# Changing the Insulin Receptor To Possess Insulin-like Growth Factor I Ligand Specificity

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ABSTRACT: To examine the role of the N-terminal part of the insulin-like growth factor I (IGF-I) receptor and insulin receptor in determining ligand specificity, we prepared an expression vector encoding a hybrid receptor where exon 1 (encoding the signal peptide and seven amino acids of the $\alpha$-subunit), exon 2, and exon 3 of the insulin receptor were replaced with the corresponding IGF-I receptor cDNA (938 nucleotides). To allow direct quantitative comparison of the binding capabilities of this hybrid receptor with those of the human IGF-I receptor and the insulin receptor, all three receptors were expressed in baby hamster kidney (BHK) cells as soluble molecules and partially purified before characterization. The hybrid IGF-I/insulin receptor bound IGF-I with an affinity comparable to that of the wild-type IGF-I receptor. In contrast, the hybrid receptor no longer displayed high-affinity binding of insulin. These results directly demonstrate that it is possible to change the specificity of the insulin receptor to that of the IGF-I receptor and, furthermore, that the binding specificity for IGF-I is encoded within the nucleotide sequence from 135 to 938 of the IGF-I receptor cDNA. Since the hybrid receptor only bound insulin with low affinity, the insulin binding region is likely to be located within exons 2 and 3 of the insulin receptor.

Insulin and IGF-I, like their respective receptors, display a high degree of homology in sequence and overall structural organization (Yarden & Ullrich, 1988; Hollenberg, 1985). Each ligand binds, but very weakly, to the receptor for the other [$K_A$ 2–3 orders of magnitude lower than for the cognate receptor (Czech, 1989)]. The recognition site is not known. Pointers come from cross-linking studies (Wedekind et al., 1989; Yip et al., 1988; Waugh et al., 1989) and observations made with anti-insulin receptor antibodies (Toyoshige et al., 1989), which suggest that bound insulin may be close to the N-terminal part of the insulin receptor $\alpha$-subunit. Furthermore, it has recently been shown, by use of mutated insulin receptors, that phenylalanine-89 is essential for full binding activity (de Meyts et al., 1990). The exon/intron structure of the gene for the human insulin receptor has been determined (Seino et al., 1989), and the identified interactions between insulin and the insulin receptor seem to reside within the region encoded by exons 2 and 3.

The use of hybrid receptors has been of value in identifying binding domains of hormone receptors. Thus, hybrids of the $\alpha_2$- and $\beta_2$-adrenergic receptors were used to locate a region conferring specificity for agonist and antagonist molecules in the seventh membrane-spanning domain (Kobilka et al., 1988). The same approach was used to identify a ligand binding domain in the EGF receptor with the aid of chicken/human EGF receptor hybrids (Lax et al., 1989).

We report here that the specificity of the insulin receptor can be changed at will by substituting a part of the IGF-I receptor corresponding to exons 2 and 3 into the insulin receptor sequence.

## Experimental Procedures

A full-length cDNA clone encoding the human insulin receptor was obtained by a combination of the Okayama and Berg (1983) cDNA cloning technique and primer extension, with use of poly(A+) RNA from the human lymphoblastoid IM9 cell line (ATCC No. CCL 159) stimulated with 1.4 $\mu$M cortisol for 20 h (Shibasaki et al., 1988). The truncated IR (sIR) cDNA was generated by insertion of the oligonucleotide duplex 5′-C CCG TCA AAT ATT GCA AAA TAA T-3′/ 3′-TG CAG GGC AGT TTA TAA CGT TTT ATT AGA TC-5′ at the *Aat*II site in position 2857, thereby introducing a termination codon followed by an *Xba*I site. DNA sequence analysis was performed by enzymatic chain termination (Sequenase, U.S. Biochemicals).

A cDNA clone encoding the soluble human IGF-I receptor was obtained by PCR.[1] First-strand cDNA was synthesized from 3.5 $\mu$g of poly(A+) RNA prepared from human term placenta with 60 units of AMV reverse transcriptase (Pharmacia), primed with specific oligonucleotides, and used as template for PCR amplification with the Gene Amp kit (Perkin-Elmer Cetus, Norwalk, CT) according to the manufacturer's instruction. The primers used for PCR contained convenient endonuclease restriction sites to allow cloning of the amplified cDNA. The oligonucleotide 3′-TGT CCT ATA CTT TTG ATT AGA TCT GAC TAG T-5′ was used to generate the 3′ cDNA of the truncated IGF-I receptor, thereby introducing a termination codon and an *Xba*I site. A similar approach was used to generate the 5′ cDNA *Bam*HI–*Eco*RI IGF-I receptor fragment equivalent to exons 1, 2, and 3 of the insulin receptor. The upstream primer 5′-C CAA ATA GGA TCC ATG AAG TCT GGC TCC GGA GG-3′ contained a *Bam*HI site followed by the initiation codon. The downstream primer 3′-CCC GAA GTA GGC CTT AAG GTC GGT CTC GT-5′ introduced an *Eco*RI site, allowing insertion directly into the insulin receptor. The *Bam*HI–*Eco*RI fragment also

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[1] Abbreviations: DMEM, Dulbecco's modified Eagle's medium; DSS, disuccinimidyl suberate; FCS, fetal calf serum; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PCR, polymerase chain reaction; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane.

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served as the 5′ end of the truncated IGF-I receptor. The amplified DNA was subcloned into appropriate vectors and subjected to sequence analysis as described above. The *Bam*HI and *Xba*I sites were eventually used for insertion of the truncated IGF-I receptor cDNA into the mammalian expression vector pZem 219b (kindly provided by E. Mulvihill, ZymoGenetics Inc., Seattle, WA). This vector also carried an expression unit for the dihydrofolate reductase (DHFR) gene to be used during selection. For expression of the soluble receptors in cultured BHK cells, the expression vectors were transfected into subconfluent cells by the calcium phosphate mediated transfection procedure (Graham & van der Eb, 1973) with subsequent glycerol shock (Scahill et al., 1983). Two days posttransfection, cells were trypsinized and diluted into selection medium containing 0.4–2.0 μM methothrexate. After 10–14 days, individual colonies were isolated and tested for insulin and IGF-I binding in the described assay.

Selected BHK colonies were grown to confluency in DMEM (Gibco) supplemented with 10% FCS (Gibco) and cultivated for 48 h in medium containing 2% FCS. Receptors secreted from the BHK cells were partially purified on a Mono Q column (HR 5/5, Pharmacia) followed by gel filtration on a Superose 6 column (HR 10/30, Pharmacia). Briefly, 10–15 mL of medium was diluted with 1 volume of 20 mM Tris-HCl (pH 8.0) and applied to the column. Bound material was eluted with a gradient from 0 to 500 mM NaCl in 20 mM Tris-HCl (pH 8.0). Fractions containing the receptors (i.e., binding activity) were subsequently applied on a Superose 6 column running in 25 mM HEPES (pH 8.0)–100 mM NaCl at 0.25 mL/min, and fractions containing binding activity were used directly for cross-linking. Fifty microliters was incubated with 0.05 μCi of $^{125}$I-insulin ($A_{14}$-monoiodinated, Novo Nordisk) (sIR) or $^{125}$I-IGF-I (Amersham) (sIGF-I-R, sIR 23) for 90 min at room temperature and cross-linked with DSS (Pierce) as described (Waugh et al., 1989) in the absence or presence of 10 μg/mL insulin (Novo Nordisk) (sIR) or 2 μg/mL IGF-I (Amersham) (sIGF-I-R, sIR 23).

Fractions from the Superose 6 column that upon cross-linking with ligand gave rise to an SDS-PAGE band at 340 kDa under nonreducing and at 125 kDa under reducing conditions were pooled and used in the binding assays. Fifty microliters was incubated with 25 000 cpm of $^{125}$I-IGF-I or $^{125}$I-insulin and various levels of unlabeled ligand in 100 mM HEPES (pH 8.0), 100 mM NaCl, 10 mM $MgCl_2$, and 0.5% bovine serum albumin for 16 h at 4 °C, followed by precipitation with 0.2% bovine γ-globulin (Sigma) and 17.5% PEG 8000 (Sigma), and the radioactivity in the pellet was counted (Marshall et al., 1985).

## Results and Discussion

The insulin receptor cDNA was isolated from an Okayama and Berg cDNA library generated with mRNA isolated from the human lymphoblastoid cell line IM9 (Figure 1A). A soluble insulin receptor cDNA of 2835 base pairs encoding 944 amino acids, including the signal peptide, was created by insertion of a stop codon at amino acid position 918, the first codon of the transmembrane region (Figure 1A). The IGF-I receptor ectodomain cDNA sequence of 2799 base pairs was generated with PCR (Figure 1A), placing a termination codon at amino acid position 903. The resulting soluble IGF-I receptor cDNA encodes a total of 932 amino acids including the signal peptide. The hybrid receptor cDNA (sIR 23) of 2811 nucleotides encodes 936 amino acids, including the signal peptide, of which the N-terminal 313 amino acids are derived from the IGF-I receptor (Figure 1A). The hybrid receptor was assembled in the *Eco*RI site located 12 base pairs upstream from the junction between exon 3 and exon 4 of the insulin receptor cDNA (Figure 1B). This results in the retention of four amino acids from insulin receptor exon 3 in the hybrid receptor; two of these, asparagine-284 and serine-286, are identical in the IGF-I and insulin receptors (Figure 1B). The insulin receptor cDNA sequence was found to be identical with the sequence published by Ullrich et al. (1985), except at amino acid positions 861 and 862 (and 1239), where the sequence was identical with the published gene sequence (Seino et al., 1989). The nucleotide sequence of the IGF-I receptor cDNA and of the IGF-I receptor cDNA fragment inserted into the insulin receptor cDNA was identical with the sequence published by Ullrich et al. (1986).

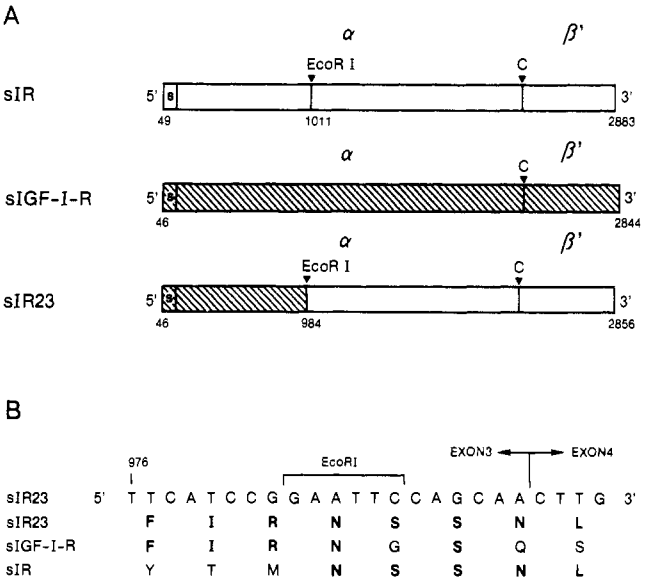


FIGURE 1: (A) Schematic presentation of the cDNA of the soluble insulin receptor (sIR), the soluble IGF-I receptor (sIGF-I-R), and the soluble insulin/IGF-I receptor hybrid (sIR 23). The signal peptide (S), proteolytic cleavage site (C), and α- and β′-subunit of the receptors are indicated. The first nucleotide of the initiation codon and the last nucleotide of the termination codon are indicated. The nucleotide numbering is according to Ullrich et al. (1985, 1986). (B) The nucleotide and amino acid sequence of the hybrid receptor (sIR 23) at the junction between the IGF-I receptor and the insulin receptor. The *Eco*RI site used for combination of the hybrid receptor is shown. The amino acid sequences derived from each of the receptors are indicated (single-letter amino acid code). The junction between exons 3 and 4 of the insulin receptor is indicated.

The cDNA constructs encoding the receptors were inserted into mammalian expression vector pZem 219b and transfected into BHK cells. The clones secreting soluble receptor were screened and selected for insulin binding activity in the medium or IGF-I binding activity in media fractionated by ion-exchange chromatography.

To avoid interference from IGF-I and IGF-I binding proteins present in the serum and secreted by the BHK cells (Blum et al., 1989), the soluble receptors were partially purified from media from transfected BHK cells, by ion-exchange chromatography and gel filtration. This procedure eliminated all IGF-I binding activity present in media from untransfected BHK control cells. The secreted receptors accumulated in the medium from transfected cells at levels of approximately 0.1–1 μg/mL.

Covalent cross-linking of iodinated ligands to the receptors (Figure 2B) revealed complexes migrating as 340-kDa bands in the absence of reducing agent; this corresponds to the tetrameric state of the receptors ($\alpha_2\beta'_2$), confirming the secretion of properly processed tetrameric soluble insulin receptors from transfected mammalian cells, as seen by others

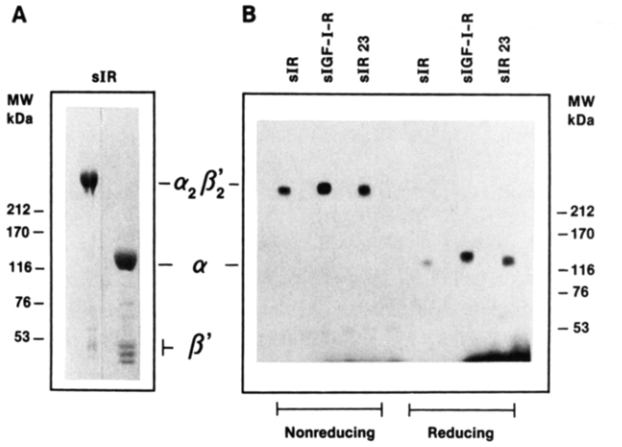


FIGURE 2: Electrophoretic mobility of the soluble receptors. (A) Coomassie-stained 3–10% SDS–PAGE of highly purified sIR: nonreduced (left lane); reduced (right lane). (B) Autoradiograph of 3–10% SDS–PAGE showing the receptors covalently cross-linked with DSS to their respective iodinated ligand: sIR cross-linked to $^{125}$I-insulin with or without 10 $\mu$g/mL insulin; sIGF-I-R and sIR 23 cross-linked to $^{125}$I-IGF-1 with or without 2 $\mu$g/mL IGF-I.

Table I: $K_D$ (M) of Receptors[a]

| | insulin | IGF-I |
|---|---|---|
| sIR | $5.0 \times 10^{-9}$ | $5.2 \times 10^{-7}$ |
| sIGF-I-R | $3.8 \times 10^{-7}$ | $4.0 \times 10^{-10}$ |
| sIR 23 | $6.0 \times 10^{-7}$ | $8.1 \times 10^{-10}$ |

[a] Dissociation constants ($K_D$) for insulin and IGF-I (M). Determined by Scatchard (1949) analysis from displacement curves of $^{125}$I-insulin and $^{125}$I-IGF-I either by insulin or by IGF-I obtained as described in the legend to Figure 3.

(Whittaker & Okamoto, 1988; Ellis et al., 1988). In the presence of reducing agents, the $\alpha$-subunit band of approximately 125 kDa appeared as expected, that of the IGF-I receptor migrating slightly slower than those of both the insulin and the hybrid receptors. For comparison, a highly purified preparation of sIR was shown to give rise to a similar pattern after SDS–PAGE and Coomassie staining (Figure 2A) [details of the purification procedure to be published elsewhere (A. S. Andersen et al.)]. Furthermore, the soluble IGF-I receptor and the hybrid receptor both had elution volumes on gel filtration that were similar to that of the highly purified soluble insulin receptor (results not shown).

The relative affinities of the soluble receptors were determined by competition assays (Figure 3). For standardized concentrations of receptors and labeled ligand, the concentration of unlabeled insulin required to half-maximally inhibit ($IC_{50}$) the binding of labeled insulin to sIR was 8 nM and of labeled IGF-I binding to sIGF-I-R and sIR 23 was 200 and 600 nM, respectively. Conversely, the $IC_{50}$ for IGF-I were 800 nM for labeled insulin binding to sIR and 0.3 and 0.6 nM for labeled IGF-I binding to sIGF-I-R and sIR 23. Scatchard analysis (Scatchard, 1949) of these data revealed linear Scatchard plots, agreeing with results obtained on a truncated insulin receptor secreted from transfected CHO cells (Ellis et al., 1988). Dissociation constants ($K_D$) derived from these plots are shown in Table I. Thus, in comparison with sIR, the insulin/IGF-I receptor hybrid, sIR 23, displays a substantial increase in IGF-I binding affinity, with a concomitant decrease in the affinity for insulin. The measured IGF-I binding affinity is not due to contaminating IGF-I binding proteins because the binding could be displaced by unlabeled insulin (Martin & Baxter, 1986; Bar et al., 1989), and only bands corresponding to receptors were apparent after SDS–PAGE upon cross-linking with the ligand (Figure 3).

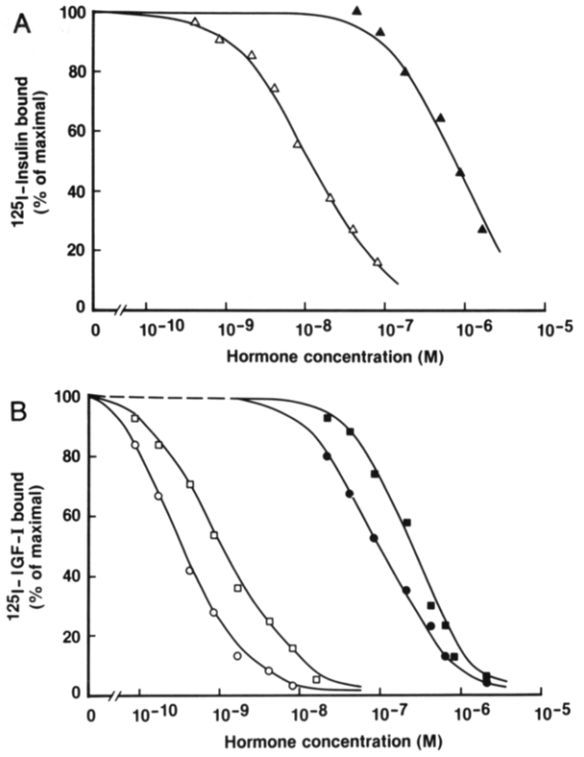


FIGURE 3: Competition curves for the soluble receptors. (A) $^{125}$I-Insulin binding to sIR: competition with unlabeled IGF-I (▲) and insulin (△). (B) $^{125}$I-IGF-I binding to sIGF-I-R (○, ●) and sIR 23 (□, ■): competition with unlabeled IGF-I (open symbols) and insulin (filled symbols).

Our results establish that the region encoded within the IGF-I receptor cDNA nucleotide sequence 135–983—corresponding to exons 2 and 3 of the insulin receptor DNA sequence—is essential for IGF-I binding. Since the hybrid receptor binds IGF-I with an affinity similar to that of wild-type IGF-I receptor, it must have retained the ability to enter the native conformation. The results further indicate that the ligand binding region of the insulin receptor lies within exon 2 and/or 3, since the hybrid receptor did not retain high affinity for insulin. The IGF-I receptor nucleotide sequence 135–983 encodes two different domains, the N-terminal domain and the cysteine-rich domain (Ullrich et al., 1986). The site defining the specificity of ligand recognition may thus contain elements of both domains, or one of the domains might alone fold to form the site conferring ligand specificity.

The insulin receptor and the IGF-I receptors have a number of similarities to the human epidermal growth factor (EGF) receptor (Yarden & Ullrich, 1988). All three contain regions rich in cysteine residues [exon 3 in the insulin receptor (Seino et al., 1989)]. The extracellular part of the EGF receptor contains two cysteine-rich domains, whereas the insulin and IGF-I receptor $\alpha$-subunits have only one (Yarden & Ullrich, 1988). The ligand binding region of the EGF receptor was reported to lie between the two cystene-rich domains (Lax et al., 1989). These observations and the results reported here imply that the loci of ligand specificity of both the IGF-I and the insulin receptors are to be found in the N-terminal domain.

Additional hybrid receptors are now being prepared with the aim of identifying the ligand binding site with higher resolution.

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# *Articles*

# Primary Structure of the Major Pepsin Inhibitor from the Intestinal Parasitic Nematode *Ascaris suum*†


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ABSTRACT: The major pepsin inhibitor from *Ascaris suum* was isolated by affinity chromatography and chromatofocusing. Its amino acid sequence was determined by automated Edman degradation of peptide fragments. Peptides were produced by chemical and enzymatic cleavage of pyridylethylated protein and were purified by reverse-phase high-performance liquid chromatography. The inhibitor consists of 149 residues with the following sequence: QFLFSMSTGP$^{10}$FICTVKDNQV$^{20}$FVANLPWTML$^{30}$EGDDIQVGKE$^{40}$-FAARVEDCTN$^{50}$VKHDMAPTCT$^{60}$KPPPFCGPQD$^{70}$MKMFNFVGCS$^{80}$VLGNKLFIDQ$^{90}$KYVR-DLTAKD$^{100}$HAEVQTFREK$^{110}$IAAFEEQQEN$^{120}$QPPSSGMPHG$^{130}$AVPAGGLSPP$^{140}$PPPSFCTVQ$^{149}$. It has a molecular weight of 16 396. All cysteines are engaged as disulfide bonds: Cys(13)–Cys(59), Cys(48)–Cys(66), and Cys(79)–Cys(146). The protein is probably composed of two domains connected by a short hydrophobic region. This is the first aspartyl protease inhibitor of animal origin that has been sequenced. The sequence has no significant homology with any other known protein.


One-fourth of the world's population and virtually all pigs are infected by the intestinal endoparasitic nematode *Ascaris* (Muller, 1979). This constitutes an important medical, agricultural, and economic problem (Levine, 1980; Mahmoud, 1989). Current control is by chemical antihelminthics directed at adult worms who are capable of propagating themselves by passing hundreds of thousands of eggs each day. As long as infected hosts live in an environment prone to fecal–oral contamination, reinfestation is imminent and chemical de-

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