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Upregulation of AKT1 Protein Expression in Forskolin-Stimulated Macrophage: Evidence From ChIP Analysis That CREB Binds to and Activates the AKT1 Promoter

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Abstract Recently, we reported that silencing *CREB* gene expression by RNAi significantly attenuates forskolin-induced activation of Akt1. We now provide evidence that forskolin-treatment causes transcriptional and translational upregulation of Akt1 in macrophages. Akt synthesis was demonstrated by [^{14}C]leucine or [^{35}S] incorporation into newly synthesized Akt1 protein. Akt protein levels increased by ~1.5-fold after only a 5 min exposure of macrophages to forskolin. Akt1 levels thereafter rapidly returned to basal values ($t_{1/2} \sim 15$ min). Maximal upregulation of Akt1 occurred in cells treated with 10 μM forskolin. Forskolin-dependent Akt1 synthesis was abolished by pretreating the cells with CREB-directed dsRNA as demonstrated at both the message and protein level, as well as by determining the synthesis of [^{35}S]-labeled Akt1 protein. The PKA inhibitor H-89, greatly attenuated forskolin-induced Akt1 synthesis. Transcriptional and translational inhibitors also greatly reduced Akt1 synthesis in forskolin-stimulated [^{14}C]leucine-labeled macrophages. Using a chromatin immunoprecipitation assay, we demonstrate that CREB binds to a CRE binding domain of the *Akt1* gene promoter. In conclusion, we show here for the first time transcriptional upregulation of Akt1 by CREB, based upon Akt1 protein synthesis and its modulation by transitional and translational inhibitors in forskolin-stimulated cells, Akt1 protein. and mRNA levels upon silencing *CREB* gene expression, and binding of CREB to the *Akt1* gene promoter. J. Cell. Biochem. 100: 1022–1033, 2007.

Key words: CREB and Akt promoter activation; transcriptional upregulation of Akt synthesis; silencing of *CREB* gene expression by RNAi and Akt1 regulation; synthesis of radiolabeled Akt1

The serine/threonine kinase Akt/PKB is activated by extracellular and intracellular stimuli to regulate growth, survival, differentiation, and metabolism. Akt is composed of three functionally distinct regions: an NH_2-terminal plekstrin homology (PH) domain, a central catalytic domain, and a COOH-terminal hydrophobic region [Datta et al., 1999; Brazil et al., 2004; Bellacosa et al., 2005; Woodgett, 2005]. The PH domain, found in many proteins, is involved in cell signaling or cytoskeletal rearrangement. Three mammalian isoforms Akt1/PKBα, Akt2/PKBβ, and Akt3/PKBγ, which share a high degree of homology, have been identified [Datta et al., 1999; Brazil et al., 2004; Bellacosa et al., 2005; Woodgett, 2005]. The basic activation process of all Akt isoforms appears similar. Growth-stimulated activation of Akt1 occurs through the binding of the PH domain to the phosphatidyl 3-kinase products PtdIns 3,4,5 P3 and PtdIns 3,4 P2 and requires phosphorylation of Thr^{308} in the activation loop and Ser^{473} in the COOH terminal activation domain by PDK1 and "PDK2" whose identity is not yet established. The tumor suppressor gene PTEN negatively regulates Akt activation by reducing the intracellular levels of PtdIns 3,4,5 P3 and PtdIns 3,4 P2 [Datta et al., 1999; Brazil et al., 2004; Bellacosa et al., 2005; Woodgett, 2005].

The three Akts differ in their activation levels and protein expression in various cell types

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[Cheng et al., 1992; Bellacosa et al., 1995; Ruggeri et al., 1998; Walker et al., 1998; Nakatani et al., 1999; Irie et al., 2005]. Akt1 and Akt2, but not Akt3, form multimeric protein complexes with TLC1 [Laine et al., 2002]. Furthermore, only Akt2, and not Akt1 or Akt3, accumulate in the cytoplasm during mitosis [Cheng et al., 1997] and in the nucleus during muscle differentiation [Calera and Pilch, 1998]. Microinjection of anti-Akt1 antibodies, but not anti-Akt2 antibodies, blocks cell cycle progression and attenuates muscle differentiation [Vandromme et al., 2001]. Differences have also been observed in the amplification of the *Akt1*, *Akt2*, and *Akt3* genes in various carcinoma cell lines. Expression of the *Akt1* gene is amplified in a gastric carcinoma cell line [Staal, 1987], whereas the *Akt2* gene is amplified in breast, ovarian, and pancreatic carcinomas [Bellacosa et al., 1995; Miwa et al., 1995; Nakatani et al., 1999]. Expression of the *Akt2* gene, but not *Akt1* or *Akt3*, results in invasion and metastasis of human breast and ovarian cancer cells [Arboleda et al., 2003]. The ability of insulin to lower blood glucose is impaired in Akt2-deficient mice, but Akt1−/− mice do not display a diabetic phenotype [Cho et al., 2001a,b; Garofalo et al., 2003]. Akt3−/− knockout mice show significant defects in total brain development [Easton et al., 2005]. The proliferation of endothelial cells in vitro is significantly impaired in Akt1−/− knockout mice [Chen et al., 2005]. Akt2 downregulation reversed 1GF-1-induced hyperproliferation and antiapoptotic activities in breast epithelial cells. In contrast, Akt1 downregulation promoted neomorphic effects and enhanced 1GF1 or EGF stimulated cell migration which was accompanied by enhanced Erk activation. These were reversed in Akt2 downregulated cells [Irie et al., 2005]. In adipocytes, TNF decreases total Akt protein levels as well as phosphorylated Akt levels [Medina et al., 2005]. Ceramides, which promote dephosphorylation of Akt^{Ser473}, also reduce Akt protein levels [Schubert et al., 2000] while reduction in ceramide levels results in a twofold increase in the expression of Akt protein in skeletal muscle [Planavila et al., 2005]. Increased expression of Akt protein may affect the ability of cells to respond to activation of signaling cascades. For example, in embryonic mouse cell cortical progenitors or 3T3 cells, increasing Akt protein expression enhances the activation of Akt in response to extrinsic signals that regulate cellular behavior [Sinor and Lillien, 2004].

The binding of many hormones and growth factors to cells induces activation of adenylyl cyclase, which catalyzes synthesis of cAMP from ATP [Myr and Montminy, 2001]. cAMP regulates a wide range of processes through its downstream effectors including PKA [Tasken and Aandahl, 2004]. Depending on the cell type, cAMP can either inhibit or stimulate cell growth in a PKA-dependent or PKA-independent manner [Stork and Schmitt, 2002; Stork, 2003]. The PKA-independent proliferative effects of cAMP occur in part through activation of Rap1 via Epac and require activation of PI 3-kinase/Akt signaling. CREB, a critical regulator of early gene transcription, is activated after phosphorylation of Ser^{133}. It then binds to cAMP-response elements (CREs) in the promoters of many cAMP-dependent genes [Myr and Montminy, 2001]. KCREB, a CREB mutant that contains a mutation of a single amino acid in the DNA binding domain, does not bind to native to CRE sequences. Cancer cells overexpressing KCREB exhibit decreased cell growth. CREB binds to many sites situated at the 5′ ends of genes as well as in internal introns [Cha-Molstad et al., 2004; Euskirchen et al., 2004]. Phosphorylation of CREB recruits HAT CBP to activate transcription [Mirwa et al., 1995]. Expression of a mutant form of CREB in human melanoma cells decreases their metastatic potential in nude mice [Robertson, 2005]. Mice lacking *Akt1* and *Akt2* genes show growth retardation and impaired adipogenesis [Chen et al., 2001; Peng et al., 2003]. Recently Park et al. [2005] have cloned and sequenced the promoter of the *Akt1* gene; however, studies on transcriptional upregulation of Akt have not been reported. Transcriptional element analysis of a 6,181 bp upstream region of the translational initiation site of the *Akt1* gene demonstrates multiple binding sites for Stat3, NFκB, AP1, and CRE. We have previously studied the regulation of CREB-dependent Akt1 phosphorylation in murine peritoneal macrophages [Misra and Pizzo, 2005]. In the current study we have examined the role of CREB in transcriptional upregulation of the Akt promoter by employing chromatin immunoprecipitation assays, determining the effect of silencing expression of the *CREB* gene with RNAi on Akt1 mRNA and protein levels, and by studies of Akt1 protein synthesis. We also

studied the effect of transcriptional and translational inhibitors on Akt1 protein synthesis. Here we report that CREB binds to the *Akt1* gene promoter and causes transcriptional upregulation of Akt1. Silencing of *CREB* gene expression reduces mRNA and protein levels of Akt1. Finally, Akt1 synthesis, as studied by incorporation of [^{35}S]-labeled amino acids or [^{14}C]leucine, peaks very rapidly (~5 min) and transiently and is inhibited by PKA inhibition, actinomycin D, or cycloheximide.

EXPERIMENTAL PROCEDURES

Materials

Cultured media were purchased from Invitrogen. Forskolin was purchased from Biomol (Plymouth, PA). Antibodies against Akt protein beta actin and CREB were purchased from Cell Signaling Technology (Beverly, MA). [^{35}S]-labeled protein lysate and [^{14}C]leucine specific activity/(306 mCi/mmol) were purchased from Perkin Elmer (Beverly, MA). Other reagents of the highest available grade used were purchased locally.

Cell Culture

The use of mice for these studies was approved by the institutional animal use committee in accordance with relevant federal regulations. Thioglycollate-elicited peritoneal macrophages were obtained from pathogen-free 6-week-old C57BL/6 mice (National Cancer Institute) in HBBSS containing 10 mM HEPES (pH 7.4) and 3.5 mM $NaHCO_3$ (HHBSS). The cells were washed with HHBSS and suspended in RPMI 1640 medium containing 2 mM glutamine, penicillin (12.5 units/ml), streptomycin (6 μg/ml) and 5% FBS; placed in 6-well plates (3×10^6 cells/well); and incubated for 2 h at 37°C in a humidified CO_2 (5%) incubator. The monolayers were washed with HHBSS three times to remove nonadherent cells, and the monolayers were incubated overnight at 37°C in the above RPMI medium before study [Reusch and Klemm, 2002].

Chromatin Immunoprecipitation (ChIP) Assay

Macrophages (1×10^6 cells/well in 6-well plates) were incubated overnight in RPMI medium as above were stimulated with either buffer or forskolin (10 μM/10 min). These studies were performed in quadruplicate. The reaction was stopped by aspirating the medium and adding a volume of RPMI medium. The cells were processed for ChIP assay using a ChIP assay Kit (Cat #17-295) available from Upstate Cell Signaling Solutions (Charlottesville, VA) [Park et al., 2005]. Other reagents required in the ChIP assay kit protocol were purchased from the same source. Briefly, after stimulation, histones and DNA were cross-linked by adding formaldehyde (27 μl of 37% formaldehyde/ml) and cells incubated for 10 min at 37°C. The medium was aspirated and cells washed twice with ice-cold PBS containing 1 mM PMSF, 1 μg/ml aprotinin, and 1 μg/ml peptstatin A. The cells were scraped into tube, centrifuged at 2,000 rpm for 4 min at 4°C and pellet lysed in 200 μl of SDS lysis buffer (ChIP assay kit) for 10 min on ice. The lysates were sonicated (Fischer Scientific Model 100 Ultrasonic Dismembrator) with 4, 10 s pulses at 1/3rd maximal power over ice. The sonicates were centrifuged for 10 min at 13,000 rpm at 4°C, supernatants transferred to new microfuge tubes and diluted 10-fold with ChIP dilution buffer (Assay kit) containing protease inhibitors as above. The nonspecific background was reduced by preclearing the sonicates by addition of 75 μl of Protein A agarose/salmon sperm DNA (50% slurry), incubation for 30 min at 4°C with rotation and centrifugation for 1 min at 1,000 rpm at 4°C. The forskolin-treated sonicate was divided into two aliquotes. One was immunoprecipitated with anti-CREB antibodies (1:50) and the other with anti-β actin antibodies (1:50), followed by the addition of 60 μl of Protein A agarose/salmon sperm DNA (50% slurry) and incubation overnight at 4°C with rotation. The tubes were centrifuged at 1,000 rpm for 1 min at 4°C and supernatants removed, the pellets washed according to the ChIP assay Kit Protocol. The immunocomplexes were eluted twice with 250 μl of freshly prepared elution buffer (1% SDS and 0.1 M $NaHCO_3$). The histone-DNA cross-linking was reversed by adding 20 μl of 5 M NaCl to eluates and incubation at 65°C for 4 h. To the eluates was now added 10 μl of 0.5 M EDTA, 20 μl of 1 M Tris-HCl buffer pH 6.5 and 20 μg proteinase K and eluates incubated for 2 h at 45°C. DNA was recovered by phenol/chloroform extraction and ethanol precipitation. Glycogen (20 μg) was added to each sample for visualizing the pellet. The purified DNA was subjected to PCR with two primers selected from the *Akt1* gene promoter sequence recently reported by Park et al. [2005]. One sequence

specific for CRE binding site within the *Akt1* gene promoter and the other nonspecific with no CRE binding motif. The sequences of the PCR primers used are as follows: sequence 1 forward (−4293) 5′-CTT CGT GAA CAT TAA CGA CAG GGCC-3′, reverse (−4143) 5′-AAT GGC CAC CCT GAC TAA GGA GTGG-13′, sequence 2 forward (−4221) 5′-AAC CCT TCA CTG GTT TCT CTT CATCC-3′, reverse (−4056) 5′-TGC TGG AAT ATC CCA CAA TCA CAGG-3′. DNA was dissolved in 20 μl of water. Each reaction mixture (50 μl) contained 3 μl of chromatin and 1× hotstart-IT-Taq Mater Mix (USB, Cleveland, OH). Amplified PCR products were resolved by 2.5% of agarose gel electrophoresis and visualized by ethidium bromide staining.

The Effects of Silencing CREB Gene Expression by RNAi on Akt Protein Levels in Forskolin-Stimulated Cells

The silencing of CREB gene expression by RNAi was performed as we reported previously [Misra and Pizzo, 2005]. Briefly, the sense (5′; GAG ACA ACA GAG AAU GAU tt 3′) and antisense (5′-UAU CAU UCU GUU GUC Uctt-3′) oligonucleotides against the target homologous gene sequence nucleotide 324–344 (5′-AAG AGA CAA CAG AGA ATG AT-3′) (SWISS PROT Entry name, AFTB MOUSE, primary session number 35451) were chemically synthesized by Ambion (Austin, TX); oligonucleotides against the target homologous gene sequence nucleotide 324–344 (5′-AAG AGA CAA CAG AGA ATG ATA-3′) (SWISS-PROT Entry name, AFTB MOUSE, Primary accession number 35451), were chemically synthesized by Ambion (Austin, TX). Other details of annealing the sense and antisense oligonucleotides, transfection of cells with CREB dsRNA (25 μg/ml/48 h), and evaluation of the magnitude of transfection by estimating the mRNA levels (RT PCR) and the protein levels of CREB (Western blotting) were the same as described previously. Forty-eight hours after transfection, cells were washed with HHBSS and stimulated with forskolin (10 μM/15 min). These transfected cells were used to examine the effects of silencing the expression of CREB gene on Akt protein levels by Western blotting.

Measurement of Akt1 mRNA Levels in Cells Transfected With CREB dsRNA by *PCR*

Qualitative measurement of the mRNA levels of Akt in cells transfected with CREB dsRNA was done according to procedures described in the preceding sections. cDNA probe for Akt1 was purchased from Gene Script Corporation (Scotch Plains, NJ). Total RNA from macrophages treated with lipofectamine or CREB ds-RNA-lipofectamine was extracted by a single-step method using an Rneasy Mini Kit (Qiagen, Chatsworth, CA) according to the manufacturer's instructions. Total RNA was reverse-transcribed with 1 μg of RNA in a 20 μl reaction mixture, using M-MLV (Moloney murine leukemia virus) reverse transcriptase (200 units) and oligo(dt) as primer for 1 h at 40°C. The resulting of cDNA (5 μl) was used as template and a 225-bp segment of the Akt cDNA was amplified using a 20-mer upstream primer (5′-CAA GAA CGA TGG CAC CTT TA-3′) and a 20-mer downstream primer (5′-GCA TTG TGC CAC TGA GAA GT-3′) complimentary to Akt 1 mRNA. A 302-bp segment of mouse β-actin (constitutive internal control) cDNA was co-amplified using a set of PCR primers provided in an R&D Systems Kit (Minneapolis, MN). Amplification was carried out in a Techne Thermal Cycler PHC-3 for 28 cycles (one cycle: 94°C for 45 s, 60°C for 45 s, and 72°C for 45 s). PCR products were qualitatively analyzed for Akt1 mRNA levels on 3% (v/v) agarose/ethidium bromide gel. To check the specificity of CREB dsRNA in silencing the expression of CREB gene, the cells were also transfected with scrambled dsRNAi (25 μg/ml/48 h) and samples processed identically for the above analyses.

Measurement of Akt Protein Levels in Cells Stimulated With Forskolin

Macrophages incubated overnight in 6-well plates (3×10^6 cells/well) were stimulated with either buffer or forskolin (10 μM) and incubated for varying periods of time as above. The reaction was terminated by aspirating the medium. The lysis of cells, lysate electrophoresis, and immunoblotting with antibodies specific for Akt1, were performed according the manufacturer's instructions. In each case, an equal amount of protein was used for electrophoresis [Misra and Pizzo, 2005]. The detection and quantification of immunoblots was done by ECF and phosphorimaging employing a Storm 860 PhosphorImager (Amersham Biosciences). The membranes were reprobed for actin as the protein loading controls, according to the

manufacturer's instructions [Misra and Pizzo, 2005].

Synthesis of Akt From [^{35}S]-Labeled Precursor Amino Acids in Forskolin-Treated Macrophages

Macrophages incubated overnight (3×10^6 cells/well in 6-well plates) in the above RPMI medium were labeled with Expre^{35}S^{35}S protein labeling mixture (300 μCi/ml) for 2 h at 37°C in an humidified CO_2 (5%) incubator. The labeling was stopped by aspirating the medium, monolayers washed four times with cold HHBSS buffer, a volume of RPMI medium added and cells incubated at 37°C for 5 min for temperature equilibration. To the respective wells, forskolin (10 μM) was added and the cells incubated as above for varying periods of time. The reaction was stopped by aspirating the medium and a volume of lysis buffer containing 50 mM Tris-HCL (pH 7.5), 120 mM NaCl, 1% (v/v) Nonidet p-40®, 25 mM sodium fluoride, 1 mM sodium pyrophosphate, 0.1 mM sodium orthovanadate, 1 mM PMSF, 1 mM benzamidine, and leupeptin (20 μg/ml) was added. The cells were lysed for 10 min over ice, scraped into tubes and centrifuged at 8,000*g* for 10 min at 4°C and lysates protein contents determined. Equal amount of lysate protein was immunoprecipitated with anti-Akt1 antibodies (1:50) at 4°C overnight with gentle rotation. Akt immunoprecipitates were washed thrice with cold lysis buffer by centrifugation at 3,000 rpm for 5 min at 4°C. To each immunoprecipitate sample buffer (30 μl) was added, samples boiled for 5 min, electrophoresed, transferred to membranes, and the membrane first autoradiographed in an PhosphorImager before probing for the Akt1 protein band by immunoblotting as described above. In experiments where the effects of silencing of CREB gene expression was studied on forskolin-stimulated Akt1 protein synthesis, it was performed by RNAi as described above [Misra and Pizzo, 2005]. Other details of labeling the cells with[^{35}S], stimulation with forskolin (10 μM/5 min at 37°C), electrophoresis, autoradiography, and immunoblotting were the same as described above.

Inhibition of Akt1 Synthesis by H-89, in [^{35}S]-Labeled Macrophages

Macrophages incubated overnight (3×10^6 cells/well) in 6-well plates in the above medium were labeled with [^{35}S] as described above. The labeling was stopped by aspirating the medium, monolayers washed four times with cold HHBSS buffer, a volume of RPMI medium added, and cells incubated at 37°C for 5 min for temperature equilibration. To the respective wells, H-89 (15 μM/90 min) was added before the addition of forskolin (10 μM/15 min), and cells incubated at 37°C. The reaction was stopped by aspirating the medium and cells lysed in lysis buffer as above. The lysates were centrifuged and equal amount of lysate protein were immunoprecipitated with anti-Akt1 antibodies (1:50) at 4°C overnight with rotation. Akt1 immunoprecipitates were washed thrice with cold lysis buffer, immunoprecipitates recovered by centrifugation (2,500 rpm/5 min), a volume of sample buffer added to the immunoprecipitated samples, these were boiled for 5 min, electrophoresed, transferred to membranes, and the membranes autoradiographed in an Phosphorimager.

Inhibition of Akt Synthesis by Actinomycin D and Cycloheximide in [^{14}C]Leucine-Labeled Macrophages

Macrophages incubated overnight (3×10^6 cells/well in 6-well plates) in the above RPMI medium were labeled with [^{14}C]leucine (2 μl/ml Perkin Elmer Spact 306 mCi/mmol) for 12 h at 37°C in a humidified CO_2 (5%) incubator. These studies were performed in triplicate. The labeling was stopped by aspirating the medium, monolayers washed four times with ice-cold HHBSS buffer, a volume of RPMI medium added, and the cells incubated for 5 min for temperature equilibration. To respective wells, actinomycin D (10 μg/ml/10 min) and cycloheximide (20 μg/ml/10 min) were added before the addition of forskolin (10 μM/10 min) and cells incubated at 37°C. The reaction was stopped by aspirating the medium, and cells lysed in lysis buffer as above. The lysates were centrifuged and lysate protein contents determined. Equal amounts of lysate proteins were immunoprecipitated with anti-Akt1 antibodies (1:50) at 4°C overnight with rotation. Akt1 immunoprecipitates were washed thrice with cold lysis buffer, immunoprecipitates recovered by centrifugation (2,500 rpm min/5 at 4°C), a volume of sample buffer added to the immunocomplex, samples boiled for 5 min, electrophoresed, transferred to membrane and autoradiographed in phosphorimager.

RESULTS

Forskolin Upregulates Akt Protein Levels

We recently reported a two- to threefold increase in Akt kinase activation in forskolin-stimulated macrophages [Misra and Pizzo, 2005]. Here we have quantified the Akt protein levels in macrophages stimulated with forskolin over times and with varying concentrations of forskolin for 10 min by Western blotting (Fig. 1). Forskolin treatment elevated the Akt protein levels ~1.5-fold reaching a maximum at ~5 min of incubation (Fig. 1A) and 10 μM of forskolin (Fig. 1B). Longer periods of incubation or incubation with higher concentrations of forskolin decreased Akt protein levels (Fig. 1A and B). The results thus demonstrate that forskolin treatment of macrophages not only increases Akt kinase activation [Misra and Pizzo, 2005] but it also increases Akt protein upregulation in forskolin-stimulated cells (Fig. 1A,B).

Forskolin Enhances Akt1 Protein Synthesis From [^{35}S]-Labeled Precursor Amino Acids in Macrophages

To understand the mechanism of increased Akt1 protein levels in forskolin treated cells, we studied the synthesis of Akt1 protein in [^{35}S]-labeled macrophages stimulated with forskolin (10 μM) for varying periods of time (Fig. 2). A two- to threefold increase in the incorporation of [^{35}S]-label into Akt protein in forskolin-stimulated cells occurred at ~5 min of incubation, but these levels rapidly declined thereafter ($t_{1/2} = 15$ min) (Fig. 2).

Forskolin-Induced Increased Synthesis of Akt1 in [^{35}S]-Labeled Macrophages Is Inhibited by PKA Inhibitor H-89

In the preceding sections, we demonstrate that forskolin treatment of macrophages upregulates Akt1 protein levels (Fig. 1), its synthesis from [^{35}S]-labeled and [^{14}C]leucine-labeled precursors (Fig. 2), and that both these events are downregulated by silencing the expression of the *CREB* gene by RNAi (Fig. 2). CREB is the downstream effector of PKA, therefore, if forskolin upregulated new synthesis of Akt1 is PKA-dependent, then inhibition of PKA by the specific inhibitor H-89 should abrogate forskolin-induced increased synthesis of Akt1. Indeed this is correct (Fig. 2C); therefore, these results demonstrate

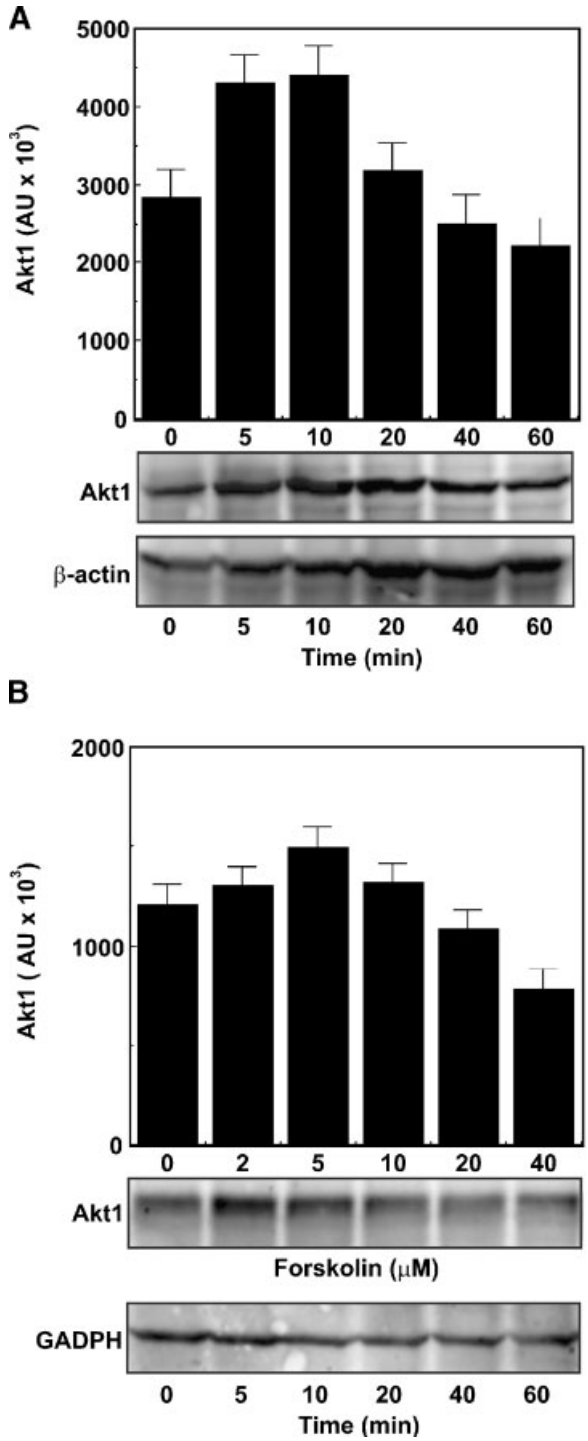
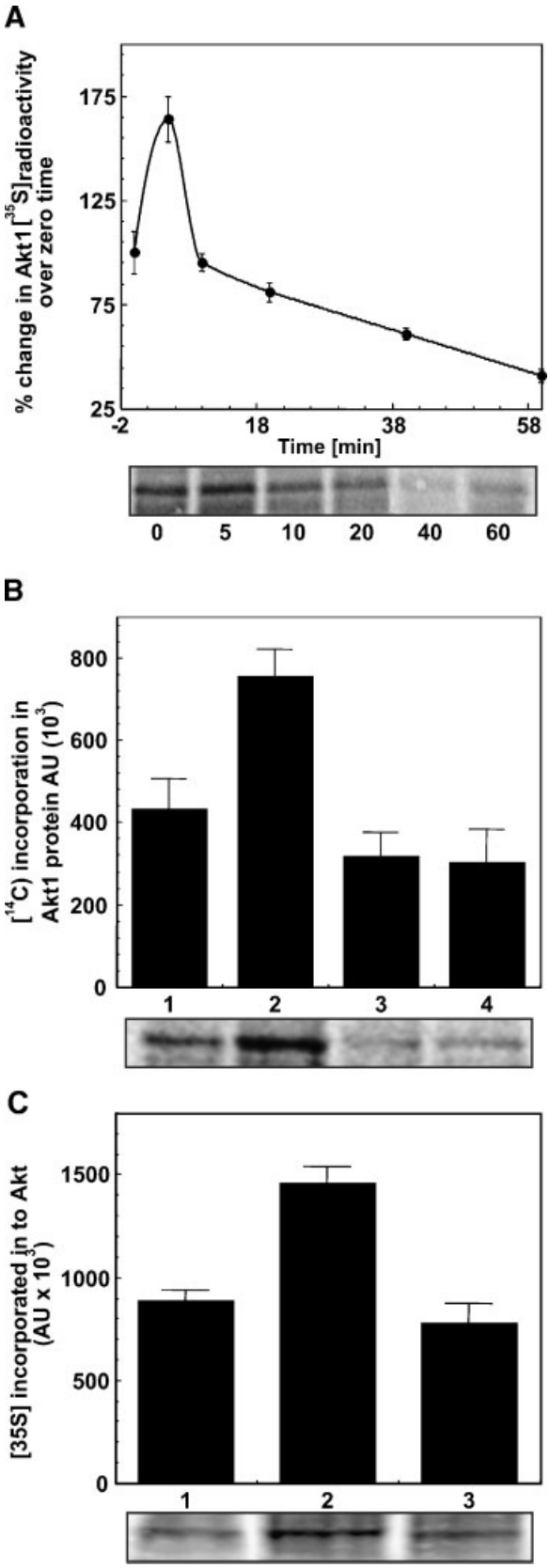


Fig. 1. Forskolin upregulates Akt1 protein levels. **Panel A**: Effect of time of incubation of cells with forskolin (10 μM) on Akt1 protein level. **Panel B**: Effect of varying concentrations of forskolin on Akt1 protein levels at 10 min of incubation. The respective immunoblots are representative of three independent experiments and are shown below the graph. Values are mean ± SE from three experiments and are expressed in arbitrary units (AU). The protein loading control, GADPH, is shown below the immunoblot.



that CREB is involved in increased Akt1 protein expression in forskolin-treated macrophages.

Actinomycin D and Cycloheximide Inhibit Forskolin-Induced Akt1 Synthesis in [14C]Leucine-Labeled Macrophages

In the next series of experiments, we examined the question of whether forskolin-induced new synthesis of Akt1 is under transcriptional and translational regulation. We treated [^{14}C]leucine-labeled cells with actinomycin D (10 μg/ml/10 min) or cycloheximide (20 μg/ml/10 min) before adding forskolin (10 μM/ml/10 min) and examined [^{14}C] radioactivity in the Akt1 immunoprecipitates (Fig. 2B). Actinomycin D and cycloheximide treatment greatly reduced new synthesis of Akt1 in forskolin-treated macrophages, showing thereby that forskolin-induced upregulation of Akt1 protein synthesis is controlled both at transcriptional translational levels.

Silencing of CREB Gene Expression Downregulates Akt1 mRNA and Protein Levels and Akt1 Protein Synthesis in Forskolin-Stimulated Cells

Forskolin elevates intracellular cAMP levels by activating adenylyl cyclase. Elevated cAMP binds to PKA and activated PKA causes transcriptional activation of several genes. PKA phosphorylates CREB at Ser^{133} and enhances its transcriptional potential. If indeed CREB regulates *Akt1* gene expression at the transcriptional level, then inhibition of *CREB* gene expression should affect the expression of Akt1 both at the mRNA and protein levels.

Fig. 2. Akt1 protein synthesis in cells stimulated with forskolin. See Experimental Procedures for details. **Panel A**: Effect of time of incubation on [^{35}S] incorporation into Akt1 protein in cells stimulated with forskolin (10 μM). **Panel B**: Effect of transcriptional and translational inhibitors on incorporation of [^{14}C] leucine into Akt protein. Cells were incubated with actinomycin D (10 μg/10 min) or cycloheximide (20 μg/10 min) prior to forskolin (10 μM) treatment for 5 min. The corresponding autoradiographs are shown below the graphs. **Panel C**: Effect of the PKA inhibitor H-89 on forskolin-induced Akt1 protein synthesis. [^{35}S]-labeled macrophages were incubated with H-89 (15 μm/90 min) before adding buffer or forskolin (10 μM/10 min). The corresponding autoradiogram is shown below the graph. Values are expressed in arbitrary units (AU) which are the mean ± SE from two experiments in each case. The bars in (Panel B) are: (1) buffer; (2) forskolin (10 μM/5 min); (3) actinomycin D (10 μg/ml/10 min) then forskolin; and (4) cycloheximide (20 μg/ml/10 min) then forskolin, and in (Panel C) are: (1) buffer, (2) forskolin (15 μM/15 min), and (3) H-89 (15 μM/90 min) then forskolin.

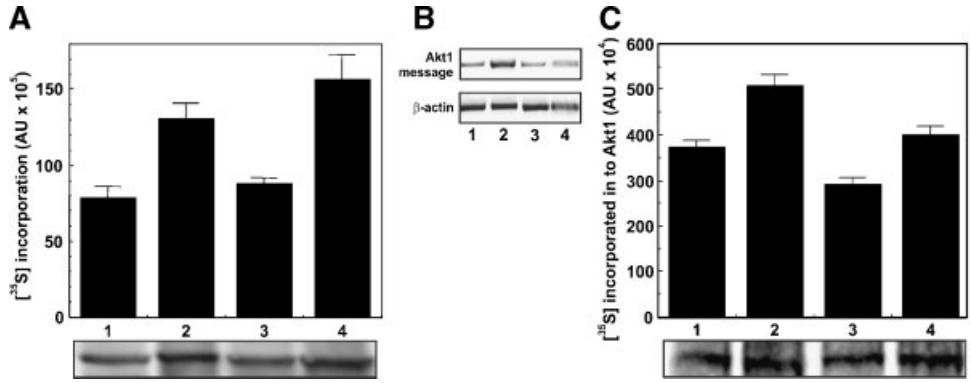


Fig. 3. Effect of silencing the expression of the *CREB* gene by RNAi on Akt1. **Panel A**: Akt1 protein levels. **Panel B**: Akt1 mRNA levels. **Panel C**: Synthesis of Akt1 protein from [^{35}S]-labeled amino acids. See Experimental Procedures for details. The bars in (Panels A, B, and C) are: (1) buffer stimulated; (2) forskolin (10 μm/5 min); (3) dsCREB-transfected + forskolin; and (4) scrambled dsRNAs + forskolin. The values in (Panel A) and (Panel C) are expressed in arbitrary units (AU) and are the mean ± SE from two experiments run in triplicate or quadruplicate. The respective immunoblots shown below corresponding graph are representative of two to three experiments.

Consistent with this hypothesis, silencing *CREB* gene expression by RNAi interference reduced both the mRNA and Akt1 protein levels by about 60% in forskolin-treated cells compared to controls (Fig. 3). The magnitude of silencing *CREB* gene expression was evaluated by quantifying CREB mRNA and CREB protein and this also was reduced between 60% and 70%. Furthermore, Akt1 synthesis in [^{35}S]-labeled macrophages where CREB gene expression was silenced was also significantly decreased when the cells were forskolin-treated (Fig. 3).

CREB Binds to a CRE Site in the Akt1 Gene Promoter

Forskolin treatment alters the expression of several genes which may or may not contain CREB binding sites. Recently Park et al. [2005] have cloned and sequenced the *Akt1* gene promoter and reported a single CRE binding site, in addition to multiple sites for Stat, NFκB, and AP1 in this promoter. To understand the mechanism by which CREB upregulates Akt1 mRNA and protein levels in forskolin-treated cells, we examined CREB binding to the *Akt1* gene promoter by ChIP assay. Indeed, the binding of CREB was observed in the *Akt1* gene promoter sequence containing the CRE site (sequence 1, Fig. 4). CREB binding was not observed in β-actin immunoprecipitate nor in sequence 2 which does not have and CRE binding site. The results thus show that CREB-stimulated transcriptional and translational upregulation of Akt1 occurs consequent to CREB binding to the *Akt1* gene promoter.

DISCUSSION

Many hormones and growth factors induce activation of adnylyl cyclase which catalyzes the synthesis of cAMP from ATP. cAMP regulates a wide range of processes through its downstream effectors PKA, cyclic nucleotide gated cation channels, and a small family of guanine nucleotide exchange factors involved in the regulation of Ras-related proteins. Depending on the cell type, cAMP can either inhibit or stimulate cell growth and proliferation in a PKA-dependent or PKA-independent manner [Misra and Pizzo, 2005 and references therein]. Recently, we have reported that forskolin stimulates mitogenesis

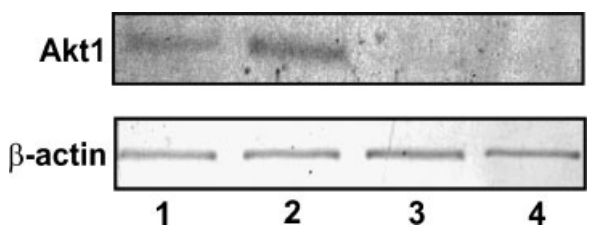


Fig. 4. Assay of binding of CREB to a single CRE binding motif on Akt1 promoter by ChIP. Experimental details are described in Experimental Procedures. The lanes in the electrophoretogram are: (1) buffer-treated cells and lysate immunoprecipitated with anti-CREB antibody; (2) forskolin-stimulated cell lysates (10 μM/10 min) immunoprecipitated with anti-CREB antibody; (3) forskolin-stimulated cell lysates (10 μM/10 min) immunoprecipitated with anti-β-actin antibody; and (4) sequence 2 of the Akt1 promoter of forskolin-stimulated cells and lysate immunoprecipitates with anti-CREB antibody.

and cell proliferation in macrophages which involves a coordinated regulation of PKA-CREB and Epac-Rap1 signaling and these two pathways converge and/or crosstalk at PKA-CREB and Epac1-Rap1 signaling [Misra and Pizzo, 2005]. By activation of PKA, cAMP regulates specific steps in cell proliferation and differentiation and cell spreading. cAMP-dependent Epac1-Rap pathway regulates cellular adhesion. cAMP-dependent signal transduction pathway also modulates apoptosis and crosstalk with PI 3-kinase signal transduction pathway is necessary for this effect. These studies are summarized in Figure 5.

The transcription factor CREB is activated by multiple signal transduction pathways in response to external stimuli including growth factors, hormones, and cytokines [Planavila et al., 2005]. Activation of CREB is mediated by the kinase inducible domain, which contains a Ser residue within a consensus phosphorylation site at position 133 and a basic leucine zipper domain that govern efficiency of CREB binding to the cognate promoter element CRE. In response to different signals, a number of kinases are involved in the phosphorylation of CREB at Ser^{133} including PKA, Ca^{2+}/calmodulation kinase II and IV, Akt, and growth factor Ras-dependent kinase 2 (RSK2) [Myr and Montminy, 2001]. CREB binding protein (CBP) is recruited selectively to phosphorylated CREB to facilitate transcriptional activation. Forskolin treatment of murine peritoneal macrophages causes an increase in proliferation rates and in cellular growth [Misra and Pizzo, 2005]. These effects of forskolin are mediated by the activation and crosstalk between the PI 3-kinase/Akt and CREB-PKA signal transduction pathways [Misra and Pizzo, 2005]. In these previous studies, we demonstrated that cAMP promotes macrophage survival by upregulating Akt1 signaling and transcriptional activation of Bcl-2 [Misra and Pizzo, 2005]. Activation of PI 3-kinase results in the two-step activation of Akt which involves its recruitment to membranes and its phosphorylation at Thr^{308} and Ser^{473} by several kinases. Activated Akt promotes cell survival

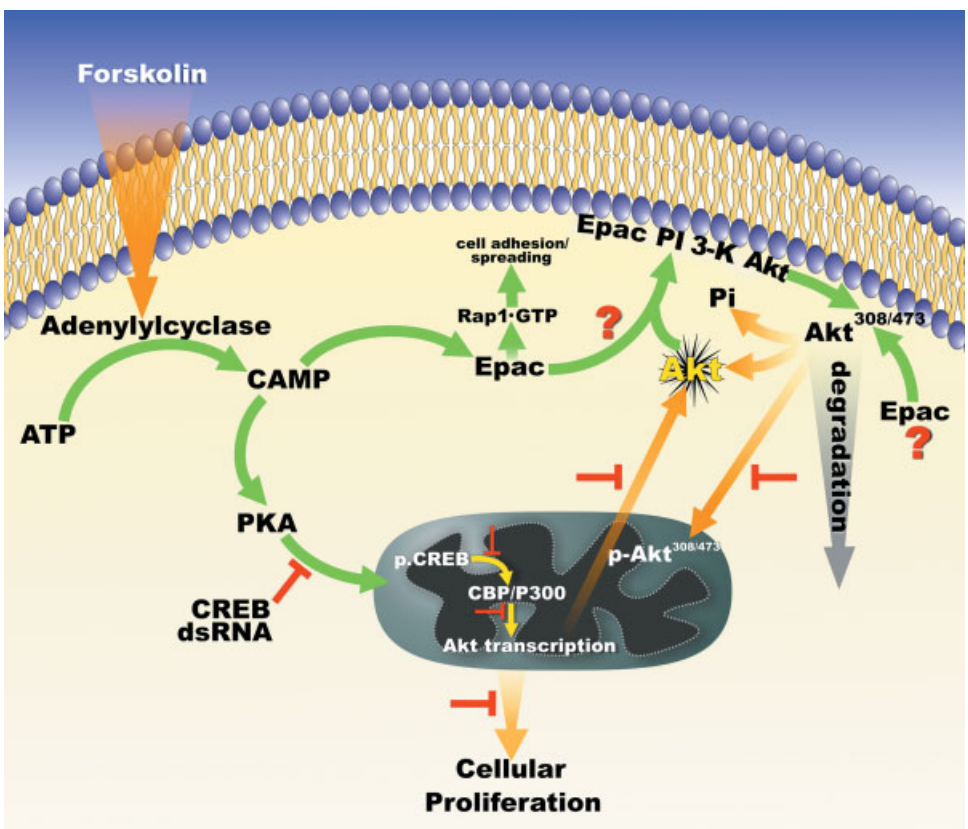


Fig. 5. A schematic representation of the possible mechanism of upregulation of Akt expression in cells treated with forskolin.

by phosphorylation-mediated inactivation of caspases and proapoptotic genes, as well as by phosphorylation-mediated activation of antiapoptotic proteins and NFκB. These effects are blunted by the PI 3-kinase inhibitors LY294002 and wortmannin as well as by silencing *CREB* gene expression, which implicates CREB involvement in stimulus-induced activation of Akt [Misra and Pizzo, 2005]. It should also be noted that Akt has been reported to phosphorylate CREB [Myr and Montminy, 2001]. Thus it appears that there is a feedback loop between Akt and CREB activation in stimulated cells.

Growth factor receptors activation leads to Akt activation which leads to pleiotropic effects of its signaling in normal and cancerous cells [Datta et al., 1999; Brazil et al., 2004; Bellacosa et al., 2005; Woodgett, 2005]. The three isoforms of Akt show structural homology and similar mechanisms of activation. However, the emerging evidences show that they exhibit distinct functions in normal and tumor cells [Irie et al., 2005], where these effects may be isoform-specific [Irie et al., 2005]. The balance between Akt isoforms activation downstream of growth factor receptor may influence the invasive or metastatic potential of tumors or tumor cell lines. The relative abundance or activation of Akt isoforms may be dynamic and may change depending on cellular context [Irie et al., 2005].

In the present study, we demonstrate that CREB activation results not only in an increase in Akt activation [Misra and Pizzo, 2005], but also Akt1 protein levels (Fig. 1). Our studies show that in forskolin-treated macrophages, there is a rapid ~1.5-fold increase in Akt protein. This increase is maximal at ~5 min after which there is also a relatively rapid return to basal values. Direct evidence for CREB involvement in regulating Akt1 levels was obtained by silencing expression of the *CREB* gene and binding of CREB to *Akt1* gene promoter. Silencing the expression of CREB gene led to significant reductions in both Akt1 message and protein levels. Modulation of Akt protein expression has been reported in different cell types expressing a variety of CREB mutants. CREB and CREB mutants which are constitutively active show higher expression levels of Akt protein whereas dominant negative CREB mutants downregulate Akt protein expression [Reusch and Klemm, 2002]. Expression of a mutant form of CREB, KCREB, in human melanoma cells decreased their tumorigenic and metastatic potential in nude mice [Robertson, 2005]. Expression of KCREB also blocked adipocyte differentiation and induced apoptosis in these cells [Reusch and Klemm, 2002]. KCREB expression increased the expression of several proapoptotic genes and decreased the expression of antiapoptotic signaling as well as downregulated the expression of Akt, a key mediator of cell survival [Reusch and Klemm, 2002]. Elevated levels of Akt protein are observed in 3T3 L1 preadipocytes treated with conventional differentiating agents or in cells which inducibly express constitutively active VP16-CREB or CREB-DIEDML [Reusch and Klemm, 2002].

In conclusion, we show here for the first time that CREB binds to CRE binding motifs in the Akt1 promoter and that this causes transcriptional and translational upregulation of Akt1 protein synthesis. Elevated cAMP levels promote PKA-mediated phosphorylation of CREB and cAMP-mediated activation of Epac [Misra and Pizzo 2005]. CREB binds CBP which upregulates Akt1 transcription. Epac-activated PI 3-kinase generates PIP 3 which causes cytosolic resident Akt1 recruitment to the plasma membranes and its phosphorylation at Thr^{308} and Ser^{473}. The activated Akt1 translocates to nuclei. Akt1 signaling is terminated upon its dephosphorylation or degradation. The Akt1 protein pool in stimulated cells is replenished rapidly by CREB-mediated upregulation of *Akt1* gene expression. Thus there appears to be a synergy between transcriptional upregulation of Akt1 by CREB and phosphorylation of CREB by Akt1 in eliciting the cell proliferative and survival effects of cAMP.

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