BMK1 Mediates Growth Factor-induced Cell Proliferation through Direct Cellular Activation of Serum and Glucocorticoid-inducible Kinase*

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Activation of the mammalian mitogen-activated protein kinase known as BMK1 is required for growth factor-induced cell proliferation. To understand the mechanism by which BMK1 mediates this cellular response, this kinase was used as bait in a yeast two-hybrid-based library screening. Here, we report the identification of serum and glucocorticoid-inducible kinase (SGK) as a cellular protein that physically interacts with BMK1. During growth factor-induced cell stimulation, BMK1 activates SGK by phosphorylation at serine 78. This BMK1-mediated phosphorylation event is necessary for the activation of SGK and, more importantly, for cell proliferation induced by growth factors.

Genetic and biochemical studies have identified the mitogen-activated protein (MAP)1 kinases as central intracellular molecules that deliver signals from activated cell surface receptors to downstream regulatory proteins. These MAP kinases have been conserved in all eukaryotes, ranging from yeast to mammals, and have a universal role in controlling cell growth through the regulation of cell cycle progression (1–6). The rate of cell cycle progression is tightly regulated by both growth factors and stress-related stimuli, and MAP kinases deliver and integrate both types of these extracellular signals to the cell cycle machinery by modulating the phosphorylation state of intracellular proteins. The MAP kinases ERK1/2, JNK1, and p38 control cell cycle progression by regulating either the expression or the activity of key molecules required for G1 to S phase transition. We have previously demonstrated that BMK1/ERK5, the newest member of the MAP kinase family (7–11), is required for growth factor-induced cell proliferation and cell cycle progression (10). Although we have established that the activity of BMK1 is required for the growth factor-mediated entry of cells into the S phase of the cell cycle (10), the downstream effector(s) of this process have not yet been reported.

To investigate the mechanism by which BMK1 mediates the entry of cells into S phase, this kinase was used as bait in a yeast two-hybrid screening of a cDNA library. Here we report the identification of serum- and glucocorticoid-inducible protein kinase (SGK) as a molecule that physically interacts with BMK1. SGK is a serine/threonine protein kinase with significant sequence homology throughout its catalytic domain with protein kinase B, ribosomal protein S6 kinase, cAMP-dependent protein kinase, and members of the protein kinase C family (12). A variety of stimuli, including glucocorticoids, hydrogen peroxide, hyperosmotic stress, serum, and insulin-like growth factor, have been shown to induce both the cellular expression and kinase activity of SGK (12–16). Similar to BMK1, the activity of SGK is closely linked to the G1/S transition of the cell cycle (17). Here, we show that BMK1 activates SGK as a result of growth factor-induced cellular activation through the phosphorylation of serine 78. Moreover, we demonstrate that the BMK1-mediated phosphorylation of SGK is critical for growth factor-induced entry of cells into the S phase of the cell cycle.

EXPERIMENTAL PROCEDURES

Two-hybrid Screening—The NdeI to BamHI fragment encoding full-length BMK1(AEF) was fused in frame with the GAL4 DNA binding domain of pGBK7T (CLONTECH) to create BD/BMK1(AEF). A human epithelial carcinoma cDNA library was separately fused with the GAL4 activation domain (AD) of the vector pGAD-GH (CLONTECH). The yeast strain PJ69-2A was co-transformed with this library along with BD/BMK1(AEF), and transformed yeast were screened for their ability to grow on plates lacking histidine and adenine according to the supplier’s protocols.

Expression Vectors and Recombinant Adenovirus—A full-length cDNA encoding human SGK was obtained from The Integrated Molecular Analysis of Genome and Their Expression (IMAGE) Consortium (clone ID 42669) and was cloned, in frame with the GST sequence, into the mammalian expression vector pEGF (18). All point mutations in the SGK gene were generated by polymerase chain reaction-based mutagenesis as described previously (8, 11). Recombinant BMK1 and MEK5(D) were produced using expression vectors encoding human BMK1 and rat MEK5(D) as described in Ref. 8. To construct recombinant adenovirus, SGK(S78A) was cloned into the Xhol/XbaI site of the vector pShuttle-CMV (Quantum). Recombinant adenoviral particles were generated as described in protocols provided by the supplier.

Copiled in Vitro Kinase Assay—HEK 293T cells were co-transfected with expression vectors encoding FLAG-BMK1 and MEK5(D), a dominant active form of the upstream kinase for BMK1 (8). Activated FLAG-BMK1 protein was purified from HEK 293T cell extracts using anti-FLAG (M2) antibody gel (Sigma) and eluted in kinase buffer containing flag peptide (1 mg/ml). Wild type and mutant GST-SGK proteins were similarly expressed in HEK 293T cells and affinity purified from cell
BMK1 Directly Activates SGK

BMK1, a recently identified mammalian MAP kinase, is activated by diverse stimuli, including hydrogen peroxide, hypomotic stress, and serum factors such as EGF (8, 10, 19). The growth factor-induced activation of BMK1 proceeds through a MAP kinase signaling cascade involving the upstream kinases MEKK3 and MEK5 (11, 20). To identify downstream targets of BMK1 activity, a dominant negative form of BMK1, in which the catalytic TEY site has been mutated to AEF (8), was used as bait in a yeast two-hybrid screen. A total of 2 x 10^7 transformants were screened from a human epithelial carcinoma cDNA library, and 83 positive clones were selected for sequence analysis based on their potential interaction with BMK1. Using the BLAST algorithm and the nucleotide data base at the National Library of Medicine, two clones were found to encode carboxyl-terminal portions of the molecule SGK (Fig. 1A). As shown, the growth of transformed yeast in media lacking histidine and adenine is completely dependent on the presence of sequences from both SGK and BMK1(AEF) in this GAL4 system. One of the clones encodes only the carboxyl-terminal 30 amino acids of SGK, indicating that this small portion of the kinase is sufficient for its interaction with BMK1.

To verify that BMK1 and SGK interact, GST-tagged portions of SGK were expressed in HEK 293T cells along with FLAG-tagged BMK1(AEF). After transfection, cell lysates were prepared, and glutathione-Sepharose was used to affinity purify GST-tagged SGK proteins. BMK1 and SGK co-purified in this system, and this interaction was dependent upon the presence of amino acid residues 401-431 of SGK (Fig. 1C). These results support the findings from the yeast two-hybrid screen and confirm that the COOH-terminal 30 amino acids of SGK are sufficient for its physical interaction with BMK1 in mammalian cells.

To identify putative site(s) in SGK that are phosphorylated by BMK1, we individually mutated the five consensus MAP kinase phosphorylation sites (Ser/Thr) followed by Pro) in SGK to alanine at positions Ser74, Ser78, Thr260, Ser369, and Ser401. All of the mutants, except SGK(S78A), were substrates for BMK1 in an in vitro protein kinase assay, indicating that Ser78 is the site in SGK that is phosphorylated by BMK1, according to Phosphoepitope mapping of wild type SGK protein treated in vitro with BMK1 revealed one phosphoepitope peptide that only contained phosphoserine upon subsequent phosphoamino acid analysis (Fig. 2, A and C). In contrast, this radioactive tryptic peptide was not detected when the mutant SGK(S78A) was used as a BMK1 substrate. These data demonstrate that BMK1 phosphorylates serine 78 of SGK in vitro.

We next examined the effect of BMK1-mediated phosphorylation on the kinase activity of SGK. Although the downstream cellular targets of SGK are currently unknown, a peptide substrate of RAC protein kinase, known as crostide, has been shown to be a substrate for SGK (21). To assess the activity of the SGK mutants in response to activated BMK1, we used crostide in a coupled in vitro protein kinase assay. The activity of all of the SGK mutants was up-regulated 5-fold by BMK1 with the exception of SGK(S78A) whose activity was unaffected.
FIG. 2. BMK1 activates SGK by phosphorylation of Ser\(^78\). A, equal amounts of dephosphorylated wild type or mutant GST-SGK proteins were incubated with activated FLAG-BMK1 protein in an in vitro protein kinase assay, as indicated. Labeled proteins were analyzed by 10% SDS-polyacrylamide gel electrophoresis followed by autoradiography. Western blot analysis using antibodies against GST were performed to verify equal loading of SGK proteins (lower panel). B, phosphopeptide mapping of wild type SGK and SGK(S78A). C, phosphoamino acid analysis of wild type SGK. D, kinase activity of SGK mutants was measured in a coupled in vitro protein kinase assay in the presence or absence of activated BMK1, as indicated. Counts were normalized to those of wild type SGK alone whose activity was taken as 1. Error bars represent the S.D. of kinase assays performed in triplicate. All assays are described under "Materials and Methods." (Fig. 2D). These results show that SGK is activated by BMK1-induced phosphorylation at Ser\(^78\).

As EGF has been shown to be a potent stimulus for BMK1 (10), we evaluated the cellular phosphorylation of SGK as a result of EGF stimulation. To this end, HeLa cells expressing GST-tagged SGK were labeled in vivo with [\(^{32}\)P]orthophosphate and treated with EGF. One EGF-inducible phosphopeptide was detected by tryptic peptide mapping (Fig. 3A). To confirm that the phosphorylation of SGK obtained in vivo and in vitro is identical, we mixed equal radiolabeled counts of SGK from these two sources followed by phosphopeptide mapping. The identical migration of the induced phosphopeptide in the mixture confirmed that SGK is phosphorylated at the same site both in vivo and in vitro. This EGF-induced phosphopeptide was absent when SGK(S78A) was expressed in this experiment, confirming that the in vivo phosphorylation site of SGK is indeed Ser\(^78\) (Fig. 3A). In agreement with this, subsequent phosphoamino acid analysis revealed that the EGF-inducible phosphopeptide of wild type SGK obtained in vivo contained only phosphoserine (Fig. 3B).

The above findings prompted us to evaluate SGK kinase activity as a result of EGF-mediated cell stimulation. EGF induced the kinase activity of wild type SGK about 3-fold but had no effect on the activity of SGK(S78A) (Fig. 3C). These results indicate that phosphorylation of Ser\(^78\) is necessary for growth factor-induced activation of SGK. To confirm that the observed cellular activation of SGK is mediated by BMK1, we tested the effects of dominant negative forms of the MAP kinases BMK1 and p98, BMK1(AEF) and p38(APF), on EGF-induced SGK activation. BMK1(AEF), but not p38(APF), completely abrogated EGF-induced cellular activation of SGK (Fig. 3D). Together, these results confirm a critical role for BMK1 in EGF-induced cellular activation of SGK.

We have shown previously that BMK1 activity is required for growth factor-mediated cell proliferation and progression into the S phase of the cell cycle (10). Therefore, we hypothesized that SGK, as a downstream substrate of BMK1, is involved in mediating this cellular function of BMK1. To test this, MCF10A cells were infected with recombinant adenovirus expressing SGK(S78A), starved in growth factor-deficient medium for 48 h, stimulated with growth factors, and assayed for cell cycle progression 15 h later. The addition of growth factors to both mock and control virus-infected cells caused the fraction of cells in S phase to increase from about 7 to 40% (Fig. 4A). In contrast, expression of SGK(S78A) prevented cells from entering S phase following the addition of growth factors. These results demonstrate that the BMK1-induced activation of SGK is required for mammalian cells to enter the S phase of the cell cycle in response to growth factors.
DISCUSSION

Previous studies in our laboratory have established that BMK1 is required for growth factor-induced cell proliferation and cell cycle progression (10). Herein, we identify SGK as a direct downstream target of BMK1 and show that upon EGF stimulation BMK1 activates SGK through phosphorylation of Ser78. Moreover, we demonstrate that Ser78 of SGK is required for growth factor-induced cell cycle progression, a finding that strongly suggests that BMK1-mediated regulation of SGK activity is critical for this cellular response. Interestingly, others have shown that SGK actively shuttles in synchrony with the cell cycle between the cytoplasm (in G1 phase) and the nucleus (in S and G2/M phase) of the cell (17). Similarly, we have found that upon activation, BMK1 actively accumulates in the nucleus of the cell (8). Thus, it is possible that these kinases are co-localized during cell cycle progression through the physical association of BMK1 with the carboxy-terminal 30 amino acids of SGK.

Members of the 3-phosphoinositide-dependent kinase (PDK) family have been shown to phosphorylate residues within the activation loop of the conserved catalytic domain of protein kinase B, ribosomal protein S6 kinase, and protein kinase Cζ (22–24). Given that the catalytic domain of SGK is homologous to that of these PDK targets (12), SGK was suspected to be a target of PDK phosphorylation. Indeed, two recent studies have reported that the PDKs phosphorylate SGK at Thr256, which resides in the activation loop of the catalytic domain of this kinase (21, 25). However, in these studies mutagenesis of Thr256 did not abrogate the total cellular phosphorylation of SGK, suggesting the existence of other phosphorylation sites within this kinase (25). Herein, we show that SGK is a downstream target of the MAP kinase family member BMK1. In contrast to PDK, changing Thr256 to alanine does not alter the BMK1-induced activation of SGK (Fig. 2D). Conversely, we have shown that changing Ser78 to alanine abrogates the phosphorylation of SGK by BMK1 (Fig. 2D). We have found that the S78A mutation has no effect on PDK-mediated phosphorylation of SGK and have confirmed that the T256A mutation abrogates this event (data not shown). Together, these results demonstrate that PDK1 and BMK1 can independently regulate SGK activity through the phosphorylation of two entirely different sites. It is noteworthy that although Ser78 is located outside the catalytic domain of SGK, there are other examples of kinase activation as a result of phosphorylation outside the catalytic region (26, 27).

In conclusion, SGK appears to be a point of convergence for at least two different signaling pathways. Given the fact that SGK activity is induced by a wide variety of cellular stimuli, it is not surprising that this kinase is regulated by more than one upstream pathway. In this regard, the PDK- and BMK1-mediated activation of SGK need not be mutually exclusive and may in fact act together to coordinate the appropriate activation of SGK under certain physiologic conditions. Until natural cellular substrates for SGK are identified, the mechanism by which the BMK1-induced activation of SGK mediates cell cycle progression in response to growth factors will remain an important area of future investigation.

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