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Requirement of the Serine-Threonine Kinase Akt for Heat Treatment-Induced Activation of p70 S6 Kinase

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p70 S6 kinase plays an important role in growth factor-induced translational control and in cell cycle progression. Although the mechanism of p70 S6 kinase regulation is not fully understood, phosphorylation of serine and threonine residues of the enzyme is essential for its activation. The possible role of the serine-threonine kinase Akt in the activation of p70 S6 kinase induced by exposure of cells to heat has now been investigated. Overexpression of a mutant Akt1 (Akt-AA) in which the phosphorylation sites (Thr³⁰⁸ and Ser⁴⁷³) targeted by growth factors are replaced by alanine was shown to exert a dominant negative effect on Akt activation induced by platelet-derived growth factor (PDGF) or by heat treatment in CHO cells. Akt-AA also inhibited p70 S6 kinase activation induced by these stimuli. However, Akt-AA had no effect on the activation of p70 S6 kinase induced by 12-O-tetradecanoylphorbol 13-acetate, which did not stimulate Akt activity in these cells. These data suggest that Akt is required for heat treatment-induced activation of p70 S6 kinase.

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p70 S6 kinase was first identified as an enzyme that catalyzes the phosphorylation of the S6 protein, a component of the 40S subunit of the eukaryotic ribosome (1, 2). Activation of p70 S6 kinase is triggered by various growth factors and is thought to be important for the control of translation of mRNAs that possess a polypyrimidine tract at their 5' ends (3). Inhibition of this kinase by a pharmacological inhibitor (4, 5) or by neutralizing antibodies (6) results in arrest of cells in the G₁ phase of the cell cycle and in inhibition of DNA synthesis, suggesting that it also plays a role in cell cycle progression.

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Activation of p70 S6 kinase by growth factors, such as platelet-derived growth factor (PDGF) or insulin, is accompanied by its phosphorylation on multiple serine and threonine residues (1, 2), and evidence suggests that this phosphorylation is essential for enzyme activation (2, 7, 8). A possible upstream regulator of p70 S6 kinase is Akt (also known as protein kinase B), a serine-threonine kinase that contains a pleckstrin homology domain and is activated in response to various growth factors (9-12). The kinase activities of a fusion construct of Akt with a viral Gag protein and of Akt tagged with a myristoylation signal sequence are greater than that of the wild-type protein, and these constitutively active mutants of Akt stimulate p70 S6 kinase activity in quiescent cells (9, 13). We have recently shown that overexpression of a mutant rat Akt1 in which the sites of ligand-induced phosphorylation (Thr³⁰⁸ and Ser⁴⁷³) are replaced by alanine inhibits insulin-induced activation of endogenous Akt in CHO cells and 3T3-L1 adipocytes (14). Furthermore, insulin-induced activation of p70 S6 kinase was inhibited in the cells expressing this mutant protein (14). Although it is not known whether Akt directly phosphorylates and activates p70 S6 kinase, these observations suggest that signaling through Akt is required, at least in part, for insulin activation of p70 S6 kinase.

Cellular stress, such as that induced by heat treatment, stimulates various protein kinases, including the p38 (HOG1) kinase, c-Jun NH₂-terminal kinase (stress-activated protein kinase), and p70 S6 kinase (15, 16). Akt has also recently been shown to be activated by exposure of cells to heat treatment or hyperosmolarity (17). We have therefore now investigated the possible role of Akt in the activation of p70 S6 kinase induced by heat treatment.

MATERIALS AND METHODS

Cells and antibodies. CHO-Akt cells, which stably express FLAG epitope-tagged rat Akt1, have been described previously (14). Mono-

clonal antibodies to the hemagglutinin (HA) epitope tag (12CA5) or to the FLAG epitope tag were obtained from Boehringer Mannheim and Kodak Scientific Imaging Systems, respectively. Polyclonal antibodies to p70 S6 kinase generated against a synthetic peptide corresponding to amino acids 2 to 23 of the rat enzyme were as described previously (14).

Adenovirus vectors. An adenovirus vector encoding an HA epitope-tagged mutant Akt1 in which Thr³⁰⁸ and Ser⁴⁷³ were replaced by alanine (AxCAAkt-AA) was described previously (14). A control virus encoding β -galactosidase (AxCALacZ) was kindly provided by I. Saito (18). Parental CHO cells or CHO-Akt cells cultured in 6-cm plates were infected with adenovirus vectors at the indicated multiplicity of infection (MOI) as described previously (14). The cells were subjected to experiments 48 h after infection.

Kinase assays. Cells were deprived of serum for 16 to 20 h, exposed to PDGF, 12-*O*-tetradecanoylphorbol 13-acetate (TPA), or heat treatment, and then immediately frozen with liquid nitrogen. Heat treatment was achieved by placing tissue culture plates into a heated, circulating water bath. For p70 S6 kinase assays, the frozen cells were lysed in a solution described previously (14), and the lysates were subjected to immunoprecipitation with polyclonal antibodies to p70 S6 kinase. After extensive washing of the immunoprecipitates, they were assayed for kinase activity with an S6 synthetic peptide (KRRRLSSLRASTSKSESSQK) as substrate, as described previously (14).

To assay the kinase activity of FLAG-tagged Akt, we lysed cells in a solution previously described (14) and subjected the lysates to immunoprecipitation with antibodies to FLAG. After extensive washing of the immunoprecipitates, they were assayed for kinase activity with histone 2B as substrate, as described previously (14). The radioactivity incorporated into histone 2B was determined with a Fuji BAS 2000 image analyzer.

RESULTS

Effects of Akt-AA on Akt Activation Induced by PDGF or Heat Treatment

CHO-Akt cells, which stably express FLAG epitope-tagged Akt1, were incubated in the absence or presence of PDGF (25 ng/ml) for 10 min at 37 °C, lysed, and subjected to immunoprecipitation with antibodies to FLAG. The immunoprecipitates were then assayed for kinase activity with histone 2B as substrate. PDGF induced an approximately fivefold increase in Akt activity (Fig. 1B), consistent with the results of previous studies (9, 10). Infection of CHO-Akt cells with an adenovirus vector encoding HA epitope-tagged Akt-AA (AxCAAkt-AA) inhibited the PDGF-induced increase in Akt activity in a virus dose-dependent manner; inhibition was >80% at an MOI of 20 plaque-forming units (PFU) per cell. The extent of inhibition paralleled the expression of the mutant Akt protein as assessed by immunoblot analysis with antibodies to HA (Fig. 1A).

Incubation of CHO-Akt cells at 45°C for 10 min resulted in an approximately threefold increase in Akt activity (Fig. 1C). Akt-AA inhibited the heat treatment-induced increase in Akt activity in a dose-dependent manner; infection of cells with AxCAAkt-AA at an MOI of 20 PFU/cell abolished the activation of Akt. Infection of CHO-Akt cells at an MOI of 20 PFU/cell with a control virus that encodes β -galactosidase (AxCALacZ)

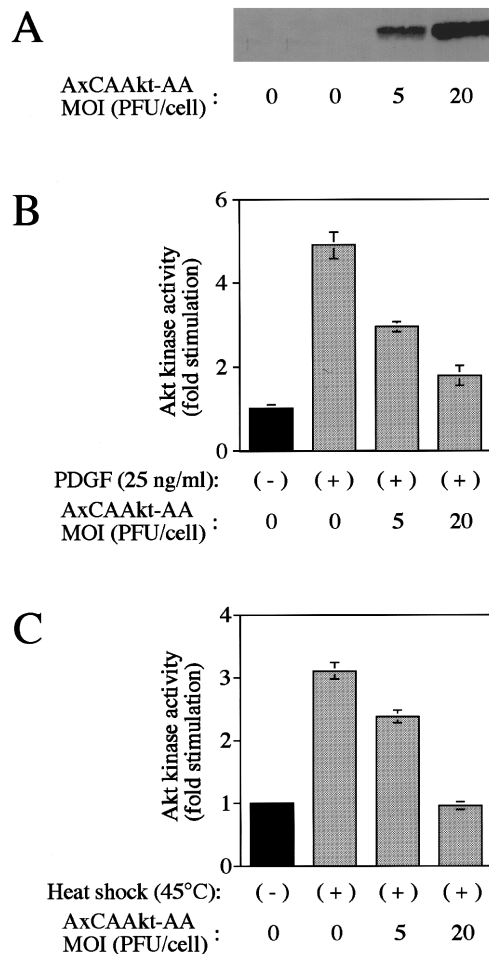


FIG. 1. Effects of Akt-AA on the activation of Akt induced by PDGF or heat treatment in CHO-Akt cells. **(A)** CHO-Akt cells were infected with an adenovirus vector encoding Akt-AA (AxCAAkt-AA) at the indicated MOI (PFU/cell). The cells were subsequently lysed, and the total cell lysates were subjected to immunoblot analysis with antibodies to HA. **(B and C)** CHO-Akt cells infected with AxCAAkt-AA at the indicated MOI (PFU/cell) were incubated for 10 min at 37 °C in the absence or presence of PDGF-BB (25 ng/ml) **(B)** or for 10 min at 37 °C or 45°C **(C)**, lysed, and subjected to immunoprecipitation with antibodies to FLAG. The immunoprecipitates were assayed for kinase activity with histone 2B as substrate. Data are expressed as fold stimulation relative to the value for uninfected, untreated cells and are means $\pm$ SE from three experiments.

had no effect on either PDGF- or heat treatment-induced activation of Akt (data not shown), suggesting that the inhibition of Akt activation by Akt-AA was not due to nonspecific effects of virus infection. PDGF or heat treatment also had no effect on the kinase activity of Akt-AA expressed in CHO cells with the use of AxCAAkt-AA (data not shown).

Effect of Heat Treatment on p70 S6 Kinase Activity

We next examined the effect of heat treatment on p70 S6 kinase activity in CHO-Akt cells. The cells were

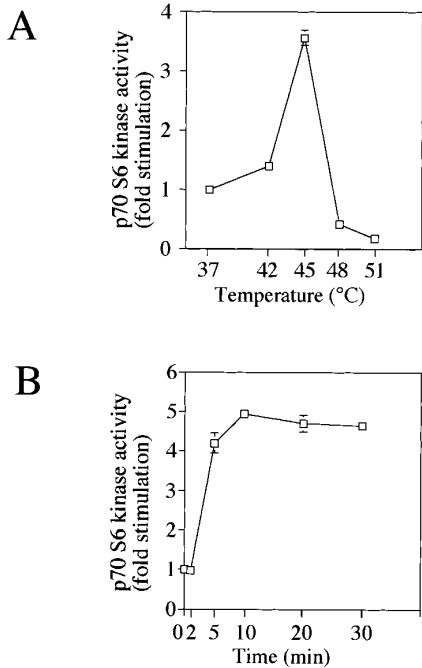


FIG. 2. Effect of heat treatment of p70 S6 kinase activity. CHO-Akt cells were incubated at the indicated temperatures for 10 min (**A**) or at 45°C for the indicated times (**B**), lysed, and subjected to immunoprecipitation with antibodies to p70 S6 kinase. The resulting immunoprecipitates were then assayed for p70 S6 kinase activity. Data are expressed as fold stimulation relative to the values for cells incubated at 37 °C (**A**) or for 0 min (**B**), and are means $\pm$ SE from three experiments.

incubated at various temperatures for 10 min, lysed, and subjected to immunoprecipitation with antibodies to p70 S6 kinase. The immunoprecipitates were then assayed for kinase activity with a synthetic S6 peptide. Although incubation of cells at 42°C had little effect on p70 S6 kinase activity, an $\sim$ 3.5-fold increase in activity was apparent in cells incubated at 45°C (Fig. 2A). Exposure of cells to temperatures of 48° or 51°C resulted in a decrease in p70 S6 kinase activity relative to that of cells incubated at 37°C. We next examined the time course of p70 S6 kinase activation at 45°C; activation was apparent within 5 min, reached a maximum at 10 min, and persisted for at least 30 min (Fig. 2B).

Effects of Akt-AA on the Activation of p70 S6 Kinase Induced by PDGF, Heat Treatment, or TPA

We next investigated the effects of Akt-AA on PDGF- or heat treatment-induced activation of p70 S6 kinase. Infection of CHO-Akt cells with AxCAAkt-AA resulted in a dose-dependent inhibition of the PDGF-induced increase in p70 S6 kinase activity (Fig. 3A); at an MOI of 20 PFU/cell, a dose that inhibited PDGF-induced Akt activation by $>$ 80%, the PDGF-induced increase in p70 S6 kinase activity was inhibited by $\sim$ 50%. Infection of CHO-Akt cells with AxCAAkt-AA also inhibited the

increase in p70 S6 kinase activity induced by heat treatment (45°C); inhibition was $\sim$ 90% at an MOI of 20 PFU/cell (Fig. 3B). Infection with AxCALacZ had no effect on the activation of p70 S6 kinase induced by PDGF or heat treatment (data not shown).

We finally examined the effect of Akt-AA on TPA-induced activation of p70 S6 kinase. Incubation of CHO-Akt cells for 20 min at 37°C with TPA (0.5 μ g/ml) resulted in an approximately threefold increase in p70 S6 kinase activity (Fig. 3C). Infection of the cells with AxCAAkt-AA had no effect on the TPA-induced activation of p70 S6 kinase, even at an MOI of 20 PFU/cell, a virus dose sufficient to inhibit the PDGF- and heat-

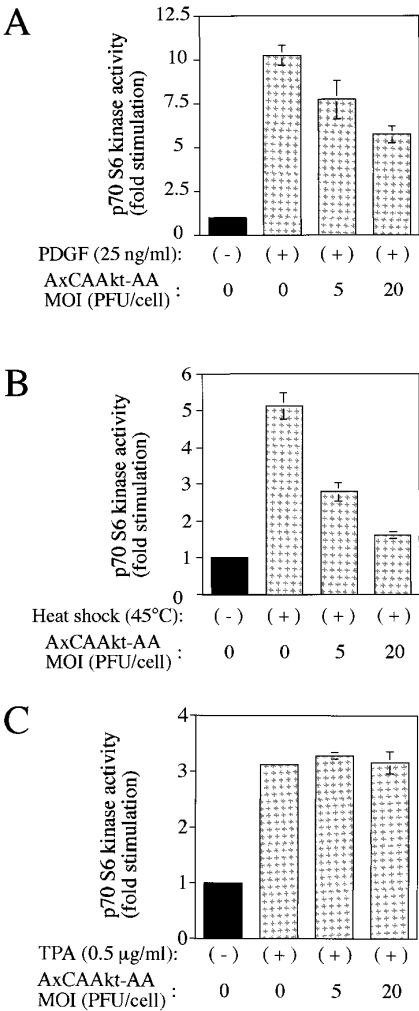


FIG. 3. Effects of Akt-AA on the activation of p70 S6 kinase by PDGF, heat treatment, or TPA in CHO-Akt cells. Cells infected with AxCAAkt-AA at the indicated MOI (PFU/cell) were incubated for 10 min at 37°C with PDGF (25 ng/ml) (**A**), for 10 min at 45°C (**B**), or for 20 min at 37°C with TPA (0.5 μ g/ml) (**C**). The cells were then lysed and subjected to immunoprecipitation with antibodies to p70 S6 kinase, and the immunoprecipitates were assayed for p70 S6 kinase activity. Data are expressed as fold stimulation relative to the value for uninfected, untreated cells and are means $\pm$ SE from three experiments.

induced increases in p70 S6 kinase activity by ~50 and 90%, respectively. Furthermore, TPA did not stimulate Akt activity in these cells (data not shown), suggesting that Akt is not required for TPA-induced activation of p70 S6 kinase.

DISCUSSION

We recently showed that insulin did not stimulate the kinase activity of a mutant Akt1 (Akt-AA) in which the phosphorylation sites targeted by growth factors are replaced by alanine (14). This mutant exerted a dominant negative effect on insulin-induced activation of endogenous Akt in CHO cells and 3T3-L1 adipocytes, whereas a mutant Akt (Akt-K179D) in which Lys¹⁷⁹ in the kinase domain was replaced by aspartate showed little such effect (14). We have now shown that heat treatment also did not stimulate the kinase activity of Akt-AA, and that heat treatment-induced activation of Akt was inhibited by overexpression of Akt-AA in CHO cells. These data suggest that the growth factor-sensitive phosphorylation sites in Akt, Thr³⁰⁸ and Ser⁴⁷³, are also important for the activation of Akt by heat treatment. The newly identified protein kinases PDK1 and PDK2 are thought to mediate the growth factor-induced phosphorylation of Thr³⁰⁸ and Ser⁴⁷³ of Akt, respectively (8, 19-21). These kinases are thus also potential mediators of heat treatment-induced Akt activation.

p70 S6 kinase was originally identified as a protein kinase activated by growth factors, but was subsequently shown also to be activated by various other stimuli, including heat treatment, TPA, sodium arsenite, calcium, and certain amino acids (1, 16, 22-24). The mechanisms by which p70 S6 kinase is activated in response to these stimuli remain less well characterized than that of growth factor-induced p70 S6 kinase activation. Although several protein kinases have been proposed to mediate activation of p70 S6 kinase by growth factors (8, 14, 25), an upstream kinase responsible for heat treatment-induced activation of the enzyme has not been identified. We have now shown that the heat treatment-induced increase in p70 S6 kinase activity was blocked in cells in which Akt activity was specifically inhibited by overexpression of Akt-AA. This observation suggests that Akt is required for p70 S6 kinase activation induced by exposure of cells to heat. Because Akt-AA had no effect on p70 S6 kinase activation triggered by TPA, which did not activate Akt, the inhibitory effect of Akt-AA on heat treatment-induced activation of p70 S6 kinase is likely due to inhibition of specific signaling through Akt.

We have previously shown that overexpression of Akt-AA inhibits insulin stimulation of p70 S6 kinase activity only partially in CHO cells or 3T3-L1 adipocytes, whereas insulin activation of endogenous Akt in these cells is inhibited by ~90% (14). These observa-

tions are consistent with our present data showing that Akt-AA inhibited PDGF-induced activation of p70 S6 kinase by ~50% in CHO cells, whereas the PDGF-induced increase in Akt activity was inhibited by >80%. One possible explanation of these results is that a small increase in Akt activity is sufficient to induce marked activation of p70 S6 kinase. Another possibility is that growth factor-induced activation of p70 S6 kinase is mediated by redundant signaling pathways, so that blocking of the Akt pathway may be insufficient for complete inhibition of p70 S6 kinase activation. The latter hypothesis is consistent with previous observations suggesting that multiple inputs from upstream regulators contribute to the growth factor-elicited activation of this kinase (2, 7, 8). The observation that activation of p70 S6 kinase by heat treatment was almost completely inhibited by overexpression of Akt-AA suggests that this effect of heat treatment is dependent on Akt to a greater extent than is that of growth factors.

In summary, we have shown that Akt participates in some, but not all, signaling pathways that lead to the activation of p70 S6 kinase. Because the activity of p70 S6 kinase is thought to be primarily regulated by phosphorylation, clarification of the phosphorylation sites of the enzyme that are targeted by heat treatment should increase our understanding of the regulation of p70 S6 kinase by this stimulus.

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