High affinity insulin binding by soluble insulin receptor extracellular domain fused to a leucine zipper

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Abstract Insulin receptors (IRs) that are truncated at the end of the ectodomain form dimers that bind insulin with different characteristics to wild type receptors. These soluble IRs have lowered affinity for insulin compared with full-length IR, and exhibit linear Scatchard plots in contrast to the curvilinear plots obtained with full-length IR, IR truncated at the C-terminus of the transmembrane region and IR ectodomains fused to the self-associating constant domains from Fc or λ immunoglobulins. In this report, we have fused the IR ectodomain to the 33 residue leucine zipper from the transcriptional activator GCN4 of *Saccharomyces cerevisiae*. This fusion protein binds insulin with high affinity in a manner comparable to native receptor. The respective dissociation constants were K_{d1} 8.2×10^{-11} M and K_{d2} 1.6×10^{-8} M for hIRedZip and K_{d1} 5.7×10^{-11} M and K_{d2} 6.3×10^{-9} M for membrane-anchored, native receptor.

Key words: Insulin receptor; Leucine zipper; Ectodomain chimera; Expression; High affinity binding

1. Introduction

The insulin receptor (IR) is synthesized as a disulfide-linked homodimer that is post-translationally cleaved to yield two α- and two β-chains. There are at least two α–α disulfide bonds linking the monomers in the dimer [1–3] and a single disulfide bond linking the α- and β-chains within each monomer [3]. The α-chain is exclusively extracellular while the β-chain contains extracellular and intracellular regions linked by a single transmembrane domain of 23 amino acids. The α-chains carry the major determinants for insulin binding with residues from four distinct regions, the L1 domain, Cys-rich region, L2 domain and the insert domain, contributing to the interaction with ligand [4–6].

Many studies of insulin binding to native, membrane bound IR have indicated the existence of high and low affinity binding states [7]. In contrast, secreted IRs that are truncated at the ectodomain/transmembrane junction form dimers with only low affinity for insulin and exhibit linear Scatchard plots, in contrast to curvilinear plots obtained for the full-length receptor [7–10]. Analysis of the binding characteristics of a series of truncated hIR receptors with progressive C-terminal deletions revealed that truncation at the C-terminal end of the transmembrane region (residues 1–952) was sufficient for the generation of curvilinear Scatchard plots, high affinity binding and negative cooperativity [11]. These data imply that a precise orientation of the C-terminal region of the ectodomain is required to facilitate the conformational changes required for high affinity. Subsequently, wild type receptor binding characteristics were obtained with soluble IR ectodomain fusion proteins, where the self-associating, constant domains from either immunoglobulin Fc (244 amino acids) or λ (103 amino acids) domains were placed at the C-terminus [12].

In order to understand the structural basis of ligand-induced signal transduction, structural information is required for different forms of the receptor. These are: the uncomplexed receptor, the low affinity complex of ectodomain or ectodomain fragments and receptor constructs capable of exhibiting the high affinity binding with ligand. In this report, we have placed a 33 amino acid leucine zipper from the GCN4 transcriptional activator of *Saccharomyces cerevisiae* [13] at the C-terminal end of the exon 11 minus form of the human IR (hIR) ectodomain. This enables the production of a soluble form of the hIR ectodomain dimer that exhibits high affinity insulin binding and curvilinear Scatchard plots. Leucine zippers have been used previously to promote the assembly of αβ heterodimers of the IL2 receptor [14] and the T cell receptor [15].

2. Materials and methods

2.1. Expression constructs hIRed and hIRedZip

The exon 11 minus form of hIR ectodomain/leucine zipper fusion (hIRedZip) was generated via the following stepwise procedure. A 4.2 kb *SalI*/*Xba* restriction fragment, isolated from the plasmid peT [16], a gift from Dr. W.J. Rutter, UCSF, which encoded hIR (exon 11+ form), was inserted into the *SalI*/*Xba*I cut and dephosphorylated pBluescript KS+ plasmid vector (Pharmacia). The resultant 7.1 kb plasmid pBluhIR contained a single *SalI*/*Xba*I inserted fragment of hIR cDNA. Single-stranded circular DNA derived from pBluhIR was modified by site-directed mutagenesis using an oligonucleotide antisense primer 5′-GGCCCATCTGGCTGGCTAGCAATATTTGACGGG-3′. This mutagenesis produced a plasmid phIRΔTM in which the nucleotide sequence that encoded both the transmembrane and the flanking Lys and Arg residues (amino acids 929–953) of hIR 11+ was replaced by a sequence which encoded a single Ser residue, and provided a unique *Nhe*I restriction site. This exon 11+ construct was converted to an exon 11− version by exchanging the 1.8 kb *Aat*II fragment from phIRΔTM with the equivalent *Aat*II fragment from plasmid pHIR/P12-1 (ATCC 57493; nucleotides 1101–2861 that encoded part of the extracellular domain and the entire cytoplasmic domain of the hIR exon 11− form [17]) to produce plasmid pEZI-1 which lacks the 36 nucleotides of exon 11. DNA which encoded the leucine zipper of the transcriptional activator GCN4 and a termination codon was amplified from *S. cerevisiae* S288C genomic DNA by

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polymerase chain reaction (PCR) using the oligonucleotide primers: 5′-TGAAGCTAGCAGAATGAAACAACTTGAAG-3′ and 5′-TGAATCTAGATCAGCGTTCGCCAAC-3′ (*Nhe*I and *Xba*I sites are underlined). The PCR fragment (~100 bp) was digested with *Nhe*I/*Xba*I and ligated to *Nhe*I/*Xba*I-digested and dephosphorylated pEZI-1 to produce the 6.1 kb plasmid pEZI-2 which contained hIR exon 11− ectodomain fused to the leucine zipper.

The mammalian expression plasmid phIRedZip was constructed by exchanging the 1.1 kb *Bam*HI/*Xba*I fragment from pEZI-2 for the corresponding fragment from plasmid pEH3, a derivative of the mammalian expression plasmid pEE14 (CellTech Ltd., Slough, UK), which contained the entire coding sequence for hIR exon 11− and from which the two *Eco*RI sites and a *Bam*HI site located outside the hIR coding region had been sequentially removed (not shown). All plasmid constructions were verified by restriction endonuclease analysis. Mutagenized regions and sequence which surrounded ligation junctions were confirmed by nucleotide sequencing.

The construction of the expression plasmid (pIRII) for the non-zippered hIR ectodomain, exon 11− form (hIRed) has been described previously [18,19].

2.2. Cell culture, DNA transfection and protein production

Chinese hamster ovary cells (CHO-K1) and the glycosylation deficient Lec8 cells were transfected with pIRII and phIRedZip using Lipofectamine (Gibco-BRL) and maintained in glutamine-free Glascow modification of Eagle's medium (GMEM) from ICN Biomedicals, Australia, and 10% dialyzed fetal calf serum (FCS) containing 25 μM methionine sulfoximine (MSX) as previously described [19,20]. Transfected cells were screened for expression by a sandwich, enzyme-linked immunosorbent assay as described previously [19,20]. Cell lines producing the highest levels of secreted receptor were cloned by limiting dilution. Selected clones (inoculum of $\sim 1.328\times10^{8}$ cells) were grown in spinner flasks packed with 10 g of Fibra-Cel disks (Sterilin, UK) as carriers and filled with 500 ml of GMEM medium containing 10% FCS and 25 μM MSX. Selection pressure, using MSX, was maintained for the duration of the culture. The expressed hIRedZip fusion protein and hIR ectodomain (hIRed) were purified from conditioned media by affinity chromatography on immobilized bis-butyloxycarbonyl-insulin (bis(BOC)-insulin) and serial elution with 0.4 M NaCl/0.2 M trisodium citrate (pH 5.0) followed by insulin in Tris-buffered saline containing 0.02% sodium azide (TBSA) pH 8.0, and 0.1 M trisodium citrate pH 3.0 into tubes containing sufficient 1 M Tris–HCl at pH 8.0 to neutralize the fractions [8]. Eluted fractions were concentrated by ultrafiltration and purified by gel filtration chromatography on a column of Superdex S200 (Pharmacia; 1×40 cm) in TBSA as described previously [20]. Final separation of monomeric hIRedZip from aggregated material was achieved by ion-exchange chromatography on Resource Q (Pharmacia). Solutions of purified hIRedZip and hIRed were stored at 4°C prior to use. Metabolic labelling, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), solid-plate insulin binding assays and Scatchard analyses were performed at least three times in triplicate as described previously [19,20]. Ligand binding data were analyzed using the LIGAND program [21].

3. Results

Purified hIRedZip protein expressed in Lec8 cells and purified by affinity chromatography on a bis(BOC)-insulin affinity column ran as two overlapping peaks of ~750 kDa and 430 kDa, respectively, on a Superdex S200 gel filtration column, indicating the presence of aggregated material (not shown). The ratio of aggregated to non-aggregated material was approximately 2:1 in the pH 5.0 eluted material and 1:2 in the insulin-eluted protein. The aggregated material could be readily separated from the non-aggregated hIRedZip protein by ion-exchange chromatography on Resource Q (Fig. 1). Rechromatography on the Superdex S200 gel filtration column showed that peak 1 from the ion-exchange column contained the aggregates while the material in peak 2 contained hIRedZip which eluted at a similar position to the non-zippered hIRed [18,20]. SDS–PAGE of purified samples of hIRedZip and hIRed under reducing and non-reducing conditions confirmed that both proteins are disulfide-linked dimers comprised of α- and β′-chains of apparent masses M_r 120 kDa and 33–50 kDa, respectively.

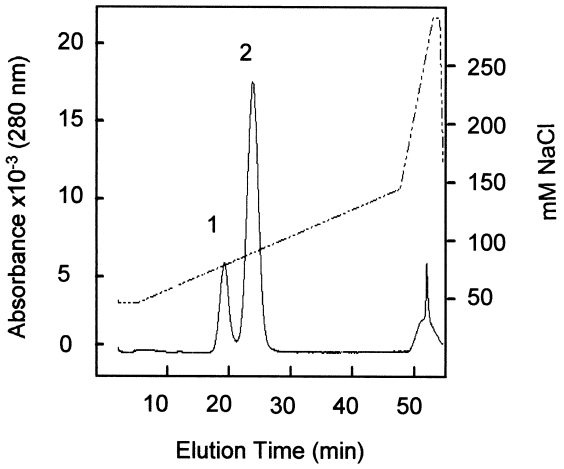

Fig. 1. Ion-exchange chromatography of affinity-purified hIRedZip. Protein obtained from the pH 5 elution as in Fig. 2 was loaded onto a Resource Q column (Pharmacia) fitted to a BioLogic L.C. system (Bio-Rad) in 20 mM Tris/pH 8.0 and eluted at 1 ml/min with a linear gradient of the same buffer against that containing 1 M NaCl as buffer B. Elution was monitored by absorbance (280 nm) and conductivity (mS/cm). Fractions eluting in peak 1 were concentrated and when this fraction was rerun over a Superdex S200 column, it eluted in the position of the ~430 kDa protein (not shown). Conversely, protein eluting in peak 2 on Resource Q was shown to correspond to protein of mass 750 kDa on S200 (not shown).

The insulin binding characteristics of zippered and non-zippered hIR ectodomains were investigated by competitive binding assays and compared with those of wild type whole receptor from CHO-T cells. Similar results were obtained with conditioned medium and highly purified protein samples produced by fermentation. The Scatchard transformations of the displacement curves are shown in Fig. 2. The non-zippered hIRed gave linear Scatchard plots and K_d of $1.1\pm0.1\times10^{-8}$ M (mean ± S.E.M.). In contrast, hIRedZip gave curvilinear Scatchard plots and K_d values of $8.2\pm1.5\times10^{-11}$ M and $1.6\pm0.2\times10^{-8}$ M for a two state fit similar to the values of $5.7\pm0.9\times10^{-11}$ M and $6.3\pm0.9\times10^{-9}$ M obtained for native whole IR (Fig. 2).

4. Discussion

In contrast to most receptor tyrosine kinases, where ligand binding induces dimerization and transphosphorylation [22], the IR already exists as a preformed, disulfide-linked dimer in its basal state. It is induced to undergo autophosphorylation following ligand binding by a process that is still poorly understood. Insulin binding to detergent-solubilized whole IR induces conformational changes (as assessed by a decrease in the Stokes radius from 9.1 nm to 7.5 nm and an increase in the sedimentation coefficient (S20.w) from 10.1 S to 11.4 S) which correlated with the onset of autophosphorylation of the kinase domain [23]. The 9.1 nm form of the $(\alpha\beta)^2$ IR is restored following dissociation of the ligand without reduction in the kinase activity of the now autophosphorylated cytoplas-

mic domains. In contrast, solubilized αβ half-receptors do not show altered conformation on binding insulin nor is their kinase activity induced [23]. Similarly, there are marked differences in the insulin binding characteristics of the $(\alpha\beta)^2$ dimer versus the αβ monomer. The $(\alpha\beta)^2$ dimer displays complex binding characteristics including curvilinear Scatchard plots, indicative of two binding states (high and low affinity) and negative cooperativity [7,23]. In contrast, the αβ half-receptors exhibit linear Scatchard plots consistent with a simple one-class insulin binding state which has relatively low affinity [23]. Collectively, these data indicate that interaction between the two αβ monomers is required for the generation of high affinity binding and ligand-induced signal transduction.

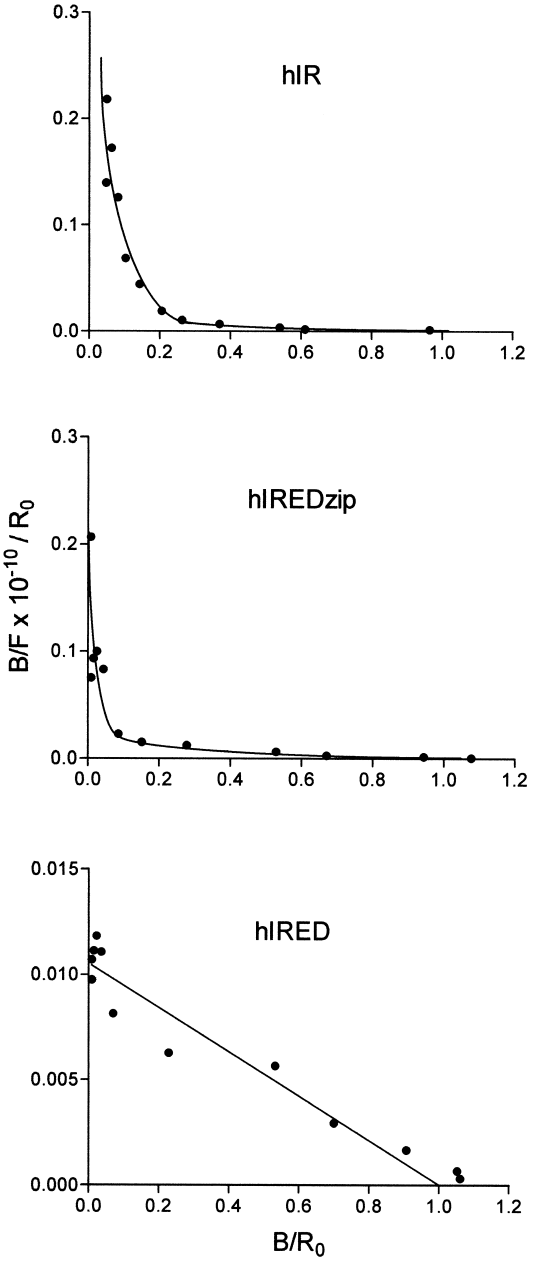

Fig. 2. Insulin binding to IR and IR ectodomain variants. Detergent lysates of CHO-T cells or culture supernatants from cells expressing hIRed and hIRedZip were incubated in microtiter plates precoated with IR specific monoclonal antibody 83-14. [^{125}I]Tyr A14 insulin and varying concentrations of unlabelled insulin were added to wells containing immobilized receptors. After thorough washing, radioactivity remaining in each well was determined and the data obtained were analyzed using the LIGAND program and displayed using the method of Scatchard (plots shown are representative of at least six analyses). To assist comparison, both bound ligand (B) and bound/free ligand (B/F) have been divided by R_0 (the total receptor content of each receptor preparation as determined by analysis of data using the LIGAND program).

Although ectodomain constructs are $(\alpha\beta')^2$ dimers, they resemble the αβ half-receptors in their binding characteristics and do not show high affinity binding [8]. In contrast, IR constructs which terminate after the transmembrane domain [11], or IR ectodomain constructs with either the Fc or λ immunoglobulin domains [12] attached to the C-terminus, exhibit curvilinear Scatchard plots similar to those found with the wild type receptor. In this report, we have shown that the leucine zipper sequence from the yeast transcriptional activator protein GCN4 can be used to regenerate the high affinity binding characteristics of whole receptor.

A common factor in all of these constructs is the presence of a 'tethering' dimerization domain (or a membrane-embedded α-helix) whose function appears to be to bring the C-termini of the adjacent monomer chains into close proximity. This intimate association appears to be a necessary precondition for both high affinity insulin binding and negative cooperativity to occur.

The molecular basis of the negative cooperativity observed with the IR [7] is still unclear but some insights into this phenomenon can be obtained from the *Salmonella typhimurium* aspartate receptor whose three-dimensional (3D) structure has been solved [24,25]. This receptor, like the IR, is a transmembrane protein that undergoes conformational change on binding ligand, resulting in the activation of a protein kinase (CheA) leading to signal transduction [26]. The aspartate receptor, like the IR, is a homodimer with two identical binding sites at the dimer interface. In the ligand/receptor complex, only one site is occupied by aspartate, which makes contacts with residues from both monomers. The effect of aspartate binding is to bring the two monomers slightly closer together and to make the second (empty) site more crowded so that it cannot accommodate ligand [24,25].

The aspartate receptor may serve as a good model for negative cooperativity in the IR family. The non-zippered hIR ectodomain has two identical binding sites and binds two insulin molecules with equal affinity that is 20 times lower than the high affinity of the whole receptor [8]. The whole IR $(\alpha\beta)_2$ dimer, like the aspartate receptor, binds only one ligand molecule with high affinity. A cross-linking model has been proposed [7,27] to explain this behavior where, in the high affinity state, insulin makes contact with distinct regions of the two monomers in the IR dimer. Negative cooperativity follows if the receptor dimer has internal symmetry [7] and binding of ligand in one site causes conformational change that precludes ligand binding at the second site.

Structural support for internal symmetry has come from electron microscopy studies that show the IR dimer has 2-fold symmetry with the 2-fold axis normal to the membrane surface [18,28]. The 3D structure of the L1/Cys-rich/L2 domains of the insulin-like growth factor-1 receptor reveals that these three domains, which contain many of the determinants of ligand binding, surround a central space of sufficient size to accommodate a ligand molecule [6]. It is tempting to speculate that, as for the aspartate receptor, ligand binding at one site

leads to contacts with key residues on both IR monomers. These contacts induce a subtle re-arrangement in the orientation of the two monomers where the corresponding contact residues in the unoccupied binding site crowd each other in such a way that a ligand molecule can no longer be accommodated at that second site. Direct structural information is required to address this point and the leucine zippered ectodomain fragment described here, or the Fc or λ constructs [12] are potential candidates for crystal production and structural analysis of high affinity receptor/ligand complexes.

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References

[1] Schaffer, L. and Ljungqvist, L. (1992) Biochem. Biophys. Res. Commun. 189, 650–653.
[2] Lu, K. and Guidotti, G. (1996) Mol. Biol. Cell 7, 679–691.
[3] Sparrow, L.G., McKern, N.M., Gorman, J.J., Strike, P.M., Robinson, C.P., Bentley, J.D. and Ward, C.W. (1997) J. Biol. Chem. 272, 29460–29467.
[4] Mynarcik, D.C., Yu, G.Q. and Whittaker, J. (1996) J. Biol. Chem. 271, 2439–2442.
[5] Kristensen, C., Wiberg, F.C., Schaffer, L. and Andersen, A.S. (1998) J. Biol. Chem. 273, 17780–17786.
[6] Garrett, T.P.J., McKern, N.M., Lou, M., Frenkel, M.J., Bentley, J.D., Lovrecz, G.O., Elleman, T.C., Cosgrove, L.J. and Ward, C.W. (1998) Nature 394, 395–399.
[7] De Meyts, P. (1994) Diabetologia 37, 135–148.
[8] Markussen, J., Halstrom, J., Wiberg, F.C. and Schaffer, L. (1991) J. Biol. Chem. 266, 18814–18818.
[9] Yip, C.C. and Jack, E. (1992) J. Biol. Chem. 267, 13131–13134.
[10] Schaffer, L. (1994) Eur. J. Biochem. 221, 1127–1132.
[11] Whittaker, J., Garcia, P., Yu, G.Q. and Mynarcik, D.C. (1994) Mol. Endocrinol. 8, 1521–1527.
[12] Bass, J., Kurose, T., Pashmforoush, M. and Steiner, D.F. (1996) J. Biol. Chem. 271, 19367–19375.
[13] O'Shea, E.K., Klemm, J.D., Kim, P.S. and Alber, T. (1991) Science 254, 539–544.
[14] Wu, Z., Johnson, K.W., Goldstein, B., Choi, Y., Eaton, S.F., Laue, T.M. and Ciardelli, T.L. (1995) J. Biol. Chem. 270, 16039–16044.
[15] Liu, J., Tse, A.G.D., Chang, H.-C., Liu, J.-H., Wang, J., Hussey, R.E., Chishti, Y., Rheinhold, B., Spoer, R., Nathenson, S.G., Sacchettini, J.C. and Reinherz, E.L. (1996) J. Biol. Chem. 271, 33639–33646.
[16] Ellis, L., Clauser, E., Morgan, D.O., Edery, M., Roth, R.A. and Rutter, W.J. (1986) Cell 45, 721–732.
[17] Ullrich, A., Bell, J.R., Chen, E.Y., Herrera, R., Petruzelli, L.M., Dull, T.J., Gray, A., Coussens, L., Liao, Y.-C., Tsubokawa, M., Mason, A., Seeburg, P.H., Grunfeld, C., Rosen, O.M. and Ramachandran, J. (1985) Nature 313, 756–761.
[18] Tulloch, P.A., Lawrence, L.J., McKern, N.M., Robinson, C.P., Bentley, J.D., Cosgrove, L., Ivancic, N., Lovrecz, G.O., Siddle, K. and Ward, C.W. (1999) J. Struct. Biol. 125, 11–18.
[19] Elleman, T.C., Frenkel, M.J., Hoyne, P.A., McKern, N.M., Cosgrove, L., Hewish, D., Jachno, K.M., Bentley, J.D., Sankovitch, S.E. and Ward, C.W. (2000) Biochem. J. 347, 771–779.
[20] Cosgrove, L., Lovrecz, G.O., Verkuylen, A., Cavaleri, L., Black, L.A., Bentley, J.D., Howlett, G.J., Gray, P.P., Ward, C.W. and McKern, N.M. (1995) Protein Expr. Purif. 6, 789–798.
[21] Munson, D. and Rodbard, P.J. (1980) Anal. Biochem. 107, 220–239.
[22] Hunter, T. (1997) Cell 88, 333–346.
[23] Florke, R.-R., Klein, H.W. and Reinauer, H. (1990) Eur. J. Biochem. 191, 473–482.
[24] Milburn, M.V., Prive, G.G., Milligan, D.L., Scott, W.G., Yeh, J., Jancarik, J., Koshland, D.E. and Kim, S.-H. (1991) Science 254, 1342–1347.
[25] Koshland, D.E. (1996) Curr. Opin. Struct. Biol. 6, 757–761.
[26] Blair, D.F. (1995) Annu. Rev. Microbiol. 49, 489–522.
[27] Schaffer, L., Kjeldsen, T., Andersen, A.S., Wiberg, F.C., Larsen, U.D., Cara, J.F., Mirmira, R.G., Nakagawa, S.H. and Tager, H.S. (1993) J. Biol. Chem. 268, 3044–3047.
[28] Luo, R.Z.T., Beniac, D.R., Fernandes, A., Yip, C.C. and Ottensmeyer, F.P. (1999) Science 285, 1077–1080.