Expression of tie-2, a member of a novel family of receptor tyrosine kinases, in the endothelial cell lineage

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SUMMARY

We are interested in the molecular mechanisms that are involved in the development of the vascular system. In order to respond to morphogenetic and mitogenic signals, endothelial cells must express appropriate receptors. To characterize endothelial cell-specific receptors, we have concentrated on receptor tyrosine kinases, because several lines of evidence suggested the importance of controlled phosphotyrosine levels in endothelial cells. A strategy based on PCR amplification using degenerate oligonucleotides and mouse brain capillaries as mRNA source, led to the identification of a novel receptor tyrosine kinase, which we designated tie-2. In situ hybridization using a tie-2-specific probe revealed an interesting spatial and temporal expression pattern. The gene was expressed specifically in the endothelial lineage. tie-2 transcripts were present in endothelial cell precursors (angioblasts) and also in endothelial cells of sprouting blood vessels throughout development and in all organs and tissues so far examined. tie-2 was down-regulated in the adult. Because of the unusual combination of immunoglobulin, EGF-like and fibronectin type III domains in the extracellular portion of tie-2 which is shared by TEK and tie, these molecules may be considered members of a new family of receptor tyrosine kinases. Signal transduction via this new class of tyrosine kinases could lead to a better understanding of the molecular mechanisms of blood vessel formation.

Key words: angiogenesis, receptor tyrosine kinase, endothelial cells, angioblasts, mouse development, vascular development

INTRODUCTION

Signal transduction by receptor and cytoplasmic tyrosine kinases is a process of critical importance in cellular proliferation and differentiation. Phosphorylation on tyrosine of cellular proteins has been detected in vivo using anti-phosphotyrosine antibodies. Proteins phosphorylated on tyrosine were abundant during embryonic development but decreased in adult tissues. Epithelial and endothelial cells were found to be the cell types that were most prominently labeled by the anti-phosphotyrosine antibodies (Maher and Pasquale, 1988; Takata and Singer, 1988; Maher, 1991).

Proliferation and invasion of endothelial cells are crucial events during the development of the vascular system. Factors probably involved in the processes of vasculogenesis and angiogenesis that lead to the formation of a vascular network (Risau, 1991) have been shown to bind with high affinity to and activate receptor tyrosine kinases. In an in vitro system of angiogenesis, molecules that regulate tyrosine phosphorylation were found to modulate the invasion and tube formation of endothelial cells (Montesano et al., 1988; Montesano and Orci, 1985). Furthermore, the expression of the polyoma middle T oncogene in endothelial cells leads to aberrant growth and morphogenetic behavior of endothelial cells in vitro and gives rise to hemangiomas in chimeric mice in vivo (Williams et al., 1988; Montesano et al., 1990). Polyoma middle T oncogene binds to and thereby constitutively activates cytoplasmic tyrosine kinases of the src family. Thus, in endothelial cells, tyrosine phosphorylation seems to play a critical role in the regulation of proliferation, invasion and morphogenesis.

Since we are interested in the molecular mechanisms of growth control and differentiation in endothelial cells, these results prompted us to identify the genes encoding tyrosine kinases in endothelial cells. We amplified DNA by the polymerase chain reaction using degenerate primers deduced from conserved regions of receptor tyrosine kinases. Most importantly, capillary fragments were isolated from postnatal day 4-8 murine brains as a source of mRNA because at this time sprouting and proliferation of endothelial cells is highest in this organ (Robertson et al., 1985). The other rationale behind this strategy was to avoid the cloning of tyrosine kinases such as FGF receptors that are abundantly expressed by capillary endothelial cells in tissue culture but not in vivo (Heuer et al., 1990; Peters et al., 1992). Recently we have reported the successful application of this strategy by the cloning and characterization of the flk-1 receptor tyrosine kinase (Millauer et al., 1993). Here
we have analyzed a different receptor tyrosine kinase, named tie-2, that was isolated by this screening.

MATERIALS AND METHODS

Animals and tissues

Balb/c mice were mated overnight and the morning of vaginal plug detection was defined as 0.5 day of gestation. Organs were removed, frozen immediately in liquid nitrogen and stored at −80°C. Capillary fragments from pooled P4-P8 mice brains were prepared according to Risau et al. (1990). For in situ hybridization and immunohistochemistry, whole embryos or organs were fixed for 12 hours in freshly prepared 4% paraformaldehyde in PBS at 4°C, rinsed for 24-48 hours in 0.5 M sucrose in PBS at 4°C, embedded in TissueTek (Miles) and stored frozen at −80°C.

RNA extraction and analysis

Total cytoplasmic RNA was isolated according to the method of Chomczynski and Sacchi (1987). Enrichment for poly(A)+-containing fractions was achieved by oligo(dT) chromatography using push columns (Stratagene). Aliquots of poly(A)+ RNA were electrophoresed in agarose gels containing 0.66 M formaldehyde and transferred to Zeta-Probe membrane (Bio Rad) in 20× SSPE. Hybridizations were performed overnight in 0.5 M sodium phosphate buffer, 5% SDS, 1% BSA, pH 7.5 at 68°C with 1×106 cts/minute/ml of probe, which had been labelled with 32P-dCTP according to the protocol of a random-primed DNA-labeling kit (Boehringer Mannheim). Membranes were washed under high-stringency conditions at 68°C in 0.1×SSPE, 0.5% SDS and autoradiographed at −80°C on Fuji films. Poly(A)+ RNA from brain capillary fragments was isolated using a QuickPrep Micro mRNA purification kit from Pharmacia.

PCR and cDNA cloning

Capillary poly(A)+ RNA was reverse transcribed and aliquots of the cDNA were used as templates in PCR reactions. In addition to those described by Wilks (1989), the following oligonucleotides were used as primers: 5′-C A C/T C G I G A C/T C/T T I G C I G C I A/C G G 3′, 5′-A T C I C T I C I A/C G I C I G A/T C C A I A C A/G T C 3′ (I stands for inosine). The amplification products were separated in acrylamide gels. Fragments of the expected size were purified, labeled and used as probes to screen a random hexanucleotide-primed cDNA library constructed with a Time Saver cDNA Synthesis Kit (Pharmacia). cDNA fragments were subcloned into Bluescript vectors (Stratagene). Sequencing was done by using nested oligonucleotide primers in combination with a 373A DNA Sequencer (Applied Biosystems) and with the conventional Sequenase System (USB).

In situ hybridization

Preparation of tissue sections and in situ hybridization with single-stranded DNA probes or single-stranded RNA probes was performed as described by Schnürch and Risau (1991). The probe for all hybridization experiments was derived from a 1179 bp DNA fragment encoding a portion of the putative tie-2 protein from amino acid 419 to amino acid 812.

Immunohistochemistry

Embedded embryos and whole organs were sectioned on a Leitz cryostat. 8 µm sections were mounted on organosilane-treated slides, dried overnight under vacuum and stored desiccated at −80°C. Sections were brought to room temperature, rehydrated in PBS for 5 minutes and incubated in 0.1% H2O2 in methanol for 15 minutes. After washing three times in PBS for 5 minutes each, unspecific antibody binding was blocked by application of 20% normal goat serum in PBS for 20 minutes. Sections were washed, incubated with a rat monoclonal antibody against mouse PECAM (a generous gift from Dr E. Dejana) for 1 hour, washed again and then incubated with a biotinylated goat anti-rat IgG (Dianova) for 1 hour. Color development was performed with a Vectastain ABC kit (Vector Laboratories) according to the vendor’s protocol. Sections were slightly counterstained with toluidine blue, dehydrated and mounted.

RESULTS
cDNA cloning and structure of the tie-2 protein

Our approach for the isolation of receptor tyrosine kinases expressed in endothelial cells of sprouting blood vessels involved the following steps. First, we purified mRNA from capillaries of pooled P4-P8 mice brains. A portion of the mRNA was reverse transcribed and used in PCR reactions with degenerate primers deduced from conserved protein regions of tyrosine kinases. Gel-purified reaction products of the expected size were radiolabelled and used directly as hybridization probes to screen a cDNA library constructed from the remainder of the capillary mRNA. Partially sequencing the inserts from positive phages revealed that 13 of the cDNAs were derived from one mRNA species. The longest of these 13 cDNAs, a fragment of 4640 bp, was sequenced completely. It contained a long open reading frame encoding a protein of 1123 amino acid residues. This deduced polypeptide has all features of a receptor tyrosine kinase: an amino-terminal signal sequence followed by a long extracellular domain, a single hydrophobic transmembrane region and a cytoplasmic portion that contains a tyrosine kinase domain (Fig. 1).

A survey for homologous proteins revealed that the predicted protein is most closely related to the two recently identified tyrosine kinases tie and tek (Partanen et al., 1992; Dumont et al., 1992). With the exception of two residues, the protein sequence published for tek is identical to the intracellular part of our polypeptide, from position 823 to the carboxy-terminal residue 1123. Both an extracellular domain and a transmembrane region are missing in the tek polypeptide (Fig. 2). It is therefore possible that tek represents a partial sequence or that the mRNA encoding tek is the result of an alternative splice event, which results in the production of a cytoplasmic tyrosine kinase. Comparison of our protein with tie reveals a high degree of similarity especially in the cytoplasmic part (76% sequence identity). In the extracellular domain and the transmembrane region, the similarity is less pronounced (33% and 37% respectively; Fig. 2). We will therefore refer to our protein as tie-2. While this manuscript was in preparation, Ziegler et al. (1993) reported the cloning of a cDNA whose translation product is a receptor tyrosine kinase with an overall similarity of more than 90% when compared to tie-2 (Fig. 2). It is therefore likely that this protein, which they called TEK, is the human homolog of tie-2. The intracellular part of tie-2 is also related to the product of the human ret protooncogene and to FGF receptors (Takahashi et al., 1989; Partanen et al., 1991; Stark et al., 1991). The similarity to both is about 43%. The intracellular part can be divided into three characteristic regions: the juxtamembrane sequence, the catalytic domain and the cytoplasmic tail. The kinase
domain, which is split by an 14 amino acid insertion contains the GxGxxG consensus sequence that is part of the ATP binding site. Typical tyrosine kinase motifs like HRDLAARN and DFGL are present.

Within the extracellular part, the FASTA program detected homologies to proteins including TAN-1 (Ellisen et al., 1991), Xotch (Coffman et al., 1990), Laminin (Sasaki et al., 1988), Delta (Vässin et al., 1987) and Perlecain (Noonan et al., 1991) (around 30% sequence identity). The structural basis for these homologies are three EGF-like repeats in the center of the extracellular portion of tie-2 (Fig. 1). EGF-like repeats consist of 30 to 40 amino acid residues often found in the extracellular parts of membrane-bound proteins or secreted proteins (Davies, 1990). A common feature of these domains are six conserved cysteine residues, which are known to be involved in disulfide bonds. The three EGF domains in tie-2 contain two additional cysteine residues at the carboxy-terminal end of each repeat.

The central EGF-like repeats are flanked by two different structural motifs, a single amino-terminal immunoglobulin (Ig) domain and a carboxy-terminal triplet of fibronectin (FN) type III domains. Although the Ig domain does not exhibit clear sequence similarities to other known proteins (except for tie and TEK), the Ig domain has the conserved features of a C2-set domain including typical residues surrounding the first cysteine residue and the canonical GxYxC found at the second cysteine residue (Williams and Barclay, 1988).

The three fibronectin type III (FN III) domains are most closely related to FN III repeats present in the protein-tyrosine phosphatases Delta (Krueger et al., 1990), LAR (Streuli et al., 1988) and DLAR (Streuli et al., 1989) and to FN III repeats of the axonal glycoprotein TAG-1 (Furley et al., 1990) (around 20% sequence identity). These domains have been described as units of approximately 90 amino acids containing hydrophobic residues at characteristic positions. In addition, a total of nine potential N-glycosylation sites are located in the extracellular part of tie-2.

tie-2 expression in brain capillaries

Our aim was to isolate receptor tyrosine kinases that are expressed in blood vessels during brain angiogenesis. In order to check the validity of the approach, we compared the amount of tie-2 in mRNA from P4-P8 brain capillaries with total P4 brain by northern analysis. To avoid artifactual results, we used the part of the cDNA that encodes the less conserved regions of the protein, namely the three FN III domains, the transmembrane region and the juxtamembrane

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![Fig. 1. Deduced amino acid sequence and structure of tie-2. (A) Amino acid sequence of tie-2 in single letter code. The potential signal sequence cleavage site is indicated by an arrowhead. Black dots mark the two cysteine residues, possibly involved in sulphydryl bonding of the immunoglobulin domain. The three EGF-like repeats are boxed. The three fibronectin type III domains are underlined. The transmembrane region is given in bold face letters. The tyrosine kinase domain is indicated by shaded boxes. The RGD triplet is marked by an asterisk. (B) Schematic diagram of the structure of tie-2. Ig, immunoglobulin domain; EGF, EGF-like repeats; FN, fibronectin type III domains; Kinase, tyrosine kinase domain; KI, kinase insertion.](image-url)
Fig. 2. Amino acid sequence comparison of tek, TEK and tie with tie-2. Amino acid residues identical to tie-2 are represented by (−). Gaps are indicated by (−).
portion. Fig. 3 demonstrates the presence of a 4.7 kb mRNA highly enriched in the capillary fraction. We could also detect this single mRNA in organs like brain, kidney and heart (data not shown).

**tie-2 expression during brain development**

Investigation of the spatial and temporal expression profile of tie-2 during brain development was performed by in situ hybridization. When we analyzed adult brain, P4 brain, E12.5 brain and E8.5 neuroectoderm, it became evident that tie-2 mRNA is exclusively synthesized in the vasculature of these tissues (Fig. 4A-H). No other brain components were labelled. In P4 brain, tie-2 expression was detected in capillaries that are about to invade the neural tissue as well as in vessels that have already reached deeper layers of the neuroectoderm. High magnifications in Fig. 4G,H clearly show the high concentration of silver grains over the vascular perikarya. In addition to capillaries, meningeal blood vessels and the choroid plexi were also found to synthesize tie-2 mRNA at comparable levels (Figs 4, 6B). At E12.5 the overall expression pattern was virtually identical to that observed at P4, although the density of labelled structures in E12.5 brain was reduced (Fig. 4C,D). This observation correlates with the less extensive vascularization of the embryonic brain (Bär, 1980). No hybridization signals were observed in the neuroectoderm of E8.5 embryos, because at that stage this tissue is still avascular (Fig. 7). In the adult brain, tie-2 expression was detectable, although it seemed to be reduced when compared to postnatal or embryonic stages. Hybridization signals persisted over larger vessels especially those of the meninges (Fig. 4A,B). The synthesis of tie-2 mRNA in smaller vessels and capillaries was barely detectable.

**Endothelial cell-specific expression of tie-2**

To identify the cellular source of tie-2 mRNA, we performed in situ hybridization and immunohistochemistry on adjacent sections. The pattern of tie-2 hybridization signals was compared with the immunohistochemical staining of a monoclonal antibody against the endothelial cell-specific adhesion molecule PECAM (CD31) (Newman et al., 1990). Fig. 5 shows a representative example of one such experiment. The antibody stains the continuous layer of endothelial cells surrounding the lumen of a medium-sized blood vessel in the head region of an E12.5 embryo. On the adjacent section, the tie-2-specific probe labels the vessel in an identical way. These results, together with the northern hybridization signal detected in RNA from a capillary fraction highly enriched for endothelial cells, provide strong evidence for endothelium-specific expression of tie-2. Fig. 6 gives a survey on the coexpression of tie-2 and PECAM in several organs and tissues. It is clear that tie-2 mRNA is present in endothelial cells all over the body. Strong hybridization signals were associated with the heart endocardium as well as with the myocardial blood vessels (Fig. 6E,F). The same holds true for the endothelium of the dorsal aorta (Fig. 6C,D), the intersomitic vasculature, the vessels surrounding the lung bronchia and the capillaries perforating the spinal cord (Fig. 6H,D). In summary, tie-2 gene expression seems to be a general feature of endothelial cells.

**tie-2 expression during early stages of development**

The possible role of tie-2 in early stages of vascular development was investigated by in situ hybridization studies with E8.5 sections. The probe detected tie-2 mRNA in the mesodermal component of the yolk sac (Fig. 7). This finding is of particular interest, because it is in the mesodermal component of the yolk sac that the first signs of blood vessel development are evident. Clusters of mesenchymal cells form the so-called blood islands. At the margin of these aggregates cells, the so-called angioblasts, adopt an endothelial-like phenotype, whereas in the center cells differentiate into embryonic hemoblasts. Fig. 7 demonstrates that the peripheral angioblasts synthesize high levels of tie-2 mRNA.

Within the embryo proper, expression was seen in the anlagen of the vascular system, e.g. in the developing endocardium, the dorsal aortae and the cardinal veins (Fig. 7A,B). It is likely that these signals stem from intraembryonic endothelial cell precursors, which are present in these structures at that stage. tie-2 expression could also be detected in the allantois and in blood vessels of the maternal decidua (data not shown).

**DISCUSSION**

We report here the isolation and characterization of tie-2, an endothelium-specific receptor tyrosine kinase with an unusual topology. So far only three other tyrosine kinases with similar features have been described, namely tie, tek and TEK. Although no studies on the developmental expression are available, tie mRNA has been detected in endothelial cells (Partanen et al., 1992). The spatial and temporal expression pattern of tek seems to overlap largely with that of tie-2. No in situ hybridization data were
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presented for TEK (Ziegler et al., 1993). However, because TEK seems to be the human homolog of tie-2, the spatial and temporal expression profiles of both genes may be similar if not identical. Obviously, there is a family of structurally related molecules synthesized by endothelial cells. The question arises how many members this family may have? Dumont et al. (1992) detected several mRNA species by northern analysis in embryonic heart tissue hybridizing with a tek probe. These findings can be explained by differential, perhaps organ-specific splice events that could lead to the production of truncated and/or cytoplasmic tyrosine kinases. Alternatively, by using a cDNA encoding the highly conserved catalytic domain as a probe, they may have detected closely related molecules. This possibility cannot be excluded, because we used the less conserved part of the tie-2 cDNA and detected only one single mRNA species. It will therefore be interesting to see whether this still small family will grow by additional members.

The fact that we could detect tie-2 mRNA in a capillary fraction highly enriched for endothelial cells gave us the first hint for an endothelium-specific expression pattern of this gene. This finding confirmed the suitability of our experimental design. Instead of cultured endothelial cells, we used freshly prepared capillary material from P4 mouse brain as a mRNA source for the initial cloning step. There were two reasons for this approach. First, the proliferation of capillaries is maximal during the first postnatal days in the rodent brain (Robertson et al., 1985). Second, receptors for FGF, although produced by endothelial cells in vitro, have not be detected in capillary endothelial cells in vivo (Heuer et al., 1990; Peters et al., 1992). Our in situ hybridization data on embryonic, postnatal and adult tissue sections confirmed that tie-2 is specifically expressed in endothelial cells. The spatial expression pattern of tie-2 was found to be identical to the immunohistochemical staining for the endothelial cell marker PECAM(CD31) (Newman et al., 1990).

The developing brain does not contain endogenous endothelial cell precursors (Noden, 1990). As a consequence, this organ is vascularized by angiogenic sprouting from the meningeal vascular plexus (Bär, 1980). The whole process consists of a complex pattern of physiological events involving sprouting, migration and proliferation of endothelial cells and finally their differentiation into tube-like structures surrounded by a basal lamina. Due to its expression pattern, tie-2 could be involved in any of this events. tie-2 synthesis in the vascular plexus could reflect the competence of endothelial cells to respond to an angiogenic stimulus. Expression both at the tip of an invading capillary and in more advanced vessels would argue for a participation in migration and proliferation. In the adult brain, tie-2 seemed to be down regulated and was detectable mainly in larger vessels of the meninges. Because endothelial cell turnover is low in the adult, persistent expression of tie-2 would favor a role in the establishment and the maintenance of an endothelium-specific phenotype in later stages of development. Since the vascular extracellular matrix also changes during embryonic development (Risau and Lemmon, 1988), tie-2 with its peculiar organization of extracellular domains may be involved in the regulation of endothelial cell-substratum adhesion.

It is unlikely that the multiple aspects of endothelial cell physiology are controlled by a single receptor. Recently, we have identified flk-1 as an endothelium-specific receptor tyrosine kinase in an identical approach as described here for tie-2 (Millauer et al., 1993). The temporal and spatial expression pattern of flk-1, which is a receptor for vascular endothelial cell growth factor (VEGF), seems to be similar, if not identical to that found for tie-2. However, both
receptors differ in topology, especially in their extracellular domains. It is conceivable that a set of receptors, whose composition changes in a time- and tissue-dependent manner, specifies endothelial cell behaviour during blood vessel development and maintenance.

From transplantation studies in the quail-chick system, it is well documented that the ability of endothelial cells to form a blood vessel system is not due to an intrinsic genetic program, but depends on environmental cues from the tissue to be vascularized (Noden, 1988; Stewart and Wiley, 1981; Ekblom et al., 1982). Given the final morphological heterogeneity of the vasculature, it is clear that recognizing and responding to different external signals must be a central aspect in endothelial cell biology. The tie-2 receptor is ubiquitously expressed in the endothelium of every organ and tissue that we have examined. Apart from brain, it was detected in heart, lung, kidney, liver and spinal cord. It is therefore likely that tie-2 is involved in a very fundamental signalling process, typical for the whole population of heterogeneous endothelial cells.

**Fig. 6.** tie-2 expression in organs and tissues of E 12.5 embryos and colocalization with immunostaining for PECAM (CD31). Adjacent sections of E12.5 embryos were stained with an antibody recognizing PECAM(CD31) (A,C,E,G) or hybridized with a tie-2 probe (B,D,F,H). The expression pattern of tie-2 was found to be identical to the staining pattern for PECAM(CD31). TE, telencephalon; LV, lateral ventricle; CP, choroid plexus of the lateral ventricle; DA, dorsal aorta; SC, spinal cord; EN, endocardium; MY, myocardium; BR, bronchus; SO, somite; Bar represents 110 μm.

**Fig. 7.** tie-2 expression at embryonic day 8.5. Transverse section of an E 8.5 embryo and adjacent yolk sac. Hybridization signals were detectable in the endocardium, dorsal aorta, cardinal vein and the mesodermal (inner layer) of the yolk sac (A,B). Arrowheads indicate hybridization signal over the marginal cells of an advanced stage blood island (C,D). Note the absence of hybridization signals in the neuroectoderm. CV, cardinal vein (head vein); BI, blood island; DA, dorsal aorta; EN, endocardial tissue; TE, telencephalon; YS, yolk sac. Bar, represents 50 μm.
The finding that tie-2 is expressed in E8.5 embryos confirms this notion and suggests a role even at the early phases of vascular development. Blood islands are mesodermal derivatives of the yolk sac with characteristic morphology. The appearance of a central cell cluster distinct from the peripheral cell layer, is the first overt sign for the separation of the hematopoietic and endothelial cell lineage. We could detect tie-2 mRNA in the peripheral endothelial cell precursors indicating that the gene could be involved in the onset of angioblast differentiation. tie-2 expression was also observed in yolk sac mesoderm outside of blood islands. The significance of this observation is not clear. However, one may speculate that tie-2 mRNA synthesis reflects the segregation of endothelial cells and hematopoietic cells on a molecular level before this process is morphologically evident.

The origin and the differentiation of endothelial cells within the embryo proper is less well understood. However, from the transplantation studies mentioned above, it is clear that endogenous endothelial cell precursors are present in most mesodermal tissues of the embryo. The development of some intraembryonic blood vessels, e.g. the dorsal aortae, has been described as an assembly of in situ differentiating endothelial cells (Meier, 1980; Pardanaud et al., 1987; Coffin and Poole, 1988; Pardanaud et al., 1989). This mode of blood vessel formation, which is called vasculogenesis, is mechanistically distinct from angiogenic sprouting (Pardanaud et al., 1987, 1989; Risau et al., 1988). We found tie-2 mRNA expressed in cells assembled like a ring in the anlagen of the dorsal aortae. The same pattern was found in the primordia of the cardinal veins. Furthermore, tie-2 mRNA was present in the developing endocardium of the heart and in scattered mesenchymal cells, which were not associated with a particular structure. This expression profile could be indicative of the presence of intraembryonic endothelial cell precursors before and at the time of differentiation. In that case, tie-2 would be an early marker for intra- and extraembryonic angioblasts and molecular probes for tie-2 would be a valuable tool for studying the early events of vascular development.

Endothelial cells are able to interpret external cues for their correct assembly into a highly diverse blood vessel system. The tie-2 receptor may be considered as part of a signaling system allowing endothelial cells to respond to soluble or matrix-bound factors and to communicate with other cells. The exact role of tie-2 on a molecular level has not been determined yet. The intracellular portion of the deduced protein contains a typical tyrosine kinase domain. The identification of substrates for phosphorylation and the question whether there exists an endothelial cell-specific signaling transduction pathway will be an area of future research. In the extracellular portion of tie-2 is a mosaic of immunoglobulin, EGF-like and fibronectin type III domains. EGF-like repeats are present in numerous, apparently unrelated proteins. The functional significance of this sequence motif is not obvious. Several copies can be present in the extracellular part of membrane-bound proteins or in polypeptides that are secreted. The three EGF-like repeats in tie-2 show about 30% sequence similarity with TAN-1 (Ellisen et al., 1991) and Xotch (Coffman et al., 1990), vertebrate homologues of the Drosophila notch gene product. Tan-1 and Xotch are integral membrane proteins, each containing 36 EGF-like repeats in the extracellular part. The molecules are thought to be involved in some sort of extracellular interaction. The A chain of laminin, a glycosylated basal lamina protein, possesses 15 EGF-like repeats, three of them having a 30% sequence identity with the EGF-like triplet of tie-2 (Sasaki et al., 1988). Unlike TAN-1, Xotch and Laminin, the EGF-like units in tie-2 (also tie and TEK) have eight instead of six cysteine residues. It is an open question whether these additional residues are also involved in disulphide bonding and whether this has functional importance for the interaction of tie-2 to its yet unidentified ligand. In any event, to our knowledge tie and tie-2 are so far the only receptor tyrosine kinases known to have EGF-like repeats in their extracellular parts.

In contrast to EGF-like repeats, immunoglobulin-like domains are structural motifs present in the extracellular portions of wide variety of receptor tyrosine kinases (Ullrich and Schlessinger, 1990; Williams and Barclay, 1988). tie-2 has a single, amino-terminal Ig domain with the characteristics of a C2-set sequence. In general, the function of Ig-containing proteins can be described as mediating heterophilic and homophilic binding events. In the case of the alpha-PDGF receptor, the three amino-terminal Ig domains determine the binding specificity for the PDGF AA ligand (Heidaran et al., 1990).

Fibronectin type III (FN III) repeats are also present in the extracellular parts of receptor tyrosine kinases including the insulin receptor, IGF-1 receptor (Ullrich et al., 1986), sevenless (Norton et al., 1990), Eph (Hirai et al., 1987), cek5 (Pasquale, 1991), axl (O’Bryan et al., 1991) and ark (Rescigno et al., 1991). Originally described as internal homologous units in the extracellular matrix (ECM) protein fibronectin (Petersen et al., 1983), FN III repeats have been identified in, for example, adhesion molecules, cytokine receptors and collagens. In the tenth FN III repeat of fibronectin, the tripeptide RGD was found to be crucial for the interaction of the ECM protein with integrins on the cell surface (Ruoslahti and Pierschbacher, 1986). The three FN III repeats of tie-2 are mostly related to those found in protein-tyrosine phosphatases and in the axonal glycoprotein TAG-1. A possible RGD cell attachment site is present between the amino-terminal Ig domain and the three EGF-like repeats of tie-2. The significance of this observation is unclear, because the tripeptide sequence is neither in a FN III repeat context nor is it conserved in tie and TEK. Interestingly, the ECM protein laminin appears to mediate its effects on endothelial cells in part by a RGD-containing sequence in the A chain (Grant et al., 1989).

Taken together, tie-2 has significant similarities to ECM proteins and adhesion molecules. Whether these homologies indicate a role for this receptor tyrosine kinase in cell-cell or cell-matrix interactions, or whether the ligand for tie-2 is a diffusible molecule remains to be determined. Due to the unusual topology of tie-2, the ligand could be a novel molecule whose identification could lead to a more complete understanding of the molecular mechanisms of blood vessel formation.
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REFERENCES


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**Note added in proof**

Dumont et al. have recently reported a full-length tek sequence that seems to be identical to tie-2 (*Oncogene* (1993), 8, 1293-1301).