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Purification, crystallization and preliminary characterization of an Eph-B2/ephrin-B2 complex

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Eph receptors and their ephrin ligands are involved in various aspects of cell–cell communication during development, including those of the axon pathfinding processes in the nervous system and cell–cell interactions of the vascular endothelial cells. The recognition and binding properties of the ligand-binding domain of EphB2 receptor and the extracellular domain of ephrin-B2 have been studied and two different cocrystals of their complex have been generated. One crystal form has space group $C2$, diffracts to 3.5 Å and has unit-cell parameters $a = 128$, $b = 88$, $c = 79$ Å, $\beta = 112^\circ$. The other crystal form grows in space group $P1$, has unit-cell parameters $a = 78$, $b = 78$, $c = 78$ Å, $\alpha = 69$, $\beta = 75$, $\gamma = 69^\circ$ and diffracts to 2.7 Å. Structure-determination experiments using the latter form are in progress. The structure of the complex will elucidate the chemical nature of the interactions between Eph receptors and ephrins, which would create the possibility of using them as targets for structure-based anticancer-drug development.

1. Introduction

Eph receptors represent the largest family of receptor tyrosine kinases (RTKs). Their ligands, the ephrins, are bound to the membrane *via* either a transmembrane domain or a short glycosyl phosphatidyl inositol (GPI) linker (Gale *et al.*, 1996; Holder & Klein, 1999). Eph receptors and ephrins are involved in various aspects of cell–cell communication during development. Eph-mediated signaling is based on the proper recognition and positioning of these cell-anchored molecules, leading to a wide array of cellular activities, including those regulating cell proliferation, survival, movement and adherence.

Originally described as important mediators of the axon pathfinding processes in the developing nervous system (Flanagan & Vanderhaeghen, 1998), the Eph RTKs and ephrins are now also known to participate in the control of other cell–cell interactions including those of vascular endothelial cells (Wang *et al.*, 1998; Gale & Yancopoulos, 1999), thus raising the possibility of using them as targets for anticancer-drug development (Dodelet & Pasquale, 2000). The membrane attachment of both Eph receptors and ephrins provides a mechanism whereby they can conduct the cell-signaling process bi-directionally, both in the direction of the receptor-carrying cells (forward signaling) and of the ligand-carrying cells (reverse signaling) (Henkemeyer *et al.*, 1996). Such bidirectional signaling appears to be unique to this ligand/receptor family.

Eph receptors and ephrins are divided in two subclasses, A and B, based on sequence conservation and on the mode of ligand attachment to the cell surface. The eight EphA receptors bind promiscuously the five A-ephrins that have a GPI cell linkage, while the six EphB receptors bind the three B-ephrins that have a transmembrane region and a short cytoplasmic domain (80 amino acids on average) (Gale *et al.*, 1996). The structural basis for the observed subclass specificity is not understood. In order to elucidate the molecular mechanisms of the Eph receptor/ephrin ligand interactions and the subsequent bidirectional signal transduction events, we have purified the ligand-binding domain of the EphB2 receptor and the extracellular domain of ephrin-B2, studied their binding characteristics and generated cocrystals of their complex.

2. Materials and methods

The sequences of the murine EphB2 ligand-binding domain (residues 28–210) and the extracellular ephrinB2 domain (residues 25–233) were subcloned by PCR into a pET32b expression vector (Novagen) and expressed in *Escherichia coli* AD494(DE3) as described in Himanen *et al.* (1998). The bacteria were grown in a 300 l fermenter in LB medium at 310 K until an OD_{600} value of 0.6 was reached, and induced with 1 m*M* IPTG at 293 K for 12 h. Selenomethionine-labeled EphB2 was expressed in the *E. coli* methionine auxotroph

B834 in 1 l flasks in the presence of 50 mg l^{-1} selenomethionine (Sigma). All attempts to express the proteins using the baculovirus system (Pharmingen) resulted in the production of aggregated forms of both the receptor and the ligand which were unsuitable for crystallization.

The recombinant proteins were purified separately using Ni-chelating chromatography and the His tags were removed by thrombin proteolysis followed by an ion-exchange chromatography step as described in Himanen *et al.* (1998). The two proteins were then mixed and the resulting receptor–ligand complex was subjected to limited proteolysis with proteinase V8 (Sigma) to remove the non-structured C-terminal region of ephrin-B2 and some of the remaining vector-derived N-terminal sequences. The resulting 28–210 fragment of EphB2 and the 25–187 fragment of ephrin-B2 both contained an additional eight N-terminal amino acids from the expression vector. The complex was concentrated to 1 m*M* and loaded onto a Superdex 16/60 gel-filtration column (Pharmacia). The final purity was 98% or greater. The elution volume of the EphB2/ephrin-B2 complex was 114 ml (Fig. 1), close to that of the 43 kDa molecular-weight marker (119 ml). However, the elution profile also displayed a minor peak containing both ligand and receptor at 104 ml, which is between the 158 kDa (94 ml) and 67 kDa (110 ml) protein standards. Since the molecular weights of the receptor and ligand preparations were 22 and 18 kDa, respectively, this observation suggests that the protein complex has a tendency to tetramerize.

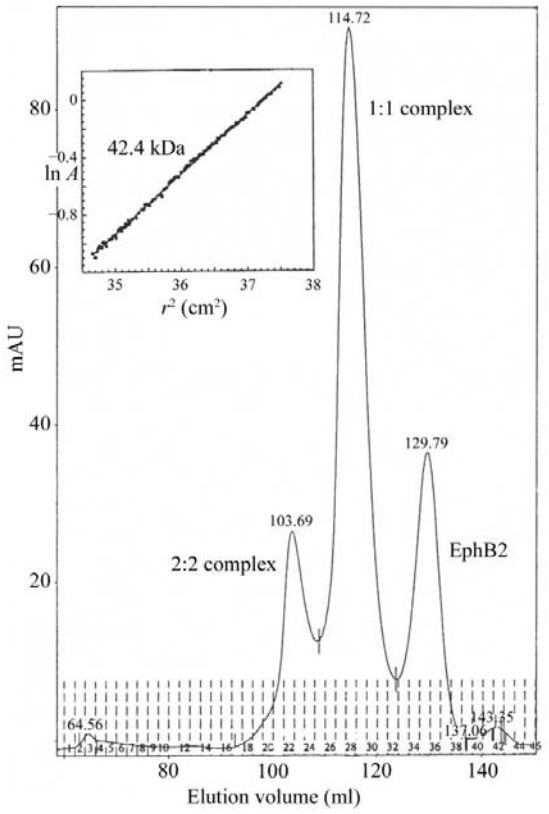

Figure 1
The elution profile of an EphB2/ephrin-B2 complex resolved on a Superdex200 16/60 column after V8 proteinase digestion. The major peak, eluting at 115 ml, corresponds to the 1:1 heterodimer complex. In addition, a 2:2 heterotetrameric complex (eluting at 104 ml), EphB2 monomer (eluting at 130 ml) and a small peak of the aggregated proteins (eluting at the void volume of the column, 65 ml) can be seen. The inset shows the determination of the molecular weight of the complex by analytical ultracentrifugation analysis.

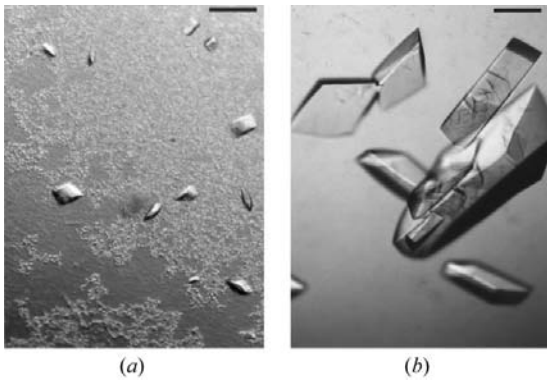

Figure 2
EphB2/ephrin-B2 crystals (*a*) before and (*b*) after treatment with V8 proteinase. A substantial improvement in the crystal quality is observed by removing the unstructured regions of the proteins. The bar corresponds to 0.1 mm.

Analytical ultracentrifugation studies over the concentration range 1–20 µ*M* were performed as described in Lackmann *et al.* (1997) and demonstrated that EphB2/ephrin-B2 exists as a high-affinity heterodimeric complex (Fig. 1). The K_d of complex formation has previously been shown to be around 10–15 n*M* (Lackmann *et al.*, 1997). The presence of a heterotetrameric species in the gel-filtration elution profile at high protein concentrations suggests that the Eph–ephrin interaction may be a two-step process, with initial formation of high-affinity heterodimers followed by formation of low-affinity heterotetramers.

The purified complex preparation was concentrated to 20 mg ml^{-1} in a buffer containing 10 m*M* KCl, 2 m*M* $MgCl_2$ and 10 m*M* HEPES pH 7.2, and crystallized in a hanging drop by vapor diffusion at 293 K. After initial screening with Hampton Research Crystal Screens I and II, crystallization conditions were found where the reservoir contained either 200 m*M* lithium sulfate, 25% PEG 4000 and 100 m*M* Tris pH 8.5 (for the non-labeled complex; space group *C*2) or 1.6 *M* Na/K phosphate, 100 m*M* Na HEPES pH 7.5 and 4% 2-propanol (for the selenomethionine-labeled complex; space group *P*1). The crystals were frozen directly under the nitrogen stream of an X-stream cooling system. Data was collected in-house on an MSC rotating-anode X-ray generator and a Rigaku R-AXIS IV imaging-plate area detector or at beamlines X9A (NSLS, Brookhaven) and F2 (CHESS, Cornell University). The oscillation photographs were processed with *DENZO* (Otwinowski & Minor, 1993).

3. Results and discussion

Since both proteins contain disulfide bridges, they were expressed in an *E. coli* AD494(DE3) strain from a pET32 vector with a thioredoxin fusion that facilitates partial formation of the disulfide bonds inside the bacterial cells (Derman *et al.*, 1993). Initially, we obtained crystals which grew from 25% PEG (see §2). The diffraction quality of these crystals was poor, but was improved substantially by performing a limited proteolysis on the complex with V8 endoproteinase prior to crystallization. The proteolysis removed some of the N-terminal vector-derived sequences and the unstructured 46 C-terminal amino acids of the ephrin-B2, resulting in crystals which grew to a size of 0.3 mm from the initial 0.05 mm maximal length (Fig. 2). Interestingly, when subcloned and expressed in *E. coli*, this shorter fragment of ephrin-B2 was in a non-functional aggregated form. These crystals grew in the *C*2 space group, with unit-cell parameters $a = 128, b = 88, c = 79$ Å, $\beta = 112^\circ$, and contained two ligand and two receptor molecules in the asymmetric unit. The diffraction resolution was, however, still quite low (3.5 Å), while the diffraction spot shape was elongated and the mosaicity was very high and non-isotropic, varying between 1.5 and 4.0 for various crystals and depending on their orientation in the beam.

Table 1
Summary of the data-processing statistics for the EphB2/ephrin-B2 crystals.

The space group is $P1$ (a = 78, b = 78, c = 78 Å, α = 69, β = 75, γ = 69°), with four receptors and four ligands in the asymmetric unit.

Data set	Crystal 1 (NSLS-X9A)	Crystal 2 (CHESS-F2)
X-ray energy (eV)	12662	12663
Resolution (Å)	30–2.9	30–2.7
Observations	344618	646135
Unique reflections	35448	43549
Data coverage (%)	98.2	96.8
$R_{\rm merge}$† (%)	7.5	7.3

† $R_{\rm merge} = \sum |I - \langle I \rangle| / \sum I$, where I is the observed intensity and $\langle I \rangle$ is the average intensity obtained from multiple observations of symmetry-related reflections.

This prompted us to search extensively for other crystallization conditions. A different crystal form was obtained from 1.6 *M* Na/K phosphate and 2-propanol with complex containing the EphB2 receptor as a selenomethionine derivative. The crystals grew in stacks of disks that were manipulated under a microscope to obtain separated crystals with dimensions of 0.03 × 0.1 × 0.2 mm. They belong to the $P1$ space group with unit-cell parameters $a = 78, b = 78, c = 78$ Å, $\alpha = 69$, $\beta = 75$, $\gamma = 69°$, display relatively low mosaicity (1.0–2.0) and diffract to 2.7 Å resolution. There are most likely to be four ligand and four receptor molecules in the unit cell. The data-processing statistics are summarized in Table 1. We plan to determine the structure by combining the selenomethionine-based MAD phasing method with molecular replacement using the coordinates from the published structure of the EphB2 receptor (Himanen *et al.*, 1998).

Our results demonstrate several aspects of X-ray crystallography important for the structure determination of biological macromolecules. First, bacterial expression systems can offer an attractive alternative to the commonly used baculovirus system for producing extracellular disulfide-bonded proteins. By using the powerful T7 RNA polymerase expression system, a special *E. coli* strain allowing the intracytoplasmic formation of disulfide bonds and easy-to-handle growth conditions in a 300 l fermenter, it was possible to obtain milligram amounts of ephrin-B2, even though 90% of it was expressed in inclusion bodies. Second, limited proteolysis of the preformed complex by a specific V8 endoproteinase resulted in the removal of unstructured regions, thus improving considerably the crystal quality, as illustrated in Fig. 2. In addition, the selenomethionine-labeled EphB2 receptor gave rise to a different crystal form with substantially improved diffraction parameters. Interestingly, we are unable to obtain this crystal form if the receptor is not selenomethionine modified.

The structure of the EphB2/ephrin-B2 complex is expected to shed light on the chemical nature of the interactions between receptors and ligands that lead to the wide array of forward and reverse signal transduction reactions. In addition, the cocrystal structure is anticipated to elucidate the biochemical basis for the strict subclass specificity of Eph receptors and ephrins. Furthermore, the participation of these molecules in the formation of new blood vessels provides an exciting possibility of using them as targets for anticancer-drug development. This will be possible, however, only after understanding the high-resolution structural details of the Eph–ephrin complex formation.

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