Role of Insulin Receptor Dimerization Domains in Ligand Binding, Cooperativity, and Modulation by Anti-receptor Antibodies

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To define the structures within the insulin receptor (IR) that are required for high affinity ligand binding, we have used IR fragments consisting of four amino-terminal domains (L1, cysteine-rich, L2, first fibronectin type III domain) fused to sequences encoded by exon 10 (including the carboxyl terminus of the α-subunit). The fragments contained one or both cysteine residues (amino acids 524 and 682) that form disulfides between α-subunits in native IR. A dimeric fragment designated IR593.CT (amino acids 1–593 and 704–719) bound 125I-insulin with high affinity comparable to detergent-solubilized wild type IR and mIR.Fn0/Ex10 (amino acids 1–601 and 650–719) and greater than that of dimeric mIR.Fn0 (amino acids 1–601 and 704–719) and monomeric IR473.CT (amino acids 1–473 and 704–719). However, neither IR593.CT nor mIR.Fn0 exhibited negative cooperativity (a feature characteristic of the native insulin receptor and mIR.Fn0/Ex10), as shown by failure of unlabeled insulin to accelerate dissociation of bound 125I-insulin. Anti-receptor monoclonal antibodies that recognize epitopes in the first fibronectin type III domain (amino acids 471–593) and inhibit insulin binding to wild type IR inhibited insulin binding to mIR.Fn0/Ex10 but not IR593.CT or mIR.Fn0. We conclude the following: 1) precise positioning of the carboxyl-terminal sequence can be a critical determinant of binding affinity; 2) dimerization via the first fibronectin domain alone can contribute to high affinity ligand binding; and 3) the second dimerization domain encoded by exon 10 is required for ligand cooperativity and modulation by antibodies.

The insulin receptor (IR)1 contains two α- and two β-subunits in a disulfide-linked β-α-α-β configuration. Ligand binding determinants reside within the extracellular α-subunit, and the cytoplasmic tyrosine kinase activity of the intracellular β-subunit is responsible for ligand-induced signal transduction to metabolic and mitogenic responses (1). The structure of the extracellular region of the insulin receptor is predicted to be composed of six distinct structural domains as follows: two homologous domains L1 and L2 flanking a cysteine-rich domain CR followed by three fibronectin type III repeats (Fn0, Fn1, and Fn2) (2). A crystal structure has been determined for the first three domains of the homologous type I IGF receptor (IGFR) (3), and the structure of the Fn domains has been modeled by comparison with similar structures in other proteins (4–6). The central fibronectin domain Fn1 includes a large inserted loop of 135 amino acids containing the site of proteolytic cleavage that generates α- and β-subunits from the proreceptor polypeptide. Disulfide links between α-subunits have been localized to Cys524 (Fn0 domain) and Cys582 (Fn1 insert), whereas the α-β-disulfide link is between Cys647 (Fn1) and Cys660 (Fn2) (7, 8).

Regions of the IR/IGFR that are involved in ligand binding have been identified by affinity cross-linking, generation of chimeric IR/IGFR, and alanine-scanning mutagenesis (9–14). Determinants of insulin specificity reside within the L1 domain of the IR and of IGF-I specificity in the CR domain of the IGFR (15–17). The carboxyl-terminal region of the α-subunit is critical for ligand binding in both receptors, as revealed by mutagenesis and generation of deletion constructs (12, 18, 19). We have shown previously that inclusion of a 16-amino acid sequence from the carboxyl terminus of the IR or IGFR α-subunit is necessary to confer ligand binding on monomeric receptor fragments containing the first three amino-terminal domains (L1/CR/L2) (20, 21). However, the affinity of such monomeric constructs for insulin is considerably lower than that of the wild type receptor (IRwt), indicating that additional structural elements are necessary to confer binding properties of wild type IR. Full-length half-receptors, generated from IRwt by disulfide reduction, similarly exhibit decreased affinity (22, 23) suggesting that dimerization plays an important part in creating a high affinity binding site.

A characteristic feature of ligand binding to wild type IR is negative cooperativity, as revealed by curvilinear Scatchard plots and accelerated dissociation of bound 125I-insulin in the presence of unlabeled insulin. It has been suggested that high affinity, cooperative binding of insulin involves contacts with both α-subunits within the native IR (24, 25). Surprisingly, a soluble dimeric construct containing the entire IR ectodomain (IREcto) does not exhibit negative cooperativity and, like monomeric fragments and half-receptors, binds ligand with decreased affinity (25). However, fusion of the IR ectodomain to self-associating domains such as immunoglobulin Fc (26) or a leucine zipper motif (27) results in high affinity insulin binding and curvilinear Scatchard plots, suggesting that the properties of dimeric constructs are highly dependent on specific dimerization domains. Previous studies from one of our laboratories

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‡ The abbreviations used are: IR, insulin receptor; IRwt, wild type insulin receptor; IGFR, insulin-like growth factor type I receptor; IGF-I, insulin-like growth factor I; CR, cysteine-rich; Fn, fibronectin type III; IREcto, soluble human insulin receptor ectodomain; HIRs-Fc, soluble human insulin receptor-immunoglobulin heavy chain chimera; mAb, monoclonal antibody; CHO, Chinese hamster ovary; DTT, dithiothreitol; PEG, polyethylene glycol.
Fragments as described previously (28). The anti-insulin receptor antibody was carried out prior to SDS-PAGE. Reduction agent DTT, followed by immunoblotting with the anti-Myc antibodies, was performed, a microtiter plate assay and a polyethylene glycol (PEG) precipitation assay. In both assay formats, conditioned medium containing soluble insulin receptor fragments or detergent lysates from cell lines overexpressing insulin receptor were diluted to bind 10–15% of added 125I-insulin in the absence of unlabeled ligand.

The microtiter plate assay was performed essentially as described previously (21) using Immulon 4 HBX plates from Dynex Technologies, coated with anti-IR 83-7 or anti-Myc 9E10 mAbs. Ligand binding experiments were performed by incubating immobilized receptors with ~6 pM 125I-insulin for 48 h at 4 °C (28) or 60 pM 125I-insulin for 16 h at 4 °C (21), together with unlabeled insulin, IGF-I, or antibodies as specified for individual experiments. Plates were then washed with cold phosphate-buffered saline, and bound radioactivity was determined in a γ-counter. IC50 values for inhibition of tracer binding by unlabeled insulin or IGF-I were determined using KaleidaGraph® software (Synergy Software, Reading, PA) according to a one-site binding model.

PEG precipitation assays were performed as described previously (29) by incubating receptor with ~60 pM 125I-insulin for 16 h at 4 °C in a final volume of 250 µl of cold binding buffer in the absence or presence of antibodies, before addition of carrier bovine gamma globulin (Sigma), precipitation with PEG M, 6000 (Sigma), and determination of radioactivity in the washed precipitates.

**RESULTS**

**Construction and Detection of Soluble Insulin Receptor Fragments**—We have described previously the construction of several monomeric and dimeric soluble insulin receptor fragments containing the L1, CR, L2, and Fn0 domains of IR (21, 28). The novel construct used in the present work, designated IR593.CT, contained the first four domains of the receptor (amino acids 1–593) fused to the carboxyl-terminal peptide sequence of the α-subunit (amino acids 704–719; amino acids are numbered according to the sequence of Ulrich et al. (35)) and a Myc/His epitope tag. The various constructs used in this study are shown diagrammatically in Fig. 1B.

The IR593.CT protein contains one of the cysteine residues (residue 524) believed to form a disulfide bridge between the α-subunits in native IR (7). To confirm that IR593.CT assembles as a dimer, as has been shown previously (21, 28) for the structurally similar IR593 and mIR Fn0 proteins, conditioned medium from clonal cell lines stably expressing either IR593.CT or IR473.CT in serum-free medium was resolved by SDS-PAGE under non-reducing or reducing conditions and immuno-blotted with an anti-Myc antibody (Fig. 2A). Under non-reducing conditions, the majority of secreted IR593.CT was detected as form of ~200 kDa consistent with a glycosylated dimer. Under reducing conditions IR593.CT was detected at ~110 kDa consistent with a glycosylated monomer, which also appeared as a minor component under non-reducing conditions. The IR473.CT protein, which lacks the Fn0 domain containing Cys524, appeared as a monomer of ~80 kDa under both non-reducing and reducing conditions.

To confirm that the secreted IR fragments were correctly folded, conditioned media were subjected to immunoprecipitation with conformation-specific monoclonal anti-receptor antibodies prior to SDS-PAGE, followed by immunoblotting with an anti-Myc antibody (Fig. 2B). The IR593.CT protein was efficiently precipitated by antibodies 83-14, 25-49, and 47-9 (reacting with the Fn0 domain of IR 83-7) (Fig. 2B). The IR473.CT protein, lacking the Fn0 domain, was precipitated only by antibodies 83-7 (the lack of reaction with 18-146 with IR473.CT and weak reaction with IR593.CT was attributed to the relatively low affinity of this antibody even for native IR). It was concluded that IR593.CT is correctly processed and folded as a dimeric mini-receptor.
Competition Assay of $^{125}$I-Insulin Binding to Soluble Insulin Receptor Fragments—Previous studies (21, 25, 28, 29) have employed various different assay conditions to estimate the apparent affinity (or IC₅₀) of receptor fragments for insulin. The use of low concentrations of $^{125}$I-insulin and long incubation times reveals a very high affinity component of insulin binding that is not apparent when higher concentrations of $^{125}$I-insulin and shorter incubation times are used (21, 29). In the present studies, we used both assay protocols so that data could be related to published work, and we compared the ligand binding properties of IR593.CT and related mini-receptors to those of the entire ectodomain, an ectodomain-immunoglobulin heavy chain chimera (HIRs-Fc) (26) and detergent-solubilized native receptors (Ex11⁻ or Ex11⁺).

Representative competition binding curves obtained using a standard protocol with 60 pM $^{125}$I-insulin as tracer are shown in Fig. 3, and deduced IC₅₀ values from replicate experiments are shown in Table I. IR593.CT exhibited a significantly higher affinity for insulin (IC₅₀ 0.21 nM) than either the very similar construct mIR.Fn0 (IC₅₀ 4.6 nM) or full ectodomain (IC₅₀ 1.7 nM). Indeed, IR593.CT appeared similar in affinity to mIR.Fn0/Ex10 (IC₅₀ 0.13 nM), HIRs-Fc (Ex11⁻ isofrom), and IRwt (Ex11⁻ isofrom), whereas the closely related mIR.Fn0 appeared more similar to monomeric IR473.CT (IC₅₀ 6.7 nM). As reported previously (21, 29), no binding of $^{125}$I-insulin tracer was detected with the IR593 dimer lacking the carboxyl-terminal sequence. The IR593.CT, mIR.Fn0, and mIR.Fn0/Ex10 constructs were all similar to the Ex11⁻ isofrom of IRwt in their affinity for IGF-1 (IC₅₀ values 3.6–7.6 nM), whereas the IR473.CT construct had much lower affinity more similar to the Ex11⁻ isofroms of HIRs-Fc and IRwt.

IC₅₀ values derived from competition binding assays do not provide a valid estimate of binding affinity (Kᵢ) if this is substantially lower than the tracer concentration. When binding assays were performed using 6 pM $^{125}$I-insulin tracer, the rank order of IC₅₀ values for the various constructs remained the same (Table I). However, compared with the assays with 60 pM tracer, the IC₅₀ values were distributed over a broader range, with lower values for the high affinity constructs. Under these conditions, it was clear that IR593.CT had a substantially higher affinity for insulin than mIR.Fn0 but not so high as mIR.Fn0/Ex10 or IRwt. It was concluded that dimerization via the Fn0 domain can create a high affinity binding site (as in IR593.CT) and that the second dimerization domain encoded by exon 10 is not essential for high affinity binding.

Dissociation of $^{125}$I-Insulin Bound to IR593.CT—To investigate negative cooperativity of insulin binding to the various mini-receptors, we examined the dissociation of previously...
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**TABLE I**

| [125I-Insulin] | IC50 \(\mu M\) | Insulin, 60 pM | IGF-I, 60 pM |
|----------------|----------------|---------------|--------------|
| IRwt(Ex11)     | 0.11 ± 0.03    | 0.015 ± 0.005 | 4.60 ± 0.75  |
| IRwt(Ex11')    | 0.11 ± 0.03    | 0.02 ± 0.01   | 33.0 ± 8.9   |
| IR593.CT       | 0.36 ± 0.15    | ND            | 48.9 ± 5.3   |
| IR593.CT       | 0.21 ± 0.07    | 0.18 ± 0.02   | 6.23 ± 0.77  |
| mIR.Fn0        | 4.62 ± 0.74    | 4.25 ± 0.35   | 7.55 ± 1.40  |
| mIR.Fn0/Ex10   | 0.13 ± 0.05    | 0.04 ± 0.01   | 3.55 ± 0.90  |
| IREcto         | 1.66 ± 0.35    | ND            | 16.5 ± 6.66  |
| IR473.CT       | 6.71 ± 0.72    | ND            | 216 ± 104*   |

* The determined IC50 value of IGF-I binding to IR473.CT was estimated from displacement curves.

bound [125I]-insulin in the absence or presence of unlabeled insulin (Fig. 4). In buffer alone, dissociation of [125I]-insulin from IR593.CT was much slower than from mIR.Fn0 (~10% versus 45% dissociation after 1 h), but in neither case was there any effect of unlabeled insulin on the dissociation rate. The mIR.Fn0/Ex10 construct and IRwt exhibited dissociation rates similar to IR593.CT in buffer alone (~10% dissociation after 1 h). As reported previously (28), the presence of unlabeled insulin accelerated dissociation from mIR.Fn0/Ex10 as it did with IRwt (~30% dissociation after 1 h in both cases). The dissociation of bound [125I]-insulin from IR473.CT was relatively rapid (~50% dissociated after 1 h) and not affected by unlabeled insulin (data not shown).

We have shown previously (36) the anti-IR monoclonal antibody 47-9 accelerates dissociation of [125I]-insulin from native IR to the same extent as unlabeled insulin. We found that this antibody also accelerated dissociation from mIR.Fn0/Ex10 but not IR593.CT or mIR.Fn0 (Fig. 4). It was concluded that the sequence encoded by exon 10 is required for negative cooperativity of ligand binding.

**Effect of Anti-IR Antibodies on Ligand Binding to Soluble Insulin Receptor Fragments**—We have characterized previously (29, 33) conformation-dependent anti-IR antibodies that, depending on epitope, either stimulate (83-7, 18-146, epitope within CR domain) or inhibit (83-14, 25-49, 47-9, epitope within Fn0 domain) insulin binding to IRwt. (In the case of 83-7, stimulation was restricted to membrane-associated IR and was not seen with detergent-solubilized IR, data not shown.) Scatchard analysis (data not shown) confirmed that stimulatory antibodies affected binding affinity rather than the number of sites, whereas inhibitory antibodies affected either affinity (83-14) or number of sites (47-9).

By having established that these antibodies bound to mini-receptors as predicted from the presence or absence of their respective epitopes (Fig. 2B), we used them as additional probes of the binding properties of the various constructs. When tested in the PEG precipitation assay format, neither the potentially stimulatory antibodies 83-7 and 18-146, nor the potentially inhibitory antibodies 25-49 and 47-9 significantly affected [125I]-insulin binding to IR593.CT or mIR.Fn0 (Fig. 5). However, antibodies 25-49 and 47-9 strongly inhibited insulin binding to mIR.Fn0/Ex10, HIRs-Fc, and IRwt. Antibodies 83-7 and 18-146 had little effect on insulin binding to mIR.Fn0/Ex10 and IRwt under these assay conditions, but 18-146 did stimulate binding to HIRs-Fc. Surprisingly, 83-7 and 18-146 modestly but reproducibly stimulated insulin binding to IR473.CT (antibodies 25-49, 47-9, and 83-14 do not react with IR473.CT). Antibody 83-14 behaved paradoxically, stimulating insulin binding to both IR593.CT or mIR.Fn0 by 1.5–2.5-fold, whereas it partially inhibited binding to mIR.Fn0/Ex10, HIRs-Fc, and IRwt. A monovalent Fab fragment of 83-14 also increased the binding of [125I]-insulin to both IR593.CT and mIR.Fn0, and monovalent Fab fragments of 83-7 and 18-146 increased binding to IR473.CT (data not shown), ruling out the possibility that the effects were a consequence of aggregation by bivalent antibody.

**Fig. 3.** Competition binding analysis of IR fragments. Representative displacement curves of [125I]-insulin binding to soluble IR fragments in the presence of competing unlabeled ligand. A, competition with unlabeled insulin; B, competition with unlabeled IGF-I. Results are expressed as the percentage of [125I]-insulin (60 pM) bound in the absence of unlabeled ligand, and the data points are the means ± range of duplicate incubations. The insulin receptors are as follows: IRwt(Ex11'), open triangles, solid line; IRwt(Ex11'), filled triangles, solid line; mIR.Fn0, open circles, dotted line; IR593.CT, filled circles, dotted line; mIR.Fn0/Ex10, open squares, dashed line; IREcto, filled squares, dashed line.
To further test whether effects of antibodies were dependent in some way on assay format, the antibodies were also examined in plate binding assays in which receptors were immuno-captured with antibody 83-7 (Fig. 6). Under these conditions the stimulatory effects of 83-14 on insulin binding to IR593.CT and mIR.Fn0 were not apparent, although other effects of antibodies (or the lack of them) were qualitatively similar to those seen in the PEG precipitation assay. To exclude the possibility that immunocapture with 83-7 influenced responsiveness to other antibodies, plate binding assays were also performed following immunocapture of IR593.CT and mIR.Fn0 were not apparent, although other effects of antibodies (or the lack of them) were qualitatively similar to those seen in the PEG precipitation assay. To exclude the possibility that immunocapture with 83-7 influenced responsiveness to other antibodies, plate binding assays were also performed following immunocapture of IR593.CT and mIR.Fn0 without the presence of either 100 nM unlabeled insulin or anti-IR mAb 47-9 was determined as described under “Experimental Procedures.” Results are expressed as the percentage of 125I-insulin bound at the commencement of the time course (t = 0). Data are the mean ± S.D. of triplicate incubations within a representative experiment. A, IRwt(Ex11); B, mIR.Fn0/Ex10; C, IR593.CT; and D, mIR.Fn0; buffer only open circles, dotted line; 100 nM unlabeled insulin filled circles, dashed line; 100 nM mAb 47-9 filled squares, solid line.

**DISCUSSION**

The present studies were undertaken to investigate the structural requirements for high affinity, negative cooperative binding of insulin to its receptor, and particularly the role of different receptor dimerization domains. Several distinct regions of IR primary sequence have been shown to be involved in ligand binding, although it remains unclear precisely how these contribute to the high affinity binding site of native IR, with its properties of negative cooperativity and modulation by anti-receptor antibodies. Although half-receptors and monomeric minireceptors bind insulin with moderate affinity, it has been proposed that the high affinity binding characteristic of native receptors involves contacts of ligand with both α-subunits (24, 25).
However, not all dimeric receptor constructs display high affinity binding and negative cooperativity (25, 28).

The L1, CR, and L2 domains contribute major determinants of insulin/IGF binding specificity (13, 15), and within the crystal structure of an amino-terminal fragment of IGFR (which is presumed to be highly homologous in structure to IR), these domains surround a cavity with dimensions appropriate to accommodate a ligand molecule (3). However, there is no detectable ligand binding either to this receptor fragment (IGFR462), the equivalent IR fragment (IR473), or a larger dimeric receptor construct containing the Fn0 domain (IR593) (18, 21). An additional peptide sequence from the carboxyl terminus of the α-subunit has been shown to make a critical contribution to binding of both insulin and IGF-I without conferring significant specificity (11, 12, 19, 20). Surprisingly, this carboxyl-terminal sequence is effective in creating a binding site of moderate affinity in monomeric mini-receptors whether fused to the three amino-terminal domains directly or via linking sequences of variable length (18, 21). Even more surprisingly, the carboxyl-terminal sequence is still effective when it is positioned at the carboxyl terminus of the presumably rigid Fn0 domain in the IR593.CT and mIR.Fn0 constructs, although the two very similar constructs differed by ~20-fold in affinity for insulin (Fig. 3 and Table I) and also differed markedly in the dissociation rate of bound insulin (Fig. 4). The significant structural difference between these constructs (apart from their epitope tags located downstream of the carboxyl-terminal sequence) is the presence in mIR.Fn0 (which might also be designated IR601.CT) of the additional sequence NPSVPLDP between the Fn0 domain and carboxyl-terminal peptide. This sequence, which constitutes a linker between Fn0 and Fn1 domains in the wild type receptor, is conserved (as a X(S/T)P(L/Q)DX consensus) in all members of the vertebrate IR/IGFR family and would be expected to form a turn that would constrain the relative positioning of flanking sequences. The IR593.CT protein instead has simply a dipeptide linker (SR) between Fn0 domain and carboxyl-terminal sequence, arising from the XbaI restriction site used in construction. We conclude that rather precise positioning of the carboxyl-terminal sequence within dimeric constructs is required to generate a binding site with high affinity as opposed to moderate affinity for insulin. It remains unclear whether L1 and carboxyl-terminal domains contribute in cis or in trans to high affinity binding within a dimeric structure.

It is common practice to use IC_{50} values from binding competition assays as a convenient measure of the affinity of ligand-receptor interaction. However, this is strictly valid only under conditions such that the receptor is far from saturated with radioligand, and true affinity can be underestimated especially when it is very high (25). The rank order of IC_{50} values for the constructs studied here was the same whether assays were conducted with higher concentrations of tracer for shorter times (21) or lower concentrations of tracer for longer times (28), although the spread of apparent affinities was greater under the latter conditions because of the underestimation of high affinities under the former conditions. Indeed the affinity of detergent-solubilized IRwt, as determined during prolonged incubation with very low concentrations of radioligand, ap-
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...pears considerably higher than the affinity of cell-associated receptor. The reason for this difference is obscure, but it does raise the question of what should be considered the true affinity of wild type IR as expressed on cells in vivo.

The significant difference in affinity between IR593.CT and mIR.Fn0 under both assay conditions, has implications for conclusions regarding the role of individual dimerization domains in creating a high affinity binding site. The fact that mIR.Fn0 has an affinity for insulin similar to monomERIC mini-receptors and substantially lower than detergent-solubilized wild type receptor has been noted previously (28). It has also been reported that dimeric deletion constructs IRA649 and mIR.Ex10, containing the Fn1 insert but not the Fn0 domain, are similar in affinity to monomERIC mini-receptors (22, 37). It was therefore concluded that the presence of both dimerization domains and their respective α-α disulfide links, as in mIR.Fn0/Ex10, was necessary to create a binding site with affinity similar to IRwt (28). However, it is clear that a single dimerization domain, as in IR593.CT, can be sufficient to confer high affinity binding more similar to wild type receptors than monomERIC forms. Moreover, as confirmed in this study, the complete ectodomain has a much lower affinity than mIR.Fn0/Ex10 (25) and behaves much more like isolated half-receptors despite possessing both α-α links. The relationship between dimerization and binding affinity is therefore not a simple one.

A surprising observation was the lack of a consistent relationship between the relative affinities for IGF-I and insulin. Both IR593.CT and mIR.Fn0/Ex10 were similar to IRwt (Ex11−isoform) in having approximately 30–40-fold lower affinity for IGF-I compared with insulin. The ratio was also similar for IR473.CT, despite much lower affinities for both ligands. As reported previously (38), the presence of the exon 11 sequence, as in HIRs-Fc (Ex11+isoform) and IRwt (Ex11−isoform), significantly decreased the affinity for IGF-I relative to insulin. The mIR.Fn0 construct behaved anomalously and exhibited little specificity for insulin versus IGF-I, having a low affinity for insulin similar to IR473.CT but a relatively high affinity for IGF-I similar to IR593.CT and mIR.Fn0/Ex10. It thus appears that the disposition of binding determinants within this construct is suboptimal for interaction with insulin but not IGF-I when compared with wild type receptor. This may reflect differences in relative importance of the L1, CR, and L2 domains in interactions with the two ligands (15–17).

In addition to high equilibrium binding affinity, a characteristic feature of insulin binding to IRwt is negative cooperativity, as manifested in curvilinear Scatchard plots and accelerated dissociation of pre-bound radioligand in the presence of excess unlabeled ligand (24). It would be expected that such cooperative behavior would depend on oligomerization, and indeed half-receptors display linear Scatchard plots (22, 37). The dimeric insulin receptor fragment mIR.Fn0/Ex10 closely resembled IRwt in its cooperative binding properties (28) although full ectodomain did not exhibit negative cooperativity (25). The specific aspects of receptor structure involved in cooperative structure of ligand binding have not been identified, although it has been suggested that the region around residue Lys460, at the L2/Fn0 boundary, may form an interface between α-subunits that is important for this phenomenon (39). It has also been suggested that interactions between both transmembrane and cytoplasmic domains may contribute to the generation of negative cooperativity (40), although the properties of the mIR.Fn0/Ex10 construct show that these are not essential.

The present data indicate that neither dimerization nor high equilibrium binding affinity is necessarily associated with negative cooperativity. Neither of the dimeric constructs IR593.CT and mIR.Fn0 exhibited accelerated dissociation of bound radioligand in the presence of unlabeled insulin or antibody 47-9. The rate of ligand dissociation from IR593.CT was similar to that of IRwt in the absence of unlabeled insulin, whereas dissociation from mIR.Fn0 was faster and similar to that of IRwt in the presence of insulin, reflecting the relative equilibrium binding affinities of the two constructs. Thus it appears that IR593.CT is constrained in a high affinity, slow dissociation state, whereas mIR.Fn0 (IR601.CT) is fixed in a low affinity, rapid dissociation state. The dimerization domain encoded by exon 10 evidently plays a critical role in allowing transition between these states and the resulting negative cooperativity of ligand binding. It can be hypothesized that the 594–601 sequence is inhibitory to high affinity binding, and this inhibition is relieved by the addition of the exon 10 sequence (amino acids 650–703). Thus the presence of both the 594–601 and 650–703 sequences (as in mIR.Fn0/Ex10) would be necessary to allow the transition between high and low affinity states. A testable prediction of this hypothesis is that an IR593.CT/Ex10 construct would display high affinity but not negative cooperativity.

The binding of insulin to its receptor can also be influenced by anti-receptor antibodies. We and others (29, 33) have shown that antibodies can either inhibit or stimulate insulin binding depending on the location of their epitopes within the receptor. These antibodies provide additional probes with which to compare different receptor constructs. Two antibodies (83-7 and especially 18-146) stimulate ligand binding to IRwt within cell membranes via epitopes in the CR domain. However, the effect of these antibodies is much less marked on detergent-solubilized receptor, enabling 83-7 to be used for immunocapture in microtiter plate assays without perturbation of insulin binding. Antibodies 83-7 and 18-146 did not significantly affect insulin binding to any of the 4-domain constructs (IR593.CT, mIR.Fn0, and mIR.Fn0/Ex10) but consistently stimulated binding to HIRs-Fc and the 3-domain IR473.CT. Thus, the stimulatory effects of these antibodies were not dependent on dimerization, and the structural basis for their effects on some constructs and not others is unclear.

Several antibodies (47-9, 25-49, and 83-14) that inhibit insulin binding to IRwt via epitopes in the Fn0 domain similarly inhibited binding to HIRs-Fc and mIR.Fn0/Ex10. Moreover, antibody 47-9, which behaves like unlabeled insulin in accelerating dissociation of 125I-insulin from IRwt (36), also accelerated the dissociation of tracer bound to HIRs-Fc and mIR.Fn0/Ex10. However, none of these antibodies significantly inhibited binding to IR593.CT or mIR.Fn0 even though they were able to immunoprecipitate these proteins. These findings suggest that the dimerization domain encoded by exon 10 may also play a role in transmitting the effects of inhibitory antibodies to the insulin-binding site, although it is unlikely to be involved directly in the binding of either antibody or insulin. The inhibitory antibodies might in principle act by sterically blocking the insulin-binding site or by allosterically inducing a low affinity conformation of the receptor. Dimerization via the exon 10 domain might therefore affect either the orientation of the Fn0 domain relative to the insulin-binding site (in a steric blockade model of antibody-mediated inhibition) or the ability of interactions in one half-receptor to influence the conformation of an associated half-receptor (in an allosteric model).

An unexpected finding was that antibody 83-14 actually appeared to stimulate insulin binding to IR593.CT and mIR.Fn0 when assessed in the PEG precipitation assay but not the microtiter plate assay. A possible explanation for this result is an increase in the precipitation of bound soluble receptors IR593.CT or mIR.Fn0 with antibody. However, this appears...
unlikely as the result was seen only with 83-14 as well as with an Fab fragment of this antibody. An alternative explanation is that 83-14 mAb may change the conformation of the dimeric 4-domain receptor fragments IR593.CT and mIR.Fn0 resulting in an increase in ligand binding, but it is unclear why this should be seen only in one assay format.

In summary, we conclude that the α-subunit carboxyl-terminal sequence is essential for ligand binding and that its location can have a major impact on binding affinity. Consistent with a cross-linking model of receptor-ligand interaction, dimeric mini-receptors can display considerably higher affinity than monomeric constructs, although the possibility cannot be ruled out that the dimerization domains contribute ligand contact sites as well as the capacity for dimer formation. Indeed, whether or not dimers display high affinity depends on structural detail rather than dimerization per se. Thus, although dimerization via the Fn0 domain alone can confer high affinity ligand binding, additional α-α contacts contributed by the Fn1 insert (encoded by exon 10) are required for cooperative interactions between α-subunits and for the inhibitory effects of anti-receptor antibodies. It remains to be determined whether cross-linking of α-subunits via the Fn1 insert affects the relative orientation of other domains within a rigid receptor structure or, alternatively, influences the transmission of conformational changes within a more dynamic structure.

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