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Role of MSK1 in the Signaling Pathway Leading to VEGF-Mediated PAF Synthesis in Endothelial Cells

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Abstract Vascular endothelial growth factor (VEGF) inflammatory effects require acute platelet-activating factor (PAF) synthesis by endothelial cells (EC). We previously reported that VEGF-mediated PAF synthesis involves the activation of VEGF receptor-2/Neuropilin-1 complex, which is leading to the activation of p38 and p42/44 mitogen-activated protein kinases (MAPKs) and group V secretory phospholipase A_2 ($sPLA_2$-V). As the mechanisms regulating $sPLA_2$-V remain unknown, we addressed the role of the mitogen- and stress-activated protein kinase-1 (MSK1), which can be rapidly and transiently activated by p38 or p42/44 MAPKs. In native bovine aortic endothelial cells (BAEC), we observed a constitutive protein interaction of MSK1 with p38, p42/44 MAPKs, and $sPLA_2$-V. These protein interactions were maintained in BAEC transfected either with the empty vector pCDNA3.1, wild-type MSK1 (MSK1-WT) or N-terminal dead kinase MSK1 mutant (MSK1-D195A). However, in BAEC expressing C-terminal dead kinase MSK1 mutant (MSK1-D565A), the interaction between MSK1 and $sPLA_2$-V was reduced by 82% and 90% under basal and VEGF-treated conditions as compared to native BAEC. Treatment with VEGF for 15 min increased basal PAF synthesis in native BAEC, pCDNA3.1, MSK1-WT, and MSK1-D195A by 166%, 139%, 125%, and 82%, respectively. In contrast, PAF synthesis was prevented in cells expressing MSK1-D565A mutant. These results demonstrate the essential role of the C-terminal domain of MSK1 for its constitutive interaction with $sPLA_2$-V, which appears essential to support VEGF-mediated PAF synthesis. J. Cell. Biochem. 98: 1095–1105, 2006.

Key words: MSK1; VEGF; PAF; $sPLA_2$-V; inflammation; angiogenesis

Pathological angiogenesis is associated with many inflammatory conditions such as atherosclerosis, cancer, rheumatoid arthritis, psoriasis, and proliferative retinopathies [Neufeld et al., 1999]. Different studies have shown that inflammation exists in a mutually dependent association with angiogenesis [Forsythe et al., 1996; Ferrara and Davis-Smyth, 1997]. Vascular endothelial growth factor (VEGF) is one of the most potent angiogenic molecule displaying strong inflammatory properties [Connolly et al., 1989; Dvorak et al., 1995]. We have previously demonstrated that the acute increase in vascular permeability produced by VEGF is mediated through the synthesis of a powerful phospholipid inflammatory mediator, platelet-activating factor (PAF) by the endothelium [Sirois and Edelman, 1997]. Since then, it has been shown that PAF is involved in pathological angiogenesis and promotes VEGF angiogenic activity [Bussolino and Camussi, 1995; Montrucchio et al., 2000]. PAF is synthesized either by the remodeling or by the de novo pathway. The remodeling pathway is the principal mechanism leading to PAF synthesis when endothelial cells (EC) are stimulated by proinflammatory mediators. Briefly, membrane phospholipids are hydrolyzed and converted into lyso-PAF by phospholipase A_2 (PLA_2) and subsequently acetylated by acetyl-CoA: lyso-PAF acetyltransferase (lyso-PAF AT) into PAF [Bussolino and Camussi, 1995; Snyder et al., 1996].

Ongoing studies in our laboratory have investigated the cell signaling mechanisms leading to endothelial PAF synthesis following VEGF stimulation. We reported that activation of VEGF receptor-2/neuropilin-1 complex

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(VEGFR-2/NRP1) by VEGF promotes the intracellular activation of both p38 and p42/44 mitogen-activated protein kinases (MAPKs), as well as group V secretory phospholipase A_2 ($sPLA_2$-V), which together, are essential to endothelial PAF synthesis [Bernatchez et al., 1999; Bernatchez et al., 2001a,b; Bernatchez et al., 2002]. However, the intracellular mechanism following the activation of both p38 and p42/44 MAPK pathways and the relation with the newly identified $sPLA_2$-V are unknown. To further elucidate the interactions between these three key enzymes, we sought to investigate the role of a kinase downstream to the p38 and p42/44 MAPK pathways: The mitogen- and stress-activated protein kinase-1 (MSK1).

As implied by its name, MSK1 is activated by a variety of extracellular signals including phorbol esters and growth factors through p42/44 MAPK activation, and by stimuli such as UV radiation, and proinflammatory cytokines through p38 MAPK activation [Deak et al., 1998; Dunn et al., 2005]. MSK1 belongs to the RSK protein kinase family, characterized by two kinase domains, joined by a short linker region, in a single polypeptide [Deak et al., 1998; Pierrat et al., 1998]. The N-terminal kinase domain of MSK1 is similar to that of members of the AGC family of protein kinases (PK) including PKA, PKC, and PKG, whereas the C-terminal kinase domain is homologous to the calmodulin-activated protein kinase family [Frodin et al., 2002]. This particular protein structure implies a complex regulation occurring in a sequential manner, which is a rate-limiting step. MSK1 is regulated by multiple phosphorylation sites and its activation requires a specific docking site for p38 and p42/44 MAPKs localized at the C-terminal end [McCoy et al., 2005]. Depending on the agonist, activation of p38 or p42/44 MAPKs leads to a first series of MSK1 phosphorylation, activating the C-terminal kinase domain and leading to subsequent autophosphorylation of N-terminal kinase domain [Dunn et al., 2005; McCoy et al., 2005]. Together, these events allow the full catalytic activity of MSK1 needed for downstream substrate phosphorylation. In addition, since MSK1 can be activated upon stimulation of EC with VEGF [Mayo et al., 2001], and that MSK1 can promote the phosphorylation of cytosolic phospholipase A_2 ($cPLA_2$) [Hefner et al., 2000], we were led to suggest that MSK1 might be involved in VEGF-mediated PAF synthesis.

In this study, we investigated the role of MSK1 in the signal transduction pathway leading to endothelial PAF synthesis induced by stimulation with VEGF. We report a constitutive protein interaction between MSK1 and $sPLA_2$-V, which appears to be specific to the C-terminal kinase domain of MSK1 and essential to VEGF-mediated PAF synthesis.

MATERIALS AND METHODS

Chemicals

All chemicals were purchased from Sigma (St. Louis, MO) or J.T. Baker (Philipsburg, NJ). Human VEGF-A_{165} (referred as VEGF) was purchased from PeproTech (Rocky Hill, NJ). SB203580 and PD98059 were purchased from Calbiochem (La Jolla, CA).

Cell Culture

Bovine aortic endothelial cells (BAEC) were isolated from freshly harvested aortas, cultured in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Pickering, ON, Canada) supplemented with 5% fetal bovine serum (FBS; Medicorp Inc., Montreal, QC, Canada), and 1% antibiotics, penicillin, and streptomycin (Invitrogen). BAEC were characterized by their cobblestone monolayer morphology and were maintained until passage 12.

Vector Constructions

cDNA encoding wild-type MSK1 (MSK1-WT), its N-terminal kinase-dead domain mutant (D195A) and the C-terminal kinase-dead domain mutant (D565A) cloned in pCMV5-FLAG were developed by Drs. Maria Deak and Dario Alessi [Deak et al., 1998] and purchased from University of Dundee, UK. Plasmids were purified and WT or mutants Flag-MSK1 cDNA fragments were recovered by EcoRI–XbaI digestion and subcloned into pCDNA3.1 vector (Invitrogen). Plasmids were amplified using the EndoFree plasmid Maxi kit as described by the manufacturer (Qiagen, Mississauga, ON, Canada).

Stable Transfections

BAEC were transfected either with the empty pCDNA3.1 plasmid, or with one of the expression vectors (MSK1-WT, MSK1-D195A, MSK1-D565A) using Lipofectin transfection reagent

(Invitrogen), under the conditions described by the manufacturer. Transfected cells having integrated the plasmid, which contains neomycin gene resistance, were selected upon a treatment with preestablished G418 concentration (500 μg/ml; Geneticin; Invitrogen), and the cells cloned by a limiting dilution method.

RT-PCR

Total RNAs were isolated using the RNeasy extraction kit (Qiagen). One microgram (μg) of total RNAs was reverse transcribed using random hexamers and the MMLV reverse transcriptase (Invitrogen) as described by the manufacturer. The PCR reactions were performed as follows: cDNAs were denatured (94°C for 5 min), submitted to 33 cycles of amplification (94°C for 45 s, primer annealing 50°C for 1 min, and 72°C for 1 min) and to a final elongation (72°C for 10 min). FLAG-MSK1 cDNA was amplified using a forward primer generated against the FLAG DYKDDDDK epitope (5′-GGACTACAAGGACGACGATGAC-3′) and a reverse primer specific for human MSK1 (5′-CCCCAACTTGTGGAGATGTTC-3′). Amplification of bovine β-actin was used as a positive control for cDNA quality using the following primers: Forward 5′-CTCGTGGTC-GACAACGGC-3′ and reverse 5′-CTTCTCAC-GGTTGGCCTTG-3′.

Immunoprecipitation and Western Blot Analysis of MSK1 Phosphorylation

Confluent BAEC (100 mm tissue culture plate) were serum starved for 18 h in DMEM, then stimulated with VEGF (1 nM) for various periods of time. In another set of experiments, BAEC were pretreated with p38 MAPK inhibitor (SB203580) or/and MEK inhibitor (PD98059), 20 min prior to stimulation with VEGF (1 nM, 15 min). After stimulation, cells were rinsed with ice-cold DMEM containing Na_3VO_4 (1 mM) and lysed with ice-cold RIPA lysis buffer (NP-40 1%, sodium phosphate 50 mM: pH 7.2, NaCl 150 mM, EDTA 1 mM, NaF 50 mM, SDS 0.1%, sodium deoxycholate 0.5%, PMSF 1 mM, benzamidine 10 mg/ml, leupeptin 5 mg/ml, trypsin inhibitor 5 mg/ml, and microcystin LR 1 mM). Cell lysate was clarified by centrifugation, and supernatant protein concentration determined using a protein assay kit (Bio-Rad, Hercules, CA). Total proteins (1.2 mg) were immunoprecipitated with a goat polyclonal antihuman MSK1 IgG (Santa Cruz Biotechnologies, Santa Cruz, CA) coupled to protein G-coated sepharose beads (Amersham Pharmacia Biotech, Upsala, Sweden), separated on a 10% SDS–PAGE and transblotted onto a PVDF membrane (Milipore, Bedford, MA). Membranes were blocked in TTBS containing 5% BSA for 1 h at room temperature, probed with three independent rabbit polyclonal antihuman phospho-MSK1 (Ser^{360}), (Ser^{376}) or (Thr^{581}) IgGs (dilution 1:500; New England Biolabs, Pickering, ON, Canada) overnight, and incubated with a secondary goat antirabbit IgG coupled to horseradish peroxidase. Afterward, membranes were stripped and reprobed with a rabbit polyclonal antihuman MSK1 IgG (dilution 1:1,000) to visualize corresponding total protein expression. Immunoreactive bands were visualized using LumiGlo™ (New England Biolabs). The density of the bands was determined using Quantity One software (Bio-Rad, Mississauga, ON, Canada).

Western Blot Analysis of MSK1-Interacting Proteins

Confluent native and transfected BAEC were treated as described above and stimulated with VEGF (1 nM, 15 min). MSK1 precipitation samples and SDS–PAGE were performed as described above to detect the proteins interacting with MSK1. The immunoblots were analyzed in parallel with the respective antibodies: Rabbit polyclonal antihuman MSK1 IgG (dilution 1:500; Sigma), rabbit polyclonal antimouse p38 MAPK IgG (1:1,000; Santa Cruz Biotechnologies), rabbit polyclonal antirat p42/44 MAPK IgG (1:1,000; New England Biolabs), and mouse monoclonal antihuman $sPLA_2$-V IgG (1:300; Cayman, Ann Arbor, MI). Blots were then treated with secondary peroxidase-conjugated goat antirabbit (1:5,000) or antimouse (1:1,000) IgGs.

Western Blot Analysis of CREB Phosphorylation

Confluent native and transfected BAEC were stimulated with VEGF (1 nM, 15 min). Supernatant proteins were prepared as described above and 75 μg of total proteins were used for Western blotting. The immunoblots were analyzed with a rabbit polyclonal antihuman phospho-CREB (Ser^{133}) IgG (dilution 1:500; New England Biolabs). Membranes were subsequently stripped and reprobed with a rabbit polyclonal antihuman CREB (dilution 1:1,000;

New England Biolabs) to visualize corresponding total protein expression.

Measurement of PAF Synthesis

PAF production in BAEC was measured by incorporation of ^{3}H-acetate into lyso-PAF as described previously [Sirois and Edelman, 1997; Bernatchez et al., 1999]. Briefly, confluent BAEC (6-well tissue culture plate) were rinsed, then stimulated for 15 min in 1 ml of HBSS/HEPES containing $CaCl_2$ (10 mM), ^{3}H-acetate (25 μCi) (New England Nuclear, Boston, MA). The reaction was stopped by the addition of acidified methanol (50 mM acetic acid). Polar lipids were isolated by the Bligh and Dyer method [Bligh and Dyer, 1959], evaporated under a stream of N_2 gas and purified by a silica-based normal-phase HPLC column. Fractions corresponding to ^{3}H-PAF were quantified by counting radioactivity with a β-counter. The authenticity of synthesized ^{3}H-PAF was confirmed by the similar HPLC elution pattern as standard ^{3}H-PAF (New England Nuclear).

Statistical Analysis

Data are mean ± SEM. Comparisons were made by analysis of variance followed by an unpaired Bonferroni *t*-test. Differences were considered significant at P values less than 0.05.

RESULTS

VEGF Effect on MSK1 Phosphorylation

In a first series of experiments, we assessed the capacity of VEGF to modulate the phosphorylation of MSK1 on Thr^{581}, Ser^{376}, and Ser^{360} residues. Treatment of native BAEC with VEGF (1 nM) induced a rapid and transient phosphorylation of MSK1-Ser^{376} and -Thr^{581} residues, which was maximal within 10–15 min as compared to control PBS-treated cells, and resumed toward its basal level within 30 min (Fig. 1A). In contrast, we did not detect any phosphorylation of MSK1-Ser^{360} residue (data not shown).

Effect of MAPK Inhibitors on MSK1 Phosphorylation

In previous studies, we reported that VEGF induces endothelial PAF synthesis upon the activation of VEGFR-2/NRP-1 complex [Bernatchez et al., 2002], and the activation of both p38 and p42/44 MAPKs, and subsequent activation of $sPLA_2$-V [Bernatchez et al., 2001a,b]. We thus assessed if the activation of MAPKs in response to VEGF were responsible for MSK1 phosphorylation. Pretreatment of BAEC, with p38 MAPK inhibitor SB203580 (10 μM; IC_{50}, 0.6 μM) [Cuenda et al., 1995], 20 min prior to the stimulation with VEGF, did not prevent MSK1-Ser^{376} and -Thr^{581} phosphorylation. In contrast, pretreatment with MEK inhibitor PD98059 (10 μM; IC_{50}, 1 μM) [Dudley et al., 1995], which prevents p42/44 MAPK phosphorylation, completely blocked VEGF-mediated MSK1-Ser^{376} and -Thr^{581} phosphorylation. Pretreatment with both MAPK inhibitors blocked VEGF-mediated MSK1-Ser^{376} and -Thr^{581} phosphorylation as observed with the MEK inhibitor (PD98059) (Fig. 1B).

Western Blot Analysis of Proteins Interacting with MSK1

In a second series of experiments, we assessed the capacity of MSK1 to interact with proteins involved in the signaling pathways leading to VEGF-mediated PAF synthesis. Native BAEC were stimulated with VEGF in a time-dependent assay and cell lysates were immunoprecipitated with an anti-MSK1 IgG. Western blot analyses were performed to identify the proteins coimmunoprecipitated with MSK1. We observed a constitutive interaction between MSK1 and p38 MAPK, p42/44 MAPK, and $sPLA_2$-V, which was not modified by VEGF stimulation (Fig. 2).

Establishment and characterization of transfected dead kinase mutants of MSK1. To clarify the role of each MSK1 N- and C-terminal kinase domains into the multiprotein complex interaction defined above, we performed stable transfections of BAEC with pCDNA3.1 plasmid expressing either the wild-type form of human MSK1 (MSK1-WT) or its N-terminal or C-terminal "dead-kinase" mutants (respectively MSK1-D195A and MSK1-D565A), all tagged with the Flag epitope [Deak et al., 1998]. Control transfection was performed with the empty pCDNA3.1 vector. Transfection efficiency was assessed by RT-PCR, using one human-MSK1 specific primer and one primer corresponding to the Flag epitope, to avoid amplification of the bovine endogenous MSK1 transcript (Fig. 3A).

The "dead-kinase" status of D195A and D565A MSK1 mutant forms was assessed by studying the capacity of VEGF to induce the

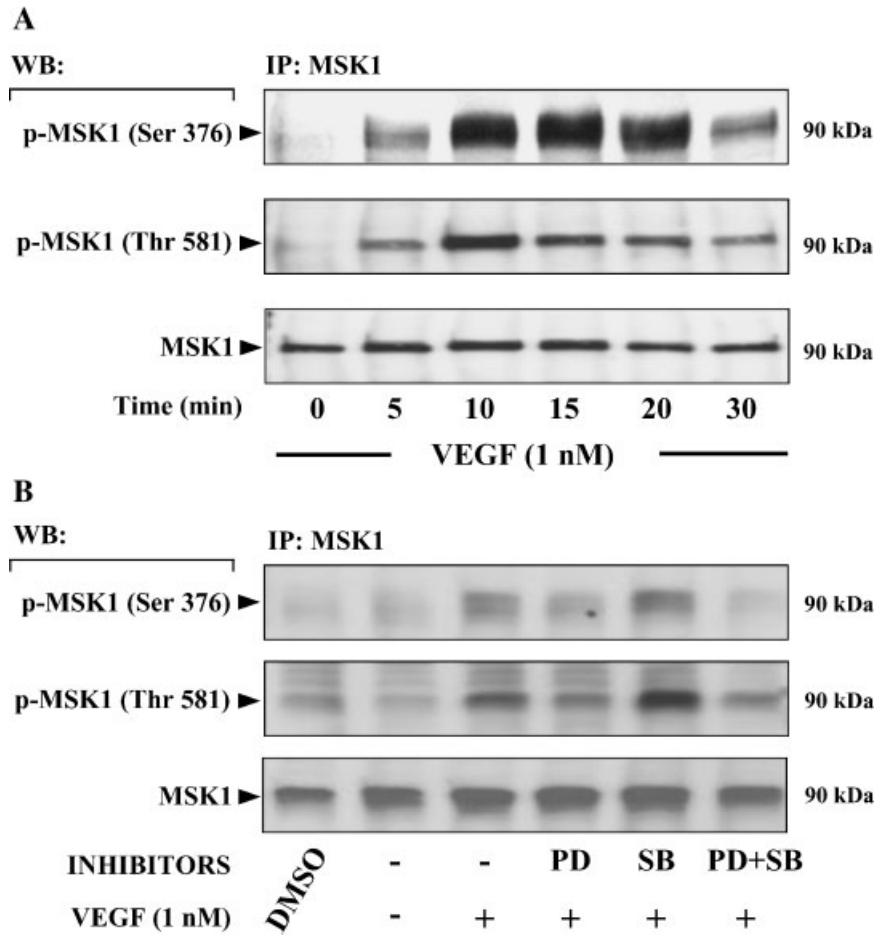

Fig. 1. Contribution of p38 and p42/44 MAPKs on VEGF-mediated MSK1 phosphorylation. Confluent BAEC were stimulated with VEGF (1 nM) up to 30 min. Cell lysates were immunoprecipitated with an anti-MSK1 IgG. MSK1 phosphorylation was assessed by immunoblotting with two antibodies detecting phospho-MSK1-Ser^{376} and -Thr^{581}, respectively. The blots were stripped and reprobed to confirm equal loading of MSK1 protein (**A**). BAEC were pretreated with p38 MAPK inhibitor (SB203580, 10 μM) (SB) or MEK inhibitor (PD98059, 10 μM) (PD) alone and combined 20 min prior to stimulation with VEGF (1 nM) for 15 min. DMSO was used to dissolve the inhibitors and inserted as negative control. Cell lysates were treated as above (**B**).

phosphorylation of CREB (cAMP-response-element-binding protein), which requires both catalytically active MSK1 kinase domains [Arthur and Cohen, 2000; Wiggin et al., 2002; Arthur et al., 2004; Delghandi et al., 2005]. Treatment with VEGF led to the phosphorylation of CREB (phospho-CREB-Ser^{133}) in native BAEC as well as in BAEC transfected with control pCDNA3.1 and WT-MSK1 (Fig. 3B). However, in BAEC transfected with the MSK1-D195A and MSK1-D565A mutants, the phosphorylation of CREB was completely abolished (Fig. 3B). It is to mention that due to the structural similarities between CREB and ATF-1, the phospho-CREB IgG recognizes the phosphorylation site of ATF-1-Ser^{63} residue [Shaywitz and Greenberg, 1999], which was detected after VEGF stimulation in native BAEC and transfected with control pCDNA3.1 and MSK1-WT, but not in BAEC transfected with MSK1-D195A and MSK1-D565A mutants (Fig. 3B).

Effect of MSK1 Dead Kinase Mutants on Protein Interactions

As described above, we observed, in native BAEC, a constitutive protein interaction between MSK1 with p38 and p42/44 MAPKs and sPLA$_2$-V. As MSK1 activation requires the activity of both kinase domains to induce the phosphorylation of its substrate CREB, we then assessed if the multi-protein complex would be affected in MSK1-D195A and MSK1-D565A mutants. The same MSK1

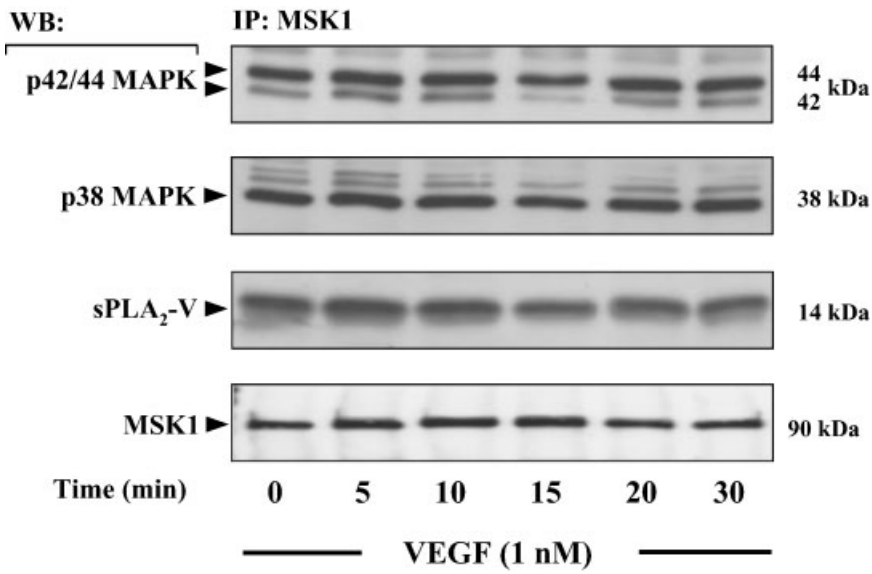

Fig. 2. MSK1 multi-protein complex formation. Confluent BAEC were stimulated with VEGF (1 nM) up to 30 min. Cell lysates were immunoprecipitated with an anti-MSK1 IgG. Coprecipitation of MSK1 with p38 and p42/44 MAPKs, and $sPLA_2$-V was assessed by Western blot analyses with corresponding antibodies.

coimmunoprecipitation experiment was, thus, achieved in native and transfected BAEC. Western blot analysis revealed that the constitutive interaction between MSK1 with p38 and p42/44 MAPKs was maintained and unchanged under VEGF stimulation in all cell types (Fig. 4). However, the coprecipitation of MSK1 with $sPLA_2$-V was almost completely abolished in BAEC transfected with the MSK1-D565A mutant, while it remained constitutive in native BAEC and in pCDNA3.1, MSK1-WT, and MSK1-D195A transfected cells (Fig. 4). The interaction between MSK1 and $sPLA_2$-V in MSK1-D565A transfected cells was reduced by 82% and 90% in PBS or VEGF-treated cells, respectively (Fig. 4).

Effect of MSK1 Dead Kinase Mutants on PAF Synthesis

Since the protein interaction between MSK1 and $sPLA_2$-V is altered in BAEC transfected with MSK1-D565A mutant, and in order to assess the possible contribution of MSK1 on PAF synthesis, we evaluated its synthesis in native and transfected BAEC. Treatment with VEGF increased basal PAF synthesis in native BAEC, pCDNA3.1, MSK1-WT, and MSK1-D195A by 166%, 139%, 125%, and 82%, respectively as compared to their respective control-PBS treated cells. In contrast, PAF synthesis mediated by VEGF was abrogated in BAEC transfected with MSK1-D565A mutant (Fig. 5). To insure that the absence of VEGF-mediated PAF synthesis in MSK1-D565A transfected cells was not due either to the absence of VEGFR-2 protein expression or altered phosphorylating capacity, we performed Western blot analyses for all BAEC subtypes under PBS and VEGF-treatment. We observed between all endothelial subtypes an equivalent VEGF capacity to promote VEGFR-2 phosphorylation as well as its corresponding protein expression level (data not shown).

DISCUSSION

We previously reported that VEGF-induced endothelial PAF synthesis requires the activation of p38 and p42/44 MAPKs and the secreted phospholipase A_2 group V ($sPLA_2$-V). However, the mechanisms involved in the regulation of $sPLA_2$-V remained unknown. In the present study, we are reporting a constitutive protein interaction between MSK1 and $sPLA_2$-V, which appears specific to the C-terminal kinase domain of MSK1 and essential for the induction of PAF synthesis.

VEGF Mediates MSK1 Phosphorylation

Parallel activation of p38 and p42/44 MAPK pathways is crucial to endothelial PAF synthesis [Bernatchez et al., 2001a]. In addition, both

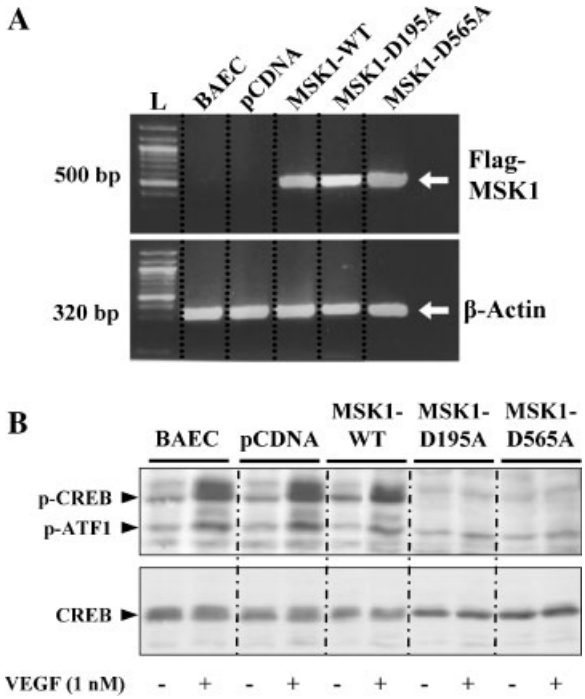

Fig. 3. Characterization of transfected BAEC. RT-PCR amplification of FLAG-MSK1 sequence was used to confirm stable transfection efficiency in BAEC expressing empty pCDNA3.1 vector (pCDNA), wild-type MSK1 (MSK1-WT), N-terminal dead-kinase domain (D195A) and C-terminal dead-kinase domain (D565A). β-Actin amplification was used as positive control for cDNA quality and quantity (**A**). Confluent native and transfected BAEC were stimulated in absence (−) or in presence (+) of VEGF (1 nM) for 15 min to assess the contribution of MSK1 N- and C-terminal kinase domain on CREB phosphorylation. Membranes were probed with an antiphospho-CREB-Ser^{133} IgG. The blots were stripped and reprobed with an anti-CREB IgG to confirm equal protein loading (**B**).

pathways can respectively activate MSK1 in response to growth factors and cellular stress stimuli [Deak et al., 1998; Dunn et al., 2005]. Since MSK1 has recently been reported to be activated by VEGF [Mayo et al., 2001], we were led to assess its contribution in VEGF-mediated PAF synthesis.

Firstly, we observed that VEGF induced a rapid and transient MSK1 phosphorylation on two different residues (Ser^{376} and Thr^{581}). These data are in agreement with previous studies, in which a similar MSK1 kinetic activation was observed upon stimulation of different cell types (Hela, SK-N-MC, PC12, cardiac myocytes and embryonic rat cortical cells) with various agonists including tumor necrosis factor, fibroblast growth factor, neuronal growth factor, endothelin-1, N-methyl-D-Aspartate and brain-derived neurotrophic factor [Deak et al., 1998; Markou and Lazou, 2002; Rakhit et al., 2005]. It has recently been defined that Thr^{581} residue in the MSK1 C-terminal kinase domain is a proline-directed site, which can be phosphorylated either by p38 or p42/44 MAPKs [McCoy et al., 2005]. Phosphorylation of Thr^{581} residue is essential for C-terminal kinase domain activation, which is then leading to an autophosphorylation cascade of Ser^{381} and Ser^{376} residues in the linker region, and Ser^{212} in the N-terminal kinase domain [McCoy et al., 2005]. In addition, they reported in HEK-293 cells, that in function of the stimuli, p38 or p42/44 MAPK activation may induce the phosphorylation of MSK1-Ser^{360} residue which constitutes another proline-directed site [McCoy et al., 2005]. However, in our study, treatment of EC with VEGF did not induce MSK1-Ser^{360} residue phosphorylation, which is in concordance with other studies, in which a treatment of myocytes and neurons with selective agonists did not support the phosphorylation of MSK1-Ser^{360} residue [Markou and Lazou, 2002; Webber et al., 2005].

Multi-Protein Complex Interaction Between MSK1 with p38, p42/44 MAPKs, and sPLA$_2$-V

MSK1 has a specific docking site localized at the C-terminal end, which has been demonstrated to be required for its activation upon its interaction with p38 and/or p42/44 MAPKs [Tomas-Zuber et al., 2001; McCoy et al., 2005]. Since VEGF-mediated PAF synthesis implies p38, p42/44 MAPKs, and sPLA$_2$-V activation [Bernatchez et al., 2001a,b], we investigated the interaction between these proteins with MSK1. By coimmunoprecipitation analyses in native EC, we detected the presence of a constitutive multi-protein complex between p38, p42/44 MAPKs, sPLA$_2$-V, and MSK1, which thus supports the possible contribution of MSK1 in VEGF-mediated PAF synthesis.

To define the role of p38 and p42/44 MAPKs on VEGF-mediated MSK1 activation, we used corresponding selective inhibitors. Treatment of EC with PD98059 abrogated the phosphorylation MSK1-Ser^{376} and Thr^{581} residues, whereas the blockade of p38 MAPK activation with S203580 did not prevent the phosphorylation of these residues. Thus, suggesting that endothelial MSK1 activation under VEGF stimulation requires p42/44 MAPK activation, whereas p38 MAPK activation does not seem to be involved at least on phosphorylation of those

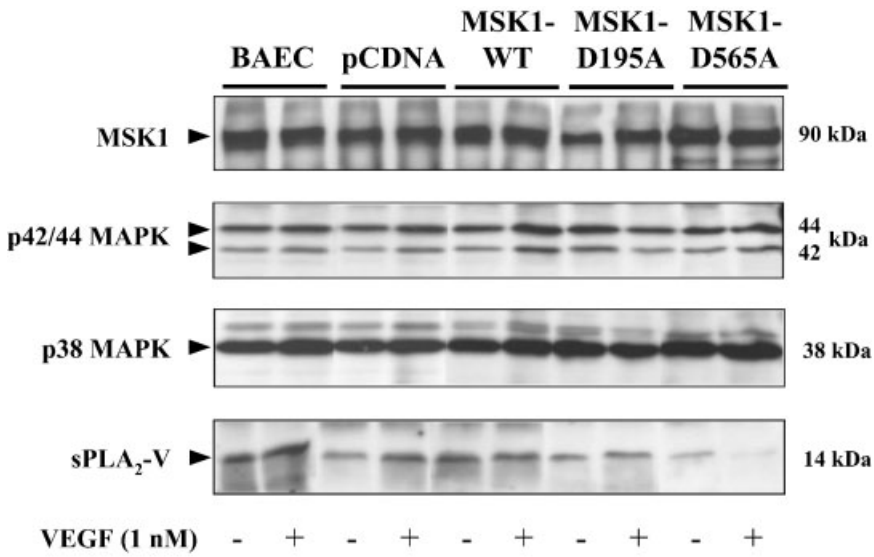

Fig. 4. Modulation of MSK1 multi-protein complex formation in transfected BAEC. Confluent native and transfected BAEC were stimulated in absence (–) or in presence (+) of VEGF (1 nM) for 15 min. Cell lysates were immunoprecipitated with an anti-MSK1 IgG. Coprecipitation of MSK1 with p38 and p42/44 MAPKs, and $sPLA_2$-V was assessed by Western blot analyses with corresponding antibodies.

MSK1 residues. Previous studies reported the contribution of p38 and p42/44 MAPKs in the activation of the $cPLA_2$ [Lin et al., 1993; Nemenoff et al., 1993], a PLA_2 essential for PAF synthesis in neutrophils [Baker et al., 2002]. In addition, it was assessed that only p38 MAPK was capable to activate the lyso-PAF acetyltransferase which converts the lyso-PAF into PAF [Nixon et al., 1999; Owen et al., 2005]. Thus, in EC, although p38 MAPK can bind to MSK1 docking site, its contribution onto PAF synthesis may reside as observed in neutrophils on its capacity to activate lyso-PAF acetyltransferase.

Contribution of MSK1 Domains on VEGF-Mediated PAF Synthesis

MSK1 possesses a particular feature by having two kinase domains, where the initial C-terminal kinase domain activation is essential to support subsequent N-terminal kinase domain activation [McCoy et al., 2005]. Furthermore, it

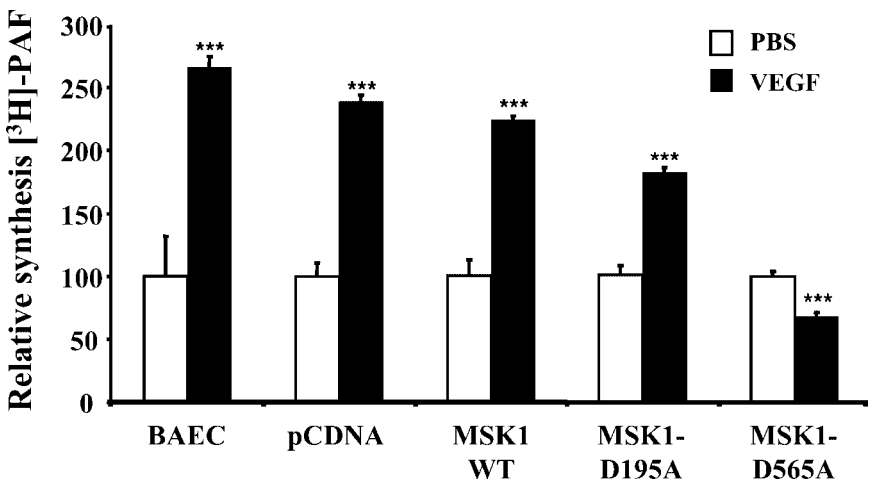

Fig. 5. Contribution of MSK1 on VEGF-mediated PAF synthesis. Confluent native and transfected BAEC were stimulated with PBS and VEGF (1 nM) for 15 min. Values are means ± SEM of at least 12 experiments and represent the incorporation of tritiated acetate (^{3}H-acetate) into lyso-PAF. ***$P < 0.001$ as compared to corresponding control-PBS treated cells.

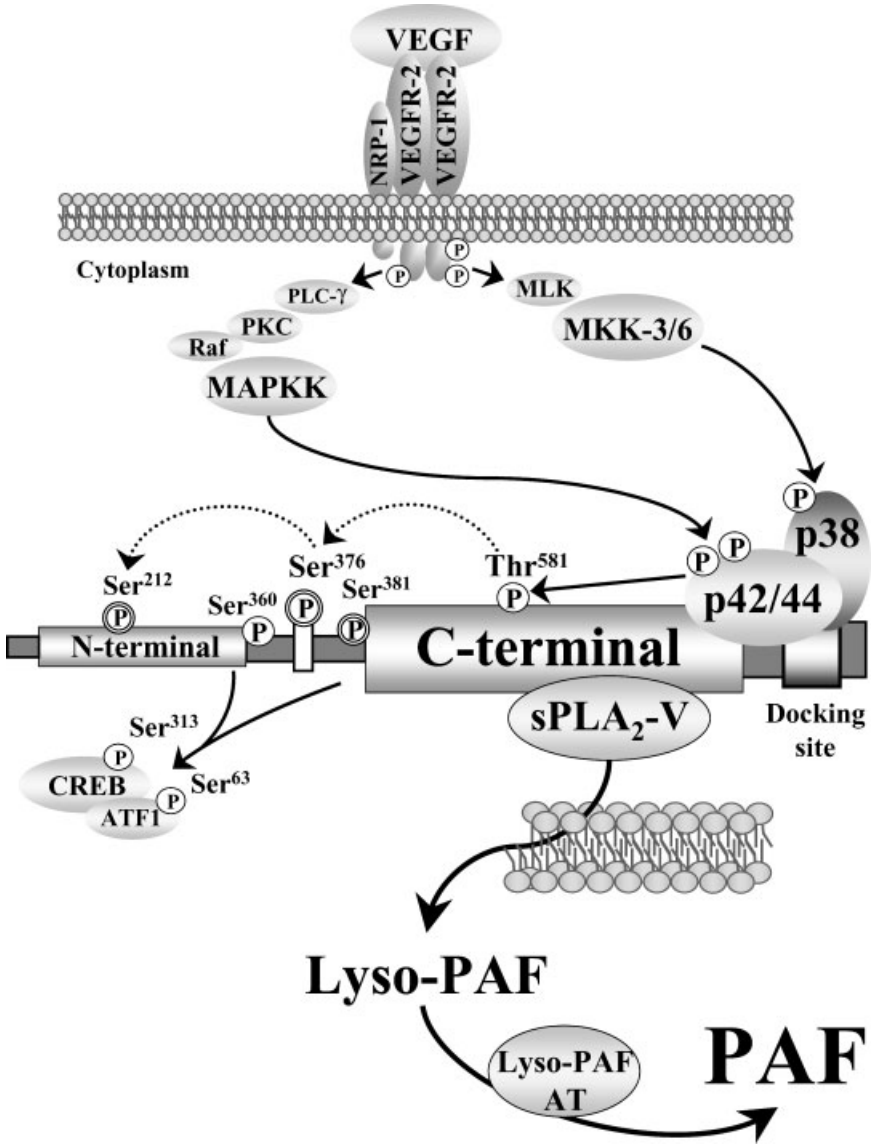

Fig. 6. Schematic illustration of VEGF-mediated PAF synthesis: Proposed contribution of MSK1. Activation of VEGFR-2/NRP-1 complex by VEGF activates p38 and p42/44 MAPKs pathways and $sPLA_2$-V, which can form a constitutive multi-protein complex with MSK1. Interaction between MSK1 and p38 and p42/44 MAPKs is supported by a specific docking site located at the C-terminal end. Activation of p42/44 MAPK promotes phosphorylation of Thr^{581} residue, which is essential for C-terminal kinase domain activation, and autophosphorylation cascade of Ser^{376} and Ser^{381} residues in the linker region, and Ser^{212} in the N-terminal kinase domain and subsequent phosphorylation of its substrate CREB. Mutation inactivating MSK1 C-terminal kinase domain as opposed to N-terminal kinase domain is silencing the protein interaction between MSK1 and $sPLA_2$-V and lyso-PAF formation, which is then converted into PAF upon the activation of lyso-PAF acetyl transferase by activated p38 MAPK. Phosphorylation and autophosphorylation of amino acids induced by MAPKs on MSK1 are represented by single and double circles, respectively.

has also been reported that the contribution of both activated MSK1 kinase domains is essential to fully support MSK1 phosphorylation catalytic activity on CREB substrate [Deak et al., 1998; Arthur and Cohen, 2000; Wiggin et al., 2002; Arthur et al., 2004; Delghandi et al., 2005]. To evaluate the role of each MSK1 kinase domain in the multi-protein complex formation on PAF synthesis, we used transfected EC expressing N- and C-terminal "dead-kinase" mutants. Firstly, we observed that the mutations inactivating either the MSK1 N- or C-terminal kinase domains prevented VEGF-mediated CREB phosphorylation. We then assessed how such mutations would affect the endogenous MSK1 multi-protein complex.

Mutations of MSK1 N- or C-terminal kinase domains did not alter the interaction between MSK1 and p38 and p42/44 MAPKs, under basal and VEGF-mediated conditions. The maintenance of these interactions could be explained by the fact that both MAPKs are binding to MSK1 within a specific docking site localized outside and at the end of the C-terminal kinase domain [Frodin and Gammeltoft, 1999; Dunn et al., 2005; McCoy et al., 2005]. However, although a constitutive interaction between MSK1 and $sPLA_2$-V was maintained in EC expressing N-terminal kinase domain (MSK1-D195A) mutant, the interaction between MSK1 and $sPLA_2$-V in EC expressing C-terminal kinase domain (MSK1-D565A) mutant was almost completely abrogated under basal and VEGF-mediated conditions. Finally, using the same molecular approach, we observed that VEGF-mediated PAF synthesis was also lost in EC expressing C-terminal kinase domain (MSK1-D565A) mutant. Together, our data demonstrate that the interaction between MSK1 and $sPLA_2$-V is driven by MSK1 C-terminal kinase domain integrity, and is essential to allow PAF synthesis. These observations are in concordance with a previous study, in which we observed that the blockade of $sPLA_2$-V activity prevented endothelial VEGF-mediated PAF synthesis [Bernatchez et al., 2001b].

In summary, the activation of VEGFR-2/NRP-1 complex with VEGF activates p38 and p42/44 MAPKs pathways and $sPLA_2$-V, which can form a constitutive multi-protein complex with MSK1. Interaction between MSK1, p38 and p42/44 MAPKs is supported by a specific docking site localized outside and at the end of the C-terminal kinase domain. Activation of p42/44 MAPK promotes phosphorylation of Thr^{581} residue, which is essential for C-terminal kinase domain activation. Mutation inactivating MSK1 C-terminal kinase domain as opposed to N-terminal kinase domain is silencing the protein interaction between MSK1 and $sPLA_2$-V, which is essential to support VEGF-mediated PAF synthesis (Fig. 6). Our data are the first one to delineate the interaction between MSK1 and $sPLA_2$-V, the pivotal role played by MSK1 C-terminal kinase domain in this protein–protein complex formation, and that MSK1 constitutes a rate-limiting step in the induction of VEGF-mediated proinflammatory activities.

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