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Structural domains of the insulin receptor and IGF receptor required for dimerisation and ligand binding

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Abstract We investigated structural requirements for dimerisation and ligand binding of insulin/IGF receptors. Soluble receptor fragments consisting of N-terminal domains (L1/CYS/L2, L1/CYS/L2/F0) or fibronectin domains (F0/F1/F2, F1/F2) were expressed in CHO cells. Fragments containing F0 or F1 domains were secreted as disulphide-linked dimers, and those consisting of L1/CYS/L2 domains as monomers. None of these proteins bound ligand. However, when a peptide of 16 amino acids from the α-subunit C-terminus was fused to the C-terminus of L1/CYS/L2, the monomeric insulin and IGF receptor constructs bound their respective ligands with affinity only 10-fold lower than native receptors.

Key words: Insulin; Insulin-like growth factor; Receptor; Ligand binding; Dimerization; Structural domain

1. Introduction

The insulin receptor (IR) and type I IGF receptor (IGFR) are disulphide-linked $(\alpha\beta)_2$ structures, assembled by dimerisation and proteolytic cleavage of the respective proreceptors [1]. Each receptor has a high affinity for its cognate ligand, and 100–1000-fold lower affinity for the converse ligand [2]. The α-subunit is wholly extracellular and contains the ligand binding site, while the transmembrane β-subunit signals via its intracellular tyrosine kinase activity. Attempts to crystallise the full extracellular portion of these receptors have been unsuccessful, but sequence analysis and molecular modelling predict six distinct structural domains, designated L1, CYS, L2, F0, F1 and F2 [3]. The L1 and L2 domains are homologous β-helix structures, linked by disulphide-bonded modules of the CYS domain, and the F domains are fibronectin type III repeats, each with a 7-stranded β-sandwich structure. The F1 domain is predicted to have a large (approximately 125 amino acids) inserted loop, containing the site of cleavage between α- and β-subunits and, in the IR, the sequence encoded by alternatively spliced exon 11. A crystal structure has been solved for a 462 amino acid N-terminal fragment of the IGFR [4] and the corresponding IR structure is presumably very similar. The IGFR structure reveals the L1, CYS and L2 domains surrounding a central space of sufficient size to accommodate a ligand molecule. However, this IGFR fragment does not bind IGF-I, and it is uncertain whether the orientation of domains in the crystal structure is the same as in native receptor.

It follows from the large size of the receptors relative to insulin/IGF-I that receptor-ligand contacts must involve only a small fraction of the receptor surface. Several approaches have been used to determine the regions of IR and IGFR which participate directly in ligand binding. Ligand cross-linking suggests that the L1, CYS, L2/F0 and F1 insert regions are all in close proximity to bound insulin [5–8]. Studies with IR/IGFR chimeras have shown that the L1 domain of IR and CYS domain of IGFR make important contributions to specific, high affinity binding of the cognate ligands [2,9]. Alanine scanning mutagenesis confirmed the importance of the L1 domain for insulin binding [10] and further revealed that conserved amino acids close to the α-subunit C-terminus (within the F1 insert) make major contributions to binding of both insulin and IGF-I [11,12]. It has been suggested that high affinity ligand binding and receptor activation involve cross-linking of the two halves of native receptors, by interaction of distinct faces of a single ligand molecule with different regions of the two α-subunits [13,14]. This model offers an explanation for the negative cooperativity of ligand binding, and electron microscopic images of insulin-receptor complexes are consistent with it [15]. However, it is not clear which of the several receptor structural domains implicated in ligand binding might contribute in *cis* and which in *trans* to a cross-linked complex.

An alternative approach to determining the minimum requirements for receptor assembly and high affinity ligand binding is to express deletion constructs lacking significant portions of the receptor ectodomain. In a C-terminal deletion series, the smallest IR fragment which bound insulin was the full-length α-subunit [16]. However, substantial internal deletions (corresponding approximately to what is now recognised as the F0 domain) within the otherwise full-length, native IR did not seriously compromise insulin binding [17,18]. More recently, it was shown that soluble, monomeric α-subunit constructs with even larger deletions (residues 469–703) bind insulin with an affinity similar to the full ectodomain [19]. To investigate further the minimum structural requirements for high affinity ligand binding, we have studied a number of IR and IGFR ectodomain fragments with either C-terminal or N-terminal deletions, and assessed the ability of F0 and F1 insert sequences to complement binding to other domains.

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Abbreviations: IGF, insulin-like growth factor; IR, insulin receptor; IGFR, type I insulin-like growth factor receptor; CYS, cysteine-rich domain; F, fibronectin type III; CHO, Chinese hamster ovary; N-terminal, amino terminal; C-terminal, carboxyl terminal; DTT, dithiothreitol

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2. Materials and methods

2.1. Materials

Insulin was purchased from Sigma and IGF-I was a generous gift from Ciba-Geigy, Basel. ^{125}I-insulin was a gift from Eli Lilly and ^{125}I-IGF-I was from Amersham Pharmacia Biotech. Restriction enzymes and other products used for molecular biology were purchased from New England Biolabs, Sigma and Stratagene. The human IR and IGFR cDNAs were gifts from Dr Leland Ellis and Dr Jonathan Whittaker respectively.

2.2. Construction of cDNAs expressing encoding insulin and IGF receptor fragments

Receptor constructs and their notations are shown in Fig. 1. Preparation and analysis of plasmid DNA was performed according to standard methods [20]. All constructs were subject to complete DNA sequencing to confirm that no sequence errors had arisen during assembly. The wild-type human IR template used to generate the various constructs contained the exon 11 and the numbering system used is that of Ebina et al. [21].

The IR593 construct was made by PCR amplification of nucleotides 1–1998 using the sense primer 5′-GCCGGGCTAGCGCCACCATGGGCACCGGGGGCCGGCGGGGG-3′ and antisense primer 5′-CGGCCTCTAGAGGTGGCATCTGTCTGGACATA-3′. The amplified fragment was digested with *Nhe*I and *Xba*I (sites underlined) and ligated into the similarly digested vector pcDNA3.1(-)/Myc-His C (Invitrogen) which had been modified by insertion of a synthetic oligonucleotide 5′-CTAGA*CTGGTGCCCCGCGGCTCCA*A-3′, encoding a thrombin site (in italics), between its *Xba*I and *Hin*dIII sites.

The IR3Fn construct was made by PCR amplification of nucleotides 1630–3006 using the sense primer 5′-CAGTGGCCCAGCCGGCCGAGTTACTTAAATTTTCTTACATTCGGACATC-3′ and antisense primer 5′-AGATGGGCCC*GGAGCCGCGGGGCACCAG*TTTTGCAATATTTGACGGGACGTCTAAATAG-3′ (*Sfi*I and *Apa*I sites underlined and thrombin site in italics). The IR2Fn construct was made by PCR amplification of nucleotides 1996–3006 using the sense primer 5′-CAGTGGCCCAGCCGGCCACCAACCCCTCTGTGCCCCTGGATCCAATC-3′ (*Sfi*I site underlined) and the same antisense primer as for IR3Fn. The IR3Fn and IR2Fn fragments were digested by *Sfi*I and *Apa*I and cloned into the similarly digested vector pSECTag2A (Invitrogen) which provides an N-terminal signal sequence and C-terminal Myc and His tags.

For constructs IR473, IR473CT, IGFR462 and IR462CT, a modified pBK.CMV vector (pBK.CMV.Myc) was used. A Myc epitope plus stop codon was introduced by ligating a synthetic oligonucleotide 5′-GATCCGAGCAGAAACATATATCAGAGGAGGACCTAAACTAGGCCGGCG-3′ into the *Bam*HI/*Eco*RI sites of pBK.CMV. The IR473 construct was made by PCR amplification of nucleotides 1–1638 of hIR cDNA using the sense primer 5′-CGAGGGGCTAGCCACCATGGGCACCGGGGGCCGGCGGGGG-3′ and antisense primer 5′-GTAAGAGGATCCAAGTAACTCATTTTCACAGGA-3′. The IGFR462 construct was made by PCR amplification of nucleotides 1–1521 of hIGFR cDNA using the sense primer 5′-CGAGGGGCTAGCCACCATGAAGTCTGGCTCCGGAGGAGGG-3′ and antisense primer 5′-GGTGAAGGATCCGACGTCACTTTCACAGGAGGC-3′. The IR473 and IGFR462 amplified fragments were digested with *Nhe*1 and *Bam*H1 (sites underlined) and cloned into the similarly digested pBK.CMV.Myc to generate the plasmids pBK.CMV.IR473 and pBK.CMV.IGFR462.

The IR473CT construct was generated by insertion of a synthetic oligonucleotide of 54 bp encoding amino acids 704–717 and 730–731 of the hIR, flanked at each end by *Bam*H1 sites (Fig. 1) into the *Bam*HI site of pBK.CMV.IR473. The *Bam*H1 linkers encode GlySer sequences at either end of the IRCT sequence. A *Stu*I site was also introduced within the oligonucleotide to allow identification of the recombinant clones, without altering the protein sequence. The same strategy was used to generate the construct IGFR462CT by inserting a synthetic oligonucleotide encoding to amino acids 691–706 of hIGFR in pBK.CMV.IGFR462.

2.3. Cell transfection

CHO cells were grown in Ham's F12 nutrient mixture (Sigma) supplemented with 10% new born calf serum (Life Technologies), 100 units/ml penicillin and 100 μg/ml streptomycin. Cells were seeded at 20–40% confluency 24 h prior to transfection of DNA by the Lipofectamine procedure for stable transfection of adherent cells (Life Technologies). Transfected cells were incubated with selection medium containing 600 μg/ml of geneticin (G418; Life Technologies) or 1 mg/ml of zeocin (Invitrogen) as appropriate for the resistance gene of the vectors. After 15–20 days, clonal lines were selected by growth at limiting dilution, expanded and analysed by SDS–PAGE and immunoblotting with anti-myc antibody 9E10 [22].

2.4. Ligand binding assays

Wells of microtitre plates (Nunc Immuno™ Modules) were coated with purified anti-receptor monoclonal antibodies (10 μg/ml, 100 μl/well) for 16 h at 4°C. The wells were washed 3 times with binding buffer (75 mM Tris pH 7.8 at 4°C, 30 mM NaCl, 0.5 mM EDTA, 0.1 mM AEBSF, 10 mM glucose, 0.1% BSA and 0.05% Triton X-100), blocking buffer (1% BSA in binding buffer) was added for 4 h at room temperature, and wells were then washed again. Cell culture media, or detergent lysates of NIH3T3.HIR3.5 cells expressing wild-type hIR [23] or IGF-1R/3T3 cells expressing hIGFR [24], were diluted in binding buffer with 200 kU/ml aprotinin and added (100 μl) to each well for 16 h at 4°C. After washing, ligand binding experiments were performed by adding ^{125}I-insulin (30 000 dpm) or ^{125}I-IGF-I (15 000 dpm) in a final volume of 100 μl for 5 h at 4°C. For competition studies with unlabelled ligand, the dilution of soluble receptors was adjusted to give 15% binding of tracer in absence of unlabelled ligand. The plates were washed with cold binding buffer and bound radioactivity was removed by adding 0.5 M NaOH and γ-counted.

3. Results and discussion

3.1. Expression of receptor fragments

Clonal cell lines expressing the various receptor constructs with C-terminal Myc epitope tags (Fig. 1) were selected by immunoblotting cell lysates with anti-Myc antibody. The receptor proteins were also readily detected in culture media.

Constructs based on N-terminal domains (IR473, IR473CT, IR593) had apparent molecular masses of 79, 81 and 110 kDa respectively on SDS–PAGE under reducing conditions (Fig. 2A), close to those predicted for the glycosylated polypeptides. When SDS–PAGE was performed under non-reducing conditions, the IR593 construct appeared mostly as a much larger protein of apparent molecular mass approximately ~200 kDa (Fig. 2B), consistent with the formation of disulphide-linked dimers, and only a very small amount of monomer was detected. In contrast, the electrophoretic mobility of the IR473 and IR473CT constructs (also IGFR462 and IGFR462CT constructs, data not shown) was little altered

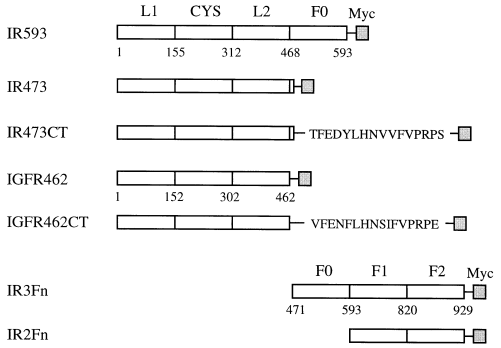

Fig. 1. Schematic representation of receptor constructs. Constructs with the normal receptor N-terminus were named according to the last C-terminal amino acid, and the presence of an additional 16-mer peptide from the C-terminal α-subunit C-terminus (CT). Constructs with N-terminal truncations were named according to their number of fibronectin type III domains. All constructs included a C-terminal Myc epitope tag represented with grey box.

under non-reducing conditions, suggesting that these proteins are secreted as monomers.

Both constructs with N-terminal deletions (IR3Fn and IR2Fn) were detected as doublets of apparent M_r 45–50 kDa by anti-Myc blotting following SDS–PAGE under reducing conditions (Fig. 2A and data not shown). The expected size of full-length, epitope-tagged IR2Fn is ~70 kDa (365 amino acids, seven glycosylation sites) and of IR3Fn ~84 kDa (487 amino acids, seven glycosylation sites). The tetrabasic sequence which marks the site of cleavage between IR α- and β-subunits is at residues 732–735 (in F1 insert), and the fragment corresponding to the tagged extracellular portion of β-subunit is expected to be approximately 42 kDa (227 amino acids, four glycosylation sites) as found for receptor expressed in COS cells [16]. We conclude that both the IR2Fn (F1/F2) and IR3Fn (F0/F1/F2) proteins are efficiently cleaved at this position. The appearance of the Myc-tagged β-subunit fragments as doublets under these conditions may reflect heterogeneity of glycosylation. Both the IR2Fn and IR3Fn constructs were detected as much larger proteins (apparent M_r 160–200 kDa) following electrophoresis under non-reducing conditions (Fig. 2B and data not shown), indicative of their secretion as dimers as for the IR593 protein. As with the IR593 protein, a trace of IR2Fn monomer was detectable under non-reducing conditions when blots were overexposed.

In the native receptor the α-subunits are linked by at least two class I disulphides, one of which involves Cys-524 (in F0 domain) and the other Cys-682/3/5 (in F1 insert), while the α- and β-subunits are linked by a single class II disulphide between Cys-647 (in F1) and Cys-872 (in F2) [25–27]. We conclude that the presence of either the F0 domain, as in the IR593 protein, or the F1 domain, as in the IR2Fn protein, is sufficient to promote class I disulphide-linked dimerisation. Thus the capacity for dimerisation is an intrinsic property of these domains which is not dependent on the integrity of other parts of the receptor structure, and in particular does not require the three N-terminal domains. Moreover, both IR3Fn and IR2Fn proteins must also contain class II α-β disulphide links, confirming the capacity of the fibronectin domains to fold and assemble independently of the rest of the receptor.

Native $\alpha_2\beta_2$ receptor heterotetramers will readily dissociate into αβ half-receptors following mild reduction of class I disulphides in vitro [28,29], indicating that non-covalent interactions between half-receptors are not strong. Half-receptors generated in this way can be induced to reform disulphide-linked $\alpha_2\beta_2$ heterotetramers by addition of ligand [30–32], although dimerisation during normal biosynthesis must obviously occur in the absence of ligand. It is surprising in the light of these in vitro observations that fragments of the receptor containing only some of the potential sites of interaction between half-receptors nevertheless assemble biosynthetically as dimers.

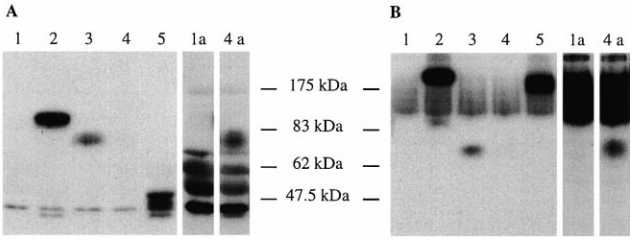

Fig. 2. Receptor constructs expressed in CHO cells. Conditioned medium from clonal cell lines was mixed with Laemmli sample buffer (with or without DTT) and loaded on 8% SDS–polyacrylamide gels. A nitrocellulose blot was prepared, probed with anti-Myc antibody 9E10, and visualised by enhanced chemiluminescence. Lane 1, untransfected cells; lane 2, IR593; lane 3, IR473; lane 4, IR473CT, lane 5, IR2Fn. Lanes 1a and 4a are overexposed versions of the corresponding lanes in the main part of the figure. Positions of prestained molecular mass markers (New England Biolabs) are indicated. A: Receptor fragments resolved under reducing conditions (+DTT). B: Receptor fragments resolved under non-reducing conditions (−DTT).

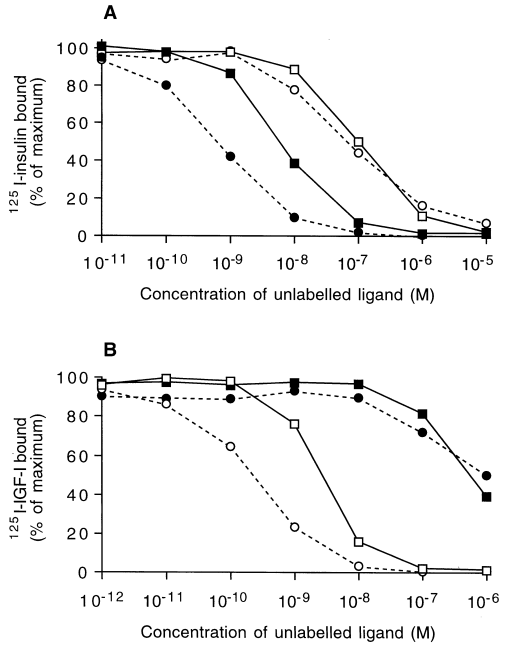

Fig. 3. Competition assay of ^{125}I-ligand binding to receptor constructs. Culture medium from clonal cell lines expressing receptor constructs, or detergent lysate of cells expressing wild-type receptors (NIH3T3.HIR3.5 or IGF-1R/3T3), was added to wells coated with anti-receptor antibody, and incubated with ^{125}I-ligand and varying concentrations of unlabelled insulin (solid symbols) or IGF-I (open symbols). Results are expressed as the percentage of ^{125}I-ligand bound in absence of unlabelled ligand, and data points are the means of duplicate determinations within a representative experiment. A: IR473CT construct (solid lines) or wild-type IR (dashed lines) added to wells coated with antibody IR 83-7 and incubated with ^{125}I-insulin. B: IGFR462CT construct (solid lines) or wild-type IGFR (dashed lines) added to wells coated with antibody IGFR 16-13 and incubated with ^{125}I-IGF-I.

3.2. Ligand binding

The capacity of the various constructs to bind their cognate

Table 1
Specificity of ligand binding to receptor constructs

Receptor	Insulin IC_{50} (nM)	IGF-I IC_{50} (nM)
IR473CT	6.5	80
NIH3T3.HIR3.5	0.6	100
IGFR462CT	600	2.5
IGF-1R/3T3	1600	0.3

Competition binding assays were performed with IR473CT and IGFR462CT constructs, or wild-type detergent-solubilised receptors, as in Fig. 3. IC_{50} values for each unlabelled ligand are means of two independent experiments, each conducted in duplicate.

ligands was assessed in a plate binding assay. Receptor fragments were captured on anti-receptor antibodies which specifically recognise epitopes in the regions 191–290 of IR (Ab 83-7, used for IR N-terminal fragments), 62–184 of IGFR (Ab 16-13, used for IGFR N-terminal fragments) or 765–770 of IR (Ab 18-44, used for IRFn constructs) [33–35]. These antibodies do not inhibit ligand binding to soluble receptors. The antibodies have high affinity for native receptors, but react very poorly with denatured receptor on blots, indicating that the epitopes are conformation dependent. It was confirmed by sequential anti-receptor immunoprecipitation and anti-Myc blotting that all the expressed receptor fragments were recognised by appropriate antibodies, and it is therefore concluded that the fragments assume a native conformation at least as regards the relevant epitopes.

Binding of ^{125}I-insulin was observed only with the IR473CT construct. No binding was detected with the IR473, IR593, IR3Fn or IR2Fn proteins, even when these were tested at 2–40 times the concentration of IR473CT as normalised by anti-Myc blotting (data not shown). Similarly, binding of ^{125}I-IGF-I was detected only with the IGFR462CT construct and not with IGFR462 (data not shown). These results confirm previous reports that similar fragments containing just the first three domains (L1/CYS/L2) of the IR and IGFR are unable to bind ligand [4,16]. Moreover the complete lack of insulin binding to the IR593 construct indicates that neither dimerisation of the four N-terminal domains nor completion of the putative contact site between amino acids 390–488 (exon 6) [7,13] is sufficient to create an effective ligand binding site.

Alanine scanning mutagenesis suggests that amino acids 704–717, close to the C-terminus of the α-subunit, make a greater energetic contribution to insulin binding than those in the L1 domain [10,11]. Our data show that this sequence alone, even in the context of the complete and appropriately processed F1 domain as in the IR3Fn and IR2Fn constructs, is not sufficient to confer detectable insulin binding at tracer concentrations. However, the data confirm that when the 704–717 sequence is taken out of its immediate context and fused to the C-terminus of the L1/CYS/L2 domains, as in IR473CT, the resulting construct binds insulin very well. Moreover, we have shown that analogous IGFR constructs behave in the same way, in that IGFR462 does not bind IGF-I at tracer concentrations but IGFR462CT binds very well.

The properties of the IR473CT and IGFR462CT proteins were further studied in competition binding assays with unlabelled insulin and IGF-I (Fig. 3). Under the conditions of these assays, with limiting amounts of receptor and tracer concentrations of radioligand, the concentrations of unlabelled ligand which inhibit tracer binding by 50% (Table 1) may be taken as a measure of the K_d. We found that both the monomeric IR473CT and IGFR462CT constructs have 8–10-fold lower affinity for their cognate ligands than the native detergent-solubilised receptors. Affinities of the constructs for heterologous ligand were not so reliably defined, but appear to be similar to those of the native receptor.

The affinity of IR473CT for insulin was similar to that reported for isolated half-receptors and free α-subunit [16,28,29]. Thus the decreased affinity of the IR473CT construct compared to native IR presumably reflects its monomeric structure rather than the absence of specific binding determinants or the fibronectin domains. Half-receptors, like soluble dimeric ectodomain [36] and native receptors with F0 deletions [17,18] not only show decreased affinity for insulin but also exhibit linear Scatchard plots indicative of simple single-site binding. Thus it has been proposed that interactions of ligand with both α-subunits in native receptors are necessary for highest affinity and negative cooperativity of ligand binding [13,14]. We have previously shown that insulin/IGF hybrid receptors have even lower affinity for insulin than isolated half-receptors [37], suggesting that an inappropriate *trans* interaction may be more deleterious to binding than no interaction at all. The situation with regard to ligand binding by the IGFR is less clear-cut. It has been reported that isolated αβ half-receptors exhibit decreased affinity relative to native $\alpha_2\beta_2$ IGFR, similarly to the situation with IR [38]. However, others have found that binding properties of IGFR αβ and $\alpha_2\beta_2$ were not significantly different [39], and we found that insulin/IGF hybrid receptors bind IGF-I with similar affinity to wild-type IGFR [37]. In the present studies, the affinity of monomeric IGFR462CT for IGF-I was clearly reduced compared to native IGFR. Interestingly, the affinities for cross-reaction of IGF-I with IR473CT or insulin with IGFR462CT were very similar to those of the native receptors, suggesting that binding of heterologous ligand does not depend on receptor dimerisation.

The simplest interpretation of the present data is that binding determinants in the L1 and C-terminal regions of the IR α-subunit cooperate in *cis* to generate an effective binding site, and that additional *trans* interactions are provided by some other portion of the α-subunit in native receptors. It remains an open question whether a precisely analogous binding mechanism operates in the IGFR. It is possible that connection of the C-terminal peptide directly to the L2 domain fortuitously mimics the positioning of the same sequence when present as part of the F1 insert in native receptor. The IR473CT construct is a little larger than the minimised receptor which has been studied previously [19], having 473 rather than 468 amino acids of the N-terminal domains, a GlySer linker between the N-terminal segment and the CT sequence, and an additional Myc tag at the C-terminus. However, the two constructs nevertheless appear very similar in their affinity for insulin. We conclude from this that precise positioning of the C-terminal sequence relative to the N-terminal portion of the IR is not essential for insulin binding. It may be that insulin itself induces an appropriate conformation providing there is some flexibility in the link between the C-terminal sequence and the rest of the receptor. It is notable that the exon 11− form (but not the exon 11+ form) of uncleaved proreceptor has a greatly reduced affinity for insulin [40], suggesting that the α-subunit C-terminus is conformationally constrained in a way which inhibits insulin binding at least in the absence of exon 11. However, while the exon 11 sequence of 12 amino acids immediately downstream of the critical C-terminal contact site enhances insulin binding to uncleaved proreceptor, the exon 11 sequence exerts a modest negative influence on the affinity of mature IR for insulin, and more especially for IGF-I [41]. Studies of further constructs involving fusions of N-terminal domains and C-terminal sequences of the IR and IGFR α-subunits should further illuminate the contribution of the C-terminus to the affinity and specificity of ligand binding.

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