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Structure of the Angiopoietin-2 Receptor Binding Domain and Identification of Surfaces Involved in Tie2 Recognition

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Summary

The angiopoietins comprise a small class of secreted glycoproteins that play crucial roles in the maturation and maintenance of the mammalian vascular and lymphatic systems. They exert their effects through a member of the tyrosine kinase receptor family, Tie2. Angiopoietin/Tie2 signaling is unique among tyrosine kinase receptor-ligand systems in that distinct angiopoietin ligands, although highly homologous, can function as agonists or antagonists in a context-dependent manner. In an effort to understand this molecular dichotomy, we have crystallized and determined the 2.4 Å crystal structure of the Angiopoietin-2 (Ang2) receptor binding region. The structure reveals a fibrinogen fold with a unique C-terminal P domain. Conservation analysis and structure-based mutagenesis identify a groove on the Ang2 molecular surface that mediates receptor recognition.

Introduction

The angiopoietins are the secreted ligands of the Tie family of protein receptor tyrosine kinases (RTK) (Davis et al., 1996, 2003; Maisonpierre et al., 1997; Ramsauer and D'Amore, 2002; Valenzuela et al., 1999). Tie2 is one of a handful of endothelial-specific RTKs that also include VEGFR. Several groups have shown Tie2 to interact with all four members of the angiopoietin family (Davis et al., 1996, Valenzuela et al., 1999). Binding studies have identified the first Ig domain and the three EGF repeats of Tie2 as essential for angiopoietin binding (Fiedler et al., 2003).

The four known angiopoietins share significant amino acid sequence identity and contain a small amino-terminal region that modulates angiopoietin clustering (superclustering region), followed by a rather large coiled-coil motif (Davis et al., 1996; Maisonpierre et al., 1997; Ramsauer and D'Amore, 2002; Valenzuela et al., 1999). A short linker separates the coiled-coil motif from a fibrinogen domain at the carboxy terminus. Binding experiments with Ang1 truncation mutants have shown that the coiled-coil region is dispensable for Tie2 binding, although it is responsible for a rather modest increase in K_d (Davis et al., 2003). It is proposed that, while the fibrinogen domain is solely responsible for receptor recognition and binding, the coiled-coil motif mediates homo- or heterodimerization (Davis et al., 2003; Fiedler et al., 2003; Ward and Dumont, 2002). While Ang2 can exist as a dimer, both Ang2 and Ang1 are observed primarily as tetramers, hexamers, and higher-order aggregates in solution (Davis et al., 1996, 2003; Fiedler et al., 2003). Thus, it is thought that the superclustering region assembles the preformed angiopoietin coiled-coil dimers into higher-order oligomers.

While Ang1 is a constitutive agonist, Ang2 is a context-dependent one. It, for example, is incapable of activating endogenous Tie2 in an endothelial cell culture system (Maisonpierre et al., 1997). Within this system, activation of Tie2 by Ang1 can be competitively inhibited by Ang2, indicating that they function by acting through the same binding site (Fiedler et al., 2003; Maisonpierre et al., 1997). This has led to the hypothesis that a general physiological role of Ang2 is to serve as a competitive antagonist, competing with Ang1 for Tie2 binding.

In order to gain a more accurate molecular understanding of angiopoietin/Tie2 interactions, we determined the three-dimensional structure of the Ang2 fibrinogen-like receptor binding domain (Ang2-RBD) at 2.4 Å resolution. The structure reveals a patch of surface residues conserved within the angiopoietin family that is likely involved in receptor recognition. Structure-based mutagenesis and in vitro binding studies corroborate the role of this molecular surface in Tie2 binding.

Results

Functional Characterization of the Angiopoietin-2 Receptor Binding Domain

The Ang2 receptor binding domain and the Tie2 ligand binding domain (Tie2-LBD) were expressed as secreted Fc-fusion proteins from stably transfected HEK293 cell lines. To assess the biological activity of our angiopoietin and Tie constructs, we employed several binding assays. First, gel filtration chromatography was used to confirm the high-affinity Ang2/Tie2 interaction, in which we monitored the retention of either Ang2 or Tie2, or a mixture of the two on an analytical size-exclusion column. When injected alone, Ang2-RBD elutes with a retention volume of 17.9 ml corresponding to an approximate molecular weight of 25 kDa (Figure 1A). Alternatively, Tie2-LBD, when chromatographed alone, elutes with a retention volume of 15.2 ml and an approximate molecular weight of 55 kDa (Figure 1B). However, when Tie2-LBD and Ang2-RBD are injected together (with Ang2-RBD in slight molar excess), two peaks are observed with retention volumes of 14.4 ml (or approximately 70 kDa) and 17.9 ml (or ~25 kDa) (Figure 1C). The first peak at ~70 kDa corresponds to a 1:1 Tie2-LBD/Ang2-RBD complex, while the second peak corresponds to Ang2-RBD alone. Samples (fractions 12–20) from each run were resolved on SDS-PAGE and are shown in Figures 1A–1C. Panel (C) clearly illustrates the comigration of Tie2 and Ang2.

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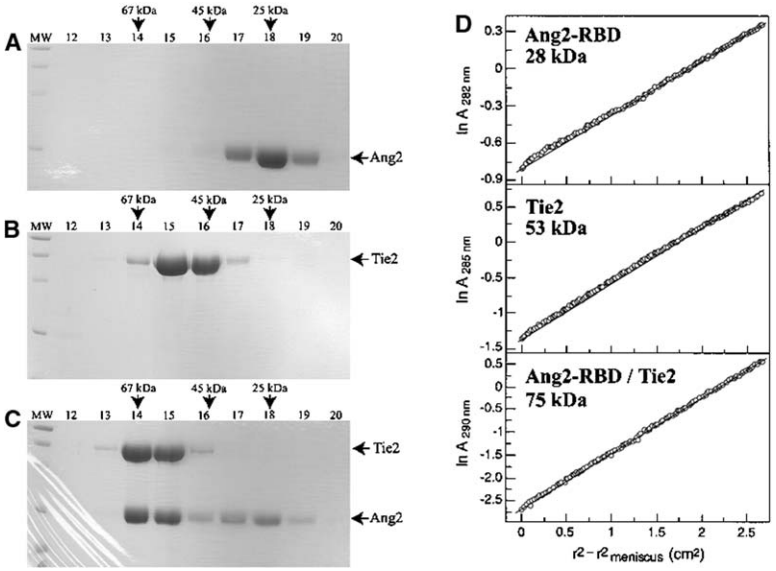

Figure 1. Gel Filtration Binding Assay Demonstrating Ang2-RBD Functional Activity

(A) Migration of monomeric Ang2-RBD on a SD-200 column. Ang2-RBD elutes at approximately 25 kDa, as judged by comparison with molecular weight standards.

(B) Tie2-LBD elutes with an apparent molecular weight of 50 kDa.

(C) Elution of the 1:1 Ang2-RBD/Tie2-LBD complex at approximately 67 kDa. Note the large shift in the elution position of Ang2. The elution positions of molecular weight standards used to calibrate the column are shown above.

(D) Sedimentation equilibrium data. The natural logarithm of the absorbance is plotted against the square of the radial position. Data were fit to an ideal single-species model.

We also investigated Ang2/Tie2 complex formation, as well as the stoichiometry of the interaction, via analytical ultracentrifugation. The results of these experiments are presented in Figure 1D. In agreement with the results obtained by gel filtration chromatography (Figures 1A–1C) and dynamic light scattering (data not shown), the analytical ultracentrifugation studies estimate the molecular weights of Ang2-RBD and Tie2 at 28 kDa and 53 kDa, respectively. In addition, these studies confirm that Ang2-RBD and Tie2 indeed form a complex with 1:1 stoichiometry (Davis et al., 2003) with an apparent molecular weight of 75 kDa. It should be noted that, as full-length Ang2 exists either as a dimer or as a multimer of dimers, via interactions mediated by the coiled-coil and superclustering domains (Davis et al., 2003), the expected stoichiometry of the complex in vivo would be 2:2 or higher.

Ang2 Structure Determination and Overall Structure Description

The structure of Ang2-RBD was determined by using a combination of Zn MAD phasing and molecular replacement (Table 1 and Experimental Procedures) from crystals grown under two distinct conditions. The final model is refined to an R factor of 22.9% (R_{free} of 28.7%) at 2.4 Å resolution. The structure of the receptor binding fibrinogen-like domain of Ang2 is illustrated in Figure 2A. Ang2-RBD is a compact, three-domain, mixed α/β protein with overall dimensions of ~50Å × 40Å × 35 Å. The structure is dominated by a large central antiparallel β sheet packing against four α helices. Ang2-RBD is structurally most similar to the blood clotting factor fibrinogen γ chain and the fibrinogen domain of the horseshoe crab Tachylectin 5A protein (Fiedler et al., 2003; Pratt et al., 1997; Yee et al., 1997; Kairies et al., 2001). In keeping with the nomenclature of the domain architecture of human fibrinogen, we will refer to the three domains of Ang2-RBD as A, B, and P (red, green, and blue, respectively, in Figure 2A). The amino-terminal domain (A) is composed of an α helix (α1), followed by two β strands (β1 and β2) that both contribute to the formation of the large central β sheet. There are three disulfide bonds connecting residues cys284–cys313, cys433–cys435, and cys437–cys450. The cys284–cys313 bond holds α1 against the small β sheet created by β1 and β2. The second domain (B) is the largest, including five β strands (β3–β6, β9) and three α helices (α2–α4). The five β strands create a large slightly twisting antiparallel β sheet that is mostly solvent exposed and forms a shallow crevice in the outer surface of the protein. The three short α helices are inserted in between strands β3 and β4 and β6 and β7. The third domain (P) contains long, extended coil regions and little secondary structure, with only two short β strands (β7–β8) and two short α helices (α5–α6). This domain packs up against the underside of the central β sheet located within the B domain and contains two disulfide bonds that serve to position residues involved in calcium binding and to brace α6 against the loop between β6 and α5. The P domain is the most divergent—both in sequence and in structure—among the fibrinogen homologs, and it is the site of ligand binding for most fibrinogen domain-containing proteins (see below).

Calcium Binding Site

Ang2 contains a calcium binding site that is similar to the one observed in Tachylectin 5A and the C-terminal fragment of the γ chain of human fibrinogen. Oxygen atoms of two side chains and several main chain carbonyl oxygens chelate the calcium ion. Specifically, the side chains of the conserved Asp429 and Asp431 each contribute one interacting oxygen, while the carbonyl oxygen atoms of Cys433 and Cys435 complete the calcium binding site (Figure 2B). Calcium binding is required for the proper function of the γ chain of human fibrinogen. Indeed, a mutation in the calcium binding loop is responsible for an inherited abnormal fibrinogen that exhibits "impaired calcium binding and fibrin poly-

Table 1. Summary of Crystallographic Analysis

Crystal	Peak	Inflection	Remote	Native	F468A/Y474A/Y475A
Resolution (Å)	3.2	3.2	3.2	2.35	2.25
Wavelength (Å)	1.282	1.282	1.255	1.74	0.979
Completeness (%)	N/A	N/A	N/A	97.2 (81.8)	99.9 (100)
Anom. Completeness (%)	92.2 (90.6)	95.7 (92.7)	96.2 (93.2)	N/A	N/A
Redundancy (fold)	3.7	4.1	4.1	2.9	3.8
I/σI	13.7	10.9	12.2	9.6	21.1
R_{merge}	6.5	8.6	6.9	7.3	4.7
Space group	$P6_122$			C2	C2
Cell dimensions (Å)	a = b = 94.06, c = 147.73			a = 74.19, b = 136.45, c = 47.73, β = 93.43	a = 140.218, b = 94.468, c = 84.673, β = 94.46

Refinement	Native	F468A/Y474A/Y475A
Resolution (Å)	8.0–2.35	8.0–2.25
Reflections (working/test)	17,641/906	48,720/2581
Non-hydrogen atoms	3699	7112
R_{crys}/R_{free}	22.9/28.7	24.6/27.8
Rmsd		
Bonds (Å)	0.007	0.008
Angles (°)	1.57	1.5

$R_{merge} = \Sigma|I - \langle I\rangle|/\Sigma I$, where I = observed intensity, <I> = average intensity obtained from multiple observations of symmetry-related reflections. Rmsds in bond lengths and angles are the respective rmsds from ideal values.

merization" (Koopman et al., 1991). Tachylectin 5A, which displays agglutinating activity against both Gram-negative and Gram-positive bacteria, is also dependent upon calcium (Kawabata and Tsuda, 2002). It is yet unknown what role calcium plays in Ang/Tie2 interactions. Ligand-receptor binding, for example, appears to be unaffected in the presence of the calcium chelator EGTA or the divalent chelator EDTA, as measured by either gel filtration or dynamic light scattering (data not shown), but such treatments often fail to remove protein-ligated calcium ions.

Homology to Other Fibrinogen Domain-Containing Proteins

Fibrinogen-like domains are interaction modules found in proteins with diverse binding partners. The structure of Ang2-RBD can be superimposed on its closest structural homologs, the fibrinogen domain of Tachylectin 5A and human fibrinogen, with rms deviations of 1.5 Å (208 equivalent Cα positions) and 1.4 Å (210 Cα positions), respectively (Figures 2C and 2D). The γ chain of human fibrinogen interacts with the A knob of fibrin following thrombin cleavage, thereby promoting fibrin polymerization, while Tachylectin 5A is a lectin involved in the innate immune response in the Japanese horseshoe crab. It is interesting to note that the major structural differences between these proteins lie within the P domain, as this is most often the site of ligand interaction (Figure 2D). For instance, the human fibrinogen γ chain contains two peptide insertions within the P domain that serve to create a deep acidic groove that is involved in binding the thrombin-cleaved α chain N termini. The carbohydrate binding region in Tachylectin 5A also consists of a hydrophobic pocket that is lined by tyrosine and histidine aromatic side chains. One side of this pocket is formed by a loop, which in Ang2 contains a single amino acid insertion, allowing it to adopt a unique, Ang2-specific conformation. Although Tachylectin 5A and the γ chain of fibrinogen interact with distinct ligands, they do share many ligand binding residues; interestingly, none of these are conserved in the Ang2 fibrinogen domain.

Identification of the Tie2-Interaction Interface

As discussed above, all fibrinogen domains that are structurally characterized contain a ligand interaction site within the P domain, close to the site of calcium binding. In the case of fibrinogen, this is the site of fibrin polymerization, while, for Tachylectin 5A, this is the site of carbohydrate binding. As residues involved in receptor binding are likely to be conserved within the angiopoietin protein family (see Figure 3A), a close examination of the Ang2 molecular surface in the context of sequence conservation could potentially identify regions responsible for Tie2 recognition. Figure 3B illustrates the molecular surface of Ang2-RBD colored according to sequence conservation. Regions colored in red are 100% conserved among all four angiopoietin family members, while regions in blue, green, and white are only 75%, 50%, or 25%-or-less conserved. The location of the ligand binding site in the fibrinogen domains of Tachylectin 5A and human fibrinogen is marked by a yellow circle. This region in Ang2 appears to be highly conserved (indicated by the blue and red coloring), and we postulate that it likely includes the molecular determinants involved in receptor recognition and binding. Interestingly, the patch highlighted in Figure 3B contains residues that are not conserved among the structurally related, but functionally distinct, angiopoietin-like proteins. The angiopoietin-like proteins are similar in overall domain architecture to angiopoietins, yet have an overall lower sequence identity and do not interact with Tie2 (Maisonpierre et al., 1997).

To investigate the role of the conserved patch in the

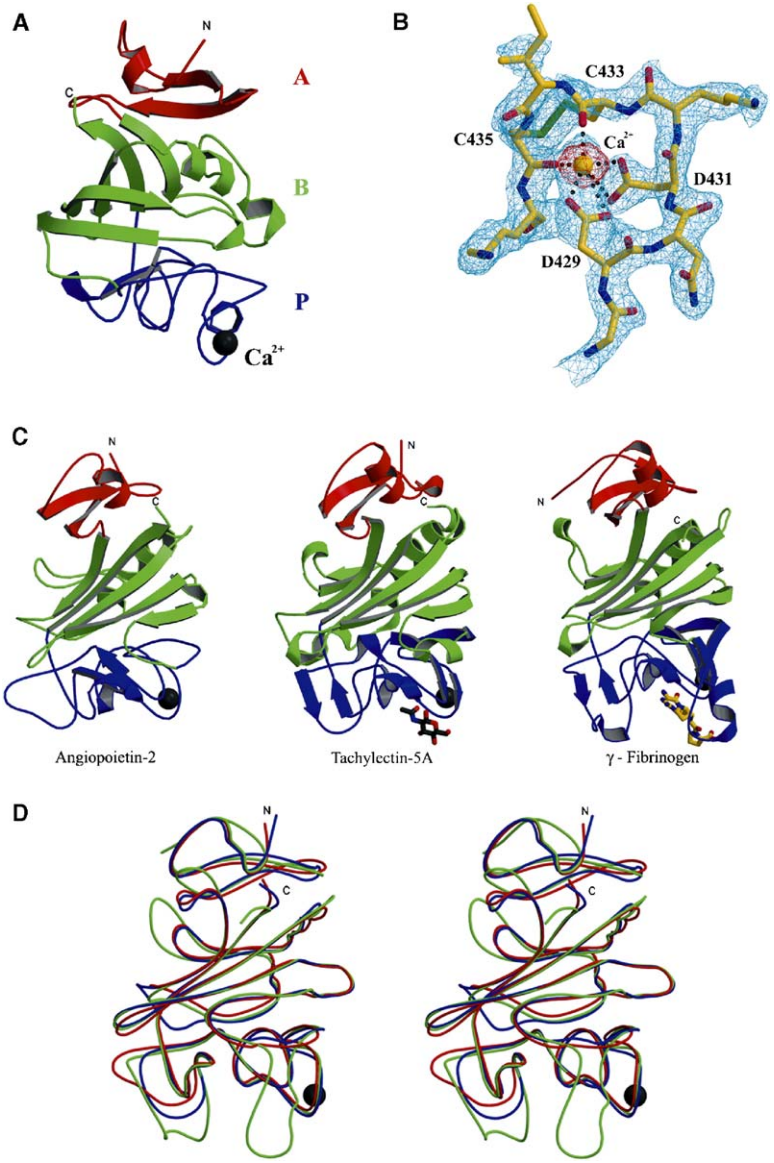

Figure 2. Structure of Ang2-RBD

(A) The refined model of Ang2-RBD with each of the three domains shown in a different color. The black sphere represents the bound calcium ion.

(B) A $2F_o - F_c$ calcium-omit map (blue) contoured at 1.5 σ depicting the clear region of density occupied by the bound calcium ion. The refined model is shown in ball-and-stick format with nitrogens in blue, carbons in yellow, oxygens in red, and sulfurs in green.

(C) Side-by-side alignment of the Ang2 structural homologs Tacylectin 5A bound to N-acetyl glucosamine (PDB ID 1JC9) and the γ chain of human fibrinogen bound to the gly-pro-arg-pro peptide (PDB ID 2FIB). The ligands are shown in ball-and-stick format.

(D) Stereoview of Ang2-RBD (red) superimposed on Tachylectin 5A (blue) and the γ chain of human fibrinogen (green). The black sphere represents the bound calcium ion in space-filling format.

P domain, we mutated several residues within this region, including K467, F468, K472, Y474, and Y475, as well as two residues that lie in a nonconserved surface region (K371E and H373A). Three Ang2 mutants were created by combining K467E and K472E into one, F468A, /Y474A, and/Y475A into a second, and K371E and H373A into a third. Gel filtration binding assays (Figures 4B and 4D) confirm that mutations made within the conserved region lead to a protein incapable of binding Tie2, while the mutations within the nonconserved Ang2 region do not affect Tie2 binding (see also below, data not shown for the K371E/H373A mutant). As all of these mutations are in surface-exposed residues, they likely do not affect the overall folding of the protein. To confirm this, we crystallized and determined the structure of the F468A/Y474A/Y475A mutant at 2.25 Å resolution. As expected, the overall structure is very similar to that of the wild-type protein (Figure 5). The rmsd between the two structures is 0.8 Å, and the only significant difference occurs within the helix containing residues Y475 and Y474. Within the F468A/Y474A/Y475A mutant, α6 adopts a slightly different conformation and packs at a somewhat altered angle relative to the main β sheet created within the B domain.

Interestingly, the angiopoietins contain two other surface patches of particularly conserved residues (Figure 3B, between the two arrows), suggesting that these might also be involved in biologically important interactions. Furthermore, the observation that both the first Ig domain and the following three EGF repeats of the Tie2 receptor are required for Ang2 binding (Fiedler et al., 2003) suggests that the Tie2-interacting region of Ang2 might be quite large and include surfaces outside of the P domain. To test this hypothesis, we generated two Ang2 mutants, I296A/T298A and R336A/E340A (Figure 3A, black dots), which alter conserved regions within the A and B domain, respectively. In vitro binding

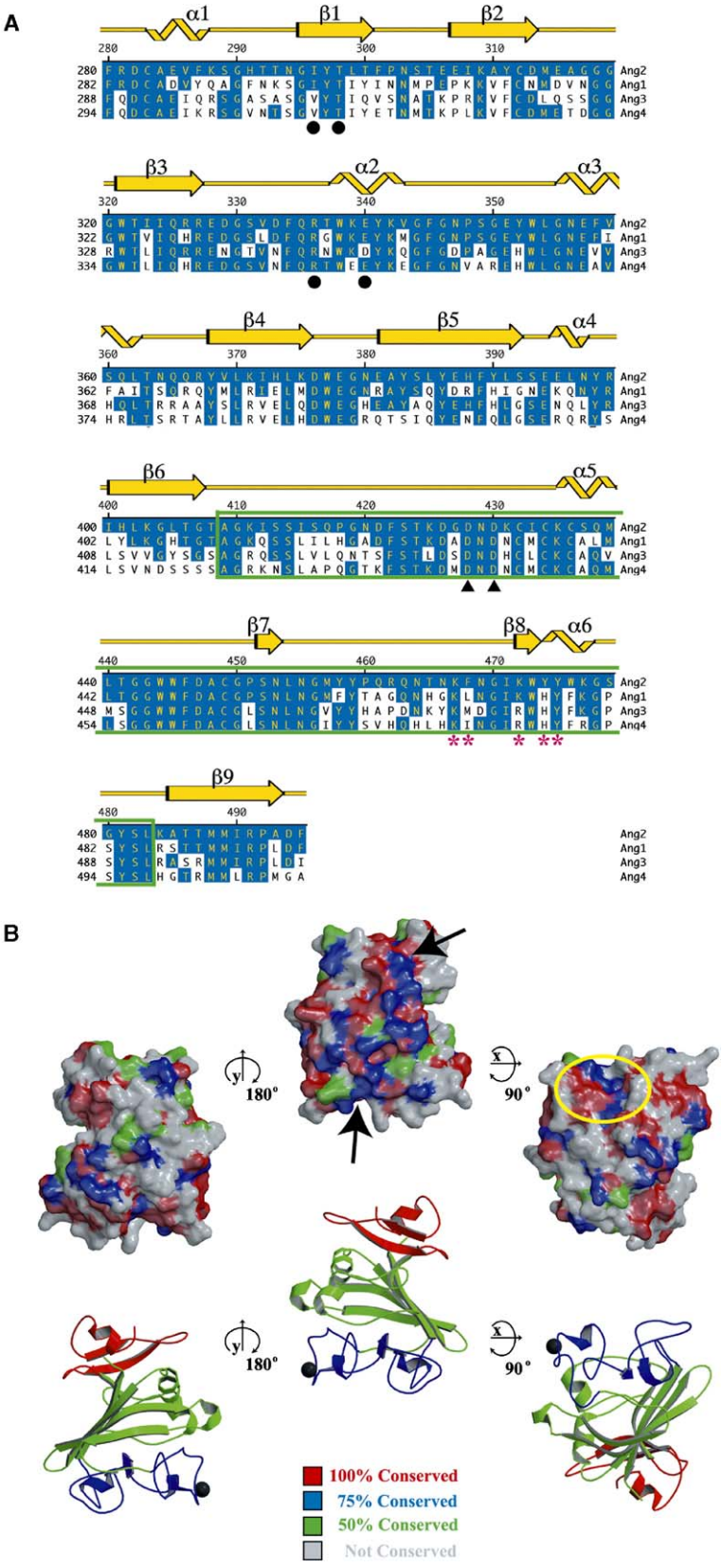

Figure 3. Sequence Conservation of the Receptor Binding Domain among Angiopoietin Family Members

(A) Sequence conservation among the four angiopoietins within the receptor binding domain. Regions of sequence identity to Ang2 are indicated. Secondary structure elements are noted above. Black triangles represent the conserved aspartic acids involved in calcium chelation. Red asterisks indicate residues involved in receptor recognition identified in this study. Black circles indicate residues within the conserved regions of the A and B domains that were also subject to mutagenesis, but found not to be involved in receptor recognition. The P domain is boxed in green.

(B) Sequence conservation mapped onto the molecular surface of Ang2-RBD. Secondary structure renderings are shown below each surface representation to orient the reader. Absolutely conserved residues (100%) are shown in red; partially conserved residues (75% or 50%) are shown in blue and green, respectively. Surfaces in gray represent residues that are not conserved among angiopoietin family members. The three views are related by 180° rotation along the y axis (left) and 90° rotation along the x axis.

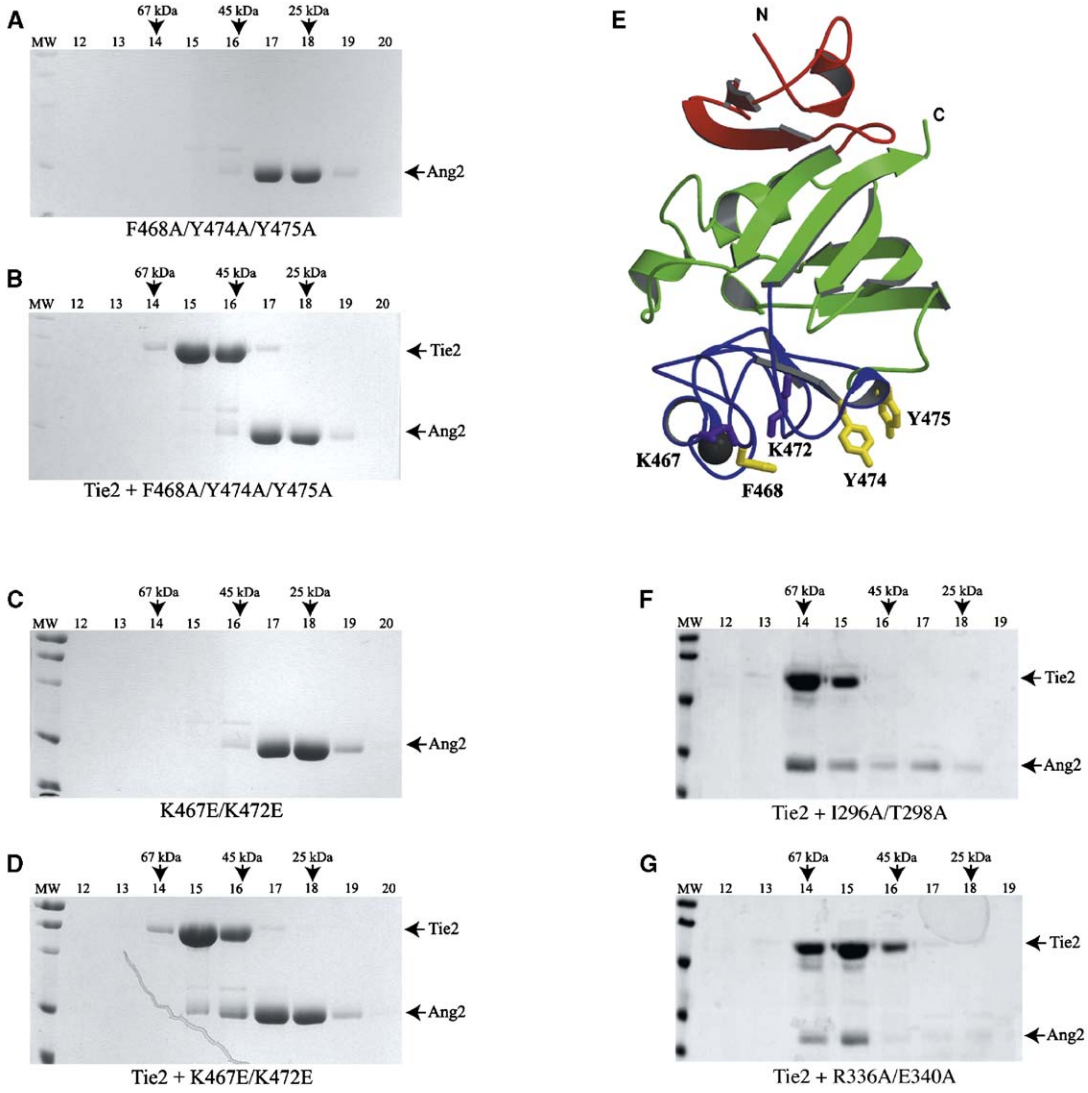

Figure 4. Gel Filtration Receptor Binding Analysis of Ang2 Mutants

(A and C) The mutants elute as monomers on gel filtration chromatography with estimated molecular weights similar to that of the wild-type protein.

(B and D) The F468A/Y474A/Y475A and K467E/K472E mutants, located within the P domain of Ang2, do not bind Tie2. The elution positions of the molecular weight standards used to calibrate the column are shown above.

(E) The structure of Ang2-RBD indicating the location of residues (ball-and-stick format) within the P domain involved in receptor recognition in yellow (aromatic residues) and purple (basic residues).

(F and G) Mutations within the conserved surface regions of the A domain (I296A/T298A) and the B domain (R336A/E340A) do not affect receptor binding.

experiments (Figures 4F and 4G) document that the mutated Ang2-RBD proteins are still able to form stable complexes with Tie2, indicating that these conserved molecular surfaces are not involved in receptor recognition. Instead, they could be involved, for example, in interactions between angiopoietin monomers, as they are normally dimerized by the coiled-coil domain in the full-length molecule. As with the proposed Tie2-interaction surface within the P domain, the surfaces within the A and B domains are conserved only in the angiopoietins, but not in the other fibrinogen-like proteins.

Discussion

Fibrinogen domains function as molecular recognition units with a diverse array of binding partners. Indeed, many of the fibrinogen domain-containing proteins are lectins, but there are also several protein-protein recognition examples, including the fibrinogen γ chain as well as the angiopoietins. It appears that the variable P domain has evolved to serve as a ligand-recognition module as elucidated by crystal structures of the γ chain of fibrinogen in complex with a peptide derived

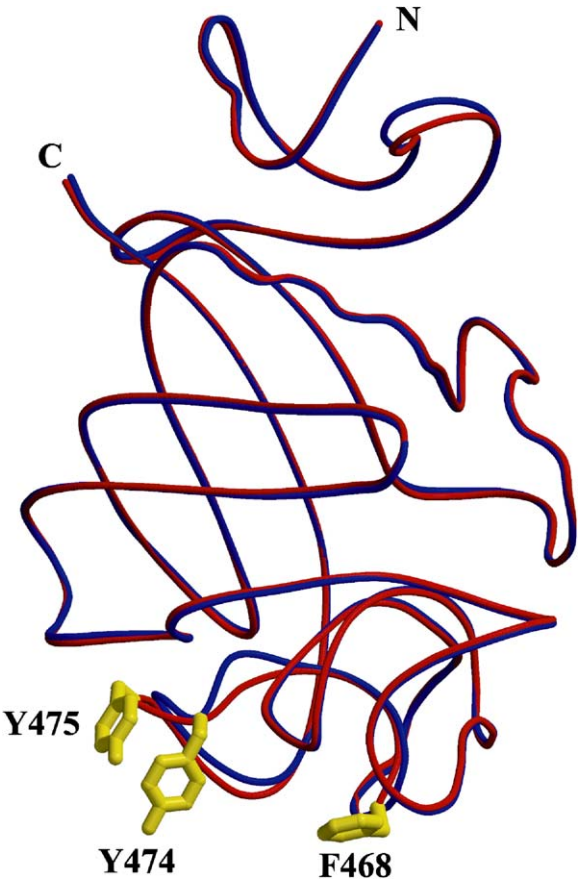

Figure 5. Structure of the F468A/Y474A/Y475A Ang2-RBD Mutant

The structure is (blue) superimposed on the structure of the wild-type protein (red). Mutated residues are shown in yellow ball-and-stick format.

from the α chain, and of Tachylectin 5A in complex with GlcNac. Our data presented here provide evidence that Angiopoietin-2, another fibrinogen domain-containing protein, utilizes the variable P domain for receptor binding. Indeed, two cluster mutations within this region completely abolish receptor binding. In addition, conservation analysis of the angiopoietin family members projected onto the three-dimensional structure suggests that other surface regions within the B domain also possess important, but distinct, biological functions.

It is noteworthy that many of the fibrinogen domain-containing proteins exist as multimers in vivo. Angiopoietin-2 is no exception, as the initial Ang1 and Ang2 dimers are further assembled in tetramers, hexamers, and higher-order complexes. In light of the location of the P domain within Ang2, our structure suggests that, upon dimer formation (mediated via coiled-coil motif interactions), the P domains would be oriented away from the dimerization interface, facilitating the simultaneous binding of two Tie2 receptors for signaling initiation.

The Ang2 structure identifies a metal binding site that is structurally conserved among fibrinogen domain-containing proteins. The close proximity of the calcium site to the receptor binding region indicates that the function of the metal ion is to stabilize the local structure for receptor binding. Indeed, calcium is likely required to keep the pocket architecturally intact and thereby accessible to receptor contact. Interestingly, although EGTA does not inhibit the Ang2/Tie2 interactions, several mutations in Ang2 designed to disrupt calcium chelation led to highly unstable proteins and very low expression (data not shown), indicating that Ca^{2+} is required for angiopoietin stability and function.

The crystal structure of an angiopoietin represents an advance in our understanding of the molecular mechanisms of Ang/Tie2 signaling and provides a solid foundation for future structural, biochemical, and cell-based studies aimed at understanding the molecular basis of the context-dependent antagonism demonstrated by this unique receptor/ligand system.

Experimental Procedures

Expression, Purification, and Mutagenesis

Ang2-RBD (amino acids 276–496) and Tie2-LBD (amino acids 1–448) were cloned as IgG fusions into a modified pcDNA3.1 vector (Invitrogen) for constitutive expression in human embryonic kidney 293 (HEK293) cells. Carboxy-terminal to the gene of interest, we placed a thrombin cleavage site followed by the constant domain of IgG to facilitate protein purification. Following clarification of the media from stable transformants, the fusion protein was purified by affinity chromatography with Protein-A Sepharose and was cleaved with thrombin. The following nine vector-derived amino acids remain at the C terminus of Ang2 following thrombin cleavage: GSASGLVPR. The cleaved fusion tag was removed by Protein-A Sepharose, and the proteins were further purified by gel filtration chromatography. N-terminal sequencing confirmed the identity of the purified products. The Tie2 receptor was expressed and directed for secretion by using the native signal sequences, while the fibrinogen domain of Angiopoietin-2 was secreted by using the efficient CD5 signal sequence. Typical expression levels were 5–10 mg per liter of culture. Site-directed mutants were prepared by using the Quikchange mutagenesis kit (Stratagene) following the manufacturers suggestions. Double- and triple-substitution mutations (rather than single substitutions) were designed and generated in order to create a larger change in the chemical nature of the targeted surfaces. While most mutations were to alanines, some lysines were mutated to glutamic acids in order to alter the local surface electrostatic potential.

Size-Exclusion Chromatography and Equilibrium Sedimentation Assays

Purified proteins were mixed at 20 μM in a buffer containing 20 mM HEPES (pH 7.4), 200 mM NaCl (HBS) and were incubated for 1 hr on ice prior to analysis. For gel filtration, 500 μl was injected onto a Superdex-200 column (10/30) (Pharmacia) preequilibrated in 20 mM HEPES (pH 7.4), 200 mM NaCl. Fractions (1 ml) were collected and resolved on SDS-PAGE.

For the analytical ultracentrifugation experiments, 10 μM protein solution was loaded in HBS and centrifuged at 20°C and 14,000 rpm in a Beckman XL-A ultracentrifuge equipped with an An-60Ti rotor. Data were fit to an ideal single-species model of absorbance versus radial distance, and they showed no systematic dependence of molecular weight on protein concentrations.

Crystallization, Data Collection, and Structure Determination

Ang2-RBD was concentrated to 15 mg/ml in a buffer containing 20 mM bis-Tris propane (pH 7.0) and 200 mM NaCl. The protein was crystallized in a hanging drop by vapor diffusion at room temperature (20°C). Two crystal forms suitable for diffraction studies were obtained: one against a reservoir containing 5 mM zinc sulfate, 100 mM MES (pH 6.25), and 22% PEG-550, and another against a reservoir containing 0.2 M MgCl2, 100 mM Tris (pH 8.5), and 30% PEG-4000.

Crystals grown in the presence of zinc were harvested and flash frozen in the cold stream of an X-Stream cooling system in the mother liquor with 30% PEG-550 as a cryoprotectant, while crystals grown in the presence of PEG-4000 were rapidly transferred to a cryo-buffer consisting of the mother liquor with an additional 20% glycerol. The data were collected at NSLS Brookhaven beamline X9A and CHESS beamline F2. Oscillation photographs were integrated, merged, and scaled by using DENZO and SCALEPACK, respectively (Otwinowski and Minor, 1997). Subsequent calculations were done with autoSHARP and the CCP4 program suite (CCP4, 1994; Evans and Bricogne, 2002). The structure was determined with a combination of MAD phasing and molecular replacement. Initially, the program AMORE was used to identify the location of the fibrinogen domain by using the horseshoe crab Tachylectin 5A (PDB ID 1JC9) as a search model (Fiedler et al., 2003; Jones et al., 1991). Simultaneously, a zinc anomalous data set was collected by utilizing a single zinc atom involved in mediating an intermolecular crystal contact. The program autoSHARP was used to identify the location of the zinc atom and to refine its position and occupancy for phase calculations. Density modification with DM improved the zinc MAD maps that proved to be of sufficient quality to confirm the placement of the fibrinogen domain (CCP4, 1994). The model was subjected to 100 rounds of improvement in the program ARP/wARP by using the phases calculated by autoSHARP and phase constraints with a blurring factor of 1.0 (Perrakis et al., 2001). Refinement proceeded with iterative rounds of model adjustments (by using the molecular graphics program O), molecular dynamics, and energy minimization in CNS (Brunger et al., 1998; Jones et al., 1991). The first model was refined at 3.2 Å resolution to R and R_{free} values of 26.9 and 32.8, respectively, for 1725 atoms and 215 amino acids.

At this point, crystals grown from $MgCl_2$ and PEG that diffracted to a higher resolution were identified. The high-resolution structure was determined by using molecular replacement with the zinc Ang2-RBD structure as a search model. The model was subjected to adjustments with O and refined in CNS. Stereochemical analysis of the refined models with PROCHECK (CCP4, 1994) revealed main chain and side chain parameters better than or within the typical range of values for protein structures.

The F468A/Y474A/Y475A mutant was crystallized from 0.2 M ammonium sulfate, 0.1 M sodium acetate (pH 5.2), 22% PEG-4000. Crystals were transferred to mother liquor with 20% glycerol for flash freezing. A 2.25 Å native data set was collected at CHESS beamline F2. The CCP4 program AMORE was used to locate four monomers in the asymmetric unit. In an attempt to keep the model unbiased within the loop containing the mutations, residues 467–476 were omitted in the initial model. The model was subsequently subjected to additional rounds of refinement within the CNS suite prior to manually building the missing region by using the graphical program O.

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Accession Numbers

Coordinates have been deposited in the Protein Data Bank (PDB accession numbers 1Z3S and 1Z3U).