Specificity of Insulin and Insulin-like Growth Factor I Receptors Investigated using Chimeric Mini-Receptors

ROLE OF C-TERMINAL OF RECEPTOR α SUBUNIT

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We have investigated the role of the C-terminal of the α-subunit in the insulin receptor family by characterizing chimeric mini-receptor constructs comprising the first three domains (468 amino acids) of insulin receptor (IR) or insulin-like growth factor I receptor (IGFIR) combined with C-terminal domain from either insulin receptor (IR) (residues 704–719), IGFIR, or insulin receptor-related receptor (IRR). The constructs were stably expressed in baby hamster kidney cells and purified, and binding affinities were determined for insulin, IGF, and a single chain insulin/IGF hybrid. The C-terminal domain of IRR was found to abolish binding in IR and IGFIR context, whereas other constructs bound ligands. The two constructs with first three domains of the IR demonstrated low specificity for ligands, all affinities ranging from 3.0 to 15 nM. In contrast, the constructs with the first three domains of the IGFIR had high specificity, the affinity of the novel minimized IGFIR for IGF was 1.5 nM, whereas the affinity for insulin was more than 3000 nM. When swapping the C-terminal domains in either receptor context only minor changes were observed in affinities (<3-fold), demonstrating that the carboxyl-terminal of IR and IGFIR α-subunits are interchangeable and suggesting that this domain is part of the common binding site.

Insulin and IGF1 mediate their effects by binding to specific tyrosine kinase receptors in the plasma membrane of target cells. The insulin receptor and IGF receptors are membrane-bound glycoproteins with intracellular ligand stimulated tyrosine kinase domains, and they display similar overall structural organization (1–3). A third member of this receptor family, the insulin receptor related receptor (IRR) was identified at the genomic level by Shier and Watt in 1989 (4), but so far no ligand for this receptor has been identified. The amino acid sequences of these receptors are approximately 50% identical (1, 2, 4), and the cysteines are conserved between these receptors and, therefore, they are thought to have same overall structure and conformation. The structure of the insulin receptor has been investigated extensively and reviewed (5, 6), it is a disulfide-linked heterotetrameric molecule with β-α-α-β receptor subunit configuration, and the disulfides responsible for α-α and α-β contacts have been identified (7, 8). Predictions of the tertiary structure of the IR ectodomain based on alignment with epidermal growth factor receptor sequences (9, 10) produced the consensus that the first 468 amino acids of IR α-subunit comprise two large homologous domains, L1 and L2, separated by a cysteine-rich region. Recently, a crystal structure of the first three domains of the IGFIR receptor (residues 1–462) has been solved confirming this domain structure (11); however, this IGFIR fragment does not bind ligand. In the IR we have shown that, in addition to the first three domains (residues 1–468), only a small peptide from the C-terminal of the α-subunit (704–719) is needed to obtain binding of insulin with the same affinity as the intact IR ectodomain (12), but a similarly minimized IGFIR has not been described.

The insulin and IGF systems share common binding epitopes on ligands as well as receptors. The two ligands, insulin and IGF, have similar amino acid sequences, but one binds weakly to the receptor for the other. However, we have demonstrated that it is possible to make a single chain hybrid between insulin and IGF that binds with high affinity to both receptors (13). This single-chain insulin/IGF hybrid (ICP) consists of insulin A and B chains connected by the C-domain from IGF. The similarity of the binding sites of the receptors have been demonstrated with chimeric receptors in which discrete domains of one receptor replaced the corresponding domain of the other receptor. The chimeric receptor studies have produced a consensus that N-terminal sequences of the insulin receptor are critical for high-affinity insulin binding, whereas the cysteine-rich domain of IGFIR is essential for high-affinity IGF binding (14, 15). The importance of the N terminus of the insulin receptor has also been demonstrated by cross-linking (16) and by alanine scanning mutagenesis (17).

In addition to N-terminal regions, the C-terminal of the IR α-subunit containing residues 704–719 has in particular been suggested to be important for ligand binding. This has been suggested by cross-linking (18) and by alanine scanning mutagenesis (19). Furthermore, alanine scanning has demonstrated that Phe-701 of IGFIR (corresponds to Phe-714 in IR) is important for the binding of IGF to IGFIR (20). Also strongly stressing the importance of this C-terminal region are our findings with the minimized IR as mentioned above (12).

To elucidate the role of the C-terminal α-subunit for ligand binding specificity in the insulin receptor family, we decided to investigate minimized IR, IGFIR, as well as chimera between these receptors also including constructs with the C-terminal region of IRR.
Experimental Procedures

**MISCELLANEOUS**—Insulin, IGF-I, and Tyr-A14-125I-insulin and Tyr-31-125I-IGF-I were from Novo Nordisk. The ICP consists of insulin with the C-domain of IGF-I, and a detailed description of this construct including purification is found elsewhere (13). DNA restriction enzymes and T4 ligase were from New England Biolabs, Pwo polymerase was from Roche Molecular Biochemicals. Preparation of plasmid DNA and agarose slabs were performed according to standard methods. For DNA minipreps, QIAprep 8 kit was used (QIAGEN). Disuccinimidyl suberate (DSS) was from Pierce Chemical Co.

**CONSTRUCTION OF cdNA EXPRESSION PLASMAIDS ENCODING RECEPTOR DELETION CONSTRUCTS**—An overview of the receptor constructs and the abbreviations used is shown in Fig. 1 and Table I. The receptor constructs comprise the first three domains of one receptor (IR or IGFIR) combined with a C-terminal sequence from either of the three receptors IR, IGFIR, or IRRR. The receptor sequences were fused to a C-terminal sequence encoding the Flag epitope (SDYKDDDDK), and inserted into the Zem expression vector (14, 21). The minimized insulin receptor IR703, which has previously been described (12), was used as template for constructing mIR which was made by extending the IR703 sequence with a Flag sequence using PCR. The antisense oligonucleotide used was 5'-TTTTTCTAGACTACTTGTCGTCGTCGTCCTTGTTAGTCAGATGCCGTTAGGCAAGAAA-3' (XhoI site underlined) and a sense primer upstream from Nhel site (amino acids 466-467). The PCR fragment was digested with Nhel and XhoI and ligated into the corresponding site of plasmid encoding IR703.

The mIGFIR that consists of IGFIR residues 1-458 and 691-706 fused to a C-terminal Flag epitope was made by PCR amplifying an IGFIR sequence similar to the described above using antisense primer: 5'- TTCTCTGACTACATGCTGTGGTGTTGGTGGTTGTTTT (Nhel site underlined) and a sense primer upstream from Nhel site (amino acids 456-457). The PCR fragment was digested with Nhel and XhoI and ligated into the corresponding site of plasmid encoding IGFIR deletion construct.

The mIR-IGFIR construct was obtained by replacing a 60-bp base pair Nhel site in IR703 with the corresponding fragment from the mIGFIR, and conversely IGFIR-IR was obtained by replacing a Nhel/XhoI fragment of mIGFIR with the corresponding fragment from mIR.

For making chimeric receptors with IRRR sequence in the C terminus, the IRRR sequence was replaced with the IGFIR sequence by taking the expression vectors were transfected into cells by the LipofectAMINETM procedure for stable transfection of adherent cells (Life Technologies Inc.). Prior to transfection, BHK cells were grown in Dulbecco’s Modified Eagle’s medium (DMEM) (Life Technologies Inc.) supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. Cells were seeded to 20-40% confluency 16-24 h prior to transfection, and the medium was replaced 1 h prior to transfection. Plasmid DNA (2 µg) was mixed with 7 µl of LipofectAMINE in a total volume of 200 µl of serum-free medium, this mixture was incubated for 30 min at room temperature before adding 0.8 ml of serum-containing medium and overlaying onto the rinsed cells. After incubation for 5 h, 1 ml of DMEM containing 20% fetal calf serum was added, and then cells were incubated for 16 h before removing DNA by replacing medium with DMEM containing 10% fetal calf serum. After an additional 48 h, cells were passaged to selection medium containing 1 µM methotrexate. After 10-14 days, individual colonies were isolated and expression was tested by insulin binding assay or by immunoblotting studies. Selected BHK clones were grown in DMEM supplemented with 10% fetal calf serum and 1 µM methotrexate.

**PURIFICATION OF FLAGGED MINI-RECEPTORS**—Receptors with C-terminal Flag epitope were purified by affinity chromatography using immobilized M2 Flag antibody from Sigma. Flag affinity gel was mixed with BHK culture supernatant and incubated at 4 °C overnight before washing with TBS (10 mM Tris (pH 7.5), 100 mM NaCl), and the bound receptor proteins were eluted with 0.1 mg/ml Flag peptide in TBS, 0.02% (w/v) sodium-azide. Purified receptors were stored in elution buffer at 4 °C. The IR703 receptor was purified as described previously (12).

**IMMUNOBLOTTING**—The expressed receptors were purified by immunoblotting using three antibodies. Flag-tagged proteins were detected using a monoclonal antibody against the M2 Flag epitope. For detecting insulin receptors, the monoclonal antibody mAb-F26 was used. This antibody was raised against a peptide corresponding to amino acids 39-75, mapping at the N terminus of the insulin receptor α-subunit. The antibody was kindly donated by Jes Thorl Clauden, Novo-Nordisk. For detecting IGFIR, the IGFIRs (N-20) from Santa Cruz Biotechnology were used, this is an affinity purified rabbit polyclonal antibody raised against a peptide corresponding to amino acids 31-50, mapping at the N terminus of IGFIR.

For blotting, samples were mixed with 0.33 volumes of loading buffer containing 100 mM dithiothreitol and incubated at 70 °C for 10 min before loading on a 4-12% polyacrylamide Bio-Tris gel (NuPAGE, Novex). After electrophoresis, proteins were blotted onto Immobilon-P membrane (Millipore). The membrane was blocked by incubating with blocking buffer (2% defatted skim milk, 2% BSA in TBS) for 16 h at 4 °C. The receptor antibodies and peroxidase-conjugated secondary antibodies were diluted in 1% BSA in TBS, and immunoreactive protein was detected using ECL reagent from Amersham Pharmacia Biotech.

**Receptor Binding Assay**—For displacement binding analysis, a suitable amount of purified receptor was incubated in a total volume of 200 µl with 15 pm of 125I-insulin or 125I-IGF-I and various concentrations of unlabeled ligand (insulin, IGF-I, or IGF, or ICRP) in binding buffer (100 mM Hepes (pH 8.0), 100 mM NaCl, 10 mM MgCl2, 0.05% (w/v) BSA, 0.025% (w/v) Triton X-100) for 16 h at 4 °C. Subsequently bound counts were recovered by precipitation with 0.2% γ-globulin and 500 µl of 30% (w/v) polyethylene glycol 8000. Bound 125I-labeled hormone was counted in a gamma counter. The concentration of receptor was adjusted to yield 10-15% binding when no competing ligand was added in the competition assay. The binding data were fitted using nonlinear regression algorithm in GraphPad Prism 2.01 (GraphPad Software Inc, San Diego, CA).

**Cross-linking of 125I-labeled Ligand to Receptors**—For chemical cross-linking, 125I-insulin or 125I-IGF-I was used, and cross-linking was performed essentially as described above (14, 21). The receptor constructs were incubated for 60 min at room temperature with labeled ligand (0.2-0.3 nM) in the presence or absence of unlabeled ligand (1 µM). DSS in dimethyl sulfoxide was added from a 10 mM stock solution to a final concentration of 0.1 µM. After 15 min on ice, the reaction was stopped by adding 0.33 volume of loading buffer containing 100 mM dithiothreitol. Samples were incubated at 70 °C for 10 min before running on a 4-12% polyacrylamide Bio-Tris gel (NuPAGE, Novex). The gel was fixed in 10% acetic acid, 20% ethanol, and a PhosphorImager screen was exposed with the dried gel.

**Gel Filtration**—The minimized IGFIR receptor (mIGFIR) was mixed with 125I-IGF-I (~200,000 cpm) allowed to equilibrate, and analyzed with the Amersham Pharmacia Biotech FPLC system, using a Superose 12 HR 10/30 gel filtration column equilibrated in 20 mM Tris, HCl buffer, pH 7.5, with 100 mM NaCl. Chromatography was performed at a flow rate of 0.5 ml/min, and 0.5 ml fractions were collected. Radioactivity in fractions was counted on a γ-counter. Aldolase (M, 158,000), BSA (M, 67,000), ovalbumin (M, 43,000), and ribonuclease A (M, 13,700) from Amersham Pharmacia Biotech were used for calibration of the column.

**RESULTS**

**CLONING AND EXPRESSION OF RECEPTOR CONSTRUCTS**—We previously expressed a minimized insulin receptor (IR703) consisting of the first three domains of the insulin receptor fused directly to 16 amino acids of the C-terminal of the α-subunit (residues 704-719) (12). In the present study the Flag epitope was added to the C-terminal of the IR703, resulting in the mIR construct, and in addition similar constructs were made from the IGFIR sequence and various chimera based on these receptors (Fig. 1, Table I). In all constructs, a Nhel restriction site was introduced corresponding to amino acids 466-467 of IR (Fig. 1), and this site allowed shuffling the C-terminal domain from one receptor to the other as described under “Experimental Procedures” (Fig. 1 and Table I). All constructs were stably expressed in BHK cells, and the C-terminal Flag epitope facilitated purification and detection of receptors using Flag antibody.
**Minimized Chimeric Insulin/IGFI Receptors**

**Fig. 1. Schematic representation of receptor constructs.** A, schematic illustration of the DNA sequence encoding minimized IR with Flag tag (DYKDDDDK). L1, Cys, and L2 are the first three domains of the insulin receptor (9); IR residues 704–719 are part of exon 10 in the C terminus of the α-subunit. The restriction sites Nhel, AvrII, and XhoI were used for shuffling these domains as described under “Experimental Procedures.” B, the receptor constructs expressed; IR domains are black boxes, IGFIR domains are white boxes, and IRRR domains are gray boxes. C, the C-terminal domains of the three receptors (704–719 from IR, 691–706 from IGFIR, and 686–697 from IRRR), the three C-terminal amino acids of IR, RPS (717–719), were inserted into the IRRR constructs. The boxes indicate residues that are identical to corresponding IR residue.

**Purification of Flagged Mini-Receptors—**Receptors with Flag epitope were purified by absorbing on anti-Flag M2 affinity column and eluting with 0.1 mg/ml of Flag peptide. This procedure enabled purification of all receptors using the same protocol. Efficient purification is required especially when investigating IGFI binding to avoid interference from IGFI and IGF binding proteins present in serum and secreted by BHK cells (23). In Fig. 2 is shown purification of two of the IGFIR-based receptors (mIGFIR and mIGFIR.IR). In both BHK supernatants (lanes 1 and 6), the dominating protein that cross-links IGFI has mobility corresponding to approximately 38 kDa which is the size expected for IGFI binding protein (30 kDa) cross-linked to IGFI (8 kDa), whereas the receptor/IGFI complex with an apparent mass of approximately 80 kDa is seen as a faint band in the BHK supernatant of mIGFIR (Fig. 2, lane 1) and is not visible at all in mIGFIR.IR (lane 6). After absorption to the anti-Flag column, the binding protein is found in the flow-through (lanes 2 and 7), whereas no bands smaller than the 80-kDa receptor/IGFI band in the eluates is apparent, demonstrating efficient elimination of binding protein using this affinity purification. In addition to the 80-kDa band comprising receptor, there seems to be a fainter band of higher molecular mass that could be a dimeric mini-receptor.

**Size Separation of Ligand Receptor Complex—**To investigate the stoichiometry further, we mixed the mIGFIR with [125I]-IGFI and then separated this complex on a Superose 12 gel filtration column. The elution profile is shown in Fig. 3. Clearly the majority of receptor bound IGFI elutes near the BSA (67 kDa) marker which is consistent with a monomeric receptor, and a smaller fraction elutes earlier corresponding to M<sub>r</sub> higher than 158,000.

**Detecting Receptors by Immunoblotting—**The purified receptor constructs were confirmed by immunoblotting. The antibody that recognizes the Flag epitope should detect all constructs except IRΔ703 (Fig. 1), whereas the antibodies specific for the N terminus of IR or IGFIR should verify that the N terminus of the constructs are correct. The immunoblots are shown in Fig. 4. In panel A is shown the proteins detected with the antibody specific for the Flag peptide sequence. The Flag sequence is detected in all the new receptor constructs (lanes 2–7). The size of the immunoreactive proteins is about 80 kDa for the IR constructs (lanes 2–4) and is somewhat lower (72 kDa) for the IGFIR constructs (lanes 5–7). This difference in part reflects that mIR is longer than mIGFIR (492 versus 482 amino acids). In addition, mIGFIR is probably less glycosylated than mIR because the mIGFIR have only seven potential N-glycosylation sites, whereas mIR has ten sites.

Immunoblotting using the receptor-specific antibodies, shown in Fig. 4B, reveals bands similar to what was detected with the Flag antibody (lanes 2–7). In addition, the IRΔ703 is now detected using the IR antibody (panel B, lane 1). We have not investigated how efficient the purification procedure is, but the immunoblots indicate that the amounts of chimeric receptors with the C-terminal IRRR domain (lanes 4 and 7) are not dramatically lower than what is found for the other mini-receptors, indicating that the IRRR domain is well tolerated in mIR as well as mIGFIR context.

As demonstrated by cross-linking (Fig. 2) and gel filtration (Fig. 3), there is a small receptor fraction that dimerizes or aggregates to molecular mass higher than 158 kDa, and this probably accounts for the faint higher molecular mass band observed on the blots (Fig. 4, panels A and B).

**Receptor Constructs, Binding of Ligands—**The purified receptors were analyzed in a competition binding assay. For the three receptors that contain the N terminus of the insulin receptor (mIR, mIR.IGFIR, and mIR.IRRR), labeled insulin was used as tracer, and for the constructs based on IGFIR (mIGFIR, mIGFIR.IR, and mIGFIR.IRRR), labeled IGFI was used. Binding curves are shown in Fig. 5, and an overview of the affinities is presented in Table I.

To evaluate the Flag tag approach, we compared mIR with the untagged IRΔ703 receptor (12). The binding affinities found for these two receptors are similar for insulin and ICP, whereas for IGFI, the mIR has slightly increased affinity (2.5-fold) compared with IRΔ703. As the Flag epitope only has minor effects on ligand binding, we decided that this system was useful for investigating members of the insulin receptor family.

The second conclusion from the binding studies is that the IRRR domain in the C-terminal abolishes binding of insulin in mIR context and abolishes binding of IGFI in mIGFIR context. As described above, the mIR.IRRR and mIGFIR.IRRR proteins are expressed and purified as judged by immunoblotting (Fig. 4), and therefore we conclude that replacing the cognate C-terminal sequence with that of IRRR directly affects the epitope that interacts with ligand.

For the constructs that contain the first three domains of IR (mIR, IRΔ703, and mIR.IGFIR), the binding affinities for either of the three ligands was comparable from one receptor to the
Minimized Chimeric Insulin/IGFI Receptors

TABLE I
Receptor domain structure and affinities for insulin, IGFI, and ICP

| Construct          | Domain structure | Binding affinities* |
|--------------------|------------------|---------------------|
|                    | N terminus (1-468) | C terminus (704-719) | Insulin | IGFI | ICP |
| mIR                | IR               | IR                  | 5.8 ± 1.5 | 15 ± 3 | 3.6 ± 1.6 |
| IRA703b           | IR               | IR                  | 5.9 ± 2.5 | 35 ± 18 | 5.3 ± 1.2 |
| mIR.IGFIR         | IR               | IGFI                | 12 ± 7.2 | 7.2 ± 1.0 | 8.1 ± 2.2 |
| mIR.IRRR          | IR               | IRR                 | 3100 ± 1700 | 1.5 ± 0.7 | 9.8 ± 3.9 |
| mIGFIR            | IGFI             | IGFI                | 3800 ± 1700 | 3.4 ± 1.0 | 6.2 ± 0.8 |
| mIGFIR.IR         | IGFI             | IR                  | 1.0 ± 0.8 | 3.9 ± 1.6 | 1.6 ± 0.3 |
| mIGFIR.IRRR       | IGFI             | IRRR                | 2.2 ± 0.8 | 1.4 ± 0.9 | 0.8 ± 0.3 |

* For displacement experiments, 125I-insulin for mIR, IRA703, and mIR.IGFIR, and 125I-IGFI for mIGFIR and mIGFIR.IR was used.

b IRA703 does not have a C-terminal Flag tag.

Fig. 2. Purification of flagged receptors. Autoradiographs illustrating purification of mIGFIR (lanes 1–5) and mIGFIR.IR (lanes 6–9). Receptors were cross-linked to 125I-IGFI using 0.2 mM DSS, and proteins were separated on a 4–12% polyacrylamide Bis-Tris gel. The following samples were analyzed: input (medium from BHK cells, lanes 1 and 6), flowthrough from anti-Flag affinity column (lanes 2 and 7), 0.1 mg/ml Flag elution fraction 1 (lanes 3 and 8), fraction 2 (lanes 4 and 9), and fraction 3 (lane 9). In adjacent lane, an 14C-labeled Rainbow marker was run, with the band sizes indicated at the left of the gel.

Fig. 3. Size separation of ligand receptor complex. The minimized IGFI receptor (mIGFIR) was equilibrated with 125I-IGFI and analyzed using a Superose 12 HR 10/30 gel filtration column. Radioactivity in each fraction is shown. Arrows indicate the location of the following size markers: aldolase (M, 158,000), BSA (M, 67,000), ovalbumin (M, 43,000), and ribonuclease (M, 13,000), other (less than 5-fold differences), and in addition these receptors displayed poor specificity, all affinities ranging from 3.6 to 35 nM. The affinities for insulin and ICP were 4–6 nM, which is similar to the affinity of the soluble IR ectodomain (sIR) for insulin (14, 21). In contrast to sIR that has approximately 100-fold specificity for binding insulin compared with IGFI (14), these minimized IR constructs only have 3–6-fold lower affinity for IGFI compared with insulin. As the affinity for insulin is unchanged, the loss in specificity must be ascribed to higher affinity for IGFI (15–35 nM) in mIR and IRA703 as compared with sIR, 500–800 nM (14, 21).

For the constructs with the first three domains of IGFI, the binding affinities of the three ligands were conserved when swapping to the C-terminal of IR (less than two-fold differences), but in clear contrast to mIR, the mIGFIR-based receptors retain high specificity (>1000-fold). The affinity of the mIGFIR for IGFI is 1.5 nM, whereas insulin has an EC50 of more than 3000 nM (<0.05%), and ICP is close to IGFI with 9.8 nM (16%). The affinities for IGFI and ICP (1.5 and 9.8 nM) are approximately 4-fold lower than has been reported for the soluble IGFI ectodomain (0.4 and 1.9 nM) (13, 14, 21), but the relative affinities are unchanged.

The binding data for chimeric receptors based on both IR and IGFI demonstrate that inserting the C-terminal domain from the non-cognate receptor does not change ligand binding char-
characteristics. In other words, the C-terminal domains are interchangeable with respect to ligand binding, and accordingly this domain appears to be part of the common binding site in these receptors.

**Cross-linking of \(^{125}I\)-labeled Ligand to Receptors**—Labeled ligands were chemically cross-linked to receptor constructs with DSS, and the gels are shown in Fig. 6. The cross-linking pattern shows specific binding of tracers to the constructs with IR or IGFIR sequence in the C terminus, all yielding bands with apparent mass between 66 and 97 kDa (lanes 1–5, 703, and mIR,IRRR), whereas the two constructs with the C-terminal domain from IRRR (lanes 7 and 13) do not present any bands, in accordance with our findings that mIR.IRR and mIGFIR.IRRR do not bind ligand in the soluble binding assay.

**DISCUSSION**

To standardize and simplify the purification of multiple receptors with varying domains and binding characteristics, we constructed the minimized receptors with a C-terminal epitope. The presence of this epitope permits a mild single-step purification of the receptors, and more importantly, this procedure efficiently eliminates the IGFIR binding proteins present in the BHK cell culture medium (23) (Fig. 2). The Flag epitope is close to the ligand binding domain that we wanted to study, and thus it could interfere with binding as has been demonstrated in IR with exon 11 (24). We have previously shown that ligand binding of the minimized IR (IR703) is not influenced by the exon11 region (12), and here we observe no difference in affinity for insulin between IR703 and mIR which has the Flag epitope. We thus concluded that the Flag epitope does not affect ligand binding in the mini-receptors.

We have previously reported that the ligand binding domain of IR can be minimized by expressing the first three IR domains (468 amino acids) fused to the C-terminal 16 amino acids of the α-subunit (residues 704–719) (12). This minimized receptor (IR703) binds insulin with affinity similar to the full-length IR ectodomain (soluble IR). In the present study, we have expressed a similar minimized IGFIR construct and four chimeric mini-receptors. The minimized IGFIR construct is particularly interesting because the x-ray structure of the first three domains of IGFIR was solved recently (11), and this structure led to suggestions of a putative central site that could accommodate IGFI. In the x-ray structure, the C-terminal of the L2 domain does not face the putative ligand binding lobe, whereas our data show that only 16 amino acids fused to this C terminus induces binding of IGFI. The binding affinity of mIGFIR for IGFI (1.5 nM) was approximately 4-fold lower than that of IGFI (37.5 nM) (14, 25), whereas in terms of specificity, it remains highly specific for IGFI compared with insulin (>1000-fold).

In terms of binding cognate ligand, the minimized IR more faithfully reflected the binding characteristics of the ectodomain; mIR and IR703 have retained the full binding activity of the IR ectodomain (sIR), all having affinities of 5–6 nM for insulin. Although mIR (and IR703) have unchanged affinity for insulin, they have increased affinity for IGFI compared with sIR, resulting in the mini-IRs having almost the same affinities...
for insulin and IGFI, with the affinity for IGFI being only 3–6-fold lower than affinity for insulin (Table I). The low specificity of the minimized insulin receptors is puzzling, we speculate that because the sIR have all the epitopes required for binding IGFI with 20 nM affinity (like mIR and IRA703), there must be something inhibiting receptor-IGFI interaction in sIR. Possibly some of the regions deleted in the minimized IRs interfere with IGFI binding in sIR, or maybe the dimeric conformation of sIR interferes with IGFI attaining the higher affinity.

Despite that the primary sequence of IRRR is as similar to IR and IGFIR as the IR and IGFIR are to each other (4), no binding of insulin, IGFI, or any other ligand has been observed with the IRRR. In the present setup we have investigated the role of the short C-terminal domain of the IRRR. In the present setup we have investigated the role of the short C-terminal domain of the IRRR α-subunit in mIR and mIGFIR context. The clear conclusion from these chimeric receptors is that the C terminus of IRRR abolishes binding of insulin and IGFI. This is somewhat surprising because only four of the residues in the C-terminal region (IR residues 704–719) are different from corresponding positions of both IR and IGFI (Fig. 1C). One of these four residues, alanine 694, has been shown by alanine scanning mutagenesis to be acceptable at corresponding position 712 in IR (19).

Chimeric receptors between IR and IRRR have been described previously, Zhang and Roth (26) found that a chimeric receptor with the extracellular domain of IR and the kinase domain of IRRR exhibited high affinity insulin binding and insulin-stimulated tyrosine kinase activity. They also made a chimeric receptor with the cysteine-rich region of IR (exon 3) replaced with the homologous region of IRRR that was shown to bind insulin with affinity similar to that of wild type IR; however, when replacing both exon 2 and exon 3 of IR with IRRR domains, no binding of insulin was detected (27).

The chimeric mini-receptors show that replacing the C-terminal domain of IR with the corresponding domain of IGFIR only has minor effect on ligand binding. This is also true for mIGFIR with the C terminus of IR. So within IR and IGFIR, the C-terminal domains are interchangeable in terms of ligand binding, suggesting that this domain is part of the common binding site of these receptors.

We have previously characterized ICP, which consists of insulin with IGFI C-domain, against various IR and IGFIR. The conclusion was that ICP binds with high affinity to IR (55–113% of insulin) and with 19–28% of IGFI affinity to IGFIR (13). Here we get similar results for relative binding affinities for mini-receptors. The affinity of ICP for mIR and IRA703 was not different from the affinity of insulin. For mIGFIR, the affinity of ICP was 16% of IGFI, and thus previous conclusions that the C-domain of IGFI is a major determinant of IGFI specificity for IGFI is true also for the minimized IGFIR.

Based on the chimeric mini-receptors, we conclude that the C-terminal domain of IRRR abolishes binding of ligand and that swapping of C-terminal sequences from IR to IGFIR or vice versa does not influence ligand binding dramatically, indicating that this region is part of the common binding site in these receptors. Finally we have demonstrated that it is possible to make a minimized IGFIR that binds IGFI, this mIGFIR receptor consists of the first three domains of IGFIR fused to 16 amino acids from the C terminus of the α-subunit. The short C-terminal domain is essential for binding ligand, whereas the crystal structure for the remaining 462 amino acids has been solved (11) and, therefore, the minimized IGFIR is a good candidate for crystallizing an IGFIR fragment with bound ligand.

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