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Biochemical and Biophysical Research Communications 347 (2006) 334–339
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Characterization of a second ligand binding site of the insulin receptor

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Received 9 June 2006
Available online 22 June 2006

Abstract

Insulin binding to its receptor is characterized by high affinity, curvilinear Scatchard plots, and negative cooperativity. These properties may be the consequence of binding of insulin to two receptor binding sites. The N-terminal L1 domain and the C-terminus of the α subunit contain one binding site. To locate a second site, we examined the binding properties of chimeric receptors in which the L1 and L2 domains and the first Fibronectin Type III repeat of the insulin-like growth factor-I receptor were replaced by corresponding regions of the insulin receptor. Substitutions of the L2 domain and the first Fibronectin Type III repeat together with the L1 domain produced 80- and 300-fold increases in affinity for insulin. Fusion of these domains to human immunoglobulin Fc fragment produced a protein which bound insulin with a K_d of 2.9 nM. These data strongly suggest that these domains contain an insulin binding site.

Keywords: Insulin; Insulin-like growth factor-I; Receptor; Binding site; Mutagenesis; Fusion protein; Affinity

Insulin interaction with its receptor is complex (for review, see Ref. [1]). Scatchard plots [2] are concave and curvilinear, indicative of the co-existence of high and low affinity binding sites. Also the dissociation rate of pre-bound labeled insulin is accelerated by the presence of unlabeled insulin in a concentration-dependent manner, suggesting possible negative cooperativity [3]. These phenomena and high affinity interactions with insulin are dependent on the dimeric structure of the native receptor and are also not observed with the recombinant secreted receptor extra-cellular domain [1]. The stoichiometry of binding appears to be one insulin molecule to one receptor dimer for the native receptor and two insulin molecules per dimer for the recombinant extra-cellular domain [4]. It has been hypothesized that the complex binding properties described above for the native receptor are the consequences of bivalent binding of insulin [5–8].

A low affinity insulin binding site of the secreted recombinant receptor has been shown to be composed of elements of the N-terminal L1 domain and the C-terminus of the α subunit [9,10]. There is evidence for a requirement for additional determinants in the L2, Fn0, and Fn1 domains of the receptor for full affinity insulin binding. However, technological limitations of these studies preclude a precise evaluation of the roles of these domains in insulin binding [11,12]. Further, they do not provide direct evidence that the L2, Fn0, and Fn1 domains contain a ligand binding site.

In the present study, we show that the substitution of the L2 and Fn0 domains of the IGF-I receptor by the corresponding domains of the insulin receptor, in conjunction with the L1 domain, produces a chimeric receptor with high affinity for insulin. Further, expression of an insulin receptor L2Fn0-Fc fusion protein which binds insulin with moderately high affinity indicates that these domains contain determinants of an insulin binding site.

Materials and methods

General procedures and materials. Molecular biological procedures were performed by standard methods [13]. Oligonucleotides were purchased from Integrated DNA Technologies. Restriction and modifying

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doi:10.1016/j.bbrc.2006.06.089

enzymes were from New England Biolabs. Recombinant human insulin and HPLC-purified [^{125}I]Tyr 31 IGF-I (IGF-I monoiodinated at Tyrosine 31) [14] were from NovoNordisk A/S [14]. HPLC-purified [^{125}I]Tyr A14 insulin (insulin monoiodinated at Tyrosine 14 of the A chain) was from Amersham. Protease inhibitors were from Roche Molecular Biochemicals. cDNAs for the insulin and IGF-I receptors were as previously described [15–17]. Both were modified for subcloning into the epitope tag expression vectors by introduction of a *Bam*HI site encoding an in-frame C-terminal Gly-Ser linker at their 3′ ends just prior to the stop codon by site-directed mutagenesis [15]. Monoclonal anti-AU5 IgG was purchased from Covance, monoclonal anti-hexahistidine antibody was from Abcam, and goat anti-human Fc polyclonal antibody was from Pierce.

Construction of receptor expression plasmids. To produce plasmids for the expression of receptor with C-terminal epitope tags, in-frame coding sequences for hexahistidine and a triple repeat of AU5 (TDFYLK) were introduced between the *Bam*HI and *Xba*I sites of the pcDNA3.Zeo+ polylinker by cassette mutagenesis. Receptor cDNAs, modified as described above, were then subcloned into the *Hin*dIII and *Bam*HI sites.

cDNAs encoding receptor chimeras were made using a modification of the mutagenesis procedures of Clackson and Winter [18] and Kirsch and Joly [15]. Oligonucleotide primers were designed according to the guidelines of Clackson and Winter [18]. Pfu Ultra (Stratagene) was used as the polymerase to minimize misincorporation. Positive mutants were identified by restriction analysis and further confirmed by sequencing of the receptor DNA coding region.

In order to construct insulin and IGF-I receptor L2, Fn0 domain-human immunoglobulin Fc fusion proteins, a human immunoglobulin Fc cDNA with a C-terminal hexahistidine tag was amplified from the plasmid pCMVHIRFc in a PCR with Pfu Ultra (Stratagene); the upstream primer contained a *Bam*HI site encoding an in-frame Gly-Ser linker at its 5′ end and the downstream primer an *Xba*I site and the anti-sense sequence for the hexahistidine tag at the 5′ end. The amplification product was digested with *Bam*HI and *Xba*I, and purified by agarose gel electrophoresis. The purified DNA was ligated into *Bam*HI, and *Xba*I digested insulin receptor cDNA in pcDNA3.Zeo+. The resulting plasmid was digested with *Sac*II and *Bam*HI and the vector fragment containing the coding sequences for the insulin receptor signal peptide and the immunoglobulin Fc fragment purified. The coding sequences for the insulin and IGF-I receptor L2 and Fn0 domains were amplified; the primers contained in-frame *Sac*II and *Bam*HI sites at the 5′ ends of the respective upstream and downstream primers. After *Sac*II and *Bam*HI digestion, the purified amplification product was ligated with the vector fragment containing the immunoglobulin Fc coding sequences. The coding sequences were verified by DNA sequencing.

Expression of receptor cDNAs. The receptor cDNAs were transiently expressed in PEAK rapid cells and conditioned medium and detergent lysates of transfected cells harvested as previously described [10].

Receptor binding assays. Insulin and IGF-I competitive receptor binding assays were performed by a micro-titer plate antibody capture assay [10]. Binding data were analyzed by a two-site sequential model [5,19] or single site models with homologous or heterologous labeled and unlabeled ligands to obtain dissociation constants [20].

Western blotting. Western blotting of proteins from lysates and conditioned media of transfected cells was performed by standard methods [21]. Blots were visualized by enhanced chemiluminescence (Pierce Supersignal Pico).

Results and discussion

Expression and characterization of chimeric insulin-IGF-I receptors

Studies from this and other laboratories have characterized a ligand binding site of the recombinant secreted insulin receptor, composed of the L1 domain and a C-terminal α subunit peptide (amino acids 705–719) [10,22,23]. Recent studies with minimized secreted recombinant insulin receptors have suggested that the L2 and Fn0 domains and the region of the insulin receptor encoded by Exon 10 of the receptor gene (X10), i.e., the α subunit component of the Fn1 domain, are essential for high affinity insulin binding comparable to that observed with the wild-type receptor [24,25]. To determine whether these regions exhibit similar properties in the holoreceptor, we have generated a series of chimeric insulin-IGF-I receptor cDNAs. In each of these chimeric receptor cDNAs, the coding sequences for either a single region or a combination of regions of the IGF-I receptor were substituted by the coding sequences for the corresponding regions of the insulin receptor (Fig. 1) .

We first transiently transfected IGFRL1HIR, IGFRL2HIR, IGFRFn0HIR, IGFRX10HIR, IGFRL2 Fn0HIR, HIR, and IGFR cDNAs in 293 PEAK cells. Expression was evaluated by determining tracer [^{125}I]Tyr A14 insulin and [^{125}I]Tyr 31 IGF-I binding to receptors isolated from detergent lysates of transfected cells. All receptor chimeras exhibited detectable specific binding of both peptides apart from IGFRX10HIR and IGFRL2Fn0HIR.

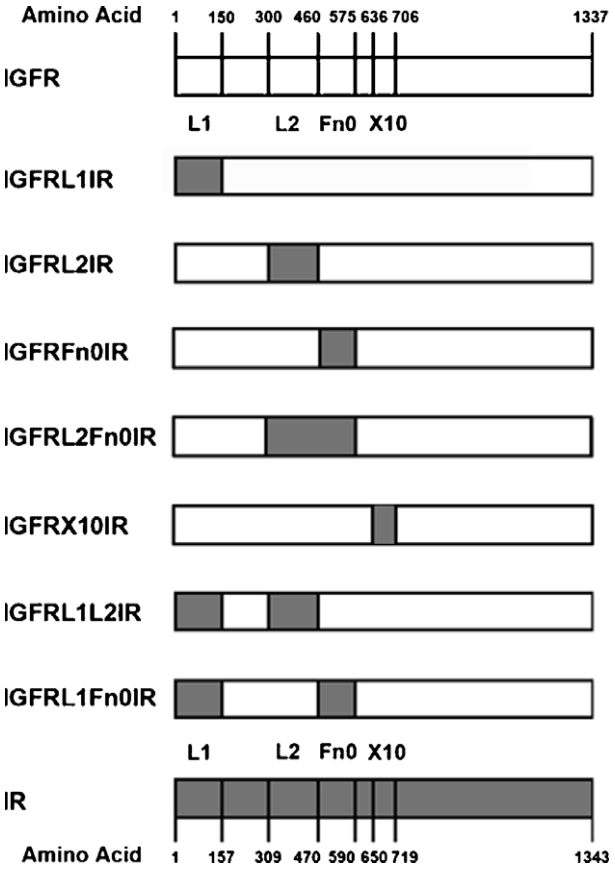

Fig. 1. Schematics of chimeric receptor cDNAs. Shown are schematics of cDNAs encoding the coding sequences of wild-type insulin receptor (HIR) and IGF-I receptor (IGFR) and their respective chimeras. Domains of the insulin receptor are represented by grey rectangles and of the IGF-I receptor by white rectangles. L1 and L2 are receptor L domains; CRD, cysteine rich domain; Fn0, N-terminal Type III fibronectin repeat; X10 region encoded by Exon 10 of the receptor gene.

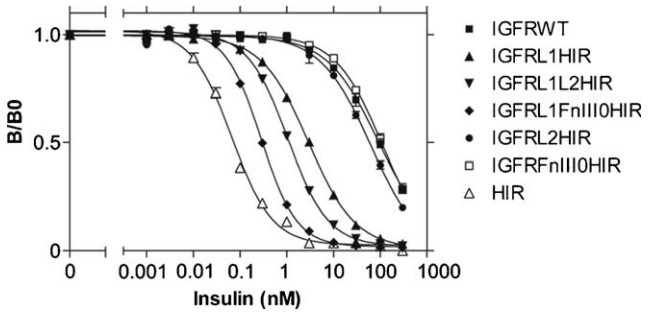

Fig. 2. Insulin binding to receptor chimeras. Receptor chimeras were transiently expressed in 293 PEAK cells. Receptors were immobilized in micro-titer plates as described in Materials and methods. Competitive binding assays were performed with [^{125}I]Tyr 31 IGF-I and varying concentrations of unlabeled insulin.

Absence of expression of these chimeras was confirmed by Western blotting with anti-AU5 antibody (data not shown). We then transfected IGFRL1L2HIR and IGFRL1Fn0HIR cDNAs. Both exhibited specific binding of [^{125}I]Tyr A14 insulin and [^{125}I]Tyr 31 IGF-I.

To evaluate the relative affinities of the expressed chimeras for insulin, competitive binding experiments were performed on receptors isolated from detergent lysates of transfected cells with unlabeled insulin and [^{125}I]Tyr 31 IGF-I. The results of these studies are shown in Fig. 1 and Table 1. Affinity of HIR for insulin was 1000-fold greater than that of IGFR. Individual substitutions of the L2 and the Fn0 insulin receptor domains into the IGFR had negligible effect on affinity for insulin. However, substitution of the insulin receptor L1 domain produced a significant increase in affinity for insulin (30-fold), consistent with previously published studies [26]. Affinity for insulin was further increased when the L1 and L2 (80-fold) and the L1 and Fn0 domains (300-fold) of the insulin receptor were substituted in combination. These findings suggest that if the combined substitution had been feasible, the resulting receptor would possibly have an affinity for insulin near that of the native receptor.

Similar results have been obtained with chimeras in which larger regions of the IGF-I receptor containing parts or all of these domains were substituted by the corresponding regions of the insulin receptor [11,12]. However, in one of these studies [11], it was found that substitution of amino acids 315–514 of the IGF-I receptor by amino acids 325–524 of the insulin receptor was sufficient to confer full affinity for insulin. It should be noted that this is not a complete domain exchange; it lacks 25 amino acids of the N-terminus of the L2 domain and 36 amino acids of the C-terminus of the Fn0 domain. It also leads to an increase in affinity for IGF-I. Thus this result could potentially be an artifact.

Brandt et al. have characterized a minimized receptor formed from, N-terminal to C-terminal, the L1, cysteine rich domains, L2 , Fn0, and a part of the receptor encoded by Exon 10 of the receptor gene including amino acids 714–719 at its C-terminus [24]. This receptor had insulin binding properties that were nearly identical to those of the native receptor, i.e., a comparable high affinity, multiple classes of binding sites, and negative cooperativity. Comparison with other minimized constructs that lacked the region encoded by Exon 10 led them to conclude that this region was also essential for high affinity insulin binding. We were unable to address this issue in the present study because of our inability to express chimeras in which this region was exchanged. However, the observation that we were able to generate a chimera with near full affinity for insulin suggests that either this region is unnecessary for full affinity or that the corresponding region of the IGF-I receptor may fulfill this function.

To further characterize the insulin binding properties of the chimeras, competitive binding experiments were performed with unlabeled insulin and [^{125}I]Tyr A14 insulin. The results of these experiments were analyzed by computerized curve fitting using a sequential two-site model [5,19] to obtain binding parameters. Binding of insulin to IGFR, IGFRL2HIR, and IGFRFn0HIR was too low to be assessed using this analysis. Results for HIR, IGFRL1HIR, IGFRL1L2HIR, and IGFRL1Fn0HIR are summarized in Table 2. The chimera with substitution of L1 of the insulin receptor into the IGFR has a high affinity dissociation constant of 1.6 ± 0.1 nM and a low affinity

Table 1
Relative affinities of chimeric receptors for insulin

Receptor	IC50 (nM)[a] (mean ± SEM, $n = 3$)	Relative affinity[b]
HIR	0.08 ± 0.01	100
IGFR	94 ± 8	0.08
IGFRL2HIR	58 ± 7	0.13
IGFRFn0HIR	105 ± 11	0.07
IGFRL1HIR	2.9 ± 0.2	2.6
IGFRL1L2HIR	1.2 ± 0.1	6.6
IGFRL1Fn0HIR	0.3 ± 0.05	24.8

[a] IC50s (concentrations producing half maximal displacement of tracer) for insulin were determined for each receptor by non-linear regression analysis of the competitive binding data from Fig. 2, using GraphPad Prism software.

[b] Relative affinities were determined from the reciprocals of the IC50s, assigning a value of 100 to the wild-type insulin receptor (HIR).

Table 2
Dissociation constants of chimeric receptors

Receptor	Membrane bound (mean ± SEM, $n = 3$)		Secreted (mean ± SEM, $n = 3$)
	K_{d1} (nM)[a]	K_{d2} (nM)[a]	K_d (nM)[a]
HIR	0.07 ± 0.02	1.1 ± 0.3	2.1 ± 0.2
IGFRL1HIR	1.6 ± 0.1	23 ± 1.8	7.4 ± 0.9
IGFRL1L2HIR	0.2 ± 0.02	7.4 ± 0.6	5.1 ± 0.4
IGFRL1Fn0HIR	0.06 ± 0.05	4.1 ± 0.6	4.9 ± 0.6

[a] Chimeric receptors were transiently expressed as either full-length or secreted recombinant receptors. Competitive binding assays were performed with [^{125}I]Tyr A14 insulin and unlabeled insulin. Dissociation constants were determined by fitting the data for the membrane bound receptors to a two-site sequential model [5,19] and the data for the secreted receptors to a single site model.

Table 3
Relative affinities of insulin analogs for the insulin receptor L2Fn0-immunoglobulin Fc fusion protein

Analog	Dissociation constant (nM)[a] (mean ± SEM, $n = 3$)	Relative affinity[b]
Insulin	2.9 ± 0.3	100
Proinsulin	2.5 ± 0.01	117
Desoctapeptide insulin	7.1 ± 0.4	41

[a] Fusion protein was isolated from conditioned medium of transiently transfected cells in micro-titer plates coated with anti-Fc IgG. Competitive binding assays were performed with [^{125}I]Tyr A14 insulin and varying concentrations of the designated analogs. Dissociation constants were determined by non-linear regression analysis of the competition data using the method of Wang [20].

[b] Relative affinities were determined from the reciprocals of the dissociation constants, assigning a value of 100 to insulin.

dissociation constant of 23 ± 1.8 nM. The additional substitutions of L2 or Fn0 result in increases in affinity mediated predominantly by a decrease in the dissociation constant of the high affinity component of binding (Table 2). We also examined the effects of these domain substitutions on the insulin binding properties of transiently expressed secreted recombinant receptors (Table 3). In contrast with the membrane bound receptor, the substitution of the L2 or Fn0 domains in addition to the L1 of the insulin receptor into the IGF-I receptor had minimal impact on affinity (Table 3). These results suggest the existence of determinants of a second ligand binding site in the L2 and Fn0 domains in the native receptor but which either has low affinity for insulin or is inaccessible in the secreted receptor.

Expression and characterization of an insulin receptor L2, Fn0-immunoglobulin Fc fusion protein

To determine whether the L2 and Fn0 domains contain determinants of a ligand binding site, we constructed cDNAs encoding hexahistidine-tagged insulin and IGF-I receptor L2, Fn0-immunoglobulin Fc fusion protein and transiently expressed them in PEAK cells. Conditioned medium from cells transfected with the insulin receptor fusion protein cDNA demonstrated detectable tracer [^{125}I]Tyr A14 binding whereas medium from cells transfected with the IGF-I receptor fusion protein cDNA and purified immunoglobulin Fc (1 mg/ml) did not (data not shown). Neither conditioned media exhibited significant tracer [^{125}I]Tyr 31 IGF-I binding. Expression of both the receptor fusion proteins was confirmed by Western blotting with a monoclonal anti-hexahistidine antibody (Fig. 3A). On reducing gels, the antibody bound to proteins of M_r 71K, which is consistent with the predicted M_r of the fusion protein monomers. In contrast, in the absence of reductant, it bound to proteins of M_r 140K, 210K, and also proteins with molecular weights too high to be accurately determined. The M_r 140K proteins are the expected fusion protein dimers, whereas the M_r 210K and higher molecular weight proteins are probably disulfide-linked trimers and

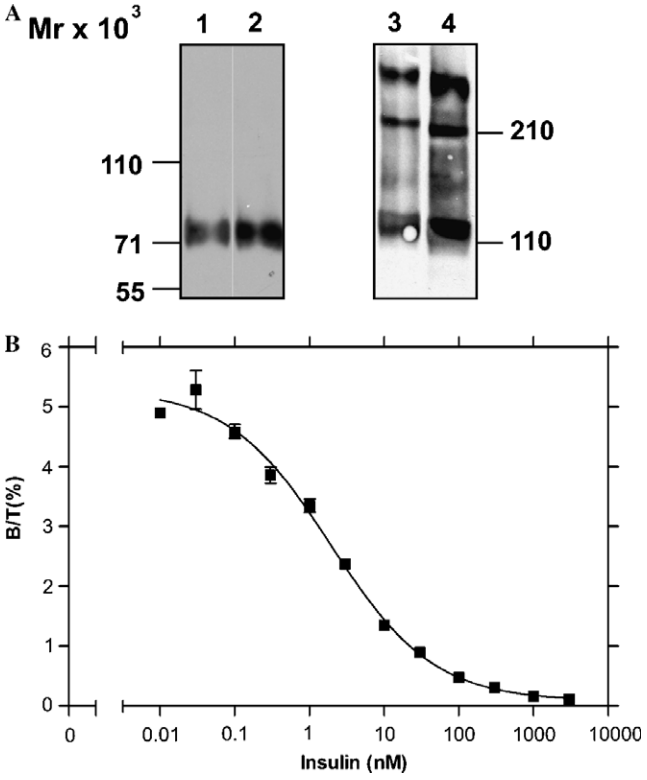

Fig. 3. Properties of insulin and IGF-I receptor. L2Fn0-immunoglobulin Fc fusion proteins. cDNAs encoding insulin and IGF-I receptor L2Fn0-immunoglobulin Fc fusion proteins were constructed as described and transiently expressed in 293 PEAK cells. (A) Western blots of insulin (lanes 1 and 3) and IGF-I receptor (lanes 2 and 4) L2Fn0-immunoglobulin Fc fusion proteins. Proteins were purified from conditioned medium by immobilized metal ion affinity chromatography and electrophoresed on a 3–8% Tris–acetate/SDS–polyacrylamide gradient gel under reducing (lanes 1 and 2) and non-reducing conditions (lanes 3 and 4). Blots were probed with an anti-hexahistidine monoclonal antibody and visualized by enhanced chemi-luminescence. (B) Competitive binding of [^{125}I]Tyr A14 insulin and unlabeled insulin to fusion protein immobilized in micro-titer plates coated with anti-Fc antibody.

higher order oligomers. Similar phenomena have been observed with other immunoglobulin Fc fusion proteins and it has been suggested to be due to aberrant disulfide bond formation between immunoglobulin Fc subunits [27].

To evaluate the ligand binding properties of the insulin receptor fusion protein, we performed competitive binding experiments with [^{125}I]Tyr A14 insulin and unlabeled insulin (Fig. 3B). These data were fitted to a single site binding model with a dissociation constant for insulin of 2.9 ± 0.3 nM (Table 3). We also evaluated the binding of proinsulin and desoctapeptide insulin to this protein. These analogs have disruptions of the structure of the "classical" receptor binding site of the insulin molecule which interacts with the binding site of the secreted receptor [28]; they have affinities for the receptor of 1% and 0.1%, respectively [28]. Their relative affinities for the fusion protein were 117% and 41%, respectively, suggesting that this binding site may interact with the novel receptor binding site of the insulin molecule recently described by De Meyts [29].

Models of insulin–insulin receptor interaction

A number of hypothetical models of insulin–insulin receptor interactions have been proposed to explain the complex nature of the interaction, high and low affinity binding sites, and negative cooperativity [3,6–8]. The model, that best explains the complexities of the interaction, is that of De Meyts et al. [3]. This proposes that the insulin molecule has two receptor binding sites located on opposite sides of the molecule, sites 1 and 2. Likewise each receptor monomer α subunit contains two distinct insulin binding sites; α1 being the cognate binding site for insulin's site 2 and α2 being the cognate site for insulin's site 1. The initial interaction of insulin with the receptor is between site 2 of insulin and site α1 of the receptor. Site 1 of the receptor bound insulin molecule then binds to the α2 of the other receptor monomer forming a cross-link. This generates the high affinity component of binding; the initial site 2-α1 interaction of a second insulin molecule represents the low affinity component. The model further proposes that there cannot be two simultaneous cross-links due to structural constraints. Thus in experiments, where labeled receptor bound insulin is allowed to dissociate in the presence of insulin, the binding of a second molecule of insulin would lead to the acceleration of dissociation of the labeled insulin by disrupting the initial cross-link.

When considered in the context of this model the binding site of the secreted receptor and the binding site in the L2 and Fn0 domains would represent the two insulin binding sites. Here, using the same assay, we have shown both to have very similar affinities; K_d 2.1 nm for the secreted receptor and 2.9 nM for L2Fn0. Both values are consistent with the experimentally determined values for the low affinity component of binding of the native insulin receptor. However, on the basis of additivity of free energies of interaction [30], it would be predicted from these findings that the K_d of the bivalently bound insulin would be 6 atto Mol/L (10^{-18}M) considerably lower than is observed experimentally. This suggests that conformational changes occur either in insulin or in the receptor during cross-link formation to account for the observed dissociation constant or the possibility that the dissociation constant of the L2Fn0 site is much higher when it is expressed in its natural context [8]. This is further supported by the observation that certain alanine mutations of the secreted receptor binding site can reduce affinity for insulin below experimentally detectable limits [10,22,23].

Summary and conclusions

In the present study, we have demonstrated that the L2 and Fn0 domains of the insulin receptor contain determinants of a second ligand binding site of the insulin receptor. Bivalent binding of insulin to this site and the previously characterized insulin binding site of the recombinant secreted receptor appear to be essential for the generation of the high affinity interactions observed in the native receptor. While these findings are supportive of hypothetical models of the insulin-receptor interaction, more detailed biophysical and probably structural studies will be necessary to fully evaluate them.

Acknowledgments

This work was supported in part by a grant from the National Institutes of Health (5 R01 DK065890) to J.W. We are grateful to Dr. Michael Weiss (Case Western Reserve University) for the gift of proinsulin and desoctapeptide insulin and to Drs. Michael Weiss and Pierre De Meyts (Hagedorn Research Institute, Denmark) for helpful discussion.

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