

Angiopoietins 3 and 4: Diverging gene counterparts in mice and humans

(angiogenesis/tie receptor tyrosine kinase/vascular endothelial growth factor)

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ABSTRACT The angiopoietins have recently joined the members of the vascular endothelial growth factor family as the only known growth factors largely specific for vascular endothelium. The angiopoietins include a naturally occurring agonist, angiopoietin-1, as well as a naturally occurring antagonist, angiopoietin-2, both of which act by means of the Tie2 receptor. We now report our attempts to use homology-based cloning approaches to identify new members of the angiopoietin family. These efforts have led to the identification of two new angiopoietins, angiopoietin-3 in mouse and angiopoietin-4 in human; we have also identified several more distantly related sequences that do not seem to be true angiopoietins, in that they do not bind to the Tie receptors. Although angiopoietin-3 and angiopoietin-4 are strikingly more structurally diverged from each other than are the mouse and human versions of angiopoietin-1 and angiopoietin-2, they appear to represent the mouse and human counterparts of the same gene locus, as revealed in our chromosomal localization studies of all of the angiopoietins in mouse and human. The structural divergence of angiopoietin-3 and angiopoietin-4 appears to underlie diverging functions of these counterparts. Angiopoietin-3 and angiopoietin-4 have very different distributions in their respective species, and angiopoietin-3 appears to act as an antagonist, whereas angiopoietin-4 appears to function as an agonist.

Two families of growth factors have been identified that are largely specific for the vascular endothelium, by virtue of having receptors that are mostly restricted to endothelial cells in their expression. These include vascular endothelial growth factor (VEGF) and its relatives, as well as the more recently discovered angiopoietins (1, 2). These two families seem to work in complementary and coordinated fashion during vascular development, with VEGF acting during the early stages of vessel development (3–5), and angiopoietin-1 (6) acting later to promote angiogenic remodeling as well as vessel maturation and stabilization (7, 8). Simply overexpressing angiopoietin-1 by using transgenic approaches leads to hypervascularization *in vivo* (9), suggesting that angiopoietin-1 is present in limiting quantities during angiogenesis *in vivo*. Angiopoietin-1 has a naturally occurring antagonist, termed angiopoietin-2, which blocks the ability of angiopoietin-1 to activate its receptor, Tie2 (10). Transgenic overexpression of angiopoietin-2 disrupts normal vascular development and results in early embryonic lethality (10). Angiopoietin-2 is the first example of a natural antagonist for a receptor tyrosine

kinase in vertebrates, suggesting that activation of the Tie2 receptor must be particularly well titrated *in vivo*. Evidence suggests that such careful regulation might be involved at sites of vascular change in an otherwise stable adult vasculature. Thus, whereas angiopoietin-1 appears to be widely expressed in the adult in a manner suggesting that it is acting to stabilize vessels, angiopoietin-2 is expressed only at sites of vascular remodeling in the adult (10). It has been suggested that this expression of the antagonist blocks an otherwise constitutive stabilizing signal provided by angiopoietin-1, leading to localized vessel destabilization (10, 11). Vessel destabilization induced by angiopoietin-2 may contribute to either vessel regression or new vascular sprouting in the adult; regression seems to occur when angiopoietin-2 is expressed in the absence of VEGF, although new sprouting occurs when angiopoietin-2 is expressed together with VEGF (10).

Remarkably, angiopoietin-2 acts only as an antagonist of angiopoietin-1 on endothelial cells. In the limited situations in which Tie2 is normally expressed outside of the vasculature, such as on primitive hemopoietic cells, or when Tie2 is ectopically expressed in nonendothelial cells, such as on fibroblasts, angiopoietin-2 acts as an agonist equivalent to angiopoietin-1 (10, 12).

The angiopoietins can be structurally divided into three domains: an N-terminal region lacking homology to any known structures, an alpha-helical rich coiled-coil segment similar to motifs found in many proteins that seem to promote multimerization, and a “fibrinogen-like domain,” thus dubbed because it is distantly related to a domain found first in fibrinogen but now noted to be in many other proteins (6). The fibrinogen-like domain (FD) represents the most conserved region of the angiopoietins, and recent studies indicate that it comprises the receptor-binding portion of an angiopoietin (S.D., T.H.A., J. Goldberg, P.C.M., N.P., T. Daly, and G.D.Y., unpublished results). In addition, all the information that determines whether an angiopoietin is an agonist or antagonist seems to reside within the fibrinogen-like domain (S.D., *et al.*, unpublished results); when chimeric molecules are made in which the fibrinogen-like domains of angiopoietin-1 and angiopoietin-2 are swapped, agonistic or antagonistic abilities track with the fibrinogen-like domains. The N-terminal and coiled-coil regions seem to serve mainly to multimerize the fibrinogen-like domains, which apparently must be clustered to be active (S.D., *et al.*, unpublished results). In fact, the N-terminal and coiled-coil regions can be substituted for by alternative motifs that

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Abbreviations: VEGF, vascular endothelial growth factor; FD, fibrinogen-like domain; Fc, constant region; BAC, bacterial artificial chromosome.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AF113707 and AF113708).

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allow clustering. Thus, the activities of angiopoietin-1 and angiopoietin-2 can be precisely mimicked by surrogates in which the FDs of these factors are fused to the constant region (Fc) of an antibody, resulting in 'FD-Fc' fusions, which can then be clustered by using secondary antibodies directed against the Fc (S.D., *et al.*, unpublished results). One practical advantage of such surrogates is that the native angiopoietins can be difficult to produce recombinantly, although the surrogates can be more easily produced.

A recent expansion in the VEGF family has been fueled by homology-based cloning approaches (13). Here we describe the use of similar approaches to expand the angiopoietin family. These efforts have led to the identification of two new angiopoietins, angiopoietin-3 cloned from mouse sources and angiopoietin-4 cloned from human sources, as well as to the identification of several more distant angiopoietin homologs that do not seem to be true angiopoietins, in that they do not bind to the angiopoietin receptors. Although angiopoietin-3 and angiopoietin-4 are strikingly more structurally diverged from each other than are the mouse and human versions of angiopoietin-1 and angiopoietin-2, they appear to represent the mouse and human counterparts of the same gene locus, as revealed in our chromosomal localization studies of all of the angiopoietins in mouse and human. The structural divergence of angiopoietin-3 and angiopoietin-4 appears to underlie diverging functions of these counterparts. Angiopoietin-3 and angiopoietin-4 have very different distributions in their respective species, and angiopoietin-3 appears to act as an antagonist although angiopoietin-4 appears to function as an agonist.

METHODS

Cloning of Mouse Angiopoietin-3, Human Angiopoietin-4, and Distant Angiopoietin Homologs. Probes spanning the full coding regions of mouse angiopoietin-1 and angiopoietin-2 were radiolabeled by PCR as described previously (14) and used separately for low stringency hybridization of replica filters of an arrayed bacterial artificial chromosome (BAC) mouse genomic library (Research Genetics catalogue no. 96050, Huntsville, AL). The filters were hybridized overnight in phosphate buffer at 55°C, washed at room temperature and then at 55, 60 and 65°C in 2× SSC (1× SSC = 0.15 M sodium chloride/0.015 M sodium citrate, pH 7), as described previously (14). DNAs from hybridizing BAC clones were subjected to Southern hybridization with probes corresponding to the fibrinogen-like domains of mouse angiopoietin-1 and angiopoietin-2; hybridizing fragments were purified, subcloned into vector pCRScript (Stratagene catalogue no. 211190-S), and sequenced. In addition to fragments corresponding to angiopoietin-1 and angiopoietin-2, fragments representing a new putative angiopoietin, angiopoietin-3, were found. These fragments were used as probes to screen a mouse uterus cDNA library in λ gt11 (CLONTECH catalogue no. ML1022B) resulting in a nearly full-length cDNA for angiopoietin-3; the region encoding the first 38 amino acids of mouse angiopoietin-3 had to be obtained subsequently from a C2C12 myoblast cDNA library by PCR amplification by using Ang3 specific primers together with library primers.

In an attempt to isolate the human counterpart of mouse angiopoietin-3, radiolabeled probes spanning the fibrinogen-like domains of mouse angiopoietin-3 and human angiopoietin-1 (as a control) were used separately to hybridize replicas of a human BAC genomic library (Genome Systems, St. Louis, catalogue no. FBAC-4432). A hybridizing BAC clone, characterized as above, seemed to encompass the entire coding region of an apparently novel angiopoietin gene, initially judged to be novel based on its divergence from angiopoietin-3, and we thus termed it angiopoietin-4. A full-length cDNA clone for Ang4 was obtained by PCR amplification

from human ovary cDNA prepared from ovary RNA (CLONTECH) by using the SuperScript Preamplification System (GIBCO/BRL).

In the course of the above screens, we identified two distant angiopoietin relatives that do not appear to be actual members of this family (see below). Human angiopoietin X was initially identified in the above screen of the human BAC genomic library, and a full-length cDNA clone for human angiopoietin X was then obtained as described above for Ang4. Mouse angiopoietin Y was initially identified during a PCR screen for new angiopoietins by using degenerate primers for conserved angiopoietin sequences PSGEYW and WWFDAC (corresponding to amino acids 351 to 356 and 447 to 452 of the angiopoietin-1 sequence in Fig. 1); a PCR fragment corresponding to mouse angiopoietin Y was then used as a probe to identify a partial cDNA clone from the mouse uterus cDNA library (CLONTECH catalogue no. ML1022B).

Northern Analysis. DNA probes for Ang3 and Ang4 (spanning amino acids 51 to 270 and 1 to 288, respectively, in Fig. 1) were used for hybridization of mouse (CLONTECH 7762-1) and human (CLONTECH 7760-1, 7759-1, and 7767-1) Poly(A)⁺ multiple tissue Northern blots.

Expression of Angiopoietin Chimeras and FD-Fc Fusions. Expression constructs were designed to express chimeric angiopoietins or FD-Fc fusions, in which the fibrinogen-like domains of Ang3, Ang4, and AngX were either substituted for those of Ang2 to make chimeric angiopoietins or fused to the Fc portion of human IgG1 to make FD-Fc fusion proteins.

Receptor Binding and Activation Studies. Binding of Tie1-Fc or Tie2-Fc to cells expressing the various angiopoietins or binding of FD-Fc fusions to cells expressing the Tie receptors or to BIAcore chips containing immobilized Tie1-Fc or Tie2-Fc was performed as previously described, as were assays of Tie2 receptor phosphorylation assays in endothelial cells and fibroblasts (6, 10).

Localization in Mouse Chromosomes. Chromosomal localization of the mouse angiopoietins was performed by interspecific backcross hybridization. Probes for the backcross hybridizations were as follows: mouse angiopoietin-1: a 350-bp segment from the 5' untranslated sequence; angiopoietin-2: an approximately 320-bp fragment from a rat cDNA corresponding to amino acids 374 to 479 in Fig. 1; angiopoietin-3: an approximately 2-kb fragment encompassing the entire coding sequence. Generation of mice used for the mapping, protocols, and strategy has been detailed previously (15, 16).

Localization in Human Chromosomes. Mapping of the human angiopoietins 1, 2, and 4 was performed by using fluorescence *in situ* hybridization by Genome Systems, by using human genomic clones as probes (hgENTL-1, an Ang1 λ clone in the EMBL-3 vector; P1 6500, an Ang2 P1 clone obtained by Genome Systems, and hBAC F221, a Ang4 BAC clone obtained from Genome Systems). In all cases, the DNA was labeled with digoxigenin dUTP and hybridized to normal metaphase chromosomes derived from peripheral blood lymphocytes.

RESULTS

Cloning of New Angiopoietins: Angiopoietins 3, 4, and Distant Homologs. Low-stringency hybridization techniques were used to identify new members of the angiopoietin family. Initially, clones from a mouse genomic BAC library array were selected that hybridized, at low stringency, to probes for both mouse angiopoietin-1 and angiopoietin-2. A short genomic fragment obtained in this manner, corresponding to a putatively novel angiopoietin, was then used to obtain a full-length cDNA encoding an angiopoietin relative we termed mouse angiopoietin-3 (Fig. 1A; see *Methods* for details).

To identify the human ortholog of mouse angiopoietin-3, we used probes corresponding to the fibrinogen-like segment of

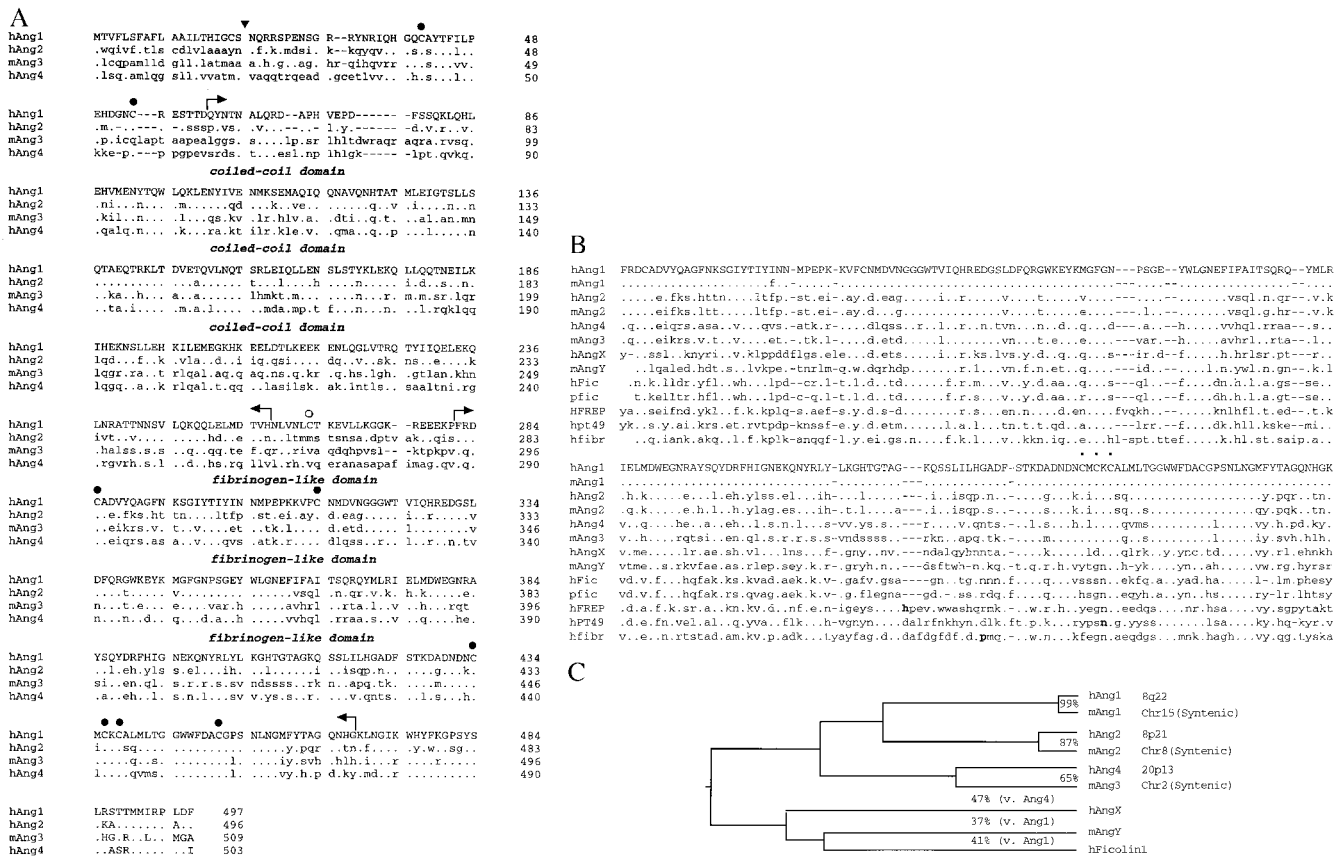


FIG. 1. Amino acid sequences and evolutionary relationships of the angiopoietins and their distant relatives. (A) Full-length sequences of the definitive angiopoietins as aligned by the CLUSTAL method by using the MEGALIGN program from DNAsStar; arrowhead marks the predicted signal peptide cleavage site, bent arrows indicate the limits of the coiled-coil and fibrinogen-like domains, closed circles denote conserved cysteines, and the open circle marks a cysteine present in Ang1 but absent in all other angiopoietins. (B) Alignment of the conserved fibrinogen-like domain of all definitive members of the angiopoietin family with the fibrinogen-like domains of the more distant angiopoietin homologs described here (AngX and AngY), as well as of previously described members of the fibrinogen superfamily (i.e., ficolin-a, ficolin-b, hFrep, hPT49, and human fibrinogen γ); dots mark the three distinctive cysteines that are present in the angiopoietin family but absent from AngX and AngY, as well as all other members of the fibrinogen superfamily. For ease of alignment, some amino acids were deleted in the alignments: in PT49 a "g" was deleted after the boldfaced "n," in FREP the sequence "dslnagnf" was deleted before the boldfaced "h," and in fibrinogen the sequence "sdkffstshng" was deleted after the boldfaced "p." (C) Cladogram (built by the CLUSTAL method by using the MEGALIGN program from DNAsStar) comparing the evolutionary relationships between members of the angiopoietin family and the distant relatives depicted in B; also indicated are the percent amino acid identity between paired orthologs or between the distant relatives and their closest angiopoietin homolog (only within the fibrinogen-like domain) as well as the chromosomal localizations of the paired ortholog in both mouse and human.

both mouse angiopoietin-3 and human angiopoietin-1 to screen replicas of a human genomic BAC library array at low stringency. Clones hybridizing to both probes were analyzed, and one clone was found to contain the entire coding region of an apparently novel angiopoietin gene, termed human angiopoietin-4; a full-length cDNA for human angiopoietin-4 was then obtained (Fig. 1A; see Methods for details). Although human angiopoietin-4 was closest to mouse angiopoietin-3 as compared with the other angiopoietins (Fig. 1C), it was initially assumed that it did not correspond to the human counterpart of mouse angiopoietin-3, as it was much more diverged from mouse angiopoietin-3 than the other human angiopoietins were from their mouse counterparts (Fig. 1C, but see below). Both angiopoietin-3 and angiopoietin-4 share all the main structural characteristics of angiopoietin-1 and angiopoietin-2; they maintain notable homology throughout the signal peptide, N-terminal region, coiled-coil segment, as well as the fibrinogen-like domain (Fig. 1A).

In addition to angiopoietin-3 and angiopoietin-4, the above screens identified a number of additional candidate angiopoietins (e.g., see sequences labeled AngX and AngY in Fig. 1B and C). In contrast to angiopoietin-3 and angiopoietin-4, these candidates were homologous to the angiopoietins only within

their fibrinogen-like domains, and they were not substantially closer to the angiopoietins within this domain than were the fibrinogen-like domains of other proteins such as Ficolin or Fibrinogen λ (Fig. 1B and C).

Angiopoietins 3 and 4, but Not Distant Angiopoietin Homologs, Bind to Tie Receptors. Angiopoietin-1 and angiopoietin-2 mediate their actions by binding the Tie2 receptor tyrosine kinase. Neither of these binds to the closely related Tie1 receptor, for which no known ligand exists. To see whether angiopoietin-3 and angiopoietin-4 or the more distant angiopoietin homologs bound to either of the Tie receptors, a variety of binding assays was performed by using either the native candidate ligands, chimeric ligands, or FD-Fc fusions of these ligands; the assays included the binding of ligands or their derivatives to cells expressing the Tie receptors or to BIAcore chips on which Tie receptors had been immobilized (Fig. 2 and data not shown). In all cases, angiopoietin-3 and angiopoietin-4, as well as chimeras or FD-Fc fusions containing the FD domains from these angiopoietins, bound to the Tie2 receptor but not the Tie1 receptor, although AngX did not exhibit binding to either Tie receptor in any assay. The binding of angiopoietin-3 and angiopoietin-4 or their chimeras could be competed by using excess amounts of soluble Tie2 receptors, but not soluble Tie1 receptors or other irrelevant soluble

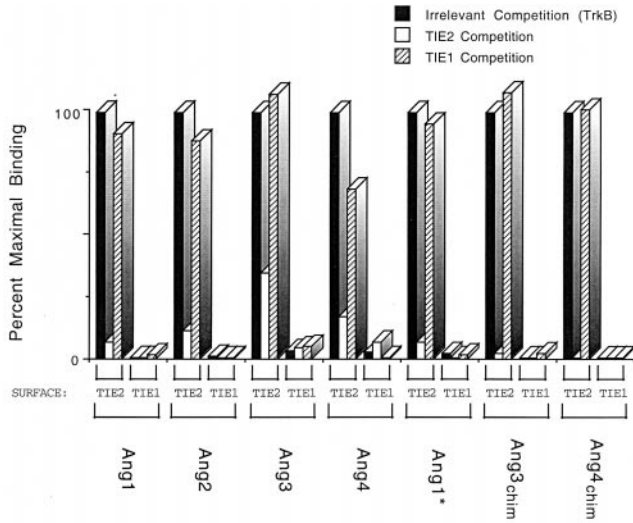


FIG. 2. BIAcore assay for evaluation of angiopoietin-3 and -4 binding to the Tie1 and Tie2 receptors. BIAcore binding assays of the indicated angiopoietins or chimeric angiopoietins (having the fibrinogen-like domains of angiopoietin-3 or -4; as noted, binding of chimeric angiopoietins is determined by the fibrinogen-like domain) to BIAcore chips coated with immobilized Tie1 or Tie2 receptors was assessed in the presence of competition with excess amounts of either an irrelevant soluble receptor (TrkB) or soluble Tie1 or Tie2 receptors (6). For all the angiopoietins, binding is noted only to the Tie2 surface and not the Tie1 surface; consistent with this observation, only soluble Tie2 receptors compete for binding to the surface.

receptors (Fig. 2 and data not shown). Thus angiopoietins 3 and 4 appear to be true members of the angiopoietin family as judged by specific binding to the Tie2 receptor, whereas the more distant homologs appear to be proteins with unrelated

functions that simply have distantly related fibrinogen domains.

Interestingly, inspection of the fibrinogen-like domains of the angiopoietins as compared with the more distant homologs reveals one distinctive structural feature that specifically distinguishes the fibrinogen-like domains in the angiopoietins from those in other proteins. Specifically, the angiopoietins all maintain a pattern of three closely spaced cysteines (Fig. 1B); AngX has two of these cysteines, whereas all other fibrinogen-like domains contain only the last of these cysteines.

Angiopoietin-3 and Angiopoietin-4 Represent Counterpart Gene Loci in Mouse and Human. As noted above, we initially assumed that mouse angiopoietin-3 and human angiopoietin-4 were not orthologs, because they were much more diverged from each other (sharing only 54% overall amino acid identity, and 65% identity within their fibrinogen-like domains) than were the mouse and human counterparts of angiopoietins 1 and 2 (which shared 99% and 87% amino acid identity within their FDs, respectively) (Fig. 1C). However, continued efforts to clone the human counterpart of angiopoietin-3 proved unproductive, repeatedly leading to recloning of angiopoietin-4; similarly, angiopoietin-3 was repeatedly cloned in attempts to obtain the mouse counterpart of angiopoietin-4. These findings raised the possibility that mouse angiopoietin-3 and human angiopoietin-4 were indeed counterparts, but counterparts that were diverging much more rapidly than those of the other angiopoietins. To explore this possibility further, we decided to determine the chromosomal positions of the genes for all the murine and human angiopoietins. The results, depicted in Fig. 3 and also summarized in Fig. 1C, reveal that angiopoietin-3 localizes to mouse chromosome 2 in a position syntenic to the human angiopoietin-4 locus at 20.13. Thus, although it remains possible that we have missed adjacent genes also located at these chromosomal positions that are the true counterparts, these mapping results together with the

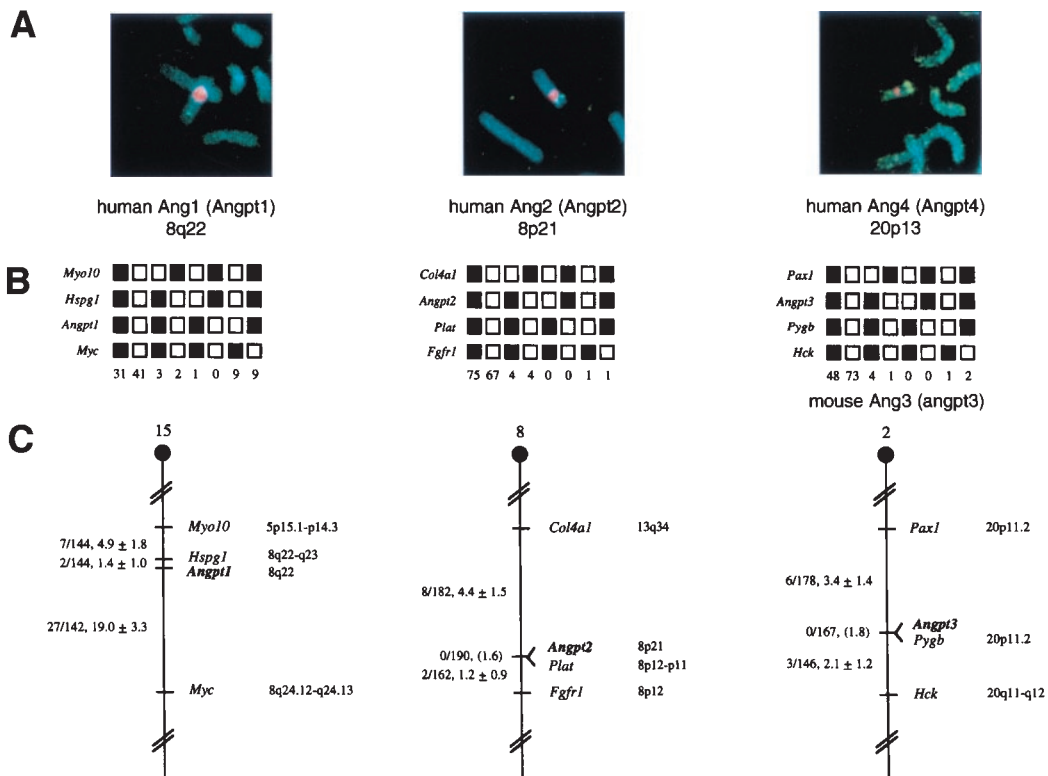


FIG. 3. Human and mouse chromosomal mapping of the angiopoietin gene family. (A) Fluorescent *in situ* hybridization images of digoxigenin-labeled probes for human angiopoietin-1, human angiopoietin-2, and human angiopoietin-4 to human chromosomes in metaphase cells from phytohemagglutinin-stimulated human peripheral blood lymphocytes. The designated name and hybridization loci are denoted underneath each panel. (B and C) Mouse chromosome linkage maps for murine angiopoietin-1, angiopoietin-2, and angiopoietin-3.

inability to identify closer orthologs strongly suggest that angiopoietin-3 and angiopoietin-4 do indeed represent counterpart gene loci that have diverged greatly in mouse and human.

Differing Tissue Distributions of Angiopoietin-3 and Angiopoietin-4. Consistent with the diverging structures of angiopoietin-3 and angiopoietin-4, the tissue distributions of the transcripts for these two genes differed dramatically. Thus, a prominent 2.5-kb transcript for angiopoietin-3 was noted in multiple mouse tissues (see Fig. 4A); also, in some tissues, a smaller transcript of about 1.1 kb can be seen. In contrast, transcripts for angiopoietin-4 were specifically noted at high levels only in human lung (Fig. 4B), with much lower levels seen in other human tissues. The differing tissue distributions of angiopoietin-3 and angiopoietin-4 suggest that in addition to their diverging structures, these two angiopoietins might be playing dramatically different roles in their respective species.

Angiopoietin-3 and Angiopoietin-4 Have Opposing Activities on the Tie2 Receptor. The first two members of the angiopoietin family were found to have differing activities, one being an activator and the other an antagonist of endothelially expressed Tie2 receptors. To determine the actions of angiopoietin-3 and/or angiopoietin-4 on Tie2 receptors, we tried to express each of them recombinantly. As noted above, since each expressed rather poorly in native form, we instead expressed each as either a chimeric angiopoietin or an FD-Fc fusion protein, in a manner we had previously shown retained the biological activity of the angiopoietin from which the fibrinogen-like domain was derived (S.D., T.H.A., J. Goldberg, P.C.M., N.P., T. Daly & G.D.Y., unpublished results). Thus, chimeras were made in which the N-terminal and coiled-coil segments of angiopoietin-2 were fused to the fibrinogen-like domain of either angiopoietin-3 or angiopoietin-4 (to generate Ang3^{Chim} or Ang4^{Chim} chimeras); such chimeras, which use the N-terminal portions of angiopoietin-2, have been shown to express quite well while retaining the biological activities of the incorporated fibrinogen-like domain (S.D., *et al.*, unpublished results). Additionally, fusions were made in which the fibrinogen-like domains of angiopoietin-3 and angiopoietin-4 were fused to the constant region of the human IgG heavy chain constant region, resulting in FD-Fc fusions that have been shown to retain their biological activities on clustering with a secondary antibody (S.D., *et al.*, unpublished results).

The chimeras and fusions involving angiopoietin-4 could clearly activate Tie2 receptors expressed in human endothelial cells, although those involving angiopoietin-3 could not (Fig. 5A and data not shown). Because angiopoietin-3 is a mouse protein, we decided to rule out the possibility that angiopoietin-3 was acting only in a mouse-specific manner, and thus we

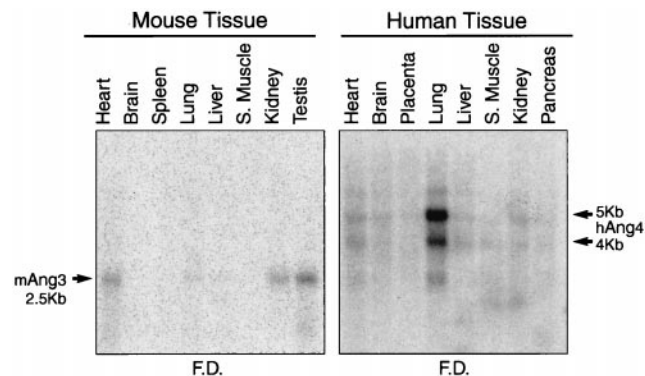


FIG. 4. Differing tissue distributions of angiopoietin-3 in mice and angiopoietin-4 in human. Depicted are Northern blots containing Poly(A)⁺ RNA from mouse tissues hybridized to a mouse angiopoietin-3 probe (Left) or from human tissues hybridized to a human angiopoietin-4 probe (Right).

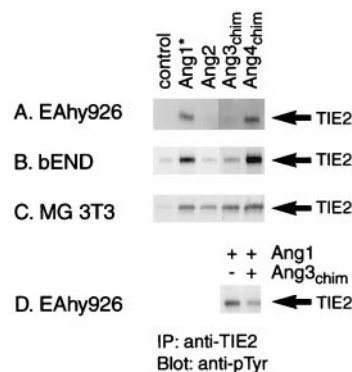


FIG. 5. Differing biological activities of mouse angiopoietin-3 as compared with human angiopoietin-4. Angiopoietin chimeras containing the angiopoietin-4 fibrinogen-like domain activate Tie2 receptors in either human (A) or mouse (B) endothelial cells as well as in mouse fibroblasts (C), whereas angiopoietin chimeras containing the angiopoietin-3 fibrinogen-like domain cannot activate Tie2 receptors in either human (A) or mouse (B) endothelial cells, although they can in mouse fibroblasts (C). Furthermore, excess amounts of chimeras containing the angiopoietin-3 fibrinogen-like domain can blunt the ability of angiopoietin-1 to activate the Tie2 receptor (D). Thus, whereas angiopoietin-4 derivatives act as agonists (like angiopoietin-1), angiopoietin-3 derivatives act as context-dependent antagonists (like angiopoietin-2).

tried similar assays on murine endothelial cells with similar results (Fig. 5B and data not shown). These results suggested that although angiopoietin-4 was an activator of the Tie2 receptor, angiopoietin-3 was not and might instead act as an antagonist. To verify that the angiopoietin-3 constructs were functional, we took advantage of the finding that angiopoietin antagonists appear to function as such only on endothelial cells, but act as Tie2 activators when the receptors are expressed on nonendothelial cells (10). Thus, we were able to show that the chimeras and fusions with either angiopoietin-3 or angiopoietin-4 were able to activate Tie2 receptors expressed in fibroblasts (Fig. 5C). Evidence that angiopoietin-3 could act as an antagonist was obtained by showing that an excess of the angiopoietin-3 chimera, Ang3^{Chim}, could blunt the ability of Ang1 to activate the Tie2 receptor (Fig. 5D); formal proof that native angiopoietin-3 can indeed act as an antagonist awaits production of high enough concentrations of the native ligand to be directly evaluated in blocking studies. However, taken together, the current evidence indicates that human angiopoietin-4 is an agonist for the Tie2 receptor, as is angiopoietin-1, although mouse angiopoietin-3 is a context-dependent antagonist, as is angiopoietin-2.

DISCUSSION

We report on the cloning of additional angiopoietins by using homology-based cloning strategies in which arrayed BAC libraries were screened under low-stringency hybridization conditions. Our efforts resulted in the cloning of mouse angiopoietin-3 and human angiopoietin-4, as well as more distant angiopoietin-related sequences that do not appear to be true angiopoietins. That is, both angiopoietin-3 and angiopoietin-4 share all the main structural characteristics of angiopoietin-1 and angiopoietin-2, maintaining notable homology throughout the signal peptide, N-terminal region, and coiled-coil segment, as well as the fibrinogen-like domain; in addition, they retain a signature cysteine-based motif in their fibrinogen-like domains that seems to mark angiopoietins and not other fibrinogen-related sequences. Furthermore, and just as importantly, angiopoietin-3 and angiopoietin-4 could both be shown to bind the Tie2 receptor. In contrast, the more distant angiopoietin-like sequences, termed AngX and AngY,

are notably homologous to the true angiopoietins only within their fibrinogen-like domains, and within this domain they are not substantially closer to the angiopoietins than they are to the fibrinogen-like domains of other proteins; in addition, they lack the signature cysteine-based motif within this domain. Furthermore, binding of these distant relatives could not be detected in either Tie receptor. Recently, AngX was independently cloned as a cornea-specific gene product termed CDT6 and termed a potential antiangiogenic factor based on its distant homology to the angiopoietins (17); however, our analysis suggests that since it is not a true member of the angiopoietin family, it is no more likely to have an angiogenic role than any of the other nonangiopoietin members of the fibrinogen domain-containing superfamily.

We initially assumed that angiopoietin-3 and angiopoietin-4 did not represent the mouse and human counterparts of the same gene, because they were much less similar to each other than were the mouse and human counterparts of angiopoietins 1 and 2. However, the repeated cloning of human angiopoietin-4 when using angiopoietin-3 as a probe, as well as the repeated cloning of mouse angiopoietin-3 when using angiopoietin-4 as a probe, forced us to reconsider the possibility that angiopoietin-3 and angiopoietin-4 might represent counterparts in mouse and human. To explore this possibility further, chromosomal localization studies of all the known angiopoietins were performed in mouse and human, leading to the realization that the angiopoietin-3 locus in mouse was indeed syntenic to the angiopoietin-4 locus in human. Although angiopoietin-3 and angiopoietin-4 appear to represent counterparts of the same gene in mouse and human, their structural divergence seems to be associated with dramatic differences in the function of the gene products in the two species. Thus angiopoietin-3 appears to be a widely expressed context-dependent antagonist, whereas angiopoietin-4 is a Tie2 receptor agonist that is expressed only in lung. It should be noted, however, that receptor activation assays for these ligands were performed by using not the native proteins, but chimeric versions that were easier to produce; formal proof of the activities of native angiopoietin-3 and angiopoietin-4 awaits production of high enough concentrations of the native ligands to be evaluated directly in receptor activation studies. Altogether, the current evidence suggests that angiopoietin-3 and angiopoietin-4 provide a striking example of structural and functional divergence of a gene locus between mouse and human.

Our exhaustive screening for angiopoietin homologs, including the low-stringency hybridization genomic screening approaches described here as well as PCR-based homology screens that have not been reported, have yielded the four definitive angiopoietins thus far described. Because these approaches have led to repetitive cloning of these four angiopoietins, as well as of significantly more distant relatives that primarily share homology only within the fibrinogen-like domains and that do not appear to bind to either of the Tie receptors, it seems quite possible that there are only four definitive members of the angiopoietin family. Notably, all the defined angiopoietins bind and act on the Tie2 receptor, whereas no angiopoietin has been described that binds to the Tie1 receptor. Although it is possible that additional angiopoietins will still be found that act primarily on the Tie1 receptor, or that binding studies using known angiopoietins to Tie1 have not been correctly performed and thus have missed

binding, it is worthwhile considering other possibilities. Thus, Tie1 may bind to angiopoietins only after they have already engaged the Tie2 receptor, resulting in heteromerization that modulates Tie2 activity. Alternatively, Tie1 may have no actual ligand, but may act in some other way to modify activity of the Tie system. In any case, further examination of the roles of the four angiopoietins and how they interact with their Tie receptors should provide important additional insights into the modulation of angiogenesis by this family of factors. In addition, definition of the chromosomal positions of these four important angiogenic regulators may allow association of angiopoietin gene defects with particular pathologic states, as has been noted for Tie2 gene defects in venous malformations (18).

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