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Activation of p38 Has Opposing Effects on the Proliferation and Migration of Endothelial Cells*[S]

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Pathological conditions such as hypertension and hyperglycemia as well as abrasions following balloon angioplasty all lead to endothelial dysfunction that impacts disease morbidity. These conditions are associated with the elaboration of a variety of cytokines and increases in p38 activity in endothelial cells. However, the relationship between enhanced p38 activity and endothelial cell function remains poorly understood. To investigate the effect of enhanced p38 MAPK activity on endothelial cell function, we expressed an activated mutant of MEK6 (MEK6E), an upstream regulator of p38. Expression of MEK6E activated p38 and resulted in phosphorylation of its downstream substrate, heat shock protein 27 (Hsp27). Activation of p38 was not sufficient to induce apoptosis; however, it did induce p38-dependent cell cycle arrest. MEK6E expression was sufficient to inhibit ERK phosphorylation triggered by growth factors and integrin engagement. MAPK phosphatase-1 (MKP-1) expression was increased upon p38 activation, and expression of a "substrate-trapping" MKP-1 was sufficient to restore ERK activity. Activation of p38 was sufficient to induce cell migration, which was accompanied by alterations in actin architecture characterized by enhanced lamellipodia. Co-expression of a mutant form of Hsp27, lacking all three phosphorylation sites, reversed MEK6E-induced cell migration and altered the cytoskeletal changes induced by p38 activation. Collectively, these results suggest that cellular decisions regarding migration and proliferation are influenced by p38 activity and that prolonged activation of p38 may result in an anti-angiogenic phenotype that contributes to endothelial dysfunction.

Under normal conditions, vascular injury triggers the proliferation and migration of normally quiescent endothelial cells, leading to repair of the injured vessel. However, several pathological conditions such as hypertension and hyperglycemia are accompanied by a dysfunctional endothelium characterized by impaired re-endothelialization. These conditions are associated with elevated levels of cytokines such as tumor necrosis factor and transforming growth factor-β as well as enhanced activation of p38 (1–5). Interestingly, high glucose levels present in diabetic patients have also been shown to induce the activation of p38 (1). These data suggest that p38 activity may play a direct role in endothelial dysfunction. The direct effect of chronic p38 activity on the proliferation and migration of endothelial cells has not been tested.

p38 is a member of the mitogen-activated protein kinase (MAPK)[1] family, which includes extracellular signal-regulated kinase (ERK) and c-Jun NH_2-terminal kinase. These serine/threonine protein kinases transmit signals from the membrane to the nucleus (6). Phosphorylation of both the threonine and tyrosine residues of the conserved Thr-*X*-Tyr motif of MAPKs is required for their activation. A variety of dual specificity protein phosphatases dampen activity by dephosphorylating these residues (7).

The MAPK family has classically been viewed as separate signaling cascades activated by distinct stimuli and upstream kinases (8). ERK is activated by MEK1/2 kinases, whereas p38 is typically activated following phosphorylation of its upstream kinases MEK3 and MEK6 (9). Functionally, these kinases are also thought of as distinct. ERK is involved in cell proliferation and survival responses in a variety of cell types, including endothelial cells (8, 10). Activation of p38 is implicated in inflammation, cell growth control, cell differentiation, cell migration, and apoptosis (6). The p38 MAPK family consists of four different isoforms including α, β, δ, and γ. The α and β isoforms are ubiquitously expressed, whereas γ expression is found predominantly in skeletal muscle, and δ expression is enriched in the lung, kidney, testis, pancreas, and small intestine (6). A growing body of evidence indicates that these isoforms can be activated differentially and may control different downstream cellular processes, depending on cell type (11–14).

The role of p38 in regulating endothelial cell function is currently not clear, and which isoforms are present in endothelial cells has not been defined. P38 can be activated by vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF), two well known angiogenic factors, and p38 activation has been shown to be critical for endothelial cell migration in

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1 (showing that MKP-1 inhibition raises phospho-p38 levels without inhibiting dephosphorylation) and 2 (showing that sustained p38 activation does not inhibit stress fiber formation in adherent endothelial cells).

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[1] The abbreviations used are: MAPK, mitogen-activated protein kinase; DNp38, dominant negative p38; ERK, extracellular signal-regulated kinase; FAK, focal adhesion kinase; FGF, fibroblast growth factor; GFP, green fluorescent protein; Hsp, heat shock protein; HUVEC, human umbilical vein endothelial cell; MEK, MAPK/ERK kinase; MKP-1, MAPK phosphatase-1; mutHsp27, mutant form of Hsp27; VEGF, vascular endothelial growth factor.

response to VEGF (15, 16). Studies utilizing pharmacological inhibitors of p38 have suggested that p38 activation may promote endothelial cell apoptosis (17, 18) and vascular permeability (19, 20) while negatively regulating the tubular morphogenesis associated with angiogenesis (17, 19). Other studies have shown that p38 is not required for neovessel outgrowths (21). Mice lacking p38α demonstrate severe defects in placental development and vascularization (22), suggesting a role in endothelial cell function. Collectively, these divergent pieces of data suggest that p38 activity is a tightly regulated component of the neovascularization response.

In the present study, we have utilized a constitutively active form of MEK6 to investigate directly the effects of sustained p38 activity in vascular endothelial cells. Our results indicate that activation of p38 is sufficient to inhibit endothelial cell proliferation while promoting migration. Our data suggest a model whereby a shift in the balance of p38 and ERK signaling, mediated in part by induction of MKP-1, alters the ability of endothelial cells to respond normally to angiogenic factors. As a result, chronic activation of p38 may contribute directly to pathological endothelial cell dysfunction.

EXPERIMENTAL PROCEDURES

Materials—VEGF was obtained from the NCI, National Institutes of Health developmental therapeutics program. Polyclonal anti-phospho-p38, anti-pan-p38 antibodies, anti-p38α, anti-p38δ, anti-phospho-Hsp27, anti-Hsp27, anti-phospho-MEK1/2, and anti-MEK1/2 antibodies were obtained from Cell Signaling Technology (Beverly, MA). Monoclonal anti-phospho-ERK and polyclonal anti-ERK1, anti-ERK2, anti-FAK, anti-p38β, and anti-MKP-1 antibodies were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA). Polyclonal anti-phospho-FAK-Tyr 397 antibody and anti-MKP-1 antibody used to show expression of MKP-1 C/S (retained activity against non-human protein) were obtained from Upstate Biotechnology (Lake Placid, NY). Monoclonal anti-FLAG antibody was obtained from Sigma. Ro-31-8220, was purchased from Calbiochem. The cDNA for dominant negative p38α (DNp38α) was from Roger Davis (University of Massachusetts Medical School). Adenoviruses were all created using the *Adeasy* system, essentially as described previously (10, 15, 23). The mutant form of Hsp27 (mutHsp27) adenovirus was a generous gift of William Gerthoffer (University of Nevada School of Medicine). The MKP-1 C/S adenovirus was a generous gift of Andrey Sorokin (Medical College of Wisconsin).

Cell Culture—Human umbilical vein endothelial cells (HUVECs) from pooled donors were supplied by VEC Technologies (Troy, NY) through the NCI, National Institutes of Health angiogenesis resource center and cultured as we have described previously (10). For experiments requiring serum deprivation, MCDB-131 supplemented with 1% penicillin/streptomycin and 2 mM L-glutamine was used as indicated.

Western Blotting—When appropriate, HUVECs were infected with an adenovirus overnight as indicated at an multiplicity of infection of 5–10. For experiments requiring growth factor stimulation, nearly confluent cells were serum-starved for 16 h prior to stimulation with 50 ng/ml VEGF or 100 ng/ml FGF for the indicated time points. Experiments conducted with subconfluent cells gave identical results. For experiments requiring integrin-induced activation, cells were trypsinized and placed into suspension for 30 min prior to replating at 50% confluency. Subsequently, whole cell lysates were collected in Laemmli sample buffer. Blotting was performed according to procedures we have described previously (10). All figures are representative of at least three independent experiments.

Apoptosis Assay—Caspase 3/7 activities were measured by an Apo-ONE™ homogeneous caspase 3/7 assay (Promega). A 96-well plate coated with 0.2% gelatin was seeded with 2×10^4 cells/well. The following day, experimental manipulations were initiated. After 48 h, an equal volume of lysis buffer containing the caspase substrate benzyloxycarbonyl-DEVD-R100 was added and incubated at room temperature for 1 h. The cell lysates at an excitation wavelength of 485 nm and an emission wavelength of 535 nm using a PerkinElmer Life Sciences HTS 7000 plus BioAssay Reader.

Cell Proliferation and Cell Migration—These experiments were conducted essentially as described previously by us (10, 15, 24).

Labeling of Actin Cytoskeleton—HUVECs seeded onto coverslips were infected with GFP or the MEK6E adenovirus and serum-starved overnight. Subsequently, the cells were treated with VEGF (50 ng/ml) for 15 min to observe the changes in actin cytoskeleton in adherent cells. To examine changes in cells actively remodeling the actin cytoskeleton, cells infected with GFP or the MEK6E adenovirus were trypsinized, and 2.5×10^4 cells were plated onto gelatin-coated coverslips. After 4 h, cells were fixed with 3.7% formaldehyde, and actin was visualized using Texas Red phalloidin (Molecular Probes) (10).

Statistical Analysis—All quantitative data were pooled from multiple independent experiments. One- or two-way analysis of variance was conducted as appropriate using Statistica software (Tulsa, OK). A Newman-Keuls *post hoc* test was performed to determine statistically significant differences ($p < 0.05$).

RESULTS

As several studies have shown chronically elevated p38 activity in disease states known to have impaired angiogenesis and endothelial dysfunction, we utilized a FLAG-tagged, constitutively active MEK6 adenovirus, MEK6E, to directly study the effects of chronic p38 activity on endothelial cells. This reagent has been described previously (25). MEK6E was expressed at ~10-fold higher amounts than the endogenous MEK6 levels (data not shown). HUVECs showed expression of the FLAG-tagged protein and induction of p38 phosphorylation at levels similar to those induced acutely by VEGF (Fig. 1*A*). Although each activates p38 to similar levels, the activation of p38 by VEGF is transient, whereas the MEK6E-induced p38 activation is sustained. P38 activation by MEK6E was sufficient to induce the sustained phosphorylation of a known substrate of p38, Hsp27 (Fig. 1, *B* and *C*), as well as the transcription factor ATF-2 (Fig. 1*C*).

MEK6 can activate all of the p38 isoforms; therefore, we tested to determine which isoforms of p38 might be present and/or activated in endothelial cells. Using commercially available isoform-specific anti-sera, we detected weak activity against p38α (data not shown). No reactivity against other isoforms was detected with the available antibodies. Because dominant negative p38 isoforms have been used to define the role of specific isoforms (14, 26), we also employed an adenovirus coding for DNp38α (15). As shown in Fig. 1*C*, co-expression of DNp38α resulted in the inhibition of both the Hsp27 phosphorylation and ATF-2 phosphorylation induced by MEK6E, suggesting that p38α is the predominant isoform activated by MEK6E.

Activation of p38 by stress signals has been shown to induce apoptosis in some systems (27), and inhibitor studies have indirectly implicated p38 in the regulation of apoptosis in endothelial cells (17, 18). In contrast, other studies have indicated pro-survival effects of p38 activation (28, 29). Therefore, we tested whether chronic activation of p38 modulated apoptosis by measuring caspase 3/7 activities. Whereas serum starvation in M199 media for 48 h induced apoptosis, no increase in caspase 3/7 activity was observed in MEK6E-infected cells. (Fig. 1*D*). However, enhanced p38 activity did enhance stress-induced apoptosis (Fig. 1*D*). It should be noted that serum starvation in MCDB-131 as a basal medium did not induce apoptosis (data not shown). Therefore, MCDB-131 was used for serum starvation protocols in all other experiments.

A requirement for re-endothelialization following injury, as well as for angiogenesis, is enhanced cell proliferation (30, 31). We investigated the effect of activating p38 on growth factor-induced DNA synthesis of endothelial cells. Analysis of [^{3}H]thymidine incorporation revealed that the increase in the number of cells entering S phase upon VEGF treatment was completely inhibited following expression of MEK6E (Fig. 2*A*). Similarly, MEK6E was sufficient to inhibit FGF-induced cell cycle progression (Fig. 2*B*), demonstrating that this effect was not specific to VEGF responses. Expression of DNp38α was sufficient to rescue the inhibition of cell proliferation induced by MEK6E (Fig. 2*B*). We also measured bromodeoxyuridine

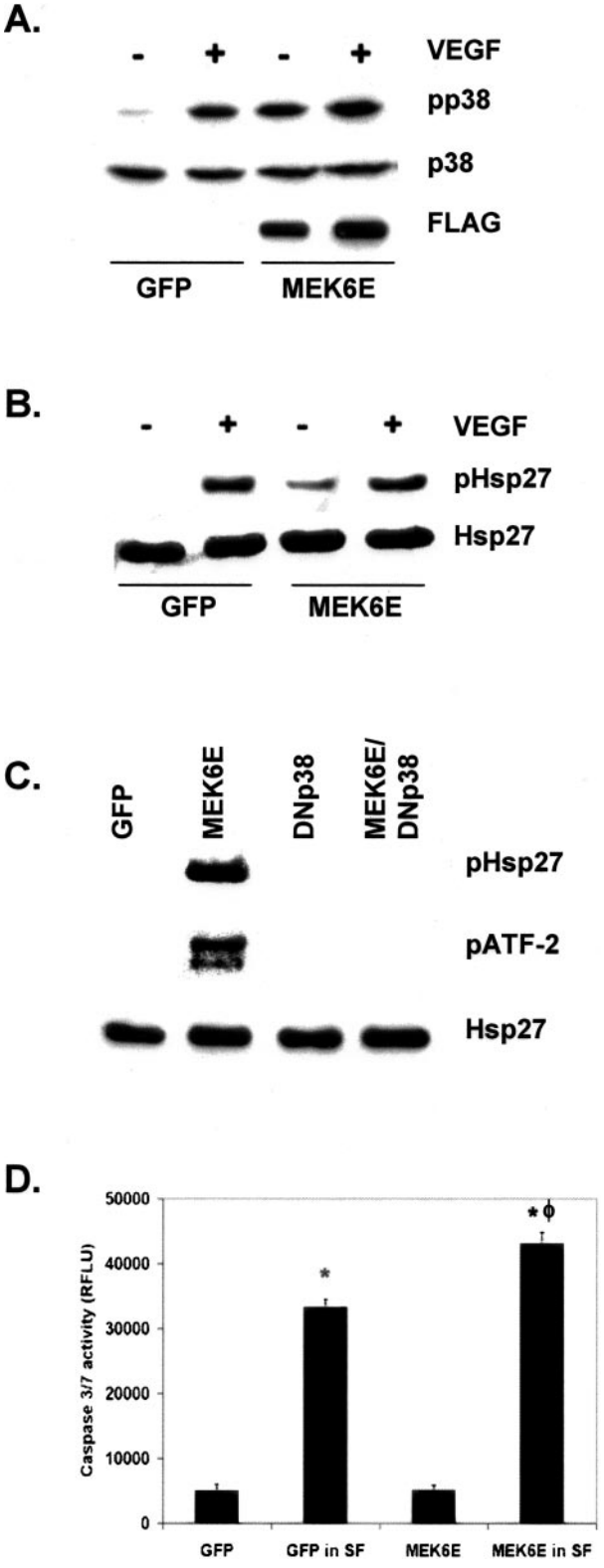

FIG. 1. **MEK6E induces p38 activation but does not induce apoptosis.** *A–C*, HUVECs infected with GFP, MEK6E, DNp38α adenovirus, or a combination were serum-starved prior to stimulation with VEGF (50 ng/ml) for 15 min where indicated. The cell lysates were analyzed by probing Western blots with phospho-p38 (*pp38*), p38, and FLAG antibodies (*A*), phospho-Hsp27 (*pHsp27*) and Hsp27 antibodies (*B*), or ATF and phospho-ATF (*pATF-2*) (*C*). *D*, HUVECs were infected with the indicated adenovirus, and apoptosis was induced by serum starvation in M199 media for 48 h (*SF*). Caspase 3/7 activity was measured by quantitating the cleavage of a profluorescent peptide. Data are plotted as mean relative fluorescent units ± S.E. *, statistical difference from GFP; ϕ, statistical difference from GFP in M199 media for 48 h ($p < 0.05$) ($n = 6$); *RFLU*, relative fluorescent light units.

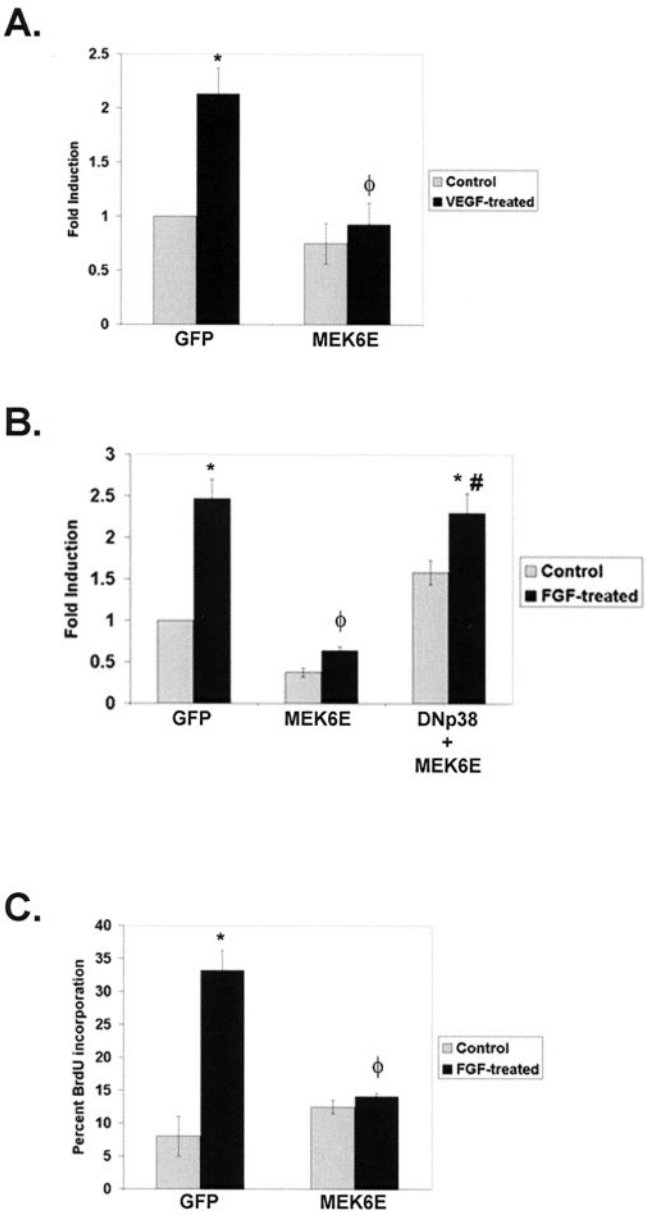

FIG. 2. **MEK6E inhibits VEGF- and FGF-induced cell proliferation.** *A*, serum-starved HUVECs were infected with either GFP or the MEK6E adenovirus prior to stimulation with VEGF (50 ng/ml) for 16 h. Cells were pulsed with [^{3}H]thymidine for 3 h prior to scintillation counting. *B*, serum-starved cells were infected with GFP, MEK6E, or co-infected with MEK6E and DNp38α adenoviruses prior to stimulation with FGF for 16 h. Incorporation of [^{3}H]thymidine was measured by scintillation counting. Data from multiple experiments were normalized and plotted as mean ± S.E. *, statistical difference from GFP; ϕ, statistical difference from GFP treated with growth factor; #, statistical difference from MEK6E treated with growth factors ($p < 0.05$) ($n = 9$). *C*, in similar experiments, cells were pulsed with bromodeoxyuridine (*BrdU*) for 3 h, and immunofluorescence was used to quantitate proliferation. Data are plotted as mean ± S.E. *, statistical difference from GFP; ϕ, statistical difference from GFP treated with FGF ($p < 0.05$) ($n = 3$).

incorporation, a method for measuring DNA synthesis independent of changes in total cell number (survival), and found similar results (Fig. 2*C*). These results clearly demonstrate that the activation of p38α by MEK6E inhibits S phase entry in response to several angiogenic factors.

ERK activity is known to be essential for proliferation in many cell types (8, 32, 33), including endothelial cells (10). Therefore, we investigated whether activation of p38 by MEK6E affected growth factor-mediated induction of ERK ac-

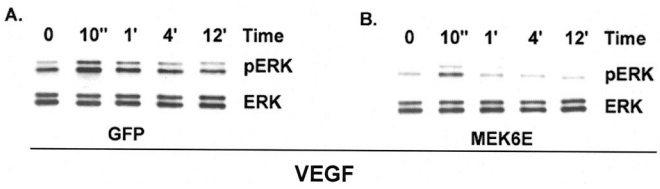

FIG. 3. **MEK6E inhibits growth factor-induced ERK activation.** Serum-starved HUVECs were infected with either GFP (*A*) or the MEK6E (*B*) adenovirus prior to treatment with VEGF (50 ng/ml). Cell lysates were made at the indicated time points and were analyzed by probing Western blots with phospho-ERK (*pERK*) and ERK antibodies. Blots are representative of three independent experiments.

tivity. Time course experiments of VEGF-mediated activation of ERK in control GFP-infected cells revealed peak activity at 5–10 min that remained elevated for ~4 h (Fig. 3*A*). In contrast, ERK phosphorylation levels in MEK6E-infected cells were reduced significantly at all time points (Fig. 3*B*), although VEGF still elevated levels above basal. Similar results were observed in FGF-stimulated cells (data not shown). These data indicate that activation of p38 reduces the basal level of ERK activity and the total level of ERK activity following growth factor stimulation.

ERK can also be activated by integrin engagement (34), and the integrin-mediated activation of ERK is critical for cell cycle progression (32). We postulated that the reduced level of ERK activity might be due to inhibition of an integrin-stimulated component and investigated the effect of MEK6E on integrin-induced ERK activity. Infected cells were placed in suspension and then seeded onto fibronectin-coated dishes for the indicated times (Fig. 4*A*) to synchronize the integrin-mediated activation of ERK. The cells expressing GFP showed a rapid induction of ERK phosphorylation within 15 min of replating. In contrast, expression of MEK6E substantially inhibited the integrin-induced ERK activity (Fig. 4*A*). Similar results were observed when experiments were performed with collagen and vitronectin (Fig. 4*B*). This finding argues that the inhibition of ERK is likely the result of inhibition of a shared adhesion-dependent activation mechanism rather than down-regulation of a specific integrin subunit or changes in matrix-integrin affinity. Integrin signaling to FAK has been reported as being an essential component of integrin-mediated ERK activation (35) and would represent a common or shared signaling protein through which p38 activation might be exerting its effects. Therefore we measured the phosphorylation of the FAK autophosphorylation site, Tyr-397, as an indicator of FAK activation. When endothelial cells were replated onto fibronectin, FAK was phosphorylated at Tyr-397, and MEK6E had no affect on this response (Fig. 4*C*). Taken collectively, these data indicate that MEK6E inhibits attachment-induced ERK activation. This inhibition is independent of the extracellular matrix ligand and occurs subsequent to FAK activation.

To gain more insight into where the down-regulation of ERK might be occurring, we examined the effect of MEK6E expression on MEK1/2 phosphorylation levels. Time course experiments of VEGF-induced MEK1/2 activity revealed peak activation at 10 min, similar to the profile of ERK activity. Surprisingly, MEK6E-infected cells had high levels of MEK1/2 phosphorylation prior to VEGF stimulation that remained elevated at all time points (Fig. 5*A*). Similar results were observed in FGF-stimulated cells (Fig. 5*B*) and in cells replated onto fibronectin (data not shown). The control experiments confirmed that MEK6E was running at a different molecular weight than the phospho-MEK1/2 and that no antibody cross-reactivity was occurring (data not shown). These data indicate that the cross-talk between p38 and ERK is occurring at the level of ERK in our system. Furthermore, these data suggest

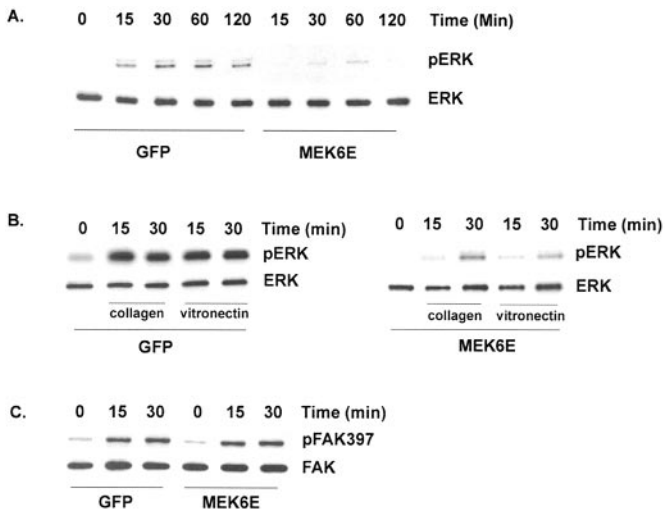

FIG. 4. **MEK6E inhibits integrin-induced ERK activation.** *A*, serum-starved HUVECs were infected with GFP or the MEK6E adenovirus. Cells were trypsinized and placed in suspension for 30 min prior to replating on fibronectin-coated dishes for the indicated time points. Cell lysates were analyzed by probing Western blots with phospho-ERK (*pERK*) and ERK antibodies. *B*, similar experiments were performed, except that cells were seeded onto collagen or vitronectin-coated dishes. *C*, FAK phosphorylation was measured in cells plated onto fibronectin by analyzing Western blots with pFAK397 and FAK antibodies. Blots are representative of experiments performed at least three times.

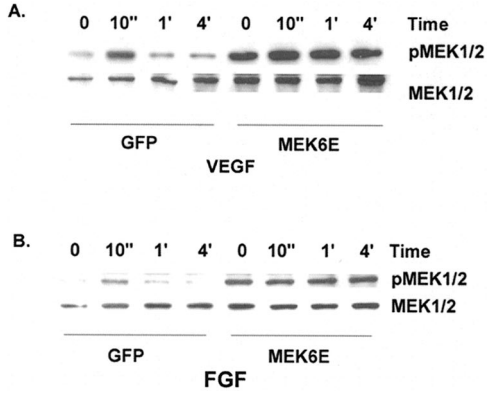

FIG. 5. **MEK6E expression results in enhanced levels of phosphorylated MEK1/2.** Serum-starved HUVECs were infected with either GFP or the MEK6E adenovirus prior to treatment with VEGF (50 ng/ml) (*A*) or FGF (100 ng/ml) (*B*). Cell lysates were made at the indicated time points and were analyzed by probing Western blots with phospho-MEK1/2 (*pMEK1/2*) and MEK1/2 antibodies. Blots are representative of three independent experiments.

ERK activation may normally provide negative feedback that results in the down-regulation of MEK1/2 phosphorylation.

Induction of phosphatase expression can regulate MAPK cross-talk in other cell types (36). The dual specificity phosphatase MKP-3 has been shown previously to be an important and relatively selective MAPK phosphatase for ERK (7). To determine whether this phosphatase was up-regulated by MEK6E expression, we measured levels of MKP-3 and observed no change in its expression levels (data not shown). Another dual specificity MAPK phosphatase, MKP-1, has also been shown to dephosphorylate ERK (37–39), and p38 has been linked to the induction of MKP-1 expression (40). Therefore, we investigated the potential role of MKP-1 in the p38-dependent down-regulation of ERK activity. Expression of MEK6E induced MKP-1 expression (Fig. 6*A*), whereas pretreatment with the p38 inhibitor, SB203580, inhibited the MEK6E-induced MKP-1 expression (Fig. 6*A*). These data suggest that MKP-1 could play a role in the MEK6E-induced down-regulation of ERK activity.

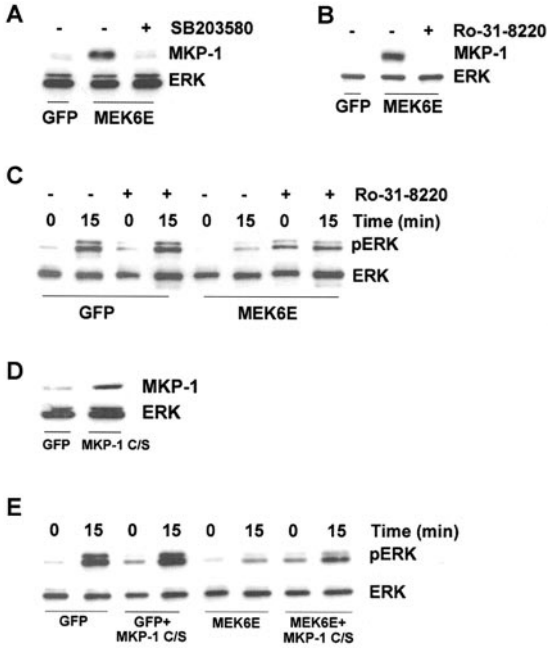

FIG. 6. **MEK6E-induced inhibition of ERK activity is mediated by MKP-1 expression.** *A*, serum-starved HUVECs were pretreated with 5 μM SB203580, an inhibitor of p38, and infected with either GFP or MEK6E. Cell lysates were analyzed by probing Western blots with the MKP-1 antibody. *B*, serum-starved HUVECs were treated with 5 μM Ro-31-8220, an inhibitor of MKP-1 expression, prior to infection with GFP or MEK6E. Western blot analysis with the MKP-1 antibody was used to examine effects on MKP-1 expression. *C*, serum-starved HUVECs were treated with 5 μM Ro-31-8220 prior to infection with GFP or MEK6E. Cells were trypsinized and placed in suspension for 30 min prior to replating on fibronectin-coated dishes for the indicated time points. Cell lysates were analyzed by probing Western blots with phospho-ERK (*pERK*) and ERK antibodies. *D*, HUVECs were infected with either the GFP or MKP-1 C/S adenovirus, and cell lysates were analyzed by probing Western blots with the MKP-1 antibody. *E*, serum-starved cells were infected with GFP and MEK6E or co-infected with MEK6E and MKP-1 C/S prior to replating onto fibronectin. Cell lysates were analyzed by probing Western blots with phospho-ERK (*pERK*) and ERK antibodies. Blots are representative of three independent experiments.

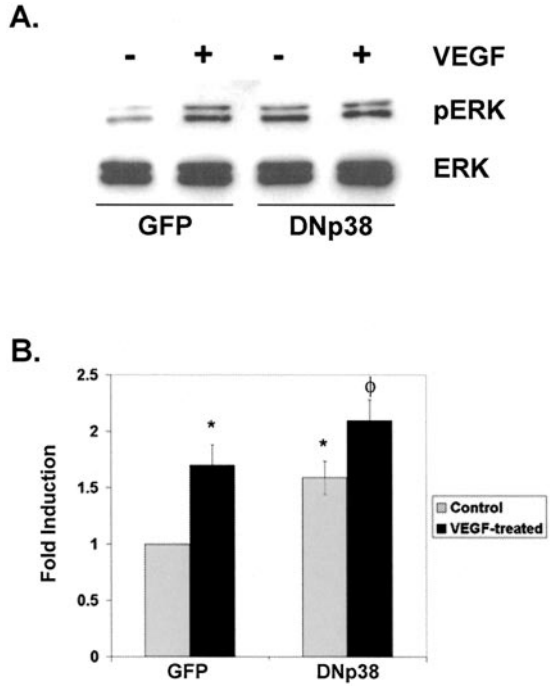

FIG. 7. **DNp38α induces ERK activity and cell proliferation.** *A*, serum-starved HUVECs were infected with either GFP or the DNp38α adenovirus prior to stimulation with VEGF (50 ng/ml) for 10 min. Cell lysates were analyzed by probing Western blots with phospho-ERK (*pERK*) and ERK antibodies. *B*, serum-starved HUVECs were infected with either GFP or DNp38α adenovirus prior to stimulation with VEGF (50 ng/ml) for 16 h. Cells were pulsed with [^{3}H]thymidine for 3 h prior to scintillation counting. Pooled data are plotted as mean ± S.E. *, statistical difference from GFP; ϕ, statistical difference from DNp38 alone ($p < 0.05$) ($n = 9$).

The compound Ro-31-8220 has been shown previously to inhibit MKP-1 expression by an indeterminate mechanism (41). We utilized this compound to determine whether the inhibition of MKP-1 could modulate ERK phosphorylation. Pretreatment with Ro-31-8220 inhibited MEK6E-induced MKP-1 expression (Fig. 6*B*) and produced an increase in the basal ERK activity in MEK6E-expressing cells; however, it was not sufficient to completely restore integrin-induced ERK activity (Fig. 6*C*). These data suggest that MKP-1 is playing a role in the modulation of ERK activity. However, studies have indicated that Ro-31-8220 can also modulate the activity of other signaling molecules (41). Therefore, we sought to confirm these results with a more specific strategy of expressing a catalytically inactive, "substrate-trapping" mutant of MKP-1 (MKP-1 C/S) (42). Infecting endothelial cells with an adenovirus coding for this mutant produced an increase in immunoreactive MKP-1, documenting expression of the mutant protein (Fig. 6*D*). MKP-1 C/S was co-infected with MEK6E prior to replating onto fibronectin. Results show that MKP-1 C/S was also able to elevate basal levels and attenuate the inhibition of integrin-induced ERK activity (Fig. 6*E*). Because MKP-1 can also inhibit p38 activation, we examined the effect of expressing MKP-1 on the level of p38 phosphorylation induced by VEGF. Predictably, expression of the dominant negative MKP-1 raised the basal levels of activated p38. Interestingly, the increased levels stimulated by VEGF returned to the basal levels over a time course similar to that of the controls (Supplemental Fig. 1). Thus, acute regulation of induced p38 activation may be controlled by an independent mechanism. Collectively, these data suggest that induction of MKP-1 expression is a prominent mechanism for regulating total cellular levels of ERK and p38 activation.

To determine whether p38/ERK cross-talk can regulate cellular signaling under conditions where MEK6 is not hyperactivated, we performed the inverse experiment. We examined the effects of DNp38α on ERK activity and proliferation. Cells expressing DNp38α showed increased levels of ERK activity that were comparable with those of control cells stimulated with VEGF (Fig. 7*A*). Surprisingly, inhibition of p38α activity alone was sufficient to increase the basal DNA synthesis rate of the cells (Fig. 7*B*). These data suggest that modulation of p38 activation may be an important element in the normal feedback control of proliferation and that a coordinated balance between p38 and ERK activity is a critical component of cell cycle control.

A second important process required for angiogenesis and repair of the endothelial cell lining following insult is cell migration. Our previous findings, as well as other studies, have indicated an essential role of p38 in VEGF-induced migration of endothelial cells (15, 16). Thus, we investigated the effect of the constitutive activation of p38 on endothelial cell migration using a modified Boyden chamber assay. We found that the activation of p38 was sufficient to enhance cellular migration even in the absence of an added chemokine (Fig. 8*A*). Co-infection with the DNp38α adenovirus was sufficient to reduce MEK6E-induced migration to basal levels (Fig. 8*B*).

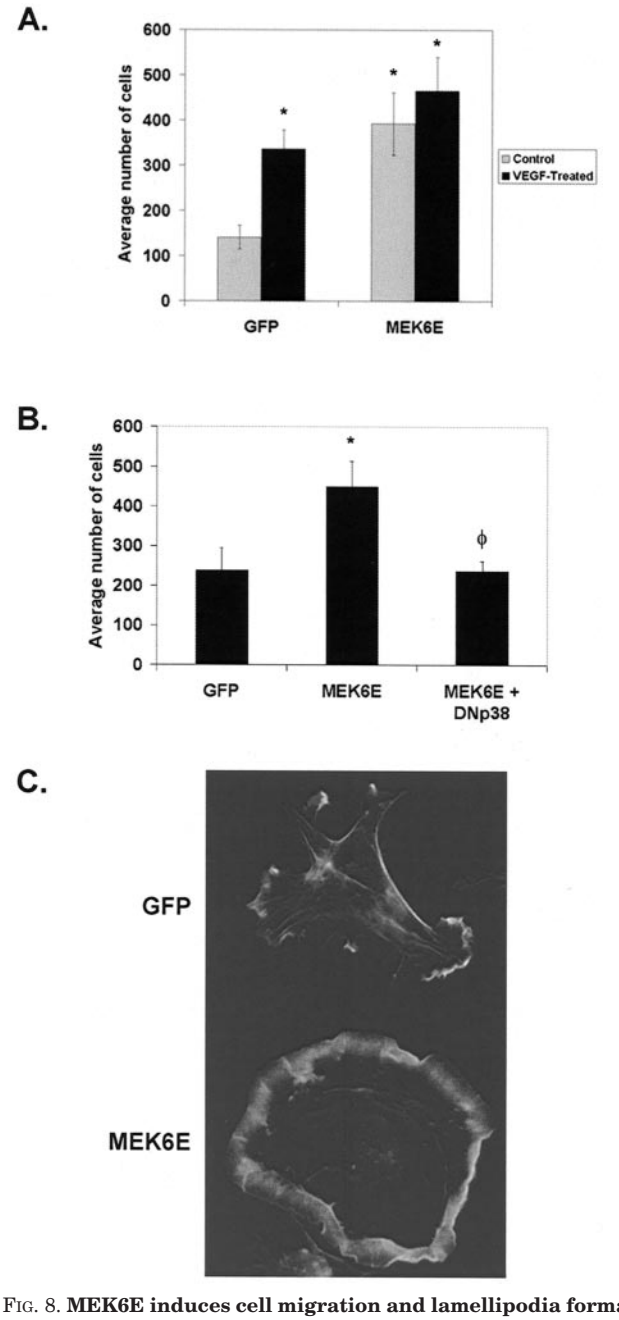

FIG. 8. **MEK6E induces cell migration and lamellipodia formation.** *A*, serum-starved HUVECs were infected with GFP or MEK6E adenoviruses prior to seeding on a Transwell filter with VEGF (25 ng/ml) in the lower chamber to induce cell migration. Pooled data are plotted as mean ± S.E. of three pooled experiments. *, data points are statistically different from GFP ($p < 0.05$) ($n = 6$). *B*, cells were infected with GFP and MEK6E or co-infected with MEK6E and DNp38α prior to seeding on a Transwell filter. Pooled data are plotted as mean ± S.E. *, statistical difference from GFP; ϕ, statistical difference from MEK6E ($p < 0.05$) ($n = 4$). *C*, infected cells were seeded onto gelatin-coated coverslips for 4 h prior to phalloidin staining to visualize actin.

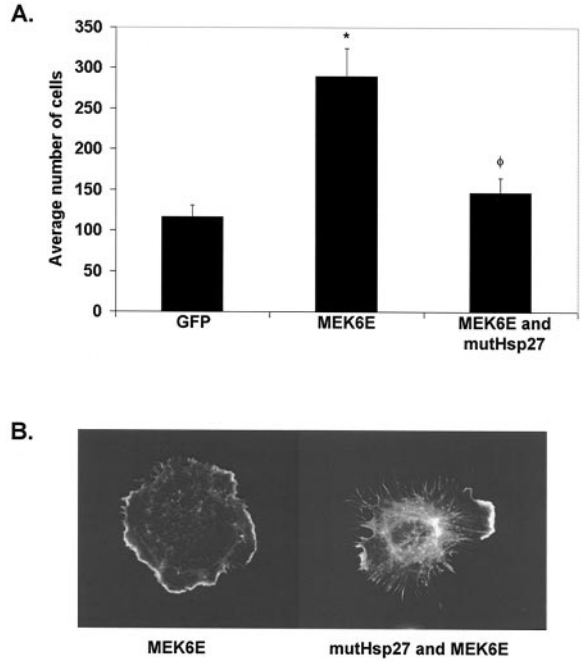

FIG. 9. **MEK6E-induced migration and actin reorganization involves Hsp27.** *A*, cells were infected with GFP, MEK6E, mutHsp27, or co-infected with MEK6E and mutHsp27 prior to seeding on a Transwell filter to measure migration. Pooled data are plotted as mean ± S.E. *, statistical difference from GFP; ϕ, statistical difference from MEK6E ($p < 0.05$) ($n = 6$). *B*, cells were infected with MEK6E or mutHsp27 and MEK6E prior to seeding onto gelatin-coated coverslips and phalloidin staining.

During cell migration, actin polymerization at the leading edge of the cell creates extensions that are critical for cell movement (43–45). We investigated the effect of p38 activation on remodeling of the actin cytoskeleton. Infected cells were plated on gelatin-coated coverslips for 4 h to mimic the conditions of the migration assay prior to phalloidin staining. The MEK6E-expressing cells appear flat and circular with an increase in lamellipodia along the periphery of the cell as compared with the control, GFP-expressing cells, which only have a few discrete cellular extensions (Fig. 8*C*). These changes in actin remodeling induced by p38 activation were much more pronounced under conditions of active remodeling such as a replated cell. In adherent cells, activation of p38 showed a modest enhancement in membrane ruffles in unstimulated cells but, importantly, showed relatively normal induction of stress fibers following stimulation with VEGF (Supplemental Fig. 2).

The downstream effector of p38, Hsp27, is an F-actin-binding protein that localizes to the basolateral membrane and controls F-actin polymerization (46). Studies have implicated Hsp27 as a potential mediator of cell migration (47, 48). We investigated the potential role of Hsp27 in MEK6E-induced migration. A mutant form of Hsp27, mutHsp27, in which the three serine residues have been mutated to alanine to prevent phosphorylation was utilized. The mutHsp27 was expressed at ~10-fold higher amounts than the endogenous levels (data not shown) and was co-infected with MEK6E prior to seeding onto the modified Boyden chamber assay. The mutHsp27 was sufficient to inhibit the MEK6E-driven migration (Fig. 9*A*). Interestingly, mutHsp27 also altered the MEK6E-induced actin phenotype by reducing the number of lamellipodia (Fig. 9*B*). These data suggest a role for Hsp27 in the actin reorganization and migration induced by MEK6E.

DISCUSSION

Several studies have indicated that increased p38 activity is observed in a variety of pathological conditions in which re-endothelialization and angiogenesis are impaired. Therefore, we investigated the effect of p38 activation on two processes critical for neovascularization and vessel repair, namely the proliferation and migration of primary endothelial cells. In the present study we have identified two opposing effects of p38 activity in which activation of p38 inhibits cell proliferation and induces cell migration, whereas inhibition of p38 leads to increases in cell proliferation and inhibits cell migration. Our

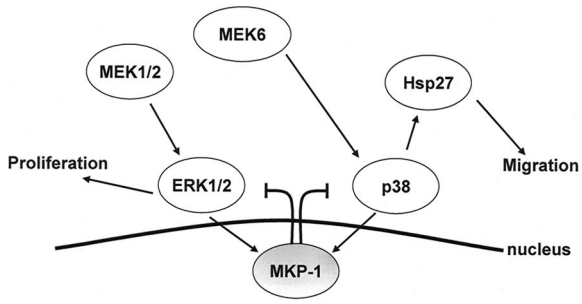

FIG. 10. **Opposing effects of p38 activity.** A potential balance of MAPK activities exists in which inhibition of p38 leads to increases in ERK phosphorylation and proliferation while preventing cell migration. In contrast, the activation of p38 leads to decreases in ERK phosphorylation and proliferation while inducing migration. This balance is regulated by induction of MKP-1 activity.

studies indicate that cross-talk between p38 and ERK may regulate these responses as a consequence of inducing MKP-1 expression. These relationships are summarized in Fig. 10. The elevated levels of p38 activation seen in hypertension, hyperglycemia, and abrasions caused by balloon angioplasty may lead to an imbalance in cellular MAPK signaling, triggering altered or inappropriate induction of MKP-1 with corresponding alterations in endothelial cell proliferation and migration that may contribute to the endothelial dysfunction associated with morbidity in these conditions.

Our data suggest that a coordinated balance between ERK and p38 activity may provide a mechanism to control cell fate decisions. An example of such a balance is found in results showing the ratio of p38 activity to ERK activity predicts tumor dormancy, with higher p38/ERK ratios leading to poor growth (49). A separate study has indicated that p38 activity in breast cancer cells controls invasiveness and motility (50). These studies support our findings of enhanced p38 activity inducing an anti-proliferative, pro-migratory phenotype. It is intriguing that our studies in primary endothelial cells are similar to results in divergent types of tumor cells. This suggests that MAPK cross-talk could be a global mechanism used to balance proliferative and migratory behavior in many cell types.

We have established that sustained activation of p38 leads to significant decreases in ERK activity, whereas MEK1/2 activation is elevated. This suggests an uncoupling of MEK1/2 and ERK. Previous studies have implicated increased expression of phosphatases in the regulation of MAPK cross-talk (36), and our data implicate induction of MKP-1 in this response in endothelial cells. Experiments investigating the effects of MKP-1 directly on endothelial cell proliferation are problematic to interpret because of the basal specificity of MKP-1 for all MAPK members. Although we see a p38-dependent induction of MKP-1, and inhibiting MKP-1 restores levels of ERK phosphorylation, we cannot rule out other parallel regulatory mechanisms. Other potential mediators of p38/ERK cross-talk include regulation by protein phosphatase 1 and protein phosphatase 2A (36, 51). To this end, inhibiting MKP-1 did not prevent dephosphorylation following VEGF-induced p38 activation. This suggests additional mechanisms of regulation that are likely MKP-1 independent.

It seems likely that induction of MKP-1 is a cellular response to dampen chronic p38 activity; however, because this enzyme has dual specificity it dampens both ERK and p38 signaling. Under these conditions the advantage is to the stronger and more persistent signal, in this case p38 activation. To this end recent studies in our laboratory have confirmed that chronic activation of ERK signaling also induces MKP-1 phosphatase levels, but under these conditions it is the induction of p38 signaling that is markedly dampened.[2] Thus, the temporal and spatial regulation of MKP-1 activity may be critically poised to regulate endothelial cell function by balancing cellular MAPK activities.

The mechanisms responsible for enhanced p38 activation in pathological conditions is currently unclear. Higher levels of inflammatory products such as tumor necrosis factor, transforming growth factor-β, interleukin-1, or oxidant radicals, all strong activators of p38, may persistently stimulate the cell. Alternatively, there may be poor p38 dephosphorylation following activation, allowing activation to persist. At least three reasons argue that the enzyme responsible for the acute dephosphorylation of p38 is not MKP-1. 1) MKP-1 is transcriptionally regulated, and the protein requires several hours after stimulation by tumor necrosis factor or other mediators to increase.[2] 2) Inhibiting the MKP-1 activity raised basal levels of p38 activity but did not affect the kinetics of VEGF induction of p38 activation and deactivation. 3) Induction of MKP-1 mRNA and protein was recently described in the atherosclerotic lesions of mice (52), arguing against failed MKP-1 induction as a causal role in p38 elevation. Investigation of other phosphatases that regulate the acute dephosphorylation of p38 should yield insight into whether these regulatory events properly function in a damaged endothelium.

Our study is the first to demonstrate that p38 activation is sufficient to induce endothelial cell migration in the absence of a chemokinetic stimulus. One of the critical steps of cell migration is the polymerization of F-actin at the leading edge of the cell to generate cell extensions such as lamellipodia. A distinct cell morphology accompanied the induction of migration in which the MEK6E-expressing cells have enhanced lamellipodia. Both this unique actin phenotype and the cellular migration were inhibited by expression of a non-phosphorylated Hsp27. Previous studies have demonstrated that phosphorylation of Hsp27 leads to dissociation of multimers and promotes actin polymerization in leading edge lamellipodia (46, 53). The mutHsp27 has been shown to exist almost exclusively as multimers, which presumably bind to actin and inhibit its polymerization (54, 55). Expression of mutHsp27 has previously been shown to inhibit growth factor-induced actin accumulation, normal lamellipodia formation, and migration (47, 48, 56). Interestingly, it does not appear to inhibit phosphorylation of endogenous Hsp27 and may even lead to increased endogenous Hsp27 phosphorylation (47). Taken collectively, these data would suggest that phosphorylation of Hsp27 and formation of lamellipodia may be a consequence of p38 activation, particularly in cells where the actin cytoskeleton is actively remodeling during reattachment.

Several studies, as well as studies in our laboratory, have shown that inhibiting p38 can inhibit stress fiber formation in adherent cells and lead to decreases in cell permeability in some cell systems (57–60). However, our data also indicate that no stress fiber formation accompanies the increase in lamellipodia formation observed in MEK6E-expressing cells replated onto gelatin. Furthermore, activation of p38 with MEK6E is not sufficient to induce stress fiber formation in adherent cells and does not inhibit stress fibers induced by VEGF in adherent cells. These data imply that p38 activation is not sufficient to induce the depolymerization of stress fibers but does suggest that regulation of actin dynamics may be complex and potentially very different in adherent cells compared with those actively challenged to remodel. To account for the differences between adherent cells and cells replated onto adhesive ligands, we propose a two-phase model of actin reorganization.

[2] M. E. McMullen and K. M. Pumiglia, unpublished observations.

In the first phase, p38 activation and Hsp27 initiate actin polymerization and lamellipodia formation. A secondary signal triggered by growth factors and likely involving Rho GTPase triggers the further polymerization of actin as well as actin bundling to promote the formation of stress fibers. This may be accompanied by the formation of Hsp27 oligomers, which stabilize microfilaments (56). Under conditions where actin is largely depolymerized (*e.g.* suspension/reattachment) and p38 is active, the balance of these phases is tipped toward the initial phase and formation of pronounced lamellipodia. This may be because the cell lacks an appropriate spatial scaffold to properly regulate the signals required for initiation of the second phase signals. Regulation of Hsp27 seems to be critical for controlling this balance, as the reintroduction of a non-phosphorylated mutant seems to decrease the membrane protrusions and enhance the formation of microfilaments.

MEK6 has been shown to activate all four isoforms of p38, whereas MEK3 preferentially activates α, δ, and γ (6). We chose MEK6E to activate p38 because MEK6 is not isoform-selective and has been reported to be activated by tumor necrosis factor, one of the cytokines known to be associated with vessel pathology (61, 62). Although we were only able to detect p38α by Western blotting, these results do not conclusively rule out the presence of other isoforms, though several other pieces of data are consistent with this finding. Our results demonstrate nearly complete inhibition of MKP-1 induction by MEK6E at 5 μM SB203580, a concentration others have reported previously as inhibiting cellular effects such as migration (16). At this concentration, SB203580 completely inhibits p38α but only partially inhibits p38β and is without effect on p38γ or δ (63). These data, coupled with our findings using DNp38α, would suggest that p38α is the principle isoform activated and that it is instrumental in controlling proliferation and migration of endothelial cells because the effects of MEK6E expression could be reversed by expression of the DNp38α.

These findings may have broad clinical implications for a variety of disease states exacerbated by poor or abnormal angiogenesis. Many studies have associated increased levels of cytokines or high glucose levels in the bloodstream with increased p38 activity and endothelial dysfunction (1–4). The mechanism controlling endothelial dysfunction is not clearly understood. Our data suggest that prolonged activation of p38 may result in an up-regulation in MKP-1 with corresponding effects on ERK activation. The decrease in ERK activation inhibits endothelial cell proliferation and, ultimately, re-endothelialization and robust neovascularization (2, 64). In addition, the pro-migratory changes we have observed in the actin cytoskeleton may contribute to an increased vascular leak in these patients, further exacerbating the condition. Indeed, a recent report has demonstrated that the administration of p38 inhibitors can enhance the angiogenic response to VEGF *in vivo* while simultaneously decreasing the VEGF-induced increases in endothelial cell permeability (19). Therefore, pharmacological inhibitors of p38 may be a useful class of drugs for modulating the endothelial dysfunction associated with diabetes and other conditions with impaired neovascularization.

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