 80 ng IR-B cDNA or 1  $\mu$ g IGFR plus 0.4  $\mu$ g IR-B cDNAs. The cells were stimulated with 10 nM insulin or IGF-I and lysates were immunoprecipitated with either anti-IR 83-14 ( $\alpha$ IR) or anti-IGFR 17-69 ( $\alpha$ IGFR) and resolved on SDS-PAGE. A) Immunoblots were probed with anti-phosphotyrosine antibody (4G10). The membrane was then stripped and reprobed with an anti-IR antibody. The blots shown are representative of three independent experiments. B) Anti-phosphotyrosine blots (as in A) were scanned and quantified using MacBAS V2.2, and values were normalised for receptor expression as determined by scanning the anti-IR blots. The results are mean  $\pm$  SE for three independent experiments. Insulin-stimulated phosphorylation was significantly less (\* $P$  < 0.02) in presence of IGFR (lanes 5 and 6) compared to IR alone (lane 4) as assessed by two-tailed paired Student's T-test.

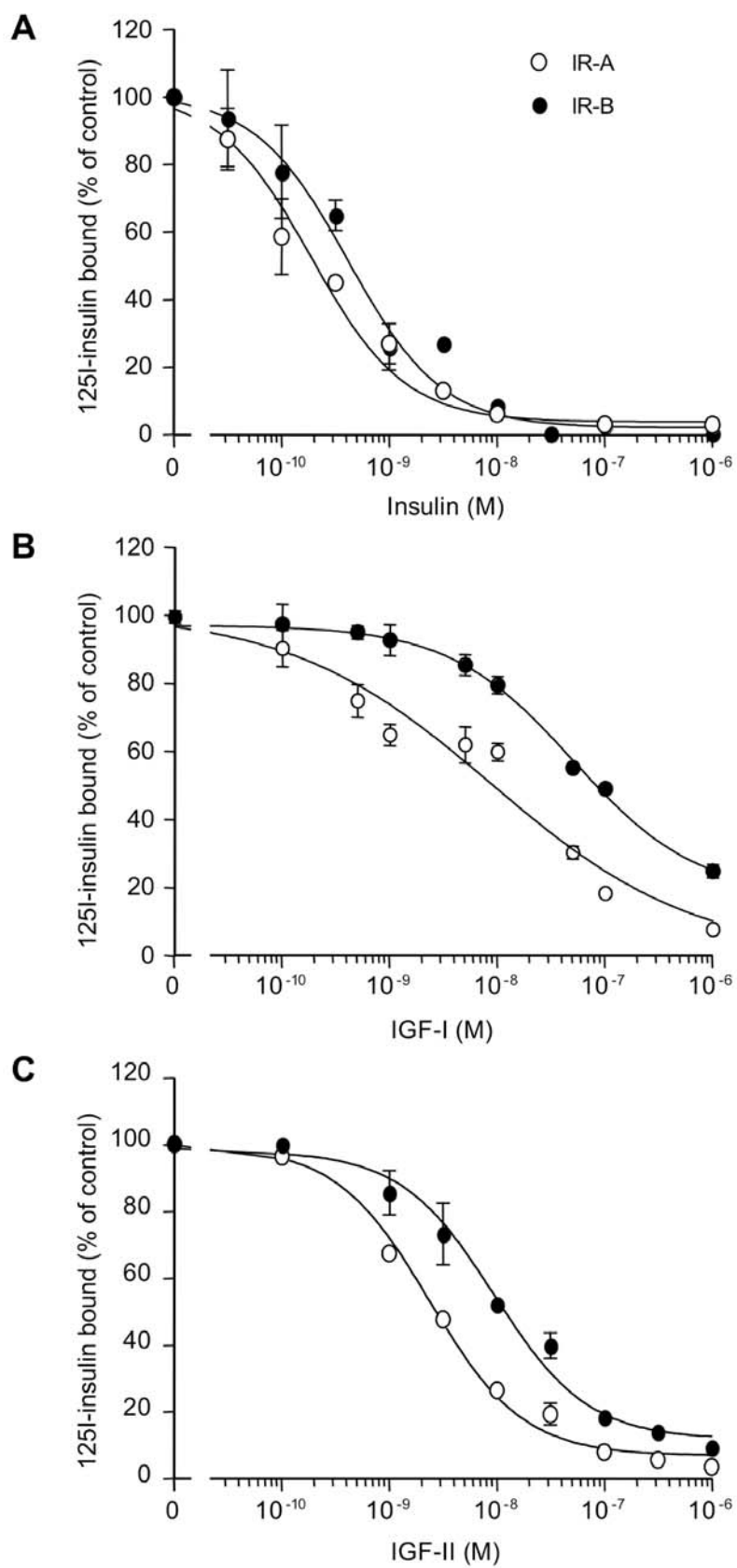
### **Figure 8: Reconstitution of high affinity insulin binding using chimeric receptors**

A) Schematic representation of the IR, IGFR and IGFR domain swap chimaeras (as previously described [35]). Domains are indicated as L1, CR (Cys-rich), L2, Fn1, Fn2, Fn3 (extracellular) and TK (intracellular), with IR open and IGFR shaded.

B) IR and chimaeric IGFR constructs were co-expressed in CHO cells as described in Methods. Cells were lysed, hybrid receptors were immunocaptured using anti-IR antibody 83-7 and binding of  $^{125}\text{I}$ -IGF1 (25 pM) was measured in the presence of unlabelled insulin. Binding is expressed as percentage of value in the absence of unlabelled ligand. Data points are the means  $\pm$  SEM of triplicate samples within a representative experiment.

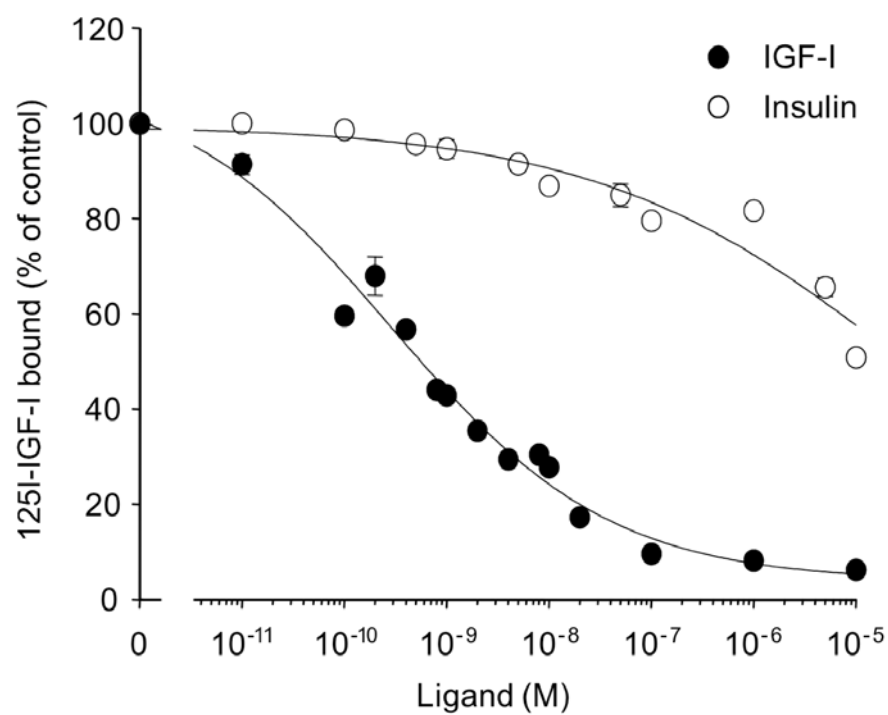
### **Figure 9: Model of ligand binding to hybrid receptors**

The model is based on that of DeMeyts and Whittaker [11] and assumes that only a single molecule of ligand binds with high affinity to homodimeric IR or IGFR, by contacting sites on both  $\alpha$ -subunits, of which site 1 contributes the greater fraction of binding energy. A and B represent binding of insulin and IGF to homodimeric IR and IGFR respectively. C and D represent two potential modes of binding to hybrid receptors, in which site 1 is contributed by the IR or IGFR half respectively. E and F represent the presumed binding modes of labelled insulin and IGF-I respectively, illustrating that  $\text{IC}_{50}$  values for competition by unlabelled insulin will be different for the two labelled ligands. The  $\text{EC}_{50}$  for receptor activation by insulin correlates with the  $\text{IC}_{50}$  for labelled IGF, suggesting that of the two possible insulin binding modes D may be more effective than C in leading to receptor activation.



**Figure 1: Ligand binding to IR-A and IR-B homodimers**





**Figure 2: Ligand binding to IGFR homodimers**

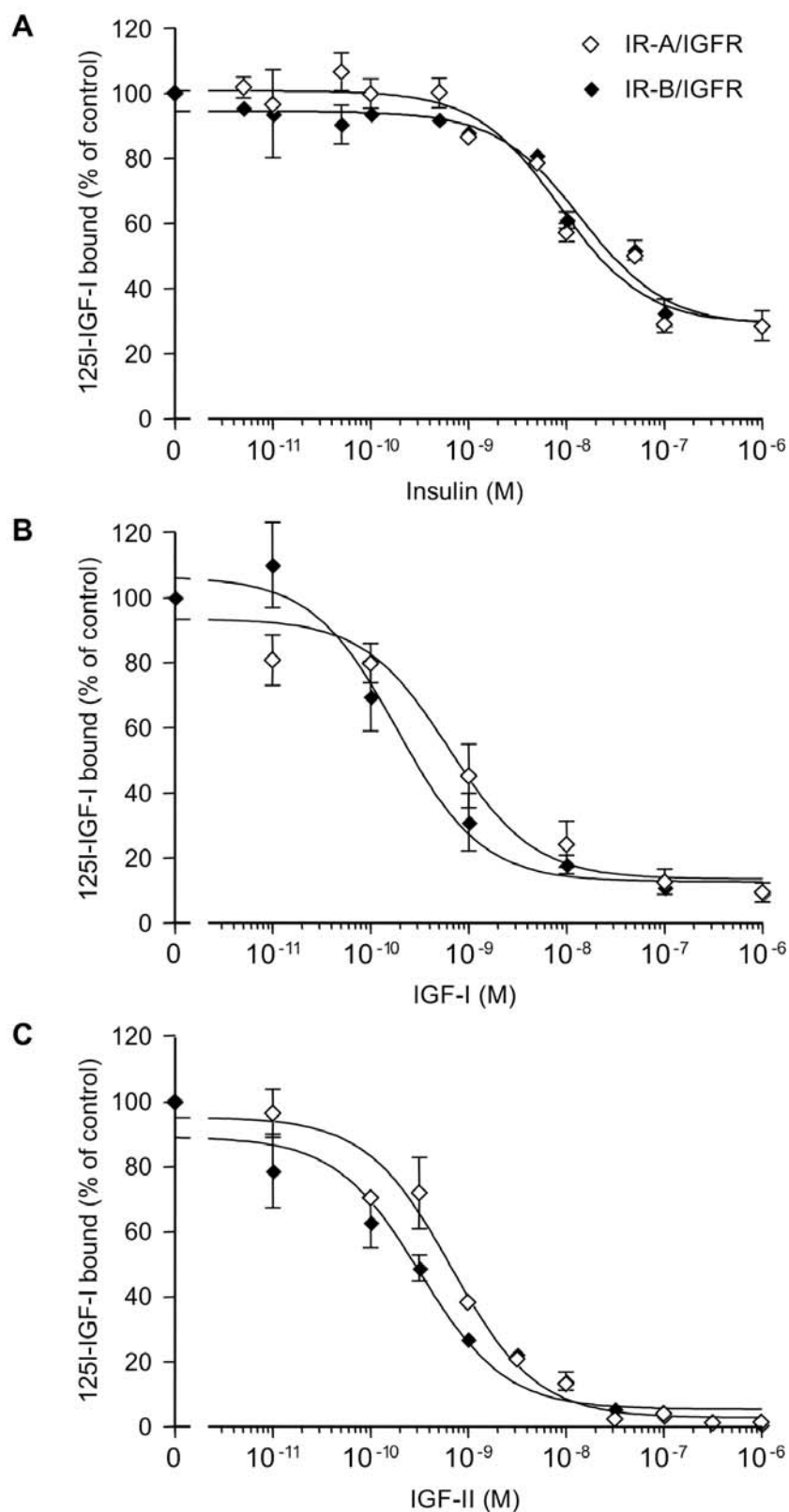
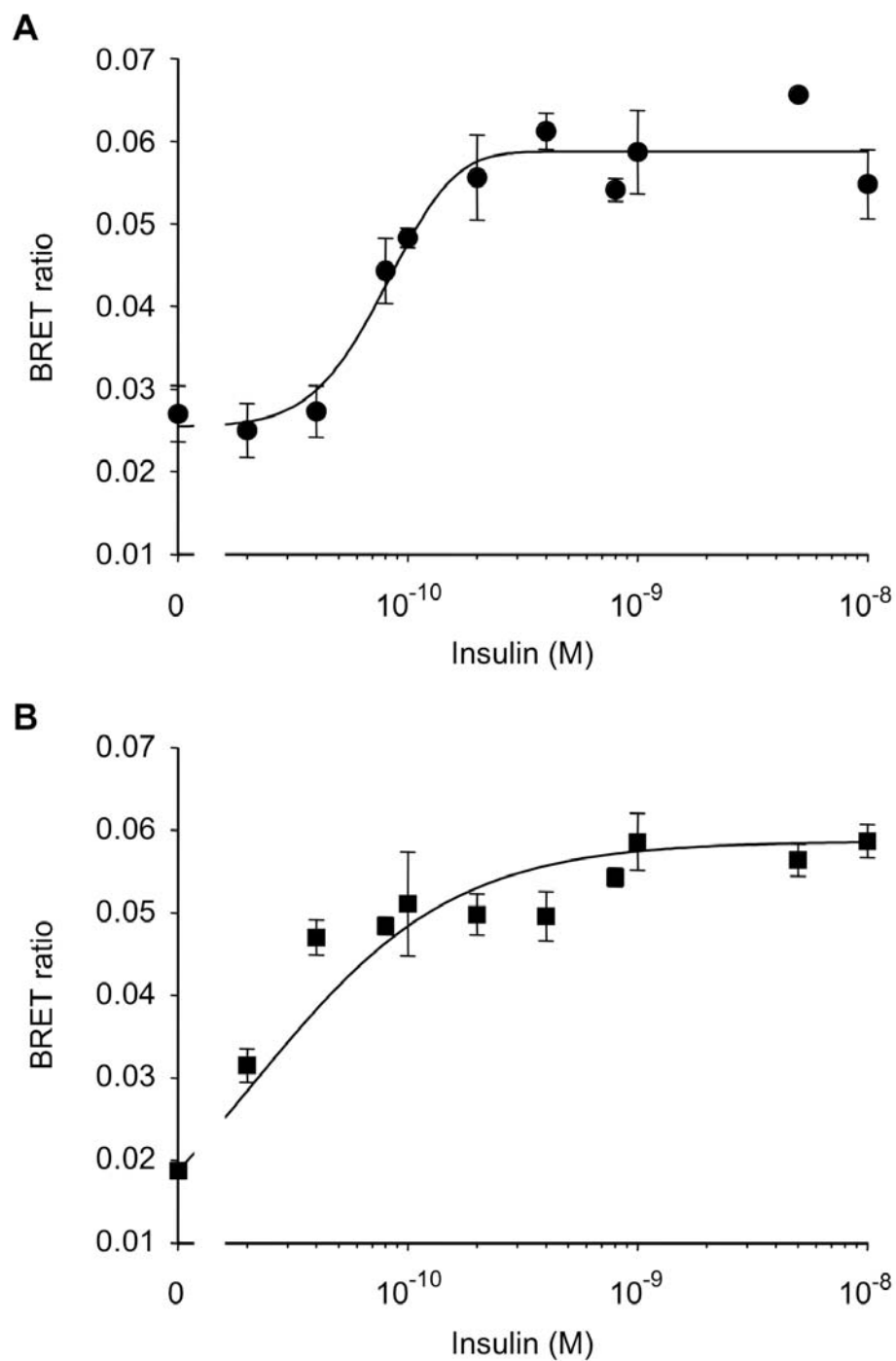


Figure 3: Ligand binding to IR-A/IGFR and IR-B/IGFR hybrids



**Figure 4: Effect of insulin on BRET of IR-B homodimers and IR-A/IR-B heterodimers**

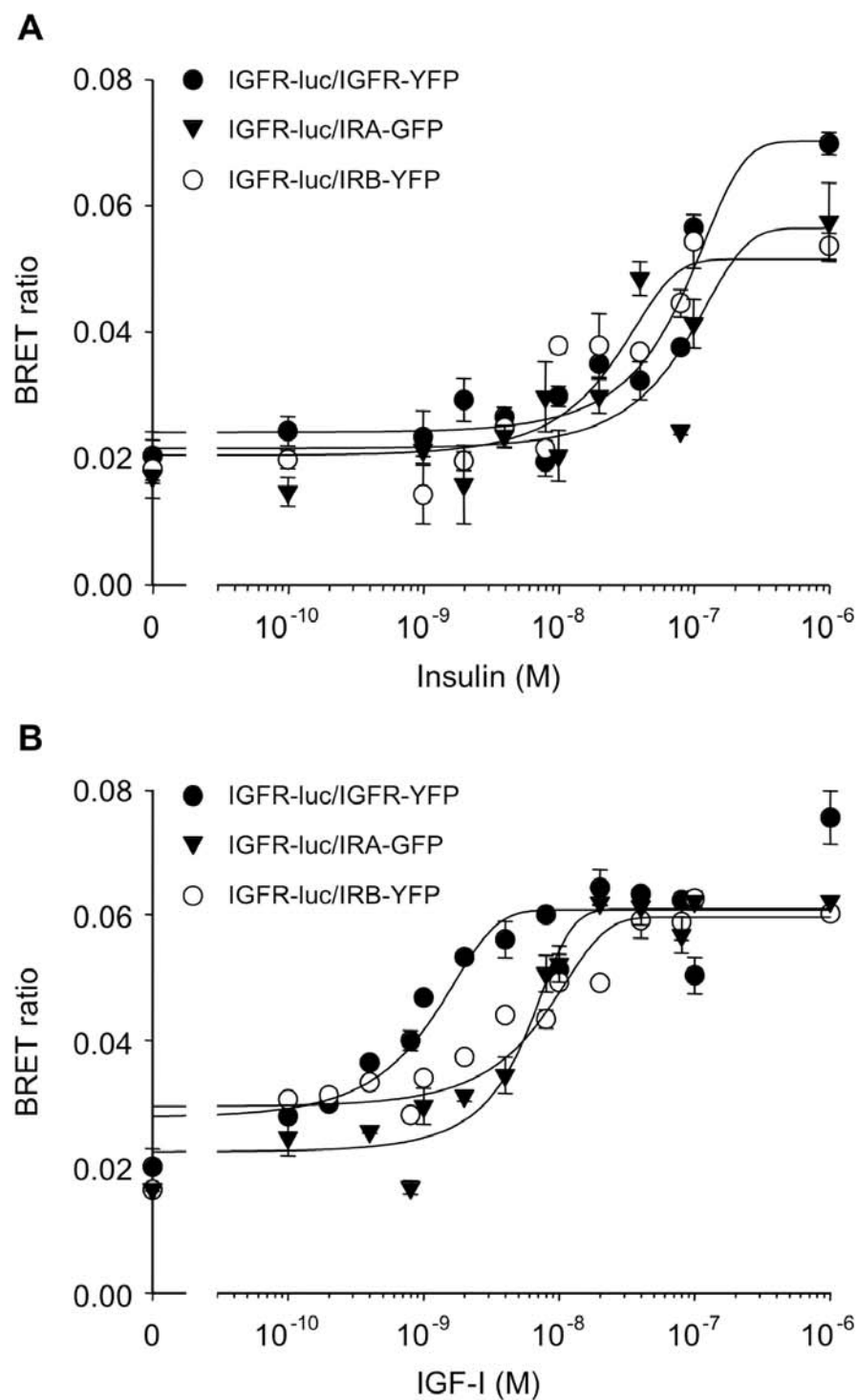


Figure 5: Effects of insulin and IGF-I on BRET of IGFR and IR/IGFR hybrids

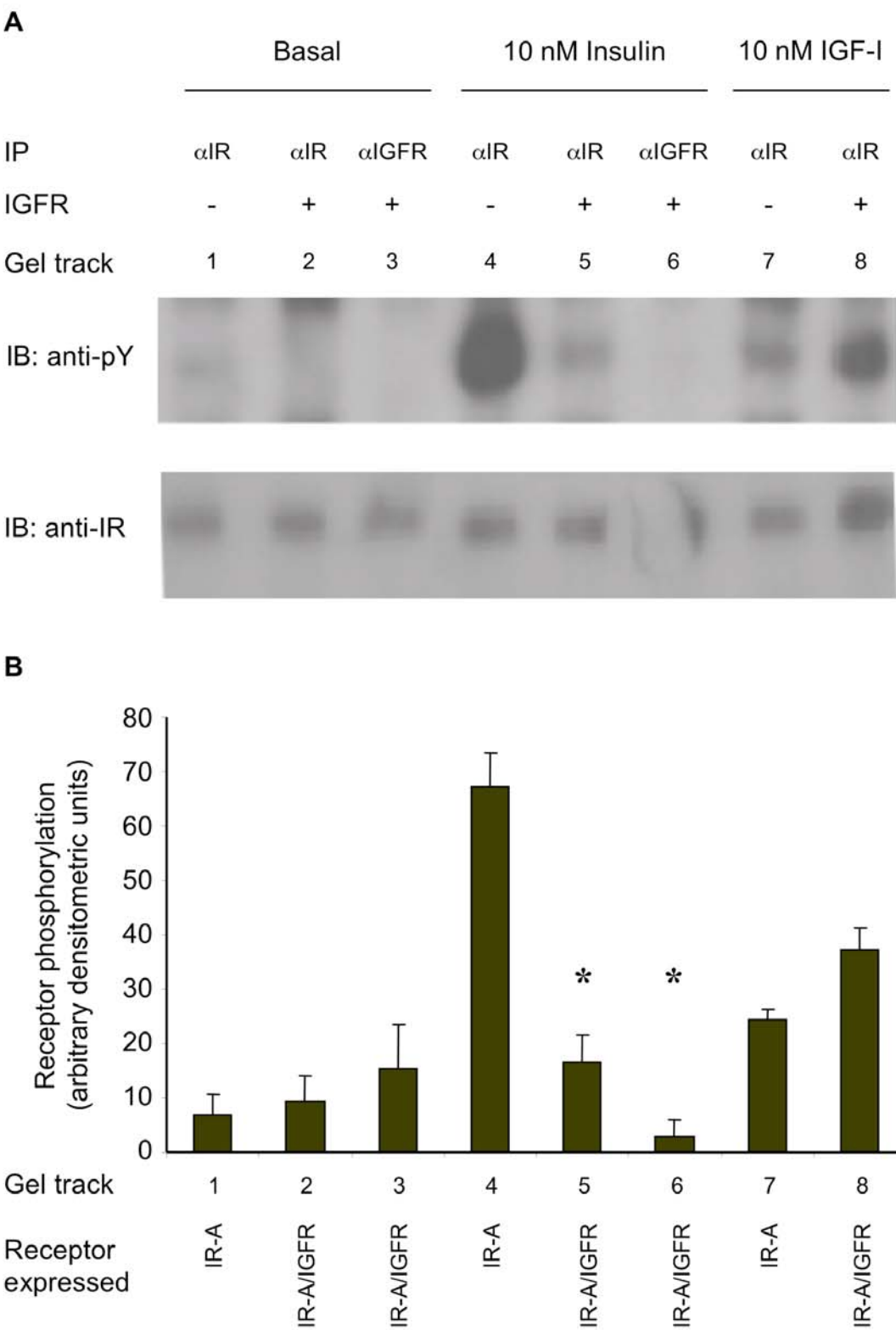
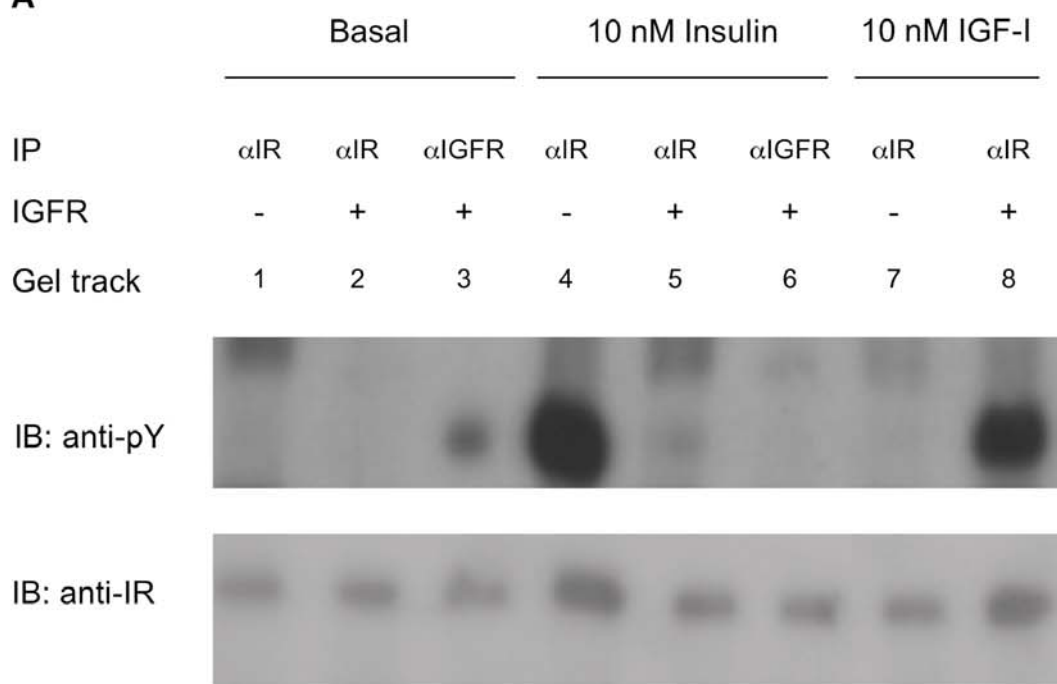
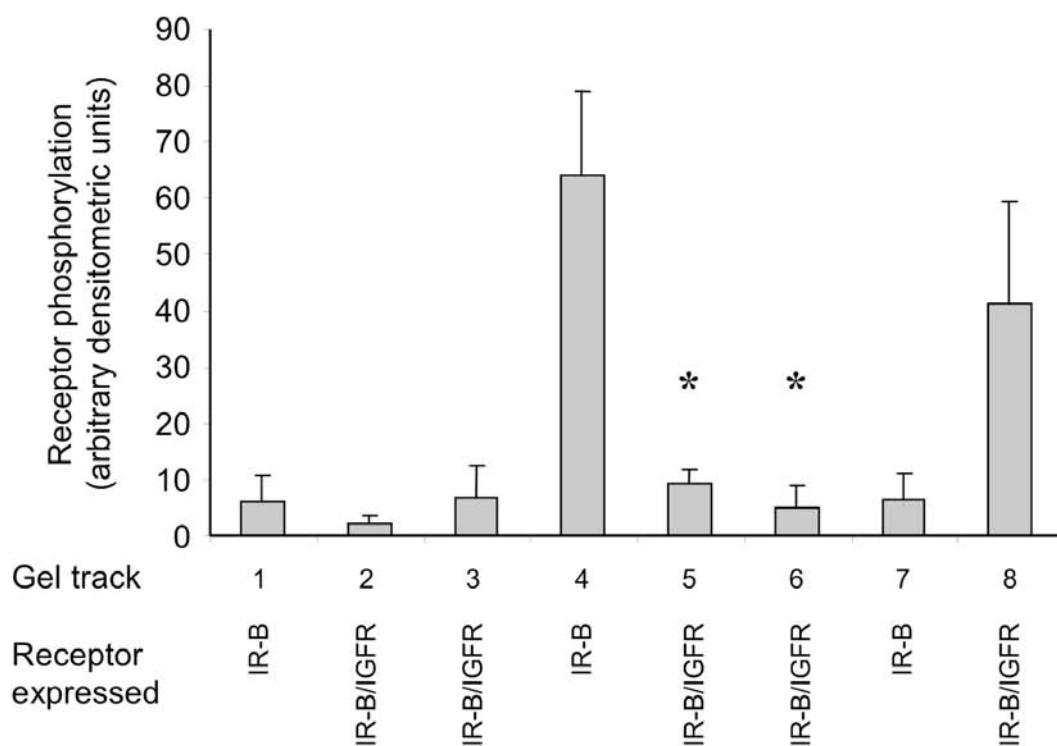


Figure 6: Phosphorylation of IR-A in homodimers and in hybrids

**A**



**B**



**Figure 7: Phosphorylation of IR-B in homodimers and in hybrids**

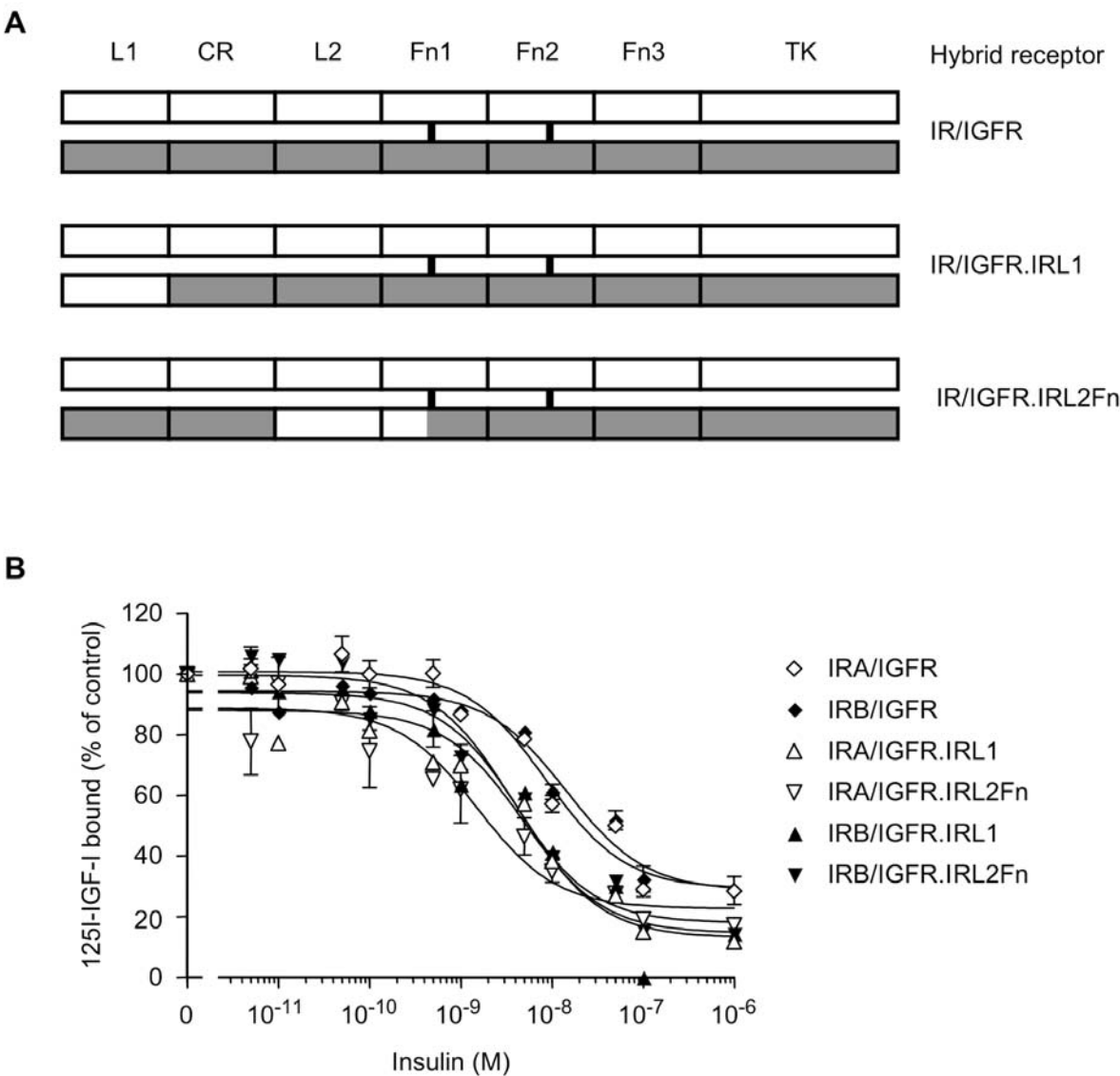
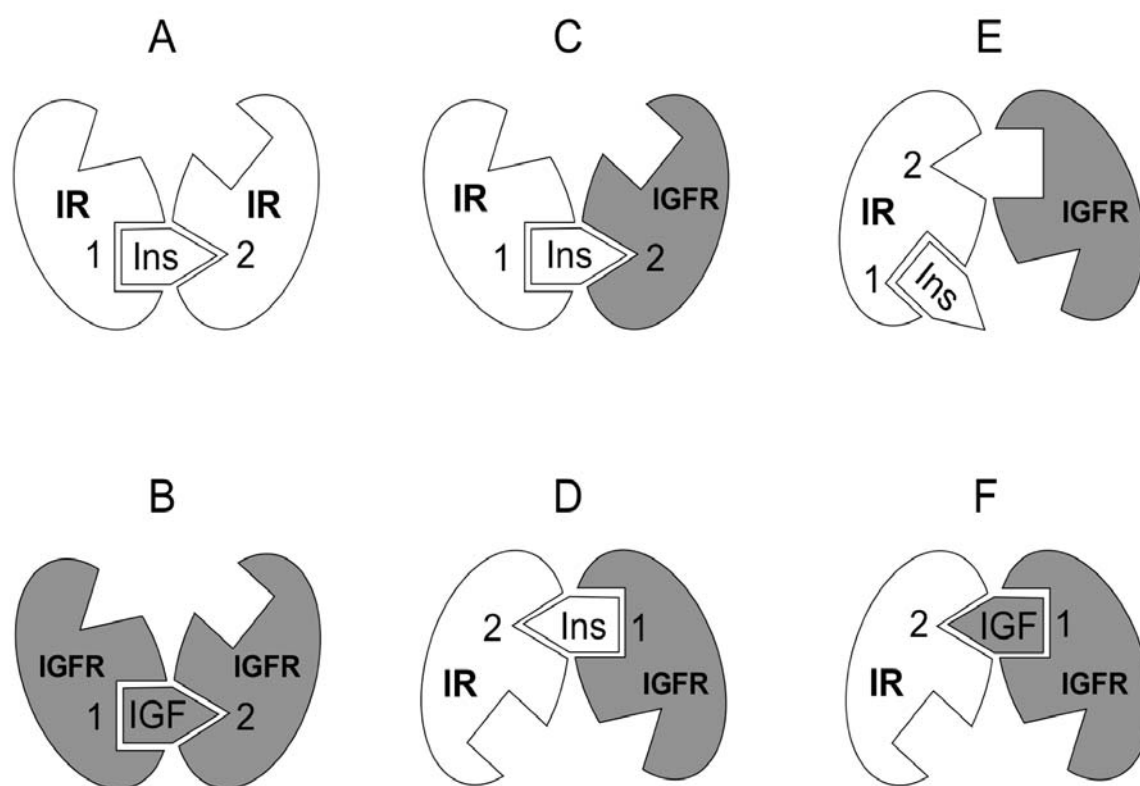


Figure 8: Reconstitution of high affinity insulin binding using chimeric receptors



**Figure 9: Model of ligand binding to hybrid receptors**