CD13/aminopeptidase N (CD13/APN) is a potent regulator of angiogenesis both in vitro and in vivo and transcription of CD13/APN in endothelial cells is induced by angiogenic growth factors via the RAS/MAPK pathway. We have explored the nuclear effectors downstream of this pathway that are responsible for CD13/APN induction. The response to serum/angiogenic growth factors mapped to a 38-bp region of the CD13/APN promoter containing an Ets-core motif that specifically binds a protein complex from nuclear lysates from activated endothelial cells. This motif and the proteins that target it are functionally relevant because mutation of this sequence abrogates CD13/APN transcription. Analysis of endothelial Ets family members showed that Ets-2, and to a lesser extent Ets-1, transactivate CD13/APN promoter activity via the Ets-core motif, whereas Fli, Erg, and NERF are ineffective. We investigated the possibility that the induction of CD13/APN is mediated by phosphorylation of Ets-2 via RAS/MAPK. A phosphorylation-defective Ets-2 mutant, T72A, failed to transactivate CD13/APN, suggesting that Ets-2 phosphorylation is obligatory for CD13/APN induction. To confirm a role for endogenous Ets-2 in CD13/APN expression, we specifically abrogated Ets-2 mRNA and protein by siRNA knockdown that significantly inhibited CD13/APN transcription. Finally, to assess the relevance of Ets-2 in endothelial cell function, we induced endothelial cells containing Ets-2 siRNA oligonucleotides to form capillary networks. Cells containing the Ets-2 inhibitory small interfering RNAs were completely incapable of forming the organized networks characteristic of endothelial morphogenesis. Thus, the phosphorylation of Ets-2 by RAS/MAPK is a prerequisite for CD13/APN endothelial induction and Ets-2 and its targets play essential roles in endothelial cell function.

CD13/aminopeptidase N (CD13/APN, EC 3.4.11.2) is a type II membrane-bound metallopeptidase originally described in studies seeking to identify lineage specific markers that would facilitate the classification of human leukemias (1). In this regard, the appearance of CD13/APN coincides with commitment to the myeloid lineage and within the hematopoietic compartment it is primarily expressed on the normal and leukemic progeny of myeloid cells (2). It is also detected on a proportion of acute lymphoid leukemias and activated T cells, and on T and B cells after cell-cell adhesion (3). Previous studies have shown that CD13/APN participates in antigen processing and presentation (4), scavenging of amino acids and catabolism of regulatory peptides (5), degradation of vasoactive (6) and neuroactive peptides (7), and tumor invasion and metastasis (8). The subsequent molecular cloning of the gene encoding this cell surface glycoprotein allowed comprehensive tissue-specific detection that extended its known range of expression beyond the hematopoietic system to include fibroblasts and epithelial cells in the liver, intestine, brain, and lung (9). Analysis of CD13/APN transcriptional regulation revealed that it is regulated by two mutually exclusive promoters, one distal (8 kb upstream of the initiation codon) that is active in myeloid cells and fibroblasts, and a second proximal promoter (adjacent to the initiation codon) that controls expression in epithelial and endothelial cells (10). We have recently identified a novel role for CD13/APN as a potent angiogenic regulator where functional CD13/APN is required for tumor growth in vivo (11) and endothelial migration and capillary network formation in vitro (12, 13). Furthermore, we demonstrated that the exclusive expression of CD13/APN on activated endothelial cells is precisely controlled by angiogenic signals present in the tumor microenvironment and it is regulated primarily at the transcriptional level (11).

The aim of the present study was to investigate the mechanism of transcriptional regulation of CD13/APN induction in response to angiogenic stimulation in endothelial cells. Angiogenesis is functionally defined as the sprouting of new vessels from pre-existing vasculature and consists of several distinct stages including endothelial cell proliferation, migration, invasion, differentiation into capillaries, and eventual maturation into blood vessels (14). Because CD13/APN belongs to the group of genes strongly induced by angiogenic stimuli, it is of interest to define those regulatory mechanisms that control its expression. Numerous transcription factors have been identified that regulate genes relevant to angiogenesis such as angiogenic growth factors (bFGF and VEGF), their receptors, and other mediators of the cellular responses to these growth factors (for review, see Ref. 15). For example, regulation of expression of...
the VEGF-R2 gene is mediated by members of the Ets, GATA, and basic helix loop helix transcription factor families (16), whereas the expression of VEGF in response to hypoxia is primarily regulated by the PAS domain family of transcription factors, including hypoxia-inducible factor 1 (17), EPAS (18), and ARNT (19). Proliferation and migration of the endothelial cells and their formation into primitive tubes is regulated at least in part by transcription factors HESR1 and peroxisome proliferator-activated receptor-γ (20, 21). Therefore, these transcription factors play key roles in the regulation of angiogenesis via activation of their target genes.

In this report, we provide evidence that transcriptional up-regulation of CD13/APN in endothelial cells in response to conditions characteristic of the angiogenic microenvironment is primarily mediated by the phosphorylation of Ets-2 by signals transmitted via the RAS/MAPK pathway. Furthermore, we demonstrate that expression of Ets-2 (and its target genes) is strictly required for endothelial morphogenesis. Whereas the precise role of CD13/APN in neovascularization is under active investigation, comprehensive understanding of the mechanisms regulating the expression of this important angiogenic regulator is fundamental to the identification of potential therapies targeting angiogenic processes.

EXPERIMENTAL PROCEDURES

Cell Culture—The human Kaposi's sarcoma endothelial cell line (KS1767) and the murine hemangioendothelioma (EOMA) cell line were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, L-glutamine (2 mM), penicillin (100 units/ml), and streptomycin (100 µg/ml).

Inhibitors, Antibodies, and Cytokines—All cells were incubated in serum-free medium 18–24 h prior to various treatments. Cells were then washed once with serum-free medium and stimulated with hGFP (50 ng/ml) or VEGF (25 ng/ml) (R&D Systems, Minneapolis, MN) in medium containing 1% serum for 24 h. The MEK inhibitor, PD098059 (22), was obtained from Calbiochem (San Diego, CA) and used at a final concentration of 30 µM. Ets-1, Ets-2, Fli-1 and Erg-2, phospho-ERK2, and ERK2 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The development of rabbit polyclonal antisem directed against Tyr5- and phosphorylated human Ets-2 was previously described (23).

Northern Blot Analysis—Total RNA was isolated using TRI Reagent (Molecular Research Center, Cincinnati, OH). 30 µg of total RNA from each sample was separated by electrophoresis in 1% agarose gel containing formaldehyde and transferred to nylon membranes. Hybridization probes (EcoRI fragments (1.8 and 1.6 kb) encompassing full-length CD13/APN cDNA (9)) were labeled with α[32P]dCTP using a random priming labeling kit (Redi-prime II, Amersham Biosciences). Membranes were hybridized in NorthernMax Prehyb/Hyb buffer (Ambion, Austin, TX). After washing, the same membrane was subsequently hybridized with human 28 S rRNA gene-specific oligonucleotide probe or a glycerol-3-phosphate dehydrogenase cDNA probe (Clontech, Palo Alto, CA) to control for loading and RNA integrity.

Details of Plasmids—Luciferase expression plasmids were constructed by cloning the 1004-bp BstXI fragment from the proximal CD13/APN promoter (10) upstream of the luciferase reporter in the promoterless luciferase reporter vector pGL2 basic (Promega). 5’-Deletions of the 1004-bp fragment were constructed by subcloning restriction fragments or PCR generated fragments of the 1004-bp BstXI promoter region upstream of the luciferase gene in pGL2 basic. RAS expression plasmids were purchased from Clontech. In the dominant-negative expression construct RAS-17, Asn has been substituted for Ser17 that inhibits RAS activation (24). In the constitutively activated RAS expression construct RAS-61, Leu was substituted for Val20, generating the constitutively active GTP-bound form of the protein (25). NERF expression plasmids were the kind gift of Dr. Peter Oettgen. Plasmids expressing the green fluorescent protein (GFP) under the control of the CD13/APN promoter were constructed by subcloning the 1004-bp BstXI fragment of the wild-type CD13/APN proximal promoter into the promoterless pEGFP vector (Null-GFP) (Promega, Madison, WI) to produce CD13wt-GFP. The GFP reporter containing the cytomegalovirus (CMV) promoter, referred to as CMV-GFP, was purchased from Clontech. Reporter constructs containing mutant versions of the CD13/APN proximal promoter were constructed by PCR amplification of sequences upstream and downstream of the 38-bp region (−1004 to −153 and −115 to −1) in separate fragments that incorporated a novel restriction site at their junction. Ligation of these two fragments into pGL2basic formed the Δ38bp mutant lacking the 38-bp region. Subsequent mutant reporter plasmids were constructed by insertion of double-stranded oligonucleotides containing specific mutations (see text) at the novel restriction site and confirmed by sequence analysis.

Transient Expression of Recombinant Plasmids and Luciferase Reporter Gene Assay—Plasmids were transfected into KS1767 cells using LipofectAMINE 2000 (Innitrogen) following the manufacturer’s protocol. Cells were harvested 48 h post-transfection and assayed for luciferase activity as described previously (12). In conditions treated with PD098059, cells were transfected, incubated with the inhibitor in 10% FBS overnight, and harvested the next day (24 h post-transfection). Luciferase values in cells co-transfected with various expression plasmids and treated with PD098059 or growth factors were expressed relative to the values obtained with cells co-transfected with matching control plasmids or untreated control conditions, respectively. The maximum effects of co-transfection of Ets family expression plasmids on CD13/APN transcription were extrapolated from experimentally generated luciferase values by a nonlinear regression analysis using GraphPad Prism software (GraphPad Software, Inc.).

Stable Expression of GFP Reporter Plasmids in Mouse Hemangioendothelioma Cells—GFP-expressing plasmid constructs were stably transfected into murine hemangioendothelioma endothelial cells (EOMA) using LipofectAMINE 2000 and neomycin-resistant pools containing each of the constructs were harvested after 4 weeks of selection.

Electrophoretic Mobility Shift Assay (EMSA)—Nuclear extracts of KS1767 cells were prepared as described using a high-salt extraction protocol (26). EMSAs were performed as described previously (27) using oligonucleotides containing the region surrounding the wild type Ets-2 binding site (12) or the next day (24 h post-transfection). Northern Blot Analysis—KS1767 nuclear extracts were prepared as described for EMSA. Lysates (25–30 µg) were separated by SDS-PAGE on a 10% gel and transferred to nitrocellulose membranes. Immunoblotting was carried out as previously described (28).

In Vitro Endothelial Morphogenesis Assay—Endothelial cells (1 × 105) were plated in 2 ml of medium in 6-well tissue culture plates coated with 0.5 ml of basement membrane matrix per well (Matrigel, BD Biosciences). Cellular morphology and fluorescence levels resulting from GFP expression were monitored by phase-contrast and fluorescence microscopy, respectively.

Xenograft Studies—EOMA cells harboring the GFP reporter driven by various promoters were injected subcutaneously into the flanks of age-matched female SCID mice and tumors were harvested when they reached 1 cm in diameter. Tumors were mechanically dissected and analyzed for the percentage of GFP-expressing cells by flow cytometric analysis. Two independent rounds of xenograft tumor production produced identical results.

Construction and Transfection of siRNA—Construction of siRNA molecules was performed using the Silencer siRNA construction kit (Ambion) according to the manufacturer’s suggestions. Oligonucleotide sequences were as follows: siRNA11, AATCAAGAATATGGACCAGGT-CCTGTCTC; siRNA18, AATATGGACCAGGTAGCCCCTCCTGTCTC; and siRNA45, A4CCTTCAGCAGGGACACTCCCTGTCTC. In vitro synthesized siRNA was transfected into KS1767 cells using Lipo-fectAMINE 2000. Cells were incubated in 10% FBS 24 h post-transfection and then plated on Matrigel for an additional 24 h as described above.

RESULTS

CD13/APN Expression in Endothelial Cells Is Induced by Serum and Growth Factor Stimulation—During the angiogenic process, oxygen deprivation in tissues induces the expression of a heterogeneous group of genes important for tissue vascularization known as angiogenic growth factors that are essential for the progression of angiogenesis (reviewed in Ref. 29). Up-regulation of these factors leads to a subsequent increase in the expression of numerous proteins that further mediate angiogenesis, including matrix metalloproteases, serine proteases, peptidases, integrins, and growth factor receptor tyrosine kinases (reviewed in Ref. 14). We have shown that CD13/APN is a member of this group of angiogenic regulatory proteins and is
induced on primary endothelial cells in response to hypoxia and angiogenic growth factors (12). To dissect the molecular mechanisms of this regulation in more detail, we have utilized the Kaposi’s sarcoma-derived KS1767 endothelial cell line as our model system. We have extensively characterized this cell line and found that it faithfully reflects the induction of CD13/APN seen in primary vascular endothelial cells in response to angiogenic signals (12, 13). Consistent with our previous findings, serum-starved KS1767 cells cultured in low or high serum concentrations (which contains functional concentrations of many angiogenic factors, Fig. 1A) or with either the bFGF or VEGF angiogenic growth factors (Fig. 1B) markedly induces CD13/APN transcription in response to serum (3.1-fold increase in promoter activity (3.92 \pm 0.36-fold) in serum-stimulated cells as compared with low serum conditions (Fig. 2). This serum responsiveness disappeared with the removal of an additional 38 bp (between −153 and −115 bp), indicating that the sequences involved in the regulation of CD13/APN transcription in response to serum are located in this region of the promoter. Inspection of the −115 to −153-bp region sequence (Fig. 3A) revealed the presence of a GGAG motif (located at approximately −130 bp) that is similar to the core consensus binding site recognized by members of the ETS family of transcription factors (GGAA/T (30)). The Ets family contains nearly 35 members, some of which participate in the expression of variety of protein involved in vascular development and angiogenesis, such as urokinase-type plasminogen activator, matrix metalloproteinases, and the integrin β3 subunit (reviewed in Ref. 30).

To test whether nuclear proteins expressed at endogenous levels in mammalian cells interact with the CD13/APN promoter at this site, we incubated nuclear extracts of KS1767 cells with an end-labeled oligonucleotide probe that spans the putative Ets recognition sequence. Nuclear extracts from serum-stimulated KS1767 cells specifically retarded the migration of the probe essentially as a single complex in an EMSA (Fig. 3B). Binding of this complex to the probe was inhibited by addition of excess unlabeled oligonucleotide (Fig. 3B, lane 3). In contrast, competition with a matching oligomer containing point mutations of the GGAG element to AAAA was not observed (Fig. 3B, lane 4). Identical EMSA results were obtained with nuclear extracts from primary endothelial cells (data not shown). Thus, the Ets-like recognition sequence appears to specifically bind nuclear protein(s) from endothelial cells, consistent with the notion that the protein(s) that constitute this complex are responsible for the induction of CD13/APN in response to angiogenic signals in endothelial cells.

If the protein complex that binds to the GGAG element regulates CD13/APN expression, mutation of this site should profoundly alter CD13/APN promoter activity. To address this question, we created reporter plasmids containing either a deletion of the functionally determined 38-bp serum responsive region (−115 to −153, Δ38bp mutant) or altered the nucleotides of the GGAG site (GGAG to AAAA, indicated as GGAG mutant) in the context of the 1-kb CD13/APN wild type promoter plasmid. Transient transfection of either the deletion

**Fig. 1.** Induction of endogenous CD13/APN in response to serum and angiogenic factors. Serum-starved KS1767 cells were stimulated with the indicated concentrations of FBS (A) or bFGF and VEGF (50 and 25 ng/ml, respectively, B) for 24 h. Relative CD13/APN mRNA levels were determined by Northern blot analysis. Levels of ribosomal 28 S RNA or glycerol-3-phosphate dehydrogenase (GAPDH) mRNA were assessed as loading controls. C, KS1767 cells were incubated in 1% FBS for 24 h before transient transfection with the CD13/APN-luc reporter plasmid, followed by stimulation with the indicated FBS concentrations. Luciferase values are presented as -fold activation over those observed in 1% FBS-treated samples.

**luciferase** reporter gene and cultured in low (1%) or high (10%) serum concentrations (Fig. 2). Constructs containing promoter sequences upstream of −115 bp consistently showed significant increases in promoter activity (3.92 \pm 0.36-fold) in serum-stimulated cells as compared with low serum conditions (Fig. 2). However, this serum responsiveness disappeared with the removal of an additional 38 bp (between −153 and −115 bp), indicating that the sequences involved in the regulation of CD13/APN transcription in response to serum are located in this region of the promoter. Inspection of the −115 to −153-bp region sequence (Fig. 3A) revealed the presence of a GGAG motif (located at approximately −130 bp) that is similar to the core consensus binding site recognized by members of the ETS family of transcription factors (GGAA/T (30)). The Ets family contains nearly 35 members, some of which participate in the expression of variety of protein involved in vascular development and angiogenesis, such as urokinase-type plasminogen activator, matrix metalloproteinases, and the integrin β3 subunit (reviewed in Ref. 30).

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plasmid or the GGAG-site mutant plasmid showed substantially lower reporter gene activity when compared with the wild type control (Fig. 4A), indicating that binding of nuclear proteins to this site is critical for CD13/APN transcription in endothelial cells.

Expression of CD13/APN increases dramatically in the angiogenic endothelial cells of the tumor vasculature in response to a combination of growth factor and cell matrix signals present in the tumor microenvironment (12). We wished to determine whether the functional GGAG element also regulates CD13/APN transcription in the more complex stimulatory setting of angiogenesis-induced endothelial morphogenesis. In this model, plating endothelial cells on a basement membrane matrix (Matrigel) induces their differentiation and they form structures resembling capillary networks characteristic of the angiogenic phenotype (12, 13). As we have previously shown (12), treatment of primary endothelial cells in this Matrigel model with the peptidase inhibitors, bestatin and amastatin, or with more specific CD13/APN neutralizing antibodies potently prevents the formation of organized networks, reiterating the requirement for functional CD13/APN in this process. To investigate the role of the GGAG element in CD13/APN induction in response to these signals, and to ensure that the observed response is not limited to KS1767 cells, we used a murine endothelioma cell line (EOMA) that expresses relatively low levels of CD13/APN in culture but markedly induces CD13/APN expression upon stimulation with serum and complex angiogenic signals such as Matrigel (12). EOMA cells were stably transfected with reporter plasmids containing either the wild type CD13/APN proximal promoter upstream of the GFP reporter gene (CD13wt-GFP), a plasmid containing point mutations replacing the GGAG motif with AAAA in the context of the 1-kb promoter fragment (CD13-GGAG-mut-GFP), the promoterless negative control (null-GFP), or a positive control containing the cytomegalovirus promoter driving GFP expression (CMV-GFP). All transfected pools formed characteristic cobblestone monolayers when cultured under unstimulated conditions and similarly, organized into capillary networks in response to the Matrigel stimulus (Fig. 4B, bright field photographs). Neither the negative control null-GFP nor positive control CMV-GFP-transfected pools showed a change in reporter gene expression in response to angiogenic signals. In contrast, transfecteds harboring the CD13/APN wild-type promoter showed significant induction of reporter gene expression upon angiogenic stimulation, forming highly fluorescent capillary networks when plated on Matrigel (Fig. 4B, CD13wt-GFP, fluorescent photographs), confirming that the sequences controlling CD13/APN induction during network formation are included in this 1-kb genomic fragment. However, mutation of the GGAG element completely abrogated reporter gene induction in response to angiogenic signals, indicating that binding of transcription factors to these sequences are essential for the induction of CD13/APN during endothelial morphogenesis.

To establish whether this GGAG motif is also necessary for CD13/APN induction during tumor progression in vivo, we injected these stable transfectants into immunocompromised mice to form tumor xenografts. Tumors were harvested, mechanically disrupted to form single-cell suspensions, and assayed for GFP expression by flow cytometric analysis (Fig. 4C). Tumors derived from the CMV-GFP and wild-type CD13/APN promoter-GFP containing EOMA lines contained comparable numbers of GFP-positive cells, indicating that the sequences controlling CD13/APN induction in tumors in vivo are contained within this 1-kb promoter fragment. The fact that most of the cells recovered from both the CMV-GFP and CD13/APN-GFP containing tumors do not express GFP suggests that these tumors contain many host-derived stromal cells. As seen in our transient transfection experiments, specific mutation of the GGAG-motif within the CD13/APN proximal promoter fragment (GGAGmut-GFP, Fig. 4C) generated considerably fewer GFP-positive cells (1%) in the resulting tumors. Therefore, those transcription factors that bind to the GGAG element in the CD13/APN proximal promoter are required for induction of CD13/APN expression in response to signals in the tumor microenvironment in vivo.

**Ets-2 Is the Most Effective Activator of the CD13/APN Proximal Promoter-driven Transcription**—Because an intact GGAG site appears to bind protein complexes that are functionally important for the induction of CD13/APN in response to angiogenic signals in vitro and in vivo, we wished to specifically identify which transcription factor is mediating these effects. The GGAG motif is similar to the well characterized GGA(A/T) consensus site for the Ets family of transcription factors (31). Vascular endothelial cells have been shown to express several members of the Ets family of transcription factors including Ets-1 and Ets-2, Fli-1, Erg-2, and NERF where they have been shown to play various roles in the expression of angiogenesis-related genes (30). We transiently cotransfected increasing concentrations of expression plasmids encoding each of these proteins along with the wild type CD13/APN reporter plasmid into KS endothelial cells to determine the effects of exogenous expression of these transcription factors on CD13/APN promoter activity (Fig. 5A). The highest activation of reporter gene expression was consistently observed with the Ets-2 expression plasmids, whereas significant activation of lesser magnitude
was detected with Ets-1 expression plasmids. Similar experiments using the Fli-1 (Fig. 5A), or NERF-1A and NERF-2 (two biologically active alternative splicing variants of NERF, data not shown) showed considerably lower levels of activation. In contrast, Erg-2 had a slight inhibitory effect, presumably because of its interference with endogenous transcriptional activators in KS1767 cells.

To test the possibility that the variations in activation of CD13/APN transcription observed with three Ets factors were not because of differences in the rate or amount of protein produced (or other factors affecting their levels in KS1767 cells), individual dose-response curves generated in Fig. 5A were extrapolated to their respective theoretical maximum values (Fig. 5B). This analysis confirmed the interpretation suggested by data generated in Fig. 5A, that Ets-2 is most effective at inducing CD13/APN expression of the family members tested (maximal relative induction by Ets-2 was shown to be 71.52 ± 13.87-fold). In contrast, comparable values for Ets-1 and Fli-1 were 34- and 3.7-fold, respectively. Western blot analysis of lysates of mock (Fig. 5C, lane 1) or expression plasmid-transfected (lane 2) KS1767 cells confirmed that transfection of the expression plasmids resulted in increased expression of the encoded proteins.

Finally, to verify that Ets-2 transactivation of the CD13/APN promoter was mediated through the functional GGAG motif in the CD13/APN promoter, CD13/APN-luc reporter plasmids containing either the wild type or Ets-site mutant (GGAG to AAAA) were transiently transfected into KS1767 cells, alone (Fig. 5C, white bars), or along with Ets-2 expression plasmids (Fig. 5D, gray bars). Mutation of the GGAG Ets-2 binding site abrogated transactivation of the CD13/APN proximal promoter by Ets-2. Taken together, these data are consistent with Ets-2 controlling CD13/APN transcription via the GGAG element.

Ets-2-mediated Induction of CD13/APN Transcription in Response to Serum Is Regulated by the RAS/MAPK Pathway—

During angiogenesis, the Ets-activated expression of specific genes is mediated primarily through the activation of the RAS/MAPK pathway (32). We have recently shown that induction of CD13/APN in response to angiogenic growth factors is also mediated via this signal transduction cascade (13). To determine whether RAS signaling pathways are involved in serum-induced CD13/APN transcriptional activation, we transiently cotransfected KS endothelial cells with reporter plasmids containing the CD13/APN promoter and either the dominant-negative expression construct, RAS-17, or a constitutively activated RAS-61 expression construct (25). As expected, expression of RAS-17 decreased the CD13/APN promoter activity in a dose-dependent manner, whereas expression of RAS-61 caused a similarly dependent activation (Fig. 6A), reiterating a role for RAS in CD13/APN induction.

Whereas experiments depicted in Fig. 6A examined the effects of mutant RAS expression on endogenous transcription

Fig. 4. Effects of mutations within the Ets recognition sequence on CD13/APN proximal promoter activity in vitro and in vivo. A, KS1767 cells were transiently transfected with 1-kb CD13/APN-luc plasmids containing either the wild type Ets recognition sequence or CD13/APN proximal promoter activity in vitro. B, individual EOMA cell lines stably transfected with either the promoterless GFP expression plasmid (Null-GFP); GFP driven by the constitutively active cytomegalovirus promoter (CMV-GFP); GFP driven by the wild-type CD13/APN proximal promoter (CD13wt-GFP), or the mutant CD13/APN promoter containing a mutated Ets recognition sequence (CD13mut-GFP). C, cells were cultured either on cell culture plastic (left panels) or Matrigel-coated plates (right panels). Confocal images (bright field, shown in black and white and fluorescence shown in color) were acquired 18 to 24 h after plating. C, cell lines from B were grown as xenografts in SCID mice and tumors were assessed by flow cytometric analysis for GFP expressing cells. Data are expressed as percent of GFP-positive cells and numbers are representative of two independent experiments.
Through phosphorylation at residue Thr72 by ERK (23), we have reported to functionally activate Ets-2 in fibroblasts mediated through Ets-2.

Similarly, treatment of KS1767 cells with PD098059, a chemical inhibitor of MEK (a downstream mediator of the Ras pathway) also inhibited the Ets-2 induction of CD13/APN promoter activity. These data substantiate the hypothesis that the Ras-dependent regulation of CD13/APN in endothelial cells is mediated through Ets-2.

Importantly, the Ras/MAPK signaling pathway has also been reported to functionally activate Ets-2 in fibroblasts through phosphorylation at residue Thr72 by ERK (23), strongly suggesting that the Ras/MAPK induction of CD13/ APN by serum may be mediated via Ets-2 phosphorylation. To address this possibility, we asked if a phosphorylation defective Ets-2 expression plasmid (T72A, in which the threonine at position 72 is replaced by alanine, thus precluding Ras-mediated Ets-2 phosphorylation (23)) could transactivate the CD13/ APN promoter. Cotransfection of KS1767 cells with increasing doses of T72A Ets-2 and CD13/APN reporter plasmids was inhibitory in a dose-dependent fashion (Fig. 6C), probably reflecting interference by the mutant protein with endogenous Ets-2 activity in KS1767 cells. These results indicate that Ets-2 must be phosphorylated in response to signals initiated by angiogenic growth factors and transmitted through the Ras/MAPK pathway to transactivate CD13/APN via the functional GGAG element.

To confirm that the Ras/MAPK pathway regulates the phosphorylation of Ets-2 in endothelial cells as it does in fibroblasts (23), we analyzed levels of phosphorylated proteins in KS1767 cells treated with serum or with the MEK inhibitor PD098059 by Western blot analysis. Whereas total ERK1/2 levels are equivalent in both samples, the levels of phosphorylated ERK1/2 are markedly reduced upon inhibition of MEK (treatment with PD098059, Fig. 6D). Similarly, using an antibody that specifically detects Thr72-phosphorylated Ets-2, Ets-2 phosphorylation is clearly inhibited upon PD098059 treatment, demonstrating that ERK participates in Ets-2 phosphorylation and consequently, its activation, in endothelial cells.

Specifi
down-regulation of Ets-2 mRNA Inhibits CD13/APN Transcription—Whereas the above results strongly suggest that Ets-2 is a primary effector of the serum-induced signal transduction pathway that regulates CD13/APN transcription in angiogenic endothelial cells, these conclusions would be strengthened by a direct assessment of the effects of targeted down-regulation of endogenous Ets-2 expression on CD13/APN. The recent adaptation of siRNA technology to mammalian cells enables the targeted degradation of specific mRNA molecules by endogenous ribonucleases, thus inhibiting the expression of corresponding genes in the appropriate cell type (reviewed in Ref. 33). To precisely evaluate the role of Ets-2 in CD13/APN transcription, we designed and synthesized three species of duplex siRNA complementary to human Ets-2 mRNA (11, 18, and 45, representing nucleotide distances from the AUG codon to the 5’ end of the 21-bp siRNA sequence). Initially, we tested these under conditions designed to optimize the efficiency of siRNA inhibition where any increase in reporter activity is because of de novo Ets-2 synthesis (i.e. directed from the Ets-2 expression vector) and so will be maximally affected by siRNA. Transient cotransfection of siRNA duplexes into KS1767 cells along with the CD13/APN promoter

**Fig. 5. Comparison of CD13/APN transcriptional activation by endothelial cell-expressed Ets family members.** A, KS1767 cells were transiently co-transfected with 0.5 μg/well 1-kb wild-type CD13/ APN-luc reporter plasmid and increasing amounts of plasmids expressing Ets family members: Ets-1, Ets-2, Fli-1, and Erg-2. B, to rule out that the observed differences in transactivation potential of the transfected expression plasmids were not because of differences in the rate or amount of protein produced their effects on CD13/APN transcription levels were extrapolated from dose curves generated in A to theoretical maximum values using GraphPad Prism software. C, protein levels produced from transiently transfected expression plasmids were determined by Western blot analysis in mock (controls, lane 1) or plasmid-transfected (transfectants, lane 2) KS1767 cells. D, CD13/APN-luc reporter plasmids containing either the wild type or Ets site point mutant were transiently transfected into KS1767 cells along with Ets-2 expression plasmids (gray bars) or vector controls (white bars). Luciferase activity values are shown as -fold activation over the activities observed in cells transfected with wild-type CD13/ APN-luc alone.
plasmids and the wild type Ets-2 expression plasmid produced mixed results among the different duplexes as is routinely observed with individual siRNA duplexes (34). SiRNA11 partially suppressed CD13/ APN induction (−36% below maximum), whereas siRNA18 and -45 completely inhibited reporter activity to below background levels (Fig. 7A), suggesting that these siRNAs substantially decrease de novo synthesized Ets-2 mRNA levels. To determine the effects of siRNA on total Ets-2 levels (pre-existing and newly synthesized), we cotransfected KS1767 cells with CD13/APN promoter plasmids and increasing concentrations of the siRNA species. Each of the three siRNAs produced a dose-dependent reduction in basal promoter activity (Fig. 7B) to levels that correlate with their relative effects on inhibition of newly synthesized mRNA (Fig. 7A, 11 is less active than either 18 or 45). The inability to completely inhibit activity under these conditions is consistent with residual transactivation of CD13/APN by endogenous Ets-2 proteins that persist after the mRNA is eliminated by siRNA. Western blot analyses to confirm that the effects observed were indeed because of reduction in Ets-2 protein levels by siRNA in KS1767 cells determined by Western blot analysis.

Fig. 6. Effects of modulating the RAS signaling pathway on CD13/APN transcriptional activation by Ets-2. KS1767 cells were transiently co-transfected with 0.5 μg/well of the wild type CD13/APN-luc plasmids and increasing amounts of plasmids expressing: A, the constitutively active form of RAS (RAS61) or dominant-negative RAS (RAS17); B, wild-type Ets-2 together with a constant amount (1 μg/well) of RAS-17, RAS-61 expression plasmids, or in the presence of the MEK inhibitor PD098059 (30 μM); C, increasing amounts of expression plasmids encoding wild type or the phosphorylation-defective T72A mutant of Ets-2. Luciferase activity is shown as -fold activation over samples transfected with the wild-type CD13/APN-luc plasmid alone. D, levels of total and phosphorylated Ets-2 and ERK2 in control and PD098059-treated KS1767 cells were determined by Western blot analysis.
bly lower levels of Ets-2 protein than mock transfected controls, whereas the less active siRNA11 was also less effective in inhibiting Ets-2 production. Importantly, the presence of these oligonucleotides had no effect on levels of the closely related Ets-1 protein (Fig 7C). Therefore, specific manipulation of Ets-2 protein levels directly affects CD13/APN promoter activity in a dose-dependent manner.

Ets-2 Is Required for in Vitro Capillary Morphogenesis—Finally, we would predict that inhibiting the induction of angiogenically relevant genes by a reduction of Ets-2 protein levels would affect endothelial cell function. To assess the significance of Ets-2 in endothelial cell function we transiently transfected the two most active siRNA species (18 and 45) into KS1767 cells and tested them in the Matrigel network assay. Cells containing either of the two siRNA species alone formed the characteristic networks associated with endothelial cell morphogenesis, similar to mock transfected controls (Fig. 7D). In contrast, cells receiving both siRNA18 and -45 aggregated, but were incapable of organizing into capillary networks despite the Matrigel stimulus (Fig. 7D). A lower magnification view illustrates the widespread and uniform extent of this effect (Fig. 7D, 10X), which did not appear to be because of increased cell death (data not shown). These data would suggest that a threshold level of Ets-2 is required to transactivate genes that are critical for endothelial cell function during angiogenesis.

DISCUSSION

The regulation of angiogenesis is a complex process involving the acquisition and integration of external signals, the modulation of the expression of numerous genes, and the proteolytic processing of multiple substrates. We have recently identified a novel role for the CD13/APN cell surface ectopeptidase as a potent angiogenic regulator where functional CD13/APN is required for tumor growth in vivo (11) and endothelial migration and capillary network formation in vitro (12). Furthermore, we demonstrated that CD13/APN is transcriptionally inactive in the quiescent endothelial cells of normal vessels but is highly up-regulated in response to angiogenic signals present in the tumor microenvironment (12). Whereas these findings
clearly identify CD13/APN as a regulator of angiogenesis, the mechanism by which it does so is under active investigation. Molecular characterization of CD13/APN expression indicated that the proximal of the two CD13/APN promoters regulates gene expression in endothelial cells in response to hypoxia, angiogenic growth factors, and signals regulating capillary network formation and xenograft tumor growth (12). The elevation of CD13/APN expression could be a direct response to hypoxia-activated transcription factors, such as hypoxia-inducible factor 1 or hypoxia-associated factor, or a secondary consequence of hypoxia-induced growth factor production. The second option appears more probable because synthesis of CD13/APN is strongly induced under normoxic conditions by treatment with serum that contains high concentrations of angiogenic growth factors. Of the specific hypoxia-induced cytokines tested, CD13/APN mRNA, protein levels, and promoter activity are increased most strikingly in response to bFGF and VEGF (12). These factors are co-expressed in a variety of physiological conditions and functionally complement each other during angiogenesis (14). The Kaposi's sarcoma-derived KS1767 cell line expresses very high CD13/APN protein and mRNA levels that correlate with the highest relative promoter activity among endothelial cell lines tested. Assay of the supernatants of cultured KS1767 cells show that this line secretes significant levels of VEGF into the culture medium, suggesting that an autocrine mechanism contributes to the high expression of CD13/APN in this cell line. Our observation that bFGF and VEGF neutralizing antibodies added together inhibit CD13/APN promoter activity in KS1767 cells more potently than either alone (12) indicates that both bFGF and VEGF (and perhaps additional angiogenic factors) may mediate the serum induction of CD13/APN in KS1767 cells. Our previous characterization of angiogenic signal transduction cascades regulating CD13/APN expression in this cell line showed that it faithfully recapitulates the mechanisms operative in primary endothelial cells (15), making it a valuable model for the present studies investigating the nuclear effectors responsible for the induction of CD13/APN in response to angiogenic signals.

To further investigate the participation of growth factors in CD13/APN induction, we identified a 38-bp region of the CD13/ APN proximal promoter that regulates its response to bFGF. Examination of the sequence in this region showed no consensus site for the hypoxia-inducible factor 1 or hypoxia-associated factor that would denote a direct hypoxic response, but a motif similar to the core binding site for the Ets family of transcription factors (GGAG-CD13/APN site; GGAT/A-Ets consensus site) (32). The Ets family of transcription factors often function in intracellular regulatory cascades and specific Ets factors that have been assigned important role(s) in vasculogenesis and angiogenesis include Ets-1 and Ets-2, Fli-1, Erg, and NERF (36). To identify the Ets transcription factor involved in regulation of CD13/APN expression we functionally examined six Ets factors that regulate genes in activated endothelial cells for their ability to induce CD13/APN transcription. Expression of either Ets-1 or Ets-2 strongly induced CD13/APN transcription in transactivation assays, with Ets-2 producing twice the reporter activity of Ets-1. The other Ets factors tested (NERF-1a, NERF-2, Fli-1, and Erg-2) were either inactive or had a marginal effect on CD13/APN induction. Whereas we have concentrated on the role of Ets-2 in CD13/APN induction in this study, the functional redundancy of Ets-1 and Ets-2 in our experiments and the fact that both are activated by phosphorylation (23), raises the possibility that both may play a role in its induction in vivo where they display complex temporal and spatial expression patterns. For example, in human embryos Ets-1 is highly expressed in endothelial cells (39), whereas Ets-2 appears to be expressed not only in endothelial cells, but ubiquitously in all proliferating tissues and cell lines examined (40), and both are expressed at equivalent levels in HUVEC primary endothelial cells (41). Expression of both Ets-1 and Ets-2 are required for proper coronary development in avian embryos (42). Studies showing that inactivation of the Ets-1 gene results in viable animals with a high rate of perinatal lethality (43), but that Ets-2-null mice uniformly die at an early embryonic stage (44), suggest that Ets-2 is more critical during early development than Ets-1. Interestingly, during Xenopus development both Ets-1 and Ets-2 are expressed in embryonic regions that are undergoing critical morphogenetic alterations, particularly in migrating cells or along their migration pathways, and it is proposed that these proteins coordinate aspects of cellular adhesion (45). Finally, the regulation of genes by Ets-2 is highly tissue and cell type-dependent. For example, mammary gland epithelia from rescued Ets-2-null animals express normal levels of MMP-3 but the levels in skin are severely depressed (44). In this regard, tight regulation of the activity of both Ets-1 and Ets-2 appears to have evolved to coordinate complex physiological processes such as angiogenesis.

The hypothesis that Ets-2 specifically contributes to CD13/ APN induction in endothelial cells was strengthened by experiments demonstrating the ability of an exogenously expressed phosphorylation-defective Ets-2 plasmid (Thr257 to Ala257 substitution) to suppress CD13/APN promoter activity. About 30% of Ets family members (including Ets-1 and Ets-2) contain a highly conserved region termed the pointed domain, homologous to the Drosophila Ets factor Pointed and containing a threonine residue that is the target of RAS/MAPK signal transduction during Drosophila eye development. Similarly, the RAS-mediated phosphorylation of threonine residues at positions 38 (in murine Ets-1) and 72 (Ets-2) controls their transcriptional activity (23, 32, 46, 47). Analogous to results seen

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using the choriconic gonadotropin-β and the heparin-binding epidermal growth factor gene promoters (46, 48), this non-phosphorylatable Ets-2 mutant is transcriptionally inactive but retains its ability to bind DNA, resulting in a dominant-negative Ets-2 protein that inhibits endogenous CD13/APN promoter activity. We have shown that, similar to fibroblasts and epithelial cells, Ets-2 is also phosphorylated on Thr72 by an ERK1/2-dependent mechanism in KS1767 endothelial cells and that this phosphorylation is necessary for optimal Ets-2 induction of CD13/APN transcription. The phosphorylation dependence would appear to be promoter-specific, because the CSF-1R promoter has been shown to be regulated by Ets-2 in a phosphorylation-independent manner (20). The fact that Ets transcription factors characteristically function in collaboration with other transcription factors and that these unique protein–protein interactions further affect specificity of each individual Ets factor (reviewed in Ref. 49) would suggest that a requirement for Ets-2 phosphorylation indicates an obligate interaction with auxiliary transcriptional proteins. In this regard, the requisite Ets-2 phosphorylation seen in CD13/APN regulation may imply that the optimal induction of CD13/APN depends on additional transcription factors.

Direct genetic confirmation of a role for Ets-2 in CD13/APN induction was obtained using siRNA designed to specifically target human Ets-2 and not other endothelial cell-expressed Ets family members (particularly Ets-1). Transfection of Ets-2-specific siRNAs resulted in down-regulation of de novo Ets-2 levels and substantial inhibition of CD13/APN promoter activity when driven by either endogenous proteins or exogenously expressed Ets-2. Furthermore, these results eliminate the possibility that our overexpression of Ets-2 dominant-negative proteins forces binding to the CD13/APN GGAG-core site (Fig. 6C), thereby interfering with binding of the bona fide transcription factor and inhibiting CD13/APN transcription. Finally, transcription of CD13/APN was inhibited but not completely suppressed in the presence of Ets-2 siRNA. This residual CD13/APN transcription is probably because of persistent levels of endogenous Ets-2 protein that are insensitive to siRNA destruction. Therefore, the results of these experiments directly demonstrate that Ets-2 is indeed a major transcription factor involved in the control of the CD13/APN gene in endothelial cells. Taken together, our results invoke a mechanism for CD13/APN induction whereby angiogenic growth factors induce the activation of RAS and the subsequent activation of ERK, leading to phosphorylation of the Ets-2 nuclear effector and allowing it to transactivate angiogenically relevant genes.

Our demonstration of a critical functional role for Ets-2 and its target genes in endothelial cell function is consistent with our previous data showing that inhibition of CD13/APN expression and enzymatic activity in vitro and in vivo inhibits several aspects of angiogenesis (11–13). Similarly, the phenotype of embryos from Ets-2 null mice suggests that angiogenic processes may be perturbed in these animals. The early embryonic lethality in these mice is the result of the inability to form embryonic vascular connections to the maternal circulation and abnormalities in placental and extraembryonic endodermal tissues that may indicate deficiencies in extracellular matrix remodeling and metabolism (44). These phenotypic alterations in embryonic angiogenesis may portend fundamental anomalies in adult angiogenic processes as well. Considering the critical requirement for functional CD13/APN in angiogenesis, decreases in CD13/APN expression most probably contribute to the observed effects of Ets-2 inhibition on capillary morphogenesis. However, because expression of other angiogenically relevant Ets-2-regulated genes (such as MMP-3, MMP-13, urokinase-type plasminogen activator, and Von Willebrand factor (44)) would also be inhibited in our system, their loss would likely contribute to impaired endothelial function as well. Our data directly show that Ets-2 plays a vital, as yet incompletely characterized role in endothelial cell function, thereby adding this family member to the list of Ets transcription factors essential to angiogenesis.

How CD13/APN facilitates angiogenesis is yet not known. Its location on the cell surface mandates that its functional activity is dictated by substrates that are available in the immediate intercellular space. Because the switch from the quiescent to angiogenic endothelial phenotype involves an alternation in the relative levels of angiogenic inhibitors and activators (35), it is intriguing to postulate a role for CD13/APN in the processing of small regulatory molecules required to initiate, maintain, or suppress the angiogenic program in tumor vessel endothelium. Because CD13/APN activity is controlled by its expression, its precise transcriptional regulation is a pivotal factor that potentially contributes to control of the switch from quiescence to the angiogenic phenotype. Transcriptional induction of CD13/APN is a marker of activated angiogenic vasculature and therefore, CD13/APN and its nuclear regulators may represent novel targets for effective antiangiogenic therapy.

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CD13/APN Transcription Is Induced by Phosphorylated Ets-2
CD13/APN Transcription Is Induced by RAS/MAPK-mediated Phosphorylation of Ets-2 in Activated Endothelial Cells
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