Role of MARCKS in regulating endothelial cell proliferation

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Zhao, Ying, Bonnie S. Neltner, and Harold W. Davis. Role of MARCKS in regulating endothelial cell proliferation. Am J Physiol Cell Physiol 279: C1611–C1620, 2000.— Myristoylated alanine-rich C kinase substrate (MARCKS), as a specific protein kinase C (PKC) substrate, mediates PKC signaling through its phosphorylation and subsequent modification of its association with filamentous actin (F-actin) and calmodulin (CaM). PKC has long been implicated in cell proliferation, and recent studies have suggested that MARCKS may function as a cell growth suppressor. Therefore, in the present study, we investigated MARCKS protein expression, distribution, and phosphorylation in preconfluent and confluent bovine pulmonary microvascular endothelial cells (BPMEC) in the presence or absence of the vascular endothelial growth factor (VEGF). In addition, we examined functional alterations of MARCKS in these cells by studying the association of MARCKS with F-actin and CaM-dependent myosin light chain (MLC) phosphorylation. Our results indicate that MARCKS protein is downregulated during BPMEC proliferation. Decreased MARCKS association with F-actin, increased actin polymerization, and CaM-dependent MLC phosphorylation appear to mediate cell shape changes and motility during BPMEC growth. In contrast, VEGF stimulated MARCKS phosphorylation without alteration of protein expression during BPMEC proliferation, which may result in reduced interaction between MARCKS and actin or CaM, leading to actin reorganization and MLC phosphorylation. Our data suggest a regulatory role of MARCKS during endothelial cell proliferation.

myristoylated alanine-rich C kinase substrate; vascular endothelial growth factor; actin; myosin light chain phosphorylation

ANGIOGENESIS, AN ESSENTIAL PROCESS of blood vessel formation, is necessary for the normal growth and development of tissues but is limited in the adult to reproduction and wound repair (13). Uncontrolled proliferation of endothelial cells is found in pathological processes of vascularization such as atherosclerosis and malignancies (39). Given the physiological and pathological importance of endothelial cell growth, several potential regulators of proliferation have been identified, among which are several growth factors. Vascular endothelial growth factor (VEGF) is unique by virtue of its direct and specific effects on endothelial cells, leading to cell proliferation and blood vessel formation (12). However, the signal transduction pathways mediating VEGF-induced cell proliferation remain unclear.

Nevertheless, activation of protein kinase C (PKC) has been demonstrated to play a vital role in cell proliferation (45). One of the most prominent intracellular substrates for PKC is myristoylated alanine-rich C kinase substrate (MARCKS), which is distributed in almost all cell types that have been investigated, including vascular endothelial cells (2, 51). Growing evidence has suggested a role for MARCKS in regulating cell proliferation. The concentration of MARCKS is attenuated in several transformed cell lines (5, 29, 31, 47). Furthermore, the level of MARCKS is low in some cell lines when they are actively proliferating but sharply increases when they stop dividing (18). This downregulation of MARCKS during proliferation appears to occur via both PKC-dependent and -independent pathways (6). Importantly, B16 cells transfected with the MARCS gene in the sense orientation grow significantly more slowly than those transfected with antisense cDNA (4). Finally, Wada et al. (46) demonstrated that myristoylation of MARCKS (an indication of MARCKS expression) increased with increasing density of C3H10T1/2 fibroblasts and suggested that MARCKS may be involved in the signaling associated with contact inhibition.

Alternatively, phosphorylation of MARCKS may influence cell growth characteristics because some tumorigenic cell lines have highly elevated basal levels of phosphorylated MARCKS (48). Stanimirovic et al. (36) demonstrated that serum-free medium conditioned by rat cortical astrocytes accelerated DNA synthesis concomitantly with an induction of MARCKS phosphorylation in rat cerebromicrovascular endothelial cells, while inhibition of PKC resulted in the loss of both MARCKS phosphorylation and DNA synthesis. In addition to PKC, mitogen-activated protein (MAP) kinase is involved in the phosphorylation of MARCKS (42). We have previously shown that several mitogenic agents such as thrombin, phorbol 12-myristate 13-acetate (PMA) (51), or lipopolysaccharide (Zhao and Davis, unpublished observations) induce the phosphorylation of MARCKS in bovine pulmonary vascular endothelial cells (BPMEC) and that both PKC and MAP kinases are involved in these phosphorylations.

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Although the biochemical and molecular characteristics of MARCKS have been studied extensively, the precise function of the protein remains unknown. In many cell types, MARCKS is targeted to the plasma membrane by an NH2-terminal myristoyl group and a basic 25-amino acid domain (PKC phosphorylation site domain; PSD). Phosphorylation by PKC results in the release of MARCKS to the cytosol (38). In addition, MARCKS cross-links filamentous actin (F-actin) and binds calmodulin (CaM) in a manner regulated by PKC-dependent phosphorylation (15, 16). Since the CaM- and actin-binding sites of MARCKS are located in the PSD, phosphorylation, actin cross-linking, and CaM binding are mutually exclusive events. Therefore, MARCKS may be involved in cell growth via mediating actin- and CaM-dependent cell functions, such as motility and contraction. The release of CaM may be especially important since CaM is involved in many aspects of cell cycle regulation (23). Indeed, Herget et al. (17) demonstrated that overexpression of MARCKS in Rat-1 cells increased the sensitivity of CaM-dependent DNA synthesis to CaM antagonists.

Because a direct role for MARCKS in regulating endothelial cell proliferation remains to be demonstrated, we investigated MARCKS expression, distribution, and phosphorylation in preconfluent and confluent bovine pulmonary microvascular endothelial cells (BPMEC). These cells were chosen since they respond better to VEGF than BPAEC and, unlike BPAEC, they undergo tube formation on Matrigel (an in vitro angiogenesis assay). In addition, we examined the functional alterations of MARCKS in these cells by studying CaM-dependent myosin light chain (MLC) phosphorylation and the association of MARCKS with F-actin.

METHODS

Reagents. Recombinant human VEGF (isoform 165) was purchased from R&D Systems (Minneapolis, MN). A polyclonal antibody to recombinant MARCKS was raised in rabbits by traditional methods. Recombinant, tagged MARCKS was prepared with the pPROEX-1 expression vector (GIBCO BRL, Gaithersburg, MD). The MARCKS coding sequence from pBS80K2A (provided by Drs. Perry Blackshear and Deborah Stumpo, Duke Univ. School of Medicine) was removed with Alu I, blunted, and ligated into the blunt-ended EcoR I site of pPROEX-1. BL-21 Escherichia coli were transformed with this vector, and MARCKS was purified according to the manufacturer’s instructions, with the exception that a subsequent step of CaM agarose chromatography was employed.

Endothelial cell culture and proliferation experimental design. BPMEC cultures (Vec Technologies, Rensselaer, NY) were maintained at 37°C in a humidified atmosphere of 5% CO2-95% air in Dulbecco’s modified Eagle’s medium (DMEM; GIBCO BRL, Grand Island, NY) supplemented with 10% (vol/vol) fetal bovine serum (Irvine Scientific, Santa Ana, CA), 1% antibiotic and antimycotic (penicillin, 10,000 U/ml; streptomycin, 10 μg/ml; and amphotericin B, 25 μg/ml; GIBCO), and 0.1 mM nonessential amino acids (GIBCO). The BPMEC grew to contact-inhibited monolayers with typical cobblestone morphology and were then passaged from each primary 75-cm2 flask into 25-cm2 flasks by trypsinization (0.05% trypsin/0.02% EDTA) and resuspended in fresh culture medium. These cells were examined at two stages of proliferation: 1) preconfluence (actively proliferating, at day 3 after plating), and 2) confluence (contact inhibition with “cobblestone” morphology at day 7 after plating). In some experiments, we treated these cells with 100 ng/ml VEGF for either short (5 min) or long (24 h) periods of time. The cells were incubated and treated in a low-serum (0.5%) DMEM medium for 48 h before termination of the experiments (i.e., for the long VEGF exposure, the cells were incubated in low serum for 24 h and then treated with VEGF for another 24 h in low-serum medium; for the control and short VEGF exposure, the cells were incubated for 48 h in the low-serum medium). Images of the cells were obtained with an Olympus inverted, phase-contrast microscope. Magnification was ×40. [3H]thymidine incorporation was carried out on both preconfluent (day 3) and confluent (day 7) endothelial cells. The cells were treated as above for the VEGF incubations except that 4 h before the termination of the experiment, 10 μCi of [3H]thymidine was added to the cells. After the thymidine labeling, the cells were washed three times with phosphate-buffered saline (PBS) and incubated in 10% ice-cold trichloroacetic acid (TCA) for 15 min to precipitate the nuclear thymidine. The cells were then washed three times with water, lysed with 1 N NaOH for 30 min at 37°C, and neutralized with an equal volume of 1 N acetic acid. Scintillation fluid was added, and the lysates were counted in a beta counter.

MARCKS expression. MARCKS protein levels in BPMEC were determined by immunoblotting at both preconfluent and confluent stages in the presence or absence of VEGF. Briefly, the cultures were washed in ice-cold PBS and harvested in 0.5 ml of MARCKS extraction buffer (25 mM Tris-HCl, pH 7.4, 5 mM EDTA, 100 mM sodium fluoride, 2 mM sodium vanadate, 10 mM sodium pyrophosphate, and 1% Igepal CA-630). After cell debris was spun down, equal quantities of protein were boiled for 5 min, and the heat-labile proteins were pelleted by centrifugation for 5 min at 4°C (MARCKS is a heat-stable protein). Supernatant proteins were separated by SDS-PAGE and transferred to nitrocellulose, and MARCKS was detected by the MARCKS antibody and quantified by densitometry.

MARCKS phosphorylation. BPMEC cultured on T-25 flasks at either preconfluent or confluent stages were loaded with 32PO4 (0.1 mCi/ml) for 7–8 h in the presence or absence of VEGF. Preliminary studies indicated that uptake of labeled PO4 reached equilibrium in ~6 h in both proliferating and confluent BPMEC. Cells were lysed with the MARCKS extraction buffer, and equal amounts of protein were immunoprecipitated with the MARCKS antibody. After isolation by SDS-PAGE and transfer to nitrocellulose, MARCKS phosphorylation was assessed by scanning densitometry of the autoradiograms and compared with scans of the immunoblots. Phosphorylated MARCKS was normalized to the quantity of MARCKS protein and expressed as a percentage of control (no treatment).

MARCKS translocation between membrane and cytosol. MARCKS translocation from membrane to cytosol was measured by ultracentrifugation of cell homogenates to separate the cytosolic and membrane fractions, followed by SDS-PAGE and immunoblotting as described previously (37). Briefly, preconfluent or confluent cells were scraped into a buffer that contained 20 mM Tris, pH 7.4, 1 mM EDTA, 330 mM sucrose, 100 mM sodium fluoride, 2 mM sodium orthovanadate, 10 mM sodium pyrophosphate, and 0.5 mM phenylmethylsulfonyl fluoride and were sonicated. The cell homogenates were subjected to ultracentrifugation to separate the cytosolic and membrane fractions, followed by SDS-PAGE and transfer to nitrocellulose, and MARCKS was detected by the MARCKS antibody and quantified by densitometry.
fraction) were dissolved in SDS-PAGE sample buffer. The supernatants (cytosolic fraction) were collected, and proteins were precipitated with 10% TCA and also dissolved in SDS-PAGE sample buffer. Both membrane and cytosol samples were run on SDS-PAGE, transferred to nitrocellulose, and probed with the MARCKS antibody. Membrane-associated and cytosolic MARCKS were quantified by densitometry and expressed as a percentage of total MARCKS protein.

**Actin polymerization and MARCKS association with the actin cytoskeleton.** A Triton X-100 extraction assay (20) was used to determine actin polymerization in preconfluent and confluent BPMEC in the presence or absence of VEGF stimulation. F-actin in nonmuscle cells is known to be resistant to Triton solubilization, whereas nonfilamentous actin (globular actin) is soluble in Triton (3). We can, therefore, monitor the recruitment of proteins to fortify the cytoskeleton. BPMEC were lysed with an extraction buffer (pH 7.4) consisting of 1% Triton X-100, 20 mM Tris, 5 mM EGTA, 20 mM sodium fluoride, 25 mM sodium pyrophosphate, 10 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 0.002% leupeptin, and 0.002% aprotinin. Both soluble and insoluble fractions were collected by centrifugation at 14,000 g at 4°C, and the proteins were separated by 10% SDS-PAGE. Some of the sample was stained with Coomassie blue to detect actin, and some was transferred to nitrocellulose and probed with the MARCKS antibody. Actin is denatured during this procedure, so only monomers are detected. The quantities of actin and MARCKS were determined by densitometry. Data are expressed as a percentage of total actin or MARCKS in the Triton-insoluble fraction.

**MLC phosphorylation.** The BPMEC in both preconfluent and confluent conditions, as described in Endothelial cell culture and proliferation experimental design, were terminated with 10% TCA, scraped into tubes, and centrifuged. We measured MLC phosphorylation by the method of Persechini et al. (30). Briefly, the pellet was solubilized in urea gel sample buffer and run on urea-glycerol gels. These gels separated native MLC from mono- and diphosphorylated MLC. The proteins were then transferred to nitrocellulose and blotted with an MLC antibody (provided by Dr. C. S. Packer, Indiana Univ. School of Medicine, Indianapolis, IN). Phosphorylation was quantified by scanning densitometry and expressed as a percentage of total MLCs.

**Other methods.** The total cellular protein concentrations of BPMEC cultured on T-25 flasks were measured with the Pierce bicinchoninic acid protein assay and used as an index...
for cell growth. SDS-PAGE was conducted as described by Laemmli (22). The method of Towbin et al. (44) was used for protein transfer to nitrocellulose and immunoblotting. The nitrocellulose sheets were probed by using a primary antibody to the specific protein and an appropriate secondary antibody conjugated to horseradish peroxidase. Results were expressed as means \( \pm \) SE. The number of observations for each condition ranged from three to eight, and duplicate samples were conducted in each experiment. Student’s t-test was used to compare the difference between two groups, and one-way ANOVA followed by t-tests were used to examine the differences between more than two groups. A significant difference was considered for \( P \leq 0.05 \).

RESULTS

Endothelial cells will grow to form a monolayer on a matrix. However, cell division and proliferation normally stop on contact with other cells. A morphological study on the monolayers of preconfluent and confluent BPMEC is shown in Fig. 1. The preconfluent cells (Fig. 1A, day 3 after plating) had lower cell density and a different cell shape (larger and spread) compared with those of confluent, cobblestone cells (Fig. 1D, day 7 after plating). VEGF (100 ng/ml) treatment for 5 min had no effect on cell density or shape in either preconfluent or confluent cells (Fig. 1B and E, respectively), but long-term (24 h) exposure to VEGF induced a striking change in cell shape, resulting in cells with long, thin protrusions under both preconfluent (Fig 1C) and confluent (Fig. 1F) conditions.

The results shown in Table 1 indicate that incorporation of [3H]thymidine into endothelial cells was \( \sim 5 \) times greater in preconfluent cells compared with confluent cells. As expected, treatment of the cells for 5 min with VEGF had no affect on thymidine incorporation into preconfluent cells. However, a modest increase in thymidine incorporation into confluent cells was detected after 5 min with VEGF incubation. Treatment of either preconfluent or confluent cells with VEGF for 24 h resulted in near doubling of thymidine incorporation, suggesting that VEGF induces proliferation even in confluent endothelial cells. These data were confirmed by cell counting and protein concentration; the flasks bearing cells at 3 days of growth con-

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Values are means \( \pm \) SE. Cells in T-25 flasks were incubated with 0.5% fetal calf serum for 24 h and then incubated for another 24 h in the presence or absence of vascular endothelial growth factor (VEGF). [3H]thymidine (10 \( \mu \)Ci) was added for the final 4 h and VEGF was added to some flasks for 5 min before the termination of the experiment, as described in METHODS. *Significantly different from control for that day, \( P \leq 0.5 \); †significantly different from day 3, \( P \leq 0.5 \).
tained only one-half the protein concentration and number of cells as those that contained confluent cells (after 7 days of growth; data not shown). Cells under these proliferation conditions were used in the present study to explore the role of MARCKS in endothelial cell growth.

MARCKS protein expression in preconfluent and confluent BPMEC is shown in Fig. 2. Proliferating cells expressed only 70% of the MARCKS per milligram of protein as confluent cells, suggesting downregulation of MARCKS during proliferation. Treatment with VEGF for either 5 min or 24 h increased MARCKS protein levels in proliferating BPMEC (to 133 and 126% of control, respectively). On the other hand, VEGF had little effect on MARCKS protein expression in confluent cells but did not affect the phosphorylation in preconfluent BPMEC. Representative autoradiographs are shown above the bar graphs.

Figure 3 shows the effects of VEGF on MARCKS phosphorylation in both preconfluent and confluent BPMEC. Although VEGF induced an increase in MARCKS protein levels in preconfluent cells, it did not alter the phosphorylation levels of MARCKS. In contrast, an increase in MARCKS phosphorylation was observed in confluent cells treated with VEGF for either 5 min or 24 h (148 and 179% of control, respectively). These results, shown in Fig. 2B and Fig. 3, suggest that VEGF regulates MARCKS function differently at distinct stages of proliferation.

Under certain conditions in some cell types, MARCKS is translocated from the cell membrane to the cytosol on phosphorylation (40). We have observed translocation with PMA but not with thrombin (51) or lipopolysaccharide (Zhao and Davis, unpublished observations). The results from the present study indicate that neither proliferation nor VEGF treatment resulted in significant translocation of MARCKS (Fig. 4), despite VEGF-induced MARCKS phosphorylation in confluent cells. These data suggest that translocation of MARCKS is not involved in endothelial cell proliferation.

F-actin within nonmuscle cells is resistant to Triton solubilization, whereas globular (nonfilamentous) actin is Triton soluble (3). Alterations in Triton-insoluble actin, thus, represent the amount of F-actin assembled into the cytoskeleton. The changes of MARCKS and F-actin in the cytoskeleton are shown in Fig. 5. The results demonstrate that preconfluent BPMEC contain higher levels of F-actin (Fig. 5B) but lower quantities of MARCKS associated with this F-actin (Fig. 5A). Treatment with VEGF for 24 h increased the amount of MARCKS observed in confluent cells treated with VEGF for either 5 min or 24 h (148 and 179% of control, respectively). These results, shown in Fig. 2B and Fig. 3, suggest that VEGF regulates MARCKS function differently at distinct stages of proliferation.

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MARCKS in the cytoskeleton without alteration of F-actin levels in the preconfluent cells. In contrast, VEGF reduced the abundance of F-actin but slightly increased the quantity of MARCKS associated with the cytoskeleton in confluent cells. Since there were changes in both the F-actin and MARCKS contents in the Triton pellet, we examined the MARCKS/F-actin ratio (an indication of the association of MARCKS with F-actin; Fig. 5C). These data indicate a substantial increase in MARCKS association with F-actin when the BPMEC became confluent and when confluent cells were treated with VEGF. A similar increase in the MARCKS/F-actin ratio was observed when preconfluent cells were treated with VEGF for 24 h.

Calcium-CaM-dependent phosphorylation of MLCs in BPMEC proliferation was also studied; the results are shown in Fig. 6. Like other agonists, VEGF had no effect on monophosphorylation of MLCs, and the proliferative state of the cells did not alter this (data not shown). However, we have previously demonstrated that diphosphorylation (and not monophosphorylation) is associated with endothelial cell contraction (14). Preconfluent cells had higher levels of diphosphorylated MLCs compared with confluent cells (28 vs. 14% of total MLCs). VEGF did not alter MLC phosphorylation in preconfluent cells but stimulated diphosphorylation in confluent cells when treated for 24 h.
preconfluent and confluent cells; *P < 0.05, compared between preconfluent and confluent cells; \(+P < 0.05\), compared with the control (no treatment) cells.

**DISCUSSION**

The present study was performed on BPMEC under different proliferation conditions: 1) preconfluent, 2) confluent (nonproliferating), and 3) VEGF-induced proliferation. These conditions were determined by the morphological study on BPMEC monolayers and thymidine incorporation study.

In this study, we have demonstrated reduced MARCKS protein levels in preconfluent endothelial cells, which is consistent with other studies on nonendothelial cells (transformed cells or tumor cells), suggesting an inverse correlation between MARCKS protein expression and cell growth. Elevation of MARCKS levels correlates with cessation of growth in Swiss 3T3 cells (18), whereas downregulation of MARCKS appears to accompany the conversion of transformed cells to a tumorigenic state (6). Importantly, cells transfected with the MARCKS gene in sense or antisense orientation showed that sense clones grew significantly more slowly than antisense clones (4). Manenti et al. (24) also reported downregulation of MARCKS protein in melanoma cells and decreased cell proliferation rate in melanocytes transfected with MARCKS. The results of several studies have suggested that two mechanisms may be involved in downregulating MARCKS protein during cell proliferation: 1) decreased MARCKS mRNA levels, because Joseph et al. (21) reported that MARCKS is transcriptionally downregulated in v-Src-transformed 3T3 cells, and 2) increased proteolytic cleavage of MARCKS protein. A specific proteolytic cleavage of MARCKS between Asn-147 and Glu-148 occurs in various tissue extracts, and inhibition of this protease activity results in an increase in the cellular concentration of MARCKS (25, 35).

The growth effects of VEGF have been demonstrated in various types of endothelial cells. Although VEGF promotes proliferation in confluent cells, VEGF-induced proliferation affects MARCKS protein expression differently than does nonstimulated proliferation, which results in downregulation of MARCKS. First, in preconfluent endothelial cells that are proliferating and have lower levels of MARCKS, VEGF increased MARCKS expression without MARCKS phosphorylation. Second, in VEGF-induced proliferating endothelial cells from confluent conditions (with higher levels of MARCKS protein), VEGF did not alter the level of MARCKS protein (failed to reduce the MARCKS protein level) but increased MARCKS phosphorylation. These results suggest that the effects of VEGF on MARCKS protein expression did not correlate with its function on endothelial cell proliferation and, therefore, did not mediate VEGF-induced proliferation. Addition of VEGF to preconfluent cells may reverse the downregulation of MARCKS protein induced by proliferation.

As mentioned earlier, MARCKS is a CaM- and actin-binding protein. CaM plays a critical role in all eukaryotic cell proliferation (23), and rearrangement of the actin cytoskeleton has been shown to be necessary for angiogenesis (19), which requires endothelial cell proliferation. Therefore, the increase in MARCKS may attenuate further proliferation by stabilizing the actin cytoskeleton or by binding up CaM. On the other hand, by inducing phosphorylation of MARCKS, VEGF may allow actin rearrangement or release of CaM to overcome contact inhibition and stimulate proliferation of confluent cells.

The increased MARCKS expression induced by a 5-min treatment with VEGF could be explained by inhibition of proteolytic cleavage of MARCKS. Spizz and Blackshear (35) reported that the cleaved fragments of MARCKS were present in significant amounts in quiescent fibroblasts and that the abundance of intact MARCKS increased within 15 s of treatment with serum. They also demonstrated that phosphorylated MARCKS was a poor substrate for this protease, whereas the nonphosphorylated form was a good substrate. Therefore, the rapid increase in MARCKS quantity induced by VEGF may be due to the inhibition of protease cleavage of MARCKS.

We have previously reported that PKC and MAP kinase may be involved in MARCKS phosphorylation in BPAEC in response to various agonists (Ref. 51; Zhao and Davis, unpublished observations). MARCKS is a well-known PKC substrate (indeed, its name was derived from this attribute), but a number of researchers have now demonstrated MAP kinase-induced phosphorylation of MARCKS in a variety of cells (33, 42, 49). VEGF-induced MARCKS phosphorylation may also result from the activation of PKC and MAP kinases, because VEGF is known to activate both of these families of kinases (11, 27, 34, 41, 50). In addition, these kinases may be at different states of activation in preconfluent and confluent cells. In fact, as we demonstrated, VEGF-induced MARCKS phosphorylation was dependent on endothelial cell density. In preconfluent cells, VEGF did not alter MARCKS phosphorylation levels but increased the protein levels, while VEGF...
induced MARCKS phosphorylation in confluent cells with no change of protein levels. Other growth factors such as platelet-derived growth factor (6) and basic fibroblast growth factor (10) also cause a rapid enhancement of MARCKS phosphorylation levels. The reason for failure of VEGF induction of MARCKS phosphorylation in proliferating endothelial cells may be due to the already high basal levels of PKC and MAP kinase activities during cell proliferation. Indeed, these high basal levels of phosphorylated MARCKS may contribute to the high rate of proliferation observed in the preconfluent cells since more free CaM and actin would be available.

MARCKS reportedly is translocated from the cell membrane to the cytosol on phosphorylation (26). Non-phosphorylated MARCKS is associated with the cell membrane and targeted to the membrane by an NH2-terminal myristoyl group and a basic 25-amino acid domain (PSD). Phosphorylation of MARCKS in the PSD has been shown to be accompanied by its translocation from the membrane to the cytosol (43). When MARCKS is dephosphorylated, it returns to the membrane where it can, once again, cross-link actin. However, in the present study, we did not detect significant translocation of MARCKS in preconfluent BPAEC or in response to VEGF treatment, indicating that MARCKS translocation between membrane and cytosol does not contribute to the actin reorganization and the changes in BPAEC morphology. Our previous studies showed that PMA, but not thrombin or lipopolysaccharide, caused MARCKS translocation from membrane to cytosol in BPAEC (51). Some recent studies have also suggested that phosphorylation of MARCKS by PKC does not correlate well with MARCKS translocation (7, 40).

Mechanical forces of endothelial cells regulate angiogenesis (19), and these forces are often similar to those observed in endothelial cell contraction, which enhances permeability through the endothelial monolayer. In fact, hyperpermeability of the endothelium precedes angiogenesis (19). Contraction is initiated by phosphorylation of the 20-kDa regulatory MLC by the CaM-dependent MLC kinase, which is required for actin-induced activation of myosin ATPase and tension development (8, 9). We have demonstrated that MLC phosphorylation is strongly correlated with the increased permeability of endothelial monolayers and that this requires activation of PKC (14). Therefore, since MARCKS is an actin- and CaM-binding protein, the alterations of MARCKS function were evaluated in this study by investigation of the association of MARCKS with F-actin and the CaM-dependent phosphorylation of MLCs, both of which have been implicated in endothelial barrier integrity, cell motility, and contraction. Therefore, they may play a role in cell proliferation. Importantly, Bachs et al. (1) have shown that MLC kinase is elevated in the nucleus of hepatocytes during proliferation and is necessary for cytokinesis. Since the binding of MARCKS with F-actin and CaM is regulated by PKC phosphorylation of MARCKS, changes of MARCKS phosphorylation levels or the abundance of MARCKS protein could affect either actin organization or CaM-dependent enzymatic activity. Our results indicate a significant decrease in the MARCKS to F-actin ratio in preconfluent compared with confluent cells that was due to both a decreased quantity of MARCKS and an increased amount of F-actin in cytoskeletal fraction. The reduced MARCKS in the actin cytoskeleton may allow for an increase in cytoskeletal actin, and the increased polymerized actin may contribute to cell shape change required for cell proliferation. On the other hand, increased free CaM due to the reduced MARCKS levels in preconfluent cells may result in activation of MLC kinase, as reported here, or the activation of other CaM-binding proteins involved in cell activation. In support of this, it has been shown that overexpression of MARCKS in Rat-1 cells increased their sensitivity to the CaM antagonists W-7 and trifluoperazine, resulting in a decrease in DNA synthesis (17).

When added to preconfluent endothelial cells, VEGF did not significantly affect MLC phosphorylation. This may be related to the lack of change in MARCKS phosphorylation. However, there was an increased MARCKS/F-actin ratio (due to VEGF-induced actin depolymerization) and MLC phosphorylation levels in VEGF-induced proliferating cells from the confluent cells. Phosphorylation of MARCKS in confluent cells treated with VEGF for 24 h may allow the increase in MLC phosphorylation (or other CaM-dependent events) via the release of CaM from MARCKS, and this increase in CaM-dependent activity may regulate increased proliferation. These results indicate that changes in MARCKS association with F-actin, actin reorganization, and MLC phosphorylation are different between preconfluent proliferating endothelial cells and VEGF-induced proliferating cells.

In summary, our studies have demonstrated the downregulation of MARCKS protein during endothelial cell proliferation. The lower levels of MARCKS protein in preconfluent endothelial cells support the idea that expression of MARCKS protein correlates with cell growth. Decreased MARCKS association with F-actin and increased actin polymerization and CaM-dependent activation of MLC or other enzymes may play an important role in cell shape changes and motility during endothelial cell growth. In contrast, VEGF stimulated phosphorylation of MARCKS without alteration of MARCKS protein expression in confluent cells, which may play a role in VEGF-induced endothelial cell proliferation via interactions between MARCKS and actin or CaM, leading to the actin reorganization and MLC phosphorylation and cell contraction. It appears that either decreased MARCKS protein expression or increased MARCKS phosphorylation or both may contribute to endothelial cell proliferation. Our results suggest a regulatory role of MARCKS in the signal transduction pathways leading to endothelial cell growth.

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