Deletion Analysis of the Human Insulin Receptor Ectodomain Reveals Independently Folded Soluble Subdomains and Insulin Binding by a Monomeric α-Subunit*

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A series of 13 deletions within the extracellular domain of the human insulin receptor delineates the boundaries of subdomains that fold de novo into stable proteins that are efficiently secreted and retain the epitopes required for interaction with two conformation-specific monoclonal antibodies. While most of these proteins fail to bind insulin, a truncation that includes only the α-subunit is secreted as a monomer that binds the hormone with an affinity only slightly less than that of the complete heterotetrameric extracellular domain. These results thus demarcate landmarks within the primary sequence which will now guide further analysis of the structure and function of this complex domain of the receptor.

The polypeptide hormone insulin elicits a variety of physiological responses in cells of target tissues. The first step in the pathway of insulin action is the binding of insulin to its cell surface receptor, an integral transmembrane glycoprotein (for review see Rosen (1987)). The insulin receptor (IR) is synthesized as a single polypeptide chain precursor (αβ) comprised of a large extracellular domain (all of the α-subunit (735 amino acids) and ~5/6 of the β-subunit (194 amino acids)), a single transmembrane domain (23 amino acids), and a cytoplasmic domain (the carboxyl-terminal ~5/6 of the β-subunit (403 amino acids)) with the features of a protein-tyrosine kinase (Ebina et al., 1985; Ulrich et al., 1985). Consistent with this deduced topology, the extracellular and cytoplasmic domains of the IR have been independently expressed as soluble proteins that are capable of autonomous function: a secreted high-affinity insulin binding protein and a cytoplasmic protein-tyrosine kinase domain, respectively (Ellis et al., 1987, 1988a, 1988b; Herrera et al., 1988; Johnson et al., 1988; Whittaker and Okamoto, 1988; Cobb et al., 1989; Sissom and Ellis, 1989). The IR precursor undergoes the addition of high mannose oligosaccharide chains, disulfide isomerization, and dimerization within the endoplasmic reticulum. Dimerization is believed to be required for export from this cellular compartment to the Golgi. Proteolytic cleavage of the precursor into α- and β-subunits and the maturation of carbohydrate chains occur within the Golgi to yield the wild-type (αβ), heterotetrameric receptor that is transported to the plasma membrane. Insulin binding is first observed for the monomeric αβ precursor in the endoplasmic reticulum (Olson et al., 1988). While insulin can be covalently cross-linked to the α-subunit, thus demonstrating the involvement of this polypeptide chain in ligand binding, the structural and functional organization of the extracellular domain in toto is rather obscure and decidedly complex; each αβ half-receptor has 4 cysteine residues and 16 potential N-linked glycosylation sites (Ebina et al., 1985; Ulrich et al., 1985). To begin to explore the organization of this complex domain of the receptor, we have engineered and expressed in a heterologous cell system a series of truncated human IR ectodomains. The efficiency of secretion, stability, and interaction of each of both insulin and a panel of monoclonal antibodies directed against eight distinct epitopes of the IR extracellular domain were assessed. These results demarcate the boundaries of soluble subdomains that fold de novo into stable secreted proteins. Furthermore, a truncation that includes only the α-subunit results in secretion of a monomeric protein that binds insulin with an affinity only slightly less than that of the secreted intact extracellular domain.

MATERIALS AND METHODS

Construction and Expression of hIR Deletion Mutants—All manipulations of DNA were according to standard procedures (Sambrook et al., 1989). Enzymes were from New England BioLabs or Boehringer Mannheim. All other reagents were reagent grade or better. Insulin receptor residues are numbered as described (Ebina et al., 1985). The recombinant plasmid pHeIR01, with a 2983-base pair cDNA fragment that encodes all but eight amino acids of the hIR extracellular domain (Ellis et al., 1988b), was used to construct expression plasmids for 12 additional deletion mutants of the hIR ectodomain. Plasmid pHeIR01 was digested with one of the enzymes listed in Fig. 1 and Table I, and 3' blunt ends were generated as described (Ellis et al., 1988). A SaI site at the 5' end of each construct was derived from the vector polylinker. The resulting DNA fragments were purified by electrophoresis in low gelling temperature agarose (SeaPlaque, FMC, Rockland, ME) and cloned into the 5'-Sal1 and 3'-XbaI (blunt) sites of the polylinker. The resulting DNA fragments were transfected into COS cells by lipofection with a calcium phosphate precipitate (DeMay and Endo, 1987). The resulting COS cells were maintained at 37 °C in an atmosphere of 5% CO2 and 95% air in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 100 units/ml penicillin G, 100 μg/ml
strepptomycin, and 10 mM Hepes (pH 7.4). Cells were transfected with supercoiled plasmid DNAs as described (Gierman, 1981). With this protocol (and without subculturing) we obtained high level expression of hIR proteins for up to 2 weeks. All short term experiments were done within 24 h of transfection.

Purification of Truncated hIR Proteins and Insulin Binding Assays—The metabolic labeling of cultured cells and the immunoprecipitation of hIR proteins with one of a panel of mouse monoclonal antibodies specific for the hIR (Soos et al., 1986) were as described (Ellis et al., 1986). Immunoprecipitated hIR proteins were analyzed by SDS-PAGE (Laemmli, 1970) on 5–15% gradient gels (1.0 mm) with an acrylamide:bisacrylamide ratio of 37.5:1. Molecular masses of standard proteins (Bio-Rad) are myosin (200 kDa), β-galactosidase (116 kDa) and phosphorylase a (97 kDa), bovine serum albumin (66 kDa), and ovalbumin (43 kDa). Fluorography was performed at −70°C using Kodak X-Omat AR film. The degree of recognition of the truncated hIR proteins by each monoclonal antibody (Table II) was estimated by scanning the fluorogram signals using a EC910 densitometer (Fisher). Transfected COS cells were maintained in 150-cm² flasks and inouated with serum-free growth medium. Conditioned medium was concentrated using a ultrafiltration stir cell apparatus (Amicon Corp.), and the concentrated hIR proteins were eluted from a wheat germ agglutinin (WGA)-agarose column with 50 mM Tris (pH 7.5), 150 mM NaCl, 5 mM KCl, 0.02% NaN₃, and 0.6 M N-acetylglucosamine. The peak of insulin binding activity eluted from the WGA-agarose column was chromatographed on a Superose-12 fast protein liquid chromatography gel filtration column (Pharmacia LKB Biotechnology Inc.) equilibrated with phosphate-buffered saline (pH 7.4). Molecular masses of standard proteins (Pharmacia or Boehringer Mannheim) are blue dextran (2000 kDa), thyroglobulin (669 kDa), ferritin (450 kDa), catalase (240 kDa), aldolase (158 kDa), bovine serum albumin (68 kDa), ovalbumin (45 kDa), and chymotrypsinogen A (25 kDa). The binding of 125I-insulin (monomeric porcine insulin (80–120 μCi/μg) was from Du Pont-New England Nuclear; unlabeled porcine insulin was from Lilly) to receptors immobilized by anti-IR monoclonal antibodies on microtiter plates was performed as described (Morgan and Roth, 1986; Ellis et al., 1986b; Sissom and Ellis, 1989). Insulin binding assays were conducted at 4°C with continuous rocking for at least 4 h, conditions sufficient to reach equilibrium. Nonspecific binding of 125I-insulin, determined in the presence of 1 μM unlabeled insulin, was always less than 2% of the total insulin binding. The autoradiogram data were analyzed with the computer program LIGAND (Munson and Rodbard, 1980) using initial parameter estimates determined by the program EBDA (McPherson, 1983). Cross-linked (with dithiobis(succinimidyl) carbonate) of 125I-insulin to hIR proteins that had been immunoprecipitated by an anti-IR monoclonal antibody was as described (Ellis et al., 1986). Cross-linked material was analyzed on 3–10% polyacrylamide gradient gels (1.0 mm) using an acrylamide:bisacrylamide ratio of 100:1. Autoradiography was performed at −70°C using Kodak X-Omat AR film and Du Pont Cronex Lightning Plus screens.

RESULTS AND DISCUSSION

The Extent of Truncation Dramatically Influences the Efficiency of Secretion—The extracellular domain of the hIR is comprised of all of the α-subunit (735 amino acids) and ∼1/3 of the β-subunit (194 amino acids, designated βo) (Fig. 1). A deletion mutant that is truncated eight amino acids from the COOH terminus (IR921 to IR883) is secreted from the membrane to the medium, with a recovery of up to 95% (Table I). The truncated hIR proteins (Table I) were readily detected by this antibody. While the intact ectodomain (IR921) is efficiently secreted, truncation within the extracellular domain of the β-subunit results in the intracellular accumulation of the expressed proteins (Fig. 2). Thus, a reduction in size from 921 residues (IR921) to 883 residues (IR883), with the concomitant deletion of a single cysteine residue (Cys-884) is within βo, results in a nearly complete loss of secretion, although the protein is reasonably stable within the cell. Furthermore, longer exposures of the autoradiogram of Fig. 2 reveal that a small amount of the IR883 protein is secreted into the medium as a truncated hIR precursor (data not shown). Further truncation to 794 residues (IR794), with the concomitant deletion of the three remaining cysteine residues (Cys-798, -807, and -872) within βo, results in a complete loss of secretion and the rapid intracellular degra-

![FIG. 1. Linear representation of the human insulin receptor. The indicated restriction enzymes were used to create deletions within the extracellular domain. α, α-subunit; β, β-subunit; βo, extracellular portion of the β-subunit. Numbering refers to amino acids in the deduced primary sequence of the insulin receptor (Ehina et al., 1985). Restriction enzyme abbreviations used are: A, AatII; A, AalII; Bg, BglII; B, BamI; B, BstXI; E, EcoRI; P, PstI; P, PstI; S, SstI; T, TthlllI; X, XmnI.](image-url)
Subdomains of the Insulin Receptor Ectodomain

**FIG. 2.** Pulse-chase analysis of truncated forms of the human insulin receptor extracellular domain expressed by transfected and metabolically labeled COS cells. COS cells were transfected and metabolically labeled for 20 min and chased for the times indicated. Non-ionic detergent (Triton X-100) extracts of cells with monoclonal antibody 83.7. Proteins were resolved on 5-15% SDS-polyacrylamide gels and visualized by fluorography. While a proteolytically processed truncated β-subunit (M, ~43,000) is observed for the IR921 construct, such processing is not observed with further truncations within the β-subunit (the IR883 and IR794 constructs, data not shown). Molecular masses (kilodaltons) of standard proteins are indicated.

**FIG. 3.** Pulse-chase analysis of truncated forms of the human insulin receptor extracellular domain expressed by transfected and metabolically labeled COS cells. Immunoprecipitated proteins were analyzed as described in the legend of Fig. 2. Note that the labeled IR728, IR682, and IR657 proteins (but not the IR883 or IR794 proteins) do accumulate in the medium when transfected cells are continuously labeled for 24 h; thus these slowly secreted proteins are reasonably stable (data not shown).
Transfection of COS cells and immunoprecipitation of metabolically labeled truncated forms of the human insulin receptor extracellular domain were performed as described under "Materials and Methods." Proteins immunoprecipitated from non-ionic detergent (Triton X-100) extracts of cells (E) or conditioned medium were resolved on 5-15% SDS-polyacrylamide gels and visualized by fluorography. The degree of recognition by each monoclonal antibody is ranked relative to the signal obtained with monoclonal antibody 83.7, which was set at 100%. Results are presented as strong (+, 67-100%), moderate (m, 34-66%), weak (w, 1-33%), or negative (−).

sensitive to truncation within the carboxyl terminus of the α- or β-subunits (Table II, antibodies 25.49 and 47.9, respectively), as both sites are quite distant from amino acid residues within the primary sequence that have been suggested recently as insulin attachment sites (Yip et al., 1988; Wedekind et al., 1989; see below).

**An Intact α-Subunit Binds Insulin with Near Wild-type Affinity**—To assess the interaction of the truncated hIR proteins with insulin, we utilized a solid-phase assay in which receptor proteins are first immunopurified with monoclonal antibody 83.7 (cf. Morgan and Roth, 1986; Ellis et al., 1988b; Sissom and Ellis, 1989). This antibody was chosen because it binds with high affinity to the receptor, does not interfere with insulin binding, and recognizes truncated hIR proteins as small as the IR296 construct. Using this assay, we have determined that the truncated extracellular domain (IR921) binds insulin with an affinity that is indistinguishable from that obtained for the wild-type receptor (Ellis et al., 1988b; Sissom and Ellis, 1989). In the present study, we find that in addition to the high level of binding observed with the IR921 protein, only the IR883 and IR728 proteins exhibit significant interaction with insulin (Table III). The binding of insulin to the IR728 protein is particularly striking because the folding of this construct to constitute a stable protein that binds insulin de novo rather than via folding of an αβ precursor as is the case for the AATIIp protein and the wild-type receptor. As expected, given the extent of the deletion, the IR728 protein is recognized in this solid-phase insulin binding assay by a subset of the panel of monoclonal antibodies (cf. IR728 versus IR921 in Fig. 4). The concentration of unlabeled insulin required for half-maximal displacement of 125I-insulin is only slightly greater (<3-fold) for the IR728 protein than it is for the IR921 protein (Fig. 5). Thus, the α-subunit can, in fact, fold de novo to constitute a stable protein which binds insulin with near wild-type affinity. The fact that a previous study failed to detect an independently synthesized α-subunit, therefore, is not due to its inherent instability (Johnson et al., 1988); rather, it is likely due to the use of antibodies that do not recognize such a truncated receptor.

**The Secreted α-Subunit Is a Monomer**—Previous analysis of the extracellular and cytoplasmic domains of the hIR, when synthesized independently as soluble proteins, has established that the propensity of the two αβ half-receptors to form the...
To assess the oligomeric state of the independently folded α-subunit as synthesized via the IR728 construct, the IR728 protein was partially purified from medium conditioned by transfected COS cells by affinity chromatography on WGA-agarose (data not shown), and its size was assessed by Superose-12 (fast protein liquid chromatography) gel filtration chromatography. A single peak of insulin binding activity was observed, with an elution volume coincident with that of aldolase (M, 158,000, Fig. 6). Identical results were obtained when aliquots of these fractions were preadsorbed onto nitrocellulose and then incubated with monoclonal antibody 83.7 followed by a colloidal gold conjugated rabbit anti-mouse antibody (data not shown). These data are consistent with a monomeric IR728 protein. In contrast, the insulin binding peak of the IR921 protein elutes just ahead of ferritin (M, 440,000), which is consistent with the (αβδ)₄ heterotetrameric structure of this protein. To assess independently the oligomeric state of the IR728 protein, we covalently cross-linked ¹²⁵I-insulin to the IR728 protein purified by WGA-agarose and Superose-12 chromatography. Analysis of the cross-linked species following SDS-PAGE under nonreducing conditions illustrates that insulin indeed binds to a monomeric IR728 protein (Fig. 7, lane 2; in fact, all of the constructs smaller than the IR728 protein are monomeric proteins, as determined by analysis of metabolically labeled proteins by SDS-PAGE with nonreducing conditions (data not shown)). In contrast, the IR921 protein migrates as a high molecular weight oligomer (Fig. 7, lane 4). The apparent lower molecular weight of the secreted IR728 protein observed with nonreducing conditions (Fig. 7, lane 2), as compared with that observed with reducing conditions (Fig. 2, lanes 10 and 11), is a consistent difference observed with all of the truncated α-subunits, and this difference suggests a compact structure for these disulfide-rich proteins. Analysis of the insulin binding data in Fig. 5 by the method of Scatchard (1949; Munson and Rodbard, 1980) reveals that each protein possesses a single high-affinity binding site for insulin (data not shown) as we have observed previously for the IR921 protein expressed in either mammalian Chinese hamster ovary (Ellis et al., 1988b) or insect SF9 (Sissom and Ellis, 1989) cells. Linear Scatchard plots also have been observed for half-receptors generated from the wild-type hIR by preferential reduction of class I (between αβ half-receptors) but not class II (between αα- and ββ-subunits) disulfides, yet the observed affinity is much lower than that obtained with the intact heterotetrameric receptor (Böni-Schnetläer et al., 1987; Sweet et al., 1987). Our results with the IR728 protein demonstrate that oligomerization is not a prerequisite for high-affinity insulin binding.

**The IR Ectodomain Is Comprised of Independently Folded Subdomains**—Based on conserved amino acid residues and secondary structure predictions, a model for the supersecondary structure of the extracellular domains of the insulin and the epidermal growth factor/c-erb-B2 receptors has been proposed (Bajaj et al., 1987). The model predicts two independently folded subdomains, designated L1 and L2 (residues 1–119 and 116–200), respectively, on either side of the cysteine-rich domain (residues 155–310) and a rather featureless domain comprised of the carboxyl-terminal ~34% of the α-subunit and the extracellular portion of the β-subunit (residues 736–929). The IR486 protein, which terminates just after the proposed L2 domain and is secreted as a stable compact monomer, is consistent with such a model. The heterogeneity observed with the smaller IR425, IR352, and IR324 proteins may derive from disruption of the proposed compact structure of the L2 domain. The apparent lack of antigenicity of the L1 and L2 domains of the receptor is consistent with such a folded and compact structure for L1 and L2 (Prigent et al., 1990). Insulin contact sites on the receptor have been mapped to residues within the amino-terminal L1 (Wedekind et al., 1989) or cysteine-rich (Yip et al., 1988) domains. The present data suggest that these amino-proximal regions per se are insufficient to constitute the insulin binding site de novo and that carboxyl-terminal sequences (i.e. at least through residue 728) of the extracellular domain are required. The involvement in insulin binding of residues within the cysteine-rich domain and the carboxyl-terminal region of the α-subunit, as well as residues within the amino-terminal portion of the β-subunit, has been invoked based on sequence divergence between the deduced primary sequences of the receptors for insulin and insulin-like growth factor I (Ullrich et al., 1986). Involvement of sequences in both the αα- and ββ-subunits in insulin binding may allow ligand-induced conformational changes in the extracellular domain to facilitate transmembrane activation of the cytoplasmic protein-tyrosine kinase.

In summary, the present experimental results demarcate new structural landmarks within the IR extracellular domain which are not obvious from consideration of the deduced primary sequence of the receptor protein (Ebina et al., 1985; Ullrich et al., 1985) or the exon-intron organization of the receptor gene (Seino et al., 1989). Furthermore, these results suggest that sequences within the carboxyl-terminal region of the extracellular domain (the β-subunit) are critical for the processing, folding, and oligomerization of this complex domain and that these sequences are therefore necessary to establish the final tertiary and quaternary structure of the receptor.

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