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The Insulin-Binding Domain of Insulin Receptor Is Encoded by Exon 2 and Exon 3

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Abstract Insulin receptors are disulfide-linked oligotetramers composed of two heterodimers each containing a 130-kDa α subunit and a 90-kDa β subunit. Insulin binds to the extracellular α subunit, and in the process stimulates the autophosphorylation of the β subunit and the expression of tyrosine kinase activity. Studies combining the use of photoaffinity labeling and immunoprecipitation with anti-peptide antibody have directly demonstrated that the cysteine-rich domain, encoded by exon 3, in the α subunit is part of the insulin-binding site of the receptor. Experiments with chimeric insulin receptors and chimeric insulin-like growth factor I receptors have confirmed that the cysteine-rich domain constitutes a part of the insulin-binding site. In addition, results from these experiments suggest that the N-terminal sequence, encoded by exon 2, in the α subunit also participates in insulin binding. In this review it is proposed that, assuming two insulin-binding sites per each holoreceptor oligotetramer, each insulin-binding domain may contain respectively two sub-domains for hydrophobic and charge contact with insulin, and that high-affinity binding would require the interaction of both subunits with the possibility of each subunit reciprocally contributing one of the sub-domains.

Key words: insulin-binding domain, insulin receptor, IGF-I receptor, chimeric receptor

As shown in Figure 1 insulin receptors are composed of two 130-kDa α subunits and two 90-kDa β subunits linked by disulfide bonds (see reviews by Czech [1985] and Goldfine [1987]). The α subunit and the β subunit encoded by 22 exons are synthesized as a precursor protein encoded by 22 exons [Seino et al., 1989]. Mild reduction of the holoreceptor gives rise to two identical heterodimers composed of one α and one β subunit. Amino acid sequence deduced from the cloned cDNA of the receptor shows the presence of a single putative membrane-spanning domain in the β subunit, suggesting that the α subunit is totally extracellular but is anchored to the cell membrane by disulfide linkage to the α subunit [Ullrich et al., 1985; Ebina et al., 1985]. Results obtained from photoaffinity labeling [Yip et al., 1980] and chemical cross-linking [Pilch and Czech, 1980] experiments have shown that insulin primarily binds to the α subunit. Insulin binding to the holoreceptor leads to the autophosphorylation of its β subunit and the expression of tyrosine kinase activity (see review by Goldfine [1987]). As is the case with other hormones, the specificity and affinity of insulin binding to its receptor determine the process of cellular activation and response. These two parameters are defined by the structural properties of the ligand-binding domain of the receptor. Two experimental approaches have been used to study the receptor insulin-binding domain, and some significant observations have been made about its nature. In one approach the ligand-binding site(s) is affinity-labeled with insulin photoprobes; in the other, chimeric receptors of insulin receptor and insulin-like growth factor I (IGF-I) receptor are analyzed for binding specificity and affinity. This communication will review these two methods of approach and the results obtained.

PHOTOAFFINITY LABELING OF INSULIN RECEPTOR LIGAND-BINDING SITES

The technique of photoaffinity labeling provided the first demonstration of the subunit structure of the insulin receptor [Yip et al., 1978]. The observation that it is the α subunit that is primarily labeled strongly suggests that insulin interacts with its receptor through bind-

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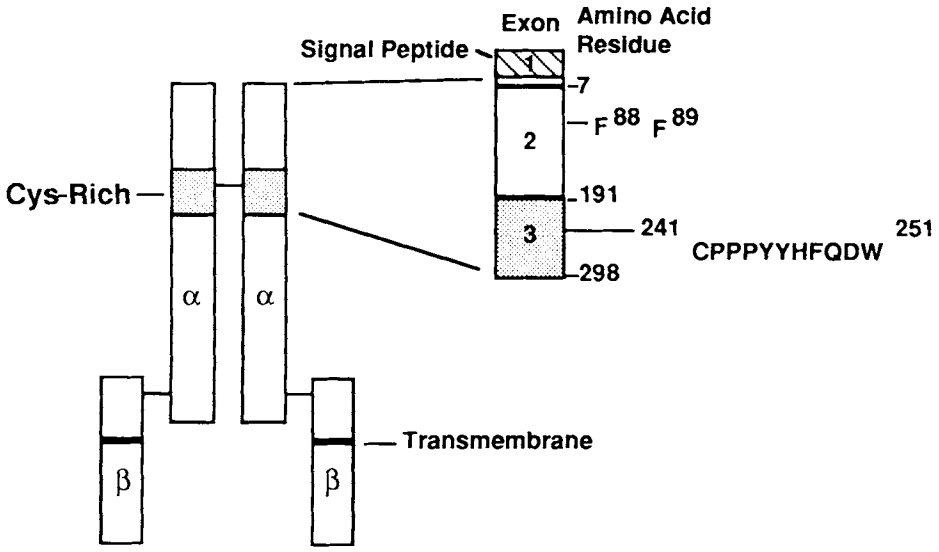

Fig. 1. Schematic representation of the structure of the insulin receptor. The ligand-binding domain encoded by exons 2 and 3 is enlarged to show specific amino acid residue and the amino acid sequence 241–251 that have been identified to be parts of the ligand-binding site.

ing to the structural domain formed by the β subunits in the holoreceptor. The insulin photoprobe, $N^{\epsilon B29}$-monoazido-benzoyl[^{125}I]-iodoinsulin, (B29-[^{125}I]-MABI), used in these studies is insulin derivatized near its putative receptor-binding region, and it retains more than 75% of the bioactivity of insulin. It is therefore reasonable to expect that photolabeled peptide fragment(s) obtained from proteolytic digestion of the photolabeled α subunit would be a part of the receptor insulin-binding domain. These considerations thus have formed the basis of the photoaffinity labeling approach to the study of the ligand-binding domain of the insulin receptor.

The use of the photoprobe, B29-MABI, to label the insulin receptor for the purpose of identifying the ligand-binding site is complicated by the fact that the insulin B chain or its carboxyl terminal fragment remains cross-linked to the receptor fragment of interest. We have therefore utilized a similar but cleavable insulin photoprobe, N-[4-[(4′-azido-3′-[^{125}I]-iodophenyl)azo]benzoyl]-(3-aminopropyl) insulin ([^{125}I]AZAP-insulin), which allows the insulin moiety to be cleaved from the labeled receptor protein after photoaffinity labeling. We thus obtained a labeled 23-kDa proteolytic fragment by endoproteinase Glu-C digestion of the α subunit isolated from the affinity-labeled receptor [Yip et al., 1988]. This labeled fragment was further digested with trypsin to a labeled fragment of less than 3 kDa. Based on several lines of indirect experimental evidence obtained at that time, we postulated that the 23-kDa fragment contained the amino acid sequence 205–316 in the cysteine-rich domain (Fig. 1). The cysteine-rich domain is encoded by exon 3 of the insulin receptor gene [Seino et al., 1989]. The high specific radioactivity (carrier-free) of the [^{125}I]AZAP-insulin has precluded its use to obtain a sufficient amount of the labeled 23-kDa or its much smaller tryptic fragment for direct identification by amino acid sequence determination. However, the origin for this fragment was subsequently confirmed by demonstrating that an antiserum (AP-II) raised against a synthetic peptide containing the sequence 241–251 was able to immunoprecipitate the labeled 23-kDa fragment [Yip et al., 1991]. It is therefore reasonable to conclude that insulin binds to the cysteine-rich domain of its receptor. Earlier studies also had suggested the possible involvement of the cysteine-rich domain in insulin binding [Boni-Schnetzler et al., 1987; Ullrich et al., 1986; Waugh et al., 1989].

A comparison between the amino acid sequence of the insulin receptor and that of the IGF-I receptor shows that the sequence 205–316 contains the longest non-homologous stretch of 18 amino acid residues. We were particularly interested in the sequence CPPPYYHFQDW (residues 241–251; Fig. 1) which could provide the necessary hydrophobic interaction with

amino acid residues FFY (B24–B26) in the receptor-binding domain of the carboxyl terminal region of the B chain of insulin [Wood et al., 1975]. Significantly we found that a synthetic peptide containing the sequence 243–251 (PPYYHFQDW) was able to bind to immobilized insulin, though with much lower affinity when compared to the insulin receptor, and that this binding was competed by solubilized insulin receptor but not by another synthetic peptide containing the down-stream sequence 260–270 [Yip et al., 1988]. The important role of the sequence 241–251 in receptor binding of insulin was further studied by site-directed mutagenesis. A mutant was constructed in which the sequence P*PP*YY-*H*FQDW was mutated to P*RR*YY*D*FQDW and the mutant receptor was expressed in rat hepatoma cells [Rafaeloff et al., 1989]. Compared to control cells expressing normal insulin receptor, cells expressing the mutant receptor showed an increase in binding affinity and an increased sensitivity to insulin in receptor kinase activity and α-aminoisobutyric acid uptake. The positive effect obtained with the mutation of this sequence is a strong indication that the mutations have directly affected the insulin-binding site.

Wedekind and colleagues [1989] have also used the photoaffinity labeling approach to identify the insulin-binding site. The insulin photoprobe used was modified at the Lys^{B29} residue to yield a derivative containing an additional lysine to which was attached a light-sensitive azidonitrophenyl group and a biotinyl group. This photoprobe was iodinated with [^{125}I]-iodine for use in photoaffinity labeling. The labeled receptor was digested with trypsin to yield a labeled "core-peptide" of 14 kDa which was purified by affinity chromatography on avidin followed by reversed phase HPLC. Ten to twenty picomoles of the core-peptide were sequenced and of the four residues detected only the last two were unambigously identified as Glu-Leu. Since insulin cannot be cleaved from the photoprobe used and its B chain should remain cross-linked to the core-peptide, the N-terminal sequence of the B chain of insulin should also have been detected and identified. Nevertheless, based on these sequence data and on where this dipeptide is found in the sequence of the subunit, it was concluded that the isolated fragment contained the N-terminal portion of the receptor α subunit and tentatively it was assigned the sequence 20–121. This result is different from ours. The difference could be due to the use of a different photoprobe. In this case, the binding affinity of the biocytinyl insulin photoprobe was reduced to 19% that of insulin, suggesting that the photoprobe might not be able to interact properly with the receptor due to the presence of the bulky lysylbiotinyl group. Thus it may have cross-linked to a site outside the insulin-binding domain. It is also possible that the sequence obtained is that of a fragment which was not labeled but was co-eluted with the labeled fragment during chromatography. This is a major problem when the separation and purification of the desired fragment by HPLC are based on its being labeled with ^{125}I-iodine.

It is possible that cross-linking could have occurred outside the insulin-binding domain with any one of the photoprobes used to identify the ligand binding of the insulin receptor. Such a possibility can be minimized by derivatizing the ligand at its putative receptor-binding site. However, modifications of the receptor-binding region of the ligand could interfere with ligand-receptor interaction resulting in the loss of binding affinity and biological activity. The antiserum AP-II that we have obtained against the sequence 241–251 provided the means to investigate if insulin was indeed cross-linked to the ligand-binding domain of the receptor. We [Yip et al., 1991] found that the antiserum did not recognize the photolabeled holoreceptor. However, insulin receptors, photolabeled with the cleavable probe (AZAP-insulin), were recognized by the antiserum after insulin was cleaved off. After similar cleavage treatment insulin receptors labeled with the non-cleavable probe (B29-MABI) remained unrecognized by the antiserum. These observations demonstrate that the sequence 241–251 was blocked by insulin bound to the receptor, further supporting the conclusion that this sequence is part of the insulin-binding domain. The antiserum also did not recognize the un-denatured receptor as shown by its inability to inhibit insulin binding and to precipitate either ^{125}I-labeled receptor or biosynthetically labeled receptor. Consequently we concluded that the insulin-binding domain may exist as a crevice accessible to insulin but not to large molecules such as immunoglobulins, and that insulin binding induces a conformational change in the binding domain such that it becomes accessible to large molecules like immunoglobulins.

LIGAND BINDING BY CHIMERIC INSULIN/IGF-I RECEPTORS

Insulin receptor and IGF-I receptor are structurally and functionally similar with respect to their oligomeric nature and ligand activation of β subunit phosphorylation and expression of tyrosine kinase activity. Although their amino acid sequences as deduced, respectively, from their cloned cDNAs are only about 50% homologous, the positions and numbers of cysteine residues and putative glycosylation sites in their α subunits are highly conserved [Ullrich et al., 1986]. Their structural and sequence homology combined with their ligand binding specificity has provided an excellent experimental approach used by several laboratories to generate chimeric insulin/IGF-I receptors to identify the insulin-binding domain. Results from affinity labeling experiments have provided the rationale to focus on exons 2 and 3 in these studies.

Gustafson and Rutter [1990] studied the binding of insulin and IGF-I by seven receptor chimeras stably expressed in Chinese hamster ovary cells. Two of these chimeras were IGF-I receptors in which the N-terminal portions of their α subunits were replaced with the corresponding portions of the insulin receptor. The others were insulin receptors in which increasing lengths of the N-terminal sequence of their α subunits were replaced with the corresponding sequence of the IGF-I receptor. Tracer ligand binding was measured on intact cells expressing each of these chimeric receptors. When the binding data were calculated as a ratio of insulin bound to IGF-I bound, a switch in preference for IGF-I was observed between one insulin receptor chimera bearing the IGF-I receptor sequence 1–225 (IGFIR225IR) and one bearing 1–286 (IGFIR286IR). It was concluded that hormone binding specificity was located between amino acid residue 230 and 285 in the insulin receptor, and between residue 223 and 274 in the IGF-I receptor. These findings are in agreement with our conclusion from photoaffinity studies that the cysteine-rich region encoded by exon 3 forms part of the insulin-binding domain. However, it is worth noting that the IGF-I receptor chimera (IR452IGFIR) bearing the N-terminal sequence 1–452 of the insulin receptor bound both ligands equally well and that the insulin receptor chimera bearing the N-terminal sequence 1–447 of the IGF-I receptor (IGFIR447IR) bound IGF-I ten times better than insulin. These observations suggest that, dependent on the receptor, other regions of the β subunit may play a role in determining binding specificity.

A different approach in the construction of chimeras was used by Andersen and colleagues [1990]. The chimeric receptor (sIR23) used was an insulin receptor truncated from the first amino acid residue of the transmembrane region of the β subunit to its carboxyl end and having exons 1, 2, and 3 replaced by those of IGF-I receptor. The soluble chimeric receptor was expressed in baby hamster kidney (BHK) cells as a soluble protein which was released into the medium. Similarly truncated insulin receptors (sIR) and IGF-I receptors (sIGF-I-R) expressed by BHK cells were used as controls. Ligand binding was carried out on highly purified receptor preparations. Their data showed that the chimeric insulin receptor sIR23 exhibited the ligand binding characteristics of sIGF-I-R, supporting the conclusion that exons 2 and 3 encode the insulin-binding domain. Since exon 1 encodes only the signal peptide and the first seven amino acid residues of the subunit, its product was not considered to be important for ligand binding. In a later study [Kjeldsen et al., 1991] the same laboratory analyzed ligand binding by several similarly truncated chimeric receptors in which the amino terminal sequences of the truncated insulin receptor and IGF-I receptor were replaced reciprocally. Their study showed that the chimeric IGF-I receptor containing the N-terminal sequence 1–68 or 1–83 of the insulin receptor bound insulin with as a high affinity (measured as IC_{50}) as the truncated insulin receptor sIR whereas its affinity for IGF-I was reduced by tenfold. However, the reciprocal chimeric insulin receptor did not bind IGF-I with high affinity although its affinity for insulin was reduced. Interestingly, compared with the truncated insulin receptor a chimeric insulin receptor containing the sequence 191–290 (essentially the sequence encoded by exon 3) of IGF-I receptor showed a higher affinity for insulin. It was concluded that common features were present in the ligand-binding site of the insulin receptor and the IGF-I receptor, but that the specificity of binding was located at different regions of the binding domain of each receptor. The study reported by Zhang and Roth [1991] in general also supports this view. They focussed on the cysteine-rich domain and analyzed the ligand binding properties of chimeric insulin receptors in which the cysteine-rich domain

(exon 3) has been replaced with that of the IGF-I receptor or the insulin receptor related receptor (IRR) [Shier and Watt, 1989], the ligand of the latter receptor being unknown. These chimeric receptors were transiently expressed in COS-7 cells, and assays of ligand binding and displacement were carried out on receptors released from cell lysis and pre-bound by anti-receptor antibody to plastic wells. Consistent with the idea that the cysteine-rich region is involved in ligand binding, the chimeric insulin receptor containing the cysteine-rich domain of IGF-I receptor was found to bind IGF-I with high affinity. However, the main finding was that the two chimeric insulin receptors showed a similar binding affinity for insulin as measured by ligand competition. In contrast to the report by Kjeldsen et al. [1991] the study by Zhang and Roth [1991] found that the chimeric insulin receptor containing the cysteine-rich domain of IGF-I receptor did not show a higher affinity for insulin than that exhibited by insulin receptors. Interestingly, like IGF-I receptor, this chimeric receptor showed the same affinity for IGF-I and IGF-II, suggesting the specificity for both IGF-I and IGF-II is determined by the same cysteine-rich domain.

The use of chimeric receptors has thus produced results confirming the cysteine-rich region as an insulin-binding domain of the insulin receptor. At the same time, however, these results also point to the N-terminal sequence encoded by exon 2 as being involved in conferring on the receptor its specificity for insulin. De Meyts and colleagues [1990] addressed this possibility by deletional and site-directed mutagenesis. They found that a deletional mutant lacking exon 2 when transiently expressed in COS-7 cells did not increase insulin binding of the host cells whereas insulin binding was increased in cells transfected with either the wild type receptor cDNA or cDNA of a receptor mutant lacking the N-terminal residues 1–66. They concluded that the insulin-binding domain probably resided between residues 66 and 190. Based on a consideration of the structure and function of insulin with respect to the role of Phe^{B24}, Phe^{B25}, and Tyr^{B26} in receptor binding, and on the expectation that these residues may contact similar hydrophobic residues in the insulin-binding domain, they identified two phenylalanines at residues 88 and 89 within the sequence 83–95 as the likely candidates. They reported that a mutation of residue 89, but not residue 88, to a leucine caused a complete loss of insulin binding, and that a mutation to tyrosine partially restored insulin binding. These findings are somewhat at odds with the results obtained by Kjeldsen et al., [1991]. As already discussed, these investigators found that, compared to insulin receptors, chimeric IGF-I receptors containing the sequence 1–68 of the insulin receptor exhibited a similar high affinity for insulin, indicating a role for this sequence in insulin binding. Further, they did not find Phe^{89} to be indispensible for insulin binding, since this chimeric IGF-I receptor and another bearing residues 1–83, both chimeras thus lacking Phe^{89} but containing Tyr^{89} of IGF-I receptor, bound insulin with an affinity equal to that expressed by the insulin receptor. It is possible that deletional or site-directed mutagenesis may have altered the structure of the receptor but not directly the receptor ligand-binding site to cause the loss of ligand binding. This consideration applies also to natural mutations found to have affected insulin binding.

PROSPECTS

On the whole, published data are consistent with the notion that the insulin-binding domain of the insulin receptor is encoded by both exon 2 and exon 3. However, these data do not provide the information on what amino acid residues in the ligand-binding domain may be interacting or in contact with insulin, except for our observation that residues 241–251 appeared to be blocked by bound insulin. X-ray crystallographic study of the insulin-receptor complex is obviously needed to provide this information. In the meanwhile, additional information can be obtained by sequencing much smaller receptor fragments that are affinity-labeled with insulin, and by studying the ligand binding properties of receptors with substitutions of smaller sequences or mutations of specific residues in the ligand-binding domain. Interpretation of the data obtained from these studies needs to be made in the context of our knowledge of the structure and function of insulin itself. In the same sense, a comparison between insulin binding and IGF-I binding to their respective receptor or to their chimeric receptors must also take into account the structure of IGF-I, which though similar to insulin is not identical. There is strong evidence that the C region of this growth factor analogous to the C-peptide in proinsulin is required for high-affinity binding to the IGF-I receptor,

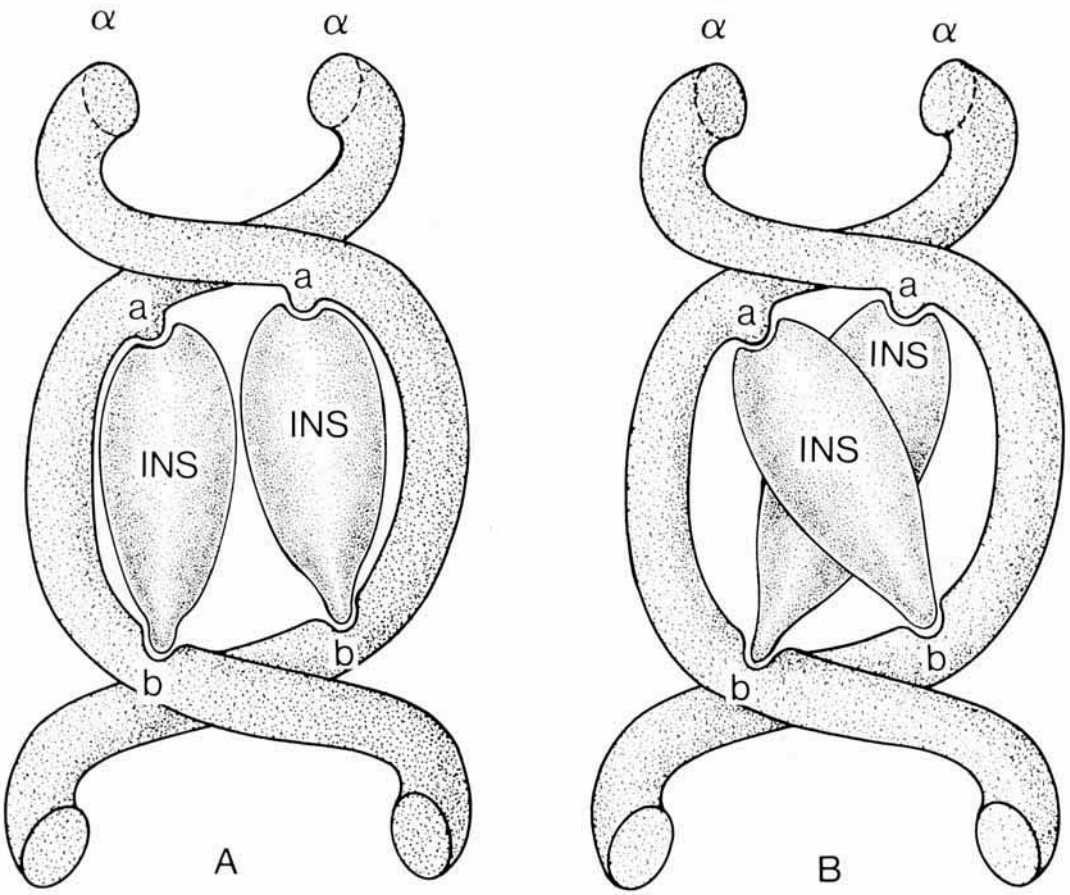

Fig. 2. Two alternative models showing how the two receptor α subunits could participate in insulin binding. It is assumed that the holoreceptor binds two molecules of insulin (INS). **A:** Each insulin molecule interacts with sub-domain a and b in each subunit. **B:** Each insulin molecule interacts with sub-domain a of one subunit and with sub-domain b of the other subunit.

but not to the insulin receptor [Bayne et al., 1988].

Many studies have shown the important role of the hydrophobic residues, particularly Phe^{B24} and Phe^{B25}, of the carboxyl end of the B chain of insulin in receptor binding [see Mirmira et al., 1991]. However it is evident that charge interactions are also involved. For example, even though these hydrophobic residues are conserved in the insulin of the guinea pig, its binding affinity to the insulin receptor is reduced by more than tenfold [Zimmerman et al., 1974]. Thus interaction between insulin and the insulin-binding domain involves more than hydrophobic contact. The importance of charge interaction is suggested by several studies. The biological activity of a truncated and amidated analogue of insulin, des-(B26-B30)-[Phe^{B25}-NH_2]insulin, was found to be comparable to that of intact insulin [Nakagawa and Tager, 1986]. Replacing Phe^{B25} with either Tyr or His in this analogue enhanced its bioactivity 2–3-fold. Charge interactions have also been suggested by the pH dependence of insulin binding [Halperin et al., 1987] and by the effect on binding when insulin receptor preparation was treated with diethyl pyrocarbonate [Pilch, 1982]. As well, the replacement of His^{B10} by Asp also increased the affinity of binding by more than tenfold [Schwartz et al., 1987; Brange et al., 1988] whereas replacement with asparagine reduced the affinity of receptor binding. Interestingly the amino acid at residue B10 in guinea pig insulin is asparagine. An insulin analogue, des-(B26-B30)-[Asp^{B10},Tyr^{B25}-NH_2]insulin, with a combination of these positive properties was superactive with an 11–13-fold increase in potency [Schwartz et al., 1989]. Our observation that the mutant receptor in which an additional net positive charge was introduced into the sequence 243–251 exhibited an increase in binding affinity and insulin response is also suggestive of a charge interaction. One can therefore

postulate that there may be two sub-domains within the receptor insulin-binding domain: one for hydrophobic and one for hydrophilic contact with insulin.

Finally, the $\alpha_2\beta_2$ oligomeric nature of the insulin receptor also must be considered in the interpretation of already available and new data. It is known that both subunits participate in the high-affinity binding of insulin. Recent studies [Finn et al., 1990; Chiacchia, 1991] have shown that there are at most two, possibly only one, so-called Class I disulfides [Massague and Czech, 1982] linking the two extracellular α subunits. Consequently the sulfhydryls in the cysteine-rich domain of each α subunit exist as intra-subunit disulfides. One can visualize a structural symmetry in the holoreceptor in which each subunit would contain an insulin-binding domain constituted by the amino acid sequence encoded by exons 2 and 3. The overall interaction between the two α subunits would give rise to the high affinity of binding. As an alternative, the insulin-binding domain in each α subunit would be cooperatively formed by both α subunits. In other words, the domain in each subunit would be constituted by the amino acid sequence encoded by exon 2 from one α subunit and by exon 3 from the other. These two alternatives are illustrated as A and B, respectively, in Figure 2. It should be possible to distinguish between them through the combined use of insulin receptor/IGF-I receptor hybrids [see Soos et al., 1990] and appropriately designed photoprobes of the two ligands.

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