Angiomotin Regulates Endothelial Cell-Cell Junctions and Cell Motility

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We have previously identified angiomotin by its ability to bind to and mediate the anti-angiogenic properties of angiostatin. In vivo and in vitro data indicate an essential role of angiomotin in endothelial cell motility. Here we show that angiostatin binds angiomotin on the cell surface and provide evidence for a transmembrane model for the topology of both p80 and p130 angiomotin isoforms. Immunofluorescence analysis shows that angiomotin co-localized with ZO-1 in cell-cell contacts in endothelial cells in vitro and in angiogenic blood vessels of the postnatal mouse retina in vivo. Transfection of p80 as well as p130 angiomotin in Chinese hamster ovary cells resulted in junctional localization of both isoforms. Furthermore, p130 angiomotin could recruit ZO-1 to actin stress fibers. The p130 but not p80 isoform could be coprecipitated with MAGI-1b, a component of endothelial tight junctions. Paracellular permeability, as measured by diffusion of fluorescein isothiocyanate-dextran, was reduced by p80 and p130 angiomotin expression with 70 and 88%, respectively, compared with control. Angiostatin did not have any effect on cell permeability but inhibited the migration of angiomotin-expressing cells in the Boyden chamber assay. We conclude that angiomotin, in addition to controlling cell motility, may play a role in the assembly of endothelial cell-cell junctions.

Angiogenesis, the formation of novel blood vessels by a process of sprouting or intussusceptive growth, has attracted considerable interest since it was shown to be crucial for tumor progression (1). Sprouting angiogenesis involves several steps: proliferation of endothelial cells, modification of the extracellular matrix, cell migration, and tube morphogenesis. The balance between specific growth factors and inhibitors controls this process (2). One of the identified inhibitors is angiostatin, a 38-kDa proteolytic fragment of plasminogen, which has been shown to potently inhibit angiogenesis and the growth of metastases in mice (3). In vitro studies have shown that angiostatin can inhibit endothelial cell proliferation (3), migration (4), and also induce apoptosis (5, 6). However, angiostatin has a short half-life in vivo and is hard to produce in sufficient amounts, which makes it difficult to utilize as a therapeutic agent (7).

We have identified angiomotin by its ability to bind angiostatin in a yeast two-hybrid screen. p80 angiomotin is a protein of 675 residues that is expressed in human endothelium and is a member of a conserved family of proteins that comprises two other proteins in human: angiomotin-like 1/1EAP and angiomotin-like 2 (8). Recent data suggest transcriptional diversity within this protein family (9).

The effects of angiomotin on transfected endothelial cells are stimulatory: the cells become more motile and invasive as determined by in vivo and in vitro assays. However, this effect can be blocked by angiostatin (10–12). Thus, angiostatin blocks tube formation of angiomotin-transfected cells in the Matrigel tube formation assay as well as migration of angiomotin-transfected cells in the Boyden chamber assay. Also, angiomotin-deficient mouse embryos exhibit impaired migration of the visceral endoderm (13). These data indicate that angiomotin may act as a promoter of angiogenesis by enhancing cell motility and migration and that angiostatin is an antagonist of angiomotin. The mechanism by which angiomotin promotes cell motility has not been defined, but in vivo and in vitro evidence shows that the C-terminal PDZ-binding motif is crucial (11). p80 angiomotin localizes to lamellipodia of migrating cells (10), and angiomotin promotes cell spreading on several different matrices, which indicates that angiomotin can control organization of the actin cytoskeleton. Angiomotin also has the ability to stabilize endothelial tubes in the Matrigel in vivo angiogenesis assay (12). Apart from angiostatin, other receptors for angiostatin have been identified, for example ATP synthase (14), the integrin αvβ3 (15), and the hepatocyte growth factor receptor c-met (16). The exact mechanism by which angiomotin down-regulates neovascularization remains to be determined.

Recently, we identified a splice form of angiomotin, p130 angiomotin. This protein differs from p80 angiomotin in that it has an N-terminal extension of 409 amino acids, which mediates the binding of angiomotin to actin stress fibers. Transfection of p130 angiomotin into mouse aortic endothelial (MAE) cells results in increased average cell size and pI60ROCK-dependant stress fiber formation. The formation of mature cell-cell contacts is a crucial step during angiogenesis. Silencing of endothelial adherence junction protein VE-cadherin leads to abnormal tight junctions, malformed vessels, and hemorrhages, and embryos die in utero within 9.5 days from fertilization (18). Endothelial tight junctions (TJ) form a seal between cells that isolates the lumen of the blood vessel from the surrounding tissue and restricts the diffusion of solutes from the blood to the surrounding cells. At the molecular level, TJs are formed by homodimerization of the specific tight junction transmembrane proteins occludin, claudin, and JAM. The cytoplasmic domains of these proteins are connected to the...

8 This work was supported in part by grants from the Swedish Cancer Society, the Swedish Research Council, Cancerforeningen i Stockholm, and Karolinska Institutet. 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 1734 solely to indicate this fact.
9 The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S3.
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4 M. Ernvikst, K. Aase, C. Ukomadu, Y. Zhou, J. Wohlschlegel, R. Blackman, N. Veitonmäki, A. Bratt, S. Fisher, A. Dutta, and L. Holmgren, submitted for publication.
5 The abbreviations used are: MAE, mouse aortic endothelial cell; ABD, angiomotin binding domain; Amot; angiomotin; TJ, tight junction; AJ, adherens junction; BCE, bovine capillary endothelial cell; bFGF, basic fibroblast growth factor; CHO, Chinese hamster ovary cell; ER, endoplasmic reticulum; HRP, horseradish peroxidase; JAM, junctional adhesion molecule; JEEP, junction-enriched and -associated protein; MAGI, membrane-associated guanylate kinase with inverted domain; P, postnatal day; PBS, phosphate-buffered saline; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate.
Angiomotin, Cell-Cell Junctions, and Cell Motility

actin cytoskeleton through a number of adaptor proteins, such as ZO-1/2/3 and MAGI-1/2/3 (19). Interestingly, angiomotin-like 1/EAP is reported to localize to TJs (20). TJs are structurally important for the cell and are linked to the cytoskeleton both physically and through signaling pathways. For example, the Rho family of GTPases controls both reorganization of the actin cytoskeleton and formation of TJs (21–24). In epithelial cells, another type of specialized junction, the adherens junction, can be distinguished. It is thought that the main purpose of AJs is to confer adhesion between cells to maintain tissue architecture. AJs are formed by homodimerization of cadherins, with VE-cadherin being the endothelial-specific cadherin. In endothelial cells, it is difficult to distinguish between TJs and AJs, because the two types of structures occur intermingled (25, 26). Here we show that p80 and p130 angiomotin are membrane proteins involved in control of permeability in cell-junctions but that the effect of angiostatin is limited to inhibiting migration of angiomotin-expressing cells.

EXPERIMENTAL PROCEDURES

Cell Culture—MAE cells stably expressing p80 and p130 angiomotin (10) were cultured in Dulbecco’s modified Eagle’s medium (Sigma). Chinese hamster ovary (CHO) cells were maintained in Dulbecco’s modified Eagle’s medium with Ham’s F-12 nutrient mixture. CHO cells stably expressing p80 or p130 angiomotin were generated by transfecting CHO cells with pcDNA3 with an insert of either p80 angiomotin or p130 angiomotin using Lipofectamine 2000 (Invitrogen) and selecting clones with G418 at 0.4 mg/ml. Bovine capillary endothelial (BCE) cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 2 ng bFGF/ml. All cell culture media were supplemented with 10% fetal calf serum (Invitrogen), 349 anti-paxillin (BD Transduction Laboratories), and monoclonal Mec 13.3 anti-CD31/PECAM (BD Pharmingen) and rabbit anti plasminogen (DAKO) were used.

Antibodies—Three different polyclonal antibodies against the following domains of angiomotin were used: the angiomotin binding domain (B3 antibody) (10), the C-terminal (TLE antibody), and N-terminal antibodies of p130 angiomotin (Fig. 3A). The following mouse monoclonal antibodies were used: 9E10 anti-Myc tag (Santa Cruz Biotechnology), M2 anti-FLAG tag (Sigma), AC-15 anti-actin (Sigma), 1A12 anti-ZO-1 (Zymed Laboratories Inc.), C060 anti-caveolin (BD Transduction Laboratories), 349 anti-paxillin (BD Transduction Laboratories), and OC-3F10 anti-occludin (BD Transduction Laboratories). Also rat monoclonal Mec 13.3 anti-CD31/PECAM (BD Pharmingen) and rabbit anti plasminogen (DAKO) were used.

Angiostatin—Angiostatin was generated by elastase degradation of plasminogen as previously described (3).

Western Blot—Proteins were separated on 7.5% Criterion SDS-PAGE gels (Bio-Rad) and transferred to Protran nitrocellulose membranes by semi-dry blotting. Membranes were blocked by incubation with PBS with 5% milk and incubated with primary antibody at 4 °C overnight followed by incubation with HRP-donkey anti-rabbit or HRP-sheep anti mouse (Amersham Biosciences) for 1 h at room temperature. The filters were washed several times in PBS plus 0.05% Tween, and the signal was visualized with Western blotting Luminol Reagent (Santa Cruz Biotechnology).

Biotinylation Experiments—~10 million confluent cells were briefly rinsed twice in PBS and incubated with NHS-Sulfo-LC-Biotin (Pierce, 0.4 mg/ml) in PBS or NHS-LC-biotin (0.4 mg/ml) in MeSO for 30 min at room temperature. The plates were then rinsed with PBS. Control plates were incubated with PBS alone. One milliliter of lysis buffer (20 mM HEPES, 140 mM KCl, 5 mM MgCl2, 10 mM β-glycerophosphate, 3% polyethylene-9-fauryl ether (Thesit), and protease inhibitor mixture, pH 7.4) was added, and the cells were harvested using a rubber policeman. Lysates were spun at 30,000 × g for 25 min, and the supernatants were subjected to immunoprecipitation by incubation with 1 μg of either B3 angiomotin antibody or paxillin antibody and 30 μl of protein G-Sepharose slurry (Pierce). The beads were washed three times in lysis buffer with 1% Thesit. Proteins were eluted with 30 μl of Laemmli buffer, and half of the material was loaded onto a 10% precast Criterion gel (Bio-Rad) and blotted onto a nitrocellulose membrane. Biotinylated proteins were detected with HRP-conjugated streptavidin (Pierce).

Trypsin Treatment—Confluent cells grown on 6-cm Petri dishes were washed twice with calcium- and magnesium-free PBS and incubated with 1 ml of sequence grade trypsin (Sigma) at 2 μg/ml or PBS alone at 37 °C for the indicated times. At 80 min a sample of cells was examined for integrity of the membrane using trypan blue, and it was found that 90% of cells had intact cell membranes. The experiment was ended by washing the cells once in PBS and adding 75 μl of Laemmli buffer. Samples were analyzed by Western blot.

Triton X-114 Phase Separation—This step was performed as described previously (28).

Angiostatin Binding Assay—Human angiostatin (kringles 1–4) was labeled with 125I by the iodogen method according to the protocol of the manufacturer (Pierce). The specific activity was estimated at 15,000 cpm/ng of protein. For binding assays HeLa cells stably expressing p80 angiomotin or empty vector were grown to confluency in 12-well plates. The cells were washed with PBS containing 1 mg/ml BSA and were incubated with 10 ng/ml radiolabeled angiostatin for 2 h. Cells were then washed five times with PBS with 1 mg/ml BSA and lysed with 1% Triton X-100 in PBS, and radioactivity was measured in a gamma counter.

Cross-linking Experiments—Confluent MAE cells grown on 15-cm plates were incubated with angiostatin (5 μg/ml) in PBS with 0.05% BSA for 100 min at 4 °C when cross-linkers BS3 and Sulfo-EGS (Pierce) were added to a final concentration of 2.5 mM for each. The cells were then incubated for additional 2 h at 4 °C. Cells were washed and lysed in 0.5% Triton X-100, 150 mM NaCl, 50 mM Tris, pH 7.5, and protease inhibitors and centrifuged at 14,000 rpm for 15 min at 4 °C. Immunoprecipitation was carried out from the supernatant with plasminogen antibody bound to protein A beads (Sigma).

In Vitro Binding Assay—His-tagged angiostatin binding domain and p80 angiominotin recombinant proteins (described in Ref. 10) were supplied by Bioinvent International AB, Lund. His-tagged Endostatin was used as negative control (kindly provided by Thomas Boehm, Children’s Hospital, Boston). 2 μg of His-tagged proteins was coupled to nickel-nitritoltriacetic acid-agarose beads (Qiagen) in binding buffer (300 mM NaCl, 50 mM Na2HPO4, and 0.05% Tween) at 4 °C for 2 h. Coupled beads were washed four times in binding buffer, and unspecific binding sites were blocked in binding buffer containing 1% milk. Kringle 4, K1–3, and plasminogen proteins were kindly provided by American Diagnostica, Greenwich, CT. Equimolar amounts of plasminogen and the different kringle fragments were added to the beads and incubated for 3 h at 4 °C. The beads were washed extensively in binding buffer. The beads were resuspended in Laemmli sample buffer and boiled for 5 min. Proteins were resolved by SDS-PAGE using a 12.5% gel, transferred electrophoretically, and visualized using electrochemiluminescence (ECL) using rabbit polyclonal antibodies against plasminogen.
Angiomotin, Cell-Cell Junctions, and Cell Motility

Immunofluorescence—Cells plated on chamber slides (Falcon) were rinsed briefly in PBS, fixed in 4% paraformaldehyde for 10 min, and (if not stated otherwise) treated with 0.05% Triton X-100 for 30 s. Cells were then incubated with 5% horse serum for 60 min, incubated with primary antibody diluted in 5% horse serum for 1 h, washed four times in PBS, and incubated with Texas red horse anti-mouse (Vector Laboratories Inc.) or FITC swine anti-rabbit (DAKO) diluted in 5% horse serum for 1 h. F-actin was visualized with Texas red phalloidin (Molecular Probes). For immunofluorescence of mouse retinas, eyes from C57BL6 mice sacrificed at P5 were fixed in 4% paraformaldehyde/PBS at 4 °C for 2–3 h and washed in PBS. Retinas were dissected as previously described (29) and incubated for 2 h at room temperature in a permeabilization/blocking buffer (PBB, PBS containing 1% BSA, 0.5% Triton X-100, and 5% normal goat serum). Retinas were then incubated at 4 °C overnight with primary antibodies diluted in PBB buffer. After six washes with PBS at room temperature, retinas were incubated 2 h at room temperature in darkness with secondary antibody diluted in PBS plus 0.5% BSA, 0.25% Triton X-100, and 5% normal goat serum. The secondary antibodies were FITC-conjugated swine anti-rabbit (Dako), Alexa Fluor 594-goat anti-mouse (Molecular Probes), and R-phycerythrin-goat anti-rat IgG mouse (Southern Biotechnology Associates). All specimens were flat-mounted in Vectashield mounting medium with 4',6-diamidino-2-phenylindole (Vector Laboratories Inc.). Pictures were captured on a Zeiss Axiosplan 2 microscope and processed with Zeiss Axiovision software and Adobe Photoshop.

Bioinformatics Analysis—Transmembrane helices were predicted with PredictProtein (30) and Tmpred (www.ch.embnet.org/software/TMPRED_form.html).

Angiomotin Induction Assay—500,000 BCE cells were plated at the indicated densities, and 24 h later cells were rinsed twice with PBS, briefly inverted on tissue paper, and lysed by addition of 100 µl of 2× SDS-PAGE loading buffer. Samples were analyzed by Western blot using the TLE antibody. Sample volume increased with increasing plate size due to residual PBS; therefore, 10% of the volume of the lysates was loaded.

Immunoprecipitation with MAGI-1—Two million CHO cells plated on 6-cm Petri dishes 1 day before were transfected with 2 µg of each plasmid DNA using Lipofectamine 2000 reagent (Invitrogen). Cells were harvested 48 h after transfection in a lysis buffer; 150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 0.5% Triton X-100, and protease inhibitors. The cell lysates were rotated end over end at 4 °C for 15 min and centrifuged at 13,000 rpm for 5 min at 4 °C. The supernatants were collected and used for the determination of total protein by the Bradford method. Lysate representing 1.5 mg of total protein were pre-cleared with 20 µl of protein A beads and then incubated with 5 µg of anti-angiomotin or anti-FLAG antibody for each IP sample for 6 h at 4 °C under rotation. Afterward 30 µl of protein A beads was added to each IP sample, and the mixture was rotated overnight at 4 °C. After incubation, beads were washed twice with lysis buffer, resuspended in Laemmli sample buffer, boiled, and resolved by 7.5% Criterion precast gel (Bio-Rad). For immunofluorescence staining, 40,000 CHO cells were plated in chamber slides and transected with 0.35 µg of each plasmid with Lipofetactamine 2000 reagent.

Aggregation Assay—CHO cells were de-attached by rinsing with Ca^{2+} and Mg^{2+} PBS twice and incubation with 0.02% EDTA (Sigma) until de-attached, resuspended in Ca^{2+}- and Mg^{2+}-free Hanks’ balanced salt solution (Sigma), washed once in Hanks’ balanced salt solution, and resuspended to 100,000 cells/ml in Hanks’ balanced salt solution supplemented with 2% fetal bovine serum dialyzed against Ca^{2+}- and Mg^{2+}-free PBS. 50,000 cells were loaded well in 24-well plates previously coated with 1% BSA. At this time the absolute majority of cells were single cells. CaCl_{2} (2 mM) and angiotatin (5 µg/ml) was added where indicated. Cells were allowed to aggregate at 37 °C for 60 min during rotation on a platform rotator at 80 rpm. The experiment was stopped by addition of glutaraldehyde to a final concentration of 5%. At least five fields from each well were photographed at 10× magnification and analyzed for cell aggregation. The total number of cells (N0) was counted, and the number of cell aggregates at 60 min (N60) as described (31). Approximately 500 cells were evaluated for each condition.

Permeability Assay—The In Vitro Vascular Permeability assay kit from Chemicon Inc., which is based on the diffusion of FITC-labeled dextran across a cell layer grown on a membrane in a 24-well plate format, was used according to the manufacturer’s instructions. Briefly, CHO cells were seeded at 12,000 cells per membrane insert and allowed to form a monolayer in 5 days. Triplicate or quadruplicate inserts were used for each condition. Where indicated, angiotatin (5 µg/ml) was added to the well 1 h before the start of the experiment. FITC-dextran was added to the upper chamber, and 100-µl samples were withdrawn at 5, 15, 60, and 120 min from the lower chamber. Fluorescence was measured on a Bio-Tek FL 600 plate reader using an excitation wavelength of 485 nm and an emission wavelength of 530 nm. The background fluorescence of cell culture medium was subtracted. The diffusion of FITC-dextran across a membrane insert without cells was measured in parallel to ensure integrity of cell monolayers.

Cell Migration Assay—A Boyden chamber migration assay was performed as described previously (10). Briefly, 30,000 cells in serum-free medium were added in each well and allowed to migrate toward serum or bFGF 20 (20 ng/ml) for 4 h. Non-migrating cells were removed, and remaining cells were fixed and stained with Giemsa stain. Three fields per well were counted under a microscope.

RESULTS

Angiomotin Is Localized on the Cell Surface—We have previously reported that MAE cells transfected with p80 angiomotin respond to angiotatin by inhibited migration and tube formation in vitro. This suggests that p80 angiomotin is a receptor for angiotatin. However, as judged by sequence analysis, angiomotin does not have an obvious signal peptide that could mediate the insertion of the protein into the membrane. To investigate whether p80 angiomotin has any extracellular domains, we incubated MAE cells stably expressing p80 angiomotin (p80 Amot MAE) with sulfo-NHS-LC-biotin, a biotin derivative with a reactive group that conjugates biotin to proteins. Sulfo-NHS-LC-biotin is water-soluble and will not penetrate intact cell membranes. After incubation with sulfo-NHS-LC-biotin we subjected cell lysates to immunoprecipitation against either angiomotin, or, as a negative control, the intracellular protein paxillin. The immunoprecipitates were judged by sequence analysis, angiomotin does not have an obvious signal peptide that could mediate the insertion of the protein into the membrane. To investigate whether p80 angiomotin has any extracellular domains, we incubated MAE cells stably expressing p80 angiomotin (p80 Amot MAE) with sulfo-NHS-LC-biotin, a biotin derivative with a reactive group that conjugates biotin to proteins. Sulfo-NHS-LC-biotin is water-soluble and will not penetrate intact cell membranes. After incubation with sulfo-NHS-LC-biotin we subjected cell lysates to immunoprecipitation against either angiomotin, or, as a negative control, the intracellular protein paxillin. The immunoprecipitates were analyzed by Western blot with HRP-conjugated avidin. As seen in Fig. 1A, p80 angiomotin was biotinylated, whereas paxillin was not. Paxillin could, however, be biotinylated with a hydrophobic, membrane-permeating analogue, NHS-LC-biotin (data not shown). Endogenous p80 angiomotin could also be biotinylated in primary cells when similar experiments were carried out with bovine capillary endothelial (BCE) cells (supplementary Fig. S1). This is of interest, because angiotatin was first identified by its ability to inhibit proliferation of these cells (3).

To verify that angiomotin has extracellular epitopes we treated MAE cells with trypsin for various times and analyzed cell lysates by Western
Angiostatin Binds Angiomotin on the Cell Surface—Previously we have shown that angiostatin-transfected HeLa cells can bind and internalize FITC-labeled angiostatin (10). To verify the binding of angiostatin to angiostatin-expressing cells, HeLa cells stably expressing angiostatin were incubated with iodinated angiostatin and scintillation was measured. Angiostatin did not bind to control cells to any great extent.

**FIGURE 2.** A, binding of iodinated angiostatin to HeLa cells expressing p80 angiostatin. HeLa cells stably expressing p80 angiostatin or empty vector was incubated with 5 μg/ml 125I-angiostatin or 5 μg/ml 125I-angiostatin plus a 100-fold excess of cold angiostatin. Cells were washed, lysed, and analyzed for radioactivity. Binding above binding to control cells not expressing angiostatin is shown. B, cross-linking of angiostatin to p80 angiostatin. MAE cells stably expressing p80 angiostatin or empty vector were incubated with angiostatin and cross-linker as indicated, lysed, and subjected to immunoprecipitation (IP) against angiostatin. Precipitates were analyzed by Western blot with antibodies against the C-terminal of angiostatin.

**FIGURE 1.** A, MAE cells stably expressing p80 angiostatin were incubated with Sulfo-NHS-LC-Biotin and subjected to immunoprecipitation (IP) with antibodies against the angiostatin binding domain of angiostatin (Amot) or paxillin (Pax.) followed by blotting (WB) with HRP-conjugated avidin. Angiostatin was biotinylated, whereas the intracellular protein paxillin was not. Trypsin treatment degrades p80 angiostatin on the cell surface. MAE cells stably expressing p80 angiostatin were incubated with trypsin for the indicated times and analyzed by Western blot using the antibody against the C-terminal against angiostatin (Amot). Overexposure of the blot revealed the appearance of a degradation product that likely represents the protected, intracellular, fragment of the C-terminal.

Trypsin degraded most p80 angiostatin in 80 min, whereas actin, which is intracellular, was not (Fig. 1B). Longer exposure of the blot revealed the appearance over time of a degradation product that likely represents a protected fragment of the C terminus, suggesting that this domain is intracellular.

Triton X-114 phase separation can be used to separate hydrophobic integral membrane proteins from hydrophilic soluble proteins. We carried out Triton X-114 fractionation of p80 Amot MAE cells. After phase separation p80 angiostatin could be detected in the aqueous fraction as well the detergent fraction, indicating that angiostatin is a integral membrane protein (Fig. 1C). VE-cadherin, a characterized transmembrane protein, also distributed to both fractions (data not shown).
and a 100-fold excess of cold angiostatin could compete with most of the binding of labeled angiostatin (Fig. 2A).

To characterize the binding of angiostatin to angiomotin, we allowed angiostatin to bind to p80 Amot MAE cells and added cross-linking agents that do not cross the cell membrane. Cell lysate was analyzed by immunoprecipitation and Western blot. Angiomotin was co-immunoprecipitated with angiostatin, and when cross-linker was added, a major shift was observed for the angiomotin band. The complex was larger than 175 kDa, indicating that the complex consisted of more than one angiostatin (38 kDa) and one p80 angiomotin (80 kDa) molecule. Cross-linker alone did not cause this complex (Fig. 2B).

**FIGURE 3. Antibody analysis of the topology of angiomotin.** A, the domain architecture of p80 and p130 angiomotin. Three different polyclonal antibodies against angiomotin were used to probe the topology of angiomotin in MAE cells: one directed against the C-terminal, one directed against the angiostatin binding domain (ABD), and one directed against the N-terminal of p130 angiomotin. The epitopes are shown as horizontal lines through the domains. These antibodies did not react with control cells expressing empty vector (not shown). In addition, Myc antibody was used to localize p80 angiomotin tagged in the N-terminal. B, the antibody against the ABD (green) binds to p80 angiomotin on the cell surface. An antibody against an intracellular epitope of caveolin (red) was used as a control for an intact cell membrane. In contrast, the antibody against the C-terminal of angiomotin (green) only displayed immunoreactivity when cells were first treated with the detergent Triton X-100. C, the antibody against the ABD (green) binds to p130 angiomotin on cells with an intact cell membrane. Here, phalloidin (red) was used as a control for an intact cell membrane. The antibody against the N-terminal of p130 angiomotin (green) did not stain without permeabilization. D, cells expressing p80 angiomotin with a N-terminal Myc tag only were stained only after permeabilization with Triton X-100. The bar represents 20 μm. E, suggested model for the topology of p80 and p130 angiomotin.
Angiomotin, Cell-Cell Junctions, and Cell Motility

The angiotatin binding domain (ABD) of angiomotin was identified by its ability to interact with angiotatin in the yeast two-hybrid screen (10). To verify the binding of angiotatin to this domain we performed in vitro pull-down assays with recombinant, His-tagged proteins. Angiotatin consist of the first four so-called “kringle” domains of plasminogen. Angiotatin kringle 1—4 as well as kringle 1—3 could be pulled down with whole p80 angiomotin as well as the ABD alone, but not with control beads (Fig. 2C). Plasminogen or kringle 4 did not interact with p80 angiomotin or the ABD in this assay. In conclusion, our data show that angiomotin is localized on the cell surface where its ABD binds angiotatin.

Topology of Angiomotin—Next, we proceeded to analyze the topology of angiomotin. By sequence analysis, p80 angiomotin contains three distinct domains. The N-terminal half is predicted to form a coiled-coil (8), and the C-terminal has a putative PDZ-binding domain, which is important for controlling cell motility (11). The angiotatin binding domain (ABD) identified in the yeast two-hybrid screen is a partly hydrophobic domain of 135 residues (10) located in the central region of the polypeptide (Fig. 3A). p130 angiomotin has an extended N-terminal domain with conserved glutamine rich motifs. We hypothesized that the ABD is extracellular and that the N-terminal coiled-coil, as well as the PDZ binding domain, are intracellular.

Generally, the cell membrane must be permeabilized for antibodies to stain intracellular epitopes in immunofluorescence studies. To investigate the transmembrane topology of angiomotin, immunofluorescence staining of subconfluent cells with antibodies directed against different domains of angiomotin in the absence or presence of prior treatment with the detergent Triton X-100 was used. We used three different polyclonal rabbit antibodies: one that is directed against the ABD (10), one directed against the most C-terminal 24 residues, and one directed against the N-terminal of p130 angiomotin (Figs. 3A and S2). The antibody directed against the ABD could stain without prior extraction of the membrane, suggesting that this domain has extracellular epitopes (Fig. 3B). As a control for an intact cell membrane we stained for the intracellular N-terminal domain of caveolin, which only stained cells treated with Triton X-100. In contrast, the antibody directed against the C-terminal of angiomotin needed permeabilization to stain (Fig. 3B). This indicates that the C-terminal is intracellular. However, one possible explanation for this result could be that the C-terminal epitope is extracellular and becomes available for the antibody after the detergent removes a masking protein, which is bound to the C-terminal. To rule out this possibility we utilized a variant of angiomotin that has an artificial signal peptide in the N-terminal, which leads to the secretion of the protein. The antibody against the C-terminal stained non-permeabilized, living cells expressing secreted angiomotin, showing that the C-terminal under these conditions was available for the antibody on the cell surface (Fig. S3A). This shows that the C-terminal epitope as such is accessible for antibody binding without Triton X-100.

To analyze the N-terminal of p80 angiomotin in the same manner we transfected MAE cells with a construct for angiomotin with a Myc tag in the N-terminal. Cells were co-transfected with a green fluorescent protein plasmid as a transfection marker. Staining for the N-terminal Myc tag required permeabilization (Fig. 3D), indicating that the N-terminal of angiomotin is intracellular. As in the case with the C-terminal, we carried out a control with secreted angiomotin with N-terminal Myc tag (Fig. S3B). Finally, the antibody against the N-terminal of p130 angiomotin only displayed immunoreactivity when cells were first permeabilized, indicating that this domain is intracellular (Fig. 3C).

Bioinformatics analysis suggests that angiomotin contains transmembrane helices in the hydrophobic part of the angiotatin binding region. The two predicted helices with the highest scores were residues 479–503 and 541–559 (probability scores in PHDhtm were 0.90 and 0.87, respectively). In conclusion, these data suggest that the N-terminal and the C-terminal of angiomotin are intracellular and that the hydrophobic regions flanking the ABD can form two transmembrane helices leaving the central part of this domain in the extracellular space (Fig. 3E).

Angiomotin Localizes to Cell-Cell Junctions in Primary Endothelial Cells and Is Up-regulated by Cell Density—To verify the extracellular staining pattern in primary cells we carried out the same experiments with BCE cells, which express p80 and p130 angiomotin in similar amounts.4 Without using membrane permeabilization, the antibody against the ABD stained the cells with a pattern similar to that of MAE cells and antibody against ZO-1 in confluent BCE cells. Confluent BCE cells were permeabilized with Triton X-100 and stained with the antibody against the C-terminal of angiomotin (green) and antibody against ZO-1 (red). Overlay of the two images is shown (merge) with co-localization appearing as yellow. C, expression of p80 and p130 isoforms of angiomotin (Amot) is regulated by cell density. Cells were plated at the indicated densities and analyzed for angiomotin expression by Western blot with the antibody against the C-terminal of angiomotin. The formation of cell-cell contacts is indicated with arrows. The bar represents 20 μm.
Angiomotin could also be detected in what appeared to be an intracellular pool, occasionally positive for ZO-1. This, together with the observation that completely confluent cells displayed no surface immunoreactivity, suggested that angiomotin is recruited to the cell surface at the place of cell-cell contacts in these cells. Treatment of confluent cells with angiostatin (5 µg/ml) did not alter the localization of angiomotin (data not shown).

The angiomotin fluorescence staining appeared stronger when BCE cells were more confluent. To investigate this BCE cells were plated at different densities and analyzed for angiomotin expression by Western blot. Expression of both p80 and p130 isoforms of angiomotin were strongly induced at 25,000 cells/cm² when cells could form contacts with each other (Fig. 4C). We conclude that formation of cell-cell contacts up-regulate angiomotin expression.
Angiomotin Localizes to Cell-Cell Junctions in Endothelial Cells in Vivo—We proceeded to analyze the localization of angiomotin in endothelial cells in vivo. In the retina of the mouse, angiogenesis occurs during postnatal days (P) 1–14 as vessels sprout from a vessel by the optic nerve toward the periphery of the retina and subsequently into deeper layers. We performed whole mount staining of retinal vessels from mice at P5 and analyzed the localization of angiomotin by immunofluorescence. Angiomotin was expressed in endothelial cells together with CD31/PECAM, a marker for endothelial cells (Fig. 5A). This shows that angiomotin expression is specific for endothelial cells in the retina. The angiomotin staining overlapped with the ZO-1 signal showing that angiomotin is localized to cell-cell contacts in endothelial cells in vivo (Fig. 5, B and C).

Angiomotin Localizes to Cell-Cell Junctions and Recruits ZO-1 in CHO Cells—We used CHO cells, which are often used as a model system for cell-cell contacts, to investigate the functionality of angiomotin in cell-cell junctions. We made stable lines of CHO cells expressing p80 angiomotin, p130 angiomotin, or empty vector. Transfection led to the aggregation of angiomotin-transfected cells (data not shown). We performed an aggregation assay and that angiostatin did not have an effect on the cell aggregation in the absence or presence of calcium in a short-term aggregation assays. These indicated that angiomotin did not mediate cell aggregation. We then turned our attention to MAGI-1, a membrane-associated guanylate kinase related to ZO-1, which has been reported to bind to the cytoplasmic domain of endothelial cell-selective adhesion molecule in endothelial cells (32). In CHO cells transiently expressing p80 or p130 angiomotin and FLAG-tagged MAGI-1 isoforms MAGI-1b and MAGI-1c, MAGI-1b could be immunoprecipitated with p130-angiomotin, but not with p80 angiomotin. Also, p130, but not p80 angiomotin, could also be immunoprecipitated with FLAG antibody (Fig. 7A). Immunofluorescence studies revealed that p130 angiomotin and MAGI-1b co-localized when expressed in CHO cells (Fig. 7B). Thus, the N-terminal domain of p130 angiomotin can associate with MAGI-1b or with proteins that interact with MAGI-1b.

Angiomotin Controls Cell Migration and Permeability—To analyze the function of angiomotin in cell-cell contacts, we carried out cell aggregation assays. These indicated that angiomotin did not mediate cell aggregation in the absence or presence of calcium in a short-term aggregation assay and that angiostatin did not have an effect on the aggregation of angiomotin-transfected cells (data not shown). We proceeded to investigate the role of angiomotin in controlling permeability of cell layers. For this purpose we used an in vitro permeability assay where diffusion of FITC-labeled dextran across a layer of cells grown on a permeable membrane was measured. CHO cells grown on the permeable membrane formed a monolayer (Fig. 8A), and there was no difference in morphology between cells expressing angiomotin or vector. When FITC-dextran had been added in the upper chamber, fluorescence increased in a time-dependent manner in the lower chamber, but markedly slower in angiomotin-expressing cells compared with control cells. After 1 h p130 angiomotin-expressing cells displayed 88% lower permeability compared with control cells, and p80 angiomotin cells displayed 70% lower permeability (Fig. 8B). This shows that angiomotin not only localizes to cell-cell junctions but can itself affect junction function. The results prompted us to investigate the role of angiostatin in affecting permeability in these cells. Angiostatin, however, did not
Angiomotin, Cell-Cell Junctions, and Cell Motility

A. morphology of CHO cells grown in permeability chambers. Bar represents 20 µm. B. permeability of CHO cell monolayers grown in permeability chambers was measured as diffusion of FITC-dextran across a cell layer over time. Rhomboids, vector; squares, p80 angiomotin; crosses, p130 angiomotin. Means and standard error of the mean from one representative experiment with triplicates from each time point are shown. When data from three experiments were pooled, standard error of the mean from one representative experiment with triplicates from each time point are shown. *** represents p < 0.001.

C. Effect of angiostatin on cell migration. CHO cells stably expressing p80 or p130 angiomotin responded to angiostatin with a 70% reduction of serum-stimulated motility. Also, BCE migration toward serum or bFGF was inhibited by angiostatin (Fig. 8E). Thus, the effect of angiostatin is limited to inhibiting cell migration and does not affect angiomotin-mediated control of cell-cell contacts.

DISCUSSION

In this report, we provide evidence that both p80 and p130 angiomotin are membrane proteins. We further show that both isoforms colocalize with ZO-1 in endothelial cells in vitro and in vivo. The data indicate that angiomotin is a novel component of endothelial cell-cell junctions and may play a functional role in the assembly of these.

We provide the following evidence for the model of angiomotin as a membrane protein: 1) angiomotin was biotinylated by cell-surface labeling of intact transfected, as well as primary, endothelial cells; 2) treatment of angiomotin-expressing cells with trypsin degraded the protein; 3) angiomotin occurs in the detergent-rich fraction after Triton X-114 partitioning; 4) angiostatin binds specifically to the surface of angiomotin-transfected cells; 5) angiomotin and angiotatin could be cross-linked by a cross-linker that does not pass through the cell membrane; and 6) antibodies directed against the angiomotin binding domain bind to angiomotin on the cell surface of transfected cells. We conclude that angiomotin can act as transmembrane protein and can serve as a receptor for angiostatin. Antibody epitope mapping suggests a transmembrane model for angiomotin, where the hydrophobic regions flanking the angiomotin binding domain can form two transmembrane helices leaving the central part of this domain exposed on the cell surface. Moreover, the C terminus contains the PDZ binding domain and such domains, as far as it is known, are always intracellular.

Angiomotin lacks a signal peptide as judged by sequence analysis, which indicates that it does not reach the membrane by the classic secretory pathway, which begins with incorporation of the peptide into the membrane of the endoplasmic reticulum during protein synthesis (33). However, proteins may insert into the membrane, or can be secreted, by other mechanisms (34). Thus, proteins can be secreted through what is usually referred to as non-classic secretion. Examples of such proteins are acidic fibroblast growth factor (35), thioredoxin (36), and TSAP6 (37). There are also examples of membrane proteins that lack signal peptides and are not inserted into the endoplasmic reticulum during protein synthesis. One group of membrane proteins, which is usually referred to as tail-anchored proteins, are post-translationally inserted into the membrane. Synaptobrevin belongs to this group of proteins (38). The model of angiomotin proposed by us, however, argues against angiomotin belonging to this class of membrane proteins. Furthermore, the protein HASPB expressed by the parasite Leish-
Angiomotin, Cell-Cell Junctions, and Cell Motility

Angiomotin, a member of the MAGI family of proteins, plays a crucial role in regulating cell-cell contacts and cell motility. Its interaction with the Rho family of GTPases and the actin cytoskeleton is significant for the regulation of cell polarity and migration.

How does angiomotin control permeability? The extracellular domain of angiomotin could participate in homotypic binding and act in vivo and in vitro and, when transfected into CHO cells, co-localizes with the TJ protein ZO-1, recruits ZO-1 to stress fibers, controls permeability, and binds to and co-localizes with the endothelial TJ protein MAGI-1. This suggests that angiomotin is a tight junction protein. However, given the close proximity between TJs and AJs in endothelial cells, we cannot rule out that the protein may also localize to AJs.

Previous data show that angiomotin expression correlates with increased cell motility and that this effect is blocked by angiotatin. This suggests that angiotatin inhibits angiogenesis by blocking angiomotin-induced cell motility (10–13). This study shows that angiomotin, in addition to controlling cell motility, localizes to cell-cell contacts in vivo and in vitro and, when transfected into CHO cells, co-localizes with the TJ protein ZO-1, recruits ZO-1 to stress fibers, controls permeability, and binds to and co-localizes with the endothelial TJ protein MAGI-1. This suggests that angiomotin is a tight junction protein. However, given the close proximity between TJs and AJs in endothelial cells, we cannot rule out that the protein may also localize to AJs.

Previously, we have reported that transgenic mice expressing dominant negative angiomotin in endothelial cells examined at embryonic day 9.5 displayed leaky blood vessels leading to severe bleeding in the brain (11). These findings are consistent with a role for angiomotin in decreasing permeability of TJs.

During angiogenesis cells migrate to the site of new vessels and then mature into a functional vessel with mature tight junctions and adherence junctions. Both migration and maturation are crucial steps during angiogenesis, and our data, together with previous findings, suggest that angiomotin can control both. In a similar manner, JAM-1, a transmembrane TJ protein, not only reduces paracellular permeability (41) but is also required for bFGF-induced motility of endothelial cells (42, 43). In this study, angiotatin specifically affected angiomotin-induced motility but not angiomotin-controlled permeability. This indicates that angiotatin exclusively affects motile endothelial cells participating in angiogenesis and not endothelial cells in established vessels. In addition, angiotatin has not been reported to increase vessel permeability in clinical trials (44). Interestingly, treatment with angiotatin can reduce permeability of tumor blood vessels (45).

How does angiomotin control permeability? The extracellular domain of angiomotin could participate in homotypic binding and act as a seal in TJs, much like occludin or claudin. Another possibility is that the effect of angiomotin is secondary and the result of signaling and/or recruitment of other proteins to TJs.

We have shown that p130, but not p80 angiomotin, interacts with the TJ protein MAGI-1b, which shows that the N-terminal extension domain of 409 residues of p130 angiomotin is necessary for this interaction. Although we have not shown a direct interaction between p130 angiomotin and MAGI-1, it is interesting to note that the N-terminal of p130 contains several proline-rich domains, including a PPXY motif, that could serve as binding motifs for the WW domains of MAGI-1 (27). However, the interaction with MAGI-1 is not necessary to target angiomotin to TJs, because p80 angiomotin by itself localizes to cell-cell contacts and controls permeability.

Cell motility and cell-cell junctions are both closely regulated by the actin cytoskeleton and the Rho family of GTPases. For example, although both Rho and Rac are recognized as two of the most important regulators of cell motility (22) both are crucial in regulating TJ formation downstream of Par-3 and Par-6, respectively (23, 24, 47). It is possible that angiomotin regulates both permeability and motility by influencing the actin cytoskeleton. Actually, there is evidence that angiotatin and angiomotin are linked to Rho signaling (48,49) and it is tempting to speculate that control of the actin cytoskeleton is at the heart of both properties of angiomotin. In line with this, MAGI-1 binds actin binding proteins α-actinin 4 and synaptopodin (47).

The encouraging results achieved with vascular epidermal growth factor antibody bevacizumab (49) not only show that we are entering the era of anti-angiogenic therapy, but also indicate that additional angiogenesis inhibitors are needed. Our results provide the rationale for designing antibodies that bind the angiostatin binding domain of angiomotin. It has been shown that anti-angiogenic antibodies that affect cell-cell interactions by blocking VE-cadherin may be designed so as to not affect permeability of mature vessels but rather to only block neo-vascularization (17). This study suggests that an angiotatin mimetic antibody may block angiogenesis by exclusively controlling cell migration without affecting the cell-cell contacts of endothelial cells in mature vessels.

Acknowledgments—We are grateful to Arne Östman for valuable discussions. MAGI-1 expression plasmids were kindly provided by Dr. Irina Dobrosotskaya, University of Texas.

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Angiomotin, Cell-Cell Junctions, and Cell Motility

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