c-Src Is Required for Oxidative Stress-mediated Activation of Big Mitogen-activated Protein Kinase 1 (BMK1)*

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Big mitogen-activated kinase 1 (BMK1) or extracellular signal-regulated kinase-5 (ERK5) has recently been identified as a new member of the mitogen-activated protein kinase family. We have shown that BMK1 is activated to a greater extent by H$_2$O$_2$ than growth factors, suggesting that in comparison with other mitogen-activated protein kinase family members, BMK1 is a redox-sensitive kinase. Previous investigations indicate that the tyrosine kinase c-Src mediates signal transduction by reactive oxygen species, including H$_2$O$_2$. Therefore, the role of Src kinase family members (c-Src and Fyn) in activation of the BMK1 by H$_2$O$_2$ in mouse fibroblasts was studied. An essential role for c-Src was suggested by four experiments. First, H$_2$O$_2$ stimulated c-Src activity rapidly in fibroblasts (peak at 5 min), which preceded peak activity of BMK1 (20 min). Second, specific Src family tyrosine kinase inhibitors (herbimycin A and CP-118,556) blocked BMK1 activation by H$_2$O$_2$ in a concentration-dependent manner. Third, BMK1 activation in the response to H$_2$O$_2$ was completely inhibited in cells derived from mice deficient in c-Src, but not Fyn. Finally, BMK1 activity was much greater in v-Src-transformed NIH-3T3 cells than wild type cells. These results demonstrate an essential role for c-Src in H$_2$O$_2$-mediated activation of BMK1 and suggest that redox-sensitive regulation of BMK1 is a new function for c-Src.

The MAP$^1$ kinases are activated by diverse stimuli to transduce signals from the cell membrane to the nucleus (1). Three MAP kinase groups may be defined based on their dual phosphorylation motifs, TEY, TPY, and TGY, which we will term ERK1/2 (2), c-Jun N-terminal kinase (JNK/SAPK) (3, 4), and p38 (5), respectively. Each MAP kinase group has relatively distinct upstream activators and substrate specificities. For example, growth factors and phorbol esters readily activate ERK1/2, but have little effect on JNK/SAPK; while UV irradiation and anisomycin are much better activators of JNK/SAPK than of ERK1/2 or p38. Similarly, ERK1/2 phosphorylates ternary complex factor/Eli-1 (6), while the transcription factor c-Jun is a specific substrate for the JNK/SAPK signaling pathway (3, 4). Common pathways also exist as shown by the fact that both JNK/SAPK and p38 are activated by apoptosis (7), and both kinases phosphorylate and regulate activating transcription factor 2 (8, 9).

A new MAP kinase family member termed BMK1 or ERK5 was cloned recently (10, 11). BMK1 has a TEY sequence in its dual phosphorylation site, like ERK1/2, but it has unique carboxyl-terminal and loop-12 domains compared with ERK1/2. We have shown that activation of BMK1 in rat aortic smooth muscle cells is distinct from activation of ERK1/2. In particular, BMK1 participates in a redox-sensitive pathway activated by H$_2$O$_2$ but not by agonists such as phorbol ester, angiotensin II, platelet-derived growth factor, and tumor necrosis factor-α (12). Specific activation of BMK1 by oxidative stress suggests that BMK1 may represent a unique redox-sensitive kinase compared with other MAP kinase family members.

Reactive oxygen species, including H$_2$O$_2$, O$_2^-$, and OH$^-$, have been implicated in the pathogenesis of cardiovascular disease, especially atherosclerosis and hypertension (13–15). Recently, it has been shown that these molecules and growth factors stimulate similar intracellular signal events including activating kinases such as c-Src and ERK1/2 (16, 17). We have demonstrated that BMK1, a member of the MAP kinase family, is specifically activated by oxidative and osmotic stress (12). Thus the upstream signal mechanisms by which H$_2$O$_2$ activates BMK1 should provide valuable insights into pathways of redox-sensitive signal transduction. Previous studies have shown that c-Src is involved in signal events stimulated by reactive oxygen species (16). Several receptors that lack intrinsic tyrosine kinase activity stimulate tyrosine phosphorylation through association with Src family kinases such as Lck, Lyn, and Fyn (18). To determine whether Src family kinases are involved in H$_2$O$_2$-mediated activation of BMK1, we investigated the role of Src kinases in cultured mouse fibroblasts using both specific Src inhibitors and cells derived from animals deficient in c-Src or Fyn. We show here that activation of BMK1 by H$_2$O$_2$ is positively regulated by c-Src, but not Fyn. Thus, the c-Src-BMK1 signaling pathway may represent a new redox-sensitive mechanism in fibroblasts.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Culture**—The Src+/+, and Fyn+/+ fibroblasts were isolated from mouse embryos, which were homozygous for disruption in Src or Fyn gene, and were immortalized with large T antigen (19, 20). Cells were kindly provided by Sheila M. Thomas (Fred Hutchinson Cancer Center, Seattle). NIH-3T3 cells and v-src-transformed NIH-3T3 cells were kindly provided by David Shalloway (21). Fibroblasts were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum as described previously (19). Cells 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 prior to use.

**Immunoprecipitation and Western Blot Analysis**—After treatment, the cells were washed with PBS, harvested in 0.5 ml of lysis buffer (50

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* The abbreviations used are: MAP kinase, mitogen-activated protein kinase; BMK1, big mitogen-activated protein kinase; JNK, c-Jun N-terminal protein kinase; PAGE, polyacrylamide gel electrophoresis; SAPK, stress-activated protein kinase; PBS, phosphate-buffered saline.
BMK1 Activation by c-Src in Fibroblasts

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To explore the role of c-Src in H2O2-mediated signal transduction in fibroblasts, we used the carboxyl-terminal antibody, which recognizes the TEY phosphorylation. A similar band shift has been reported for the p42 and p44 MAP kinases when they undergo phosphorylation on T and Y (24).

Previous investigators have suggested that c-Src may be an upstream mediator of redox-sensitive signal transduction (16). To explore the role of c-Src in H2O2-mediated signal transduction in fibroblasts, we measured c-Src kinase activity by an immune complex assay with BMK1 carboxyl-terminal antibody, which recognizes the TEY phosphorylation. A similar band shift has been reported for the p42 and p44 MAP kinases when they undergo phosphorylation on T and Y (24).

Because it was difficult to quantitate BMK1 kinase activity by Western blot analysis, we performed an in vitro kinase assay based on BMK1 autophosphorylation activity. The time course for BMK1 autophosphorylation (Fig. 2, B and C) was similar to that observed for changes in electrophoretic mobility (Fig. 2A) with peak activation at 20 min. The H2O2-induced increase in BMK1 autophosphorylation was not due to changes in BMK1 protein expression. There was a good correlation between the magnitude and time course of BMK1 autophosphorylation and in vitro phosphorylation of myelin basic protein. Because the autophosphorylation assay was more reproducible and of greater magnitude it was chosen for subsequent experiments. The role of autophosphorylation of BMK1 in functional activation is unknown at this time.

H2O2 Activates c-Src in Fibroblasts—Previous investigations have suggested that c-Src may be an upstream mediator of redox-sensitive signal transduction (16). To explore the role of c-Src in H2O2-mediated signal transduction in fibroblasts, we measured c-Src kinase activity by an immune complex assay with c-Src autophosphorylation. c-Src activity increased within 2 min in response to 200 μM H2O2 (Fig. 3) with a maximum 3.1 ± 1.0-fold increase 5 min after H2O2 stimulation. No dif-

FIG. 1. Analysis of BMK1 kinase phosphorylation by Western blot mobility shift and autophosphorylation. Growth-arrested mouse fibroblasts were stimulated for 20 min with 200 μM H2O2. Cell lysates were prepared and BMK1 immunoprecipitated. An immune complex protein kinase assay using [γ−32P]ATP was performed, protein was separated on 8% SDS-PAGE, and then analyzed by Western blotting (A) and subsequent autoradiography for autophosphorylation (B). Hyperphosphorylated (pBMK1) and unphosphorylated (BMK1) forms of BMK1 are indicated. Note: to achieve adequate separation, the gel front was run for 4 h after the dye front had reached the bottom.

**RESULTS**

BMK1 Kinase Phosphorylation Is Associated with a Mobility Shift—As reported previously (10), BMK1 resembles other members of the MAP kinase family in that it has a conserved TXY phosphorylation motif (specifically TXY) that upon dual phosphorylation is associated with stimulation of kinase activity. However, the highest molecular weight band has the same apparent molecular weight as the autophosphorylated protein band shown in subsequent figures, suggesting that these BMK1 proteins have undergone TEY phosphorylation.}

Because it was difficult to quantitate BMK1 kinase activity by Western blot analysis, we performed an in vitro kinase assay based on BMK1 autophosphorylation activity. The time course for BMK1 autophosphorylation (Fig. 2, B and C) was similar to that observed for changes in electrophoretic mobility (Fig. 2A) with peak activation at 20 min. The H2O2-induced increase in BMK1 autophosphorylation was not due to changes in BMK1 protein expression. There was a good correlation between the magnitude and time course of BMK1 autophosphorylation and in vitro phosphorylation of myelin basic protein. Because the autophosphorylation assay was more reproducible and of greater magnitude it was chosen for subsequent experiments. The role of autophosphorylation of BMK1 in functional activation is unknown at this time.
A difference in c-Src protein expression was observed in lysates from control and H2O2-stimulated cells as determined by immunoprecipitation and Western blot analysis with anti-Src antibody.

Both Herbimycin A and CP-118,556 Inhibit BMK1 Activation by H2O2—To determine whether c-Src is an upstream signaling mediator of BMK1, we studied the effect of two different inhibitors, herbimycin A and CP-118,556, on H2O2-mediated BMK1 activity (Fig. 4). These two reagents have been shown to exhibit specificity for inhibition of c-Src (25, 26). Herbimycin A, a benzoquinone ansamycin antibiotic, inhibits Src family kinases by covalent interactions with sulfhydryl groups (25) and by disrupting Src interactions with heat shock proteins (especially HSP90) (27). CP-118,556, a pyrazolopyrimidine, interacts specifically with Src family kinases and is a competitive inhibitor of ATP (25). CP-118,556 inhibits Src family kinases preferentially compared with ZAP-70, JAK2, and the epidermal growth factor receptor (25), although the relative specificity for inhibition of individual Src family kinases is not established. Herbimycin A and CP-118,556 caused a concentration-dependent inhibition of H2O2-mediated BMK1 activation with approximate IC50 values of 0.3 and 1 μM, respectively.

Activation of BMK1 in Response to H2O2 Is Dependent on c-Src, but Not Fyn—The ability of herbimycin A and CP-118,556 to inhibit BMK1 activation by H2O2 suggested an important role for c-Src. To verify that activation of BMK1 occurred by a c-Src-dependent mechanism, we utilized cells derived from mice deficient in c-Src kinase family members (19). There was no immunoreactive c-Src in Src−/− cells, while immunoreactive Fyn was expressed equally (or to a greater extent) than in wild type cells (Fig. 5, top). Likewise, there was no immunoreactive Fyn in (Fyn−/−) cells, while there was no change in expression of c-Src in Fyn−/− cells compared with the wild type cells (Fig. 5, top). H2O2 stimulated BMK1 activity in wild type fibroblasts that was maximal at 20 min (Fig. 6, right). In contrast, in (Src−/−) fibroblasts, H2O2 failed to stimulate BMK1 activity at any time (Fig. 6, left). Interestingly, in
Fyn knock-out (Fyn−/−) cells were harvested and Western blot analysis was performed on whole cell lysates using anti-Src antibody (top: left panel) and anti-Fyn antibody (top: right panel). Total cell lysates from non-transformed NIH-3T3 (WT) and v-Src-transformed NIH-3T3 (v-Src) cells were immunoprecipitated with anti-Src antibody. Protein precipitates were analyzed by immunoblotting with anti-Src antibody (bottom panel).

Western blot analysis of total cell lysates showed that adhesion to fibronectin (a ligand for \( \alpha_{3,4,5,\beta_1}, \alpha_{\beta_3}, \alpha_{\beta_5}, \) and \( \alpha_{\beta_6} \) integrins) stimulated tyrosine phosphorylation of 72–75- and 110-kDa proteins, and there was no significant difference in the tyrosine phosphorylation of these proteins in Src−/− cells compared with the wild type cells (Fig. 8). To investigate the role of Src further we studied NIH-3T3 wild type (WT-3T3) and v-Src-transformed (v-Src-3T3) cells. There was significantly more immunoreactive Src in the v-Src cells (Fig. 5, bottom). In addition, there was significantly greater BMK1 activation (7.1 ± 1.4-fold increase) in the v-Src cells compared with wild type fibroblasts (Fig. 9).

The major finding of this paper is that \( \text{H}_2\text{O}_2 \)-mediated BMK1 activation requires c-Src. Redox-sensitive regulation of BMK1 is thus a new function for c-Src. Data that support an essential role for c-Src in \( \text{H}_2\text{O}_2 \)-mediated BMK1 activation include the following. 1) The time course for c-Src activation was rapid (peak at 5 min) and preceded BMK1 activation (peak at 20 min). 2) c-Src inhibitors, herbimycin A and CP-118,556, blocked BMK1 activation by \( \text{H}_2\text{O}_2 \) at concentrations consistent with a specific effect on c-Src. 3) In Src−/− fibroblasts, there was no BMK1 activation in response to \( \text{H}_2\text{O}_2 \). In contrast, in Fyn−/− fibroblasts, \( \text{H}_2\text{O}_2 \) stimulated BMK1 activation significantly. 4) In v-Src-transformed NIH-3T3 cells BMK1 activation was increased relatively to wild type fibroblasts. Our results are the first to show that c-Src, but not Fyn, is involved specifically in oxidative stress-mediated BMK1 activation.

Previous investigators have suggested that c-Src may be an upstream mediator of redox-sensitive signal transduction based on findings with UV irradiation (16). There are at least nine members of the Src family of cytoplasmic protein kinases. Three family members (c-Src, Fyn, and Yes) are expressed...
ubiquitously and studies suggest that their functions may be at least partially overlapping (28). In the present study we observed that c-Src, but not Fyn, was required for H$_2$O$_2$-mediated BMK1 activation. This result suggests that unique activators of c-Src, but not Fyn, are generated by H$_2$O$_2$ in fibroblasts. Future studies will be required to define the precise nature of these activators.

Several investigators (29–32), including our group (17, 33), have suggested that reactive oxygen species regulate cell function by stimulating many of the same signal transduction pathways utilized by growth factors. For example, both platelet-derived growth factor and superoxide activate ERK1/2 in vascular smooth muscle cells, stimulate c-fos and c-myc expression, and increase DNA synthesis (17, 33). Other investigators have shown that reactive oxygen species stimulate increases in intracellular calcium and raise intracellular pH (31). However, several results suggest that activation of BMK1 by reactive oxygen species does not occur via a pathway shared by growth factors. First, we showed previously that activation of BMK1 occurred in response to H$_2$O$_2$ and sorbitol but not to growth factors such as platelet-derived growth factor and phorbol esters (12). Second, growth factors stimulate ERK1/2 in rat aortic smooth muscle cells, while H$_2$O$_2$ does not (17). Finally, growth factors such as angiotensin II and platelet-derived growth factor stimulate paxillin phosphorylation in smooth muscle cells and fibroblasts (34), but H$_2$O$_2$ does not (data not shown). These findings suggest that H$_2$O$_2$ uses a unique signal pathway to activate BMK1 which is different from growth factors.

Activation of BMK1 by H$_2$O$_2$ likely reflects a balance between positive and negative regulators as shown for other members of the MAP kinase family. For example, ERK1/2 is positively regulated by MAP kinase/ERK kinase and negatively regulated by the MAP kinase phosphatase-1. To date the phosphatase responsible for inactivating BMK1 has not been identified. c-Src itself is positively and negatively regulated by dephosphorylation and phosphorylation. One of the residues that appears to be critical for regulation of c-Src is Tyr$^{530}$, which is not present in v-Src. Phosphorylation of Tyr$^{530}$ by Csk (C-Src kinase) family members inhibits c-Src activity (35), whereas dephosphorylation of this residue appears to be an activating mechanism. Phosphorylation of Tyr$^{418}$ in the catalytic domain may be an activating signal, although the kinase(s) responsible have not been identified. Future work, including analysis of Csk function and phosphorylation of specific c-Src residues, will be necessary to identify the mechanisms for c-Src activation in response to H$_2$O$_2$.

In summary, we have shown that c-Src and BMK1 are activated by oxidative stress in fibroblasts. The fact that BMK1 is not activated by growth factors indicates that c-Src-mediated activation of BMK1 represents a growth-independent function of c-Src. Furthermore, the demonstration that H$_2$O$_2$-mediated activation of BMK1 required Src, but not Fyn, suggests that these two Src family kinases serve different intracellular functions with respect to oxidative stress and that the c-Src-BMK1 signaling pathway may involve novel intracellular mediators.

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