Proteomic Analysis of Vascular Endothelial Growth Factor-induced Endothelial Cell Differentiation Reveals a Role for Chloride Intracellular Channel 4 (CLIC4) in Tubular Morphogenesis

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Formation of new vessels from pre-existing capillaries demands extensive reprogramming of endothelial cells through transcriptional and post-transcriptional events. We show that 120 protein spots in a two-dimensional isoelectric focusing/electrophoretic analysis were affected during vascular endothelial growth factor-A-induced endothelial cell tubular morphogenesis in vitro, as a result of changes in charge or expression level of the corresponding proteins. For about 22% of the spots, the protein products could be identified, of which several previously have been implicated in cytoskeletal reorganization and angiogenesis. One such protein was heat shock protein 27, a chaperone involved in β-actin rearrangement that was identified as regulated in degree of serine phosphorylation. We also identified regulation of chloride intracellular channel four (CLIC4), the expression of which decreased during tubular morphogenesis. CLIC4 was expressed at high levels in resting vessels, whereas expression was modulated during pathological angiogenesis such as in tumor vessels. The subcellular localization of CLIC4 in endothelial cells was dependent on whether cells were engaged in proliferation or tube formation. Antisense- and small interfering RNA-mediated suppression of CLIC4 expression led to arrest in tubular morphogenesis. Our data implicate CLIC4 in formation of a vessel lumen.

Angiogenesis, the formation of new blood vessels from the preexisting vasculature (1, 2), arises as a result of endothelial cell activation by increased production of angiogenic growth factors such as vascular endothelial growth factor (VEGF-A) or decreased production of angiogenesis inhibitors (3). VEGF-A is a principal mediator of angiogenesis (4) and gene targeted inactivation of only one of the VEGF-A alleles leads to arrest in vascular development and embryonic death by day E8.5 (5, 6). The angiogenic effect of VEGF-A is dependent on binding to and activation of VEGF receptor-2 (7). Mechanistically, the formation of new vessels from preexisting ones can be divided into at least two categories, capillary sprouting and intussusception (splitting of vessels). In capillary sprouting, the endothelial cells respond to VEGF-A by producing proteases that degrade the basement membrane. The detached endothelial cells migrate to form cord structures headed by a nondividing tip cell (8). This is followed by proliferation of cells in the stalk of the cord and later differentiation to form a lumen-containing vessel. Circulating endothelial cell precursors may also contribute to the formation of new vessels (9).

A number of different in vitro assays of angiogenesis have been shown to model specific steps (proliferation, migration, cord formation, and lumen creation) of the capillary sprouting process (10). In the three-dimensional collagen gel assay, endothelial cells are induced to migrate and fuse to form a cord of cells. This is followed by rudimentary lumen formation in a manner closely mimicking the pattern of vessel formation in a collagenous matrix in vivo (11) making it a suitable model for analysis of early regulation of angiogenesis. Indeed, the vessel structures assembled in a collagen matrix have the ability to anastomose with the pre-existing vasculature and to form functional vessels in vivo (12). The three-dimensional collagen assay has been employed in analyses of gene regulation during the sprouting process (13, 14), but until now, post-transcriptional events critical in capillary formation have not been identified. We have therefore undertaken a proteomic approach to identify key regulatory events at the protein level, in tubular morphogenesis. One of the identified VEGF-A-regulated endothelial cell proteins was chloride intracellular channel four (CLIC4).

The CLIC proteins have the unusual capacity to translocate from the cytoplasm to various cellular membranes (15, 16). Overexpression of CLIC4 promotes plasma membrane localization where it is associated with anion channel activity (17). CLIC4 has also been shown to engage in complex formation with cytoskeletal components such as β-actin, tubulin, and dynamin 1 (18). The expression of CLIC4 is regulated in a p53-dependent manner (16), and it contributes to p53-regulated apoptosis (19). CLIC4 has also been described as involved in the differentiation of fibroblasts into myofibroblasts (20). In Caenorhabditis elegans, interfering RNA; CLIC4, chloride intracellular channel 4; PBS, phosphate-buffered saline; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; TIME, telomerase-immortalized dermal microvascular endothelial; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; MS, mass spectrometry.
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CLIC-like proteins have been implicated in formation of the excretory tube (21). In this study, we demonstrate a role for CLIC4 in endothelial cell tubular morphogenesis.

EXPERIMENTAL PROCEDURES

Tube Formation Assay—Human telomerase-immortalized dermal microvascular endothelial cells (TIME) (22) were maintained on gelatinized dishes in endothelial microvascular EB Mv2 growth medium with the following supplements: 5 ng/ml endothelial growth factor, 0.2 μg/ml hydrocortisone, 0.5 ng/ml VEGF-A, 10 ng/ml basic fibroblast growth factor, 20 ng/ml insulin-like growth factor-1 (Promocell). The cells were starved overnight in 1% serum in EB Mv2 without growth supplements. Collagen gels were prepared by mixing 10× Ham’s F-12 medium, 0.1 M NaOH, collagen type 1 (1:1:8), supplemented with bicarbonate solution (Invitrogen) to 0.117% and Glutamax-1 (Invitrogen) to 1%. The gels were allowed to set at 37 °C overnight. To allow cell proliferation, tissue culture plastic was coated with overnight fibronectin at 20 μg/ml (Sigma). Cells were seeded on collagen or fibronectin matrices at 6.3 × 10^5 cells/cm². The cells were allowed to set for 2 h at 37 °C on the collagen gel before a second layer of collagen was added on top. After top gel polymerization (1 h), the cells were kept in medium containing serum and VEGF-A to final concentrations of 1% and 50 ng/ml, respectively.

Two-dimensional Gel Electrophoresis and Protein Identification—At the indicated time points, cells in collagen gels were harvested by disrupting the gel with 2.5 mg/ml collagenase SC2674 (Sigma) for 13 min in the presence of 1 mM proteasome inhibitor MG-132 (Calbiochem), 10 μg/ml cycloheximide, and 50 μM Na3VO4 to prevent protein degradation, new protein synthesis, and dephosphorylation, respectively. The cells were collected by centrifugation and washed with ice-cold PBS to remove collagen monomers. The cell pellet was lysed in a modified RIPA buffer (1% Triton X-100, 40 mM Tris-HCl, pH 8 (Amersham Biosciences), 0.1% SDS, and 1× Complete protease inhibitor mixture (Roche Applied Science)). Cells on fibronectin were washed once with PBS before lysis in RIPA buffer. Whole cell protein extracts were prepared for two-dimensional gel analysis by using a two-dimensional clean-up kit following the manufacturer’s instructions (Amersham Biosciences). Immediately thereafter, proteins were precipitated and washed before being dissolved in isoelectric focusing compatible buffer containing 8 M urea, 2% CHAPS, and 40 mM dithiothreitol (Amersham Biosciences). Protein concentration was determined using the two-dimensional Quant kit (Amersham Biosciences). Protein identification by two-dimensional gel electrophoresis and protein identification by mass spectrometry were carried out by the WCN Expression Proteomics Facility (Department of Medical Biochemistry and Microbiology, Uppsala University). Carrier ampholytes (0.5%) and bromphenol blue (0.002%) were added, and the sample was loaded on an isoelectric focusing strip (Amersham Biosciences) by “in-gel rehydration loading” and run essentially according to the manufacturer’s instructions (Amersham Biosciences). Protein samples were separated on analytical gels (220-μg sample) or on preparative gels (500-μg or 1-mg samples). Prior to the second dimension separation, proteins in the immobilized pH gradient strips were reduced and alkylated using dithiothreitol and iodoacetamide, respectively. The second dimension SDS-PAGE was carried out using the Ettan DALTsix vertical gel electrophoresis system (Amersham Biosciences) with 12.5% acrylamide gels. Proteins were subsequently stained with SYPRO ruby (Molecular Probes) essentially according to the manufacturer’s instructions. The gel image was recorded by a Typhoon 9400 scanner (Amersham Biosciences). Regulated proteins were identified and extracted by in gel digestion essentially as described (23). The generated peptides were analyzed by MALDI-MS using α-cyano-4-hydroxy-trans-cinnamic acid as matrix and MS spectra recorded on an Ultraflex TOF/TOF mass spectrometer (Bruker Daltonics). Spectra were internally calibrated with trypsin autolysis products (m/z = 842.51, m/z = 1045.56, and m/z = 2211.10). Finally, proteins were identified by peptide mass fingerprinting using the Mascot search engine (Matrix Science).

Confirmation of Regulated Proteins on Protein Blots—Whole cell protein samples from collagen and fibronectin matrices were subjected to SDS-PAGE in 10% acrylamide gels, followed by transfer onto Hybond-C filters (Amersham Biosciences). The protein expression was detected using rabbit anti-HSP27 and phospho-HSP27 (Upstate) and rabbit anti-CLIC4 (24). Primary antibody reactivity was visualized by secondary horseradish peroxidase-conjugated anti-rabbit IgG antibody, followed by enhanced chemiluminescence (Amersham Biosciences).

Real Time PCR Analysis—Total RNA was prepared from cells cultured in collagen or on fibronectin using the RNeasy mini kit (Qiagen). The cell lysis buffer containing guanidine isothiocyanate completely dissolved the collagen matrix, allowing the RNA to be purified directly from the cells. Contaminating genomic DNA was removed with DNase I (Amersham Biosciences), and 0.5 μg of DNase-treated RNA was used to prepare cDNA using oligo(dT) primers and the Advantage RT-PCR kit (Clontech). PCR was performed by mixing the cDNA with primers and SYBR Green PCR master mix (Applied Biosystems). CLIc4 primer sequences are as follows: 5’-GGTGATTC-TGAACCTTGGCCTCA-3’ and 5’-TCCCTCTTTGTAGCCCTCCAC-CT-3’ (Invitrogen). The PCR was run in an ABI Prism 7700 instrument (Applied Biosystems).

Immunofluorescence—Induction of angiogenesis in mouse embryonic bodies was performed as described previously (25). Collagen gels, with tube-forming TIME cells and embryoid bodies, were fixed overnight at 4 °C in zinc fix (0.1 M Tris-HCl, pH 7.5, 3 mM calcium acetate, 23 mM zinc acetate, 37 mM zinc chloride), permeabilized in Triton X-100, and blocked using 3% bovine serum albumin in Tris-buffered saline for 1 h at room temperature. Frozen T241 tumor and normal C57Bl/6 kidney sections were fixed in ice-cold methanol and blocked in 3% bovine serum albumin in PBS. Collagen gels, embryoid bodies, and tumor sections were incubated with the primary antibodies rabbit anti-CLIC4 and rat anti-CD31 (BD) as indicated and then with appropriate secondary antibodies (anti-rabbit-Fab2-Alexa-488 and anti-rat IgG-Alexa-555; Invitrogen). Collagen gels were transferred to microscope slides, and coverslips were mounted using Fluoromount-G (Southern Biotechnologies) and examined using a Nikon Eclipse E1000 microscope or an LSM 510 META confocal microscope (Carl Zeiss).

CLIC4 Antisense Transfections—CLIC4 antisense construct (24) was transfected into TIME cells by electroporation using the human umbilical vein endothelial cell nucleofector kit (Amaza Biosystems) following the manufacturer’s instructions. In brief, 1 × 10⁶ cells per condition were mixed with electroporation solution and 8 μg of plasmid and electroporated to transfer the DNA into the nucleus. The cells were resuspended in EB Mv2 medium and seeded on collagen gels or on fibronectin-coated plastics. Cells in three-dimensional collagen were fixed after 24 h with Zn-fix and incubated with 0.8 units/ml Texas Red-phalloidin (Molecular Probes) and 1 μg/ml Hoechst 33342. Tube structures were defined as structures containing four or more connected cells and were counted in nine fields per well and in three gels per condition. Statistical analyses were performed using the Statistica® software and an independent t test.
RESULTS

Identification of Proteins Regulated in Angiogenesis—Primary endothelial cells are known to undergo tubular morphogenesis and form capillary-like structures in three-dimensional collagen I gels in response to treatment with VEGF-A (26, 27). We have studied tubular morphogenesis of TIME cells, which retain the characteristics of the primary human dermal endothelial cells from which they were derived (22). TIME cells express several markers for vascular endothelial cells and lack lymphatic endothelial cell markers (22). The cells continue to divide for at least 200 population doublings, whereas parental primary cells will become senescent on average within 35–45 doublings. TIME cells between passage 21 and 25 were seeded between two layers of collagen I and induced with VEGF-A to form capillary structures (Fig. 1A). Gradually, cells would send out fine branches and make contact with neighboring cells, followed by swelling and fusion of cells into tubes. By 24 h, mature vessel-like structures had formed in which the actin cytoskeleton was lined up in parallel bundles seemingly expanding across cell borders (Fig. 1). These structures have been shown previously to contain rudimentary lumens and be growth-arrested (28, 29). In contrast, cells seeded on a fibronectin matrix proliferated and failed to form vessel-like structures (Fig. 1B).

To detect proteins regulated during different steps of vessel formation, cells were harvested and proteins isolated at specific time points corresponding to the discrete morphological steps in vessel formation. Whole cell protein lysates were generated from cells spreading on collagen 2 h after seeding and at 1, 3, 8, 16, and 24 h after onset of VEGF-A treatment. The relative expression patterns of total cellular proteins were analyzed by two-dimensional gel electrophoresis and compared between different time points on collagen, and also between differentiating cells on collagen and proliferating cells on fibronectin. Three gels for each time point were analyzed to identify consistent changes in protein expression pattern. Initially, two-dimensional gels with a broad isoelectric focusing range (pH 3–10) were used to visualize as many proteins as possible (Fig. 2A). Because most proteins have an isoelectric point between 4 and 8, rare proteins may be masked by more abundant ones. Therefore, we also separated the protein extracts on two-dimensional gels with a more narrow isoelectric focusing range (pH 4–7). This strategy allows better separation and loading of more protein. Guided by the analytical gels, we selected certain time points for preparative two-dimensional gels and spot identification as follows: 2 h after seeding but before addition of VEGF-A (time point 0), and after 1 and 16 h in the presence of VEGF-A. Selected spots were subjected to in-gel trypsin digestion, and the resulting peptide mix was analyzed by MALDI-TOF MS. The resulting mass spectra were run against the Mascot search engine (Matrix Science) for protein identification.

Over 120 protein spots were identified as differentially regulated on the two-dimensional gels, but reliable sequence identification could only be generated for 27 spots (TABLE ONE). Twelve of the identified proteins have been implicated in regulation of the cytoskeleton, reflecting the dramatic cytoskeletal rearrangements required to form new vascular tubes. Five of the proteins were classified as involved in metabolism and three proteins as involved in protein turnover, which indicates the need for reprogramming in transition from proliferation to differentiation. Some of the proteins identified belong to protein families already associated with a role in angiogenesis, such as HSP27 (30, 31) and annexins (32). The family of chloride intracellular protein families already associated with a role in angiogenesis, such as proliferation to differentiation. Some of the proteins identified belong to protein families already associated with a role in angiogenesis, such as HSP27 (30, 31) and annexins (32). The family of chloride intracellular

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with time on collagen, but the relative level of phosphorylated HSP27 increased 2.6 times at 16 h (in relation to β-actin expression levels). Because both HSP27 (33) and CLIC4 (18) are known to be associated with the cytoskeleton, cells were lysed with a harsher SDS-containing lysis buffer to identify potential changes in protein subcellular localization, to a more insoluble compartment, with time. Similar levels of CLIC4 protein were generated independent of the lysis technique, indicating that the apparent decrease in CLIC4 expression level at 16 h was not because of trapping of protein. The decrease in HSP27 expression at the 16-h collagen gel time point also persisted under harsher lysis conditions, as did the relative increase in serine phosphorylation. On fibronectin cultures, increased levels of HSP27 protein were detected after lysis in SDS buffer compared with RIPA buffer indicating relocalization of HSP27.

**CLIC4 Down-regulation through Post-translational Mechanisms**—To determine whether the observed decrease in CLIC4 expression in cells kept in three-dimensional collagen gels for 16 h was dependent on transcriptional regulation, total mRNA was prepared from the indicated time points.

**FIGURE 2. Identification of proteins regulated during endothelial cell differentiation.** A, proteins were isolated from endothelial cells in collagen gels at time point 0 (after seeding on the lower collagen gel layer but before addition of the second collagen layer and addition of VEGF-A) and at time point 16 h (16 h of VEGF-A treatment). Proteins were separated and visualized by two-dimensional isoelectrofocusing and electrophoresis. Three proteins with different expression patterns are indicated. The level of enolase was unchanged; CLIC4 was down-regulated and the expression of the indicated HSP27 isoform increased. B, validation of changes in CLIC4 and HSP27 regulation by immunoblotting. C, real-time PCR analysis of CLIC4 in relation to β-actin showed that the decrease in CLIC4 protein levels seen at 16 h of differentiation was not caused by decreased transcript levels.
and the levels of CLIC4 transcripts were analyzed by real time PCR (Fig. 2C). No changes in transcript levels could be observed at different time points, suggesting that the decrease in CLIC4 protein involved post-translational mechanisms.

**CLIC4 Is Expressed by Vessels in Vivo**—Expression of CLIC4 in endothelial cells has not yet been studied despite the intriguing link to lumen formation for the CLIC homologue EXC-4 in *C. elegans*. In other cell types, CLIC4 has been linked to differentiation (myofibroblasts) (20), and apoptosis (keratinocytes and tumor cells) (19). To gain insight into the role of CLIC4 in vessels of different angiogenic status *in vivo*, we examined CLIC4 immunoreactivity in normal mouse kidney vessels, in developing murine embryonic vessels, and in T241 fibrosarcoma tumor vessels (Fig. 3). In normal mouse kidney, in which CLIC4 is known to be abundantly expressed in a constitutive manner (16), CLIC4 was expressed in all vessels, and relative to the surrounding stroma, the expression seemed high. CLIC4 was also expressed at high levels in endothelial cells in sprouting vessels in a mouse embryo body model of angiogenesis (34). In contrast, in T241 mouse fibrosarcoma, where vessels are characterized by continuous neoangiogenesis and regression (35), expression of CLIC4 showed a pattern of selective expression in a subset of vessels. CLIC4 was also to some extent expressed by the surrounding T24 fibrosarcoma tumor cells.

**CLIC4 Subcellular Localization during Endothelial Cell Differentiation Versus Proliferation**—Reports on the subcellular localization of CLIC4 *in vitro* does not form a coherent pattern; CLIC4 may be localized in the endoplasmic reticulum membrane (36), in large dense core vesicles in neurons (37), in the cytoplasm and in mitochondria (16), and in the nucleus (24). It is likely that changes in the subcellular localization of CLIC4 are critical in regulation of its function. The subcellular localization of CLIC4 in endothelial cells in three-dimensional collagen I gels and on fibronectin was analyzed by confocal microscopy. As shown in Fig. 4, the matrix and the time spent on the matrix clearly influenced the subcellular localization of CLIC4. In proliferating cells on fibronectin, high CLIC4 immunoreactivity was observed in prominent circular vesicles in neurons (37), in the cytoplasm and in mitochondria (16), and in the nucleus (24). The large dense core vesicles were most abundant at 1 h after addition of VEGF-A. In endothelial cells in collagen I gels and on fibronectin the actin cytoskeleton did not reveal close association between CLIC4 and actin, as has been reported to occur under certain circumstances (18) (data not shown).
Intact Levels of CLIC4 Is Necessary for Optimal Tube Formation—To investigate whether CLIC4 has a direct role during endothelial vessel formation, CLIC4 antisense was introduced into TIME cells (19) (Fig. 5). As judged from parallel transient transfection of GFP cDNA, routinely about 60% of the cells were transfected. In antisense transfected cells, the level of CLIC4 expression decreased by an average of 57% (Fig. 5A). As not all cells expressed the antisense, the actual CLIC4 expression in the total cell population probably ranged from 100 to 0% of the control cells. TIME cells with and without antisense were seeded in collagen I gel or on fibronectin. As a consequence of introduction of CLIC4 antisense, the TIME cells displayed a decreased ability to form tube structures in three-dimensional collagen gels (Fig. 5B). Quantification by counting the number of structures containing four or more cells demonstrated a significant decrease in tube length in the CLIC4 antisense culture compared with control (Fig. 5C). In contrast, introduction of CLIC4 antisense did not affect the growth of endothelial cells on fibronectin (data not shown). These data were validated by introduction of two independent CLIC4-specific siRNAs that reduced CLIC4 transcript levels by 85–95%, thereby causing a significant decrease in the ability of TIME cells to form tubular structures (see Supplemental Material).

DISCUSSION

This study demonstrates changes in expression or charge, as visualized by isoelectric focusing and electrophoretic separation, of more than 120 proteins in human endothelial cells undergoing tubular morphogenesis. Of these, the molecular nature of 27 proteins could be unequivocally identified, which indicates that sensitivity still poses a considerable limitation in this strategy. Moreover, most identified proteins were associated with a function in the regulation of the cytoskeleton and expressed at relatively high levels in cells. The cytoskeletal association reinforces the strict need for extensive modulation in cell shape during the formation of an elongated, highly structured tube. Several of the proteins identified in this study have been implicated in the regulation of angiogenesis, such as HSP27 (31) and annexins (32, 38), thus validating the strategy. It is noteworthy that the regulatory changes of proteins such as HSP27 and CLIC4 identified in this study would not have been selected in a microarray analysis of mRNA regulation. Generally, there is poor correlation between studies reporting on the regulation of protein expression on the one hand and transcript levels on the other, at least in part due to post-transcriptional modifications (39, 40).

HSP27 is a stress-induced heat shock protein that is involved in the response of endothelial cells to angiogenesis inhibitors (31). In this study, HSP27 was identified as a protein undergoing changes in mobility in the two-dimensional gel analysis, because of a change in charge during tubular morphogenesis. Using a phosphospecific HSP27 antibody,
we confirmed that the change in charge involved serine phosphorylation. Phosphorylation of HSP27 regulates its association with the cytoskeleton (41), which is in accordance with a role for HSP27 in the dramatic changes in the actin cytoskeleton required in neo-vessel morphogenesis. In the nematode C. elegans, tubular formation of the excretory tube requires a conserved 55-amino acid domain (21). Conceivably, the folding of this domain regulates insertion into the membrane. We examined the localization of CLIC4 in endothelial cells forming tubular structures in three-dimensional collagen gels, and we observed a general cytosolic staining as well as staining of small, potentially pinocytotic vesicles. In cells proliferating on a fibronectin matrix, CLIC4 was found in striking, large vesicular structures. Most intriguingly, CLIC4 is localized in somewhat similar structures in hippocampal neurons, i.e. in large dense core vesicles, which is a type of secretory vesicle that exists in nerve endings (37). We sought to determine whether the CLIC4-positive vesicles observed by us corresponded to secretory Weibel-Palade bodies that exist in endothelial cells. Endothelial cells were immunostained to detect von Willebrand factor, a component of the Weibel-Palade bodies. However, co-localization of Weibel-Palade bodies and CLIC4-positive vesicles was not detected (data not shown). Moreover, endothelial cells were labeled with the cell tracker dye CM7000 (Molecular Probes), which is taken up by lysosomes, to determine potential co-localization with the CLIC4-positive large vesicles, but again this was not detected (data not shown). Currently, we do not understand the nature of these CLIC4-positive vesicles. It has been reported previously that CLIC4 can be localized to a complex containing actin isolated from rat brain cytosol (18). We could not detect co-localization of CLIC4 and actin stress fibers or focal adhesions in endothelial cells (data not shown).

CLIC4 has been shown to be expressed in many different cell types in vivo. A role for CLIC4 in endothelial cell function has not been reported before, and we therefore analyzed expression of CLIC4 in different types of vessels. By immunostaining, we showed that CLIC4 is expressed at high levels in normal resting kidney vessels, in developing vessel structures in embryoid bodies, and in vessels engaged in pathological angiogenesis in growing fibrosarcomas. CLIC4 expression in fibrosarcoma cells showed a nonuniform expression pattern with low or no expression in certain vessels. Tumor vessels undergo constant rearrangements where vessels form and dissolve; therefore, all stages in vessel formation may be represented. In conclusion, our data show that CLIC4 is expressed at relatively high levels in resting vessels in vivo but that pathological angiogenesis that occurs in tumors may be accompanied by down-regulation of CLIC4. This would be in accordance with the decreased expression of CLIC4 recorded during endothelial cell tube morphogenesis.

To test the function of CLIC4 in endothelial cell tube formation, an antisense construct was introduced by transient transfection. This resulted in a severe loss in tube-forming capacity. Generally, the antisense expressing cells spread out on collagen but did not migrate and fuse to the same extent as control cells. Data in excellent agreement were obtained when testing the effects of two independent CLIC4-spe-
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cific siRNAs, which also suppressed the tube-forming ability of the endothelial cells (Supplemental Material). Given the high degree of homology between the different CLIC family members, we cannot exclude that the effects of the antisense was exerted through a block also of other CLIC family members (43); however, the siRNAs specifically target CLIC4 (data not shown). We also attempted to overexpress CLIC4 in endothelial cells, but consistently, only very modest levels of overexpression were achieved (data not shown). Thus, our data indicate a need for strict regulation of CLIC4 expression levels during the formation of the vascular tube. CLIC4 has been implicated in apoptosis, and long term suppression of CLIC family proteins may induce apoptosis in cancer cells (43). On the other hand, short term CLIC suppression (similar to the approach used in this study) counteracted p53-dependent apoptosis (19). Moreover, introduction of CLIC4 antisense did not induce apoptosis in proliferating endothelial cell cultures on fibronectin (data not shown). The role of CLIC4 in vessel formation is therefore likely not dependent on apoptosis. Instead, we favor the hypothesis that there is a direct evolutionarily conserved role for CLIC proteins in the architecture of tubular structures, possibly dependent on their chloride channel function. CLIC4 could potentially be involved in fusion of pinocytotic vesicles into larger vacuoles that eventually form the new lumen (44).

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