CREB Is a Regulatory Target for the Protein Kinase Akt/PKB*

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The nuclear factor CREB stimulates the expression of cellular genes following its protein kinase A-mediated phosphorylation at Ser-133. Ser-133 phosphorylation, in turn, activates target gene expression by recruiting the co-activator CBP. Recent studies showing that CREB and its paralog CREM are required for survival of certain cell types prompted us to examine whether CREB is a nuclear target for activation via the growth factor-dependent Ser/Thr kinase Akt/PKB. When overexpressed in serum-stimulated cells, Akt/PKB potently induced Ser-133 phosphorylation of CREB and promoted recruitment of CBP. Correspondingly, Akt/PKB-stimulated target gene expression via CREB in a phospho(Ser-133)-dependent manner. Akt/PKB induced CREB activity only in response to serum stimulation, and this effect was suppressed by the phosphatidylinositol 3-kinase inhibitor LY 294002. Our results support the notion that Akt/PKB promotes cell survival, at least in part, by stimulating the expression of cellular genes via the CREB/CBP nuclear transduction pathway.

Originally characterized on the basis of its sequence homology with the v-Akt oncogene and with protein kinase A (1–3), the Ser/Thr kinase Akt has been shown to block cellular apoptosis and to promote cell survival in response to growth factor induction (reviewed in Ref. 4). Akt/PKB-mediated phosphorylation of BAD, for example, blocks cellular apoptosis by promoting binding of BAD to the 14-3-3 protein and thereby sequestering BAD from Bcl-XI, (5–7). Following activation by PI3-K,1 Akt/PKB translocates to the nucleus where it is thought to regulate specific genetic programs by catalyzing the phosphorylation of specific nuclear factors (8, 9).

A number of growth factors and hormones have been shown to stimulate the expression of cellular genes by inducing the phosphorylation status of the nuclear factor CREB at Ser-133 (reviewed in Ref. 10). Originally characterized as a target for PKA-mediated phosphorylation (11), CREB is also recognized by other cellular kinases including protein kinase C (12), PKA-mediated phosphorylation (11), CREB is also recognized by other cellular kinases including protein kinase C (12), CREB and its paralog CREM are important for cell survival. CREM-deficient mice, for example, exhibit a spermatogenesis defect in secondary to enhanced apoptosis of germ cells (17, 18). Overexpression of a dominant negative CREB transgene, moreover, induces apoptosis in T cells, following growth factor stimulation (19). The involvement of CREB family members in cell survival and the resemblance of Akt/PKB to protein kinase A, not only in primary sequence but also in its apparent substrate specificity, prompted us to examine whether CREB is a regulatory target for Akt/PKB. Here we demonstrate that Akt/PKB promotes phosphorylation of CREB, stimulates recruitment of CBP to the promoter, and activates cellular gene expression via a CRE-dependent mechanism. Our results suggest that CREB may contribute importantly to cell survival in response to growth factor stimulation.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfections—293T cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. For transfection assay, cells were plated at 1.5 × 10^6 cells/well 24 h prior to transfection. Cells were transfected with Lipofectin reagent supplied by Life Technologies, Inc. For assays with GAL4 CREB expression vector, each transfection contained 0.5 μg of G5B CAT, 0.5 μg of HA-tagged Akt/PKB expression plasmid, 0.25 μg of GAL4-CREB expression plasmid, plus 0.5 μg of RSV β-galactosidase expression vector as internal control. Total amount of plasmid was adjusted to 1.75 μg with empty CMV expression vector. Cells were harvested 36–40 h post-infection. For PI3-K inhibitor studies in Fig. 3C, transfected cells were treated with 10% serum plus Me2SO vehicle or 10% serum plus 20 μM LY 294002 for 24 h. CAT activity was quantitated with a Phosphor Imager after normalizing to β-galactosidase activity.

For mammalian two-hybrid studies 293T cells were transfected with 0.25 μg of GAL4-KID, 0.25 μg of KIX VP16, 0.5 μg of Akt/PKB expression vector, and 0.5 μg of RSV β-galactosidase as normalization control. In assays where KIX VP16 was omitted, VP16 expression vector was added in place.

In Vitro Kinase Assays—293T cells (1 × 10^6) were transfected with 8 μg of HA-tagged wild-type or kinase-inactive Akt/PKB expression plasmid using Lipofectin reagent. Cells were harvested 36 h post-transfection and lysed in buffer containing 20 mM HEPES (pH 7.5), 420 mM NaCl, 5 mM MgCl2, 1% Nonidet P-40, 10% glycerol, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, and 1 mM okadaic acid. After removing cell debris by centrifugation at 13,500 for 10 min, lysates were pre-cleared by incubation withagarose-protein A/G resin and then incubated with anti-HA monoclonal antibody (16B12, Babco) for 4 h. Anti-HA Akt/PKB complexes were then collected by incubation with agarose-protein A/G resin, and these immunoprecipitates were used in in vitro kinase assays containing [γ-32P]ATP and 1 μg of recombinant CREB protein. Reactions were terminated by addition of 2× SDS loading buffer.

RESULTS AND DISCUSSION

To determine whether CREB is a regulatory target for Akt/PKB, we performed in vitro kinase assays with HA-tagged wild-type and mutant forms of Akt/PKB following transfection in 293T cells. Immunoprecipitates of HA-tagged Akt/PKB phosphorylated recombinant CREB protein in vitro (Fig. 1). Lower levels of Ser-133 kinase activity were recovered from Akt/PKB immunoprecipitates prepared from serum-deprived cells compared with serum-stimulated cells, indicating that Akt/PKB must be activated to phosphorylate CREB (Fig. 1). These results are consistent with previous reports showing that serum stimulation activates Akt/PKB kinase activity (9). By contrast, no CREB kinase activity was recovered from immunoprecipitates of 293T cells expressing kinase-inactive K179M mutant

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Akt/PKB phosphorylates CREB in response to serum stimulation. A, top, autoradiogram of 32P-labeled recombinant CREB following incubation with immunoprecipitates of wild-type Akt/PKB (WT) or kinase-inactive (MT) Akt/PKB prepared from transfected 293T cells. Immunoprecipitates of Akt/PKB from serum-treated (+) or serum-deprived (−) cells indicated over each lane. Bottom, Western blot of whole cell lysates from transfected cells using anti-HA antiserum (α HA) to detect HA-tagged wild-type Akt/PKB and mutant Akt/PKB polypeptides. B, top, autoradiogram of 32P-labeled recombinant wild-type CREB (WT) and Ser-133 → Ala (MT) CREB polypeptides following incubation with immunoprecipitates of wild-type Akt/PKB from transfected 293T cells. Bottom, Coomassie Blue-stained gel of recombinant wild-type CREB and Ser-133 → Ala mutant CREB polypeptides used for phosphorylation assay above.

HA-Akt/PKB (20), indicating that the effect of Akt/PKB on CREB phosphorylation is likely to be direct (Fig. 1).

To test whether Akt/PKB-mediated phosphorylation of CREB is sufficient to stimulate recruitment of CBP, we performed mammalian two-hybrid studies in 293T cells using relevant interaction domains in CREB and CBP, referred to as KID and KIX, respectively. Overexpression of wild-type Akt/PKB stimulated recruitment of a KIX-VP16 fusion protein to GAL4-KID about 10-fold, as evaluated on a G5β CAT reporter containing five GAL4 recognition sites (Fig. 2A). By contrast, kinase-inactive mutant Akt/PKB had no stimulatory effect on G5β CAT reporter activity, demonstrating the importance of Akt/PKB-mediated phosphorylation for induction of the CREB-CBP complex.

Based on its ability to promote recruitment of CBP to CREB, we examined whether Akt/PKB correspondingly induces target gene expression via this pathway. When overexpressed in 293T cells, wild-type Akt/PKB but not kinase-inactive Akt/PKB stimulated a CRE-CAT reporter about 15-fold (Fig. 2B). To rule out regulatory contributions from other CRE binding proteins and to evaluate the importance of Ser-133 phosphorylation for induction via Akt/PKB, we performed transfection assays with GAL4 CREB polypeptides in which the CREB trans-activation domain (amino acids 1–283) is fused to the GAL4 DNA-binding domain (amino acids 1–147). Following transfection into 293T cells, wild-type Akt/PKB stimulated GAL4-CREB activity 20-fold relative to kinase-inactive Akt/PKB in 293T cells (Fig. 3A). By contrast, wild-type Akt/PKB had no effect on the activity of a Ser-133 → Ala GAL4 CREB (GAL4-M1) mutant polypeptide, indicating that this kinase stimulates CREB activity via phosphorylation at Ser-133. Indeed, Western blot analysis of whole cell lysates from transfected 293T cells revealed that the wild-type GAL4-CREB polypeptide was phosphorylated in cells expressing wild-type Akt/PKB but not kinase-inactive Akt/PKB (Fig. 3B).

In keeping with its effects on CREB phosphorylation in vitro, serum treatment promoted Akt/PKB induction of Gal4 CREB activity in 293T cells about 3–4-fold compared with serum-deprived cells (Fig. 3C). Following serum stimulation, Akt/PKB appears to be activated by PI3-K (9, 20). To test whether CREB activation proceeds via a similar mechanism, we treated 293T cells with the PI3-K inhibitor LY 294002. Compared with serum-stimulated cells, 293T cells blocked with LY 294002 compound showed far lower GAL4 CREB activation in response to Akt/PKB, demonstrating that the PI3-K pathway is indeed required for this effect (Fig. 3C).
via CREB/CBP is unclear, but current data favor a secondary phosphorylation event on CREB that would inhibit complex formation with CBP. In this regard, Maurer and colleagues have observed that secondary phosphorylation of phospho(Ser-133) CREB at Ser-142 strongly inhibits target gene activation (14). It will be interesting to determine whether and to what degree Ser-142 is phosphorylated in vivo following stimulation via pathways that do or do not promote target gene activation.

Recent studies indicate that CREB functions importantly in promoting cell survival. Targeted disruption of the CREB gene, for example, leads to a defect in spermatogenesis secondary to germ cell apoptosis (17, 18). Overexpression of a dominant negative CREB transgene, moreover, induces T cell apoptosis in response to growth factor stimulation (19). Characterizing target genes that are activated via CREB will further clarify the mechanism by which Akt/PKB promotes cell survival.

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