The insulin receptor (IR) is a dimeric receptor, and its activation is thought to involve cross-linking between monomers initiated by binding of a single insulin molecule to separate epitopes on each monomer. We have previously shown that a minimized insulin receptor consisting of the first three domains of the human IR fused to 16 amino acids from the C-terminal of the α-subunit was monomeric and bound insulin with nanomolar affinity (Kristensen, C., Wiberg, F. C., Schäffer, L., and Andersen, A. S. (1998) J. Biol. Chem. 273, 17780–17786). To investigate the insulin binding properties of dimerized α-subunits, we have reintroduced the domains containing α-α disulfide bonds into this minireceptor. When inserting either the first fibronectin type III domain or the full-length sequence of exon 10, the receptor fragments were predominantly secreted as disulfide-linked dimers that both had nanomolar affinity for insulin, similar to the affinity found for the minireceptor. However, when both these domains were included we obtained a soluble dimeric receptor that bound insulin with 1000-fold higher affinity (4–8 pM) similar to what was obtained for the solubilized holoreceptor (14–24 pM). Moreover, dissociation of labeled insulin from this receptor was accelerated in the presence of unlabeled insulin, demonstrating another characteristic feature of the holoreceptor. This is the first direct demonstration showing that the α-subunit of IR contains all the epitopes required for binding insulin with full holoreceptor affinity.

Insulin mediates its effects by binding to tyrosine kinase receptors in the plasma membrane of targets cells. The primary sequence of the IR was cloned in 1985 (1, 2), and the exon-intron structure of the IR gene was described in 1989 by Seino et al. (3). The IR protein is a dimer of two identical αβ monomers covalently linked by disulfide bonds (4). Each monomer is synthesized as a heavily glycosylated single chain proreceptor, which is proteolytically cleaved by furin yielding a synthesized as a heavily glycosylated single chain proreceptor, with low affinity and displays linear Scatchard plots (25). To account for these ligand binding properties, it has been suggested that high affinity binding involves one molecule of insulin that cross-links to distinct sites on opposite αβ-subunits (23, 24) and that siRNA targets this communication between α-subunits (23).

Several groups have shown that mammalian cells expressing the IR ectodomain secrete a soluble and properly processed dimeric receptor (sIR) that binds insulin in the nanomolar range (17–20). However, this affinity is considerably lower than that typically found with the full-length holoreceptor (hIR) (18, 21–23). In addition to its high affinity for insulin, hIR exhibits nonclassical receptor binding properties suggestive of negative cooperativity or site-site interactions between the two receptor halves, namely accelerated dissociation of labeled insulin in the presence of unlabeled insulin and curvilinear Scatchard plots (24). In contrast, sIR binds two molecules of insulin with low affinity and displays linear Scatchard plots (25). To account for these ligand binding properties, it has been suggested that high affinity binding involves one molecule of insulin that cross-links to distinct sites on opposite α-subunits (23, 24) and that siRNA lacks this communication between α-subunits (23).

There have been some reports on soluble IR ectodomains attaining better than nanomolar affinity, for insulin, either by certain purification procedures (26) or by expressing sIR as a fusion with self-associating proteins such as the immunoglobulin Fc and α domains (27) or a leucine zipper (28) placed at the C terminus of the truncated β-subunit.

In the present study we have made dimeric fragments of the
**High Affinity Soluble Insulin Receptor**

**Fig. 1. Insulin receptor constructs.** A, structure of the insulin receptor gene and the relative spans of exons and encoded domains. Domains are the homologous L1 and L2 domains separated by the cysteine-rich domain (CYS) followed by three fibronectin type III domains (Fn0, Fn1 and Fn2), the transmembrane domain (TM), and the intracellular kinase domain. Notably, nearly all domain boundaries correspond to boundaries between exons. The L1/CYS/L2 domains are encoded by exons 2–6, Fn0 is encoded by exons 7–9, Fn2 is encoded by exons 13–14, TM is encoded by exon 15, and the kinase domain is encoded by exons 16–22. B, the holoreceptor (hIR) is shown at the left, mIR, mIR.Ex10, mIR.Fn0, mIR.Fn0/Ex10, and sIR comprises the last 70 residues (residues 650–719) of the alpha-subunit encoded by exon 12. Open triangles show the approximate location of the beta and alpha-alpha disulfide bonds. The length of the soluble ectodomain (sIR) is shown, with the left, mIR.Ex10, mIR.Fn0, and mIR.Fn0/Ex10 are depicted in their dimeric forms showing the expected arrangement of disulfide bonds between inserted domains. Ex10 comprises the last 70 residues (residues 650–719) of the alpha-subunit encoded by exon 10 and residues 718–719, the first two residues encoded by exon 12. C-terminal FLAG-epitope tags are shown as open triangles. 

**EXPERIMENTAL PROCEDURES**

**Miscellaneous—** Insulin and [125I-TyrA14]insulin were from Novo Nordisk. DNA restriction enzymes and T4 DNA ligase were from New England Biolabs, Pwo polymerase was from Roche Molecular Biochemicals. Preparation of plasmid DNA and agarose gel electrophoresis were performed according to standard methods. For DNA mini-preps Qiagen kit was used (Qiagen). DSS (Disuccinimidyl suberate) was obtained from Pierce Chemical Co., and other chemicals were from Sigma-Aldrich. The insulin receptor monoclonal antibodies F12 and F26 were raised and characterized as described previously (18, 21, 29). BN gel electrophoresis and immunoblotting were performed according to standard methods. For DNA minipreps the QIAprep 8 kit was used (Qiagen). Disuccinimidyl suberate was from aprotinin (50 μg/ml), 0.5 mM phenylmethylsulfonyl fluoride (4 ml lysis buffer/1 × 10^6 cells). The lysate was cleared by centrifugation at 15 min at 35,000 × g, concentrated three-fold on Microcon-100 (Millipore) and stored at −80 °C. Unless stated otherwise, samples of all other receptors constructs were culture supernatants from BHK cells electroporated with the receptor.

mIR.Ex10 that consists of residues 1–468 and 650–719 fused to the FLAG epitope was made by PCR amplification of exon 10 using DNA encoding wild-type human IR as template with the sense primer 5’-G-CAACAGGGTCAGTGCGTGGCTGAAAGGCC-3’ (Nhel site underlined) and antisense primer 5’-TTTTCTCATGGGACGAAACACAG3’ (AvrII site underlined). The PCR fragment was digested with Nhel and AvrII and ligated into the corresponding sites in the plasmid encoding mIR (29). For making mIR.Fn0 that consists of IR residues 1–601 and 704–719 fused to the FLAG epitope, two complementary oligonucleotides, 5’-GATCCACGTTGAGATCTCAGCTCGAACTGTTGTGCAGGTAATCCTC-3’ and 5’-CTAGGAGGAAAAACCACTTGTGCGAAATCCTCACAAACCCTGG-3’ (both with phosphorylated 5’-ends) were hybridized to produce a double-stranded fragment encoding residues 704–717 with BamHI and AvrII compatible ends. This fragment was ligated with a 3.5-kilobase AvrIII/AvrII vector fragment from plasmid encoding mIR, which has the C-terminal FLAG sequence downstream from the AvrII site, and a 3.2-kilobase MfeI/BamHI fragment from the IRwt construct described previously (16) having the N-terminal IR residues 1–601 upstream from the BamHI site. Finally, mIR.Fn0/Ex10 consisting of residues 1–601 and 650–719 fused to the FLAG epitope, was made by PCR amplification of exon 10 using the mIR.Ex10 construct as template with the sense primer 5’-CGCTATCGCGGATCTCAGGCTGAAGCTGCCC-3’ and an antisense primer downstream from the FLAG epitope encoding sequence and its flanking XbaI site. The PCR fragment was digested with BamHI and XbaI and ligated into the corresponding sites in the plasmid encoding mIR.Fn0.

**Receptor Competition Binding Assays—** Two types of competition binding assays were used, a polyclonal glycidyl (PEG) precipitation assay and a microtiter plate assay. For both assays the concentration of receptor was adjusted to yield ~10% binding of tracer when no competing insulin was added. This corresponds to receptor concentrations 10-fold lower than the IC50 obtained for the given receptor. Moreover, the amount of tracer and duration of incubation was adjusted depending on the affinity of the receptor, so that low concentrations of tracer (~3 pm) and longer incubation times (>4 h) were used for receptors with high affinity binding (picomolar range). For the PEG assay a suitable dilution of receptor sample was incubated for 16–60 h at 4 °C in a total volume of 200 μl with 3–12 pM of [125I-TyrA14]insulin and various concentrations of unlabeled insulin in binding buffer (100 mM Hepes, pH 8.0, 100 mM NaCl, 10 mM MgCl2, 0.25% (w/v) BSA, 0.025% (w/v) Triton X-100). Subsequently bound counts were recovered by precipitation with 0.2% polyethylene glycol 6000 (w/v) and 740 μl of 30% (w/v) polyethylene glycol M, 8000. Bound 125I-labeled insulin was counted in a gamma-counter.

The microtiter plate assay was performed essentially as described in the literature (30). For immobilization of receptor fragments, an insulin receptor specific antibody, F12, was used. F12 was raised against purified mini-receptor IRA70s (16) and shown to recognize an epitope within residues 1–468 (data not shown). First, microtiter plates (Wellco C8, maxisorp from Nunc) were coated with goat anti-mouse IgG antibody (Pierce); for each well was used 50 μl of a 20 μg/ml solution in TBS (0.01 M Tris, pH 7.5, 100 mM NaCl). Plates were incubated for 1 h at room temperature before washing twice with TBS and blocking with 250 μl of Superblock (Pierce). Then 50 μl of affinity-purified F12 antibody (1.2 μg/ml) was added to each well. Plates were incubated for 2 h at room temperature before washing three times with binding buffer followed by the addition of 50 μl of a suitable dilution of receptor sample. After a 2-h incubation at room temperature, plates were washed three times, and binding experiments were performed by adding a total volume of 150 μl of binding buffer with 3–10 pM of 125I-labeled insulin and varying concentrations of insulin. After 16–60 h at 4 °C, unbound ligand was removed by washing once with cold binding buffer, and the tracer bound in each well was counted in a gamma-counter.

**Receptor Saturation Binding Assay—** For saturation binding experiments, receptor samples were incubated in a total volume of 250 μl with various insulin concentrations in binding buffer for at least 60 h at 4 °C. Subsequently, bound counts were recovered by precipitation with 0.2% γ-globulin and 740 μl of 30% (w/v) polyethylene glycol M, 8000. Bound 125I-labeled insulin was counted in a gamma-counter. For each tracer concentration, nonspecific binding was determined by measuring bound 125I-insulin in the presence of 1 μM of unlabeled insulin.

**Immunoblotting—** The expressed receptor proteins were detected by autoradiography.
immunoblotting using the monoclonal antibody F26. This antibody was raised against a peptide corresponding to residues 39–75 near the N terminus of the insulin receptor α-subunit. For immunoblotting, samples were mixed with 0.33 volumes of 4× LDS loading buffer (Novex). Reduced samples were mixed with loading buffer containing 100 mM dithiothreitol and incubated at 95 °C for 5 min before loading 15 μl on a 4–12% polyacrylamide Bis-Tris gel (NuPAGE, Novex). After electrophoresis in MOPS-running buffer (Novex), proteins were blotted onto Immobilon-P membrane (Millipore). The membrane was blocked by incubating with blocking buffer (2% defatted skim milk, 1% BSA in TBS) for 1 h at room temperature. The receptor antibody F26 (diluted in TBS, 1% BSA) was added to the membrane and incubated for 1 h at 4 °C. The membrane was washed with TBS before incubating with peroxidase-conjugated secondary antibody (Dako, Denmark) diluted in 1% BSA in TBS. Finally the blot was washed with TBS, and immunoreactive proteins were detected by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech) and visualized using the FujiFilm CCD camera, and the Image Gauge software (Fuji Photo Film Co).

**Chemical Cross-linking of 125I-labeled Insulin to Receptors—**Chemical cross-linking was performed essentially as described (18, 31). Receptor samples were incubated for 60 min at room temperature with [125I]-A14insulin (0.2–0.3 nM) in the presence or absence of unlabeled insulin (1 μM). Disuccinimidyl suberate (DSS) in dimethyl sulfoxide was added from a 10 mM stock solution to a final concentration of 0.1 mM. After 15 min on ice, the reaction was stopped by adding 0.53 volume of 4× LDS loading buffer, and samples were separated by SDS electrophoresis as described for immunoblotting. The gel was fixed in 10% acetic acid, 20% ethanol, and a phosphorimagery screen was exposed with the dried gel.

**Dissociation of 125I-labeled Insulin From Receptors—**For investigating dissociation of labeled insulin, receptors were immobilized in microtiter wells with the antibody F12 as described for the competition assays. After immobilization of the receptors, 150 μl of binding buffer containing labeled insulin (20 nM) was added and allowed to equilibrate by incubating for 2 h at room temperature, followed by washing with binding buffer. Dissociation of tracer was followed under two conditions by adding either binding buffer alone or binding buffer with 0.5 μM unlabeled insulin. At various time points, wells were washed once with ice-cold binding buffer, and the bound tracer was counted in a γ-counter.

**Purification and Gel Filtration of mIR.Fn0/Ex10—**Insulin receptor fragments were purified from transfected BHK cell culture supernatant by affinity chromatography using immobilized insulin as described previously (25). After affinity purification, the dimeric form of mIR.Fn0/Ex10 was separated from the monomer using a Sepharyl S300 High Resolution column (Amersham Pharmacia Biotech: 2.5 × 89 cm) equilibrated in 0.2 mM Tris, HCl buffer, pH 7.8. Chromatography was performed at a flow rate of 0.2 ml/min. Fractions containing the dimer were pooled and concentrated on Centriprep-10 (Millipore). Fractions containing the monomer were pooled, concentrated, and subsequently rerun over the column for further purification, and a fraction was selected for characterization. The purified receptor fragments were stored at 4 °C.

**RESULTS**

**Cloning and Expression of Receptor Constructs—**We previously expressed a minized insulin receptor (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-terminal of the α-subunit and the FLAG epitope (29). In the present study three new constructs were made on the basis of mIR as described under “Experimental Procedures.” All three constructs contained all residues of mIR and in addition either the first fibronectin type III domain, Fn0 (mIR.Fn0), the remaining residues of exon 10 (mIR.Ex10), or both these regions (mIR.Fn0/Ex10). All constructs were stably expressed in BHK cells, and the culture supernatants were used for the various assays, except for hIR for which samples were made from solubilized cells. An overview of all receptor constructs used in the present study is shown in Fig. 1 and in Table I.

**Binding of Insulin Competition Assays—**The affinities of the recombinant receptors for insulin were determined in two competition binding assays: a soluble assay where receptors were precipitated with polyethylene glycol and a microtiter plate assay in which receptors were immobilized using a receptor-specific monoclonal antibody. Representative binding curves for the competition assays are shown in Fig. 2. In all cases, the binding curves were fitted to a one-site binding model from which the binding affinities (IC50) were determined. An overview of all binding affinities is presented in Table I. In the PEG assay the control receptors mIR, sIR, and hIR yielded affinities similar to what has been found in previous studies; that is 5–7 nM for mIR and sIR whereas the affinity for hIR was 0.17 nM. For the new receptors, there was a slight increase in affinity compared with mIR when inserting either the Fn0 domain or the full-length sequence of exon 10, mIR.Fn0 and mIR.Ex10 yielding affinities of 3.5 and 3.0 nM respectively. However, the insertion of both these domains led to a 1000-fold increase in binding affinity; the affinity of mIR.Fn0/Ex10 was 0.002 nM, which is slightly better than the 17 nM found for the holoreceptor (Table I).

The affinities obtained with the immobilized assay were similar to those found in the soluble PEG assay (Table I), demonstrating that the F12 antibody binds the various receptor fragments without affecting binding affinity for insulin.

**Saturation Binding of Labeled Insulin—**The high insulin binding affinity of mIR.Fn0/Ex10 allowed us to do saturation binding experiments with labeled insulin only. Fig. 3 shows representative saturation binding curves for mIR.Fn0/Ex10 and hIR. Both of these curves fitted to a one-site binding model giving an equilibrium dissociation constant (Kd) of 0.004 ± 0.002 nM for mIR.Fn0/Ex10, which is slightly better than found

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**Table I**

| Construct                     | PEG assay (nM) | Microtiter assay (nM) | Saturation assay (nM) |
|-------------------------------|---------------|-----------------------|-----------------------|
| mIR                           | 7.3 ± 1.6     | 5.6 ± 1.3             | —                     |
| mIR.Ex10                      | 3.0 ± 0.9     | 3.8 ± 0.1             | —                     |
| mIR.Fn0                       | 3.5 ± 0.9     | 2.1 ± 1.0             | —                     |
| mIR.Fn0/Ex10                  | 0.008 ± 0.001 | 0.007 ± 0.004         | 0.004 ± 0.002         |
| sIR                           | 5.6 ± 1.5     | 4.7 ± 1.3             | —                     |
| hIR                           | 0.017 ± 0.004 | 0.020 ± 0.01          | 0.014 ± 0.006         |

**Fig. 2.** Competition binding of recombinant receptors. Displacement curves for 125I-labeled insulin displaced with unlabeled insulin obtained with samples of mIR (○), mIR.Ex10 (●), mIR.Fn0 (◇), mIR.Fn0/Ex10 (△), sIR (□), and hIR (○) in the soluble PEG assay. All curves are best fits for a single binding site.
for the horeceptor, hIR, 0.014 ± 0.006 nM. The saturation binding experiment clearly confirms the high affinities that were obtained for mIR.Fn0/Ex10 in the competition binding experiments (Table I). Thus, in all binding assays mIR.Fn0/Ex10 resembles hIR in having a binding affinity for insulin in the low picomolar range.

Detecting Recombinant Receptors by Immunoblotting—An antibody, F26, that recognizes the N terminus of the insulin receptor α-subunit, and thus recognizes all receptors in the present study, was used to detect soluble insulin receptors secreted into culture medium from transfected BHK cells. For comparison detergent lysates of cells expressing hIR were also analyzed. Immunoblotting was performed on reduced as well as nonreduced samples of each receptor. The immunoblots are shown in Fig. 4. On the reduced gel (Fig. 4B) the antibody detects the full-length α-subunit of 130 kDa in the samples of sIR and hIR (lanes 5–6), whereas the truncated α-subunit of mIR (lane 1) shows an apparent mass of 80 kDa in accordance with previously published data (16). When inserting domains into mIR we obtain larger α-subunit fragments and accordingly mIR.Ex10 (lane 2), mIR.Fn0 (lane 3), and mIR.Fn0/Ex10 (lane 4) showed apparent masses of 95–105 kDa on the gels.

On the nonreduced gel (Fig. 4A), the size of mIR was found to be the same as for the reduced sample (80 kDa) in accordance with the minireceptor being a monomer, whereas sIR and hIR (lanes 5–6) gave high molecular mass bands of more than 220 kDa corresponding to the dimers of disulfide-linked α- and β-subunits. In samples of mIR.Ex10, mIR.Fn0, and mIR.Fn0/Ex10 (lanes 2–4), two bands were recognized by the antibody. The prominent upper bands in these samples had an apparent mass corresponding to a dimeric receptor (190–210 kDa) consistent with the introduction of regions containing the cysteine residues that are involved in the α-α disulfide connections in these receptor constructs (Fig. 1). The relative intensities of the dimer band versus monomer band were between 1:1 and 1.8:1.

Chemical Cross-linking of 125I-Insulin to Receptors—Labeled insulin was chemically cross-linked to recombinant receptors using DSS and then separated by SDS-gel electrophoresis under reducing and nonreducing conditions (Fig. 5). All receptor fragments detected by immunoblotting could cross-link labeled insulin, and the cross-linking gels show a pattern that is similar to the immunoblotting patterns (Fig. 4). The only exception is that the 105-kDa band corresponding to the monomeric form of mIR.Fn0/Ex10 is not detected by cross-linking in the nonreduced gel (Fig. 5A, lane 7). This observation indicates that the high affinity binding property of mIR.Fn0/Ex10 is associated with the dimeric form only. For mIR.Ex10 and mIR.Fn0 both dimeric and monomeric forms were cross-linked (Fig. 5A, lanes 3 and 5), showing that both these forms bind insulin. Because
all binding curves for these two receptors fit to a one-site model, we presume that the monomeric and dimeric forms of these receptors bind insulin with similar affinities, implying that binding of insulin to mIR.Ex10 or mIR.Fn0 is not influenced by α-α disulfide contact(s). On the reduced gel (Fig. 5B), faint bands corresponding to the size of the dimers are seen for all samples except for mIR. These are most likely caused by cross-linking between the two α-subunits in the dimeric receptor fragments.

Dissociation of Labeled Insulin from Receptors; Effect of Unlabeled Insulin—One of the characteristic features of the insulin holoreceptor hIR is that dissociation of bound tracer is markedly accelerated in the presence of unlabeled insulin in the buffer (32). To examine mIR.Fn0/Ex10, we investigated the dissociation of insulin from receptors immobilized with the F12 antibody. The resulting dissociation curves of hIR, mIR.Fn0/Ex10, and sIR are shown in Fig. 6. The curves for hIR and the new high-affinity receptor mIR.Fn0/Ex10 clearly show accelerated dissociation in the presence of 0.5 μM unlabeled insulin. After 20 min, about 85% of initially bound tracer is still bound in the absence of unlabeled insulin whereas less than 40% is bound in the presence of unlabeled insulin. In contrast, no accelerating affect of unlabeled insulin is seen for the soluble receptor sIR. When comparing the dissociation curves in the absence of unlabeled insulin, the dissociation is clearly faster from sIR than from hIR or mIR.Fn0/Ex10, probably reflecting the lower affinity of sIR.

Characterizing Monomeric versus Dimeric Forms of mIR.Fn0/Ex10—mIR.Fn0/Ex10 was purified by affinity chromatography, and subsequently the two forms of the receptor were separated by gel filtration. The immunoblot in Fig. 7A shows that the mixture of the two forms found in the BHK cell culture supernatant (lane 1) have been separated into dimeric (lane 2) and monomeric (lane 3) forms. The sample of purified dimer (Fig. 7A, lane 2) appears to contain an additional band migrating slightly faster than the dimer band present in the culture supernatant (Fig. 7A, lane 1). At present we do not know what this receptor band represents, but it is also seen as a weak band in the monomer fraction (Fig. 7A, lane 3) indicating that the monomer sample still contains traces of higher molecular weight proteins. Chemical cross-linking of labeled insulin to the same three samples (Fig. 7B) shows only one band of ~200 kDa in the cell supernatant (lane 1) and in the dimer sample (lane 3). The monomer fraction (Fig. 7B, lane 5) shows two bands of which the 100-kDa band corresponds to the monomer, whereas the weaker 200-kDa band probably represents a trace of the dimeric form of mIR.Fn0/Ex10.

Insulin binding experiments with the two purified forms of mIR.Fn0/Ex10 (Fig. 7C) clearly show that the dimer has a much higher affinity (IC50 of 0.010 ± 0.002 nM) than the monomer (IC50 of 0.9 ± 0.3 nM). The poor fit of binding data for the purified monomer at the top of the binding curve indicates that there is a small fraction of high affinity receptor present in the sample. This high affinity receptor is probably related to the presence of small amounts of dimer that was also seen in the cross-linking experiment (Fig. 7B, lane 5).

DISCUSSION

We have previously identified a minimized IR α-subunit (mIR) that binds insulin with the same nM affinity as the soluble ectodomain, sIR (16, 29). The aim of the present study was to investigate the insulin binding properties of dimeric α-subunit fragments. We have employed a strategy of reintroducing domains that contain the cysteines involved in α-α disulfide bonds into the minimized insulin receptor. These domains are the first fibronectin type III domain F0 that contains Cys524 and the region encoded by exon 10 that contains Cys682, Cys683, and Cys685.

When inserting only one of these domains we obtained mIR.Fn0 and mIR.Ex10, which were both predominantly secreted as dimers (Fig. 4A). A receptor fragment similar to mIR.Fn0 was also reported by Molina et al. (33), who found that a fragment comprising the first four domains (L1/CYS/L2/Fn0) was predominantly secreted as a dimer. However, this fragment did not bind insulin, probably because of the lack of essential residues from the C-terminal of the α-subunit (16). We found that mIR.Fn0 and mIR.Ex10 bound insulin with...
affinities in the low nanomolar range. This is slightly better than what is found for the monomeric minireceptor, mIR, and the soluble ectodomain, sIR (Table I). When both domains were inserted into mIR, we obtained mIR.Fn0/Ex10 that bound insulin with an affinity that was 1000-fold higher than that found for mIR or the receptors with only one domain inserted (Table I). This high affinity in the low picomolar range was similar to what was found for the solubilized holoceptor.

In the chemical cross-linking experiments insulin was found to be associated with the dimeric form of mIR.Fn0/Ex10 only (Fig. 5A), whereas immunoblotting showed that the BHK cells secreted a mixture of monomers and dimers (Fig. 4A). This indicates that the picomolar affinity binding property is associated only with the dimeric structure of the α-subunits. This was confirmed by characterizing the two receptor forms individually after separating them by gel filtration (Fig. 7). The binding data clearly showed that the purified dimer bound insulin with high affinity (−10 pm) whereas the monomer bound with an affinity of ∼1 nM. Schäffer (23) and De Meyts (24) have proposed models for the interaction between insulin and its receptor involving an initial binding of insulin to one site on one α-subunit followed by cross-linking of the insulin molecule to a distinct site on the other α-subunit. The initial binding event involves binding of insulin with nanomolar affinity (αIR binding mode) whereas the high affinity is obtained only when the insulin molecule also binds to the second site on the opposite α-subunit resulting in the cross-linking effect. Thus, according to these cross-linking models, the formation of dimers is a prerequisite for high affinity binding of insulin. Supporting these models are stoichiometric studies showing that hIR binds only one insulin molecule with high-affinity (34–36), whereas αIR monomers formed by controlled reduction bind one insulin molecule per monomer with lower affinity (nM) (34, 35, 37). The present data on the receptor fragments containing either the Fn0 domain (mIR.Fn0) or the Ex10 domain (mIR.Ex10) show that dimerization of mIR per se does not increase binding affinity dramatically and therefore suggest that both these domains are important for high affinity binding of insulin.

The binding results for mIR.Fn0/Ex10 demonstrate for the first time that the α-subunit of IR contain all epitopes required for picomolar affinity binding of insulin. It is possible that a second insulin contact site has been restored by the introduction of one or both of the Fn0 and exon 10 domains into mIR. For instance, putative contact sites have been proposed to reside in the region of the L2/Fn0 junction that is disrupted in mIR. This has been suggested by photoaffinity labeling (residues 390–490, Ref. 38), chimeric receptors (residues 325–524, Ref. 39) and by antibodies that inhibit insulin binding (residues 450–601, Ref. 40) or activates the insulin receptor (residues 469–592, Ref. 41). Thus, this putative site could represent the second binding site necessary for high affinity binding as proposed by cross-linking binding models. However, the fact that this putative contact site is also restored in mIR.Fn0 that bind insulin with low affinity, indicates that properties of the additional sequences of exon 10 are also needed for the generation of high affinity receptors.

Several groups have used electron microscopy techniques to study the structure of the insulin receptor and its domain organization. These studies have suggested either extended T- or Y-shaped receptor conformations (11, 42, 43) or more globular conformations (44–46) but no clear consensus has emerged. The high affinity obtained with mIR.Fn0/Ex10 suggests that the first four domains, L1/Cys/L2/Fn0 and the Ex10 domain are in close proximity in the IR structure and that these most likely are the only domains interacting with the insulin molecule. The model based on electron microscopy and three-dimensional reconstruction suggested by Ottensmeyer et al. (46) is in accordance with our observations in having a single insulin molecule bound to a distinct central core composed of the two set of contiguous domains from L1 to Fn0 and additional residues encoded by exon 10.

Previous attempts to characterize the full-length IR α-subunit have been difficult because of very low yields of secreted material (20, 47). In contrast, mIR.Fn0/Ex10 was found to be efficiently secreted although this fragment only differs in having a 48-residue deletion of the exon 9 region. In the native receptor, the deleted region is in direct contact with the β-domain via the Cys647 that is disulfide-linked to Cys860 in the β-subunit (4). Furthermore this region contains the first five of the seven strands of the predicted β-sandwich structure of Fn1, the other strands being located in the β-subunit (11). Thus, it may be that the full-length α-subunit is poorly expressed because of problems with proper folding of the exon 9 region that lacks its counterparts from the β-subunit.

There have been some reports on soluble insulin receptors that had higher affinity for insulin than sIR but in all cases these included the entire extracellular domain including the N-terminal part of the β-subunit (27, 28). The fact that sIR when purified under certain conditions can obtain picomolar affinity (26) indicates that the IR ectodomain in itself has the full potential for high affinity binding of insulin. It has been reported that ectodomain constructs can be stabilized in a high affinity conformation via a C-terminal self-associating fusion partner. Baas et al. (27) fused sIR to immunoglobulin domains, whereas Hoyne et al. (28) used a 33-amino acid leucine zipper sequence. In contrast to the fusion proteins that had molecular masses of 300–400 kDa, our approach yielded a much smaller high affinity α-subunit construct with an apparent mass of only 200 kDa.

In conjunction with the property of high affinity binding of insulin, hIR is characterized by having nonclassical binding features that suggest negative cooperativity or site-site interaction between separate binding sites. Negative cooperativity has been demonstrated directly by accelerated dissociation of labeled insulin from the receptor when unlabeled insulin is present, or indirectly by Scatchard analysis yielding curvilinear plots (24, 32). In the present study, we demonstrated accelerated dissociation of bound labeled insulin in the presence of unlabeled insulin both for the soluble high affinity receptor mIR.Fn0/Ex10 and for hIR (Fig. 6). In contrast the dissociation of labeled insulin from the soluble ectodomain, sIR was unaffected by the presence of unlabeled insulin. To our knowledge this is the first time that dissociation experiments have been reported for this receptor. In the present study the binding curves for hIR and mIR.Fn0/Ex10 (Fig. 2) were best fitted to a one-site binding model and accordingly the Scatchard transformations resulted in linear plots. Summarizing all the binding data, we conclude that the soluble high affinity receptor mIR.Fn0/Ex10 has the same ligand binding properties as the holoceptor hIR, in all aspects of ligand binding analyzed here. All recombinant insulin receptors previously reported to bind insulin with high affinity (pM) have included the entire extracellular part of the β-subunit. Here we demonstrate that a dimeric fragment of the α-subunit binds insulin with full holoceptor affinity and also exhibits the dissociation properties of the holoceptor, making this minimized soluble insulin receptor an attractive candidate for detailed structural analysis.

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