Big Mitogen-activated Protein Kinase 1 (BMK1) Is a Redox-sensitive Kinase

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Mitogen-activated protein (MAP) kinases are a multigene family activated by many extracellular stimuli. There are three groups of MAP kinases based on their dual phosphorylation motifs, TEF, TPY, and TGY, which are termed extracellular signal-regulated protein kinases (ERK1/2), c-j un N-terminal kinases, and p38, respectively. A new MAP kinase family member termed Big MAP kinase 1 (BMK1) or ERK5 was recently cloned. BMK1 has a TEY sequence similar to ERK1/2 but has unique COOH-terminal and loop-12 domains. To define BMK1 regulation, its activation in cultured rat vascular smooth muscle cells was characterized. Angiotensin II, phorbol ester, platelet-derived growth factor, and tumor necrosis factor-α were the strongest stimuli for ERK1/2 but were weak activators of BMK1. In contrast, H₂O₂ caused concentration-dependent activation of BMK1 but not ERK1/2. Sorbitol activated both BMK1 and ERK1/2. BMK1 activation by H₂O₂ was calcium-dependent and appeared ubiquitous as shown by stimulation in human skin fibroblasts, human vascular smooth muscle cells, and human umbilical vein endothelial cells. These findings demonstrate that activation of BMK1 is different from ERK1/2 and suggest an important role for BMK1 as a redox-sensitive kinase.

The mitogen-activated protein (MAP) 1,2 kinase cascade is a multi-substrate signal transduction system in which extracellular stimuli are transduced into intracellular responses. Extracellular stimuli are converted into intracellular responses (1). Extracellular signal-regulated protein kinases (ERKs) 1 and 2 were the first of the ERK/MAP kinase subfamily to be cloned, and these kinases are activated by diverse extracellular stimuli and by intracellular pro-oxidants that induce proliferation or differentiation (2). Other related mammalian MAP kinases have been identified including two ERK3 isoforms (3, 4), ERK4 (5), J un N-terminal kinases/stress-activated protein kinases (JNK/SAPK) (6, 7), and p38 MAP kinase (8). MAP kinases are activated by phosphorylation on T and Y residues within a TXY phosphorylation motif, where X can be Glu (E), Pro (P), or Gly (G). Three classes of dual specificity MAP kinases may be defined based on their motifs (TEY, TPY, and TGY), which we will term ERK1/2, JNK/SAPK, and p38, respectively.

Relative activation of the three classes of MAP kinases is characteristic for particular stimuli. For example, growth factors such as phorbol myristate acetate (PMA) and epidermal growth factor activate ERK1/2 strongly but JNK/SAPK and p38 weakly (10). Hypersmolar stress is a strong stimulus for p38 (8), and this stimulus also activates ERK1/2 and JNK in rat ventricular myocytes (11). In VSMC we have shown that growth factors and angiotensin II (AngII) are powerful activators of ERK1/2 (12). Recently we found that oxidative stress activated ERK1/2 when the oxidative stress was superoxide but not H₂O₂ (13). In other systems, oxidative stress has been demonstrated to activate JNK strongly (14). The specificity for MAP kinase activation is determined, in part, by members of the MAP kinase/ERK kinase (MEK) family, which exhibit unique pairing with downstream MAP kinases. For example, MEK1 and MEK2 activate ERK1/2, MKK3 activates p38, and MKK4 (i.e. SEK1 the murine homolog of MKK4) activates JNK/SAPK. These data suggest that cell-specific and stimulus-specific events regulate the activities of the three classes of MAP kinases.

The specificity of activation of MAP kinases by individual stimuli is reiterated by specific substrates for each class. Common substrates for the MAP kinases are transcription factors that upon phosphorylation may be activated and induce changes in gene expression. ERK1/2 phosphorylate ternary complex factor/Erk-1 on sites essential for transactivation (15), which regulates c-fos induction. JNK/SAPK phosphorylates N-terminal c-j un and increases its transcriptional activating potential (6, 7, 16–18). Activating transcription factor 2 is phosphorylated and activated by both JNK and p38 (19, 20).

A new human MAP kinase gene termed Big MAP kinase 1 (BMK1) or ERK5 was recently cloned by Lee et al. (21) and Zhou et al. (22). Because the primary structure of this MAP kinase is quite unique from ERK1/2 (21), the name BMK1 will be used in this paper. BMK1 has a TEY sequence in its dual...
phosphorylation site like ERK1/2 but has unique C-terminal and loop-12 domains compared with ERK1/2, suggesting that its regulation and function may be different from those of ERK1/2. To define the regulation of BMK1, we have characterized its activation in cultured rat VSMC, which we have previously shown to have robust ERK activity in response to several stimuli. We show here that activation of BMK1 by hormonal and chemical stimuli is clearly distinct from activation of ERK1/2. In particular, BMK1 appears to participate in a redox-sensitive pathway activated by H₂O₂ but not by agonists such as PMA, AngI, PDGF, and TNF-α.

EXPERIMENTAL PROCEDURES

Cell Culture—Vascular smooth muscle cells (VSMC) were isolated from 200–250-g male Sprague-Dawley rats and maintained in 10% calf serum/Dulbecco's modified Eagle's medium as described previously (23). Passage 5–15 VSMC at 70–80% confluence in 100-mm dishes were grown arrested by incubation in 0.4% calf serum/Dulbecco's modified Eagle's medium for 48 h to use. HUVEC were obtained from umbilical veins as described previously (24). Cells at passage 3 were grown in RPMI 1640 medium supplemented with 20% fetal bovine serum and were deprived of growth factors by incubation in serum-free RPMI 1640 containing 0.4% bovine serum albumin for 24 h. Human arterial smooth muscle cells (HASM) and human skin fibroblasts (HSF) were a kind gift from Dr. R. Ross and Dr. J. F. Oram, respectively, and were maintained in subconfluent state as described (25, 26). In brief, human newborn (13 days) arteries were obtained from the thoracic aortas of infants on accidental death and cultured. Normal human skin fibroblasts were grown from explants of punch biopsies of skin from the inner thighs of normal volunteers in plastic tissue flasks containing Dulbecco's modified Eagle's medium plus 10% fetal bovine serum. Both HASM and HSF were maintained in Dulbecco's modified Eagle's medium/0.4% calf serum for 2 days before experiments.

BMK1 Antibody—The peptide sequence used to generate rabbit anti-human BMK1 antibody was keyhole limpet hemocyanin-EGHGMN-PADIE. The keyhole limpet hemocyanin-peptide immunogen was emulsified by mixing with an equal volume of Freund's adjuvant and injected into three to four subcutaneous dorsal sites, for a total volume of 1 ml (0.1 mg of peptide) per immunization. Two weeks after the third boost, blood was allowed to clot, and serum was collected by centrifugation. The anti-peptide antibody titer was determined with an enzyme linked immunosorbent assay with free peptide on the solid phase. The anti-peptide antibodies were negative. The anti-peptide antibodies were negative (25). The assay employed a commercial blocking buffer and preimmune serum (26). Samples were analyzed by 10% SDS-PAGE and Western blot analysis with BMK1 antibodies. A single band of 90 kDa is present. Analysis of cell lysates after immunoprecipitation demonstrated that >90% of BMK1 immunoreactive protein was precipitated. Above equal amounts of protein (5–10 μg) were separated by SDS-PAGE through a gel containing 0.4 mg/ml myelin basic protein (MBP).

RESULTS

Immunodetection of BMK1—An antibody was prepared against the recently cloned MAP kinase, BMK1, as described under "Experimental Procedures" (21). As shown in Fig. 1, immunoprecipitation and Western blot analysis with BMK1 antibody revealed a prominent 110-kDa protein band in cultured rat VSMC. Preimmune serum showed no band except IgG.

BMK1 Is Poorly Activated by the VSMC Agonists AngII, PDGF, and TNF-α—To determine which known VSMC agonists activated BMK1, we stimulated growth arrested VSMC with AngII, PDGF-BB, PMA, and TNF-α. We have previously shown that these agonists are potent stimuli for activation of ERK1/2 (12, 13), which contain a TEF dual phosphorylation site identical to that present in BMK1. The results presented below indicate that activation of BMK1 by these agonists is very different from activation of ERK1/2.

As shown in Fig. 2A, AngII (100 nm) caused only a small activation of BMK1, approximately 2-fold greater than control at 5 min. In contrast, AngII caused a potent activation of ERK1/2 with a 10-fold increase in activity at 5 min. PDGF-BB (10 ng/ml) caused only a small increase in BMK1 (Fig. 2B),
BMK1 is a Redox-sensitive Kinase

**Fig. 2. Angiotensin II and PDGF-BB weakly stimulate BMK1.** Growth arrested VSMC were stimulated with 100 nM Ang II (A) and 10 ng/ml PDGF-BB (B) for the indicated times, cells were harvested, and ERK1/2 and BMK1 activities were determined. Top, ERK1/2 were measured by an in gel kinase assay using MBP as substrate. MBP phosphorylation was detected after SDS-PAGE by autoradiography. Middle, BMK1 activity was measured by an immune complex protein kinase assay using MBP as substrate. MBP phosphorylation was detected after SDS-PAGE by autoradiography. Bottom, the results were quantified by densitometry of autoradiograms using NIH Image 1.49.

**Fig. 3. Phorbol ester and TNF-α weakly stimulate BMK1 activity.** Growth arrested VSMC were stimulated with 200 nm PMA (A) and 10 ng/ml TNF-α (B) for the indicated times, cells were harvested, and ERK1/2 and BMK1 activities were determined as described in the legend to Fig. 2.

**Fig. 4. H2O2 and osmotic stress stimulate BMK1 activity.** Growth arrested VSMC were stimulated with 200 μM H2O2 (A) and 0.4 M sorbitol (B) for the indicated times, cells were harvested, and ERK1/2 and BMK1 activities were determined as described in the legend to Fig. 2.

approximately 1.7-fold at 20 min. In contrast, PDGF-BB was a potent activator of ERK1/2, stimulating an 9-fold increase in activity at 20 min. PMA (200 nm) failed to stimulate BMK1 (Fig. 3A) but caused a 4.5-fold increase in ERK1/2 at 5 min. Finally, TNF-α failed to stimulate BMK1 (Fig. 3B) but caused an 11-fold increase in ERK1/2 activity at 20 min. Thus, these four hormonal agonists, which are potent stimuli for ERK1/2 in VSMC, caused minimal or no activation of BMK1.

BMK1 Is Stimulated by H2O2 and Sorbitol in VSMC—Because agonists known to activate ERK1/2 strongly were weak agonists for BMK1, we determined whether agonists known to activate JNK and p38 kinase could activate BMK1. Oxidative stress has previously been shown to activate JNK (14), and hyperosmolar stress (e.g. 0.4 M sorbitol) has been shown to activate p38 (8). Using the same experimental protocol described for Figs. 2 and 3, we assayed BMK1 activity in response to 200 μM H2O2 and 0.4 M sorbitol. As shown in Fig. 4A, H2O2 was a potent stimulus for BMK1, causing a 3.8-fold increase in activity at 5 min that was sustained for 60 min. In contrast, ERK1/2 was not significantly activated by H2O2. Of interest, sorbitol was a potent stimulus for both BMK1 and ERK1/2 stimulating 10- and 5.7-fold increases in activity at peak time, respectively (Fig. 4B) These data suggest that the regulation of BMK1 may be more similar to JNK and p38 than ERK1/2.

**H2O2 and Sorbitol Stimulate BMK1 in a Concentration-dependent Manner—**We determined the concentration dependence for BMK1 activation by H2O2 and sorbitol. As shown in Fig. 5A, H2O2 stimulation of BMK1 was maximum at 200 μM with half-maximal effect at 50–100 μM. These concentrations are similar to those previously reported for H2O2-mediated stimulation of c-fos mRNA and DNA synthesis in VSMC (30, 31). Sorbitol also stimulated a concentration-dependent increase in BMK1 activity, which was maximal at 0.8 M (Fig. 5B).

**H2O2 Stimulation of BMK1 Kinase Activity Is Calcium-dependent in VSMC—**We previously found that H2O2-mediated c-fos expression was dependent on both calcium and protein kinase C (30). Because BMK1 appeared not to be activated by protein kinase C-dependent mechanisms (Fig. 3A), we determined whether BMK1 activation by H2O2 was calcium-dependent. To deplete intracellular calcium, we used thapsigargin (10 μM for 10 min). Following thapsigargin treatment, H2O2 was no longer able to stimulate BMK1 (Fig. 6). We also used BAPTA-AM to chelate intracellular calcium as previously reported (28). However, BAPTA-AM treatment caused a significant increase in BMK1 activity in unstimulated cells, confounding analysis of the results (not shown). Based on these findings it appears that calcium-dependent mechanisms are likely to be involved in regulation of BMK1 in VSMC.

**BMK1 Is Activated by H2O2 in Several Cell Types—**To determine whether activation of BMK1 by H2O2 was a ubiquitous characteristic, we determined the response to H2O2 in several different cell types. Cell lysates were prepared from HUVEC, HASM, HSF, and RASM cells, and Western blot analysis was performed. As shown in Fig. 7A, a band of 110 kDa was present in all cell types studied with the greatest relative expression in HSF and RASM. In addition, a band of 112 kDa was present in the HASM. Next the response to H2O2 was determined. As shown in Fig. 7B, exposure to 200 μM H2O2 for 5 min stimulated increases in BMK1 activity in both HSF and RASM. Smaller responses were observed in HUVEC and HASM. Thus
Raingeaud et al. (22) cloned a new member of the MAPK/ERK kinase family termed MEK5. This kinase interacted with a kinase identical to BMK1 in the yeast two hybrid screen, which was termed ERK5 by these authors. They showed that MEK5 specifically interacted with BMK1 and that MEK5 was unable to interact with BMK1, suggesting that the MEK5/ERK1 pathway and the MEK5/BMK1(ERK5) pathways have different functions. The results of the present study support their concept. There also appear to be important differences in the activation of BMK1 compared with JNK and p38. For example, Raingeaud et al. (20) showed that TNF-α was a powerful stimulus for both JNK and p38 in Hela cells, whereas we observed no activation of BMK1 in VSMC treated with TNF-α. Thus the upstream kinases that regulate MKK3 and MKK4 are likely different from those that regulate MEK5.

The fact that H₂O₂ was able to activate BMK1 but not ERK1/2 is of particular interest. We have previously demonstrated that oxidative stress, generated by xanthine and xanthine oxidase, stimulates VSMC DNA synthesis (32). We also showed that H₂O₂ was able to stimulate c-fos expression (33). However, H₂O₂ was unable to activate ERK1/2 (13), suggesting that another kinase pathway was responsible for H₂O₂-mediated gene expression. BMK1 appears a likely mediator based on its rapid activation by H₂O₂ (peak at 5 min), its concentration dependence (peak at 200 μM H₂O₂, similar to the peak effect on c-fos induction), and its ability to stimulate BMK1 in multiple cell types. Thus BMK1 is a new candidate as a redox-sensitive kinase.

We previously showed in VSMC that H₂O₂ and superoxide induced proto-oncogene mRNA expression in a protein kinase C-dependent manner (32, 33). In addition, activation of ERK1/2 by superoxide is protein kinase C-dependent. In contrast, BMK1 was not activated by PMA, suggesting that a protein kinase C-independent pathway may be involved. Other investigators have reported that H₂O₂ and superoxide cause myocardial injury with intracellular calcium overload (34). Depletion of intracellular calcium stores by thapsigargin treatment caused nearly complete inhibition of BMK1 activation by H₂O₂. Thus calcium-dependent tyrosine kinases, such as the recently described PYK2 (35), may be important upstream activators of BMK1. Finally, previous investigators have suggested that Src may be an upstream mediator of redox-sensitive signal transduction (14). Future work will be necessary to identify upstream mediators of H₂O₂-stimulated BMK1 activity.

In summary, we have shown that BMK1 is present in VSMC and activated by both H₂O₂ and hyperosmolar stress. The hormonal and chemical mediators that activate BMK1 clearly differ from the mediators that activate ERK1/2, suggesting that these two classes of MAP kinases serve different intracellular functions. The exciting finding that BMK1 is activated by H₂O₂, whereas ERK1/2 are not suggests that BMK1 may represent a new class of redox-sensitive kinases.
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BMK1 Is a Redox-sensitive Kinase