Loss of responsiveness to chemotactic factors by deletion of the C-terminal protein interaction site of angiomotin

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Summary

We have recently identified a novel protein, named angiomotin, by its ability to bind the angiogenesis inhibitor angiostatin in the yeast two-hybrid system. Angiomotin belongs to a family with two other members, AmotL-1 and -2 characterized by coiled-coil and C-terminal PDZ binding domains. Here we show that the putative PDZ binding motif of angiomotin serves as a protein recognition site and that deletion of three amino acids in this site results in inhibition of chemotaxis. Furthermore, endothelial cells expressing mutant angiomotin failed to migrate and form tubes in an in vitro tube formation assay. To study the effect of angiomotin on embryonic angiogenesis, we generated transgenic mice expressing wild-type angiomotin and the C-terminal deletion mutant driven by the endothelial cell-specific receptor tyrosine kinase (TIE) promoter. Expression of mutant angiomotin in endothelial cells inhibited migration into the neuroectoderm and intersomitic regions resulting in death at embryonic day 9.5. In contrast, mice expressing wild-type angiomotin developed normally and were fertile. These results suggest that the putative PDZ binding motif of angiomotin plays a critical role in regulating the responsiveness of endothelial cells to chemotactic cues.

Key words: Endothelium, Neovascularization, Migration, Plasminogen, Receptor

Introduction

Angiostatin is a circulating inhibitor of angiogenesis originally isolated as a result of its ability to inhibit the growth of established metastases (O'Reilly et al., 1994). In vitro, angiostatin inhibits endothelial cell migration and reduces endothelial cell growth in proliferation assays; this effect is specific for endothelial cells (Ji et al., 1998). Furthermore, it has been shown that angiostatin induces endothelial cell-specific apoptosis in vitro (Claesson-Welsh et al., 1998; Lucas et al., 1998). Although the mechanism of angiostatin action remains unknown, several molecules have been identified as possible angiostatin-binding sites. Angiostatin was initially purified by its affinity to heparin-sepharose and has also been shown to bind the chondroitin sulfate proteoglycan that may inhibit its anti-angiogenic activity (Goretzki et al., 2000; Chekenya et al., 2002). Moser et al. have shown that alpha-beta subunits of ATP synthase, present on the cell surface, can act as an angiostatin-binding protein (Moser et al., 1999) and proposed, that ATP metabolism may play a role in the action of angiostatin. In addition, angiostatin has been shown to bind αvβ3 integrin, annexin II and c-met on the endothelial cell surface (Tarui et al., 2001; Tuszynski et al., 2002; Wajih and Sane, 2002).

We have recently identified a novel gene, angiomotin, via its interaction with angiostatin in the yeast two-hybrid system (Troyanovsky et al., 2001). Angiomotin belongs to a new protein family with only two additional members characterized by conserved coiled-coil domains and C-terminal PDZ binding motifs (Bratt et al., 2002; Nishimura et al., 2002). One of the members of this family, JEAP/AmotL-1, was identified by its localization to tight junctions in exocrine epithelial cells (Nishimura et al., 2002). The expression pattern of angiomotin differs from that of JEAP as it is expressed in endothelial cells of tissues undergoing angiogenesis such as extra-embryonic tissues and human tumors. Angiomotin is also expressed in cytotrophoblasts of the placenta as well as in polymorphic mononuclear cells [the latter of which was recently reported to respond to angiostatin (Benelli et al., 2002)]. At a cellular level, endogenous as well as transfected angiomotin localizes to the leading front of lamellipodia of migrating cells. A role in cell migration is suggested by the findings that overexpression of angiomotin in endothelial cells resulted in increased cell-migration. Furthermore, angiostatin inhibited migration and tube formation in angiomotin-transfected cells whereas control cells were unaffected. These data suggest that angiostatin is an antagonist of angiomotin activity in endothelial cells.

In the present study, we found that the putative PDZ binding motif of angiomotin serves as a protein recognition site and binds to a 90 kDa protein. Deletion of three amino acids in the C-terminal binding domain abrogated protein-protein interaction and resulted in inhibition of endothelial migration in vitro and in vivo.

Materials and Methods

Cell lines

Spontaneously immortalized mouse aortic endothelial (MAE) cells (Bastaki et al., 1997) and ecotropic retrovirus producing Phoenix Eco

cells (provided by Dr G. Nolan, Stanford University, Palo Alto, CA) were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 1% penicillin and 1% glutamine.

Peptide affinity columns

The following peptides were synthesized: CTLERKTPIQ ILGQEPDAEM VEYLI, CTLERKTPIQ ILGQEPDAEM VE (Innovagen AB, Lund, Sweden). The first cysteines were added to facilitate immobilization of the peptides to the sulfolink coupling gel (Pierce, according to the protocol of the manufacturer). For peptide affinity purification, mouse aortic endothelial cells were grown to 80% confluency in 14.5 cm Petri dishes. Before harvesting, cells were washed twice in cold PBS and scraped off in 3 ml lysis buffer (20 mM Hepes, 140 mM KCl, 5 mM MgCl, 10 mM β-glycerophosphate and 3% thesit and proteinase inhibitor cocktail pH 7.4; Sigma). The lysates were spun at 30,000 ***g*** for 25 minutes at 4°C. The supernatants were loaded to the peptide affinity column that had been equilibrated with lysis buffer. The column was washed with eight volumes of lysis buffer. Bound protein was eluted with 100 mM glycine buffer at pH 3.0. The volume of the collected fractions was reduced in a speedvac before analysis by SDS-PAGE. Protein bands were visualized by Coomassie staining.

Generation of deletion mutants

The angiomotin-pBabe vector was generated by blunt-end cloning a 3054 bp fragment of angiomotin cDNA generated by *Hin*dIII digestion into the *Eco*RI site of the pBabe vector. Deletion variants were created by PCR, using the angiomotin cDNA as template. The following primers were used: forward: 5′-CGGAATT-CAGGCCAGCGCAGGACATC-3′

Reverse primer for the 3′ of: Del 1, 5′-GGTTATTCCAGAG-TATTGGAGT-3′, Del 2, 5′-GGTTAGGGAGTTTTTCTTTCCAG-AGT-3′, Del 3, 5′-GGTTAAGGCTCTTGTCCC-AGGATCT-3′, Del 4, 5′-GGTTATTCCACCATCTCTGC-ATCA-3′. The PCR-product was cloned using the AdvanTAge PCR cloning Kit (Clontech), then cloned as an *Eco*RI fragment into the pBabe vector. To direct expression to endothelial cells during development we used the TIE-1 promoter (kindly provided by Dr K. Alitalo). Angiomotin (wild type and Δ4) were blunt-end cloned into the *Apa*I site of the TIE-vector using either the *Hin*dIII fragment (wild type angiomotin) or the *Eco*RI fragment (Δ4 angiomotin).

Western blotting

Parental MAE cells, transfected with pBabe angiomotin deletions were subjected to electrophoresis in SDS-PAGE gel under non-reducing conditions, transferred onto nitrocellulose membrane and non-specific binding was blocked overnight in PBS containing 5% dried milk. Angiomotin polyclonal antibodies were added for 1 hour at room temperature, washed three times for 5 minutes each in PBS and then incubated for 60 minutes with horseradish peroxidase goat anti-rabbit antibodies (Amersham Life Science). Bands were visualized using the ECL detection system (Amersham Life Science).

Migration assay

Migration assays were performed in a modified Boyden chamber using a 48-well chemotaxis chamber (Neuroprobe Inc., Gaitherburg, MD). Eight-micron Nucleopore polyvinylpirrolidine-free polycarbonate filters were coated with 100 μg/ml of collagen type 1 (Cohesion, Palo Alto, USA) in 0.2 N acetic acid for 24 hours and air-dried. The filter was placed over the bottom chamber containing basic fibroblast growth factor (bFGF; Pharmacia and Upjohn), vascular endothelial growth factor (VEGF; R&D systems) or lyso phosphatidic acid (LPA; Calbiochem). MAE cells were suspended in DMEM and 30,000 cells in 50 μl were added to each well in the upper chamber. In order to test the inhibitory activity of angiostatin on angiomotin-transfected cells, MAE cells were pre-incubated with 2.5 μg/ml of angiostatin for 1 hour. The assembled chemotaxis chambers were incubated for 5 hours at 37°C with 10% CO_2 to allow cells to migrate through the collagen-coated polycarbonate filter. Non-migrated cells on the upper surface of the filter were removed by scraping with a wiper tool (Neuro Probe, Inc., Gaitherburg, MD) and cotton swab, and the filter was stained with Hematoxylin (Mayer). The total number of migrated cells per field was counted at 200× magnification; each sample was tested in quadruplicate.

In vitro tube formation assay

MAE cells, transfected with angiomotin and angiomotin deletions (1.5×10^5 cells) were seeded on a layer of polymerized Matrigel as previously described (Troyanovsky et al., 2001). Matrigel cultures were incubated at 37°C. After 16 hours changes in cell morphology were captured through a phase-contrast microscope and photographed.

Immunofluorescent stainings

MAE transfected cells were fixed in 4% paraformaldehyde for 7 minutes at room temperature and blocked with 5% horse serum in PBS for 30 minutes. Cells were then incubated with angiomotin antibodies for 1 hour at room temperature. Antibody binding was detected with FITC-labeled anti-rabbit antibodies (Dako). F-actin was visualized with rhodamine-phalloidin (Molecular Probes). Images were collected using a Hamamatsu CCD camera and the Openlab software.

Transgenic animals

Transgenic mice were generated as described previously (Hogan et al., 1995). Briefly, TIE-angiomotin or TIE-Δ4 DNA constructs were injected into the pronucleus of fertilized C57BL/CBA mouse eggs. The eggs were then transferred into pseudopregnant mothers and transgenic embryos and adult mice were identified by Southern blot analysis. Genomic DNA was cleaved with *Ssp*I, generating a 1.8 kb fragment containing both the TIE promoter and approx. 1 kb of the 5′ part of angiomotin, which is the same in both wild-type and Δ4 constructs. Positive transgenes were verified using a probe spanning the same region, labeled by random priming (Megaprime kit, Amersham).

PECAM whole mount staining and sectioning

Both TIE-angiomotin and TIE-Δ4 embryos were dissected at E9.5 and fixed in 4% paraformaldehyde for 2 hours on ice. After washing in PBS, the embryos were incubated in 17% sucrose overnight, washed again and then dehydrated stepwise in methanol (25%, 50%, 75% and 100% for 15 minutes each) at room temperature. The embryos were bleached in 5% H_2O_2 for 5 hours, followed by stepwise rehydration to PBS. To block non-specific binding the embryos were incubated with 0.5% bovine serum albumin (BSA; Sigma) and 1% Triton X-100 (Sigma) for 1 hour at room temperature. Next they were incubated overnight at 4°C with rat anti-mouse PECAM1 antibody (Pharmingen, cat no. 553370) diluted 1:250 in 0.5% BSA, 0.5% Triton X-100 (BSA-Tx). Following washing in PBS/0.1% Triton X-100 five times for 1 hour each the embryos were incubated with a secondary antibody (biotinylated goat α-rat IgG, Vector laboratories) overnight at a dilution of 1:200 in BSA-Tx. The embryos were then washed again and incubated overnight with the ABC complex (Vector laboratories) diluted to 1:500 in BSA-Tx. After washing five times for 1 hour each the embryos were developed

with diaminobenzidine tetrahydrochloride (DAB; Sigma). The embryos were then embedded in paraffin and 5 μm sections were stained with Hematoxylin and Eosin.

Results

The C terminus of angiomotin contains a putative PDZ-binding motif

Angiomotin belongs to a protein family characterized by coiled-coil domains and C-terminal PDZ binding motifs (Bratt et al., 2002). Fig. 1A shows the eight amino acids of the C terminus of human and mouse angiomotin are aligned with glycophorin C, a protein with a characterized PDZ binding motif. The putative PDZ binding motif is 100% conserved between mouse and man. The last three amino acids of C-terminal PDZ binding motifs have previously been shown to confer the specificity of binding to proteins contain PDZ domains (Songyang et al., 1997). We wanted to analyze whether the putative PDZ-binding motif could serve as a protein interaction site. For this purpose we generated a peptide identical to the C-terminal 20 amino acids of angiomotin that was immobilized to agarose to generate an affinity column. A protein of a molecular mass of 90 kDa could be purified from mouse aortic cell lysate (Fig. 1B, lane 1). In contrast, no proteins could be isolated using an affinity column with a peptide lacking the last three amino acids (YLI).

Angiomotin promotes, whereas C-terminal deletion mutants inhibit, the response to chemotactic factors

In order to test the importance of the putative PDZ-binding motif in the control of endothelial cell migration we generated a series of deletions in the C terminus. The angiomotin cDNA contains a 2025 bp open reading frame, predicted to encode a 675 amino acid protein. The last 21 amino acids from the COOH end of angiomotin were divided into four parts: deletion 1 encoded amino acids (aa) 1-654; deletion 2, aa 1-658; deletion 3, aa 1-66; deletion 4, aa 1-672 (Fig. 1C). Wild-type angiomotin or one of these angiomotin mutant cDNAs were inserted into pBabe vector and used to generate retroviruses. MAE cells were infected with retroviruses, containing vector, wild-type angiomotin, or angiomotin mutants. Protein expression was verified by western blot analysis of cell lysates (Fig. 1D). No significant difference in proliferation rate could be detected as analyzed in a 3-day cell-doubling assay (data not shown). We have previously shown that angiomotin can promote cell migration in vitro. We therefore tested the migratory response of the cells transfected with the C-terminal angiomotin deletions using the Boyden chamber assay. Angiomotin-expressing cells exhibited increased migration towards bFGF, VEGF and LPA, all of which have been shown to induce migration in endothelial cells. In contrast, expression of the delta 1-4 mutant cDNAs in MAE cells resulted in inhibition of the migratory response to bFGF (Fig. 2A). Peptides lacking the last YLI amino acids of angiomotin do not bind the p90 protein as shown in figure 1. Furthermore, the Δ4 mutants did not exhibit any significant increase in migration in response to VEGF or LPA (Fig. 2B and C). These data show that deletion of as little as 3 amino acids of the C-terminal region of angiomotin results in repression of migration. We chose to focus on mutation 4 as we wanted to correlate cell migration to the functionality of the C-terminal protein interaction site.

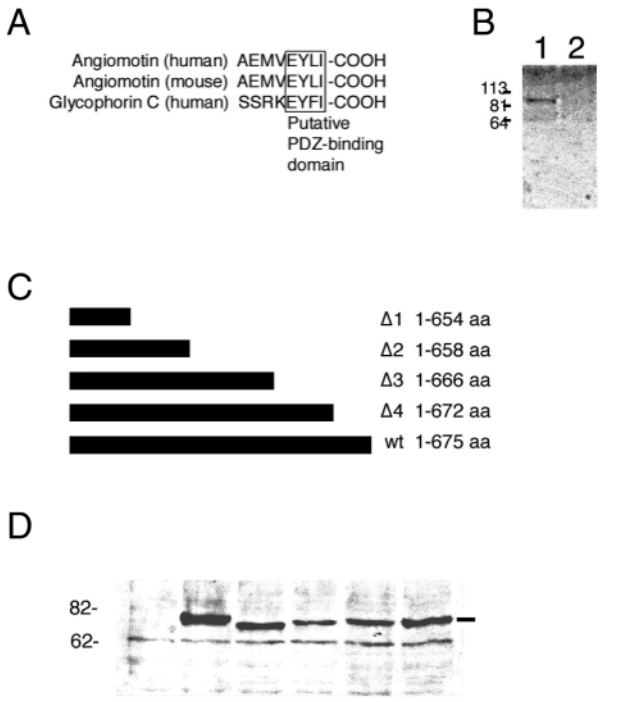

Fig. 1. The C terminus of angiomotin contains a conserved putative PDZ-binding motif. (A) Eight amino acids of the C terminus of human and mouse angiomotin are aligned with glycophorin C, a protein with a characterized PDZ binding motif. The last four amino acids that determine the binding specificity to PDZ-containing proteins are boxed. (B) Coomassie staining of SDS-PAGE showing specific binding of a 90 kDa protein isolated by peptide affinity chromatography using the last 20 amino acids of angiomotin (lane 1). The protein did not bind the angiomotin peptide that lacked the C-terminal amino acids YLI (lane 2). (C) Generation of 3′ deletion mutations of the angiomotin gene. A series of 3′ deletions were generated by PCR amplifications from the angiomotin cDNA as described in the Materials and Methods. The deletions ranged from 20-3 amino acids and were cloned into the pBabe retroviral vector. (D) Western blot analysis showing expression levels of angiomotin and the Δ1-4 mutants after transfection into MAE cells.

Next we investigated the behavior of the angiomotin transfectants in an in vitro tube formation assay. When plated on matrigel, extracellular matrix MAE cells attach, migrate and form multicellular capillary-like tubular structures. No significant difference in the capacity to form tubes in vitro could be detected between angiomotin and vector-transfected MAE cells (Fig. 3). However, mutants Δ1-3 all showed perturbed tube formation in that tubes were formed but collapsed resulting in a lower total tube length (Fig. 3 and data not shown). A more dramatic effect was observed after plating Δ4-transfected cells. Here, the cells did not spread and migrate to form tube structures but remained as single cells. These data show that removal of the C-terminal three amino acids of angiomotin results in repression of cell migration and tube formation.

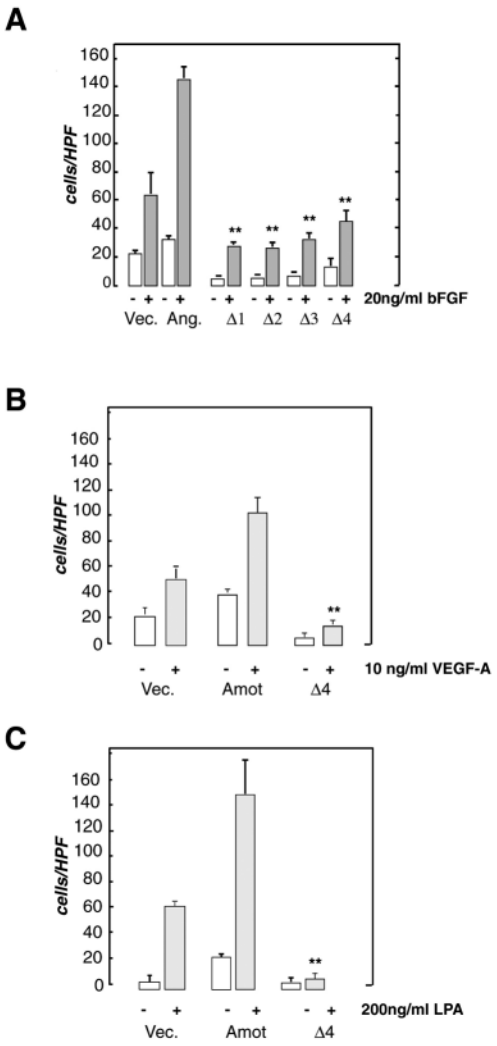

Fig. 2. Defective chemotactic response by cells transfected with the C-terminal deletion mutants. Mouse aortic endothelial cells (MAE) transfected with vector (Vec), angiomotin (Amot) or deletion mutants (Δ1-4**)** were stimulated by addition of (A) 20 ng/ml bFGF, (B) 10 ng/ml VEGF or (C) 200 ng/ml LPA. The differences between angiomotin and the deletion mutants were statistically significant (asterisks denotes $P<0.01$, error bars=s.d.). Cell migration was stimulated by the addition the chemo-attractant to the lower chamber in a modified Boyden chamber. The number of cells migrating through a collagen 1-coated filter was estimated after 5 hours. All assays were performed in quadruplicate.

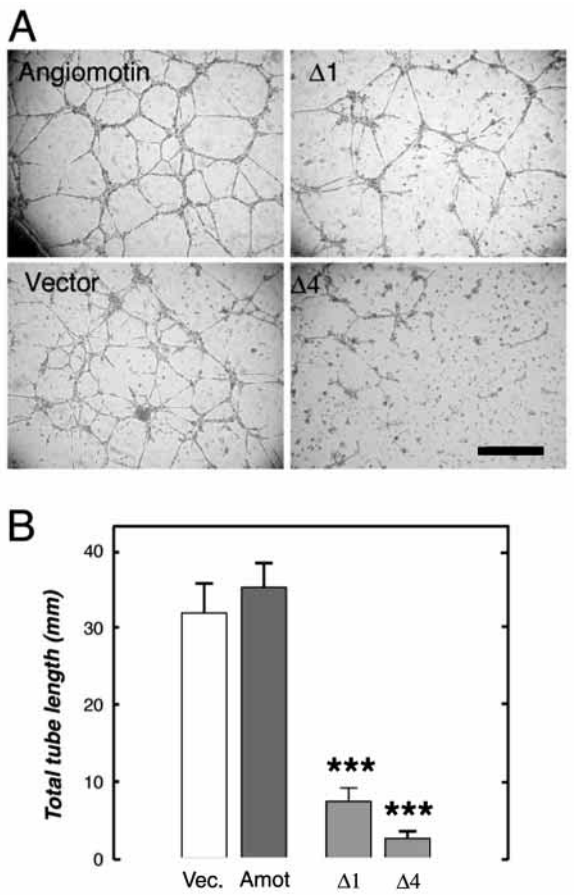

Fig. 3. Deletion mutants of angiomotin inhibit endothelial tube formation. Tube formation of vector, angiomotin and Δ1 and 4 transfected MAE cells was analyzed by in the matrigel assay. (A) Images show tube formation 16 hours after the start of the experiment. Scale bar: 300 μm. (B) Total tube length formed by vector, angiomotin and Δ1 and 4 transfected MAE cells. All analyses were performed in triplicate. The differences between vector and the Δ1 and Δ4 mutants were statistically significant (asterisks denotes $P<0.001$, error bars=s.d.).

The putative PDZ-binding motif of angiomotin is not required for localization to lamellipodia

Angiomotin localizes to lamellipodia of migrating cells. Therefore we wanted to assess whether the three amino acid deletion of the angiomotin C terminus affected the sub-cellular localization of the protein. It is possible that removal of the C-terminal end would either change protein conformation or remove a targeting signal that confers localization to lamellipodia. The angiomotin antibodies preferentially stained the lamellipodia of motile MAE angiomotin-transfected cells (Fig. 4). No difference in the cellular localization of the Δ4-angiomotin could be detected indicating that the last three amino acids are not required for angiomotin localization to the lamellipodia.

The angiomotin Δ4 mutant inhibits embryonic angiogenesis

Next we wanted to assess whether the Δ4 mutation could inhibit endothelial cell migration and angiogenesis in vivo. For this purpose, we generated transgenic mice expressing wild-type angiomotin or the Δ4 mutant driven by the endothelial cells-specific TIE promoter that is activated at embryonic day 8 (Fig. 5A). Expression of transgenic constructs was detected by Southern blot and RT-PCR analysis from DNA and RNA extracted from the tails. The DNA analysis revealed that 15% (8/57) of the offspring from TIE-angiomotin injections carried the transgene (Fig. 5B). No aberrant phenotype could be detected in these animals that were fertile when crossed with wild-type C57B6 mice. Two independent TIE-angiomotin transgenic lines were generated. Analysis of transgenic embryos derived from these lines at E 9.5, E13.5 and at birth

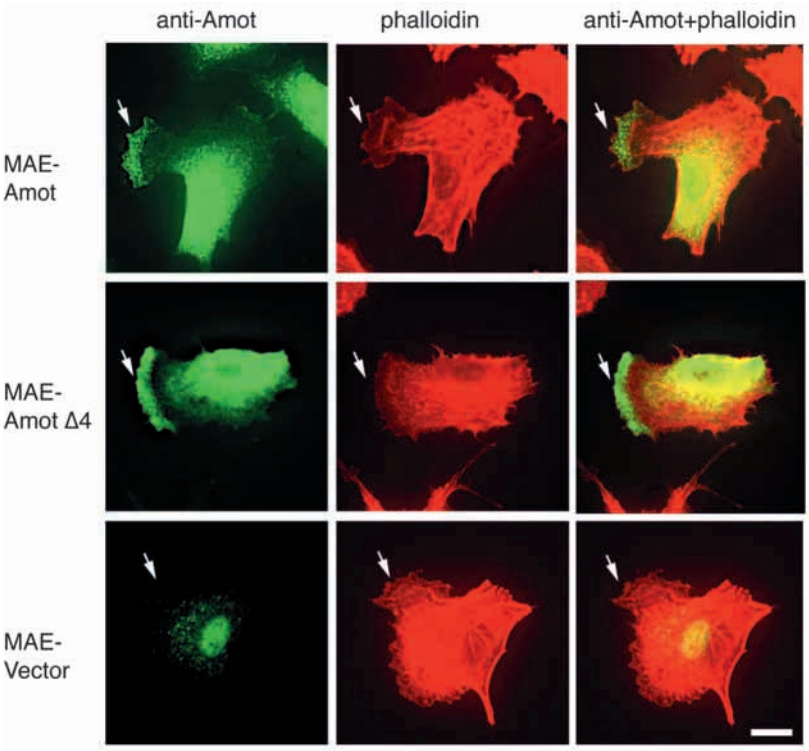

Fig. 4. Similar localization of angiomotin and Δ4-angiomotin in MAE cells. Immunofluorescent staining with a polyclonal antibody against angiomotin shows that both angiomotin (top panel) and Δ4 (middle panel) localizes to the central area of the cell and at the leading front of lamellipodia. Arrows and phalloidin stainings indicate location of the lamellipodia. Vector control cells (bottom panel) show some background staining in the nucleus. Scale bar: 10 μm.

did not reveal any detectable vascular defects (Table 1 and Fig. 5C,D).

In contrast, injections of the TIE-Δ4 construct resulted in no live transgenic mice (0/38). We therefore analyzed embryos at E9.5 for possible vascular abnormalities. Embryos carrying the transgene were identified by RT-PCR analysis of mRNA extracted from the placenta (Fig. 5B). A total of 14 transgenic embryos were identified, each embryo representing an individual transgene with a unique integration site. Analysis of these embryos revealed that 13 out of 14 Δ4 transgenes displayed severe vascular deficiencies at this time (Fig. 5H). The non-transgenic littermates (n=86) had no detectable vascular insufficiencies (Table 1). The transgenic embryos 1-9 were similar in size to wild-type embryos but suffered from severe bleeding in the brain as well as from intersomitic vessels (Table 2). The other embryos were smaller than the wild-type littermates. These embryos did not suffer from any detectable bleeding. We visualized the vascular endothelial cells in wild-type and TIE-Δ4 mice by whole-mount immunostaining using antibodies against mouse PECAM1. The TIE-Δ4 embryos had a defective cranial vascularization (Fig. 6). The localization of the vessels in the TIE-Δ4 angiomotin mice was assessed histologically. In normal brains at E9.5, capillaries were juxtaposed or migrating into the neuroepithelium (Fig. 7A,C). The TIE-Δ4 mice had cranial aneurysms and dilated vessels were found in the cephalic mesenchyme (Fig. 7B,D). These vessels did not align properly and fewer capillaries could be observed within the neuroepithelium. In addition, some of the dilated vessels showed leakage of nucleated blood cells into the surrounding mesenchyme (Fig. 7D). A more severe phenotype is illustrated in Fig. 7E,F in which no capillaries migrated into the neuroepithelium and the vessels adjacent to the neuroepithelium were grossly dilated.

Table 1. Analysis of vascular defects TIE-angiomotin transgenic mice

Age of mice	n	TIE-angiomotin	Phenotype
E9.5	19	+	No detectable phenotype
E13.5	48	+	No detectable phenotype
New born pups	130	+	No detectable phenotype, fertile
Total	197	+	No detectable phenotype

Heterozygote TIE-angiomotin mice were mated and the DNA from the resulting embryos and newborn pups was analyzed by Southern blot for transgene integration. The vascular phenotype of embryos was analyzed by whole-mount PECAM staining. The animals used in this experiment stem from two independent founder transgenic lines.

Discussion

Cell migration is an essential component in many biological

Table 2. Vascular defects in TIE-Δ4 angiomotin transgenic mice

Embryo	TIE-Δ4 angiomotin (Southern blot)	Phenotype
1	+	Cranial hemorrhage, cranial aneurysms
2	+	Cranial hemorrhage, cranial aneurysms
3	+	Cranial hemorrhage
4	+	Cranial hemorrhage
5	+	Cranial hemorrhage
6	+	Cranial hemorrhage
7	+	Cranial hemorrhage, not viable
8	+	Cranial aneurysms
9	+	Cranial aneurysms
10	+	Cranial and cardiac malformation
11	+	Cranial malformation
12	+	Not viable
13	+	Not viable
14	+	No detectable phenotype
15-100	Negative – litter mates	No detectable phenotype

No viable offspring could be generated from pronuclear injections with the TIE-Δ4 angiomotin construct. The vascular phenotype of embryos was therefore analyzed at E9.5. The DNA from the embryos was analyzed by Southern blot for transgene integration. 100 pronuclear injections resulted in 14 transgenic embryos. Note that each of these embryos reepresents an independent founder animal with a specific transgene integration site. The vascular phenotype of embryos was analyzed by whole-mount PECAM staining. The appearance of vascular defects was statistically significant (P<0.001) as analyzed by the Chi-square exact test.

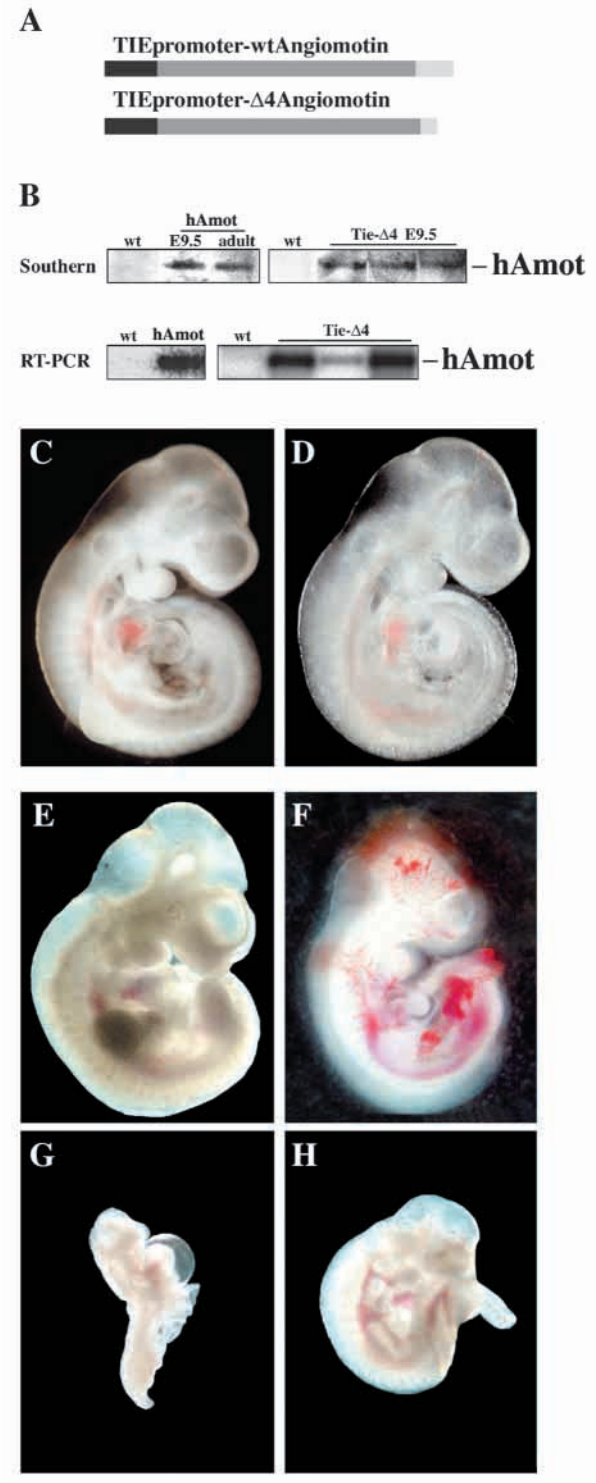

Fig. 5. Expression of wild-type and Δ4-angiomotin in endothelial cells of transgenic mice. (A) TIE-angiomotin and TIE-Δ4 angiomotin constructs. (B) Southern blot showing integration of TIE constructs in angiomotin (TIE-amot) and Δ4-angiomotin (TIE-Δ4) transgenic embryos (E9.5) and adult mice, and expression of the transgenes, analyzed by RT-PCR, showing angiomotin expression in angiomotin (TIE-ang) and Δ4-angiomotin (TIE-Δ4) in transgenic embryos (E9.5) and adult mice (hAmot, human angiomotin). (C-H) Phenotype of TIE-angiomotin and TIE-Δ4 angiomotin embryos at E9.5. TIE-angiomotin embryos (D) are phenotypically indistinguishable from their wild-type littermates (C). (E) Wild-type littermate of the TIE-Δ4 angiomotin embryos. (F) Cranial hemorrhage in TIE-Δ4 angiomotin-expressing embryo. (G,H) Embryonic malformations in TIE-Δ4 angiomotin embryos.

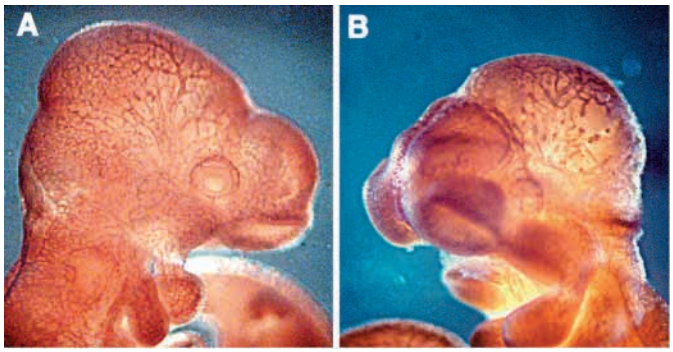

Fig. 6. PECAM whole-mount staining of TIE-angiomotin (A) and TIE-Δ4 angiomotin embryos (B). PECAM whole-mount staining shows aberrant cranial vascularization in the TIE-Δ4 angiomotin embryo.

processes such as immune activation, neurogenesis and angiogenesis. In this report, we show that the putative PDZ-binding motif of angiomotin is involved in protein-protein interaction and plays a critical role in regulating the response to chemotactic factors. Expression of wild-type angiomotin in endothelial cells increases the migratory response to chemotactic factors such as bFGF, VEGF and LPA indicating a role for angiomotin in cell motility. We could further show that deletion of the terminal three amino acids abrogates the response to extra-cellular chemotactic signals.

Angiomotin belongs to a novel protein family with two additional members, AmotL1 and Amotl2 (Bratt et al., 2002). These proteins are characterized by coiled-coil domains and contain a consensus motif for the binding of PDZ domains in the C terminus. The interaction between PDZ binding motifs and PDZ-containing proteins has been shown to play an important role in the targeting of proteins to specific membrane compartments (reviewed by Sheng and Sala, 2001). However, immunofluorescent stainings of angiomotin and the Δ4 mutant did not reveal any detectable difference in cellular localization. Both proteins were localized to areas of lamellipodia formation. Furthermore, both proteins are accessible to antibody staining and can be biotinylated on the cell surface (A.B., data not shown). Another role of PDZ proteins is to organize protein scaffolds for signaling complexes associated to membrane receptors. There are several examples indicating that PDZ proteins binding to membrane receptors may control the cellular response to migratory cues. For example, the cytoplasmic protein PDZ-RGS3 binds to the C terminus of transmembrane B ephrins and modulates the response of cerebellar granule cells to chemoattractant signaling via heterotrimeric G proteins (Lu et al., 2001). Furthermore, disruption of the PDZ binding domain of syndecan 4 inhibits migration, tube formation and exerts a dominant-negative effect on bFGF signaling in endothelial cells (Horowitz et al., 2002). In analogy, we show that the putative PDZ binding motif

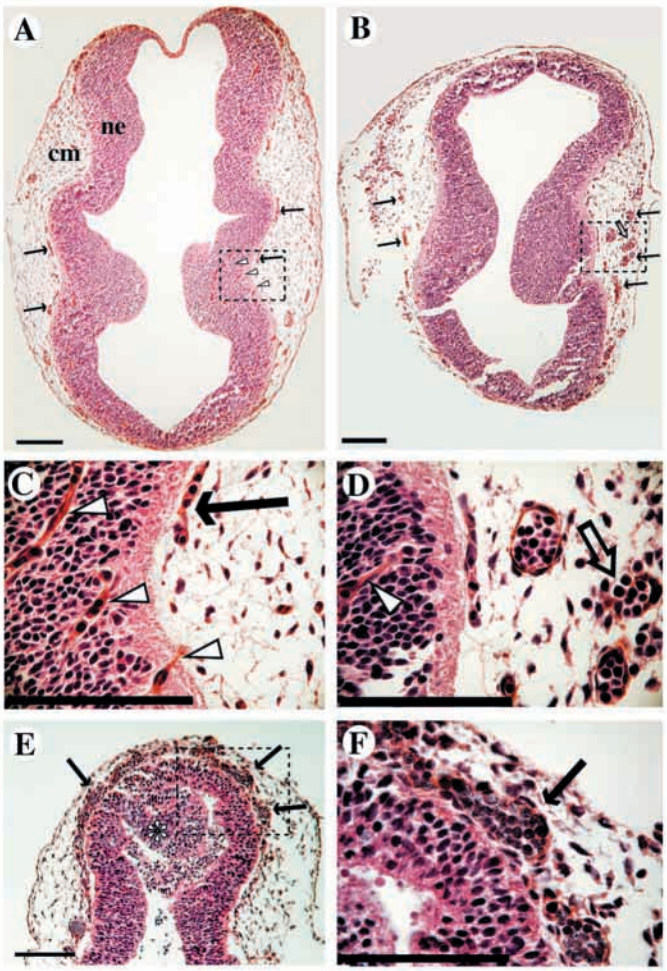

Fig. 7. Transverse histological sections of wild-type and TIE-Δ4 angiomotin E9.5 brains. Whole-mount PECAM-stained embryos were sectioned and stained with Hematoxylin and Eosin. (A) In wild-type brain, blood vessels align the neuroepithelium (arrows). (B) In contrast, the TIE-Δ4 angiomotin brain has dilated blood vessels within the cephalic mesenchyme (arrows). This panel is representative of embryos 1-9 in Table 1. (C) Magnification of boxed area in A, showing capillaries residing and entering the neuroepithelium (arrowheads). (D) Magnification of boxed area in B, showing the presence of dilated blood vessels (open arrow) within the mesenchyme and with only a few capillaries entering the neuroectoderm (arrowhead). Note also the ruptured vessel (open arrow) with nucleated fetal blood cells leaking into the mesenchyme. (E) A more severe phenotype of a TIE-Δ4-angiomotin mutant embryo displaying sack-like structures adjacent to the neuroepithelium (arrows), which has collapsed into the brain vesicle (asterisk). (F) Magnification of the boxed area in E, showing the dilated blood vessels. No vessel enters the neuroepithelium. cm, cephalic mesenchyme; ne, neuroepithelium. Scale bars: 125 μm.

of angiomotin binds to a 90 kDa protein. Deletion of the last three amino acids specifically abrogates both protein-protein interaction and results in loss of responsiveness to migratory stimulators.

We used the TIE promoter to express the angiomotin gene and the Δ4 mutant in the endothelial cell lineage in mice. The TIE-angiomotin mice were viable and fertile and did not exhibit any detectable impairment in blood vessel formation. Expression of TIE-Δ4 resulted in embryonic lethality, as it was thus not possible to generate any viable transgenic offspring. The TIE-Δ4 mice undergo normal vasculogenesis as could be expected since the TIE promoter is activated between E8-8.5 and is expressed in differentiating angioblasts as well as in endothelial cells. During normal development VEGF produced by the neuroectoderm stimulates the ingrowth of capillaries from the perineural vascular plexus (Breier et al., 1992). The TIE-Δ4 mice exhibited severely impaired blood vessel formation in the brain as well as in inter-somitic regions. The phenotype varied from cranial vessel dilation and hemorrhage to severe impairment of blood vessel formation in the embryos resulting in malformation and reduced size. The dilation and leakage of vessels is usually indicative of abnormalities in the basement membrane, cell-cell adhesion or other supporting structures. Several reports show that inactivation of components of the vascular extra-cellular matrix may cause hemorrhage. For example, inactivation of laminin α4 or fibulin, both components of the vascular wall, result in hemorrhage during embryonic development (Thyboll et al., 2002; Kostka et al., 2001). Another cause of hemorrhage is the failure of endothelial cells to recruit pericytes to form a vessel wall characteristic of mature vessels. PDGF-B-deficient mice exhibit dilation of brain vessels and post-natal hemorrhage. The defects are probably caused by defective migration of pericytes that depend on the chemotactic signaling from endothelial cells. The phenotype observed in the TIE-Δ4 mice occurs earlier than that observed in PDGF-B mice, that is before the recruitment of pericytes in the brain (Lindahl et al., 1997). Analysis of vessel morphology by histological sectioning revealed that vessels in transgenic mesenchyme were dilated and did not align properly with the neuroectoderm. Furthermore, the ingrowth of capillaries into the neuroectoderm was inhibited. We therefore suggest that the aberrant vascular phenotypes detected in TIE-Δ4 mice are caused by the inability of endothelial cells to respond to local chemotactic factors.

In conclusion, we provide evidence that inactivation of the putative PDZ domain switches angiomotin from promoting to repressing cell motility. Further understanding of the signaling pathways involved may yield important clues on how to inhibit endothelial cells responding to local chemotactic factors and thereby block the formation of new vessels.

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