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A Novel Endothelial Cell Surface Receptor Tyrosine Kinase with Extracellular Epidermal Growth Factor Homology Domains

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Endothelial cell surfaces play key roles in several important physiological and pathological processes such as blood clotting, angiogenic responses, and inflammation. Here we describe the cloning and characterization of *tie*, a novel type of human endothelial cell surface receptor tyrosine kinase. The extracellular domain of the predicted *tie* protein product has an exceptional multidomain structure consisting of a cluster of three epidermal growth factor homology motifs embedded between two immunoglobulinlike loops, which are followed by three fibronectin type III repeats next to the transmembrane region. Additionally, a cDNA form lacking the first of the three epidermal growth factor homology domains was isolated, suggesting that alternative splicing creates different *tie*-type receptors. Cells transfected with *tie* cDNA expression vector produce glycosylated polypeptides of 117 kDa which are reactive to antisera raised against the *tie* carboxy terminus. The *tie* gene was located in chromosomal region 1p33 to 1p34. Expression of the *tie* gene appeared to be restricted in some cell lines; large amounts of *tie* mRNA were detected in endothelial cell lines and in some myeloid leukemia cell lines with erythroid and megakaryoblastoid characteristics. In addition, mRNA in situ studies further indicated the endothelial expression of the *tie* gene. The *tie* receptor tyrosine kinase may have evolved for multiple protein-protein interactions, possibly including cell adhesion to the vascular endothelium.

Tyrosine phosphorylation is one of the key modes of signal transduction across the plasma membrane. Several currently known protein tyrosine kinase genes encode transmembrane receptors for polypeptide growth factors and hormones, such as epidermal growth factor (EGF), insulin, insulinlike growth factor I, platelet-derived growth factors (PDGF-A and PDGF-B), and fibroblast growth factors (FGFs) (26, 61). Also, the receptors of several hematopoietic growth factors are tyrosine kinases; these include the colony-stimulating factor 1 receptor (52) and c-*kit*, a primitive hematopoietic growth factor receptor (28). Growth factor receptors of endothelial cells are of particular interest because of the possible involvement of growth factors, such as FGFs, in angiogenesis, wound healing, arteriosclerosis, and inflammatory reactions (18).

On the basis of structural similarities, the receptor tyrosine kinases can be divided into evolutionary subfamilies (61). EGF receptor-like (subclass I) and insulin receptor-like (subclass II) kinases contain repeated homologous cysteine-rich sequences in their extracellular domains. A single cysteine-rich region is also found in the extracellular domains of the *eph*-like kinases (27, 34, 35). PDGF receptors as well as c-*fms* and c-*kit* receptor tyrosine kinases can be grouped in subclass III, while the FGF receptors form subclass IV. Typical of the members of both of these subclasses are extracellular folding units stabilized by intrachain disulfide bonds. These so-called immunoglobulin (Ig)-like folds are found in the proteins of the Ig superfamily, which contains a wide variety of other cell surface receptors having either cell-bound or soluble ligands (65).

Although the ligands of these receptor tyrosine kinases are relatively small soluble polypeptides, many of these, such as EGF, transforming growth factor α, amphiregulin, colony-stimulating factor 1, and the c-*kit* ligand (the product of the mouse steel locus), are produced by the cleavage of larger plasma membrane-bound precursors (12, 22, 33, 43, 47, 51). Furthermore, it has been reported that a mutated form of the transforming growth factor α precursor that cannot be cleaved to produce the mature growth factor is able to stimulate the EGF receptor (6, 66). This kind of growth factor precursor-receptor interaction can also mediate cell-cell adhesion (2, 17).

We have previously studied tyrosine kinases expressed in the K562 chronic myeloid leukemia cell line, which has the capacity to differentiate into both erythroid and megakaryoblastoid directions. Using a polymerase chain reaction application (64), we identified several novel members of the tyrosine kinase gene family from these cells (41). In this study, we have further characterized the JTK14 gene, renamed *tie* (tyrosine kinase with Ig and EGF homology domains), which represents a novel type of endothelial cell receptor tyrosine kinase.

MATERIALS AND METHODS

Isolation and characterization of cDNA clones. An oligo(dT)-primed human erythroleukemia (HEL) cell cDNA library in bacteriophage λgt11 (a kind gift from Mortimer Poncz, Children's Hospital, Philadelphia, Pa. [44]) and a random-primed human endothelial cell cDNA library (Clon-

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tech catalog no. 1070b) were screened with the JTK14 cDNA fragment amplified by polymerase chain reaction from the polyadenylylated RNA of K562 leukemia cells (41). Positive plaques were identified and purified as previously described (48). cDNA inserts of bacteriophage lambda were isolated as *Eco*RI fragments and subcloned into the GEM3Zf(+) plasmid (Promega). The entire *tie* protein coding region was isolated from both libraries. Two overlapping clones isolated from the HEL library (HE11-1, nucleotides 62 to 3845 [see Fig. 1], and 12a, nucleotides 1 to 2446) were sequenced by the dideoxy chain termination method with oligonucleotide primers designed according to the sequences obtained. All portions of the cDNAs were sequenced on both strands. Sequence analyses were performed with the Genetics Computer Group package programs (13) and the Prosite program for the Apple Macintosh.

Preparation of antisera. A *tie* cDNA fragment encoding 196 carboxy-terminal amino acids was inserted into the pEX2 bacterial expression vector (55) by using an internal *Xho*I site. The resulting β-galactosidase fusion protein was produced in bacteria and partially purified by preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Polypeptide bands were cut from the gel, minced, mixed with Freund's adjuvant, and used for the immunization of rabbits. Antisera were used after the third booster immunization. A peptide corresponding to 15 amino acids from the carboxyl terminus of the predicted *tie* protein was synthesized and coupled to keyhole limpet hemocyanin. The immunizations were performed as described above.

Expression in COS-1 cells and immunoprecipitation. The full-length *tie* protein coding sequence (combined from two overlapping clones, HE11-1 and 12a) was inserted into the *Eco*RI site of the pSVpoly mammalian expression vector (54) (construct SV14-2). The construct SV14-1 lacks the first seven amino acids from its signal sequence, but it is initiated from an ATG codon present in the pSVpoly vector. The constructs (SV14-2 and SV14-1) were introduced into COS-1 cells by the DEAE-dextran transfection method (38). Two days after transfection, the cells were labeled for 4 h with [^{35}S]methionine in the presence or absence of 10 μg of tunicamycin per ml. The cells were washed with phosphate-buffered saline (PBS) and scraped into immunoprecipitation buffer (10 mM Tris [pH 7.5], 50 mM NaCl, 0.5% sodium deoxycholate, 0.5% Nonidet P-40, 0.1% SDS, 0.1 trypsin inhibitor units of aprotinin per ml). The lysates were sonicated, centrifuged for 15 min at 10,000 × *g*, and incubated overnight on ice with 3 μl of antiserum. Protein A sepharose (Pharmacia) was added, and the incubation was continued for 30 min with rotation. The precipitates were washed four times with the immunoprecipitation buffer, once with PBS, and once with water before analysis by SDS-PAGE.

Expression in NIH 3T3 cells and immunoblotting. The full-length *tie* cDNA was subcloned under the control of the Moloney murine leukemia virus long terminal repeat promoter. This construct was used to cotransfect NIH 3T3 cells with the pSVneo1 marker plasmid, and G418-resistant clones were analyzed for *tie* expression. For immunoblot analyses, cells on one confluent plate were lysed in 2.5% SDS–125 mM Tris, pH 6.5. Cell lysates were electrophoresed by SDS-PAGE and electroblotted to nitrocellulose membranes. The membrane was incubated with the antipeptide antiserum against the *tie* carboxy terminus, and bound antibodies were visualized by using horseradish peroxidase-conjugated swine anti-rabbit antiserum (Dako) and ECL reagents (Amersham). Proteins phosphorylated on tyrosine residues were immunoprecipitated as described previously (19). Briefly, cells on one confluent 9-cm-diameter plate were lysed in extraction buffer (1% Triton X-100, 10 mM Tris [pH 7.6], 5 mM EDTA, 50 mM NaCl, 100 μM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride), and the lysates were incubated with rotation for 2 h on ice with agarose-conjugated antiphosphotyrosine antibodies (1G2-A; Oncogene Science). The immunoprecipitates were washed four times with extraction buffer, and proteins phosphorylated on tyrosine residues were eluted with 1 mM phenyl phosphate. Eluted proteins were analyzed by immunoblotting as described above.

Chromosomal in situ hybridization. Metaphase spreads from normal human male peripheral blood buffy coat leukocytes were prepared and hybridized essentially as previously described (25). For in situ hybridization, about 1 μg of HE11-1 cDNA insert was labeled by nick translation by using four ^{3}H-labeled nucleoside triphosphates. The labeled DNA had a specific activity of about 4×10^7 to 8×10^7 cpm/mg. After hybridization, slides were washed in a solution of 50% formamide and 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 μ sodium citrate) at 39°C and exposed to Kodak NTB2 nuclear track emulsion for 12 days at 4°C. The slides were developed with Kodak Dektol developer and Kodafix solution, and chromosomes were first G banded with Wright-Giemsa stain (8) and, if necessary, rebanded by the trypsin-Giemsa technique.

mRNA extraction and Northern (RNA) blot analyses. The leukemia cell lines used in this study have been reported previously, as follows: K562 (36), HL-60 (9), HEL (37), Dami (23), MOLT-4 (39), Jurkat (50), U937 (58), KG-1 (31), JOK-1 (1), ML-2 (21), and RC-2A (7). The leukemia cells were grown in RPMI medium containing 10% fetal calf serum and antibiotics. Dami cells were cultivated in Iscove's modified Dulbecco's medium with 10% horse serum. A permanent hybrid cell line (EA · hy926) obtained by fusing first-passage human umbilical vein endothelial cells with A549 lung carcinoma cells (14) was cultured in Dulbecco's modified Eagle medium–hypoxanthine-aminopterin-thymidine medium containing 10% fetal calf serum and antibiotics. The porcine aortic endothelial (PAE) cells were grown in Ham's F12 medium containing 10% fetal calf serum.

Poly(A)$^+$ RNA was extracted from the cell lines as previously described (48). Five micrograms of the poly(A)$^+$ RNA samples was electrophoresed in agarose gels containing formaldehyde and blotted by using standard conditions (48). The insert of the HE11-1 cDNA clone was labeled by the random-priming method and hybridized to the blots. Hybridization was carried out in 50% formamide, 5× Denhardt's solution (100× Denhardt's solution is 2% [each] Ficoll, polyvinylpyrrolidone, and bovine serum albumin), 5× SSPE (3 M NaCl, 200 mM $NaH_2PO_4 \cdot H_2O$, 20 mM EDTA [pH 7.0]), 0.1% SDS, and 0.1 mg of sonicated salmon sperm DNA per ml at 42°C for 18 to 24 h. The filters were washed at 65°C in a solution of 1× SSC (pH 7.0) and 0.1% SDS and exposed to Kodak XAR-5 film.

In situ hybridization for RNA in tissues. Selected fragments of cloned human *tie* cDNA outside the tyrosine kinase domain exhibiting a low degree of homology to other receptor tyrosine kinases were used as in situ hybridization probes to detect the *tie* mRNA. Specifically, we used a *Sma*I fragment (nucleotides 268 to 1767) of the full-length cDNA clone corresponding to the extracellular domain of the *tie* clone, further digested to smaller fragments with *Pst*I and *Sal*I. The probe was labeled with ([^{35}S]thio)dATP for the in situ hybridizations (16). *Bgl*I-generated 100- to 790-bp frag-

ments of bacteriophage λ DNA were labeled similarly and used as a negative control probe.

All specimens from fetal abortuses were obtained with the permission of the joint ethical committee of the University Central Hospital and the University of Turku (Turku, Finland). In situ hybridizations were carried out as described previously (49). In brief, tissue samples of 15- to 19-week human fetuses obtained from therapeutic abortions were fixed with formalin and embedded in paraffin for sectioning. The sections were pretreated with proteinase K and HCl and acetylated. The hybridizations were carried out at 42°C for 24 h by using ([^{35}S]thio)dATP-labeled probes and were followed by washing, autoradiography at +4°C for 5 to 25 days, and staining of the sections with hematoxylin.

Nucleotide sequence accession number. The nucleotide sequence of the *tie* cDNA has been deposited in the EMBL data base (accession no. X60957).

RESULTS

Structure of the *tie* protein deduced from its cDNA. The 200-bp-long *tie* cDNA fragment (JTK14) isolated by a polymerase chain reaction cloning method from K562 cell cDNA (41) was used as a molecular probe to screen an oligo(dT)-primed HEL cell cDNA library (44) and a random-primed human endothelial cell cDNA library. Several overlapping clones were identified from both libraries. Nucleotide sequence analysis of clones HE11-1 and 12a isolated from the HEL library revealed an open reading frame of 1,138 amino acid residues (Fig. 1). The translational initiator (methionine) indicated in Fig. 1 is surrounded by a typical consensus sequence (32) and followed by a hydrophobic amino acid sequence characteristic of signal sequences for translocation into the endoplasmic reticulum.

Beginning with amino acid residue 214 of the reading frame, there is a region of 130 amino acid residues containing 24 cysteine residues altogether. This region can be aligned into three repeated homologous domains containing eight cysteine residues each (Fig. 2A). The figure also shows the comparison of the *tie* cysteine-rich domains with the EGF and CRIPTO growth factor proteins, the EGF-like repeats of the laminin A chain, the notch and *lin*-12 developmental control proteins of *Drosophila melanogaster* and *Caenorhabditis elegans*, respectively, and blood coagulation factor IXa. Significant structural similarities, which allow the inclusion of the cysteine-rich repeats of *tie* in the EGF repeat family, can be observed. However, the *tie* repeats are more closely related to each other than to other members of the EGF repeat family. This is particularly evident when the amino-terminal ends of the repeats, whose three cysteine residues are not conserved in other EGF repeats (Fig. 2A), are examined. In addition to several *tie* cDNA molecules encoding three EGF repeats, a cDNA clone which lacked the first of the EGF repeats (marked between the arrowheads in Fig. 1) without any other changes to the reading frame was isolated from the HEL cell cDNA library.

The most amino-terminal region of the *tie* extracellular domain shows weak but significant homology to the amino terminus of chicken N-CAM protein (10). As in N-CAM, a pair of cysteine residues surrounded by consensus motifs characteristic for the proteins of the Ig superfamily (65) is found in this region (Ig 1 in Fig. 1). In addition, two pairs of cysteine residues are located on the carboxyl-terminal side of the three EGF repeats. The amino acid sequence around the first cysteine pair shows additional homology to Ig domains (Ig 2 in Fig. 1). The extracellular region following the Ig 2 domain (including one of the cysteine pairs) can be aligned into three segments that are homologous to fibronectin type III (FNIII) repeats. The three repeats of the *tie* protein and their comparison with the FNIII repeats present in the human LAR phosphotyrosine phosphatase (57) are shown in Fig. 2B. Interestingly, the second of these three fibronectin repeats (FN 2) contains a pair of cysteine residues as well as some other features of an Ig domain (Fig. 2B) and thus represents an intermediate of a FNIII repeat and an Ig domain. Five consensus sites for potential N-linked glycosylation (NXS/T, where X = any amino acid) can be distinguished in the extracellular domain. None of these appear in the EGF repeats.

Amino acid residues 761 to 786 form a hydrophobic stretch of sequence, which is likely to function as the transmembrane domain of the receptor, followed by several basic residues on the putative cytoplasmic side of the polypeptide. The juxtamembrane domain is 50 residues long before the beginning of tyrosine kinase sequence homology at amino acid 837. With the interruption of homology in the kinase insert sequence of 14 amino acids (indicated by italics in Fig. 1), this homology is first lost at the beginning of the 31-amino-acid carboxyl-terminal tail of the receptor. A search for related tyrosine kinase domains in amino acid sequence data bases (Swissprot and NBRF) identifies the FGF receptor 1 and the *ret*, c-*fms*, PDGFR, and c-*kit* receptor tyrosine kinases as the closest homologs of *tie* (about 40% amino acid sequence identity in the tyrosine kinase domain).

The *tie* cDNA directs the synthesis of a 117-kDa glycoprotein in COS cells. The structural predictions of the *tie* cDNA sequence were tested by cloning the full-length *tie* protein coding region into the *Eco*RI site of the pSVpoly expression vector (54) (constructs pSV14-2 and pSV14-1), and these constructs were transfected into COS cells. The proteins produced by these two constructs differ in their signal sequences (see Materials and Methods), but the predicted mature protein products are identical. After 2 days, the cells were metabolically labeled and immunoprecipitated with antibodies generated against a β-galactosidase–*tie* fusion protein containing 195 carboxyl-terminal amino acid residues of the predicted *tie* protein (antiserum HI) or against a 15-amino-acid peptide corresponding to the *tie* carboxyl terminus (antiserum MI). Figure 3 shows analysis of the immunoprecipitated radioactive polypeptides by SDS-PAGE. As can be seen from Figure 3A, the HI immune serum precipitated some weakly labeled polypeptides from untransfected COS cells. These polypeptides were probably not related to *tie*, because the COS cells do not express its mRNA (data not shown). Cells transfected with a pSV14-1 construct show an additional specific polypeptide of 117 kDa (labeled "*tie*" in Fig. 3). This *tie* polypeptide was not precipitated with the preimmune serum or the antiserum blocked with the immunogen.

The 117-kDa polypeptide was also recognized by the MI antiserum against a carboxyl-terminal peptide (Fig. 3B). Immunoprecipitation of *tie* polypeptides from transfected COS cells metabolically labeled in the presence of tunicamycin to prevent N-linked glycosylation of proteins gave a specific polypeptide with an apparent molecular mass of approximately 105-kDa (labeled "*tie**" in Fig. 3B).

The *tie* protein expressed in NIH 3T3 cells is tyrosine phosphorylated. Stable cell lines expressing *tie* protein were produced by transfecting the *tie* cDNA under the control of the Moloney murine leukemia long terminal repeat promoter into NIH 3T3 cells. In these cell clones, the 117-kDa *tie* protein could be detected by immunoblotting with the anti-

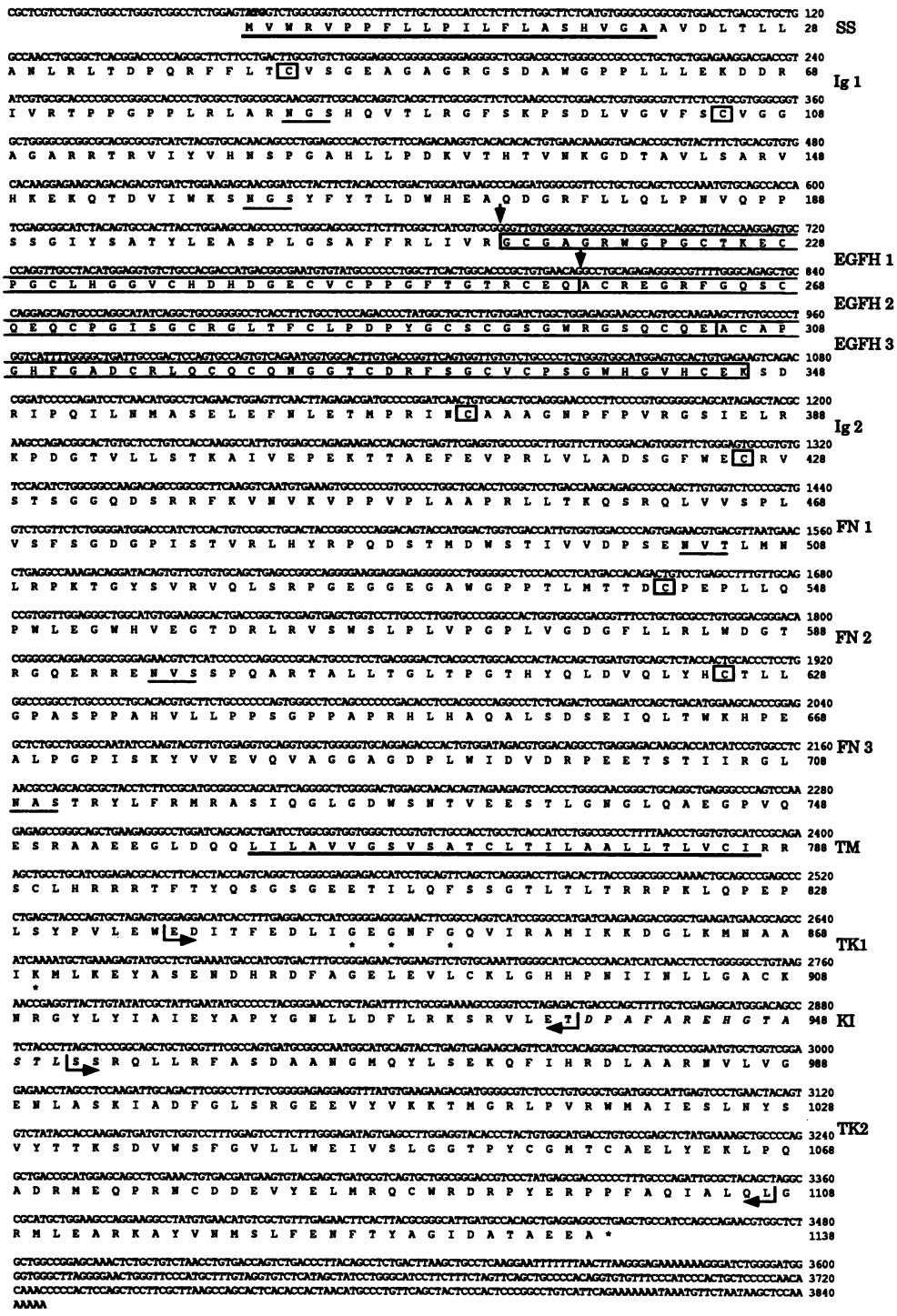

FIG. 1. Nucleic and deduced amino acid sequences of the *tie* cDNA. The 3,845-bp nucleotide sequence compiled from two overlapping cDNA clones isolated from the HEL cell cDNA library contains an open reading frame of 1,138 amino acids (the single-letter code is used). The hydrophobic signal sequence (55) and the putative transmembrane (TM) domain are underlined (thick lines), as are the sites for potential N-linked glycosylation (thin lines). Cysteine residues found in the extracellular domain have been boxed; the tyrosine kinase (TK) domain is shown by arrows, and the kinase insert (KI) is shown in italics. The three cysteine-rich segments having homology to EGF-like domains are also boxed (EGFH 1 through 3). Their alignment is shown in Fig. 2. The first of the EGF repeats missing in clone 3a is indicated by arrows.

serum raised against the peptide corresponding to the *tie* carboxyl terminus (Fig. 4). In addition, endogenous *tie* protein with a similar molecular mass could be detected in PAE cells. The *tie* protein was also detected in antiphospho tyrosine immunoprecipitates of the *tie*-transfected cells.

Chromosomal mapping of the *tie* locus. In situ hybridization of the radiolabeled *tie* probe to normal human metaphase chromosomes localized *tie* sequences to chromosome

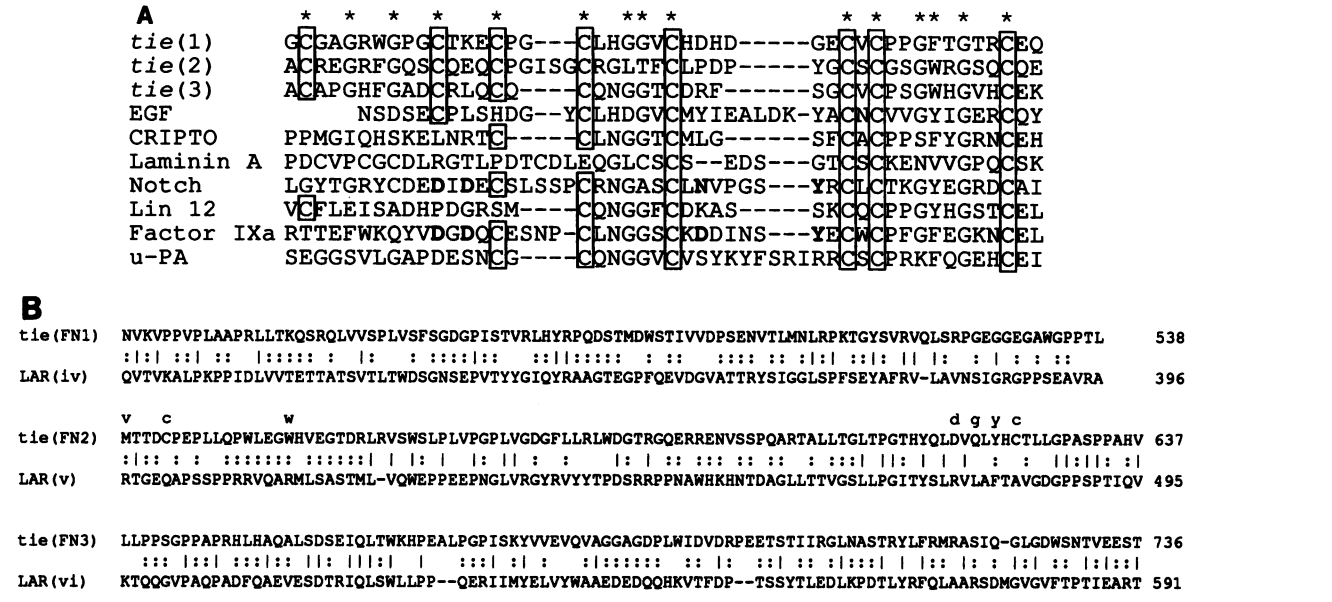

FIG. 2. (A) Alignment of the EGF-like domains of *tie* with human EGF sequence (aa 1 to 44) and homologous sequences in the growth factor CRIPTO (aa 67 to 108), laminin A chain (aa 1092 to 1138), *D. melanogaster* Notch (aa 897 to 945) and *C. elegans lin*-12 (aa 204 to 246) developmental control proteins, human blood coagulation factor IXa (aa 83 to 130), and mouse urokinase-type plasminogen activator (u-PA) (aa 18 to 65). The asterisks indicate conserved residues, and the conserved cysteine residues are boxed. The consensus residues for β-hydroxylation present in the repeats of notch and factor IXa are printed in boldface. (B) Comparison of the three FNIII repeats of the *tie* protein and the first three FNIII repeats of the human LAR receptor phosphotyrosine phosphatase. The cysteine residues as well as some other consensus residues typical of Ig domains are shown above the second FNIII repeat of the *tie* protein.

1. A total of 317 chromosomally localized grains were scored on 145 metaphases. A total of 34% (109 of 317) of the grains were on chromosome 1, with 69% (75 of 109) of chromosome 1 grains localized to 1p33 to 1p34. Grain localization on chromosome 1 is illustrated schematically in Fig. 5, in which each dot represents three grains. This narrows the localization to 1p33 to 1p34, with the highest concentration of grains close to the border between bands 1p33 and 1p34. Chromosomal localization with a panel of somatic mouse-human hybrid cell lines also placed the *tie* locus on human chromosome 1, supporting the in situ hybridization results (data not shown).

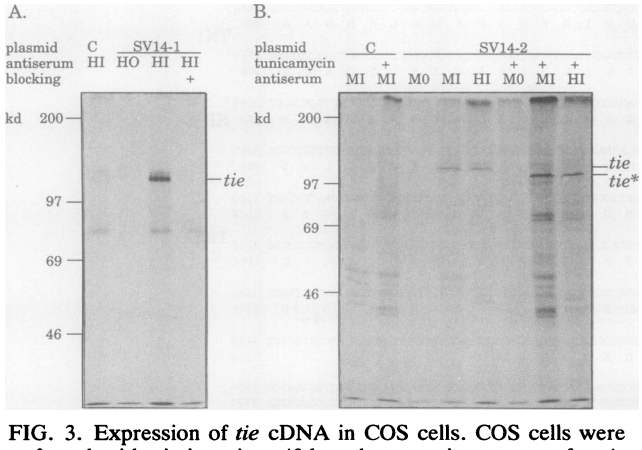

FIG. 3. Expression of *tie* cDNA in COS cells. COS cells were transfected with simian virus 40-based expression vectors for *tie* (SV14-1 and SV14-2) and FGF receptor 4 (C) (42), labeled with [^{35}S]methionine, lysed, and immunoprecipitated as described in Materials and Methods. Autoradiograms of the SDS-PAGE analysis of the precipitated proteins are shown. (A) Identification of *tie* polypeptides expressed in the COS cells. HI, immune serum against the β-Gal-*tie* fusion protein; and HO, preimmune serum. The immune serum was blocked with the antigen where indicated (+). (B) Effect of tunicamycin on the molecular mass of the *tie* protein. MI, immune serum against a carboxyl-terminal *tie* peptide; and MO, preimmune serum. Where indicated (+), the transfected cell cultures were labeled in the presence of tunicamycin. Mobilities of the molecular weight markers are shown on the left.

Expression of the *tie* mRNA in leukemia cell lines and endothelial cells. Figure 6 shows the results of the analysis of *tie* mRNA expression in 11 leukemia cell lines. Only the HEL cells, the KG-1 myeloid leukemia cells, and the Dami megakaryoblastic leukemia cells expressed a 4.4-kb *tie* mRNA, as detected with the 3.8-kb *tie* cDNA probe. The

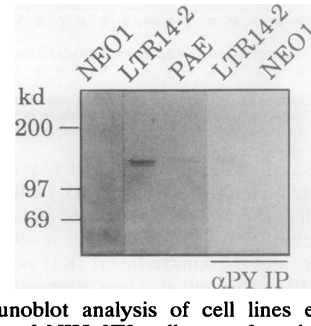

FIG. 4. Immunoblot analysis of cell lines expressing the *tie* protein. Lysates of NIH 3T3 cells transfected (LTR14-2) or not transfected (NEO1) with a *tie* expression vector, as well as PAE cells, were analyzed by immunoblotting with antiserum against a carboxyl-terminal *tie* peptide. The samples in the two rightmost lanes (αPY IP) were immunoprecipitated with antiphosphotyrosine antibodies prior to immunoblotting.

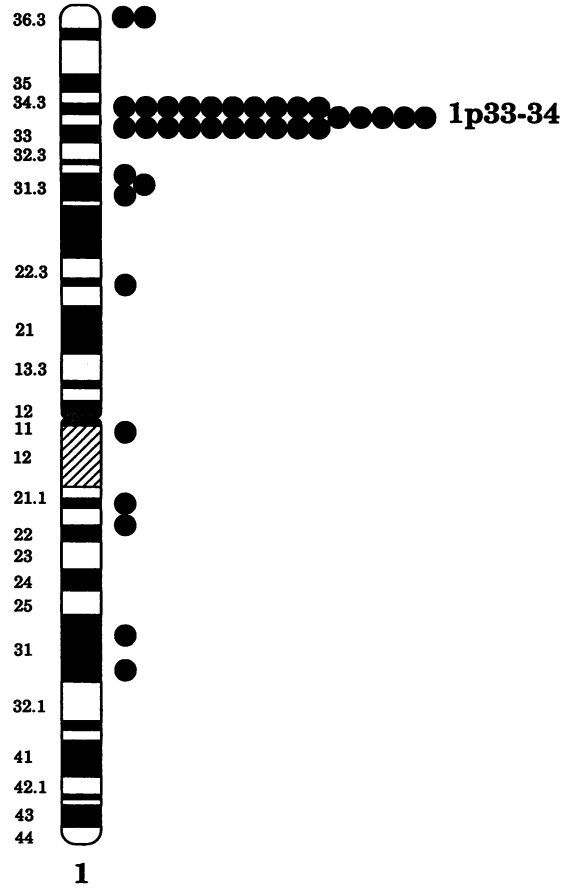

FIG. 5. Chromosomal mapping of the *tie* locus. Radiolabeled JTK14 DNA was hybridized to normal human male metaphase preparations; slides were washed, developed after exposure, and G banded to distinguish individual chromosomes. Grain localization is illustrated on the schematic chromosome 1, where each dot represents three grains. Some nonspecific background signal was detected for the other chromosomes; 12.6% (40 of 317) for other chromosomes of group A, 8.5% (27 of 317) for chromosomes of group B, 29.6% (94 of 317) for C-group chromosomes, and 14.8% (47 of 317) for the other chromosome groups.

Jurkat and MOLT-4 T-cell leukemia cells, as well as the HL-60 promyelocytic leukemia cells, the U937 and RC-2A monocytic leukemia cells, the JOK-1 hairy-cell leukemia cells, and the ML-2 myeloid leukemia cells, were negative

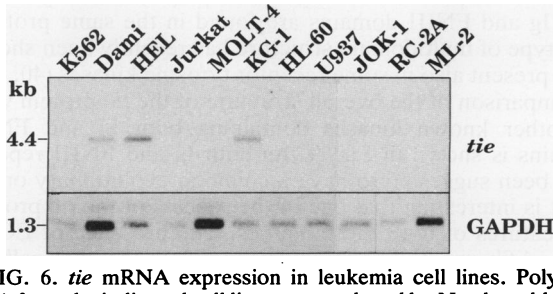

FIG. 6. *tie* mRNA expression in leukemia cell lines. Poly(A)$^+$ RNA from the indicated cell lines was analyzed by Northern blotting and hybridization with the *tie* cDNA probe. Hybridization with the glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) probe was used as an internal control for the loading of even amounts of RNA for analysis.

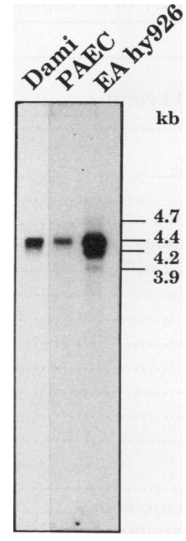

FIG. 7. *tie* mRNA expression in endothelial cell lines. Northern blot analysis of *tie* mRNA expression in PAE cell and EA · hy926 endothelial cell lines. A lane containing poly(A)$^+$ RNA from Dami cells was included as a positive control.

for the *tie* mRNA. As reported earlier (41), the *tie* mRNA is induced after tetradecanoyl phorbol acetate treatment of the K562 cells, when the cells undergo megakaryoblastoid differentiation. Interestingly, PAE cells, as well as a hybrid human endothelial cell line, EA · hy926, which has been reported to express several endothelial markers in vitro (14, 15), expressed *tie* mRNA abundantly (Fig. 7). The EA · hy926 cell line was created by the fusion of human umbilical vein endothelial cells with A549 lung carcinoma cells. The A549 cells were negative for *tie* mRNA expression (data not shown). In addition to the 4.4-kb mRNA, the EA · hy926 cells expressed *tie* mRNA species of 3.9, 4.2, and 4.7 kb. The results of Northern blot analyses of the *tie* mRNA expression in cell lines are summarized in Table 1.

Expression of *tie* in blood vessels. *tie* mRNA expression in tissues was further studied by mRNA in situ hybridization of 15- to 19-week-old human fetal tissues. In agreement with the *tie* expression in endothelial cell lines, *tie* mRNA was seen to be located in the walls of medium and large vessels (Fig. 8). Labeled lambda DNA used as a negative control did not give any hybridization signal above the background signal (data not shown).

DISCUSSION

The *tie* tyrosine kinase is yet another example of the tendency of evolution to create new molecules by combining already existing motifs. Containing EGF-like, Ig-like, fibronectinlike, and tyrosine kinase domains, *tie* belongs to four different gene superfamilies. A combination of motifs from Ig, fibronectin, and EGF homology superfamilies in the extracellular domain is a unique feature among known receptor tyrosine kinases.

The EGF-like domain is a commonly found structural motif in cell surface and extracellular proteins involved in protein-protein interactions (11). Many transmembrane receptors for either soluble or cell-bound ligands contain EGF repeats. Furthermore, two of the six EGF repeats of thrombomodulin, an endothelial cell surface glycoprotein, have

TABLE 1. Summary of *tie* expression in cell lines

Cell line	Expression[a]
Endothelial	
EA · hy926, endothelial hybrid	++
PAE	++
Leukemia	
Dami, megakaryoblastic	++
HEL, erythroleukemic	++
KG-1, acute myelogenous	++
K562, chronic myelogenous	−
K562 + TPA[b]	+
HL-60, promyelocytic	−
U-937, monocytic	−
MOLT-4, T cell	−
Jurkat, lymphoma	−
JOK-1, hairy-cell	−
ML-2, myeloid	−
RC-2A, monocytic	−
Others	
A549, lung carcinoma	−
U1690, small-cell lung carcinoma	−
H358, small-cell lung carcinoma	−
MCF-7, breast adenocarcinoma	−
Y79, retinoblastoma	−
SK-NEP-1, Wilms' tumor	−
Kelly, neuroblastoma	−
PA-1, ovarian teractocarcinoma	−
RD, rhabdomyosarcoma	+
A204, rhabdomyosarcoma	−
NIH 3T3, mouse fibroblast	−
COS-1, monkey kidney fibroblast	−

[a] −, no expression, +, moderate expression; ++, abundant expression.
[b] TPA, tetradecanoyl phorbol acetate.

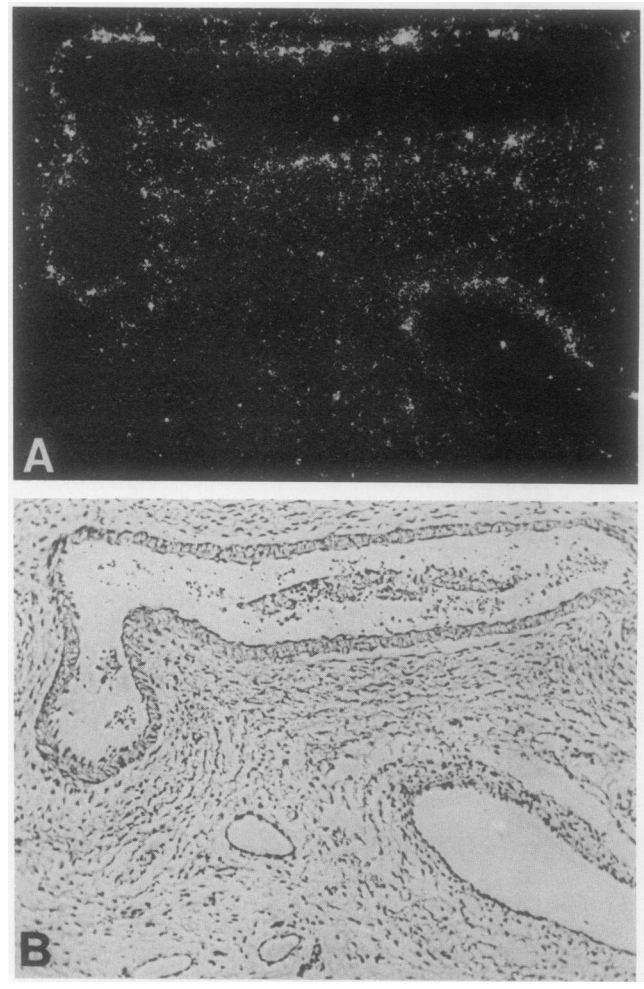

FIG. 8. Location of *tie* mRNA in kidney vessels by in situ hybridization. A dark-field image showing the hybridization signal (A) and the corresponding phase-contrast micrograph (B) are shown.

been reported to be responsible for thrombin binding (56), and the EGF domain of the lymph node homing receptor has been implicated in the adhesion of lymphocytes to high endothelial venules (53). Also, some homeotic genes, such as notch, delta, and crumbs of *D. melanogaster* (59, 62, 63) and *lin*-12 and *glp*-1 of *C. elegans* (68, 69), encode large transmembrane proteins containing several EGF-like repeats. These proteins participate in several cell fate decisions, requiring cell-cell communication. Genetic evidence further suggests that the distinct EGF motifs function in different protein-protein interactions (29, 45). Multiple EGF repeats are found also in extracellular matrix proteins mediating cell adhesion, such as laminin and tenascin. In addition, EGF repeats are a common motif in secreted proteins involved in blood clotting, including coagulation factors VII, IX, and X, proteins C and S, and tissue and urokinase-type plasminogen activators (20). The EGF-like domain of the urokinase-type plasminogen activator has been shown to be responsible for its receptor binding (3).

The EGF-like repeats of *tie* contain eight cysteine residues instead of the usual six. Although eight cysteines are also found in the EGF repeats of laminin, the *tie* repeats are clearly most closely related to each other. None of the repeats of *tie* contains the consensus residues required for asparagine or aspartate β-hydroxylation and calcium binding. The finding of a *tie* cDNA clone which encodes a protein lacking the first of the EGF-like repeats further suggests that these domains are located in separate exons and that the repeat structure was presumably created by exon duplication in the course of the molecular evolution of the *tie* receptor tyrosine kinase. In addition, the observation of several *tie* mRNA forms in EA · hy926 cells supports the notion that various forms of the *tie* receptor are produced, presumably because of differential splicing.

The Ig and fibronectin superfamilies also consist of glycoproteins implicated in extracellular protein-protein interactions with either soluble or cell-bound molecules (65). Many receptor tyrosine kinases such as the PDGF and colony-stimulating factor 1 receptors, the c-*kit* proto-oncogene, and the FGF receptors contain Ig-like loops (61). In many cases, both Ig and FNIII domains are found in the same protein. This type of multidomain structure has recently been shown to be present also in some receptor tyrosine kinases (40, 46). A comparison of the overall structure of the *tie* protein with the other known kinases containing both Ig and FNIII domains is shown in Fig. 9. As both Ig and FNIII repeats have been suggested to have a common evolutionary origin (4), it is interesting that one of the repeats of the *tie* protein has features of both these classes. The presence of EGF-, Ig-, and fibronectinlike structural motifs in the extracellular domain of the *tie* protein suggests that the *tie* receptor might interact with several different extracellular molecules.

The *tie* protein expressed in NIH 3T3 cells could be immunoprecipitated by using antiphosphotyrosine antibodies, indicating that the *tie* protein is tyrosine phosphorylated

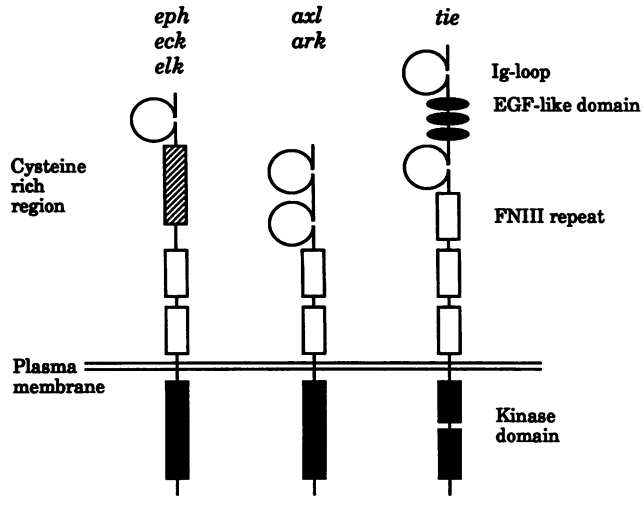

FIG. 9. Comparison of the structure of the *tie* protein with structures of some other receptor tyrosine kinases containing Ig and FNIII repeats. The open circles represent Ig loops, the open boxes represent FNIII repeats, and the filled ovals represent EGF homology domains. The shaded box represents the cysteine-rich region of the *eph*-like kinases. The cytoplasmic tyrosine kinase domains are represented by black boxes.

in these cells. This result supports the prediction that *tie* is a tyrosine-specific protein kinase autophosphorylating itself. We cannot, however, exclude the possibility of transphosphorylation of the *tie* protein by another kinase. We have not been able so far to detect kinase activity of the immunoprecipitated *tie* protein. The kinase activity might be low in the absence of the appropriate ligand.

The regional localization of the *tie* gene at 1p33 to 1p34 demonstrates that the *tie* locus is on the telomeric side of the *jun* locus, since the PB5-5 hybrid which is negative for *tie* is positive for *jun* (24). The chromosomal region 1p32 to 1p34 is involved in deletions in neuroblastomas, malignant lymphomas, gliomas, and other malignancies (cited in reference 60). It will be interesting to analyze in further detail the fate of the *tie* alleles in these tumor-specific chromosomal rearrangements.

Our earlier and present experiments indicate that the mRNA for the *tie* receptor is expressed in only a few tumor cell lines in culture. In contrast, expression was evident in the analysis of isolated RNA from all mouse and human fetal tissues studied (reference 41 and our unpublished results). This pattern of expression is compatible with the possibility that the signal obtained from the tissues is derived from endothelial cells, as suggested by the finding of *tie* mRNA in the EA · hy926 and PAE cell lines as well as in primary cultured human endothelial cells (kindly provided by Ari Ristimäki) (our unpublished results). Furthermore, in situ hybridization analyses of *tie* expression in human as well as in mouse tissues (31a) indicate that *tie* mRNA is present in endothelial cells.

The findings for *tie* mRNA expression suggest that its protein product is characteristic of the bipotential hematopoietic cell lineage retaining erythroid and megakaryoblastic differentiation capacities, as well as of the endothelial cell lineage. Several differentiation antigens shared between megakaryoblastic and endothelial cells have been shown to exist, one example being the platelet glycoprotein IIIa (5, 30, 67). The observed expression pattern of *tie* mRNA is rather intriguing, as EGF motifs are a common theme of proteins controlling hemostasis as well as proteins mediating associations with the endothelium. We are studying the possible role of the *tie* receptor tyrosine kinase in hematopoietic cell differentiation and/or in blood cell-endothelial cell interactions.

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