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# Isolation of Angiopoietin-1, a Ligand for the TIE2 Receptor, by Secretion-Trap Expression Cloning

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## Summary

**TIE2 is a receptor-like tyrosine kinase expressed almost exclusively in endothelial cells and early hemopoietic cells and required for the normal development of vascular structures during embryogenesis. We report the identification of a secreted ligand for TIE2, termed Angiopoietin-1, using a novel expression cloning technique that involves intracellular trapping and detection of the ligand in COS cells. The structure of Angiopoietin-1 differs from that of known angiogenic factors or other ligands for receptor tyrosine kinases. Although Angiopoietin-1 binds and induces the tyrosine phosphorylation of TIE2, it does not directly promote the growth of cultured endothelial cells. However, its expression in close proximity with developing blood vessels implicates Angiopoietin-1 in endothelial developmental processes.**

## Introduction

Embryonic vascular development involves a complex series of events during which endothelial cells differentiate, proliferate, migrate, and undergo morphologic organization in the context of their surrounding tissues (Risau, 1991, 1995). Vascular development is generally classified into two successive phases. The first, known as vasculogenesis, refers to the process whereby newly differentiated endothelial cells spontaneously coassemble into tubules that fuse to form a rather homogeneous primary vasculature in the embryo. Subsequent remodeling of this primary vascular network into large and small vessels brings into play a different process, termed angiogenesis. Angiogenesis in the embryo also leads to the sprouting of vessels into initially avascular organs, such as the brain. In the adult, angiogenesis accounts for neovascularization that accompanies the normal processes of ovulation, placental development, and wound healing, as well as various clinically significant pathologic processes such as tumor growth and diabetic retinopathy (Ferrara, 1995; Folkman, 1995; Hanahan and Folkman, 1996).

Intercellular signaling mechanisms that govern the formation of blood vessels have only recently begun to be studied at the molecular level. Two families of receptor tyrosine kinases have been identified whose expression is largely restricted to endothelial cells and which are essential for normal development of blood vessels (Mustonen and Alitalo, 1995). One family includes Flt-1, Flt-4, and Flk-1/KDR, all of which are members of the vascular endothelial growth factor (VEGF) receptor family. The requisite roles of Flt-1 and Flk-1 during vascular development, as well as that of VEGF, have been confirmed by analysis of genetically engineered mice lacking these proteins (Fong et al., 1995; Shalaby et al., 1995; Carmeliet et al., 1996; Ferrara et al., 1996). The more recently discovered TIE receptor family (Dumont et al., 1992; Partanen et al., 1992; Iwama et al., 1993; Maisonpierre et al., 1993; Sato et al., 1993; Schnurch and Risau, 1993; Ziegler et al., 1993), consisting of TIE1 and TIE2 (also termed Tek), also have been found to be critically involved in the formation of vasculature (Dumont et al., 1994; Puri et al., 1995; Sato et al., 1995). Mice deficient in TIE1 die between embryonic day 13.5 (E13.5) and birth and display edema and hemmorhage resulting from poor structural integrity of the endothelial cells (Puri et al., 1995; Sato et al., 1995). In contrast, mice deficient in TIE2 have an earlier lethal phenotype and die by E10.5 (Dumont et al., 1994; Sato et al., 1995). The most prominent defects observed in these mice include the failure of the endothelial lining of the heart to develop properly, the failure of remodelling of the primary capillary plexus into large and small vessels, and the lack of capillary sprouts into the neuroectoderm. In addition to their expression by endothelial cells, the TIEs are also specifically expressed in early hemopoietic stem cells (Iwama et al., 1993; Batard et al., 1996; Hashiyama et al., 1996), perhaps reflecting the origin of both lineages from a common hemangioblast precursor (Shalaby et al., 1995); however, the early death of mice lacking the TIEs has limited the use of these mice in elucidating the precise roles of the TIEs in hemopoiesis (Rodewald and Sato, 1996). Because the TIE receptor family is critically involved in angiogenesis and may play a role in hemopoiesis as well, we initiated a search for ligands that may activate these receptors. Here we describe the use of a novel expression cloning strategy to identify a secreted ligand for the TIE2 receptor, which we designate Angiopoietin-1 to reflect not only its requisite role in angiogenesis (Suri et al., 1996 [this issue of *Cell*]) but also its potential actions during hemopoiesis.

Searches for the ligands for orphan receptors have traditionally proceeded by several routes, depending on the type of ligand that is sought. In the case of secreted ligands, two major approaches have been used. The first uses soluble forms of the receptors to effect affinity purification of the ligands, followed by protein sequencing and cloning of cDNAs containing the desired peptides (e.g., Stitt et al., 1995). Alternatively, expression cloning strategies involve the construction and screening of "pooled expression libraries" (e.g., Lok et al., 1994). In these strategies, many small pools of cDNAs are individually transfected into cells, and conditioned media from the individual transfections are then separately assayed for their ability to produce activities that stimulate receptor-bearing reporter cells. A sensitive and simple assay must be available, since tens of thousands of pools often must be screened, particularly if the desired cDNA is present only at low abundance.

Less labor-intensive approaches, such as those based on the yeast two-hybrid system for cloning interacting partners (Chien et al., 1991), cannot be used since growth factor–receptor binding depends on a large interacting surface between two essentially full-length and correctly folded proteins (de Vos et al., 1992), the folding of which depends on correct disulfide pairing, which usually occurs efficiently only during secretion from mammalian cells. The yeast two-hybrid system takes advantage of interactions between short stretches of peptides that can occur within the reducing environment found within the yeast cell cytoplasm.

The expression cloning of cDNAs encoding membrane-bound (as opposed to secreted) ligands for orphan receptors has been far easier. Transfection of entire cDNA libraries into large numbers of cells can be used and still allow very rapid screening, since an individual cell containing the desired cDNA is uniquely marked on its surface by expression of the desired ligand. This rare cell can be individually detected within a background of millions of other cells and thus allows isolation of the ligand-encoding cDNA (most efficiently by Davis et al., 1994) using soluble and epitope-tagged forms of the orphan receptor for the detection by a process that is formally identical to methods previously used to clone receptors using epitope-tagged ligands (Davis et al., 1991). Here we introduce an extension of this method, termed "secretion-trap expression cloning," that allows rapid expression cloning of secreted ligands based only on receptor–ligand binding and that we have exploited in the isolation of Angiopoietin-1 as a ligand for the TIE2 receptor. This approach should be useful not only for isolating ligands for other orphan receptors but also for identifying interacting partners for the plethora of uncharacterized secreted proteins being discovered in the attempt to sequence the mammalian genome.

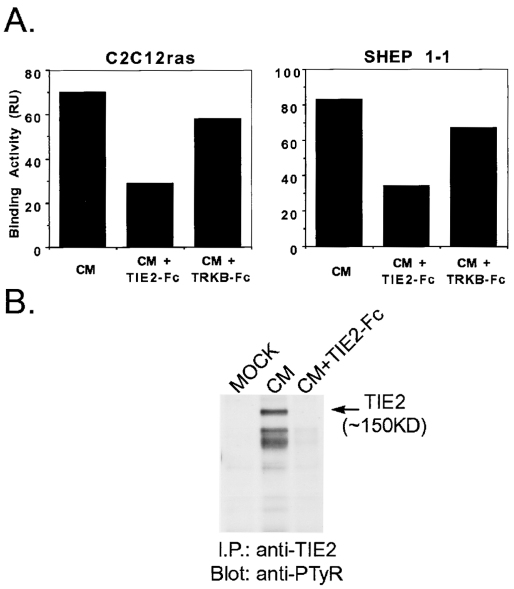


Figure 1. Identification of TIE2 Binding and Phosphorylating Activity in Conditioned Media of C2C12ras Cells and SHEP1–1 Cells

(A) BIAcore assay of TIE2 binding activity. Conditioned media (CM) from each cell line was passed over a BIAcore sensor chip that had TIE2-Fc covalently coupled to it. Specificity of binding was assessed by the inclusion of excess competing soluble TIE2-Fc or TrkB-Fc during the measurement. Binding to the sensor chip is given as resonance units (RUs).

(B) Phosphorylation of TIE2 receptor is induced by C2C12ras conditioned medium. ABAE cells were either unchallenged (MOCK) or challenged with 10-fold–concentrated conditioned medium from C2C12ras cells that had previously been depleted with either beads alone (CM) or with beads coated with TIE2-Fc (CM + TIE2-Fc). Lysates of cells were immunoprecipitated (I.P.) with anti-TIE2 antibodies and analyzed for levels of tyrosine-phosphorylated TIE2 receptor.

## Results

### Identification of a Source for a TIE2 Binding and Phosphorylating Activity

To identify potential sources for TIE2 ligands, we constructed a probe molecule consisting of the ectodomain of TIE2 fused to the Fc portion of human IgG1 as well as control fusion proteins in which the ectodomain of TrkB or TIE1 replaced the TIE2 sequences (designated TIE2-Fc, TrkB-Fc, and TIE1-Fc, respectively). These molecules were purified and coupled to the surface of a BIAcore sensor chip (as per Stitt et al., 1995), which was then used to screen conditioned media from a variety of cell lines for binding activity that was specific for TIE2. Two cell lines, the human neuroepithelioma cell line SHEP1–1 and the mouse myoblast cell line C2C12ras, were found to have binding activity that could be competed by the addition of soluble TIE2-Fc but not TrkB-Fc (Figure 1A). In addition, conditioned media from these cell lines could induce tyrosine phosphorylation of the TIE2 receptor in endothelial cells (Figure 1B). The component of the conditioned media that induced phosphorylation was presumably the same as the component that specifically bound TIE2-Fc because the phosphorylating activity could be depleted by beads coated with TIE2-Fc (Figure 1B). Despite the detectable binding in their conditioned media, no binding of TIE2-Fc to the surface of either SHEP1–1 or C2C12ras cells could be detected, indicating that both cell lines were producing a secreted as opposed to a membrane-bound ligand for TIE2.

### Secretion-Trap Expression Cloning of a TIE2 Ligand

We have previously shown that receptor-Fc fusion proteins can be effectively used for the expression cloning of cDNAs encoding membrane-bound ligands, in particular by using them to surface-label rare ligand-bearing cells individually within a large population of library-transfected cells (Davis et al., 1994); after detection of the rare cell expressing the desired ligand on its surface, the cDNA encoding the ligand was rescued from the labeled cell (Davis et al., 1994). We devised an extension of this method (Figure 2A) that would be applicable to secreted ligands, based on the notion that a secreted ligand is, at least temporarily, trapped inside vesicular compartments of the cell until it is exocytosed. We reasoned that if cells were appropriately fixed and permeabilized, it might be possible to detect the ligand en route to secretion by using the receptor-Fc fusion protein as

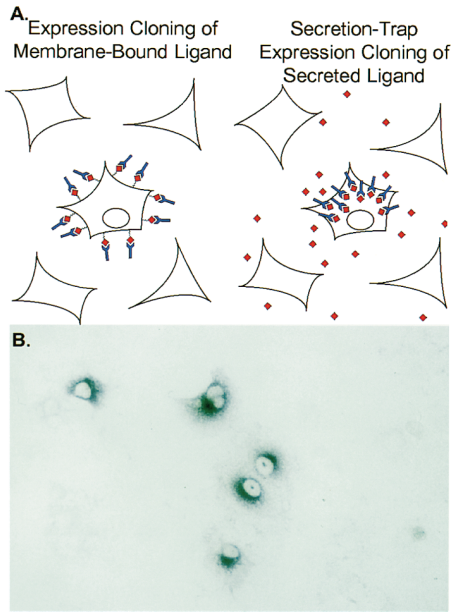


Figure 2. Secretion-Trap Expression Cloning

(A) Schematic diagram of the strategy used for expression cloning of secreted ligands (right), an extension of methods used for expression cloning of membrane-bound ligands (left).

(B) COS cells expressing intracellular Angiopoietin-1 stained with TIE2-Fc. COS cells depicted were transfected with plasmid DNA rescued from a positively stained cell in the primary library screen, and then stained with TIE2-Fc as described in Experimental Procedures; about 20% of the cells are positively stained.

an immunohistochemical probe. This approach would allow the identification of rare cells expressing the ligand within a large population of cells transfected with an expression library; plasmid DNA encoding the ligand could then be directly rescued from these rare cells.

cDNA expression libraries were constructed from human SHEP1–1 and mouse C2C12ras cells, both of which are sources of TIE2 ligand, and were screened according to the strategy outlined above. In each case, rare cells ($\sim$1 in $10^6$) were identified when COS cells transfected with the libraries were stained with TIE2-Fc fusion proteins. For each of these stained cells, plasmid DNA was rescued, electroporated into bacterial cells, and amplified to produce an enriched pool of much lower complexity than the original library; each of these primary pools of plasmids was then used to retransfect COS cells. In contrast to the rare TIE2-Fc stained cells seen following library transfections, cells transfected with the primary pools displayed many TIE2-Fc stained cells, as shown in Figure 2B (each with the typical appearance of intra-Golgi staining, as would be expected for a protein detected en route to secretion), demonstrating that considerable enrichment for plasmids encoding TIE2 ligands had been achieved. Examination of individual plasmids derived from the primary pools revealed that a high percentage were positive for TIE2-Fc staining following transfection into COS cells and therefore contained cDNAs encoding either human (from SHEP1–1 cells) or mouse (from C2C12ras cells) versions of a ligand for TIE2. We designate these ligands human and mouse Angiopoietin-1, respectively, since additional related ligands have also been isolated (P. F. J. et al., unpublished data).

The DNA sequence of both the human and mouse Angiopoietin-1 cDNA clones were determined, revealing open reading frames encoding 498 amino acids and sharing 97.6% identity (Figure 3A). Additional cDNA clones were also derived by hybridization screening of a human fetal lung library and of a library of the human cell line T98G. The sequences of all of the human clones were identical, except that the clone derived from T98G carried a short three-base deletion, which led to the deletion of a single glycine residue at position 269 of the amino acid sequence. RNAse protection assays revealed that mRNAs carrying the three-base deletion were present as a minority population in all of the RNA samples examined that were positive for Angiopoietin-1 (data not shown). It is not known whether this deletion has functional significance. However, the sequence at the deletion point matches the consensus sequence of a splice junction after the completion of splicing, so it seems possible that the deletion is simply a consequence of some type of "exon slippage."

As expected, there were hydrophobic sequences at the N-terminal regions of Angiopoietin-1 typical of secretory signal sequences. Database homology searches revealed that the Angiopoietin-1 contained two regions that bore similarity to known proteins (Figure 3B). The first region, consisting of residues 100–280, showed weak homology to myosin and its relatives in the regions of these proteins where they are known to possess coiled-coil quaternary structure. An analysis of the amino acid sequence, using algorithms developed to estimate the probability of coiled-coil structure in a given protein sequence, revealed a high probability of coiled-coil structure in this region. The second region, consisting of residues 280–498, had strong similarity to a family of proteins, including fibrinogen, tenascin, hfrep, ficolin, and the Drosophila protein SCABROUS, that share a region of homology about 200 amino acids long (Figure 3C).

### Biochemical Characterization of Angiopoietin-1

To examine the biochemical properties of Angiopoietin-1, we expressed it in COS cells and studied the conditioned media produced by the transfected cells. Western blot analysis revealed that Angiopoietin-1 was a 70 kDa protein, larger than would be predicted from its amino acid sequence (Figure 4A). Because the sequence contained several potential glycosylation sites, we examined the effect of treatment with PNGase-F, which removes sugar residues. This treatment did indeed reduce the molecular weight to the expected $\sim$55 kDa, thus confirming that Angiopoietin-1 is a glycoprotein (Figure 4A).

To determine whether recombinant Angiopoietin-1 could bind in secreted form to the TIE2 receptor, we examined conditioned media from COS cells transfected with Angiopoietin-1 cDNAs for binding activity on BIAcore sensor chips coupled to TIE2-Fc, TIE1-Fc, or TrkB-Fc. Angiopoietin-1–containing conditioned media displayed binding activity only to the TIE2-Fc surface

A.

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human Ang-1 MTVFLSFAFL AAILTHIGCS NQRRSPENSG RRYNRIQHGQ CAYTFILPEH DGNCRESTTD QYNTNALQRD APHVEPDFSS QKLQHLEHVM ENYTQWLQKL ENYIVENMKS EMAQIQQNAV  120
mouse Ang-1 .........f .......... ....n...g. .......... .......... .......a.e .......... .......... .......... .......... .......... ..........  120

human Ang-1 QNHTATMLEI GTSLLSQTAE QTRKLTDVET QVLNQTSRLE IQLLENSLST YKLEKQLLQQ TNEILKIHEK NSLLEHKILE MEGKHKEELD TLKEEKENLQ GLVTRQTYII QELEKQLNRA  240
mouse Ang-1 .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ...s...f.. .......s..  240

human Ang-1 TTNNSVLQKQ QLELMDTVHN LVNLCTKEGV LLKGGKREEE KPFRDCADVY QAGFNKSGIY TIYINNMPEP KKVFCNMDVN GGGWTVIQHR EDGSLDFQRG WKEYKMGFGN PSGEYWLGNE  360
mouse Ang-1 .n...i.... .......... ..s....... .......... .......... .......... ...f...... .......... .......... .......... .......... ..........  360

human Ang-1 FIFAITSQRQ YMLRIELMDW EGNRAYSQYD RFHIGNEKQN YRLYLKGHTG TAGKQSSLIL HGADFSTKDA DNDNCMCKCA LMLTGGWWFD ACGPSNLNGM FYTAGQNHGK LNGIKWHYFK  480
mouse Ang-1 .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........  480

human Ang-1 GPSYSLRSTT MMIRPLDF  498
mouse Ang-1 .......... ........  498
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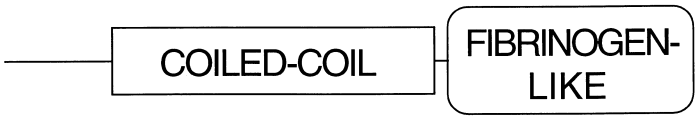
B.




C.

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Angiopoietin-1 RDCADVYQAGFNKSGIYTIYIN-NMPEPKKVFCNMDVNGGGWTVIQHREDGSLDFQRGWKEYKMGF--------GN----PSGEYWLGNEFIFAITSQR--QYMLRIELMD-WEGNRAYSQYD    390
ficolin-α      .t.kelltr.hil..wh...lp-d-cq.lt.l.d..td......f.r.s...v..y.d.aa..r..--------.s----ql..f....dh.h.l.a.g--tne..vd.v.-f...hqfak.r    218
ficolin-β      .t.kelltr.hfl..wh...lp-d-cq.lt.l.d..td......f.r.s...v..y.d.aa..r..--------.s----ql..f....dh.h.l.a.g--tse..vd.v.-f...hqfak.r    221
tenascin       k..sqamln.dtt..l....l.gdkaqale...d.tsd....i.flr.kn.ren.yqn..a.aa..--------.d----rre.f...ldnlnk..a.g--..e..vd.r.--h.et.fav..   2087
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hpT49          k..s.y.ai.krs.et.rvtpd-pknssfe.y.d.etm......l.a.l...tn.t.t.qd..a..--------..----lrr.f....dk.hll.ksk--emi...d.e.-fn.vel.al..    317
SCABROUS       h..se.-ht--qtd.lhl.apa-gqrh.lmth.ta.----...tv.r.f...a..n.s.ad.aq..--------.a----.g..f.i...qlhhl.ldn--csr.qvqmq.iydnvwv-ae.k    640
fibrinogen-γ   k..q.iank.akq..l.f.kpl-kanqqfl.y.ei.gs.n....f.k.l...v..kkn.iq..e..--------.hlspttt..f.....k.hl.st.saip.a..v..e.-.n.rtstad.a    289
fibrinogen-β   ke.eeiirk.get.em.l.qpd-ssvk.yr.y.d.nten.......n.q...v..g.k.dp..q..gnvatntd.kycglnp.......dk.sql.rmg--pte.l..me.-.k.dkvkah.g    357

Angiopoietin-1 RFHIGNEKQNYRL-YLKGHTGTA----------GKQSSL---ILHGAD-FSTKDADNDNCMC-KCALMLTGGWWFDACGPSNLNGMFYTAGQNHGKL-NGIKWHYFKGPSYSLRSTTMMIRPLDF  498
ficolin-α      s.qvad.aek.m.-v.-.afveg----------nagd.---ts.nnsl.t...q...qyas-n..vlyq.a..yns.hv.....r-.lg.-s..sf-..vn.ssg..yn..ykvse.kf.a    322
ficolin-β      s.qvag.aek.k.-v.g.flegn----------agd-..---ss.rdqf.....q....hsg-n..eqyh.a..yn..hs.....r-.lr.lhtsya-..vn.rsgr.yn..yqvse.kv.    324
tenascin       k.sv.da.tr.k.-kve.ys...----------.-d.ma---yhn.rs-...f.k.t.sait-n...s-.r.f.yrn.hrv..m.r-.-gdn..s---q.vn.fhw..heh.iqfae.kl..sn.  2189
hfrep          n.kv.d..nf.e.-nigeys...gdslagnfhpev.wwa---shqrmk-...w.r.h..yeg-n..eedqs....nr.hsa....vy.sgpytaktd-...v.ytwh.ww...k.vv.k...n.    306
hpT49          q.yva..flk...-hvgnyn...----------.dalrfnkhyn.dlkf.t.p.k...rypsgn.g.yyss.......lsa....ky.hq-kyr.vr-...f.                        407
SCABROUS       ..y.ssradg...-hiaeys.n.----------sdaln----yqq.mq-..ai.d.r.isqt-h..anye.....sh.gha....r-.nl.ltwfda-arne.iav.ss                729
fibrinogen-γ   m.kv.p.adk...t.ayfag.d.----------.dafdg---fdf.d.-p.d.fftsh.g.q-fstwdndndkfegn.aeqdgs.-wwmnkcha.h.-..v---.yg.gt..-kas.          385
fibrinogen-β   g.tvq..ank.qi-svnkyr...----------.--na.----md..                                                                                  385
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Figure 3. Sequence Analysis of Angiopoietin-1

(A) Comparison of human and mouse Angiopoietin-1. Asterisk, variable glycine.

(B) Schematic diagram of the structure of Angiopoietin-1. The coiled-coil domain begins approximately 50 amino acids from the beginning of the mature protein.

(C) Comparison of the fibrinogen-like domain of Angiopoietin-1 with homologous domains of related proteins.

(Figure 4B), and this binding could be competed only with the simultaneous addition of excess soluble TIE2-Fc but not TrkB-Fc or TIE1-Fc (Figure 4B), together demonstrating that Angiopoietin-1 is specific for TIE2. The binding affinity was estimated at $K_D$ = 3.7 nM based on an assay in which the ligand was immobilized on nitrocellulose and probed with varying amounts of TIE2-Fc (Figure 4C).

To examine whether cloned Angiopoietin-1 could induce tyrosine phosphorylation of TIE2, we challenged bovine and human endothelial cells with COS cell conditioned media containing Angiopoietin-1. Angiopoietin-1 could induce tyrosine phosphorylation of TIE2 in all of the endothelial cell lines examined (Figures 4D and 4E), and this induction could be blocked by the addition of excess soluble TIE2-Fc but not TrkB-Fc (Figure 4D). Thus, recombinant Angiopoietin-1 both binds and activates TIE2.

### Angiopoietin-1 Does Not Induce Growth Responses in Endothelial Cells

Factors involved in angiogenesis often have proliferative effects on cultured endothelial cells. We compared the proliferative potency of several of these factors with the effects of Angiopoietin-1 on cultured endothelial cells. When human endothelial cells were treated with basic fibroblast growth factor, VEGF, or epidermal growth factor, proliferation was readily induced (Figure 5A and data not shown). However, under the same culture conditions, concentrations of Angiopoietin-1 that are higher than necessary to induce robust tyrosine phosphorylation of TIE2 did not elicit a detectable growth response (Figure 5B). In another assay typically used to evaluate angiogenic factors, in which factors such as basic fibroblast growth factor or VEGF induce endothelial cell tubules to form within collagen matrices, chronic addition of Angiopoietin-1 was unable to induce tubule formation (data not shown).

Because Angiopoietin-1 did not appear to display the biologic activities typical of other endothelial cell growth factors, we decided to verify that other ways of activating the TIE2 catalytic domain also did not lead to proliferative responses. We thus constructed a chimeric receptor consisting of the ectodomain of TrkC fused to the cytoplasmic domain of TIE2, which thereby allowed activation of the TIE2 kinase domain following NT3 addition

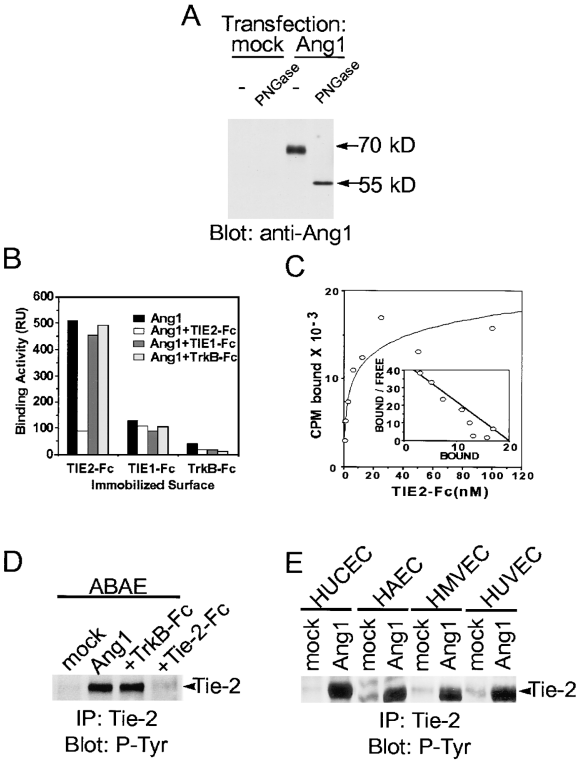


Figure 4. Biochemical Characterization of Angiopoietin-1

(A) Molecular weight and glycosylation of Angiopoietin-1. Supernatants from mock-transfected or Angiopoietin-1–transfected COS cells were treated with PNGase-F and Western blotted with an antibody to Angiopoietin-1. Deglycosylation reduces the apparent molecular weight of Angiopoietin-1 from 70 kDa to the size predicted from the amino acid sequence, 55 kDa.

(B) Binding of TIE2 receptors by cloned Angiopoietin-1. Angiopoietin-1–containing COS cell supernatants were assayed for binding activity to BIAcore sensor chips to which TIE2-Fc, TIE1-Fc, or TrkB-Fc were immobilized. In each case, excess soluble TIE2-Fc, TIE1-Fc, or TrkB-Fc was added to assess the specificity of binding. RU, resonance units.

(C) Scatchard analysis of binding of Angiopoietin-1 to TIE2-Fc. (Inset) Binding data plotted in Scatchard format. BOUND is given in CPM $\times 10^{-3}$, and BOUND/FREE is given in CPM $\times 10^{-2}$/nM.

(D) Competable activation of TIE2 receptors by cloned Angiopoietin-1 in ABAE cells. ABAE cells were untreated or treated with conditioned media from either mock-transfected or Angiopoietin-1–transfected ($\sim$125 ng/ml) COS cells and then assayed for tyrosine phosphorylation of TIE2. Phosphorylation activity was blocked by the addition of excess soluble TIE2-Fc but not TrkB-Fc.

(E) Activation of TIE2 receptors by cloned Angiopoietin-1 in human endothelial cells. Four human endothelial cell types (HUCEC, HAEC, HMVEC, and HUVEC) were treated with conditioned media from either mock-transfected or Angiopoietin-1–transfected COS cells and then assayed for tyrosine phosphorylation of TIE2 receptors.

Ang1, Angiopoietin-1; IP, immunoprecipitation.

(Figure 5D, inset). We transfected this chimeric receptor into MG87 fibroblasts, which display proliferative or survival responses following activation of many introduced receptor tyrosine kinases (e.g., Glass et al., 1991); control cells were transfected with an expression vector containing full-length TrkC. Both TrkC/TIE2 cells and TrkC cells possess endogenous fibroblast growth factor (FGF) receptors and proliferate similarly in response to FGF (Figure 5C). In contrast, when treated with NT3, the TrkC cells proliferated, whereas the TrkC/TIE2 cells did not (Figure 5D), even though tyrosine phosphorylation of the chimeric receptor was clearly induced in these cells by NT3 (Figure 5D, inset).

Taken together, our results indicate that Angiopoietin-1 activation of TIE2 does not induce the typical growth responses seen with other endothelial cell growth factors, suggesting that it may have actions that are not revealed by classic in vitro assays previously used to characterize endothelial growth factors. Consistent with this possibility, in an accompanying article (Suri et al., 1996) we report that disruption of the Angiopoietin-1 gene leads to distinctive defects in embryonic vascular development unlike those seen in embryos lacking VEGF (but similar to those in embryos lacking TIE2), suggesting that Angiopoietin-1 plays a unique collaborative and sequential role with VEGF.

### Localization of Angiopoietin-1 in Developing Embryos

To obtain insight into the possible role of Angiopoietin-1 during vascular development, its expression in developing embryos was examined by in situ hybridization. To compare the distribution of Angiopoietin-1 with the distribution of endothelial cells, the in situ hybridization analysis was performed on parallel sections with an endothelial-specific probe. (Although both TIE1 and TIE2 probes were used to detect endothelial cells, only hybridizations with TIE1 are depicted because they provided better images.) Earlier in development, at E9 to E11, Angiopoietin-1 is found most prominently in the heart myocardium surrounding the endocardium (Suri et al., 1996), consistent with findings that the primary defects in the developing vasculature of mice lacking either TIE2 (Sato et al., 1995) or Angiopoietin-1 (Suri et al., 1996) involve endocardial branching and folding. Later in development, Angiopoietin-1 becomes much more widely distributed, most often in the mesenchyme surrounding developing vessels, in close association with endothelial cells (Figures 6A and 6B). For example, in E13 embryos, Angiopoietin-1 could be detected in the vicinity of the intersegmental vessels (Figures 6C and 6D), in mesenchyme surrounding several vessels in the peritoneum (Figures 6E and 6F), and in the developing eye adjacent to developing vasculature (Figures 6G and 6H); in all cases, signals with the TIE1 probe are much more distinct and punctate since they reflect detection of endothelial cells themselves, whereas those with Angiopoietin-1 are more diffuse owing to signal in surrounding mesenchyme.

The embryonic expression patterns of Angiopoietin-1 suggest that it plays a particularly important role in the heart early in development and an increasingly widespread role as the rest of the vasculature matures.

### Discussion

We have previously shown that a "single-cell" expression cloning technique could be used for the efficient isolation of membrane-bound ligands (Davis et al.,

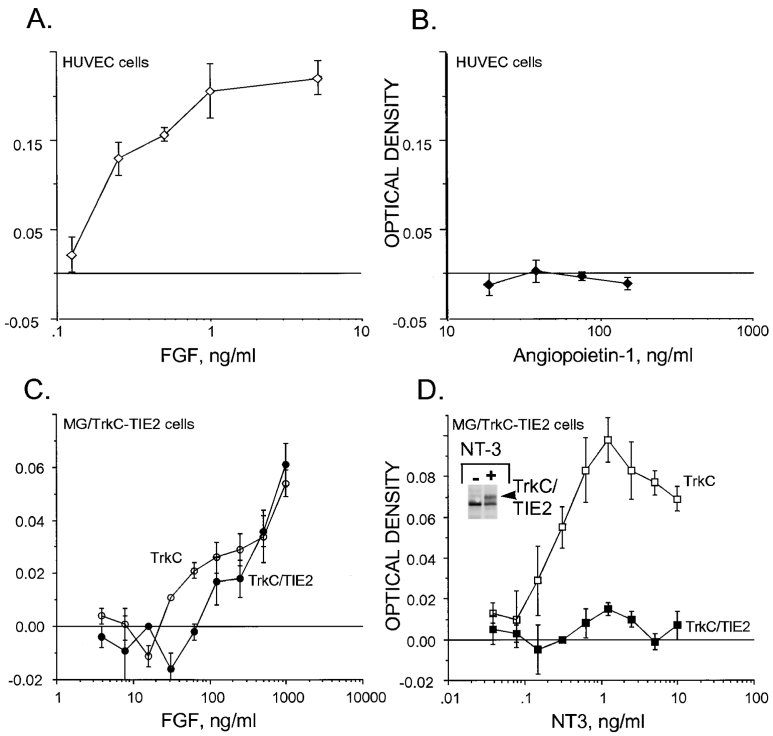


Figure 5. Lack of Proliferative Effects of TIE2 Activation in Endothelial Cells and in Fibroblasts

(A and B) Effects of FGF and Angiopoietin-1 on the proliferation of endothelial cells. HUVEC cells were treated with the indicated amounts of FGF or Angiopoietin-1 and assayed for proliferative activity. Angiopoietin-1 was provided as conditioned media of Angiopoietin-1–transfected COS with known amounts of Angiopoietin-1; similar amounts of conditioned media from mock-transfected COS cells produced no effects on HUVEC cell proliferation (data not shown).

(C and D) Effects of activation of the TIE2 kinase domain in fibroblasts. MG cells ectopically expressing either TrkC or TrkC/TIE2 chimera were treated with the indicated amounts of FGF or NT3 and assayed for proliferative activity. (Inset) Induction of tyrosine phosphorylation of the TrkC/TIE2 chimera by NT3, indicating that the chimeric receptor is functional.

1994). This method provided considerable simplification over iterative panning methods or techniques based on construction of pooled libraries, which entails screening of individual pools followed by subdivision and rescreening of positive pools. Here we have extended the single-cell method to include secreted ligands, in a method we have termed secretion-trap expression cloning, thus providing an alternative route to the more time-consuming traditional approaches to this difficult problem. We describe application of secretion-trap expression cloning for the cloning of a novel secreted ligand for the TIE2 receptor. We anticipate that this method may be useful not only for the cloning of secreted ligands for other orphan receptors but also for the identification of interacting partners for the many secreted proteins of unknown function currently being discovered in large sequencing projects.

The structure of Angiopoietin-1 is unlike that of any other angiogenic factor or any other factors that bind to receptor tyrosine kinases. Numerous proteins are known to possess domains resembling the C-terminal domain of fibrinogen, but in most cases little is known about the functions of these proteins. It is noteworthy that all known examples of this type of protein have the fibrinogen-like domain at the C-terminus and that most are accompanied by N-terminal domains that have coiled-coil structure (one exception is the ficolins, which have collagen-like N-terminal regions); it is not known whether these shared structures form the basis for some common functional theme. However, it is known that coiled-coil regions typically are involved in the assembly of the protein into multimeric structures; of note, the collagen-like N-terminal region of ficolin is also thought to be involved in multimerization. It seems reasonable that the coiled-coil domain of Angiopoietin-1 may be responsible for assembling the fibrinogen-like domain into some type of multimeric structure. Preliminary results (data not shown) indicate that this possibility is indeed the case. It will be relevant to determine the precise structure of Angiopoietin-1, to identify the portions of it that are required for binding to TIE2, and to determine whether multimeric structure is necessary for binding or activation. Because some proteins with coiled-coil structure are found as heteromultimers of similar proteins rather than as homomultimers (e.g., fibrinogen [Doolittle, 1984]), the possibility exists that Angiopoietin-1 can also form heteromeric structures with other, closely related proteins; indeed, several such relatives of Angiopoietin-1 have been identified (P. F. J. et al., unpublished data). Different combinations of Angiopoietin-1–related molecules may have different receptor specificities.

In contrast to previously characterized angiogenic factors such as VEGF and FGF, Angiopoietin-1 is, under the conditions tested here, unable to elicit some of the responses that are typical of angiogenic factors. Apparently, Angiopoietin-1 serves some function other than proliferation or tubule formation in endothelial cells. Because vascular development evidently is a complex, multistep process that requires appropriate formation and integration of the vasculature into its surrounding tissue, it would not be surprising that some factors might regulate aspects of endothelial maturation that occur after the initial stages of proliferation and tubule formation. The expression of Angiopoietin-1 in the immediate vicinity of developing blood vessels implicates this ligand in processes that are important for vascularization. The later expression of TIE2 as compared to that of the VEGF receptors (Dumont et al., 1995), together with our findings that Angiopoietin-1 expression seems to become more widespread as development proceeds, is

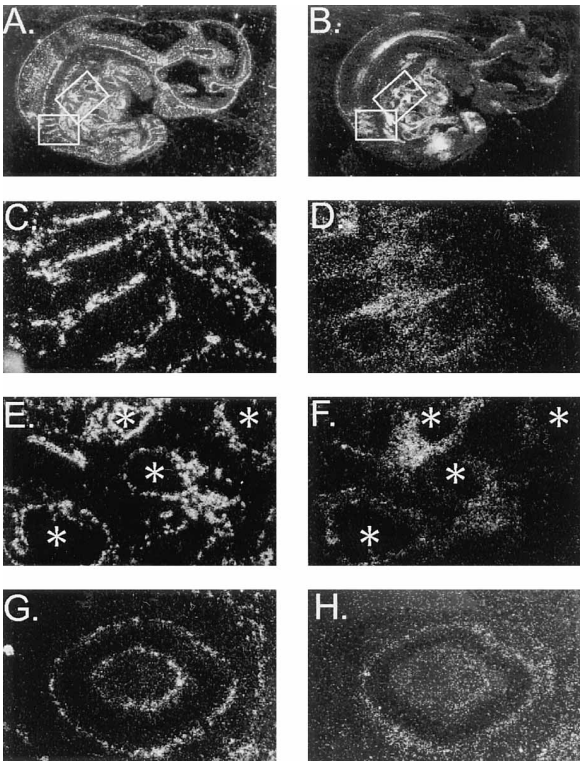


Figure 6. In Situ Analysis of Angiopoietin-1 Gene Expression in Developing Embryos

(A, C, E, and G) were probed with TIE1 to localize endothelial cells. (TIE2 in situ analyses, of inferior quality and thus not shown, revealed similar staining patterns.) (B, D, F, and H) were probed with Angiopoietin-1.

(A and B) Saggital cross section of rat embryos showing general correlation between endothelial cells and Angiopoietin-1 expression in surrounding mesenchyme.

(C and D) Enlargement of lower left insets in (A) and (B), highlighting expression of Angiopoietin-1 in the vicinity of the intersegmental vessels.

(E and F) Enlargement of upper right insets in (A) and (B), highlighting expression of Angiopoietin-1 in mesenchyme surrounding several vessels (asterisks) in the peritoneum.

(G and H) Expression of Angiopoietin-1 in the developing eye, adjacent to developing vasculature.

consistent with the notion that Angiopoietin-1 plays an angiogenic role later and distinct from that of VEGF. Indeed, the findings of our accompanying study (Suri et al., 1996), in which embryos lacking Angiopoietin-1 were analyzed for their vascular defects, are consistent with this notion. The availability of a recombinant ligand for TIE2 will make it possible to investigate more directly the functions of the TIE2 receptor in vivo and to develop in vitro systems in which the role of Angiopoietin-1 can be revealed and studied. This work will be relevant not only for understanding vascular development, but also, since the TIEs mark the earliest of hemopoietic precursors (Iwama et al., 1993; Batard et al., 1996; Hashiyama et al., 1996), for understanding the role of the TIEs in hemopoiesis.

Angiopoietin-1 may also have potential therapeutic utility independent of its usual biologic functions. Because TIE2 apparently is upregulated during the pathologic angiogenesis that is requisite for tumor growth (e.g., Kaipainen et al., 1994), Angiopoietin-1 may provide a means for specifically targeting tumor vasculature, for example by conjugating it to a cytotoxin, as has been suggested for VEGF (Burrows and Thorpe, 1994). In contrast to clinical situations in which attacking neovascularizing endothelia might be desirable, there are situations in which it would be useful to promote angiogenic processes, such as to induce collateral vascularization in an ischemic heart or limb (Ferrara et al., 1995). Precise understanding of the biologic roles of Angiopoietin-1 and its relatives and of how they may collaborate with members of the VEGF family may lead to novel and therapeutically significant strategies for promoting or inhibiting neovascularization.

## Experimental Procedures

### Cell Culture and Production of Conditioned Media

SHEP1–1 cells were obtained from ATCC. C2C12ras cells were derived from C2C12 cells by stable transfection with an expression plasmid containing the *ras* oncogene. Cells were grown in defined medium without added serum, and conditioned media were harvested and clarified by centrifugation. These media were brought to 1 mM phenylmethylsulfonyl fluoride and 0.14 U/ml aprotinin, concentrated approximately 10-fold with 3000 MWCO membranes (Amicon), and stored at −80°C, as previously described (Stitt et al., 1995).

### Production and Purification of Receptor-Fc Fusion Proteins

The ectodomains of each of TIE2, TIE1, and TrkB were fused to the hinge, $C_H2$, and $C_H3$ regions of human IgG1 via a bridging sequence (glycine–proline–glycine) as previously described (Davis et al., 1994). The fusion proteins were produced according to standard protocols in Sf-21AE cells infected with baculovirus vectors bearing the respective fusion constructs (O'Reilly et al., 1992). Recombinant fusion proteins were then purified by protein A–Sepharose (Pharmacia) chromatography.

### BIAcore Analysis

Binding activity measured by use of BIAcore biosensor technology (Pharmacia Biosensor) was performed essentially as previously described (Stitt et al., 1995); specific binding to the TIE2 surface was determined by incubating the samples with 25 μg/ml of TIE2-Fc, TIE1-Fc, or TrkB-Fc prior to assay.

### Expression Cloning

cDNA libraries of SHEP1–1 and C2C12ras cells were constructed in the expression vector pJFE14 as described (Davis et al., 1994). COS-7 cells were transfected with the libraries (1 μg/100 mm dish). Two days after transfection, cells were washed with PBS and then fixed with PBS/1.8% formaldehyde for 15 min. Cells were then permeabilized and blocked with assay buffer (PBS/10% calf serum) containing 0.1% Triton X-100 for 15 min. Cells were then treated with assay buffer containing 1 μg/ml TIE2-Fc for 30 min. After two PBS washes, cells were incubated with assay buffer containing alkaline phosphatase–conjugated goat anti-human antibodies for 30 min. After two additional PBS washes, substrate buffer containing NBT/BCIP and 1 mM levamisole was added, and development was allowed to proceed until positive cells could be recognized over the background. Development time must be determined empirically for different types of receptor-Fc protein. Dishes were scanned for positively stained cells. Positive cells were scraped from the dish and treated with a buffer containing 100 mM EDTA, 10 mM Tris (pH 8), 0.1% SDS, and 100 μg/ml proteinase K at 55°C overnight. Samples were extracted with phenol/chloroform and ethanol precipitated with carrier tRNA. Samples were resuspended in 5 μl of water, and portions of this were used for electroporation of electrocompetent bacteria. After electroporation the recovered bacterial cell suspension was either cultured overnight or plated on ampicillin plates to produce individual colonies. Minipreps arising from cultures or derived by culturing individual colonies were transfected into COS

cells and then assayed as described above for the presence of TIE2-Fc–staining cells.

### Biochemical Analysis

To identify Angiopoietin-1 on Western blots, antisera were raised against a synthetic peptide (NQRRNPENGGRRYNRIQHGQ) derived from the N-terminal region of Angiopoietin-1. These antisera were affinity purified with the same peptide. Supernatants from COS cells transfected with Angiopoietin-1 were treated with PNGase-F (New England Biolabs) according to the manufacturer's instructions and acetone precipitated. Samples were electrophoresed on a polyacrylamide gel, transferred to an Immobilon membrane, and probed with Angiopoietin-1-specific antibodies. An HRP-conjugated anti-rabbit antibody coupled with ECL reagents was used to visualize Angiopoietin-1–specific bands.

To measure the binding affinity, Angiopoietin-1 was immobilized on nitrocellulose strips (~200 ng per dot). After blocking with PBS containing 10% calf serum, the strips were incubated for 1 hr with varying amounts of TIE2-Fc in PBS containing 2.5% calf serum and 0.1% Tween 20, followed by 2 × 10 min washes in PBS containing 0.1% Tween. Strips were then incubated for 1 hr in a goat anti-human $^{125}$I-labeled second antibody (New England Biolabs, 1:250 dilution) in the same buffer, followed by 3 × 5 min washes, after which bound radioactivity was determined. For each concentration of TIE2-Fc, nonspecific binding in the absence of ligand was subtracted from the total binding in the presence of ligand.

### Cell Culture and Phosphorylations

Human umbilical vein (HUVEC), human aortic (HAEC), and human dermal microvascular (HMVEC) endothelial cells were from Clonetics, Inc. (San Diego, CA); endothelial cells from human subcutaneous fat pads, isolated by two rounds of cell selection with an anti-PECAM antibody (HUCEC) were kindly provided by J. Springhorn, Alexion Pharmaceuticals. Human endothelial cells were maintained according to the supplier's instructions (Clonetics, Inc.). Adult bovine aortic endothelial cells (ABAE) were from ATCC (Rockville, MD) and grown in high glucose DMEM, 10% bovine calf serum, 2 mM L-glutamine, and 1% each of penicillin and streptomycin. TIE2 receptor activation assays were done with late-confluency T-75 flasks of endothelial cells that were serum starved in high glucose-DMEM for 2–4 hrs. Conditioned media were placed on cells for 5–10 minutes at 37°C. Cell lysis, TIE2 immunoprecipitations, and phosphotyrosine immunoblotting were performed largely as described (Stitt et al., 1995), except that TIE2 immunoprecipitations were performed using a rabbit antiserum made to the conserved last 16 amino acids of mammalian TIE2 (RG133). To deplete C2C12 conditioned media prior to challenge of ABAE cells, 10 ml of 10-fold– concentrated media was incubated with rocking for 90 min together with 220 μl of protein G-Sepharose beads that had been previously coated with 150 μg of TIE2-Fc. The beads were spun down and the remaining supernatant was used for challenge. For competitions, 50 μg/ml of the desired receptor-Fc fusion protein was added to the challenge media.

### Proliferation Assays

HUVEC cells were trypsinized for 10 min at 37°C in CMF buffer, and then serum-containing medium was added to neutralize the trypsin. Cells were harvested, spun down, and washed several times with serum-containing medium. Three thousand cells per well were cultured in 96-well tissue culture plastic plates in DMEM containing 2% FBS with added penicillin, streptomycin, and glutamine. Cells were added to wells that already contained doses of FGF or Angiopoietin-1 (provided in conditioned media from transfected COS cells) diluted in assay medium. After 3 days in vitro, cell number was assessed using the vital dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as described (Stitt et al., 1995). Values shown are the mean ± SD of triplicate wells from a single typical experiment. Proliferation assays using MG-EGFR/TIE2 reporter cells were performed largely as described above except that 5000 cells per well were plated in serum-free DMEM with added penicillin, streptomycin, and glutamine and that FGF or NT3 was diluted into the assay medium.

### In Situ Analysis

Fresh-frozen E13.5 rat embryos were sectioned by cryostat and probed with $^{35}$S-labeled cRNAs as described (Valenzuela et al., 1993). Probes were, for TIE1, a 1.3 kb fragment of rat TIE1 spanning the last 309 codons and 375 bp of 3′ untranslated sequence, and for Angiopoietin-1, a 770 bp fragment of rat Angiopoietin-1 spanning the last 253 codons and 12 bp of 3′ untranslated sequence. Sections were immersed in radiographic emulsion (NTB-2, Kodak) and exposed for 5–10 days.

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