Molecular Characterization of the Mouse Tem1/endo-sialin Gene Regulated by Cell Density in Vitro and Expressed in Normal Tissues in Vivo*

Received for publication, June 7, 2001, and in revised form, August 3, 2001
Published, JBC Papers in Press, August 6, 2001, DOI 10.1074/jbc.M105241200

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Human tumor endothelial marker 1/endo-sialin (TEM1/endo-sialin) was recently identified as a novel tumor endothelial cell surface marker potentially involved in angiogenesis, although no specific function for this novel gene has been assigned so far. It was reported to be expressed in tumor endothelium but not in normal endothelium with the exception of perhaps the corpus luteum. Here we describe the cDNA and genomic sequences for the mouse Tem1/endo-sialin homolog, the identification and characterization of its promoter region, and an extensive characterization of its expression pattern in murine and human tissues and murine cell lines in vitro. The single copy gene that was mapped to chromosome 19 is intronless and encodes a 92-kDa protein that has 77.5% overall homology to the human protein. The remarkable findings are 1) this gene is ubiquitously expressed in normal human and mouse somatic tissues and during development, and 2) its expression at the mRNA level is density-dependent and up-regulated in serum-starved cells. In vitro, its expression is limited to cells of embryonic, endothelial, and preadipocyte origin, suggesting that the wide distribution of its expression in vivo is due to the presence of vascular endothelium in all the tissues. The ubiquitous expression in vivo is in contrast to previously reported expression limited to corpus luteum and highly angiogenic tissues such as tumors and wound tissue.

Angiogenesis, a tightly regulated formation of new blood vessels, plays an essential role in embryonic development, normal growth, wound healing, and other physiological processes (1–3). In addition, this process is critically involved in patho-

* This work was supported in part by NCI, National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) AF388572 and AF388573.

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1 The abbreviations used are: EGF, epidermal growth factor; DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-PCR; BAC, bacterial artificial chromosome; bp, base pair(s); kb, kilobase pair(s); GFP, green fluorescent protein; PBS, phosphate-buffered saline; aa, amino acid(s); GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
normal somatic cells and embryonic tissue. These findings alter the previous view that this gene is preferentially associated with tumor endothelium; however, they do not rule out the possibility that the expression of this gene is elevated in tumor tissue. Of further significance, and perhaps even more important, is our finding that Tem1:endosialin mRNA is regulated by cell culture density.

MATERIALS AND METHODS

Cell Cultivation—All cell lines were kept at 37 °C with 5% CO<sub>2</sub>. Cell lines SVEC4–10, 2P-E4, 3T3-L1, BALB/c3T3–2, SR, C2C12, 3T3, and KLN205 were purchased from ATCC (Manassas, VA). NIH3T3, V-2, SVEC4–10, IP2-E4, and 3T3-L1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% FCS (Life Technologies, Inc.). BALB/c3T3–2 cells were maintained in DMEM containing 1.5 g/liter sodium bicarbonate, 1 mM sodium pyruvate, and 10% fetal bovine serum. SR-498T cells were kept in McCoy's 5a medium with 10% FCS. Stromal cell line MC3T3-G2/Pa6 (Pa6) (13) was provided by Kevin Holmes and maintained in a minimal essential medium supplemented with 10% FCS. A primary culture of rat brain capillary endothelial cells was prepared and kept as described previously (14).

Human umbilical vein endothelial cells and media for their maintenance were obtained from Clonetics Corp. (Walkersville, MD). Endothelial cell growth medium contained endothelial cell basal medium supplemented with human recombinant epidermal growth factor, hydrocortisone, gentamycin, amphotericin B, bovine brain extract, and 2% FCS. Endothelial cell growth medium-2 contained endothelial cell basal medium supplemented with human recombinant epidermal growth factor, basic human fibroblast growth factor, human vascular endothelial growth factor, ascorbic acid, R3-IGF-1 human recombinant insulin-like growth factor, heparin, gentamycin, amphotericin B, and 2% FCS. Cells were used at passage 3.

NIH3T3 fibroblasts transformed with v-ras, v-raf, and v-src were provided by Douglas Lowy and William Vass. NIH3T3 cells transformed with v-mos were prepared using plasmid pM1 (15). pM1 plasmid DNA was tranferred into the 2- packaging cell line, and the supernatant was used for infection of NIH3T3 cells.

To generate sparse, semiconfluent, and confluent cell cultures, logarithmically growing cells were seeded on 100-mm diameter tissue culture dishes (4 x 10<sup>5</sup> cells/dish) and incubated at 37 °C for 24, 48, or 72 h. Serum-starved cell cultures were incubated for 24 h in 10% FCS/DMEM, washed and resuspended in 0.2% FCS/DMEM, and incubated for 24 h. For some experiments, the cells were refeed with 10% FCS/DMEM and grown for an additional 24 h. At the end of the incubation period, the cells were harvested and immediately subjected to RNA isolation as described below.

Screening of cDNA and Genomic DNA Libraries—A mouse thymus cDNA library cloned in pCMV6-XL4 (Rapid-Screen Arrayed cDNA Library Panel; OriGene Technologies, Rockville, MD) was screened by PCR using primers F1 (5'-TTCCAGTCCTCAAATCAGTG-3') and R1 (5'-GCGGATTTCTGAGTTCG-3'). PCR products were generated using 50 ng of pSK-Xb13 as a template and a probe.

Mouse liver genomic DNA was isolated by fluorography. Mouse liver genomic DNA was isolated using the Plasmid Midi Kit (Qiagen).

Positive clones RPCI-22 364N1, RPCI-22 466N19, and RPCI-22 336N20 were obtained from Clontech Corp. (Walkersville, MD). Endothelial cell growth medium contained endothelial cell basal medium supplemented with 10% FCS. A primary culture of rat brain capillary endothelial cells was prepared using the BigDye Terminator Cycle Sequencing kit (Applied Biosystems). DNA from positive clones was performed on single bacterial colonies. DNA from positive clones was subjected to RNA isolation.

The TNT T7-coupled reticulocyte lysate system (Promega, Madison, WI) was used for in vitro translation as recommended by the manufacturer. Briefly, 1 μg of pSK-Plasmid DNA was incubated with a rabbit reticulocyte lysate for 1 h at 30 °C in the presence of T7 RNA polymerase and 40 μCi of [35S]methionine. The reaction was terminated by the addition of SDS sample buffer, and aliquots were analyzed by SDS-PAGE and visualized by fluorography.

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to map the Tem1/endosialin locus. DNA isolation, restriction enzyme digestion, agarose electrophoresis, Southern blot transfer, and hybridization were performed essentially as described (25). All blots were prepared with Hybond-N+ nylon membrane (Amersham Pharmacia Biotech). The probe (2.6-kb cDNA) was labeled with [α-32P]dCTP using a random primed labeling kit (Stratagene). Stringency of 1.0× SSC, 0.1% SDS, 65 °C. Fragments of 1.9 and ~0.5 kb were detected in Tag1-digested C57BL/6J DNA, and a fragment of 2.7 kb was detected in Tag1-digested M. spretus DNA. The presence or absence of the 2.7-kb Tag1 M. spretus-specific fragment was followed in the backcross mice. A description of the probes and RFLPs for the loci linked to Tem1/endosialin is included in Table A1, and was reported previously (26). Recombination distances were calculated using Map Manager, version 2.6.5. Gene order was determined by minimizing the number of recombination events required to explain the allele distribution patterns.

Northern Blot Analysis—Poly(A)+ and total RNA were prepared using an Oligotex Direct mRNA Midi/Maxi kit and RNeasy Mini kit (Qiagen, Valencia, CA), respectively, according to the instructions of the manufacturer. Samples containing 2 μg of poly(A)+ or 5 μg of total RNA were electrophoresed on 1.2% agarose gels containing 0.7% formaldehyde. RNA was blotted onto Nytran SuPerChargeNylon membrane (Schleicher & Schuell), UV-cross-linked using Stratalinker 1800 (Stratagene), and hybridized by standard techniques with 32P-labeled probes. Immunobots were transferred from nylon membranes to Clontech and used as recommended by the manufacturer. A 1.1-kb human TEM1/ endosialin probe was prepared by PCR screening of a human kidney cDNA library (OriGene Technologies) using human gene-specific primers hF1 (5'-GAGCAAGACCCCAACAGAAC-3') and hR1 (5'-TGGGTGATTCCCTTGTCGCAA-3').

RT-PCR Analysis—Expression of the mouse Tem1/endosialin was analyzed by RT-PCR using the Mouse Rapid-Scan Gene expression panel (OriGene Technologies). For this analysis, primers F1 and R1 were utilized under conditions similar to those used above for screening of the cDNA library. Four panels containing 1000×, 100×, 10×, and 1× concentrations of cDNA from 24 different mouse tissues were used as templates. Five μl of PCR products was separated on a 1% agarose gel followed by Southern blot analysis using the 10/5 insert as a probe. Expression of the human TEM1/endosialin was assessed by RT-PCR analysis of human I, II, and cardiovascular multiple tissue cDNA panels (Clontech) using the HotStarTaq Master Mix kit (Qiagen). One ng of cDNA was used in combination with primers hF1 and hR1 under the following conditions for PCR amplification: 95 °C for 15 min and 30 cycles consisting of 94 °C for 30 s, 63 °C for 30 s, and 72 °C for 90 s. Aliquots from reactions were taken after 30, 34, 38, and 42 cycles, and the presence of the 272-bp DNA fragment was determined followed by electrophoresis on agarose gels. Reactions with GAPDH-specific primers were used as a control according to the manufacturer’s instructions.

Luciferase Reporter Assays—Plasmid DNA for transient transfections was isolated using the Plasmid Maxi Kit (Qiagen). NIH3T3, PA6, and IP2-E4 cells were plated at a density of 3 × 104 cells/60-mm plate and 1 × 104 cells/35-mm dish. Transfection was performed using the Calcium Phosphate Transfection System (Life Technologies) according to the manufacturer’s instructions. A total of 10 μg of TEM1/endosialin promoter construct and 0.1 or 0.5 μg of pRL-TK Renilla luciferase vector (Promega) were used for each transfection. The pRL-TK luciferase activity was used to control transfection efficiency. Each transfection experiment was performed in triplicate and repeated at least two or three times with two different DNA isolates. For experiments performed to determine the sensitivity of the promoter to cell density, cells were split 18–20 h after transfection and plated in 12-well plates to achieve rapid confluence. Transfections were carried out using the Calcium Phosphate Transfection System (Life Technologies) according to the manufacturer’s instructions. A total of 10 μg of TEM1/endosialin promoter construct and 0.1 or 0.5 μg of pRL-TK Renilla luciferase vector (Promega) were used for each transfection. The pRL-TK luciferase activity was used to control transfection efficiency. Each transfection experiment was performed in triplicate and repeated at least two or three times with two different DNA isolates. For experiments performed to determine the sensitivity of the promoter to cell density, cells were split 18–20 h after transfection and plated in 12-well plates to achieve rapid confluence (dense cultures) or 60-mm plates to obtain sparse cultures. For transfection of SR-4987 cells, 10 μg of promoter construct along with 0.1 μg of plasmid encoding GFP were transfected into cells by electroporation with the addition of DEAE-dextran at a final concentration of 10 μg/ml. Cells (2 × 106) in a volume of 0.3 ml were mixed with DNA, transferred into 0.4-cm electroporation cuvettes, and transfected by electroporation using Gene Pulser II (Bio-Rad) (250 V, 950 microfarads). After electroporation, cells were plated on 60-mm tissue culture dishes with fresh medium and analyzed 24 h later. Firefly luciferase and Renilla luciferase activities were performed using the Dual-Luciferase Reporter Assay System (Promega) 24–48 h after transfections. Both luciferase activities were measured in the same tube by a luminometer TD-20e (Turner Designs, Sunnyvale, CA).

Production of Polyclonal Sera—Rabbit antisera were custom made by Primmblabe (Cambridge, MA) using a peptide, NH2-RITDCYR-WYTHAGNKSSTPPMPR-COOH, encoded by TEM1/endosialin ORF (amino acid residues 729–752).

Western Blot Analysis—Cells (5 × 106) were transfected with 20 μg of the pMH-Tem construct using the Calcium Phosphate Transfection System (Life Technologies) according to the manufacturer’s instructions. 48 h after transfection, the cells were washed and lysed in RIPA buffer (10 μg/ml Tris, pH 7.4, 0.15 M NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS) with 10 μg iodoacetamide, 1 μg phenylmethylsulfonyl fluoride, and Complete protease inhibitor mixture (Roche Molecular Biochemicals). The amount of protein was determined by the Bio-Rad DC (detergent-compatible) Protein Assay kit. Twenty-five μg of whole-cell lysate protein was resolved on 7.5% SDS-PAGE gels and transferred with 1.5 μg of Tem-GFP, pMH-Tem plasmid, and control GFP vector, respectively, using the calcium phosphate method. For observation by confocal microscopy of the signal produced by GFP-Tem protein, the cells were washed twice with PBS, fixed for 30 min in 4% paraformaldehyde, and washed twice with PBS before mounting in Antifade (DAKO Corp., Carpenteria, CA).

Intracellular Localization and Confocal Microscopy—To determine intracellular localization of Tem-1, NIH3T3 cells were grown on cover slips in 35-mm dishes and transfected with 1.5 μg of Tem-GFP, pMH Tem plasmid, and control GFP vector, respectively, using the calcium phosphate method. For observation by confocal microscopy of the signal produced by GFP-Tem protein, the cells were washed twice with PBS, fixed for 30 min in 4% paraformaldehyde, and washed twice with PBS before mounting in Antifade (DAKO Corp., Carpenteria, CA).

For immunocytochemistry, cells were washed three times with PBS and fixed with ice-cold methanol for 15 min. After thorough washings, primary anti-Myc (1:200 dilution) or anti-His6 (1:200 dilution) (Invitrogen) and secondary fluorescein isothiocyanate-conjugated anti-mouse IgG1 and IgG3 antibodies (1:200–1:5000 dilution; Roche Molecular Biochemicals) in PBS were used for detection of Myc- or His6-tagged proteins by 30-min incubations at 37 °C followed by three washes with PBS. Then, cover slips were incubated with a 1:50 dilution of FITC-conjugated secondary antibody (1:1000 dilution) and washed twice with PBS.

Western blot analysis and confocal microscopy of the signal produced by GFP-Tem protein, the cells were washed twice with PBS, fixed for 30 min in 4% paraformaldehyde, and washed twice with PBS before mounting in Antifade (DAKO Corp., Carpenteria, CA).

RESULTS

Mouse Tem1/endosialin cDNA—The GenBank™ data base contains several mouse expressed sequence tag clones that possess high homology to human TEM1/endosialin (accession number AF279142) and originate from different tissues. We used an expressed sequence tag clone, IMAGE number 355868, to design primers F1 and R1 for screening of a mouse thymus cDNA library (Rapid-Screen Arrayed cDNA library panel; OriGene Technologies). This approach allowed us to isolate two clones designated A1 and A2 that contained the mouse Tem1/endosialin cDNA inserts with approximate sizes of 2.5 and 2.6 kb, respectively. Sequence analysis revealed that the 5′-end of the A2 cDNA insert is extended by 139 nucleotides compared with A1. As depicted in Fig. 1, the assembled mouse Tem1/endosialin cDNA contains 2553 bp and is composed of a short 5′-untranslated region of 3 bp, followed by a coding region of 2295 bp and a 3′-untranslated region of 243 bp. The open reading frame starts from ATG at position 4, ends at TAG at position 2299, and has the phenylalanine codon specified by the 5′-end translation signal. The polyadenylation signal AATAAA is localized 189 bp downstream of a stop codon and 25 bp upstream of a poly(A) tail. The sequence was submitted to the GenBank™ data base under accession number AF388572. The overall amino acid composition of Tem1/endosialin is acidic, with a predicted isoelectric point of 5.28. The protein is rich in prolines (13.73%),
FIG. 1. Nucleotide and deduced amino acid sequences of mouse Tem1/endosialin cDNA. An open reading frame of 2295 bp encodes the 765-aa protein. The translation initiation codon and polyadenylation signal are shown in boldface, underlined letters. Predicted signal peptide and
Murine Tem1/endosialin

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Abstract

Tem1/endosialin is a transmembrane protein of the C-type lectin family that is expressed in a variety of normal tissues and tumors. The protein contains several well-known protein motifs: C-type lectin domain, sushi (aa 164–230), three EGF-like domains (aa 234–272, 274–311, 315–351) and sialomucin-like region. This modular architecture, as well as the homology to both thrombomodulin and C1qRp complement receptor, have already been described for human Tem1/endosialin.

Human Tem1/endosialin cDNA was cloned into the pBluescript SK vector and used as a positive control. The size of the product agrees with the predicted molecular weight of the mouse Tem1/endosialin core protein (Fig. 2A). To determine the molecular mass of protein in eukaryotic cells, we transiently transfected NIH3T3 cells with an expression vector carrying Tem1/endosialin cDNA fused to a C-terminal Myc-His6 tag. Western blot analysis using anti-Myc antibody revealed a band of ~95 kDa (Fig. 2B). Since the fusion protein contains a 3.1-kDa tag, the estimated molecular mass for the Tem1/endosialin protein was 92 kDa. Western blotting of untransfected NIH3T3 cells using rabbit antiserum raised against the intracellular portion of Tem1/endosialin demonstrated that the cells contain an endogenous protein of 92 kDa (Fig. 2C). However, the analysis also revealed several additional bands of higher molecular weight presumably representing different glycosylated forms of the protein as has been described previously for the human Tem1/endosialin (9).

Distribution of Tem1/endosialin in Vivo and in Vitro—Availability of the cDNA allowed us to perform a survey of a broad range of mouse and human tissues. This was important with respect to the previously reported absence of the human TEM1/endosialin expression in normal tissues (9, 10). First, we analyzed a collection of tissues in a mouse Rapid-Scan panel (OriGene). F1 and R1 gene-specific primers were employed in an RT-PCR amplification, and the products were detected by Southern blot analysis with a labeled cDNA fragment (Fig. 3A). Surprisingly, we found that Tem1/endosialin mRNA can be detected in all mouse tissues involved in the panel, with the highest levels present in the heart, kidney, stomach, skin, alanines (9.41%), glycines (8.24%), and leucines (8.1%). It appears to be a type I membrane protein with a putative signal peptide of 17 amino acids (aa) and a long extracellular part of 676 aa followed by a single membrane spanning domain of 21 aa and a short cytoplasmic tail of 51 aa. The extracellular region contains several well known protein motifs: C-type lectin domain (aa 22–157), sushi domain (aa 164–230), three EGF-like domains (aa 234–272, 274–311, 315–351) and sialomucin-like region. This modular architecture, as well as the homology to both thrombomodulin and C1qRp complement receptor, have already been described for the human TEM1/endosialin (9).

Using the PROSCAN program, we found that the mouse Tem1/endosialin extracellular domain contains a potential N-glycosylation site (at position 636) and many putative O-linked glycosylation sites, while the cytoplasmic tail possesses a number of potential phosphorylation sites and several N-myristoylation sites. The utilization and importance of these modification sites remain to be assessed. Since the human TEM1/endosialin protein has been shown to be heavily O-glycosylated, we would expect that many of the predicted sites of O-glycosylation in the mouse homolog would also be true sites of post-translational modification (9).

Alignment of the mouse and human amino acid sequences showed an overall 77.5% identity (Fig. 1). The least conserved part of the molecule is a region from aa 478 to 610 on the mouse sequence. Interestingly, the putative transmembrane region is 100% identical between human and mouse Tem1/endosialin, and there are only four amino acid substitutions out of a total of 51 amino acids in the cytoplasmic tail, suggesting that these two regions may possess conserved function.

To prove that the Tem1/endosialin cDNA described here encodes a protein with the expected molecular weight, we cloned the full-length cDNA into the pBluescript SK vector and used this plasmid as a template for T7-primed TNT-coupled in vitro transcription and translation. The reaction yielded a unique translation product migrating on SDS-polyacrylamide gel electrophoresis as a band of ~82 kDa, which is in excellent agreement with the predicted molecular weight of the mouse Tem1/endosialin core protein (Fig. 2A). To determine the molecular weight of protein in eukaryotic cells, we transiently transfected NIH3T3 cells with an expression vector carrying the mouse cDNA fused to a C-terminal Myc-His6 tag. Western blot analysis using anti-Myc antibody revealed a band of ~95 kDa (Fig. 2B). Since the fusion protein contains a 3.1-kDa tag, the estimated molecular mass for the Tem1/endosialin protein is 92 kDa. Western blotting of untransfected NIH3T3 cells using rabbit antiserum raised against the intracellular portion of Tem1/endosialin demonstrated that the cells contain an endogenous protein of 92 kDa (Fig. 2C). However, the analysis also revealed several additional bands of higher molecular weight presumably representing different glycosylated forms of the protein as has been described previously for the human Tem1/endosialin (9).

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pancreas, uterus, embryo at day 19, and virgin breast. Our data indicate a role for the gene in embryonic development, since expression was detected in all samples spanning embryonic days 8.5–19. The finding of high expression in numerous tissues is contradictory to data published before for human TEM1; therefore, we also looked at the expression of TEM1/endosialin in a collection of normal human tissues. Hybridization of a multiple tissue Northern blot, with a 1.1-kb human Tem1/endosialin cDNA fragment as a probe, proved that TEM1/endosialin is transcribed in most normal human tissues (Fig. 4A). Similar data were obtained by RT-PCR analysis (Fig. 4B). The highest levels of TEM1/endosialin mRNA were detected in the placenta, ovary, heart, skeletal muscle, small intestine, and various parts of cardiovascular system. Although Tem1/endosialin is widely expressed in preparations of whole tissue, examination of various murine cell lines by a Northern blot analysis revealed that its in vitro expression is limited to cells of very specific origins (Fig. 3B). These include the cell lines derived from embryonic fibroblasts (NIH3T3, BALB/3T12–3, and SC1), preadipocytes (3T3L1 and PA6), and endothelial cells (SVEC4–10 and IP2-E4). The cell lines that represent other cell types, such as hepatocytes (AML12), carcinoma cells of mammary gland (C127:LT), breast (JC), and lung (KLN205); stromal cells of the bone marrow (SR-4987); keratinocytes; and various cell lines of hematopoietic origin were negative (data not shown). These data suggest that the ubiquitous expression of Tem1/endosialin in tissues is conferred by their endothelial cell components.

Based on this assumption, we tested two available primary endothelial cell lines for the presence of Tem1/endosialin transcripts and found that the rat primary brain capillary endothelial cells express Tem1/endosialin specific RNA. On the other hand, the human umbilical vein endothelial cells do not express the gene, even when grown in the presence of angiogenic growth factors (data not shown). This lack of expression is in agreement with previously published data (10). These results suggest that the expression of TEM1/endosialin might be restricted to microvascular endothelial cells.

In summary, these analyses of Tem1/endosialin steady state expression indicate that mRNA for this gene is produced in a diversity of tissues in the adult and throughout development. However, the only cell lines identified here as positive for Tem1/endosialin expression were of either embryonic, endothelial, or preadipocyte origin. The distribution of Tem1/endosialin and its human relative as determined by us is much broader than originally reported.

**Intracellular Localization of C-terminally Tagged Tem1/endosialin**—The domain composition of Tem1/endosialin predicts that it is a type 1 receptor subject to intracellular transport along the secretory pathway and exposure at the plasma membrane. In order to investigate whether the mouse Tem1/endosialin is indeed associated with the plasma membrane, we generated a fusion antigen with the GFP at its carboxyl terminus. Confocal microscopy revealed that the Tem1/endosialin-GFP transiently expressed in NIH3T3 cells was predominantly located in a perinuclear area of the cytosol, while a minor fraction of the fusion protein was observed in association with the plasma membrane (Fig. 5A). The cytosolic fluorescence was not uniformly spread but showed a granular pattern reminiscent of that seen with the proteins associated with endoplasmic reticulum or Golgi vesicles. On the other hand, the wild-type GFP displayed a rather different distribution, being evenly spread over the whole cytosolic and nuclear areas (data not shown).

Because the addition of a relatively large GFP moiety to the C terminus of Tem1/endosialin protein could affect its conformation and thereby the capacity for proper transport and cell surface localization, we also examined a Tem1/endosialin fused to a shorter Myc-His6 tag. However, the immunofluorescence using anti-Myc antibody (Fig. 5B) or anti-His6 antibody (Fig. 5C) displayed a similar pattern as described above for the GFP fusion protein, supporting the view that only a small portion of the C-terminally tagged Tem1/endosialin was associated with the plasma membrane.

**Cell Density-dependent Expression Tem1/endosialin in Culture**—The domain architecture of Tem1/endosialin suggests that the protein might be involved in cell-cell interaction; in particular, it has a C-lectin domain and EGF domains, both of which are found in selectins (27, 28). Since many intercellular communication events rely upon direct contact, their regula-
tory pathways frequently exert density-dependent modulation. On this basis, we decided to investigate a possible effect of the cell density on expression of endogenous Tem1/endosialin in NIH3T3 cells. Cells were plated at a density of $5 \times 10^4$ per 100-mm dish and harvested for analysis after 24 h (while they were still sparse), after 48 h (at medium density), and after 72 h (at full confluence). Northern blot analysis revealed that the sparse NIH3T3 cells express a relatively low level of Tem1/endosialin RNA, but transcription is strongly induced when the cells reached full confluence (Fig. 6A).

Similar cell density-dependent induction of Tem1/endosialin transcription was observed in the IP2-E4 mouse endothelial cell line (Fig. 6A) as well as in other cell lines including BALB3T12-3 and the bone marrow-derived preadipocyte cell line PA6 (not shown). The effect of cell density on expression of the Tem1/endosialin protein was confirmed by Western blot analysis of whole cell extracts prepared from the sparse, semi-confluent, and confluent NIH3T3 cells using the rabbit polyclonal Tem1/endosialin antiserum (Fig. 6B).

Molecular signals involved in the induction of Tem1/endosialin expression in dense cells could be related either to establishment of direct contacts between the plasma membranes of the neighboring cells or to depletion of serum growth factors during the extended cultivation period. Interestingly, serum starvation for 24 h resulted in elevated transcription of Tem1/endosialin in NIH3T3 cells, although the induction was not so dramatic as that caused by increased density. On the other hand, the refed cells displayed a similar level of the transcript as the nonstarved control culture (Fig. 6A).

Potent viral oncoproteins such as v-src, v-ras, v-mos, and v-raf, when expressed in NIH3T3 cells, cause both a diminished requirement for growth factors and abnormal intercellular contacts. In particular, they are refractive to normal contact-inhibitory signals. We wished to see if any of these oncogenes, which are involved in signal transduction, could prevent the contact-inhibited up-regulation of Tem1/endosialin. NIH3T3 cells expressing these oncogenes were examined for density-dependent induction of Tem1/endosialin mRNA. Cells expressing v-src, v-ras, and v-raf, still showed density dependent expression of Tem1/endosialin (Fig. 6C). In contrast and to our surprise, v-mos-transformed NIH3T3 cells expressed high levels of Tem1/endosialin transcript regardless of the cell density (Fig. 5C). This suggests that v-mos, instead of blocking the up-regulation of Tem1, activates a pathway leading to the Tem1/endosialin expression.

Density-stimulated expression of Tem1/endosialin could be related to the proliferative status of the cells, since the sparse cells proliferate more rapidly than the cells approaching confluence. This raised the possibility that Tem1/endosialin could be cell growth-inhibitory. To investigate this, we performed a series of experiments in which we analyzed growth properties of NIH3T3 cells that overexpressed Tem1/endosialin. Cells transfected with pcDNA3.1 containing Tem1/endosialin cDNA, in sense and antisense orientation, and mock-transfected cells were compared for their growth capacity in colony formation assays. In addition, Tem1/endosialin was expressed conditionally using a lac operon system (Stratagene). Although we observed high induction of Tem1 mRNA, we were unable to show any growth inhibitory effects of the gene (data not shown). Moreover, clonal cell lines that were derived from transfected SR4987 cells and constitutively overexpressed Tem1/endosialin did not display any changes in their proliferative rate. Thus, at present we have no evidence that Tem1/endosialin is directly involved in the regulation of cell proliferation; however, these experiments do not completely rule out this possibility.

**Isolation and Structure of the Mouse Tem1/endosialin Gene**—To further our knowledge of the molecular properties...
and regulation of mouse Tem1/endosialin expression, we began a characterization of its genomic sequence including the related transcriptional control region. For the isolation of the Tem1/endosialin gene, we used a 650-bp fragment from the original expressed sequence tag clone (accession number AF279142) to screen a mouse BAC library. Positive clones were analyzed by Southern blot hybridization using the full-length Tem1/endosialin cDNA as a probe and all displayed a similar digestion pattern (data not shown). This indicated that the isolated BAC clones contained the Tem1/endosialin gene and that no major rearrangements had occurred during cloning and propagation of the BAC library.

Interestingly, the entire coding sequence appeared to be located on a 13-kb XbaI fragment that was subcloned into pBluescript II SK for in depth characterization. Genomic sequence of 8245 bp was obtained from both strands using the primers initially selected from the full-length Tem1/endosialin cDNA and then completed by primer walking. The sequence was submitted to the GenBank™ data base under accession number AF388573. A BLAST search against the current GenBank™/EMBL data base revealed substantial sequence homology only to human TEM1/endosialin. Alignment of the genomic sequence with the full-length Tem1/endosialin cDNA revealed that the gene is intronless throughout its coding region (Fig. 7A). Two differences were detected between the genomic and cDNA sequences. One was a substitution of T for C in position 459 of the cDNA sequence, which did not result in an amino acid change and was present only in clone A2 of the cDNA, suggesting that this substitution was introduced during a preparation of the cDNA library. A second difference was a substitution of T for C at position 1637 of the cDNA sequence and resulted in a change of proline to serine at position 525. This substitution, which introduced a SalI site, was found to be present in both cDNA clones and presumably represents a polymorphism between different mouse strains used for construction of cDNA and genomic libraries. Digestion by SalI can be potentially used as a marker to identify different mouse strains.

Southern blot analysis revealed that the Tem1/endosialin gene is a single copy gene (Fig. 7B). A polyA+ RNA was prepared from sparse (S), confluent (C), serum-starved (SE), and refed (RE) NIH3T3 cells or IP2-E4 cells that were sparse or confluent. Two μg of RNA was examined by Northern blot hybridization with Tem1/endosialin cDNA and reprobed with GAPDH. B, whole cell extracts were prepared from sparse (P), semi-confluent (SC), and confluent (C) NIH3T3 cells. The samples were analyzed by immunoblotting with anti-Tem1/endosialin rabbit polyclonal antiserum. C, Northern analysis of Tem1/endosialin expression in sparse (S) and confluent (C) NIH3T3 cells transformed by viral oncogenes v-mos, v-src, and v-raf was performed as described above.

Chromosomal Localization of Tem1/endosialin Gene—The mouse chromosomal location of Tem1/endosialin locus was
determined by interspecific backcross analysis using progeny from matings of (C57BL/6J × Mus spretus)F1 × C57BL/6J mice (24). C57BL/6J and M. spretus DNAs were digested with several enzymes and analyzed by Southern blot hybridization for informative restriction fragment length polymorphisms using a mouse cDNA probe. The 2.7-kb TaqI M. spretus restriction fragment length polymorphism was used to follow the segregation of the Tem1/endosialin locus in the backcrossed mice. The mapping results indicated that Tem1/endosialin is located in the proximal region of mouse chromosome 19 linked to the Gal, Adrbk1, and Cd5 genes (Fig. 8). Although 118 mice were analyzed for every marker and are shown in the segregation analysis (Fig. 8), up to 191 mice were typed for some pairs of markers. Each locus was analyzed in pairwise combinations for recombination frequencies using the additional data. The ratios of the total number of mice exhibiting recombinant chromosomes to the total number of mice analyzed for each pair of loci and the most likely gene order are: centromere-Gal-1/191-Adrbk1-0/190-Tem1-3/121-Cd5. Recombination frequencies (expressed as genetic distances in centimorgans ± S.E.) are: -Gal-1.1 ± 0.7- [Adrbk1,Tem1]-2.5 ± 1.4-Cd5. No recombinants were detected between Adrbk1 and Tem1 in 190 animals typed in common suggesting that the two loci are within 1.6 centimorgans of each other (upper 95% confidence limit).

We have compared our interspecific map of chromosome 19 with a composite mouse linkage map that reports the map location of many uncloned mouse mutations (provided from the Mouse Genome Database, a computerized database maintained at the Jackson Laboratory, Bar Harbor, ME). Tem1/endosialin mapped in a region of the composite map that lacks mouse mutations with a phenotype that might be expected for an alteration in this locus (data not shown).

The proximal region of mouse chromosome 19 shares a region of homology with human chromosome 11q, and accordingly the human TEM1/endosialin gene was recently mapped to chromosome 11q13 (9, 10).

Sequence Analysis of 5’-flanking Region and Promoter Activity—In an effort to determine the position, structure, and activity of the transcriptional control region of the mouse Tem1/endosialin gene, we analyzed the sequence of its 5’-flanking region and examined the capacity of deletion fragments to drive transcription of a reporter gene.

**Fig. 7. Genomic region and restriction endonuclease digestion pattern of Tem1/endosialin gene.** A, schematic illustration of 8269 bp of genomic sequence with indicated sites of selected restriction endonucleases. The large arrows represent the intronless region that is transcribed. cDNA clones A1 and A2 are depicted below. A 5’-flanking fragment is drawn as a thick dashed line, and the position of a CpG island is shown by an open box. B, Southern blot of genomic DNA isolated from mouse liver, digested with indicated restriction enzymes and probed with A2 cDNA clone. The fragments in NheI, SstI, and HindIII-digested DNA correspond in size to those on the restriction map of the intronless Tem1/endosialin sequence shown in A and representing the intronless gene.
from the (C57BL/6J chromosome identified in the backcrossed progeny that was inherited chromosome 19.

Tem1/endosialin were typed for all loci are shown at the “...”.

features of a CpG island, with an average observed-to-expected the CpG plot program has shown that this region displays proposed ATG initiation codon (Fig. 9). Further analysis using

ment revealed the presence of a CG-rich region overlapping the portion of the 5'-end of the Tem1/endosialin gene at position 83. However, in accordance with the GC-rich character of this region, we were able to identify an exact transcription initiation site(s) either by 5'-rapid amplification of cDNA ends or by primer extension analysis (not shown). Therefore, we decided to study the promoter activity of the whole 1.8-kb flanking region starting immediately upstream of ATG.

Toward this aim, we performed PCR amplification of the −1829/−5 fragment and generated a series of nested deletion mutants fused to the luciferase reporter gene in the promoterless pGL3-Basic vector. Altogether, we prepared nine constructs containing the fragments in the sense orientation (pGL-TemA to -F and pGL-TemJ to -L) and two constructs with the fragments in the antisense orientation (pGL-TemG and -H).

The constructs were transiently transfected into cells that express the endogenous Tem1/endosialin gene (NIH3T3, PA-6, and IP2-E4) as well as into nonexpressing SR-4987 cells, and luciferase activity was measured after 24 h.

An evaluation of the complex profile of the luciferase activity produced by different constructs in NIH3T3 cells (Fig. 10A) shows that the promoter of the mouse Tem1/endosialin gene is located in the region −263/−65 and that this region contains important positive regulatory elements. In support of this, elimination of the promoter region from the longest fragment almost fully abrogated its activity. The antisense fragments were inactive.

Interestingly, the cell lines that express a lower level of Tem1/endosialin RNA displayed lower promoter activity, albeit with an analogous profile (not shown), suggesting that the initiation of transcription critically contributes to regulation of Tem1/endosialin expression. The transfected promoter constructs were also active in SR-4987 cells that do not express Tem1/endosialin mRNA (Fig. 10B), suggesting that negative regulatory mechanism(s) operative at the endogenous chromosomally localized gene were not functional on the non-integrated constructs.

Insight into the promoter sequence with regard to the presence of putative regulatory elements revealed binding sites for several transcription factors, including SP1, AP2, RREB1, WT1, and Ets1 sites and one site for the serum response factor (Fig. 10A). Some of these elements have been shown to play an important role in transcriptional control of angiogenesis-related genes (29, 30), but their actual contribution to the regulation of the Tem1/endosialin gene remains to be determined.

Since the Tem1 gene is up-regulated in high density cells, it was important to determine if this expression is due to regulation of transcription at the level of the promoter. Therefore, NIH3T3 cells were transfected individually with pGL-TemA and pGL-TemF as well as the pGL3-Basic vector and separated into dense and sparse cultures. The luciferase assay demonstrated a significant increase in promoter activity in dense cultures compared with sparse cultures as shown in Fig. 10C. Similar density-dependent luciferase activity was also demonstrated for construct pGL-TemC (data not shown). This strongly indicates that transcription initiation contributes at least partially to the augmented expression of Tem1 observed in dense cell cultures.

**DISCUSSION**

The data presented here indicate for the first time that Tem1/endosialin plays not only a role in tumor-specific endothelium, but a more general role in vascular endothelium both in the adult and during embryonic development. Although previous data suggested that its expression is restricted to tumors (9), we propose that it may simply be expressed at higher levels during the formation of new vessels in tumors. In support of this idea, others have found that it is expressed during angiogenic states, such as in the corpus luteum and granular tissue of healing wounds (8). Although this may not represent a qualitative difference between normal endothelium and tumor endothelium as suggested previously, it may certainly reflect a quantitative difference. Quantitative differences are likely in tumor tissue known to have imbalances in the levels of regulators of angiogenesis. Since Tem1/endosialin’s expression is most likely elevated in tumor tissues undergoing formation and reorganization of vessels, it could provide a target for antiangiogenic therapy for neoplastic disease.

Endothelial cells demonstrate heterogeneity among and within tissues (1). Interestingly, the distribution of Tem1/endosialin varied among tissues with higher levels in the heart...
and pancreas and lower expression in the spleen and liver in both mice and humans. In addition, the placenta, ovary, and uterus were demonstrated to have high levels. This observation, along with the fact that the gene was expressed in rat capillary cells, but not in large vessel cells (human umbilical vein endothelial cells), suggests that this gene may be important in some subtype(s) of microvascular endothelial cells.

As we have demonstrated in this study, Tem1/endosialin mRNA is up-regulated as cells increase in density. This was shown for NIH 3T3 cells as well as a mouse endothelial cell line, IP2-E4, and was confirmed at the protein level in NIH3T3 cells. Because its up-regulation may be associated with contact inhibition, we performed experiments to test whether, under conditions of forced expression, the gene could induce growth arrest. However, we were not able to demonstrate such a function for Tem1/endosialin. We hypothesize, therefore, that up-regulation of this gene occurs either in conjunction with increases in cell density or growth arrest but does not induce growth arrest itself. The fact that Tem1/endosialin is also up-regulated in serum-starved cells suggests that this induction is not simply triggered by the contact between cells per se. Alternatively, there may be more that one mechanism for its induction. Our data using luciferase reporter constructs show that the density-dependent expression is at least in part due to differences in activity at the promoter level. Many potential transcription factor-binding sites have been identified in the promoter region (Fig. 9), and it will be of interest to determine which of these sites functions to activate Tem1 transcription.

The Tem1/endosialin protein might serve one of several roles in the vasculature including signaling from cell to cell or serving as a receptor for soluble ligands. Its potential role in cell-cell interactions is suggested by the structure within its extracellular domain. Like many selectins that are involved in cell-cell interactions, it has a C-lectin domain and EGF domains (28). Its potential cell-cell interactions could include homotypic interactions or heterotypic interactions with periendothelial cells (pericytes in small vessels) that lie adjacent to endothelial cells in vivo (3). One cannot rule out the additional possibility that Tem1/endosialin is involved in interactions with components within the extracellular matrix. In any case, its specific function in the vasculature is beyond the scope of this discussion and will be subject of future investigations.

Tem1/endosialin shares, in its extracellular domain, homology with thrombomodulin. This is a cell surface molecule that is widely expressed on normal endothelial cells and under certain conditions in smooth muscle cells. It is best known for its role in the interaction with thrombin in the coagulation cascade (9, 31). Tem1/endosialin, however, lacks homology to...
thrombomodulin in the thrombin interaction domains (EGF repeats 5 and 6). An additional role for thrombomodulin is evident from its ability to inhibit arterial smooth muscle cell proliferation (32, 33) and modulate mitogenic responses of endothelial cells to thrombin (34). Indeed, embryonic lethality in homozygous thrombomodulin knockout mice has been proposed to be due to thrombin-independent functions (35). Parallel investigations on this related protein may help us to begin to understand the function of Tem1/endosialin.

Our findings concerning the localization of Tem1/endosialin in the cell support its predicted cell surface association. Following transfection and immunofluorescence of permeabilized cells, we found a proportion of the protein in the vicinity of the cell membrane, although a large proportion was also found in the perinuclear region. It is interesting to point out that this localization is similar to that seen for thrombomodulin following transient transfection and permeabilization of cells (36). We realize that the GFP or Myc-His6 tag at the carboxyl terminus used in this study could potentially alter the localization of the protein. Although we wanted to confirm our result by looking at endogenous Tem1/endosialin, our antibody raised to the protein did not function well in this assay.

The intracellular tail of Tem1/endosialin contains several putative phosphorylation sites that could serve in transduction of extracellular signals. This domain could also function in the subcellular localization of the protein, as was shown to be the case for thrombomodulin (37).

An interesting outcome of our analysis of the genome structure of murine Tem1/endosialin was the finding that the coding region for the gene is intronless. Only a small proportion (at
most 5% of genes lack introns. A family whose diverse members are usually intronless are the G-protein-coupled receptor family genes. Among other human cell surface receptor genes, about 35% are found to be intronless (38). It has been proposed that an advantage of being intronless may be that transcription can occur more efficiently and with greater abundance, but in some cases experimental evidence indicates that introns actually increase gene expression levels (39).

In conclusion, our data indicate that Tem1/endosialin may be functionally involved in angiogenesis or vascular function, not only in pathological situations, but under physiological conditions as well. Although information about pathways leading to only in pathological situations, but under physiological conditions as well. Although information about pathways leading to introns as well. Although information about pathways leading to some cases experimental evidence indicates that introns actually occur more efficiently and with greater abundance, but in some cases experimental evidence indicates that introns actually increase gene expression levels (39).

Acknowledgments—We thank Douglas Lowy, William Vass, and Kevin Holmes for providing cell lines and plasmids and Deborah B. Householder for excellent technical assistance. In addition, we thank Jaromir Pastorek for critical reading of the manuscript.

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