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FOR THE RECORD

Crystallization of the first three domains of the human insulin-like growth factor-1 receptor

NEIL M. McKERN,[1] MEIZHEN LOU,[2] MAURICE J. FRENKEL,[1] AMANDA VERKUYLEN,[1] JOHN D. BENTLEY,[1] GEORGE O. LOVRECZ,[1] NEVA IVANCIC,[1] THOMAS C. ELLEMAN,[1] THOMAS P. J. GARRETT,[2] LEAH J. COSGROVE,[1,3] AND COLIN W. WARD[1]
[1]CSIRO Division of Molecular Science, 343 Royal Parade, Parkville, Victoria, 3052 Australia
[2]Biomolecular Research Institute, 343 Royal Parade, Parkville, Victoria, 3052, Australia

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Abstract: The insulin-like growth factor-1 receptor (IGF-1R) is a tyrosine kinase receptor of central importance in cell proliferation. A fragment (residues 1–462) comprising the L1-cysteine rich-L2 domains of the human IGF-1R ectodomain has been overexpressed in glycosylation-deficient Lec8 cells and has been affinity-purified via a c-myc tag followed by gel filtration. The fragment was recognized by two anti-IGF-1R monoclonal antibodies, 24–31 and 24–60, but showed no detectable binding of IGF-1 or IGF-2. Isocratic elution of IGF-1R/462 on anion-exchange chromatography reduced sample heterogeneity, permitting the production of crystals that diffracted to 2.6 Å resolution with cell dimensions a = 77.0 Å, b = 99.5 Å, c = 120.1 Å, and space group $P2_12_12_1$.

Keywords: crystallization; glycosylation; IGF-1 receptor; insulin receptor; Lec8 cells; purification; X-ray diffraction

The growth-promoting actions of the insulin-like growth factor-1 (IGF-1) and IGF-2 are mediated by the IGF-1 receptor (IGF-1R), a tyrosine kinase receptor whose sequence (Ullrich et al., 1986) and genomic structure (Abbott et al., 1992) are similar to those of the insulin receptor (IR). IR and IGF-1R are homodimers, composed of two extracellular α and two membrane-spanning β-chains, linked by disulphide bonds (Schäffer & Ljungqvist, 1992; Sparrow et al., 1997). Sequence analyses indicate that each monomer in the ectodomains of IGF-1R and IR consist of two homologous domains (L1 and L2) separated by a single cysteine-rich region (Bajaj et al., 1987; Ward et al., 1995), followed by a connecting region linking L2 to two fibronectin-type III domains, the first of which contains an insert domain that includes the α-β cleavage site (O'Bryan et al., 1991; Schaefer et al., 1992).

Although there have been numerous analytical and functional studies of ligand binding to IGF-1R and IR (see De Meyts, 1994), the mechanisms of ligand binding and subsequent transmembrane signalling have not been resolved. Crystal structures of the ectodomains of these receptors, together with the known 3D structure of the IR tyrosine kinase domain (Hubbard et al., 1994), should provide valuable data in this regard. However, several factors hamper macromolecular crystallization including sample selection, purity, stability, solubility (McPherson et al., 1995; Gilliland & Ladner, 1996), and the nature and extent of glycosylation (Davis et al., 1993). Initial attempts to obtain structural data from soluble IGF-1R ectodomain (residues 1–906) protein, expressed in Lec8 cells (Stanley, 1989) and purified by affinity chromatography, produced large, well-formed crystals (1.0 × 0.2 × 0.2 mm), which gave no discernable X-ray diffraction pattern (unpub. obs.). Similar difficulties have been encountered with crystals of the structurally related epidermal growth factor receptor (EGFR) ectodomain, which diffracted to only 6 Å, insufficient for the determination of an atomic resolution structure (Weber et al., 1994). This prompted us to search for a fragment of IGF-1R that was more amenable to X-ray crystallographic studies. The data reported here demonstrate the successful outcome of this research.

The fragment expressed (residues 1–462) comprises the L1-cysteine-rich-L2 region of the ectodomain. The selected truncation position at Val_{462} is four residues downstream of the exon 6/exon 7 junction (Abbott et al., 1992) and occurs at a position where the sequences of the IR and the structurally related EGFR families diverge markedly (Lax et al., 1988; Ward et al., 1995), suggesting it represents a domain boundary. The expression strategy included use of the pEE14 vector (Bebbington & Hentschel, 1987) in glycosidase-defective Lec8 cells (Stanley, 1989), which produce N-linked oligosaccharides lacking the terminal galactose and

Reprint requests to: Colin Ward, CSIRO, Division of Molecular Science, 343 Royal Parade, Parkville, Victoria, Australia, 3052; e-mail: colin.ward@molsci.csiro.au.
[3]Current address: CRC for Tissue Growth and Repair, Adelaide, South Australia, 5000, Australia

Abbreviations: DMEM, Dulbecco's modified Eagle medium; EGFR, epidermal growth factor receptor; ELISA; enzyme-linked immunosorbant assay; GMEM, Glasgow minimum essential medium; IGF-1, insulin-like growth factor-1; IGF-2, insulin-like growth factor-2; IGF-1R, insulin-like growth factor-1 receptor; IR, insulin receptor; Mab, monoclonal antibody; MSX, methionine sulfoximine; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TBSA, Tris-buffered saline containing 0.02% sodium azide.

N-acetylneuraminic acid residues (Davis et al., 1993; Liu et al., 1996). The construct contained a C-terminal c-myc affinity tag (Hoogenboom et al., 1991), which facilitated immunoaffinity purification by specific peptide elution and avoided aggressive purification conditions. These procedures yielded protein that readily crystallized after a gel filtration polish. This provided a general protocol to enhance crystallization prospects for labile, multidomain glycoproteins.

The structure of this fragment is of considerable interest because it contains the major determinants governing insulin and IGF-1 binding specificity (Andersen et al., 1990; Gustafson & Rutter, 1990; Schumacher et al., 1991; 1993; Schäffer et al., 1993; Williams et al., 1995) and is very similar to an IGF-1R fragment (residues 1–486) reported to act as a strong dominant negative for several growth functions and which induces apoptosis of tumor cells in vivo (D'Ambrosio et al., 1996).

Expression, purification, and characterization of IGF-1R/462: The expression plasmid pEE14/IGF-1R/462 was constructed by inserting the oligonucleotide cassette:

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   AatII
5' GACGTC  GACGATGACGATAAG  GAACAAAAACTCATC
    D V     D  D  D  D K      E  Q  K  L  I
           (EK cleavage)      ( c-myc tail
        S  E  E  D  L  N  Stop)
        TCAGAAGAGGATCTGAAT TAGAATTC GACGTC 3'
                           EcoRI   AatII
```

encoding an enterokinase cleavage site, c-myc epitope tag (Hoogenboom et al., 1991) and stop codon into the *Aat*II site (within codon 462) of IGF-1 receptor cDNA in the mammalian expression vector pECE (Ebina et al., 1985; generously supplied by W. J. Rutter, UCSF, USA), and introducing the DNA comprising the 5′ 1521 bp of the cDNA (Ullrich et al., 1986) ligated to the oligonucleotide cassette into the *Eco*RI site of the mammalian plasmid expression vector pEE14 (Bebbington & Hentschel, 1987; Celltech Ltd., UK). Plasmid pEE14/IGF-1R/462 was transfected into Lec8 mutant CHO cells (Stanley, 1989) obtained from the American Tissue Culture Collection (CRL:1737) using Lipofectin (Gibco-BRL). Cell lines were maintained after transfection in glutamine-free medium (Glascow modification of Eagle's medium (GMEM; ICN Biomedicals, Australia) and 10% dialyzed FCS (Sigma, Australia) containing 25 μM methionine sulphoximine (MSX; Sigma, Australia) as described (Bebbington & Hentschel, 1987). Transfectants were screened for protein expression by Western blotting and sandwich enzyme-linked immunosorbant assay (ELISA) (Cosgrove et al., 1995) using monoclonal antibody (Mab) 9E10 (Evan et al., 1985) as the capture antibody and either biotinylated anti-IGF-1R Mab 24-60 or 24–31 for detection (Soos et al., 1992; gifts from Ken Siddle, University of Cambridge, UK). Large-scale cultivation of selected clones expressing IGF-1R/462 was carried out in a Celligen Plus bioreactor (New Brunswick Scientific, USA) containing 70 g Fibra-Cel Disks (Sterilin, UK) as carriers in a 1.25 L working volume. Continuous perfusion culture using GMEM medium supplemented with non-essential amino acids, nucleosides, 25 μM MSX, and 10% FCS was maintained for one to two weeks followed by the more enriched DMEM/F12 without glutamine, with the same supplementation for the next four to five weeks. The fermentation production run was carried out three times under similar conditions and resulted in an estimated overall yield of 50 mg of receptor protein from 430 L of harvested medium. Cell growth was poor during the initial stages of the fermentation when GMEM medium was employed, but improved dramatically following the switch to the more enriched medium. Target protein productivity was essentially constant during the period from ~100 to 700 h of the 760-h fermentation, as measured by ELISA using Mab 9E10 as the capture antibody and biotinylated Mab 24–31 as the developing antibody.

Soluble IGF-1R/462 protein was recovered from harvested fermentation medium by affinity chromatography on columns prepared by coupling Mab 9E10 to divinyl sulphone-activated agarose beads (Mini Leak; Kem En Tec, Denmark) as recommended by the manufacturer. Mini-Leak Low- and Medium-affinity columns with antibody loadings of 1.5–4.5 mg/mL of hydrated matrix were obtained, with the loading range of 2.5–3 mg/mL giving optimal performance (data not shown). Mab 9E10 was produced by growing hybridoma cells (American Tissue Culture Collection) in serum-free medium in the Celligen Plus bioreactor and recovering the secreted antibody (4 g) using protein A glass beads (Prosep-A, Bioprocessing Limited, USA). Harvested culture medium containing IGF-1R/462 protein was adjusted to pH 8.0 with Tris-HCl (Sigma), made 0.02% (w/v) in sodium azide and passed at 3–5 mL/min over 50 mL Mab 9E10 antibody columns at 4 °C. Bound protein was recovered by recycling a solution of 2–10 mg of the undecamer c-myc peptide EQKLISEEDLN (Hoogenboom et al., 1991) in 20 mL of Tris-buffered saline containing 0.02% sodium azide (TBSA). Between 65 and 75% of the product was recovered from the medium as estimated by ELISA, with a further 15–25% being recovered by a second pass over the columns. Peptide recirculation (~10 times) through the column eluted bound protein more efficiently than a single, slower elution. Residual bound protein was eluted with sodium citrate buffer at pH 3.0 into 1 M Tris HCl pH 8.0 to neutralize the eluate, and columns were re-equilibrated with TBSA.

Gel filtration over Superdex S200 (Pharmacia, Sweden), of affinity-purified material showed a dominant protein peak at ~63 kDa, together with a smaller quantity of aggregated protein (data not shown). The peak protein migrated primarily as two closely spaced bands on reduced sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; data not shown), reacted positively in the ELISA with both Mab 24–60 and Mab 24–31, and gave a single sequence corresponding to the N-terminal 14 residues of IGF-1R (data not shown). No binding of IGF-1 or IGF-2 by the IGF-1R/462 fragment or a larger IGF-IR fragment (residues 1–580, not shown) could be detected in the solid plate binding assay (Cosgrove et al., 1995). This confirms that while the major determinants for specificity are contained within IGF-1R/462 based on analyses of IR/IGF-1R chimeras (Andersen et al., 1990; Gustafson & Rutter, 1990; Schumacher et al., 1991, 1993; Schäffer et al., 1993; Williams et al., 1995), additional determinants required for binding reside in the C-terminal region of the α-chain, as demonstrated for IR by direct chemical cross-linking (Kurose et al., 1994) and for IR and IGF-1R by alanine scanning mutagenesis (Myrnacik et al., 1996, 1997a, 1997b).

The IGF-1R/462 fragment was further purified by ion-exchange chromatography on Resource Q (Pharmacia, Sweden). Using shallow salt gradients, protein enriched in the slowest migrating SDS-PAGE band was obtained (data not shown), which formed relatively large, well-formed crystals (see below). Isoelectric focusing showed the presence of one major and two minor isoforms. Protein purified on Resource Q with an isocratic elution step of 0.14 M NaCl in

20 mM TrisCl at pH 8.0 (fraction 2, Fig. 1) showed less heterogeneity on isoelectric focusing (Fig. 1, inset) and SDS-PAGE (data not shown) and produced crystals of sufficient quality for structure determination (see below).

Crystallization and data collection: Crystals were grown by the hanging drop vapor diffusion method using purified protein concentrated in Centricon 10 concentrators (Amicon Inc, USA) to 5–10 mg/mL in 10–20 mM Tris-HCl pH 8.0 and 0.02% (w/v) azide, or 100 mM ammonium sulfate and 0.02% (w/v) azide. A search for crystallization conditions was performed initially using a factorial screen (Jancarik & Kim, 1991) and subsequently optimized. Crystals were cryo-cooled in a mother liquor containing 20% glycerol and examined at −160 °C on an M18XHF rotating anode generator (Siemens, Germany) equipped with Franks mirrors (MSC, USA), a low-temperature system (MSC, USA), and RAXIS IIC and IV image plate detectors (Rigaku, Japan).

From the initial crystallization screen of this protein, crystals of about 0.1 mm in size grew in one week. Upon refining conditions, crystals of up to 0.6 × 0.4 × 0.4 mm could be grown from a solution of 1.7–2.0 M ammonium sulfate, 0.1 M HEPES pH 7.5. The crystals varied considerably in shape and diffraction quality, growing predominantly as rhombic prisms with a length-to-width ratio of up to 5:1, but sometimes as rhombic bipyramids, the latter form being favored when using material that had been eluted from the Mab 9E10 column at pH 3.0. Each crystal showed a minor imperfection in the form of very faint lines from the center to the vertices. Protein from dissolved crystals did not appear to be different from the protein stock solution when run on an isoelectric focusing gel (data not shown). Upon X-ray examination, the crystals diffracted to 3.0–4.0 Å and were found to belong to the space group $P\,2_12_12_1$ with a = 76.8 Å, b = 99.0 Å, c = 119.6 Å. In the diffraction pattern, the crystal variability noted above was manifest as a large (1–2°) and anisotropic mosaic spread, with concomitant variation in resolution. This mosaic spread was not due to the cryo-cooling, as crystals at room temperature showed a similar diffraction pattern. To improve the quality of the crystals, they were grown in the presence of various additives or were recrystallized. These methods failed to substantially improve the crystal quality although bigger crystals were obtained by recrystallization. The variability in crystal quality appeared to be due to protein heterogeneity, as demonstrated by the observation that more highly purified protein, eluted isocratically from the Resource Q column and showing one major band on isoelectric focusing (Fig. 1, inset), produced crystals of sufficient quality for structure determination. These crystals diffracted to 2.6 Å resolution with cell dimensions, a = 77.0 Å, b = 99.5 Å, c = 120.1 Å and mosaic spread of 0.5°. Heavy metal derivatives of the IGF-1R/462 crystals have been obtained and are leading to the determination of an atomic resolution structure of this fragment, which contains the L1, cysteine-rich and L2 domains of human IGF-1R. Such information will provide valuable insight into the structure of the corresponding domains of the IR and insulin receptor-related receptor as well as members of the related EGFR family (Bajaj et al., 1987; Ward et al., 1995).

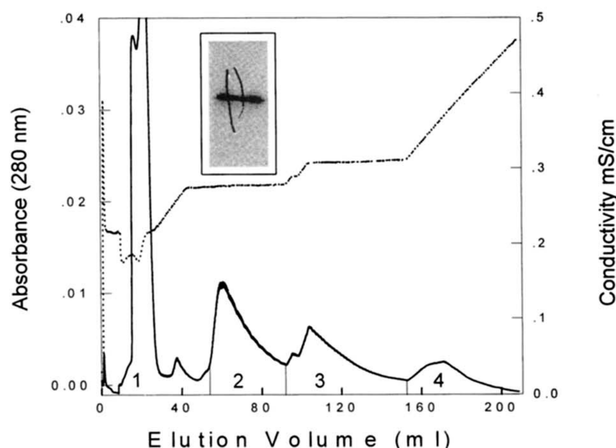

Fig. 1. Ion exchange chromatography of affinity-purified, truncated IGF-1R ectodomain. A mixture of gradient and isocratic elution chromatography was performed on a Resource Q column (Pharmacia) fitted to a BioLogic System (Bio-Rad), using 20 mM Tris/pH 8.0 as buffer A and the same buffer containing 1 M NaCl as buffer B. Protein solution in TBSA was diluted at least 1:2 with water and loaded onto the column at 2 mL/min. Elution was monitored by absorbance (280 nm) and conductivity (mS/cm). Target protein (peak 2) eluted isocratically with 20 mM Tris/0.14 M NaCl pH 8.0. (**Inset**) Isoelectric focusing gel (pH 3–7; Novex Australia Pty Ltd) of fraction 2. The pI was estimated at 5.1 from standard proteins (not shown).

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