We have previously shown that a minimized insulin receptor (IR) consisting of the first 468 amino acids of the insulin receptor fused to 16 amino acids from the C terminus of the α-subunit (CT domain) bound insulin with nanomolar affinity (Kristensen, C., Wiberg, F. C., Schäffer, L., and Andersen, A. S. (1998) J. Biol. Chem. 273, 17780–17786). In the present study, we show that a smaller construct that has the first 308 residues fused to the CT domain also binds insulin. Insulin receptor fragments consisting of the first 468 or 308 residues did not bind insulin. However, when these fragments were mixed with a synthetic peptide corresponding to the CT domain, insulin binding was detectable. At concentrations of 10 μM CT peptide, insulin binding was fully reconstituted yielding apparent affinities of 9–11 nM. To further investigate the minimum requirement for the length of the N terminus of IR, we tested smaller receptor fragments for insulin binding in the presence of the CT peptide and found that a fragment consisting of the first 255 amino acids of IR was able to fully reconstitute the insulin binding site, yielding an apparent affinity of 11 ± 4 nM for insulin.

Insulin mediates its effects by binding to tyrosine kinase receptors in the plasma membrane of targets cells. The IR protein is a dimer of two identical α/β monomers covalently linked by disulfide bonds in a β-α-α-β conformation (1). The IR structure function has recently been reviewed (2). Predictions of the structure of the IR ectodomain have been based on sequence alignments with homologous domains in other receptors, reviewed by Marino-Busjes et al. (3). The consensus from these alignments is that the first 468 residues of the α-subunit contain two large homologous domains L1 and L2 separated by a cysteine-rich (CYS) region (4, 5). A crystal structure of the protein is a dimer of two identical α/β monomers covalently linked by disulfide bonds in a β-α-α-β conformation (1). The IR structure function has recently been reviewed (2). Predictions of the structure of the IR ectodomain have been based on sequence alignments with homologous domains in other receptors, reviewed by Marino-Busjes et al. (3). The consensus from these alignments is that the first 468 residues of the α-subunit contain two large homologous domains L1 and L2 separated by a cysteine-rich (CYS) region (4, 5). A crystal structure of the L1-CYS-L2 region of the homologous IGFIR receptor (IGFIR) has been solved, confirming this domain structure (6). However, this construct does not bind ligand, whereas studies on minimized receptors show that, when fusing the L1-CYS-L2 domain to either of the C-terminal α-subunit sequences, IGFIR residues 691–706 or IR residues 704–719 (CT domains), the construct does bind ligand (7). The affinity of the minimized insulin receptor (mIR) for insulin is ~5–10 nM or similar to what is found for the soluble IR ectodomain (sIR) (8). The solubilized holoreceptor (hIR) binds insulin with an affinity that is almost 1000-fold better than mIR and sIR (9). Recently, we reported that, when expressing a receptor construct in which 48 residues of the exon 9 region of the α-subunit were deleted, we obtained a dimeric α-α receptor fragment that bound insulin with full holoreceptor affinity. The data showed that the picomolar affinity was associated with the dimeric structure of the α-subunits, whereas monomers had nanomolar affinity for insulin (9). Moreover the dimeric construct (mIR.Fn0/Ex10) also displayed accelerated dissociation of labeled insulin in the presence of excess unlabeled insulin, which is another characteristic of the hIR. Accelerated dissociation can be explained by site-site interaction assuming that there are two insulin binding sites on the IR (10, 11). One binding site on the receptor is proposed to interact with the classical binding site (site 1) on the insulin molecule, whereas the other receptor binding site is thought to interact with residues in the hexamer surface including the leucine residues in A13 and B17 (site 2) (10). To obtain full picomolar affinity, it has been suggested that the insulin molecule cross-links the two α-subunits with the site 1 bound to receptor binding site 1 in one subunit and site 2 bound to receptor binding site 2 in the other subunit (10). The nanomolar affinity obtained with mIR is believed to represent the interaction between the receptor binding site 1 located within mIR and the classical binding site of insulin, whereas higher affinity requires a dimeric construct that allows a cross-interaction with receptor binding site 2. This binding site 2 on IR may include ligand contact sites located within residues 469–703 of the α-subunit that are not present in mIR (9).

In the present study we focused on monomeric insulin receptor fragments trying to delineate the minimum requirements for obtaining nanomolar affinity binding of insulin. Initially, we deleted the L2 domain from mIR and showed that this construct, consisting of the first 308 residues fused to the CT domain, also bound insulin with nanomolar affinity, whereas when we removed the CT domain from either mIR or IR308, there was no measurable binding of insulin. Second, we found that insulin binding could be fully reconstituted from these non-binding receptor fragments by mixing them with a large excess of a free synthetic CT peptide. Finally, we used the strategy of reconstitution to demonstrate that a fragment corresponding to the first 255 residues of IR binds insulin with
nномар appear apparent affinity when mixed with the CT peptide. The implications of these findings for the binding reaction are discussed.

EXPERIMENTAL PROCEDURES

Miscellaneous Materials—Insulin and 125I-labeled Tyr-A14-substituted insulin were from Novo Nordisk. DNA restriction enzymes and T4 DNA ligase were from New England Biolabs. Pco polymerase was from Roche Molecular Biochemicals. Preparation of plasmid DNA and agarose gel electrophoresis were performed according to standard methods. Disuccinimidyl suberate (DSS) was from Pierce, and other chemicals were from Sigma.

Construction and Expression of Insulin Receptor Fragments—An overview of the receptor constructs is shown in Fig. 1 and Table I. IR468.CT is the minimized α-subunit construct (mIR) comprising the first 3 domains of IR (residues 1–468) fused to a 16-amino acid peptide from the C terminus of the α-subunit (residues 704–719) followed by the Flag epitope (DYKDDDDK). This receptor has been described previously (7).

All DNA constructs were inserted in the pZem expression vector (12) and stably expressed in baby hamster kidney (BHK) cells. Cells were grown in Dulbecco’s modified Eagle’s medium (Invitrogen). Cell transfection procedures and culture conditions were described in detail previously (7). Unless stated otherwise, samples of the receptor fragments were culture supernatants from BHK cells expressing the receptor construct.

The construct IR468 consists of IR residues 1–468 fused to the Flag epitope via the short peptide linker PRPS (716–719) followed by the Flag epitope (7). In the present study we made five additional modified CT peptides were used as explained in the figure legend. All peptides were made by standard Fmoc solid phase peptide chemistry. Peptide amides were synthesized on TentaGel RAM resin (Rapp Polymer, Tübingen, Germany). Samples of 100–200 mg resin portions were coupled with 8 eq of Fmoc-l-amino acids (Novabiochem) for 1–3 h, using 8 eq of diisopropycarbodiimide and 8 eq of 1-hydroxy-7-azabenzotriazole in N-methylpyrrolidone as activation.

For all experiments, except that shown in Fig. 4B, the CT peptide used comprised residues 704–719 of IR (NH₂-TFEDYLHNVFVFPERS-COOH). For the experiment shown in Fig. 4B, four additional modified CT peptides were used as explained in the figure legend. For all experiments, except that shown in Fig. 4B, the CT peptide used comprised residues 704–719 of IR (NH₂-TFEDYLHNVFVFPERS-COOH). For the experiment shown in Fig. 4B, four additional modified CT peptides were used as explained in the figure legend. All peptides were made by standard Fmoc solid phase peptide chemistry. Peptide amides were synthesized on TentaGel RAM resin (Rapp Polymer, Tübingen, Germany). Samples of 100–200 mg resin portions were coupled with 8 eq of Fmoc-l-amino acids (Novabiochem) for 1–3 h, using 8 eq of diisopropycarbodiimide and 8 eq of 1-hydroxy-7-azabenzotriazole in N-methylpyrrolidone as activation.

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Cloning and Expression of Receptor Constructs—We previously expressed mIR consisting of the first three domains of the human insulin receptor (L1-CYS-L2, residues 1–468) fused directly to residues 704–719 from the C terminus of the α-subunit and the Flag epitope (7). In the present study we made five new constructs based on this minimized receptor, mIR (called IR468.CT in the present study), as described under “Experimental Procedures.” Including IR468.CT a total of six constructs were stably expressed in BHK cells, and the culture supernatants were used for the various assays. An overview of the constructs is shown in Fig. 1.

Detecting Recombinant Receptor Fragments by Immunoblotting—To be able to detect receptor fragments that were poorly expressed, the constructs were initially purified by affinity chromatography using an anti-FLAG column as described under “Experimental Procedures.” The purified proteins were then analyzed by immunoblotting using the receptor antibody, F26, which recognized all the receptor fragments. The immunoblots are shown in Fig. 2.

The apparent molecular mass of IR468.CT and IR468 was ~80 kDa (lanes 1 and 2), whereas masses of ~50 kDa were found for the IR908.CT and IR908 constructs (lanes 3 and 4) and 35 kDa for the IR255.CT and IR255 fragments (lanes 5 and 6). Comparison with the predicted molecular mass for the glycosylated polypeptides. The faint band seen for the IR255.CT construct (lane 5) reflects its very low level of expression as compared with the other fragments.

Binding of Insulin: Competition Assays—The affinities of the recombinant receptors and mixtures of fragments and CT peptides were determined in a soluble competition assay where...
receptors were precipitated with PEG. Representative binding curves are shown in Fig. 3, and an overview of the binding affinities is presented in Table I. Moreover, Fig. 4 shows displacement curves for 125I-insulin displaced with unlabeled insulin. IR468 was mixed with various concentrations of CT peptide. Concentrations of CT peptide in the assay were 10 μM (□), 1 μM (●), 0.1 μM (▲), and 0.05 μM (○).

![Image](http://www.jbc.org/)

**TABLE I.** Insulin affinity of reconstituted receptors

| Receptor (mixture) | Affinities + 10 μM CT |
|--------------------|----------------------|
| IR468.CT (mIR)     | 11 ± 1.0 13 ± 1.3    |
| IR308.CT           | 45 ± 21 21 ± 2.8    |
| IR255.CT           | ND 10 ± 5.5        |
| IR468              | ND 9.3 ± 0.6       |
| IR308              | ND 11 ± 1.6        |
| IR255              | ND 11 ± 3.8        |
| IR468 + 5 μM CT    | 9.1 ± 1.4          |
| IR468 + 1 μM CT    | 16 ± 2.8           |
| IR468 + 0.1 μM CT  | 68 ± 21            |
| IR468 + 0.05 μM CT | 88 ± 38            |
| No receptor        | ND ND              |

Adding 10 μM CT peptide to IR468 reconstituted insulin binding, as we obtained approximately the same nanomolar affinity (9.3 ± 0.6 nM) as found for the IR468.CT (Fig. 3). The apparent affinity determined was dependent on the concentration of the CT peptide. The affinity increased from ~100 nM to 10 nM when increasing the concentration of the CT peptide from 0.05 to 5 μM, whereas further increase to 10 μM did not improve the apparent affinity any further (Fig. 3). Functional reconstitution was also obtained when mixing IR308 with 10 μM CT peptide, resulting in an apparent affinity of 11 ± 2 nM. The addition of CT peptide to IR468.CT did not have any significant influence on its insulin affinity, whereas the apparent affinity of IR308.CT in the presence of 10 μM CT peptide increased by a factor of 2, yielding an IC50 of 21 ± 3 nM. Subsequently, we tested even smaller N-terminal fragments of the insulin receptor for their ability to bind insulin and found that the IR255.CT and IR255 constructs consisting of the 255 first residues of IR with or without CT domain both bound insulin with an apparent affinity of ~10 nM in the presence of 10 μM CT peptide. None of these two constructs could be detected to bind insulin in the PEG precipitation assay in the absence of added CT peptide (Fig 4A), despite the fact that the IR255.CT constructs appeared to be able to cross-link insulin in the chemical cross-
linking experiment shown below (Fig. 5). As can be seen from the experiment shown in Fig. 4A (no receptor), addition of 10 μM CT peptide alone did not result in any increase in precipitation of labeled insulin in the PEG precipitation assay. In summary, the binding data indicate that residues 309-468 of IR, which include approximately one third of the CYS domain and the whole L2 domain, do not play a major role in the binding of insulin to IR468.CT (mIR) and that both the presence as well as the positioning of the CT domain are critical determinants for the ability of insulin to bind to the N-terminal part of the insulin receptor.

Effect of Mutating the CT Peptide on Reconstitution of Insulin Binding to IR468—We have shown previously that a chimeric IR468.CT containing the CT sequence from the corresponding domain of the IGF IR receptor bound insulin, whereas its replacement with the CT domain from the IRRR completely abolished binding of insulin (7). To see whether the comparable changes in the CT peptide sequence would have a similar effect on its ability to reconstitute insulin binding, we tested two new variants of the CT peptide in the PEG binding assay with IR468 (Fig. 4B). The result clearly shows that the IGFIR-like CT peptide was able to reconstitute insulin binding, whereas binding was undetectable with the IRRR-like CT peptide. Only four of the residues in the IRRR-like peptide are different from corresponding positions in both the IR and IGFIR-like CT peptides (see sequences in Fig. 4B). Of these four positions, the insulin receptor phenylalanine 714, which is conserved as phenylalanine 701 in the IGFIR, is particularly interesting, as it has been shown by alanine scanning mutagenesis that alanine in this position is disruptive for ligand binding in both receptors (15, 16). Accordingly, we synthesized a new CT peptide comprising residues 703-719 of IR, in which the phenyalanine in position 714 had been replaced by alanine, and we tested this peptide for its ability to reconstitute binding in the PEG binding assay. As this peptide also had an extra N-terminal lysine corresponding to IR position 703, the corresponding wild type CT peptide was also included as a positive control. As shown in Fig 5B, binding was undetectable with the F714A mutated CT peptide, suggesting that the phenyalanine in position 714 plays a very important role in the establishment of a functional binding site for insulin within the IR.

Chemical Cross-linking of 125I-Insulin to Receptors—Labeled insulin was chemically cross-linked to anti-Flag tag-purified receptor fragments or mixtures using DSS and separated by SDS-gel electrophoresis under reducing conditions (Fig. 5). When no CT peptide was added, cross-linking could only be demonstrated with the IR468.CT construct (lanes A) and the IR255.CT constructs (lanes E), showing bands of ~80–90 and 40 kDa, respectively. Cross-linking is also expected to have occurred with the IR308.CT construct (lanes C), but in this case detection seems to be obscured by co-migration of non-specifically cross-linked albumin (~55–65 kDa). Albumin originates from the insulin tracer preparation and gives rise to bands seen in all lanes, including the control with no receptor (lanes G). When 10 μM free CT peptide was added to the samples, cross-linking of insulin tracer could be seen for all six receptor fragments including the three constructs, IR468, IR308, and IR255, lacking the CT domain (lanes B, D, and F). The appearance of these three bands probably reflects the functional reconstitution of an insulin binding site involving residues within the first 255 amino acids of these fragments. In all cases the cross-linked tracer could be displaced with unlabeled insulin, demonstrating the specific cross-linking of insulin to the N-terminal insulin fragments. For the constructs IR308.CT (lanes C) and IR255.CT (lanes E), the intensity of the corresponding bands of approximately 55 and 40 kDa were seen to increase as a result of addition of CT peptide, probably reflecting the increase in insulin binding affinity also seen in the PEG precip-
Fig. 6. *Ternary complex kinetic model.* This model describes the reversible interaction of the N-terminal fragments of the insulin receptor with the CT peptide to explain insulin binding properties of the insulin receptor. $K$ denotes the affinity constant of insulin ($I$) for the N-terminal fragment of IR (exemplified by IR468), and $K_{CT}$ denotes the affinity constant of the CT peptide ($CT$) for IR468. $K'$ and $K_{CT}'$ denote the affinity constants for binding of insulin and CT peptide to IR468*CT and IR468*I, respectively, and IR468*CT*I is the resulting ternary complex.

**DISCUSSION**

Previously, we identified a monomeric minimized IR α-subunit (mIR/IR468.CT) that binds insulin with the same nanomolar affinity as the soluble ectodomain, sIR (7, 8). In the present study we have produced even smaller insulin receptor fragments trying to further delineate the minimum requirements for nanomolar binding affinity for insulin.

The nanomolar binding affinity obtained with IR468.CT is believed to reflect the interaction between binding site 1 on the receptor and the classical binding site on the insulin molecule described in the binding models proposed by Schäffer (17) and De Meyts (11). These models imply that, in addition to this site, there is another receptor binding site 2 on the other receptor monomer, which is required for the full holoreceptor affinity when insulin binds to both sites in a cross-linking mechanism.

To further characterize the nanomolar binding site, we first made the IR308.CT construct, which is IR468.CT deleted of the L2 domain, and found that this construct also bound insulin but with an apparent affinity ~4-fold lower than IR468.CT (Table I). In contrast, the corresponding constructs without the CT domain, IR468 and IR308, did not bind insulin. This supports previous findings that the presence of the CT domain is essential for insulin binding but that it can be effective from different positions in the primary sequence (8, 18). Therefore, we investigated whether it was possible to reconstitute insulin binding by mixing the IR468 and IR308 fragments with free synthetic CT peptide. We found that insulin binding could be fully reconstituted by mixing IR468 or IR308 with 10 μM free CT peptide, resulting in apparent affinities for insulin of ~10 nM, which is similar to that found for IR468.CT (Table I). Subsequently, we could demonstrate that even smaller N-terminal fragments, IR255.CT and IR255, could bind insulin with nanomolar affinity in the presence of free CT peptide. Accordingly, we suggest that the epitopes of IR468.CT that interact with insulin must be located within these first 255 amino acids and/or in the CT peptide. This is somewhat in contrast with previous suggestions that the L2 domain of IR (residues 310–468), or sequences spanning the L2-Fn0 junction, is essential for interaction with insulin. The information suggesting a role for the L2 domain in insulin binding is the Ser-323 → Leu mutation in IR that has been described in severely insulin-resistant patients with Rabson-Mendenhall syndrome (19).

This mutant IR is processed normally and transported to the plasma membrane but has very low binding affinity for insulin, indicating that Ser-323 forms part of the insulin binding site or stabilizes its conformation. Other lines of evidence are chemical cross-linking experiments, in which insulin was shown to associate with an IR fragment beginning at Gly-390 in the middle of the L2 domain and extending to Arg-488 in the first fibronectin domain (Fn0) (20). Additionally, data on chimeric receptors suggest that major insulin binding determinants are located within the region that comprises most of the L2 and first part of the Fn0 domain (amino acids 325–524) (21). It cannot be excluded that these reports address binding site 2 mechanisms that we have recently ascribed to the L2, Fn0, and/or the 650–703 region of exon 10 (9), whereas our monomeric constructs probably only involve site 1.

Based on the crystal structure of the first three domains of the IGFIR, the L2 domain was proposed to be involved in IGF binding (6). The fact that this molecule adopted a C-shaped structure with L1 and L2 domains at either end, leaving sufficient space between the three domains to accommodate the ligand (IGF1), led to suggestions that the binding mechanism involved interaction between the ligand and all three flanking domains: L1, CYS, and L2. However, the L1-CYS-L2 fragment did not bind ligand, whereas, in the minimized IR and IGFIR, where the CT domains are fused to the C terminus of the L2 domain, ligand binding is restored (7, 8). In the crystal structure the C terminus of the L2 domain is facing away from the putative binding pocket, and therefore in IR468.CT, which binds ligand as a monomer (7), the position of the L2 domain must adopt a different orientation than found in the crystals of the non-binding L1-CYS-L2 fragment of the IGFIR. However, in the holoreceptor it may be that the nanomolar binding site comprises epitopes from L1 on one monomer and the CT peptide domain from the other receptor monomer, which would be consistent with an antiparallel association in the dimer, as suggested by some electron microscopy studies of the insulin receptor (22, 23). In the present study we have only investigated IR fragments; therefore, further studies are needed to demonstrate whether reconstitution is feasible with the minimized IGFIR. In particular in the IGFIR the loop corresponding to IR 269–277 appears to play a role for discriminating between insulin and IGF binding (24). This loop is not present in our shortest functional IR fragment IR255.

Although the CT domain appears to be able to support insulin binding from different locations in the primary sequence, its positioning may be a critical determinant of binding affinity (18). In the present study, this was demonstrated by the IR308.CT and IR255.CT constructs. The positioning of the CT domain in the IR308.CT construct gave a 4-fold lower affinity than for the corresponding IR308 + CT peptide mixture. However, the IR308.CT could not be fully reconstituted by the addition of 10 μM free CT peptide yielding only an IC50 of 21 ± 3 μM, which is slightly poorer than the 11 ± 1.6 obtained for the mixture of IR308 and 10 μM CT peptide, suggesting that the CT domain in IR308.CT makes a less favorable insulin binding site as compared with IR308 in the presence of high concentrations of free CT peptide. In the case of the IR255.CT construct, we were not able to detect insulin binding in the PEG precipitation binding assay, but in this case the binding of insulin could be fully reconstituted by the addition of 10 μM free CT peptide, yielding an apparent affinity of ~10 nM similar to that found for the corresponding mixture of IR255 and CT peptide. This result indicates that the positioning of the CT domain in IR255.CT is not favorable for making a fully functional insulin binding site. Still, the fact that we did observe insulin associating to IR255.CT in the chemical cross-linking experiment.
Functional Reconstitution of Insulin Receptor Binding Site

(Fig. 5, lanes E) indicates that the construct does bind insulin. However, this binding cannot be detected by the PEG assay, probably because of to low concentration of the receptor fragment and/or to low affinity (IC50 > 200 nM).

In the insulin proreceptor, the CT domain is either followed directly by the tetrabasic cleavage site at the α-β-subunit junction or by a stretch of 12 amino acids encoded by exon 11. Interestingly, it has been shown that mutations in the tetrabasic cleavage site that lead to a defect in the proteolytical processing of the proreceptor into mature subunits results in receptors with markedly reduced insulin affinity, but primarily in the receptor isoform that lacks exon 11. Thus, it is suggested that, in this proreceptor isoform, cleavage results in the alleviation of structural constraints leading to increased insulin binding (25). Our studies imply that this constraint keeps the CT domain from docking into a region within the N-terminal 255 amino acids. Proreceptor processing subsequently allows for proper docking of the CT domain, thereby creating a functional insulin binding site within the receptor.

The binding mechanisms involved in the functionally reconstituted receptors are complex. The CT peptide most likely interacts with an epitope in the N-terminal 255 amino acids and probably also interacts directly with insulin. The present data on mutated CT peptides (Fig. 4B) strongly suggest that the phenylalanine in position 714 may play a key role in one or both of these interactions. The evidence for direct contact between the CT domain and insulin is chemical cross-linking (26) and alanine scanning mutagenesis (15). However, it has not been definitely proven that the CT domain participates directly in hormone binding. Alternatively, it may stabilize the conformation of the binding site.

In this study we have shown that the apparent affinity of the reconstituted receptors improves with increasing CT peptide concentration. This behavior can, as a working hypothesis, be explained by using a ternary complex kinetic model (Fig. 6). In this model tracer insulin can bind to the N-terminal fragments of IR and to the complex between the N-terminal fragments and the CT peptide with affinity constants, K and K*, respectively. However, these singular affinity constants are effectively replaced by an apparent affinity constant Kapp that is defined by the following equation: Kapp = (K + K* × KCT × (CT))/((1 + KCT × (CT)) (27). Kapp thus changes hyperbolically from K in the absence of CT peptide to K* in the presence of receptor-saturating concentrations of CT peptide. Because ~10 μM CT peptide is needed to obtain receptor saturation and one can approximate the concentration of receptor in the assay to 0.1 × IC50 (1 nM), the affinity of the CT peptide for the N-terminal fragments of IR appears to be in the low micromolar range.

Functional reconstitution of a protein was first described for the bovine pancreatic ribonuclease A (RNase A). RNase A may be cleaved by subtilisin to give the inactive S peptide fragment (residues 1–20) and S protein (residues 21–124), and these fragments can be reconstituted to give the catalytically active complex RNase S (28).

We have here demonstrated that the minimized insulin re-

ceptor can be functionally reconstituted from fragments that have no measurable binding affinity. The structural implications of the present data are that the nanomolar insulin binding site has been further narrowed down to 271 amino acids: the first 255 residues of IR combined with the 16 residues of the CT domain. The mechanisms involved may now be studied using various CT peptide alterations, and reconstituted receptors may also be amenable to detailed structural analysis of the insulin binding domains.

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