Monomeric Structure of the Human EphB2 Sterile α Motif Domain*

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The sterile α motif (SAM) domain is a protein module found in many diverse signaling proteins. SAM domains in some systems have been shown to self-associate. Previous crystal structures of an EphA4-SAM domain dimer (Stapleton, D., Balan, I., Pawson, T., and Sicheri, F. (1999) Nat. Struct. Biol. 6, 44–49) and a possible EphB2-SAM oligomer (Thanos, C. D., Goodwill, K. E., and Bowie, J. U. (1999) Science 283, 833–836) both revealed large interfaces comprising an exchange of N-terminal peptide arms. Within the arm, a conserved hydrophobic residue (Tyr-8 in the EphB2-SAM structure or Phe-910 in the EphA4-SAM structure) is anchored into a hydrophobic cleft on a neighboring molecule. Here we have solved a new crystal form of the human EphB2-SAM domain that has the same overall SAM domain fold yet has no substantial intermolecular contacts. In the new structure, the N-terminal peptide arm of the EphB2-SAM domain protrudes out from the core of the molecule, leaving both the arm (including Tyr-8) and the hydrophobic cleft solvent-exposed. To verify that Tyr-8 is solvent-exposed in solution, we made a Tyr-8 to Ala-8 mutation and found that the EphB2-SAM domain structure and stability were only slightly altered. These results suggest that Tyr-8 is not part of the hydrophobic core of the EphB2-SAM domain and is conserved for functional reasons. Cystallographic evidence suggests a possible role for the N-terminal arm in oligomerization. In the absence of a direct demonstration of biological relevance, however, the functional role of the N-terminal arm remains an open question.

The sterile α motif (SAM)1 domain is approximately 70 amino acids long and conserved in over a hundred diverse proteins, including the Eph family of tyrosine kinase receptors (1, 2). This family of receptors has been implicated in many developmental processes including axon guidance and bundling (3–8), segmentation of the developing brain (9, 10), angiogenesis, and cell migration (11). SAM domains are found at the N-termini. This motif is recognized by the PDZ domains in the tyrosine phosphatase binding. Stein et al. (23) reported that the binding of low molecular weight protein-tyrosine phosphatase to the EphB1 receptor tyrosine kinase is abrogated by a Y929F mutation in the SAM domain (23). A role for SAM domains in protein-tyrosine phosphatase binding is also supported by results of Serra-Pages et al. (24), who showed that the LAR (leukocyte common antigen related) protein-tyrosine phosphatase binds to a region of LIP (LAR-interacting protein) that consists of three tandem SAM domains. Third, the same Y929F mutation in EphB1 also abolished binding of the SH2-containing adapter protein Grb10, suggesting that SAMs may also bind SH2 domains (25).

Four SAM domain structures have now been reported, including the SAM domain from the Ets-1 transcription factor, the SAM domains from two Eph receptors, EphA4 and EphB2, and a C-terminal SAM domain from the p53 homolog, p73 (16, 26–28). Although the Ets-1-SAM and p73-SAM solution structures are monomeric, both the EphA4-SAM and EphB2-SAM structures revealed substantial intermolecular contacts in the crystals, providing possible models for SAM domain quaternary structure. In both models, N-terminal peptide arms on adjacent subunits are swapped into a hydrophobic cleft on the surface of a neighbor, burying approximately 2000 Å² of surface area.

Although the intermolecular SAM domain interactions seen in the Eph receptor SAM domain crystal structures are much larger than expected for crystal contacts, the associations in solution are very weak. Stapleton et al. (16) were able to detect dimerization of the EphA4-SAM domain, but the dissociation constant was in the range of 0.5–5 mM. In the case of EphB2-SAM and several other SAM domains described here, the interactions are even weaker. Certainly, a large dissociation constant in solution does not preclude productive association when the domains are tethered to the bilayer, their natural state (29). Nevertheless, the biological importance of the observed oligomeric structures remains unknown. Here we have solved the structure of a new crystal form of the human EphB2-SAM.

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1 The abbreviations used are: SAM, sterile α motif; r.m.s.d., root mean square deviation.
domain, in which no substantial intermolecular interfaces are formed.

EXPERIMENTAL PROCEDURES

**Eph Domain Cloning and Expression**—All reagents were supplied by Sigma or Fisher unless otherwise noted. The genes encoding the SAM domains from the EphB1 receptor (residues 901–976), and the EphB2 receptor (residues 905–981) were polymerase chain reaction-amplified from a human renal microvascular endothelial cell cDNA library obtained as a gift from Tom Daniel. Each polymerase chain reaction-amplified from a human renal microvascular endothelial cell cDNA library obtained as a gift from Tom Daniel. Each polymerase chain reaction fragment was subcloned into a modified version of the vector pET-3a, pMEKTR. This vector adds the amino acid leader sequence MEKTR to the beginning of the protein. Each pMEKTR-Eph-SAM construct was transformed into the *Escherichia coli* strain BL-21. To express the protein, a saturated 500-ml overnight culture of pMEKTR-Eph-SAM/BL21 grown in LB media containing 100 μM IPTG was added to 10 liters of LB-A. The culture was incubated with shaking at 37 °C until the *A*$_{600}$ reached 1.0. At this point, expression was induced by adding isopropyl-β-D-galactopyranoside to a final concentration of 200 μM, and incubation was continued for 5 h. Approximately 30–40 g of wet cells were collected/10 liters of culture used and stored at −80 °C.

**Eph-B1 SAM Purification**—The Eph-B1 SAM domain was purified from inclusion bodies. To prevent oxidation of cysteine residues, 5 mM β-mercaptoethanol was added to all buffers. For a typical preparation, 30 g of cells were suspended in 300 ml of 100 mM HEPES (pH 7.0) and lysed by sonication. Cell debris and inclusion bodies were pelleted by centrifugation at 17,000 rpm for 20 min in a Sorvall SS-34 rotor. The inclusion bodies were washed twice by resuspending the pellet in 100 mM HEPES (pH 7.0), 2 mM urea, and 5% Triton X-100 using a Waring tissue homogenizer, followed by centrifugation at 17,000 rpm for 20 min in a Sorvall SS-34 rotor. The inclusion bodies were then solubilized in 6 M guanidine-HCl, 100 mM phosphate (pH 8.0) and diluted to an *A*$_{280}$ of 0.25. The protein was refolded by dialysis into 50 mM phosphate (pH 7.0), 100 mM ammonium sulfate, 300 mM NaCl, 10% glycerol, and 0.1% sodium azide. At this point, the sample was passed through two disposable Waters Sep-Pak QMA and two Waters Sep-Pak CM columns. The flow-through was pooled and dialyzed into 50 mM phosphate (pH 9.0). The sample was then passed through another disposable Sep-Pak QMA column, and the flow-through was collected. The sample was then dialyzed into 50 mM phosphate (pH 6.0) and loaded onto a Pharmacia XK-26/20 column (Amersham Pharmacia Biotech) packed with Waters CM resin. The protein was eluted from the column using a gradient ranging from 0 to 0.7 M NaCl over 70 min at a flow rate of 1 ml/min, and peak fractions were collected. The protein was dialyzed into 20 mM Tris (pH 9.0), concentrated to 12.5 mg/ml by ultrafiltration, and snap-frozen at −80 °C in 100-μl aliquots. The protein was greater than 99% pure at this point.

**Eph-B2 SAM Purification**—Approximately 20 grams of wet cells were resuspended in buffer A (50 mM HEPES (pH 7.0), 5 mM EDTA). Cells were lysed by sonication and spun down at 17,000 rpm for 20 min in a Sorvall SS-34 rotor. The supernatant was loaded onto a Pharmacia XK-26/20 column packed with Waters CM resin equilibrated in buffer A. The protein was eluted from the column using an increasing NaCl gradient ranging from 0 to 0.7 M NaCl in 70 min at a flow rate of 1 ml/min. Peak fractions were pooled and dialyzed into buffer B (50 mM Tris (pH 9.0), 5 mM EDTA). The sample was then loaded onto a Pharmacia XK-26/20 column packed with Waters QMA resin equilibrated in buffer B. The protein was eluted using a gradient ranging from 0 to 0.7 M NaCl in buffer B over 70 min at a flow rate of 1 ml/min. Peak fractions were pooled and dialyzed into buffer C (100 mM phosphate (pH 6.0), 5 mM EDTA) and loaded onto a Pharmacia XK-26/20 column packed with Waters CM resin equilibrated in buffer C. The protein was eluted using a gradient ranging from 0 to 0.7 M NaCl in buffer B over 70 min at a flow rate of 1 ml/min. Peak fractions were pooled and snap-frozen at −80 °C.

**Eph-B2 SAM Purification**—For a typical preparation, 35 g of thawed cells were suspended in 350 ml of 50 mM phosphate (pH 8.0) containing protease inhibitors 200 μM EDTA, 0.5 mg/liter leupeptin, and 100 μM phenylmethylsulfonyl fluoride. The protease inhibitors were added to all buffers. The cells were lysed by sonication, and cell debris was removed by centrifugation at 17,000 rpm for 20 min in a Sorvall SS-34 rotor. Ammonium sulfate was slowly added to the supernatant to a final concentration of 40% (w/v). The precipitate was removed by centrifugation at 17,000 rpm for 20 min. The ammonium sulfate concentration of the supernatant was then raised to 60% (w/v), and the precipitate was

| SAM     | $M_r$ (calc) | C | $M_r$ (vis) | $M_r$ (EM) |
|---------|-------------|---|-------------|-------------|
| EphA2   | 9233        | 0.66 | 8160        | 8020        |
| EphB1   | 8964        | 1.90 | 8080        | 7860        |
| EphB2   | 9394        | 2.80 | 9080        | 8840        |

**TABLE I**

Equilibrium sedimentation of EphA2-SAM, EphB1-SAM, and EphB2-SAM

$M_r$ (calc) is the calculated molecular weight based on the amino acid composition of each protein. $M_r$ (vis) and $M_r$ (EM) are the apparent molecular weights at rotor speeds of 24,000 and 30,000 rpm, respectively. Each protein fit well to a monomeric model at concentrations up to 300 μM. In addition to the results displayed in the table, EphB2-SAM was examined at 3.0 mg/ml and a rotor speed of 18,000 rpm. At the higher concentration, EphB2-SAM had an apparent molecular weight of 10,000, NM, not measured.
recovered by centrifugation. The pellet was resuspended in 20 ml of 50 mM phosphate (pH 7.0) and dialyzed into 50 mM phosphate (pH 7.0). The sample was then passed through two disposable Waters Sep-Pak QMA and two Waters Sep-Pak CM columns, and the flow-through was retained. The sample was then dialyzed into 50 mM Tris, pH 8, and loaded onto a Pharmacia XK-26/20 column packed with Waters QMA resin. The protein was eluted from the column using a gradient ranging from 0 to 0.7M NaCl over 70 min at a flow rate of 1 ml/min. Peak fractions were pooled, concentrated by 75% ammonium sulfate precipitation, and redissolved in 2 ml of 50 mM Hepes (pH 8.5). The sample was then loaded onto a Pharmacia XK-26/100 column packed with Sephacryl S-100 gel filtration media equilibrated in 50 mM Hepes (pH 8.5) and developed at a flow rate of 0.5 ml/min. Peak fractions were collected and pooled. At this point, the protein was greater than 95% pure. To remove a residual contaminant, the protein was dialyzed into 50 mM Hepes (pH 8.5) to 50 mM phosphate (pH 6) over 30 min at 1 ml/min. The pure protein was dialyzed into 10 mM Tris (pH 8.0), concentrated to 20 mg/ml by ultrafiltration, and stored in small aliquots at −80 °C.

Analytical Ultracentrifugation Experiments—Sedimentation equilibrium runs were performed at 4 °C on a Beckman Optima XL-A analytical ultracentrifuge using absorption optics at 280 nm. A 12-mm path length, six-sector cell was used for protein concentrations of 0.77 mg/ml, and a 3-mm path length double sector cell was used for protein concentrations of 3.0 mg/ml. All samples were in 150 mM NaCl, 10 mM Tris (pH 8.0). Sedimentation equilibrium profiles were measured at 18,000 and 24,000 rpm for EphB2-SAM at 3.0 mg/ml and at 24,000 and 30,000 rpm for EphB2-SAM at 0.77 mg/ml. The data were fitted with a nonlinear least squares exponential fit for a single ideal species using the Beck-
EphB2-SAM is shown in the maximum likelihood target function (34, 35). Restrained individual model was refined with crystallography and NMR system (CNS) using 44.4 with a correlation coefficient of 60.6 for data from 40–3.0 Å. The structure as the search model. The R-factor of the initial solution was residues 12 through 76 of chain A of the previously solved EphB2-SAM molecular replacement solution was found using AMORE (33) using 100 K. The data were reduced using Denzo and Scalepack (32). The Laboratory on beamline X12C using a Brandeis CCD area detector at 3

Monomeric EphB2-SAM Structure

Crystallography—Crystals of EphB2-SAM were grown by the hanging drop method. The drop contained 20 mg/ml EphB2-SAM in 50 mM HEPES, pH 7.0, 87.5 mM LiSO₄, 15% polyethylene glycol-6000. The reservoir contained 100 mM HEPES, pH 7.0, 175 mM LiSO₄, and 30% polyethylene glycol-6000. The crystals grew reliably after a 1–2-day incubation at room temperature to dimensions of approximately 0.4 × 0.4 × 5 mm. The x-ray data were collected at Brookhaven National Laboratory on beamline X12C using a Brandeis CCD area detector at 100 K. The data were reduced using Denzo and Scalepack (32). The molecular replacement solution was found using AMORE (33) using residues 12 through 76 of chain A of the previously solved EphB2-SAM structure as the search model. The R-factor of the initial solution was 44.4 with a correlation of 60.6 for data from 40–3.0 Å. The model was refined with crystallography and NMR system (CNS) using the maximum likelihood target function (34, 35). Restricted individual B-factor refinement, anisotropic B-factor correction, and a bulk solvent model were used in the refinement. Additional crystallographic data are given in Table II.

RESULTS

Eph-SAM Domain Cloning, Purification, and Characterization—SAM domains from the human EphB1, EphB2, and EphA2 receptors were cloned, expressed in E. coli, and purified as described under “Experimental Procedures.” Circular dichroism (CD) spectra and thermal melting curves were collected on an AVIV 62DS spectropolarimeter. Spectra were collected at 4 °C in 0.1-mm path length quartz cuvettes with 1.5 mg/ml protein in 50 mM phosphate (pH 7.0). A set of 20 scans was averaged for each spectrum. The scans were collected at 1.0-nm intervals with a 1.0-nm bandwidth and a 1-s time constant. Thermal melts were performed in 1.0-cm path length cells at a protein concentration of 0.1 mg/ml in 50 mM phosphate (pH 7.0). The ellipticity at 222 nm was measured every 2.5 °C, equilibrating for 1 min at each temperature and recording for 30 s.

RESULTS

Eph-SAM Domain Cloning, Purification, and Characterization—SAM domains from the human EphB1, EphB2, and EphA2 receptors were cloned, expressed in E. coli, and purified as described under “Experimental Procedures.” Circular dichroism spectra of all the Eph receptor SAM domains are quite...
similar (Fig. 1). The estimated helical content, based on the ellipticity at 208 nm (36), ranges from 55% for EphA2-SAM to 65% for EphB2-SAM. The helical content observed in solution is in reasonable agreement with the 60% helical content seen in the EphB2 crystal structure. These results indicate that each of the Eph receptor SAM domains can fold, independent of the intact receptor.

Analytical ultracentrifugation was used to evaluate the oligomeric state of the three isolated Eph receptor SAM domains (Table I). At concentrations up to 300 μM, each Eph-SAM is a monomer in solution. At higher concentrations, EphB2-SAM showed a small concentration-dependent increase in its average molecular weight, e.g., at 24,000 rpm the molecular weight increased from 9220 at 0.77 mg/ml to 9760 at 3.0 mg/ml. There was also a small decrease in molecular weight on increasing rotor speed, as would be expected if some molecular weight heterogeneity were present. If this increase arose from a monomer-dimer equilibrium, it would correspond to a dissociation constant of approximately 6 mM. This is comparable to the dissociation constant for the EphA4-SAM dimer to be 0.5–5 mM. Thus, none of the Eph receptor SAM domains show a strong tendency to self-associate in solution.

**EphB2-SAM Crystallization and Structure Determination**—Although we had previously solved the structure of the human EphB2-SAM domain with seleno-methionine incorporated (27), we also obtained crystals of the natural protein in a different space group. Given the large structural differences in the subunit interactions observed so far, we were interested in comparing the structures in alternate crystal forms. The new crystal environment. As shown in Fig. 4, however, there are significant deviations at the N- and C-terminal ends, which are involved in intermolecular contacts in previously determined structures.

The mEphB2-SAM structure shows a much larger overall deviation of 2.6 Å r.m.s.d. from the more distantly related Ets-1-SAM structure. Three substantial structural differences occur between the Eph receptor SAMs and the Ets-1-SAM (16, 26, 27). First, as diagrammed in Fig. 5, helix 1 is much longer in the Ets-1-SAM. This helix is 7 residues long in EphB2-SAM (residues 14–20) and 14 residues long in Ets-1 (residues 46–59). Second, helix 2 in EphB2-SAM is replaced by a long loop in the Ets-1 SAM structure (Fig. 5). This loop in EphB2-SAM is 12 residues long (residues 62–73). Finally, the N-terminal peptide that extends out in the Eph receptor SAM structures instead folds back into the core of the domain in the Ets-1-SAM structure. Thus the N-terminal region may play a structural role in the Ets-1-SAM domain.

**The N-terminal Peptide Arm**—Although the overall folds of all five SAM structures are similar, the structural comparison displayed in Fig. 3 highlights the presence of large conformational differences in N-terminal peptide arms (residues 6–13 in the EphB2-SAM structure). In the mEphB2-SAM structure, the arm is completely solvent-exposed and has relatively high temperature factors (average B = 84.14 Å²). The N-terminal arm in p73-SAM is also solvent-exposed (28).

As shown in Fig. 5, one of the conserved hydrophobic residues in the SAM domain family is in the N-terminal peptide arm (1, 2). This position corresponds to Tyr-8 in the EphB2-SAM structure. Usually, conservation of hydrophobicity at a position indicates that the residue is part of the hydrophobic core (39). The fact that conserved residue Tyr-8 is not part of the hydrophobic core in EphB2-SAM suggests that it may be conserved for functional reasons.

To examine whether Tyr-8 is involved in the folding and stability of the monomeric protein, we prepared a Y8A-mutant EphB2-SAM domain. The CD spectrum of the Y8A mutant is shown in Fig. 1. The ellipticity of Y8A mutant spectrum at 208 nm corresponds to a helical content of about 56%. Although the helical content of the Y8A mutant is slightly lower than the wild-type EphB2-SAM domain, it clearly must be substantially folded. Thermal melting of both EphB2-SAM variants was fully reversible, and the melting curves are shown in Fig. 6. For the wild-type EphB2-SAM we found a Tm of 69 °C, whereas the Y8A mutant had a Tm of 68 °C. At 68 °C, the difference in unfolding free energy is only 0.2 kcal/mol. The reduced helical content in the CD spectrum of Y8A and the slight reduction in stability indicate that Tyr-8 may partially fold back into the SAM domain as seen in the Ets-1-SAM structure. Nevertheless, the contribution to stability is much less than would be expected for such a large alteration in hydrophobic core residue (40).

**DISCUSSION**

The results presented here suggest that the SAM domain from the EphB2 receptor contains a flexible N-terminal arm that does not play a role in the tertiary structure of the protein. The conserved residue Tyr-8 is not an important structural residue. The presence of a conserved hydrophobic position in this part of the protein that does not contribute to protein stability (Tyr-8) implies that the N-terminal arm may have a functional role. This role remains to be determined.

In the previously solved Eph receptor SAM domain structures, the N-terminal arm is involved in intersubunit contacts. The conserved hydrophobic position (Tyr-8 in EphB2-SAM and Phe-910 in EphA4-SAM) is anchored in a conserved hydropho-
bic cleft on the surface of a neighboring subunit. In these structures, Tyr-8 and Phe-910 bury the most surface area in the arm-exchange interfaces (not shown). Interestingly, the position analogous to Tyr-8 and Phe-910 in the PcG protein ph is a Trp, and when mutated to Ala, binding to other PcG-SAMs is abolished (12). Together these results suggest a functional role of the N-terminal peptide arm in SAM domain oligomerization.

Enthusiasm for the previously described models of SAM domain quaternary structure must be tempered in view of the weak associations of SAM domains seen in solution. It remains possible that Eph-SAM oligomerization is entropically favored by association with the bilayer (29). Alternatively, receptor clustering imparted by the binding of ephrin (23) or other proteins (22) could provide the driving force for oligomerization. Nevertheless, in the absence of further experiments, the functional role of the N-terminal arm in Eph receptor signaling cannot be assigned.

Note Added in Proof—While this paper was in press, the solution structure of another EphB2-SAM domain was reported (Smalla, M., Schmieder, P., Kelly, M., Ter Laak, A., Krause, G., Ball, L., Wahl, M., Bork, P., and Oschkinat, H. (1999) *Protein Sci.* 8, 1954–1961). Concentration-dependent resonance shifts are consistent with an arm-exchange type dimer interface.

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