Angiopoietin-1 and -2 Coiled Coil Domains Mediate Distinct Homo-oligomerization Patterns, but Fibrinogen-like Domains Mediate Ligand Activity*

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Activity of endothelial Tie2 receptor tyrosine kinase is modulated by two naturally occurring, secreted ligands, angiopoietin-1 and -2, which have opposing effects on its phosphorylation. Receptor tyrosine kinase activation requires receptor dimerization/multimerization, which, for many receptors, is mediated by homo-oligomeric ligands binding to and bridging receptor molecules. We show here that angiopoietin-1 and -2 form distinct arrays of disulfide-linked homo-oligomeric complexes. Their mobilities on nonreducing gels suggest that angiopoietin-2 exists predominantly as a homodimer but also forms higher order multimers. In contrast, angiopoietin-1 forms some homotrimers, but predominantly exists in higher order multimers. These two structurally related, 60% homologous ligands are predominantly composed of an amino-terminal coiled coil domain and a carboxyl-terminal fibrinogen-like domain. We show that their distinct oligomerization patterns are determined by their coiled coil domains and, furthermore, that their coiled coil domains, but not their fibrinogen-like domains, are sufficient to mediate formation of disulfide-linked homo-oligomers. In contrast, the differential effects of these ligands on endothelial Tie2 phosphorylation is mediated by their fibrinogen-like domains. We conclude from these studies that the coiled coil and fibrinogen-like domains of the angiopoietins have distinct functions with the coiled coil domain mediating ligand homo-oligomerization and the fibrinogen-like domain mediating ligand activity.

The endothelial cell-restricted receptor tyrosine kinase, Tie2, plays a critical role in developmental angiogenesis (1–3) and is thought to be an important modulator of both normal physiology (4, 5) and pathologic angiogenesis (6–8). Tie2 activity is regulated by two naturally occurring secreted ligands, an agonistic ligand, angiopoietin-1 (Ang1)† (9), that induces Tie2 phosphorylation and a second ligand, angiopoietin-2 (Ang2) (4), that has a tissue-specific modulatory effect on Tie2 activity.

Ang2 blocks the ability of Ang1 to activate Tie2 in endothelial cells, but it activates Tie2 expressed in hemangioblast precursor cells or ectopically expressed in fibroblasts (4, 10–12). The mechanism by which Ang1 and Ang2 differentially modulate Tie2 activity has not been defined.

Tie2 activation and phosphorylation are presumed to occur through mechanisms common to previously described receptor tyrosine kinases, i.e. by inducing receptor dimerization, which leads to trans-phosphorylation. In various systems, receptor tyrosine kinase activation can result from either homo- or heterodimerization, and dimerization has been shown to occur through a variety of mechanisms (13). Many soluble ligands form covalently or noncovalently linked homo- or hetero-oligomers (13–19), thereby creating complexes with multiple receptor binding sites that can act to bridge receptor molecules. For example, disulfide-linked homodimerization of vascular endothelial growth factor creates a complex with two symmetric vascular endothelial growth factor receptor binding sites formed by loop 2 of one vascular endothelial growth factor monomer and loop 3 of the other, thus facilitating the bridging of two receptor molecules (20–22). In contrast, human growth hormone exists as a monomer but has two binding sites capable of binding two growth hormone receptor molecules to induce dimerization (23). A third mechanism of receptor tyrosine kinase activation has been described for the epidermal growth factor receptor in which the binding of an epidermal growth factor monomer induces a conformational change in the receptor’s extracellular domain that stabilizes interactions between two occupied epidermal growth factor receptor molecules (24–27). Finally, ligands for the EPH-related family of receptor tyrosine kinases do not function as conventional soluble factors but must be presented in membrane-bound form, possibly facilitating ligand dimerization or aggregation (28). Which of these mechanisms, if any, mediates Tie2 activation is unclear.

We have begun structural analysis of Ang1 and Ang2 to determine the mechanism by which these two ligands have opposing effects on endothelial Tie2. These two structurally related glycoproteins are predominantly composed of an amino-terminal coiled coil domain and a carboxyl-terminal fibrinogen-like domain. Here we show that Ang1 and Ang2 form distinct arrays of homo-oligomeric complexes mediated by their coiled coil domains but that their differential effects on endothelial-Tie2 phosphorylation is mediated by their fibrinogen-like domains.

EXPERIMENTAL PROCEDURES

Cloning of Ang1 and Ang2—Murine Ang1 cDNA was cloned from cytoplasmic RNA from C2C12 murine myoblasts by oligo(dT)-primed reverse transcription with Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.) followed by polymerase chain reaction amplified with oligonucleotide primers, 5'-GCCTGGCCAGTA-CAAATGACAGT-3' and 5'-GGGGGATCTTGTGACCTCA-3' with Pfu DNA polymerase (Stratagene). The Ang1 cDNA sequence was con-
human IgG1, an XhoI site was introduced downstream of the penultimate Ang1 codon of pAng1 by site-directed mutagenesis using the GeneEditor™ in vitro site-directed mutagenesis system (Promega Corp., Madison, WI), and the Ang1 coding region was ligated into the NotI/XhoI site of IgG/EF-BOS (a gift from P. Kincade (31)). pAng2-Fc was created by introducing a G/C codon at position 20 into pAng2 and ligating the Ang2 coding region into the NotI/BamHI site of IgG/EF-BOS. To facilitate construction of Ang1 deletion mutants, chimeras, and epitope-tagged constructs, in-frame HindIII sites were introduced into pAng1 by site-directed mutagenesis 3′ to Ang1 codons 20 and 273 to create plasmid pAng1H/H273. HindIII sites were similarly introduced into pAng2H/H273 to create pAng2H and introduced 3′ to Ang2 codons 20 and 268 to create plasmid pAng2H/H268. pAng1fib and pAng2fib were then created by deleting the HindIII fragments of pAng1H/H273 and pAng2H/H268, thus deleting codons 21–273 of Ang1 and codons 21–268 of Ang2. pH-A Ang2, a plasmid encoding Ang2 N-terminally tagged with the hemagglutinin (HA) epitope, YPYDVPDYA, was created by ligating self-annealed primers HA-F and HA-R (see below) into the HindIII site 3′ to codon 20 in pAng2H, pMyc-Ang2, which encodes Ang2 N-terminally tagged with the Myc epitope, EQKLL-SEEDL, was similarly created using self-annealed primers Myc-F and Myc-R. pAng1/2 was created by ligating the HindIII fragment of Ang1H/H273 into pAng2fib and encodes a chimeric protein in which the Ang1 coiled coil domain is fused to the Ang2 fibrogen-like domain. pAng2/1 was similarly created by ligating the HindIII fragment of pAng2H/H268 into pAng1fib. pAng1c and pAng2c were created by introducing a premature termination codons downstream of Ang1 codon 265 in plasmid, pAng1, and downstream of Ang2 codon 269 in plasmid, pAng2, respectively, by site-directed mutagenesis. To create pTie2-Fc, a BamHI site was engineered downstream of the mouse Tie2 ectodomain, 3′ to codon 744, in plasmid ptcp (gift from D. Dumont (32)), and the Tie2 ectodomain was ligated into the NotI/BamHI site of IgG/EF-BOS. The primers used were as follows: HA-F, 5′-AGCTTGCCTACCATCGA- CTGCTCTACAGCTATGGTGTCTCTCA-3′; HA-R, 5′-AGCTTCTATAGCGTGTCTGGGACGTCGTATGGGTAC-3′; Myc-F, 5′-AGTCGGACGCTGACCATTTTGAGAGAAGGGACTTCGATATGGG-3′; and Myc-R, 5′-AGTCGGACGCTGACCATTTTGAGAGAAGGGACTTCGATATGGG-3′.

### Cell Culture and Transfections—
293T human embryonic kidney cells and MS-5 murine vascular smooth muscle cells (ATCC, Manassas, VA) were maintained in 10% fetal bovine serum (Life Technologies, Inc.) in Dulbecco’s modified Eagle’s medium (BioWhittaker) supplemented with penicillin and streptomycin (each at 100 μg/ml). 293T cells were transiently transfected using calcium phosphate, and conditioned media were prepared by culturing transiently transfected cells in serum-free Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, streptomycin, and ITS (Collaborative Biomedical). At 48 h, conditioned medium was harvested and filtered, and protease inhibitors were added (aprotinin (10 μg/ml), leupeptin (5 μg/ml), pepstatin A (2 μg/ml), and phenylmethylsulfonyl fluoride (1 mM)). Transiently transfected cells were cultured in cysteine- and methionine-free Dulbecco’s modified Eagle’s medium (Life Technologies) supplemented with ITS (insulin transferrin and selenite) and 125 μg/ml Easy Tag Express乒乓球Protein Labeling Mix (NEN Life Science Products) to create 35S-labeled Ang1, Ang2, or their mutants.

### Immunoprecipitation and Western Analysis—
293T cells transiently transfected with pTie2-Fc or IgG/EF-BOS was used as a source of Tie2-Fc or the Fc fragment alone, respectively. Ang1, Ang2, and their mutants were precipitated from conditioned medium with Tie2-Fc (5 μg), separated by SDS-polyacrylamide electrophoresis under reducing (5% 2-mercaptoethanol) or nonreducing (no 2-mercaptoethanol) conditions and transferred to a Protran membrane (Schleicher and Schuell). Membranes were probed with antibodies against the amino or carboxyl terminus of Ang1 or Ang2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and an HRP-conjugated anti-goat antibody coupled with enhanced chemiluminescence (ECL) was used to visualize bands. An anti-Myc antibody produced by the murine hybridoma, 9E10 (ATCC), was used for immunoprecipitation of Myc-Ang2. An antibody reactive with the HA epitope, 12CA5 (Roche Molecular Biochemicals), was used in Western analyses to detect HA-Ang2 and imaged using an HRP-conjugated anti-mouse secondary antibody and ECL. The relative abundance of different oligomers of Ang1 or Ang2 was determined by scanning densitometry using a Bio-Rad Gel Doc System with Molecular Analyst software and analysis using ImageQuant software.

**Tie2 Tyrosine Phosphorylation Assay—** MS-1 cells grown to late confluence and serum-starved overnight in Dulbecco’s modified Eagle’s medium with ITS were incubated for 10 min with medium conditioned by transiently transfected 293T cells. After stimulation, cells were
Anti-Ang2 antibody reactive with the Ang2 N terminus (ECL. Ang1 and Ang2 monomers and oligomers are and imaged using an HRP-conjugated anti-goat secondary antibody and bracketed). 

FIG. 2. Ang1 and Ang2 bind Tie2 as disulfide-linked oligomers. 293T cells were transiently transfected with pAng1 (Ang1), pAng2 (Ang2), or vector control (Ctrl). Tie2-Fc (T) or Fe (C) immunoprecipitations were performed on conditioned medium, and Western analysis was performed on immunoprecipitates electrophoresed under reducing (Red) or nonreducing (NR) conditions. Blots were probed using an anti-Ang1 antibody reactive with the Ang1 N terminus (left panel) or an anti-Ang2 antibody reactive with the Ang2 N terminus (right panel) and imaged using an HRP-conjugated anti-goat secondary antibody and ECL. Ang1 and Ang2 monomers and oligomers are bracketed.

Soluble Ang1 and Ang2 Exist in an Array of Disulfide-linked Oligomeric Complexes—Since Tie2 is presumed to require dimerization or multimerization for activation, we examined the oligomeric structure of Ang1 to determine whether Ang1 may act to bridge receptor molecules. Ang1 was precipitated from conditioned medium of 293T cells transiently transfected with pAng1 using Tie2-Fc, a Tie2-immunoglobulin fusion protein in which the Tie2 ectodomain was fused to the hinge region and Fe domain of human IgG1. Western analysis using an antibody reactive with the N-terminus of Tie2 (Santa Cruz Biotechnology), Western analysis was performed on immunoprecipitates, and Tie2 phosphorylation was analyzed by probing blots with the anti-phosphotyrosine antibodies, 4G10 (Upstate Biotechnology, Inc.) and PY20 (Santa Cruz Biotechnology). After stripping, blots were reprobed for Tie2 with an anti-Tie2 antibody.

RESULTS

Soluble Ang1 and Ang2 Exist in an Array of Disulfide-linked Oligomeric Complexes—Since Tie2 is presumed to require dimerization or multimerization for activation, we examined the oligomeric structure of Ang1 to determine whether Ang1 may act to bridge receptor molecules. Ang1 was precipitated from conditioned medium of 293T cells transiently transfected with pAng1 using Tie2-Fc, a Tie2-immunoglobulin fusion protein in which the Tie2 ectodomain was fused to the hinge region and Fe domain of human IgG1. Western analysis using an antibody reactive with the N-terminus of Tie2 (Santa Cruz Biotechnology), Western analysis was performed on immunoprecipitates, and Tie2 phosphorylation was analyzed by probing blots with the anti-phosphotyrosine antibodies, 4G10 (Upstate Biotechnology, Inc.) and PY20 (Santa Cruz Biotechnology). After stripping, blots were reprobed for Tie2 with an anti-Tie2 antibody.

To determine whether Ang2 also forms disulfide-linked oligomeric structures, Ang2 precipitated by Tie2-Fc from conditioned medium of transiently transfected 293T cells was examined by Western analysis using an antibody reactive with the Ang2 amino terminus. Under reducing conditions, Ang2 migrated as a single 65-kDa band (Fig. 2, right panel). Like Ang1, under nonreducing conditions, multiple bands of higher molecular mass were observed (Fig. 2, right panel). In contrast to Ang1, the Ang2 band of greatest mobility migrated at 140 kDa, and, by scanning densitometry, a greater fraction of total Ang2 was in this oligomeric complex when compared with Ang1 (69% compared with 33%). Thus, both soluble Ang1 and Ang2 exist in arrays of disulfide-linked oligomeric complexes, but the different mobilities of these oligomeric complexes on nonreducing gels suggest that these ligands have different oligomeric structures.

Ang1 and Ang2 Form Disulfide-linked Homo-oligomeric Complexes—To determine the composition of Ang1 and Ang2 complexes, Ang1- and Ang2-expressing 293T cells were cultured in the presence of [35S]cysteine/methionine, and the radiolabeled ligands were precipitated from conditioned medium using Tie2-Fc. Under nonreducing conditions, Ang1 and Ang2 migrated as complexes comparable with those seen on Western analysis (Fig. 3), which again demonstrated the distinctive oligomerization patterns of these two ligands. Of note, the higher order multimers of Ang1 and Ang2, as well as the faster mobility oligomers, have distinct mobilities on nonreducing gels. Under higher reducing conditions, the Ang1 oligomers migrated as a single 70-kDa band, and the Ang2 oligomers migrated as a single 65-kDa band (Fig. 3), suggesting that the complexes seen under nonreducing conditions are composed either of homo-oligomers of these ligands or of hetero-oligomers in which Ang1 and Ang2 complex with other factors of similar mobility.

To determine whether Ang1 homo- or hetero-oligomers, we created pAng1-Fc, which encodes an Ang1-immunoglobulin chimera in which Ang1 was fused to the hinge region and Fc domain of human IgG1. Ang1-Fc, unlike Ang1, can be precipitated by protein A-agarose (Fig. 4A). When Ang1-Fc was precipitated by protein A-agarose from conditioned medium of 293T cells co-transfected with pAng1-Fc and pAng1, Ang1 was coprecipitated, demonstrating that Ang1 and Ang1-Fc hetero-oligomerize and strongly suggesting that soluble Ang1 complexes are composed of homo-oligomers of Ang1 (Fig. 4A). To determine if Ang1 also hetero-oligomerizes with another 70-kDa factor, Ang1-Fc-expressing 293T cells were cultured in the presence of [35S]cysteine/methionine, and radiolabeled Ang1-Fc was precipitated from conditioned medium using protein A-agarose. Reducing SDS-PAGE revealed a single predominately 105-kDa band, demonstrating that Ang1-Fc does not hetero-oligomerize with another 70-kDa factor (Fig. 4B). The faint, nonstoichiometric faster mobility bands seen in this lane represent proteolytic decay products of Ang1-Fc (data not shown). However, precipitation of Ang1-Fc from conditioned medium of cells co-transfected with pAng1-Fc and pAng1 co-precipitated an additional 70-kDa factor, consistent with the observation that Ang1-Fc and Ang1 hetero-oligomerize (Fig. 4B). We conclude from these observations that soluble Ang1
Ang1 or Ang2 were cultured in the presence of Ang1-Fc (Ang1-Fc), pAng1 (Ang1), or pAng1-Fc and pAng1 (Ang1-Fc + Ang1). Protein A-agarose precipitations were performed on conditioned medium, and SDS-PAGE and Western analysis were performed on precipitates. The blot was probed using an anti-Ang1 antibody reactive with the Ang1 N terminus and imaged using an HRP-conjugated anti-goat secondary antibody and ECL. The arrows identify Ang1-Fc and Ang1. B, transiently transfected 293T cells were cultured in the presence of [35S]cysteine/methionine. Tie2-Fc immunoprecipitations or protein A-agarose precipitations were performed on conditioned medium, and precipitates were separated by reducing SDS-PAGE.

exists in homo-oligomeric complexes and that the molecular mobilities of these complexes on nonreducing SDS-PAGE are most consistent with homotrimers and higher order oligomers.

To examine the oligomeric composition of Ang2, we created pMyc-Ang2 and pHA-Ang2, which encode Ang2 tagged at the amino terminus with a Myc or hemagglutinin epitope, respectively. (An Ang2-Fc chimera was poorly expressed, necessitating an alternate strategy for determining the oligomeric composition of Ang2.) To determine if Ang2 homo-oligomerizes, 293T cells were co-transfected with pMyc-Ang2 and pH-Ang2, and Myc-Ang2 was immunoprecipitated from conditioned medium using an anti-Myc antibody, 9E10. Western analysis of immunoprecipitates probed for the HA epitope demonstrated that HA-Ang2 was co-precipitated with Myc-Ang2 (Fig. 4, lane 4). 9E10 did not immunoprecipitate HA-Ang2 or Ang2 (Fig. 4, lane 2, and data not shown), demonstrating that HA-Ang2 was co-precipitated by Myc-Ang2. Furthermore, HA-Ang2 co-precipitated by Myc-Ang2 formed disulfide-linked oligomeric complexes comparable with Ang2 under nonreducing conditions (Fig. 4, lane 6). We conclude from these data that Ang2, like Ang1, forms homo-oligomeric complexes. Interestingly, Myc-Ang2 was also shown to hetero-oligomerize with and co-precipitate HA-Ang1, demonstrating that Ang1 and Ang2 have the potential to hetero-oligomerize (data not shown). Whether or not Ang1 and Ang2 hetero-oligomerize in vivo is unknown, and the activity of such hetero-oligomers in modulating Tie2 is unknown.

Ang1 and Ang2 Oligomeric Structures Are Modulated by Their Coiled Coil Domains—Nonreducing gels suggest that the oligomeric structures of Ang1 and Ang2 are different (Fig. 3), raising the possibility that this distinguishing feature might be an important determinant of their effect on Tie2. Whereas the mobilities of Ang1 complexes are most consistent with Ang1 existing as trimers and higher order oligomers, mobilities of Ang2 complexes suggest that it forms trimers and higher order oligomers. To determine whether elements in the coiled coil domain of the fibrinogen-like domain determine the pattern of oligomerization, we created pAng1/Fc and pAng1/Fc, which encode chimeric glycoproteins containing the Ang1 coiled coil domain fused to the Ang2 fibrinogen-like domain or the Ang2 coiled coil domain fused to the Ang1 fibrinogen-like domain, respectively (see Fig. 1). 293T cells transiently transfected to express Ang1/2 or Ang2/1 were cultured in the presence of [35S]cysteine/methionine, and radiolabeled Ang1/2 and Ang2/1 were precipitated from conditioned medium using Tie2-Fc. Under reducing conditions, Ang1/2 migrated at 70 kDa, comparable with Ang1, while Ang2/1 migrated at 65 kDa, comparable with Ang2 (Fig. 6, lanes 1–4). Under nonreducing conditions, Ang1/2 yielded an oligomerization pattern virtually identical to that yielded by Ang1, while Ang2/1 yielded a pattern comparable with that of Ang2 (Fig. 6, lanes 11–14), demonstrating that Ang1 and Ang2 oligomerization patterns are determined by their coiled coil domains.

Ang1 and Ang2 Coiled Coil Domains, but Not Their Fibrinogen-like Domains, Are Sufficient to Mediate Oligomerization—That the coiled coil domains determine the pattern of Ang1 oligomerization, we created pAng1/Fc and Ang1/Fb, which encode the Ang1 coiled coil domain and its fibrinogen-like domain, respectively. To determine if either domain alone was sufficient to mediate Ang1 oligomerization, 293T cells were co-transfected with pAng1/Fc and either pAng1/Fc or pAng1/Fc. Ang1/Fc was not precipitated by protein A-agarose from conditioned medium of cells transfected with pAng1/Fc, however, Ang1/Fc was co-precipitated with Ang1/Fc from conditioned medium of cells co-transfected with pAng1/Fc and pAng1/Fc (Fig. 7A, compare lanes 3 and 4). Furthermore, under nonreducing conditions, Ang1/Fc migrated with the disulfide-linked oligomers (Fig. 7A, lane 6). In contrast, Ang1/Fc did not co-precipitate Ang1/Fc from conditioned medium of cells co-transfected with pAng1/Fc and Ang1/Fc (Fig. 7B, lane 4), although easily detectable levels of Ang1/Fc were contained in the conditioned medium (Fig. 7B, lane 5). These observations demonstrate that the Ang1 coiled
FIG. 6. Ang1 and Ang2 oligomerization patterns are modulated by their coiled coil domains. 293T cells transiently transfected with pAng1 (Ang1), pAng2 (Ang2), pAng1/2 (Ang1/2), pAng1cc (Ang1cc), or vector control (Ctrl) were cultured in the presence of [35S]cysteine/methionine. Tie2-Fc or Fc immunoprecipitations were performed on conditioned medium, and immunoprecipitates were electrophoresed under reducing or nonreducing conditions.

FIG. 7. The Ang1 coiled domain, but not its fibrinogen-like domain, is sufficient to mediate Ang1 oligomerization. A, 293T cells were transiently transfected with pAng1-Fc (Ang1-Fc), pAng1cc (Ang1cc), pAng1-Fc and pAng1cc (Ang1-Fc + Ang1cc), or vector control (Ctrl). Protein A-agarose precipitations were performed on conditioned medium, and SDS-PAGE and Western analysis were performed on precipitates. The blot was probed using an anti-Ang1 antibody reactive with the Ang1 N terminus and imaged using an HRP-conjugated anti-goat secondary antibody and ECL. The arrows identify Ang1-Fc and Ang1cc. B, 293T cells were transiently transfected with pAng1-Fc (Ang1-Fc), pAng1fib (Ang1fib), pAng1-Fc and pAng1fib (Ang1-Fc + Ang1fib), or vector control (Ctrl). Protein A-agarose precipitations were performed on conditioned medium, and SDS-PAGE and Western analysis were performed on precipitates. The blot was probed using an anti-Ang1 antibody reactive with the Ang1 C terminus and imaged using an HRP-conjugated anti-goat secondary antibody and ECL. The arrows identify Ang1-Fc and Ang1fib.

coil domain, but not the fibrinogen-like domain, is sufficient to mediate Ang1 disulfide-linked homo-oligomerization. The Ang2 coiled domain, but not its fibrinogen-like domain, was similarly found to be sufficient to mediate its homo-oligomerization (data not shown).

Ang1 Fibrinogen-like Domain Modulates Tie2 Activity—To examine the possibility that the different oligomeric structures of Ang1 and Ang2 determine their effect on Tie2, MS-1 murine endothelial cells were stimulated with Ang1, Ang2, Ang1/2, or Ang2/1. Tie2 was immunoprecipitated from cell lysates using an anti-Tie2 antibody, and Western blots of immunoprecipitates were probed for phosphotyrosine and for Tie2. Background phosphorylation of Tie2 was never entirely eliminated, even when cells were serum-starved overnight. However, as previously demonstrated (4, 9), Ang1, but not Ang2, induced phosphorylation of Tie2 an average of 3-fold in three separate experiments (Fig. 8). Interestingly, Ang2/1, but not Ang2/2, also induced Tie2 phosphorylation an average of 3-fold, comparable with Ang1 (Fig. 8). Ang1/2 binds Tie2 comparably to both Ang1 and Ang2 (Fig. 6 and data not shown); therefore, lack of Tie2 induction does not result from disruption of its Tie2 binding domain. These data demonstrate that the effect of Ang1 and Ang2 on Tie2 is linked to their fibrinogen-like domain. Furthermore, they suggest that the different oligomerization patterns of Ang1 and Ang2 do not determine their effect on Tie2 activity.

DISCUSSION

Here we have shown that secreted Ang1 and Ang2 exist in distinct arrays of disulfide-linked homo-oligomeric complexes. The smallest Ang1 oligomer has a mobility most consistent with a homotrimer but predominantly exists in higher order oligomers. The smallest Ang1 oligomer migrates slightly faster than would be expected for a homotrimer (apparent molecular mass of 196 kDa compared to the expected mass of 210 kDa). Gel mobility is a function of both size and shape; therefore, this aberrant mobility is probably caused by molecular constraints that multiple intermolecular disulfide bonds place on the ability of Ang1 to migrate as linear polypeptide strand. In contrast, the smallest Ang2 oligomer has a mobility most consistent with a homodimer, and only a minority of Ang2 exists in higher order oligomers. We have shown that the coiled coil domains of Ang1 and Ang2, but not their fibrinogen-like domains, are sufficient to mediate oligomerization and furthermore that the distinct oligomerization patterns of Ang1 and Ang2 are linked to their coiled coil domains. Finally, we have shown that the Tie2-agonist property of Ang1 is linked to its fibrinogen-like domain but not its coiled coil domain.

That both Ang1 and Ang2 form higher order disulfide-linked oligomers demonstrates that multiple cysteine residues form intermolecular disulfide bonds. Since Ang1cc forms disulfide bonds with Ang1-Fc, at least one of the cysteine residues flanking the coiled coil domain, at position 41, 54, or 265 of Ang1, must form an intermolecular disulfide bond. Similarly, at least one of the coiled coil domain-flanking cysteines at positions 41 and 53 of Ang2 must form intermolecular disulfide bonds in order for Ang2cc to disulfide-link to Ang2. Although more than one of these cysteine residues may form disulfide bonds, we...
have not ruled out the possibility that cysteine residues located in the fibrinogen-like domain also form intermolecular disulfide bonds. However, since the fibrinogen-like domain alone did not mediate oligomerization, any such disulfide bonds formed by cysteine residues within this domain must require the presence of the coiled coil domain.

The coiled coil domains of Ang1 and Ang2 are 63% identical and 91% similar, allowing for conservative amino acid substitutions, and both domains were predicted by the MultiCoil program to oligomerize (33). The probability that the Ang1 coiled coil oligomerizes was predicted to be greater than 95%, while Ang2 was scored as having approximately an 80% probability of oligomerizing. However, both Ang1 and Ang2 are predicted to dimerize and not to form trimers. It is, therefore, surprising that the smallest molecular weight Ang1 oligomer on nonreducing gels appears to migrate as a homotrimer and has a mobility that is clearly distinct from the smallest Ang2 oligomer, although we have not formally ruled out the possibility that Ang1 forms homodimers that migrate aberrantly on polyacrylamide gel electrophoresis. The higher molecular weight Ang1 and Ang2 multimers also have distinct mobilities, and the different relative amounts of lower and higher molecular weight oligomers suggests that Ang1 has a greater propensity to multimerize than Ang2. Some oligomerization domains show a greater propensity to oligomerize or polymerize at higher concentrations (34), and we have not examined whether Ang1 and Ang2 oligomerization patterns are concentration-dependent. However, such concentration dependence would not explain the greater propensity of Ang1 to multimerize, because the concentration of Ang2 in conditioned medium of transiently transfected cells was approximately 10-fold greater than that of Ang1 (data not shown).

The homo-oligomeric structure of Ang1 suggests that it activates Tie2 by bridging receptor molecules. However, simply bridging receptor molecules appears to be insufficient to activate endothelial-Tie2, because Ang2 homo-oligomers, which presumably bridge Tie2 receptors, do not activate endothelial-Tie2. Furthermore, the specific pattern of oligomerization was not linked to the ligands’ ability to induce Tie2 phosphorylation.

Ang1/2 had an oligomerization pattern indistinguishable from Ang1 yet did not induce Tie2 phosphorylation, while Ang2/1 had an oligomerization pattern similar to Ang2 yet did not induce Tie2 phosphorylation, while Ang1/2 had an oligomerization pattern indistinguishable from Ang1 yet did not induce Tie2 phosphorylation. The differences in oligomerization patterns raise the possibility that Ang1 and Ang2, besides binding and bridging of receptor molecules, do not activate endothelial-Tie2 (33).

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