ATF-1 Mediates Protease-activated Receptor-1 but Not Receptor Tyrosine Kinase-induced DNA Synthesis in Vascular Smooth Muscle Cells*  

Previously we have demonstrated that activation of p38 mitogen-activated protein kinase (MAPK) and induction of DNA synthesis in response to receptor tyrosine kinase (RTK) and G protein-coupled receptor (GPCR) agonists require NADH/NADPH-like oxidase activity in vascular smooth muscle cells (VSMC). Here we tested the role of p38 MAPK in RTK and GPCR agonist-induced DNA synthesis in VSMC. Platelet-derived growth factor (PDGF)-BB and thrombin (RTK and GPCR agonists, respectively) activated p38 MAPK in a time-dependent manner in VSMC. Inhibition of p38 MAPK led to a 50% decrease in the DNA synthesis induced by thrombin but not PDGF-BB. ATF-1 was found to be the predominant member of the cyclic AMP response element (CRE)-DNA complex formed in VSMC in response to PDGF-BB and thrombin, and both agonists induced its phosphorylation. Regardless of this, inhibition of p38 MAPK reduced only thrombin- but not PDGF-BB-induced ATF-1 phosphorylation. Similarly, inhibition of p38 MAPK caused a 50% decrease in thrombin- but not PDGF-BB-induced CRE promoter-dependent transcription. Ectopic expression of an inhibitory anti-ATF-1 single-chain antibody fragment, ScFv, significantly interfered with DNA synthesis induced by thrombin but not PDGF-BB. Together, these results suggest the following conclusions. 1) Both RTK and GPCR agonists activate p38 MAPK and induce CRE promoter-dependent transcription; 2) both RTK and GPCR agonists induce ATF-1 phosphorylation, and ATF-1 is a predominant member in the CRE-DNA complexes formed in response to these agents; and 3) p38 MAPK-dependent ATF-1 phosphorylation and CRE promoter-mediated transcription are associated with GPCR agonist-induced VSMC growth.

Increased vascular smooth muscle cell growth is a contributing factor in the pathogenesis of atherosclerosis and restenosis (1). Increased levels of mitogens such as platelet-derived growth factor (PDGF)1 and fibroblast growth factor (FGF) were reported in atherosclerotic arteries compared with normal (1–4). These mitogens modulate VSMC growth both in an autocrine and paracrine manner in VSMC (1). In addition, in vitro studies have shown that a variety of receptor tyrosine kinase (RTK) and G protein-coupled receptor (GPCR) agonists and of oxidants are potent mitogens to VSMC (5–10), suggesting that many of the molecules that are produced at the site of arterial injury by various cell types can account for the increased VSMC growth during the formation of these lesions. Towards understanding the role of specific mitogens in the induction of VSMC growth during the formation of these lesions, several investigators have used neutralizing antibodies and antisense oligonucleotides (4, 7, 10, 11). Use of neutralizing antibodies against PDGF or FGF resulted only in partial inhibition of VSMC growth and lesion progression (4, 10). Similarly, the use of antisense oligonucleotides against a molecule that appears to be critical in the mitogenic signaling events, such as c-Myc, resulted only in partial inhibition of VSMC growth and lesion progression (11). Thus, inhibition of individual mitogens or proto-oncogenes has so far resulted in minimal reduction in VSMC growth and lesion progression in animal models of atherosclerosis and restenosis.

Identification of molecules that are critical in the signaling pathways of several mitogens may provide more successful targets. Mitogen-activated protein kinases (MAPK) are a group of serine/threonine kinases that are ubiquitously expressed (12–14). These are grouped primarily into three major categories, namely 1) extracellular signal-regulated kinases, 2) Jun N-terminal kinases, and 3) p38 MAPKs (13, 14). Furthermore, differences were observed in the responsiveness of different groups of MAPKs to various external stimuli. Specifically, extracellular signal-regulated kinases have been reported to respond preferentially to agents that induce cell growth and differentiation (15–20), whereas Jun N-terminal kinases and p38 MAPK have been reported to be potently activated by cellular stressors and cytokines (21–24). Despite these differences in responsiveness, cross-talk between different groups of MAPK has been observed in mediating cellular responses to certain agonists (25). In addition, activation of one or all three groups of MAPKs are involved in the regulation of activities of various transcriptional factors, including activator protein 1 (AP-1) and nuclear factor κB (NFκB) (12). Previously we have
demonstrated that activation of p38 MAPK and induction of DNA synthesis in response to RTK and GPCR agonists require NADH/NADPH-like oxidase activity in VSMC (26). The purpose of the present investigation was to study the role of p38 MAPK in the induction of growth by both RTK and GPCR agonists in VSMC. We show that 1) both RTK and GPCR agonists activate p38 MAPK in VSMC; 2) although activation of p38 MAPK is not required for PDGF-BB-induced DNA synthesis, it is involved in thrombin-induced DNA synthesis; 3) thrombin- but not PDGF-BB-induced p38 MAPK activation is required for ATF-1 phosphorylation and CRE promoter-dependent transcription; 4) thrombin-stimulated p38 MAPK-dependent ATF-1 phosphorylation and CRE promoter-mediated transcription are associated with growth in VSMC; and 5) disruption of ATF-1 activity by intracellular expression of an inhibitory anti-ATF-1 single-chain antibody fragment, ScFv, interfered with only thrombin but not PDGF-BB-induced DNA synthesis in VSMC. Together, these findings reveal that ATF-1 plays a role in thrombin- but not PDGF-BB-induced VSMC growth.

MATERIALS AND METHODS

Reagents—Acetyl-coenzyme A, aprotinin, diithiothreitol, HEPES, leupeptin, phenylmethylsulfonyl fluoride, poly(dI-dC), sodium orthovanadate, sodium deoxycholate, and thrombin were purchased from Sigma. Recombinant human PDGF-BB was bought from R&D Systems Inc. (Minneapolis, MN). Anti-c-Fos (SC-052), anti-Fos-B (SC-048), anti-Fra-1 (SC-183), anti-Jun-B (SC-73), anti-Jun-D (SC-74), anti-ATF-1 (SC-270), anti-ATF-2 (SC-187), and anti-CAMP response element-binding protein-1 (CREB-1) (SC-271) antibodies and consensus oligonucleotides for AP-1 (5'-CGGTGTAGCTACCGCAGGAA-3') (SC-2501) and CRE (5'-AGAGATCGCCGTCAGTAG-3') (SC-2504) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Phospho-specific anti-CREB (9191S) and anti-p38 MAPK (9211S) antibodies were obtained from Cell Signaling Technology (Beverly, MA). SB203580 was bought from Calbiochem Corp. (San Diego, CA). Tyrosine protein-kinase isoenzyme was procured from Invitrogen. pCRE-LUC plasmid was from Stratagene (La Jolla, CA). Luciferase assay kit was bought from Promega (Madison, WI). [γ-32P]ATP (3000 Ci/mmol) and [3H]thymidine (20 μCi/μmol) were obtained from PerkinElmer Life Sciences. v-Tris-chloroacetate (1-14)chloramphenicol (85 μCi/μmol) was purchased from Amersham Biosciences.

Cell Culture—VSMC were isolated from the thoracic aortas of 200–300-g male Sprague-Dawley rats by enzymatic dissociation as described previously (5). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin, containing 0.1% (v/v) HEPES. Cultures were maintained at 37 °C in a humidified 95% air and 5% CO2 atmosphere. Cells were growth-arrested by incubation in DMEM containing 1% (v/v) FBS for 24 h and used to perform the experiments unless otherwise stated.

DNA Synthesis—VSMC with and without appropriate treatments were pulse-labeled with 1 μCi/ml [3H]thymidine for the indicated times. After labeling, cells were washed with cold PBS, trypsinized, and collected by centrifugation. The cell pellet was suspended in cold 10% (v/v) trichloroacetic acid and vortexed vigorously to lyse cells. After standing on ice for 20 min, the cell lysate mixture was passed through a glass fiber filter (GF/C, Whatman). The filter was washed once with cold 5% HCl, twice with cold 0.1 M glycine, and once with cold 70% (v/v) ethanol. The filter was rinsed with cold phosphate-buffered saline (PBS) and frozen immediately in liquid nitrogen. Cells were lysed by thawing in 250 μl of lysis buffer (PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/ml phenylmethylsulfonyl fluoride, 1 mg/ml aprotinin, 1 mg/ml leupeptin, and 1 mM sodium orthovanadate) and scraped into 1.5-ml Eppendorf tubes. After standing on ice for 20 min the cell lysates were cleared by centrifugation at 12,000 rpm for 15 min at 4 °C. The protein content of the supernatants was determined using Micro BCA protein assay reagent kit (Pierce). Cell lysates containing equal amounts of protein were resolved by electrophoresis on 10% SDS polyacrylamide gels. The proteins were transferred electrophoretically to a nitrocellulose membrane (Hybond, Amersham Biosciences). After blocking in 10 mM Tris-HCl buffer, pH 8.0, containing 150 mM sodium chloride, 0.1% Tween 20, and 5% (w/v) nonfat dry milk, the membrane was treated with appropriate primary antibodies followed by incubation kit following the supplier's protocol (Invitrogen).

Generation of ScFv-Anti-ATF-1.—The construction of ScFv-anti-ATF-1 has been described previously (27). The plasmid pRSV-KCREB was kindly provided by Dr. Richard H. Goodman (Oregon Health Science Center, Portland, OR).

Transfection and Reporter Gene Assays—VSMC were plated evenly onto 60-mm dishes the day before transfection and grown in DMEM containing 10% (v/v) heat-inactivated PBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were transfected with appropriate plasmid DNA (20 μg/60-mm dish) using calcium phosphate precipitation as described previously (26). Cells were washed with PBS 16 h after transfection and incubated in DMEM containing 0.1% (v/v) FBS for 36 h at 37 °C. Cells were then treated with and without PDGF-BB (20 ng/ml) or thrombin (0.1 unit/ml) for the indicated times, and cell lysates were prepared. Cell lysates normalized for protein were assayed for either CAT activity using [14C]chloramphenicol and acetyl-coenzyme A as substrates or luciferase activity using Luciferase assay system (Promega) and a Turner luminometer (TD-20/20).

Western Blot Analysis—After appropriate treatments, VSMC were rinsed with cold phosphate-buffered saline (PBS) and frozen immediately in liquid nitrogen. Cells were lysed by thawing in 250 μl of lysis buffer (PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/ml phenylmethylsulfonyl fluoride, 1 mg/ml aprotinin, 1 mg/ml leupeptin, and 1 mM sodium orthovanadate) and scraped into 1.5-ml Eppendorf tubes. After standing on ice for 20 min the cell lysates were cleared by centrifugation at 12,000 rpm for 15 min at 4 °C. The protein content of the supernatants was determined using Micro BCA protein assay reagent kit (Pierce). Cell lysates containing equal amounts of protein were resolved by electrophoresis on 10% SDS and 10% polyacrylamide gels. The proteins were transferred electrophoretically to a nitrocellulose membrane (Hybond, Amersham Biosciences). After blocking in 10 mM Tris-HCl buffer, pH 8.0, containing 150 mM sodium chloride, 0.1% Tween 20, and 5% (w/v) nonfat dry milk, the membrane was treated with appropriate primary antibodies followed by incubation.
with horseradish peroxidase-conjugated secondary antibodies. The antigen-antibody complexes were detected using a chemiluminescence reagent kit (Amersham Biosciences).

Statistics—All the experiments were repeated at least three times with similar results. Data on luciferase activity and [3H]thymidine incorporation were presented as mean ± S.D., and the treatment effects were analyzed by Student’s t test. p values < 0.05 were considered to be statistically significant. In the case of CAT assay, EMSA, and Western blot analysis, one representative set of data is shown.

RESULTS

Previously we have demonstrated that activation of p38 MAPK and induction of DNA synthesis in response to RTK and GPCR agonists require NADH/NADPH-like oxidase activity in VSMC (26). Here we have tested the role of p38 MAPK in RTK and GPCR agonist-induced VSMC growth. Lysates of VSMC that were treated with and without PDGF-BB (20 ng/ml) or thrombin (0.1 unit/ml) in the presence and absence of SB203580 (10 μM) for 24 h, and DNA synthesis was measured by pulse-labeling cells with 1 μCi/ml [3H]thymidine for the last 2 h of the 24-h incubation period and counting the trichloroacetic acid-precipitable radioactivity. *, p < 0.01 versus control; **, p < 0.01 versus thrombin treatment.

thrombin (0.1 unit/ml) were analyzed by Western blotting for phosphorylated p38 MAPK using its phosphospecific antibodies. Both PDGF-BB and thrombin activated p38 MAPK in VSMC in a time-dependent manner with a maximum effect of 10-fold at 30 min and declining thereafter (Fig. 1A). To confirm these observations, lysates of VSMC that were treated with and without PDGF-BB (20 ng/ml) or thrombin (0.1 unit/ml) for 30 min were immunoprecipitated with anti-p38 MAPK antibodies and the immunocomplexes were assayed for p38 MAPK activity using recombinant ATF-2 and [γ-32P]ATP as substrates in the presence and absence of SB203580 (10 μM), a potent inhibitor of p38 MAPK. As expected, both PDGF-BB and thrombin increased p38 MAPK activity, and it was completely inhibited by SB203580 (Fig. 1B). To learn whether p38 MAPK plays a role in PDGF-BB- and thrombin-induced VSMC growth, we tested the effect of SB203580 on DNA synthesis induced by these agonists. Quiescent VSMC were treated with and without PDGF-BB (20 ng/ml) or thrombin (0.1 unit/ml) for 30 min were immunoprecipitated with anti-p38 MAPK antibodies and the immunocomplexes were assayed for p38 MAPK activity using recombinant ATF-2 and [γ-32P]ATP as substrates in the presence and absence of SB203580 (10 μM), and DNA synthesis was measured by [3H]thymidine incorporation into trichloroacetic acid-precipitable material. PDGF-BB and thrombin stimulated VSMC DNA synthesis 3–5-fold, and SB203580 inhibited 50% of the thrombin-induced, but not PDGF-BB-induced, DNA synthesis (Fig. 2).

Earlier studies from other laboratories have reported that p38 MAPK plays a role in c-Fos gene induction in response to cytokines and ultraviolet B light in some cell types (28–30).

FIG. 2. Effect of SB203580 on PDGF-BB- and thrombin-induced DNA synthesis in VSMC. Quiescent VSMC were treated with and without PDGF-BB (20 ng/ml) or thrombin (0.1 unit/ml) in the presence and absence of SB203580 (10 μM) for 24 h, and DNA synthesis was measured by pulse-labeling cells with 1 μCi/ml [3H]thymidine for the last 2 h of the 24-h incubation period and counting the trichloroacetic acid-precipitable radioactivity. *, p < 0.01 versus control; **, p < 0.01 versus thrombin treatment.

FIG. 3. Effect of SB203580 on PDGF-BB- and thrombin-induced expression of the Fos and Jun family proteins in VSMC. Equal amounts of protein from VSMC that were treated with and without PDGF-BB (20 ng/ml) or thrombin (0.1 unit/ml) in the presence and absence of SB203580 (10 μM) for 2 h were analyzed by Western blotting for the Fos and Jun family proteins using their specific antibodies.

FIG. 4. Effect of SB203580 on PDGF-BB- and thrombin-induced AP-1-DNA binding activity and its composition. Panel A, equal amounts of nuclear protein from VSMC that were treated with and without PDGF-BB (20 ng/ml) or thrombin (0.1 unit/ml) in the presence and absence of SB203580 (10 μM) for 2 h were incubated with 100,000 cpm of [32P]-labeled AP-1 consensus oligonucleotide probe, and the protein-DNA complexes were separated by PAGE and subjected to autoradiography. In panels B–G, all conditions were the same except that antibodies to the indicated Fos and Jun family proteins were added to the protein-DNA complexes and incubation continued for an additional 2 h and the complexes were separated by PAGE and subjected to autoradiography.
Therefore, to understand the mechanism by which p38 MAPK participates in thrombin-induced DNA synthesis, we studied its role in PDGF-BB- and thrombin-induced expression of the Fos and Jun family proteins. Lysates of VSMC that were treated with and without PDGF-BB (20 ng/ml) or thrombin (0.1 unit/ml) in the presence and absence of SB203580 (10 μM) for 2 h were analyzed for Fos and Jun family proteins using their specific antibodies. PDGF-BB and thrombin induced expression of c-Fos, Fra-1, c-Jun, and Jun-B by 10- to more than 100-fold (Fig. 3). Interestingly, Fos-B expression was induced more potently (100-fold) by thrombin than by PDGF-BB. Other RTK agonists, such as epidermal growth factor (50 ng/ml) and basic FGF (50 ng/ml), also failed to induce the expression of Fos-B (data not shown), indicating that GPCR agonists specifically induce the expression of this proto-oncogene product. Jun-D is expressed constitutively in VSMC, and its levels were increased 3-fold in response to PDGF-BB and thrombin. SB203580, while having no effect on c-Fos, Fos-B, and c-Jun induction, inhibited Fra-1 expression induced by both PDGF-BB and thrombin and Jun-B and Jun-D expression induced by thrombin only. The Fos and Jun family proteins form the transcriptional factor, AP-1, which has been shown to play an important role in cell proliferation, differentiation, and apoptosis (31). Because inhibition of p38 MAPK depleted the levels of Jun-B and Jun-D in thrombin-treated cells, we suspected a role for AP-1 in p38 MAPK-dependent thrombin-induced growth in these cells. To address this we first determined the composition of AP-1 in PDGF-BB and thrombin-treated VSMC. Quiescent VSMC were treated with and without PDGF-BB (20 ng/ml) or thrombin (0.1 unit/ml) for 2 h, and nuclear proteins were isolated. Equal amounts of nuclear protein from control and agonist-treated VSMC were analyzed for AP-1-DNA binding activity by EMSA, using a 32P-labeled AP-1 consensus oligonucleotide probe. AP-1-DNA binding activity was increased by both PDGF-BB and thrombin compared with control (Fig. 4A). Supershift EMSA for each of the Fos and Jun family proteins showed the presence of c-Fos, Fos-B, Fra-1, Jun-B, and Jun-D in the AP-1 complexes formed in response to both PDGF-BB and thrombin, although their levels differed between the two treatments (Fig. 4, B–D, F, and G). Fra-1, Jun-B, and Jun-D were present in the AP-1 complexes formed in response to both PDGF-BB and thrombin. Fos-B was present more abundantly in thrombin-induced AP-1 complexes. Consistent with the Western blot analyses, inhibition of p38 MAPK by SB203580 resulted in reduced amounts of Fra-1 and Jun-B in the AP-1 complexes formed in response to PDGF-BB and thrombin. Previously, we have reported that Jun-B forms the majority of the AP-1 complex in VSMC in response to PDGF-BB and thrombin and in concert with c-Fos drives AP-1-responsive reporter gene expression (26). Because SB203580 significantly reduced the expression of Jun-B and Fra-1 and thereby their levels in AP-1 complexes, we wanted to examine whether these decreases have any impact on PDGF-BB- and thrombin-induced AP-1-responsive reporter gene expression. VSMC were transiently transfected with an AP-1-responsive reporter plasmid, pCOLL-CAT, growth-arrested, treated with and without PDGF-BB (20 ng/ml) or thrombin (0.1 unit/ml) in the presence and absence of SB203580 for 4 h and cell lysates prepared. Cell lysates containing equal amounts of protein were assayed for CAT activity using acetyl-coenzyme A and [14C]chloramphenicol as substrates. As shown in Fig. 5, both PDGF-BB and thrombin induced AP-1-responsive CAT expression in VSMC by 1.8–2.4-fold. Inhibition of p38 MAPK, while causing a 50% reduction in thrombin-induced DNA synthesis, attenuated only the basal but not the PDGF-BB- or thrombin-induced AP-1-responsive reporter gene expression. This result suggests that p38 MAPK mediates thrombin-induced VSMC growth independent of its effects on AP-1.

Earlier studies have reported that MAPKs via phosphorylating CREB/ATF1 play a role in CRE-dependent gene expression (32, 33). To test the role of p38 MAPK in CRE-dependent gene expression and its role in VSMC growth, we next studied the effect of PDGF-BB and thrombin on CRE activity. Quiescent VSMC were treated with and without PDGF-BB (20 ng/ml) or thrombin (0.1 unit/ml) in the presence and absence of SB203580 (10 μM) for 2 h, and nuclear proteins were prepared and analyzed by EMSA and supershift EMSA for CRE-DNA binding activity and CRE constituents, respectively. Both
PDGF-BB and thrombin induced CRE-DNA binding activity, and SB203580 inhibited ~60% of the thrombin-induced, but not PDGF-BB-induced, CRE-DNA binding activity (Fig. 6). Supershift EMSA using anti-ATF-1 antibodies revealed that ATF-1 constitutes most of the PDGF-BB- and thrombin-induced CRE-DNA complexes. Use of anti-CREB-1 or anti-ATF-2 antibodies neither abolished nor caused a supershift of the CRE-DNA binding activity (data not shown). However, the lack of effect of anti-CREB-1 or anti-ATF-2 antibodies to cause a supershift or abolish the CRE-DNA binding activity does not rule out the presence of these transcriptional factors in the complexes. To confirm CRE-dependent transactivation, VSMC were transfected with a CRE-LUC reporter plasmid, growth-arrested, treated with and without PDGF-BB (20 ng/ml) or thrombin (0.1 unit/ml) for 4 h, and cell lysates prepared. Both PDGF-BB and thrombin induced CRE-dependent luciferase activity (Fig. 7). *p < 0.01 versus control; **p < 0.01 versus thrombin treatment.

DISCUSSION

The important findings of the present study are as follows. 1) p38 MAPK plays a selective role in GPCR agonist-induced DNA synthesis in VSMC, and 2) phosphorylation of ATF-1 appears to be the likely mechanism for the selectivity of p38 MAPK. A role for p38 MAPK in cell growth has been reported previously (34). The present study, however, shows that p38 MAPK plays a differential role in the induction of VSMC growth by RTK and GPCR agonists. The most intriguing find-
ing of the present study is the phosphorylation of ATF-1, a transcriptional factor that belongs to the leucine zipper family of proteins (35, 36). ATF-1, like CREB, binds to a consensus motif $5'$-TGACGTCA-3'$ in the promoter region of genes and activates transcription. These transcriptional factors contain a kinase-inducible transactivation domain that possesses consensus phosphorylation sites for several kinases including protein kinase A (37). In fact, several kinases, including calcium calmodulin kinase IV (38) and p90 ribosomal S6 kinase 2 (39), have been reported to phosphorylate and activate CREB. Although CREB/ATF-1 family proteins have been extensively studied for their role in cAMP and Ca$^{2+}$-responsive gene regulation, their role in cell growth is less clear. The absence of ATF-1 in normal melanocytes and its detection in metastatic melanoma cells, however, suggest that this transcriptional factor may be involved in the regulation of cell growth (40). The most convincing evidence for the role of ATF-1 and its related protein CREB in cell growth comes from depletion studies (27, 41, 42). Ectopic expression of single-chain antibody fragment of ATF-1 or dominant negative mutant of CREB-1 inhibited the tumor growth and metastasis of human melanoma cells and the survival of these cells, respectively (27, 41).

CREB/ATF-1 transcription factors form homodimers or heterodimers with members of the Jun family proteins, and they preferentially bind to CRE (31, 43). Despite the presence of both CREB-1 and ATF-1 in VSMC, only ATF-1 was found in the CRE-DNA complexes formed in response to PDGF-BB and thrombin in these cells. This result implies that ATF-1 exists as either homodimers or heterodimers with other transcriptional factors, such as Jun-B or Jun-D. Although both PDGF-BB and thrombin stimulated ATF-1 phosphorylation, only thrombin-induced phosphorylation was sensitive to p38 MAPK. This finding also suggests that ATF-1 phosphorylation induced by PDGF-BB is independent of p38 MAPK. Regardless of the mechanisms of its phosphorylation, ATF-1 is present both in PDGF-BB- and thrombin-induced CRE-DNA complexes. If it was involved in growth induced by both agonists, then inhibition of its activity via ectopic expression of its single-chain antibody should have interfered with the DNA synthesis induced by both PDGF-BB and thrombin and not the latter only. It is possible that, although it is the predominant component in PDGF-BB- and thrombin-induced CRE-DNA complexes, it may exist as heterodimers with different Jun family proteins in response to PDGF-BB and thrombin and that the ATF-1 complex in GPCR agonist-treated cells is involved in growth induction.

PDGF-BB and thrombin also stimulated the phosphorylation of CREB-1 in a p38 MAPK-dependent manner. CREB-1 phosphorylation by PDGF-BB and thrombin, although mediated by p38 MAPK, appears not to be on the path to growth stimulation. The lack of a role for p38 MAPK-dependent CREB-1 phosphorylation in the induction of growth was further confirmed by the inability of its dominant negative mutant to suppress either PDGF-BB- or thrombin-induced DNA synthesis in VSMC. It was recently reported that CREB plays an important role in VSMC differentiation-specific gene regulation (44). This study further showed that CREB is a negative regulator of VSMC growth. The present findings are consistent with these observations.

CREB/ATF-1 act as survival factors in metastatic melanoma cells (27, 41, 42). In addition, the expression of the thrombin receptor (protease-activated receptor-1) is directly correlated with the metastatic ability of human melanoma cells (46, 47). Our observation that thrombin-induced p38 MAPK-dependent...
ATF-1 phosphorylation is involved in DNA synthesis may provide a mechanism by which thrombin receptor (protease-activated receptor-1) contributes to the growth of VSMC and tumor cells. In summary, in the present study we demonstrate for the first time that p38 MAPK plays a selective role in GPCR agonist-induced VSMC DNA synthesis via phosphorylating and activating ATF-1.

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Addendum—While this paper was under review, a study from other laboratories reported that p38 MAPK plays a role in angiotensin II- and 12(S)-hydroxyeicosatetraenoic acid-induced hypertrophy in VSMC via a mechanism involving CREB (45). Thus, the above study and ours have independently demonstrated a role for p38 MAPK in GPCR agonist-induced VSMC growth involving CREB/ATF-1 transcriptional factors.

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