Akt (Protein Kinase B) Negatively Regulates SEK1 by Means of Protein Phosphorylation*

Akt, also known as protein kinase B, is a serine-threonine kinase that plays an important role in a variety of biological processes including cell survival, cell growth, gene expression, and oncogenesis (1). Various peptide growth factors, including insulin and insulin-like growth factor I, induce the activation of Akt as a result of their triggering of the phosphatidylinositol 3-kinase (PI3K) signaling pathway (2–4). The binding of such growth factors to their cell surface receptors results in the recruitment of PI3K to the plasma membrane (4) and the consequent phosphorylation of phosphatidylinositol, which generates phosphatidylinositol 3-phosphate, phosphatidylinositol 3,4-bisphosphate, and phosphatidylinositol 3,4,5-trisphosphate (5, 6). The interaction of these phosphoinositides with the pleckstrin homology domain of Akt is responsible for the translocation of this enzyme from the cytoplasm to the plasma membrane (1, 5, 7), where it is activated as a result of phosphorylation by 3'-phosphoinositide-dependent kinase 1 and 2 (2, 3, 8–10). Akt activated by the growth factor receptor signaling promotes cell survival by phosphorylating and inactivating various pro-apoptotic proteins, including Bad, Forkhead family transcription factors, caspase-9, and ASK1 (4, 11–14). Identification of additional target proteins of Akt should provide greater insight into the precise role of this kinase in the regulation of cell survival and apoptosis.

Akt, also known as protein kinase B, mediates cell survival signaling initiated by various growth-promoting factors such as insulin. Here we report that SEK1 is a target of Akt in intact cells. Insulin inhibited the anisomycin-induced stimulation of both endogenous SEK1 and its substrate c-Jun N-terminal kinase (JNK), but not that of the upstream kinase MEKK1, in 293T cells. The inhibitory action of insulin on SEK1 or JNK1 activation was prevented by the phosphatidylinositol 3-kinase inhibitor LY294002. Expression of a constitutively active form of Akt also inhibited both SEK1 and JNK1 activation, but not that of MEKK1, in transfected 293T cells. Co-immunoprecipitation analysis revealed that endogenous Akt physically interacted with endogenous SEK1 in cells and that this interaction was promoted by insulin. In vitro and in vivo 32P labeling indicated that Akt phosphorylated SEK1 on serine 78. The SEK1 mutant SEK1(S78A) was resistant to Akt-induced inhibition. Finally, activated Akt inhibited SEK1-mediated apoptosis, and this effect of Akt was prevented by overexpression of SEK(S78A). Taken together, these results suggest that Akt suppresses stress-activated signaling by targeting SEK1.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—Human embryonic kidney 293T and human cervical carcinoma HeLa cells were routinely maintained at 37 °C in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. For DNA transfection, cells were plated at a density of 2 × 10^5 cells/100-mm dish, grown overnight, and transfected for 48 h with appropriate expression vectors with the use of LipofectAMINE (Invitrogen) or by the calcium phosphate method (21). The transfected cells were then treated with the indicated agents and harvested for further experiments.

Immunoblot Analysis—Cell lysates were subjected to centrifugation at 12,000 × g for 10 min at 4 °C, and the resulting soluble fraction was subjected to SDS-PAGE. The separated proteins were transferred to a nitrocellulose membrane, which was then incubated for 1 h at room temperature with Tris-buffered saline (pH 7.4) containing 0.1% Tween 20 and 5% nonfat dried milk. The blots were probed with various

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antibodies including rabbit polyclonal antibodies to Akt (New England Biolabs) and a mouse monoclonal antibody to SEK1 (PharMingen). Immune complexes were detected with horseradish peroxidase-conjugated secondary antibodies to rabbit or mouse IgG (Amer sham Biosciences, Inc.) and an enhanced chemiluminescence system (Pierce).

Immunocomplex Kinase Assays—Cultured cells were harvested in a lysis buffer (22), and the resulting cell lysates were subjected to centrifugation at 12,000 × g for 10 min at 4 °C. The soluble fraction was subjected to immunoprecipitation with appropriate antibodies. The resulting immunoprecipitates were rinsed three times with lysis buffer and then twice with 20 mM Hepes buffer (pH 7.4). Immunocomplex kinase assays were performed by incubating the immunoprecipitates for 30 min at 30 °C with 2 μg of substrate protein in 20 μl of a kinase reaction buffer (22). Phosphorylated substrates were resolved by SDS-PAGE, and protein phosphorylation was quantified with the use of a Fuji phosphorimager. Mouse monoclonal antibodies used for immunocomplex kinase assays included those to JNK1 (PharMingen), to SEK1 (PharMingen), to MEKK1 (Santa Cruz Biotechnology), to the hemagglutinin epitope (HA) tag (Roche Molecular Biochemicals), and to the Flag epitope tag (Stratagene). Protein concentration was determined by the Bradford method (Bio-Rad).

Site-directed Mutagenesis—Site-directed mutagenesis of mouse SEK1 was performed with a QuikChange™ kit (Stratagene). The SEK1(578A) mutant cDNA was generated with the oligonucleotides 5′-GAGACTGAGAACACACgcCATTGAGTCATCAGGAAAAC-3′ (mismatches with the wild-type SEK1 template are indicated by lowercase letters).

Metabolic Labeling with 32P—293T cells were transfected to phospho-free Dulbecco’s modified Eagle’s medium (Invitrogen) containing [32P]orthophosphate (100 μCi/ml) and were incubated for 3 h. Where indicated, the cells were treated with 100 nM wortmannin for 30 min and subsequently with 100 nM insulin for 20 min. The cells were lysed and subjected to immunoprecipitation with a mouse monoclonal anti-SEK1 antibody. The immunoprecipitates were subjected to SDS-PAGE on a 12% gel, and phosphorylation of SEK1 was examined with a Fuji phosphorimager.

Apoptotic Cell Death—HeLa cells were transfected with pEGFP together with expression vectors encoding the indicated proteins. At 48 h of transfection, the cells were fixed with 0.25% glutaraldehyde and stained with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI). The DAPI-stained nuclei in GFP-positive cells were analyzed for apoptotic morphology by fluorescence microscopy. The percentage of apoptotic cells was calculated as the number of GFP-positive cells with apoptotic nuclei divided by the total number of GFP-positive cells.

RESULTS

Akt Suppresses the JNK/SAPK Signaling Pathway—To investigate possible effects of Akt on the JNK/SAPK signaling pathway, we first examined whether stress-induced activation of JNK in 293T cells was influenced by pretreatment of the cells with insulin, which activates the PI3K-Akt pathway (2–4). Exposure of 293T cells to anisomycin induced a marked increase in the activity of JNK1, and insulin pretreatment inhibited this effect of anisomycin (Fig. 1A). The inhibitory action of insulin on JNK1 activation was blocked by the PI3K inhibitors wortmannin (Fig. 1A) and LY294002 (data not shown). Furthermore, overexpression of a constitutively active myristoylated Akt (Akt-CA; Ref. 25) resulted in inhibition of anisomycin-induced JNK1 activation (Fig. 1B). These results suggest that the PI3K-Akt pathway mediates the inhibitory action of insulin on JNK1 activation.

SEK1 Is a Target of Akt—The JNK signaling cascade consists of JNK and upstream kinases that include MAP2K such as SEK1/JNKK/MKK4 and MAP3K such as MEKK1 (15, 16, 26–28). To identify the possible target(s) of Akt in the JNK pathway, we first transfected 293T cells with expression vectors encoding Akt-CA and Flag-tagged MEKK1 (Fig. 1C). Exposure of the transfected cells to anisomycin resulted in activation of MEKK1, and the anisomycin-induced MEKK1 activation was not inhibited by overexpression of Akt-CA. In contrast, Akt-CA inhibited the activation of JNK induced by MEKK1, a constitutively active form of MEKK1 (Fig. 1D). Akt-CA also inhibited the ΔMEKK1-induced stimulation of a transcription-stimulating activity of c-Jun (data not shown). Phosphorylation of c-Jun by JNK increases the transcription-stimulating activity of c-Jun (29). Together, these results suggest that Akt inhibits the activity of a component downstream of MEKK1 in the JNK/SAPK signaling pathway.
We next examined the effect of Akt on SEK1 activity in 293T cells transfected with plasmid vectors expressing HA-Akt-CA and GST-SEK1. Exposure of the cells to anisomycin resulted in an increase in SEK1 activity, and this effect was inhibited by overexpression of Akt-CA (Fig. 2A). Overexpressed Akt-CA also inhibited SEK1 activation induced by other cellular stresses including UV light, sorbitol, and hydrogen peroxide (data not shown). In comparison, overexpressed Akt-CA did not affect anisomycin-induced MKK7 activation (Fig. 2B). The effect of insulin on the activities of endogenous SEK1 and MEKK1 in 293T cells was also investigated (Fig. 3). Insulin prevented the anisomycin-induced increase in SEK1 activity, and the inhibitory effect of insulin on SEK1 activation was blocked by 20 μM LY294002 for 30 min prior to insulin treatment. Cell lysates were then analyzed for SEK1, MEKK1, and JNK3 activities (Fig. 3B). The effect of insulin on the activities of endogenous SEK1 and MEKK1 in 293T cells was also investigated (Fig. 3). Insulin prevented the anisomycin-induced increase in SEK1 activity, and the inhibitory effect of insulin on SEK1 activation was blocked by 20 μM LY294002 for 30 min prior to insulin treatment. Cell lysates were then analyzed for SEK1, MEKK1, and JNK3 activities (Fig. 3B).

**Fig. 2.** Overexpression of a constitutively active form of Akt inhibits SEK1 activation in transfected 293T cells. A, 293T cells were transfected with expression vectors encoding HA-Akt-CA and GST-SEK1, as indicated. After 48 h of transfection, the cells were incubated for 20 min in the absence or presence of 10 μg/ml anisomycin. Ectopic GST-SEK1 was isolated from the cell lysates with glutathione-Sepharose beads and assayed for kinase activity with GST-JNK3(K55R) as a substrate. B, 293T cells were transfected for 48 h with expression vectors encoding HA-Akt-CA and MKK7-Flag. The transfected cells were incubated for 20 min in the absence or presence of 10 μg/ml anisomycin, and then cell lysates were subjected to immunoprecipitation with the use of anti-Flag antibody. The resulting immunoprecipitates were examined for MKK7 activity by immunocomplex kinase assay. In A and B, cell lysates were also subjected to immunoblot analysis with the use of anti-GST, anti-HA, and anti-Flag antibodies, respectively.

**Fig. 3.** Insulin suppresses the activation of SEK1, but not MEKK1, in intact cells. 293T cells were untreated or treated with insulin for 20 min, and then incubated for 20 min in the absence or presence of 10 μg/ml anisomycin. Where indicated, the cells were pre-treated with 20 μM LY294002 for 30 min prior to insulin treatment. Cell lysates were then analyzed for SEK1 (A) or MEKK1 activity (B) by immunocomplex kinase assays with antibodies to SEK1 or MEKK1, respectively.

**Fig. 4.** Insulin enhances a physical interaction between Akt and SEK1 in intact cells. A, 293T cells were transiently transfected with expression vectors encoding GST-SEK1 and HA-Akt, as indicated. At 48 h of transfection, the cells were untreated or treated with 100 nM insulin for 20 min. Cell lysates were subjected to immunoprecipitation with anti-HA antibody, and the resulting immunoprecipitates were subjected to immunoblot analysis with anti-GST antibody. Cell lysates were also immunoblotted with antibodies to GST and to HA. B, 293T cells were incubated for 20 min in the absence or presence of 100 nM insulin, and then the cell lysates were subjected to immunoprecipitation with anti-Akt (left panel) or anti-SEK1 antibody (right panel), respectively. The resulting immunoprecipitates were subjected to immunoblot analysis with anti-Akt (left panel) or anti-Akt antibody (right panel). Cell lysates were also immunoblotted with antibodies to SEK1 and Akt, as indicated.

Immunoblot analysis using anti-Akt antibody of the SEK1 immunoprecipitates also showed the insulin-induced increase in the interaction between the two endogenous proteins.

**Akt Phosphorylates SEK1 on Ser78**—Akt phosphorylates its substrate proteins at the conserved sequences of RXRXX(S/T) (4, 7, 30). A consensus Akt phosphorylation sequence (RLRTHS) is present at amino acids 73–78 of mouse SEK1 (or amino acids 75–80 of human SEK1). We therefore determined whether Akt phosphorylates SEK1. We first carried out in vitro phosphorylation experiments with HA-Akt immunoprecipitates (Fig. 5A). HA-Akt immunoprecipitates prepared from im-
FIG. 5. Akt phosphorylates SEK1 on Ser78. A and B, 293T cells were transiently transfected with HA-Akt construct for 48 h and then incubated for 30 min in the absence or presence of 100 nM insulin. Cell lysates were subjected to immunoprecipitation with anti-HA antibody, and the resulting immunoprecipitates were assayed for kinase activity with the following GST-fusion proteins (2 μg protein/assay) as substrates: GST, GST-c-Jun(1–79), GST-JNK3(K55R), and GST-SEK1(K129R) (panel A), or GST-SEK1(K129R) and GST-SEK1(S78A) (panel B). C, 293T cells were labeled for 3 h with [32P]orthophosphate (100 μCi/ml), incubated for 30 min without or with 100 nM wortmannin, and then incubated for 20 min in the absence or presence of insulin. Cell lysates were subjected to immunoprecipitation with anti-SEK1 antibody, and the resulting immunoprecipitates were analyzed by SDS-PAGE and autoradiography. D, 293T cells were transfected with expression vectors encoding HA-Akt-CA and either GST-SEK1(K129R) or GST-SEK1(S78A). After 48 h of transfection, the cells were untreated or treated with 100 nM wortmannin and then incubated for 20 min in the absence or presence of 100 nM insulin and then for an additional 20 min in the absence or presence of 10 μM anisomycin. In A and B, cell lysates were subjected to immunoprecipitation with anti-GST antibody and then the immunoprecipitates were analyzed for SEK1 activity by an immunocomplex kinase assay with GST-JNK3(K55R) as substrate.

Fig. 6. Resistance of SEK1(S78A) to Akt-induced inhibition in intact cells. A, 293T cells were transfected with expression vectors encoding GST-SEK1(S78A) and HA-Akt-CA, as indicated. At 48 h of transfection, the cells were untreated or treated with 10 μg/ml anisomycin for 20 min. B, 293T cells were transfected with plasmid vector expressing GST-SEK1(S78A). After 48 h of transfection, the cells were incubated for 20 min in the absence or presence of 100 nM insulin and then for an additional 20 min in the absence or presence of 10 μM anisomycin. In A and B, cell lysates were subjected to immunoprecipitation with anti-GST antibody and then the immunoprecipitates were analyzed for SEK1 activity by an immunocomplex kinase assay with GST-JNK3(K55R) as substrate.

of site-directed mutagenesis, we showed that replacement of Ser78 of SEK1 with alanine prevented the in vitro phosphorylation of the recombinant protein by HA-Akt immunoprecipitates prepared from insulin-treated cells (Fig. 5B).

We next examined whether Akt phosphorylates SEK1 in vivo. Insulin increased the extent of phosphorylation of endogenous SEK1 protein in 293T cells metabolically labeled with [32P]orthophosphate, and this effect of insulin was blocked by wortmannin (Fig. 5C). We next transiently transfected 293T cells with vectors encoding HA-Akt-CA and either GST-SEK1(K129R) or GST-SEK1(S78A), and then metabolically labeled the transfected cells with [32P]orthophosphate. Expression of Akt-CA resulted in an increase in the extent of phosphorylation of GST-SEK1(K129R), but not in that of GST-SEK1(S78A), in the transfected cells (Fig. 5D).

SEK1(S78A) Is Resistant to Akt-induced Inhibition—We next determined whether replacement of Ser78 of SEK1 with alanine affected the inhibitory action of Akt on SEK1 activity. Exposure of 293T cells transfected with a vector encoding GST-SEK1(S78A) to anisomycin induced the stimulation of the kinase activity of the recombinant protein (Fig. 6). The anisomycin-stimulated kinase activity of GST-SEK1(S78A) was neither inhibited by expression of Akt-CA (Fig. 6A), nor by insulin (Fig. 6B). The Ser78 residue of SEK1 thus appears to be essential for the inhibitory action of Akt.

We next investigated whether the Akt-mediated phosphorylation of SEK1 on Ser78 could affect an interaction between SEK1 and its substrate JNK1 in intact cells. 293T cells were transfected with a combination of plasmid vectors expressing GST-SEK1, JNK1-Flag, and HA-Akt-CA, and a physical interaction between GST-SEK1 and JNK1-Flag in the transfected cells was examined (Fig. 7A). Immunoblot analysis using anti-Flag antibody of the GST pull-down precipitates showed a physical interaction between GST-SEK1 and JNK1-Flag in the transfected cells. The interaction between GST-SEK1 and
JNK1-Flag was abolished in the cells cotransfected with HA-Akt-CA (Fig. 7A). In contrast, HA-Akt-CA failed to suppress the interaction between GST-SEK1(S78A) and JNK1-Flag in the cotransfected cells (Fig. 7B). These data suggest that the Akt-directed phosphorylation of SEK1 on Ser78 interferes with the interaction between SEK1 and JNK, thereby inhibiting SEK1-catalyzed JNK phosphorylation.

Akt Activation Attenuates SEK1-mediated Apoptosis——We next examined whether Akt, by phosphorylating SEK1 on Ser78, could suppress SEK1-mediated apoptosis (Fig. 8A). Transfection of HeLa cells with a vector encoding ΔMEKK1, GST-SEK1(S78A), HA-Akt-CA, and SEKI(K129R). At 48 h of transfection, the cells were fixed and stained with DAPI. Where indicated, the cells were exposed to UV (80 J/m²) after 36 h of transfection. In A and B, GFP-positive cells were analyzed for the presence of apoptotic nuclei with a fluorescence microscope. The results shown are representative of three independent experiments.

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phosphorylation on Ser\(^{78}\) results in a decrease in the binding of SEK1 to JNK1 in cells. Thus, inhibition of the interaction between SEK1 and JNK may be a possible mechanism by which Akt-mediated SEK1 phosphorylation inhibits SEK1 activity. Other possibilities could be also proposed. For instance, Ser\(^{78}\) phosphorylation might directly inhibit the catalytic mechanism of SEK1, or interfere with the activation of SEK1 by a MAP3K. Further studies are needed to clarify these possibilities.

The JNK/SAPK signaling pathway mediates many cellular events including cell death (15–17). Recent studies have suggested that Akt negatively regulates the JNK signaling pathway and JNK-mediated apoptosis (31–34). However, the molecular mechanism by which Akt suppresses the JNK signaling pathway has remained unclear. Activated Akt has been shown recently to phosphorylate and inhibit ASK1, a MAP3K that stimulates JNK and p38 mitogen-activated protein kinase signaling pathways (14). Our data now suggest that SEK1 is another target of Akt in the stress-activated protein kinase pathways. Thus, Akt may tightly regulate stress-activated signals through phosphorylation of both SEK1 and ASK1 in the JNK/SAPK signaling cascade. In summary, our study demonstrates that the inhibition of SEK1 by Akt may be an integral component of the mechanism by which Akt functions as a survival factor and as a negative regulator of the stress-activated signals.

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