

Inhibition of cAMP-response Element-binding Protein Activity Decreases Protein Kinase B/Akt Expression in 3T3-L1 Adipocytes and Induces Apoptosis*

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White adipose tissue mass is governed by competing processes that control lipid synthesis and storage, the development of new adipocytes, and their survival. We have shown that the transcription factor cAMP-response element-binding protein (CREB) participates in adipogenesis, with constitutively active forms of CREB inducing adipocyte differentiation and dominant negative forms of CREB blocking this process. In other cell types, CREB and related factors have been shown to play important roles in survival and apoptosis. Here we demonstrate that reduction of CREB activity by ectopic expression of the dominant negative CREB, KCREB, induces apoptosis of mature 3T3-L1 adipocytes in culture. Death by apoptosis was confirmed by increased nuclear condensation, changes in membrane morphology, and increased DNA fragmentation. Gene microarray analysis indicated that KCREB expression increased expression of several pro-apoptotic genes like Interleukin Converting Enzyme and decreased the expression of the anti-apoptotic signaling molecule, Akt/protein kinase B. Finally, introduction of constitutively active CREB, CREB-DIEDML, blocked death of mature adipocytes treated with TNF- α . The data indicate that CREB plays a central role in adipocyte survival, perhaps by regulating the expression of certain pro- and anti-apoptotic genes. These results not only extend the role of CREB in adipocyte biology but also highlight the general developmental and survival role of this factor in numerous cell and tissue types.

In addition to its primary role in energy storage, white adipose tissue plays significant roles in overall energy homeostasis and metabolic regulation and, in part, regulates satiety and insulin sensitivity (1). Diseases or dysfunction of white adipose tissue are observed with increasing frequency in clinical situations. For example, overweight and obesity and related conditions, including diabetes and cardiovascular disease, are reaching epidemic proportions worldwide (2–4). Loss or redistribution of adipose tissue associated with acquired or congen-

ital lipodystrophies, or due to aggressive antiretroviral therapy or subcutaneous insulin injection, also constitutes a growing health concern (5–8). Although these syndromes reflect the action of numerous interacting processes, they all are characterized by changes in adipose tissue mass.

Adipose tissue mass is governed in part through competing processes that either increase or decrease the size, number, and “maturity” of fat cells (9). Adipocyte size increases via increased storage of triacylglycerol from dietary sources or generated by lipogenic pathways; new fat cells may arise via the proliferation and differentiation of pre-adipocytes or adipoblasts to mature adipocytes (10). Alternately, decreases in adipose tissue mass generally involve the loss of stored lipids by lipolytic processes. There is now evidence that decreases in adipose tissue mass may also involve the loss of mature fat cells through programmed cell death or apoptosis in certain situations (8, 11–17).

In culture, adipocytes from rodents or cell lines like 3T3-L1 undergo apoptosis upon exposure to TNF- α ¹ or HIV protease inhibitors (8, 17). Ectopic overexpression of constitutively active MKK6, an activator of p38 MAPK, also induces apoptosis and necrosis of 3T3-L1 adipocytes (11). Other studies in rodents have demonstrated a decrease in adipose tissue DNA content due to loss of adipocytes in response to starvation or streptozotocin-induced diabetes (14). More recently, intracerebroventricular administration of leptin was also shown to decrease fat pad weight and DNA content (16). Apoptotic features, including DNA laddering and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining accompanied these changes. Apoptosis of adipose tissue cells has also been observed in human adipose tissue explants subjected to growth factor deprivation, elevated temperature, or TNF- α exposure as determined by changes in cell morphology and DNA laddering (12, 15). Similar results were reported for adipose tissue explants retrieved from cancer patients (13).

Previously, we have shown that the transcription factor CREB is a target for extracellular agents and intracellular signaling systems that induce adipogenesis (18, 19). Ectopic expression of a constitutively active, chimeric VP16-CREB protein was sufficient to induce adipogenesis, whereas expression

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¹ The abbreviations used are: TNF- α , tumor necrosis factor- α ; HIV, human immunodeficiency virus; MAPK, mitogen-activated protein kinase; CRE, cAMP-response element; CREB, cAMP-response element-binding protein; KCREB, dominant negative form of CREB; CREB-BL, cAMP, dibutyryl cyclic AMP; PI3K, phosphatidylinositol 3-kinase; FCS, fetal calf serum; PBS, phosphate-buffered saline; RIP, receptor interacting protein; ICE, interleukin 1 β converting enzyme; FACS, fluorescence-activated cell sorting; IGF-1, insulin-like growth factor 1; ERK, extracellular signal-regulated kinase; ATF, activating transcription factor.

of a dominant negative form of CREB, KCREB, blocked the adipogenic program. Similar roles for CREB have been observed in neuronal cells where it participates in differentiation and neurite outgrowth (20–22). CREB also serves as a potent survival factor in neurons preventing apoptosis due to neurotrophin withdrawal (23–26). When KCREB is expressed in mature adipocytes, we observe a loss of triacylglycerol vesicles and cells with typical adipocyte morphology over a 4- to 8-day period. Is this loss due to lipolysis and dedifferentiation of fat cells or is it due to adipocyte apoptosis? Here we show that ectopic expression of KCREB leads to apoptosis of mature adipocytes, which are replaced by undifferentiated pre-adipocytes in culture. Apoptosis was concomitant with an increase in several pro-apoptotic genes and down-regulation of the anti-apoptotic protein kinase B/Akt. We also demonstrate that ectopic expression of constitutively active forms of CREB block adipocyte apoptosis in response to exposure to TNF- α . These results indicate that CREB acts as a survival factor in mature adipocytes and may play out a role in regulating adipose tissue mass and controlling insulin sensitivity.

EXPERIMENTAL PROCEDURES

Materials—All standard chemicals were from Sigma Chemical Co. (St. Louis, MO), and anti-Akt antibody was from Cell Signaling (Beverly, MA). All supplies and reagents for SDS-PAGE were from Novex/Invitrogen (Carlsbad, CA). Cell culture media and supplies were from Invitrogen (Beverly, MA) and Gemini Bioproducts (Gaithersburg, MD). The Ecdysone-inducible expression system (pIND, pVgRXR vectors, zeocin, and Ponasterone A) was from Invitrogen. The PI3K inhibitor was purchased from Calbiochem, and the adenoviral expression vector for Akt(K179M) was provided by Dr. Carol Sable (VA Medical Center, Denver, CO).

Cell Culture—3T3-L1 fibroblasts were grown to confluence in fibroblast growth medium (Dulbecco's modified Eagle's medium containing 5.5 mM glucose, 10% fetal calf serum (FCS), and 0.5 mM glutamine). Differentiation was initiated by addition of medium containing 10% FCS, 1 mM glutamine, 500 μ M isobutylmethylxanthine (or 300 μ M Bt₂cAMP), 1 μ M dexamethasone, and 1 μ g/ml insulin. After 2 days, cells were transferred to adipocyte growth medium containing 25 mM glucose, 0.5 mM glutamine, 10% FCS, 1 mM glutamine, and 1 μ g/ml insulin and re-fed every 2 days. Differentiation of fibroblasts into mature adipocytes was confirmed by Oil Red O staining (18).

Transfection Procedures—Plates of 3T3-L1 fibroblasts were grown to 70–80% confluency and transfected with the indicated plasmids with Superfect Reagent (Qiagen, Valencia, CA) according to the manufacturer's recommendations. Cells stably transfected with the plasmid pVgRXR were selected in conventional medium containing 500 μ g/ml zeocin, and cells stably transfected with pIND-KCREB, pIND-VP16-CREB, pIND-CREB-DIEDML, or pIND-LacZ plasmids were selected in medium containing 500 μ g/ml Geneticin. Large, rapidly growing, well-separated colonies were isolated 10–12 days after selection was begun with either antibiotic. Isolated clones were passaged in low glucose Dulbecco's modified Eagle's medium containing 10% FCS, 1 mM L-glutamine, and 500 μ g/ml each of zeocin and Geneticin. KCREB, VP16-CREB, CREB-DIEDML or LacZ expression was induced through the addition of 5 μ M Ponasterone A to the growth medium. Assays were performed on cells growing on eight-chamber microscope slides. Ten days following the initiation of differentiation, the cells were stained with Oil Red O and counterstained with hematoxylin to visualize cell morphology. Cells were observed by bright-field microscopy, and representative fields were photographed with Kodak 200 film. Alternately, cells growing on multiwell slides were lysed directly in Laemmli SDS gel loading buffer, and the lysates were subjected to Western blot analysis for marker protein expression.

Ecdysone-inducible Expression System—The Ecdysone-inducible expression system was employed to prepare stably transfected 3T3-L1 cells in which we could induce the expression of KCREB, CREB-DIEDML, and LacZ as described previously (18, 19).

Western Blot Analysis—After correcting for protein concentrations, lysates from 3T3-L1 fibroblasts and adipocytes treated as described in the figure legends were prepared in Laemmli SDS loading buffer, resolved on 10% polyacrylamide-SDS gels, and transferred to nitrocellulose. The nitrocellulose blots were blocked with phosphate-buffered

saline containing 5% dry milk and 0.1% Tween 20 and then treated with antibodies that recognize Akt, CREB, or VP16. The blots were washed and subsequently treated with goat anti-rabbit IgG conjugated to alkaline phosphatase. After the blots were washed, specific immune complexes for each target protein were visualized with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium.

Analysis of Nuclear Condensation—Nuclear condensation in apoptotic cells was determined by microscopic observation of cells stained with Hoechst 33342. Control cells (LacZ expressing), KCREB-expressing cells, or cells treated with TNF- α were washed with PBS and fixed in PBS containing 3.7% formaldehyde for 10 min. The cells were washed in PBS, and stained in PBS containing 5 μ g/ml Hoechst 33342 for 15 min at ambient temperature. The monolayer were washed three times with PBS and observed by fluorescence microscopy (fluorescein isothiocyanate filter set). Images were captured on Kodak Royal Gold 200 film and scanned into a Macintosh G4 computer using an Agfa T1200 scanner.

Flow Cytometry—Essentially, 3T3-L1 cells were lifted from culture containers by gentle trypsinization and washed twice in PBS. The cells were resuspended in a buffer containing 100 mM Tris-HCl, pH 7.6, and 1 mM EDTA at 4° C. Immediately, an equal volume of 100% ethanol was added dropwise while vortexing, and the cells were fixed for 3 h at 4° C. After fixing, an additional volume of 100 mM Tris-HCl, pH 7.6, containing 0.1% Nonidet P-40, 20 units/ml RNase A, and 75 μ M propidium iodide was added while vortexing, and the suspension was placed under refrigeration overnight. The following day the suspensions were subjected to flow cytometry on a Becton Dickinson FACScan linked to a Macintosh G3 computer running CellQuest software.

Atlas cDNA Array Analysis—Total RNA was extracted from cells using the Atlas Pure Total RNA Labeling kit from CLONTECH (Palo Alto, CA). Single-strand cDNA probes were generated from total RNA using [³²P]dATP. These probes were used for hybridization with separate Mouse cDNA array membranes using protocols and reagents provided by the manufacturer (CLONTECH). Arrays were subjected to autoradiography at –80° C using Kodak Lightning Plus screens. Scanned arrays were analyzed using Atlas Image software (CLONTECH), comparing relative intensities of specific cDNA “spots,” which were corrected for differences in the relative intensities of housekeeping genes between membranes prior to analysis. Three separate Array analyses were performed with probes generated from RNA resulting from two different experiments. Results for cDNAs depicted below are the mean of these three separate determinations. Data is presented as mRNA content in KCREB-expressing cells relative to mRNA content in LacZ-expressing cells.

RESULTS

We have reported that CREB participates in the induction of adipogenesis. As part of the studies, we noted that CREB phosphorylation and activity were stimulated by conventional differentiation-inducing agents in an acute fashion, but were also transiently elevated at later times during adipogenesis. This suggested that CREB might also play a role in later stages of the differentiation process. In addition, our results in adipocyte cell lines parallel similar results in neurons wherein CREB not only participates in differentiation but also acts as a survival factor preventing apoptosis in the absence of neurotrophins. These factors led us to hypothesize that CREB may also play roles in the later stages of adipocyte differentiation or in maintaining the mature adipocyte phenotype.

As an initial test of this hypothesis, we blocked the activity of endogenous CREB in mature adipocytes through the ectopic expression of the dominant negative CREB protein, KCREB (27). KCREB inhibits the action of endogenous CREB by forming heterodimers with the endogenous protein that are incapable of binding target DNA sequences. KCREB differs from CREB by one amino acid in the DNA binding domain, and antibodies capable of differentiating between CREB and KCREB do not exist. However, using antibodies that recognize both proteins we were able to detect Ponasterone A-induced KCREB expression in 3T3-L1 adipocytes by Western blot (Fig. 1A). Inhibition of endogenous CREB activity by KCREB was verified by demonstrating that KCREB expression blocked

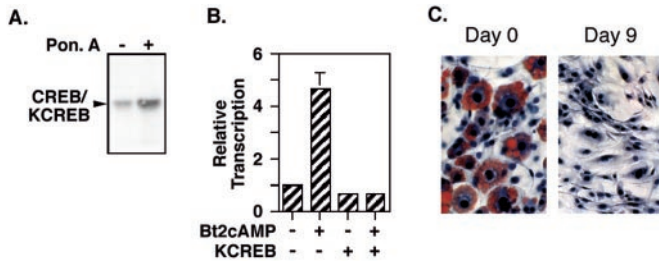


FIG. 1. Inducible expression of KCREB in 3T3-L1 adipocytes inhibits CRE-mediated transcription and elicits fat cell loss. 3T3-L1 pre-adipocytes were transfected with an Ecdysone-inducible KCREB expression system as described under "Experimental Procedures." The cells were then propagated to confluence, and adipogenesis was induced with Bt_2cAMP , insulin, and dexamethasone. **A**, inducible expression of KCREB protein was verified by preparing nuclear extracts from mature adipocytes (day 10 in post-induction) treated with $1.0 \mu M$ Ponasterone A (*Pon. A*) for 24 h or untreated, control cells. $25 \mu g$ of extract protein from each extract was resolved on 10% polyacrylamide-SDS gels and transferred to nitrocellulose. After the blots were blocked, they were incubated with antibody to total CREB, which recognizes both CREB and KCREB. The panel shows a representative Western blot of induced KCREB expression. **B**, mature adipocytes, stably transfected with the inducible KCREB expression system, were transfected with a plasmid containing the CRE-containing *somatostatin* gene promoter linked to a luciferase reporter gene using Superfect reagent. As indicated, KCREB expression was induced with $10 \mu M$ Ponasterone A (*KCREB + lanes*) overnight. The following day, the cells were treated with $0.3 mM$ Bt_2cAMP for 4 h as indicated. Luciferase expression was measured in lysates as an index of transcriptional activity, and levels are shown relative to luciferase activity in cells not treated with Ponasterone A or Bt_2cAMP . Levels were corrected for transfection efficiency by co-transfecting cells with a plasmid containing the Rous sarcoma virus (RSV) long terminal repeat linked to a β -galactosidase reporter. The data shown are averaged from three separate assays. **C**, mature adipocytes were treated with $10 \mu M$ Ponasterone A on day 0 to induce KCREB expression. The cells were refed every 3 days with complete medium containing $10 \mu M$ Ponasterone A for 9 days. Duplicate wells of cells were stained with Oil Red O and counterstained with hematoxylin on days 0 and 9. The panels show representative photomicrographs of cells on days 0 and 9 of treatment.

dibutyryl-cAMP (Bt_2cAMP)-stimulated transcription from the CRE-containing *somatostatin* gene promoter (Fig. 1B). We found that within 6–9 days following the initiation of KCREB expression in mature adipocytes, no triacylglycerol vesicles were visible in the cells, and in general, the cells lacked the characteristic rounded morphology of adipocytes (Fig. 1C). Similar results were obtained when adipocytes were infected with adenoviral vectors expressing other dominant negative CREBs, including CREBm1 and ACREB (data not shown).

We initially proposed that the changes elicited by KCREB reflected lipolysis of triacylglycerol stores and partial dedifferentiation. However, close examination of the cultures revealed a substantial number of non-adherent cells suggesting that the loss of mature adipocytes may have been due to cell death. To test the later hypothesis we expressed KCREB in mature adipocytes over several days and looked for apoptotic nuclear condensation by Hoechst staining. As shown in Fig. 2A, KCREB expression produced a time-dependent, transient increase in cells containing brightly staining nuclei indicative of nuclear condensation. The number of cells with apoptotic nuclei reached an apparent maximum on day 3 and then declined over the following 72 h. During the 6-day period, cells with dimly staining non-apoptotic nuclei, fibroblast morphology, and lacking lipid vesicles increased in number. These data suggested that KCREB expression induced the death of mature adipocytes, which were replaced in culture by undifferentiated pre-adipocytes. In addition to nuclear condensation, we also noted changes in membrane morphology consistent with apoptosis, including membrane retraction (Fig. 2B) and the formation of "blebs" (Fig. 2C).

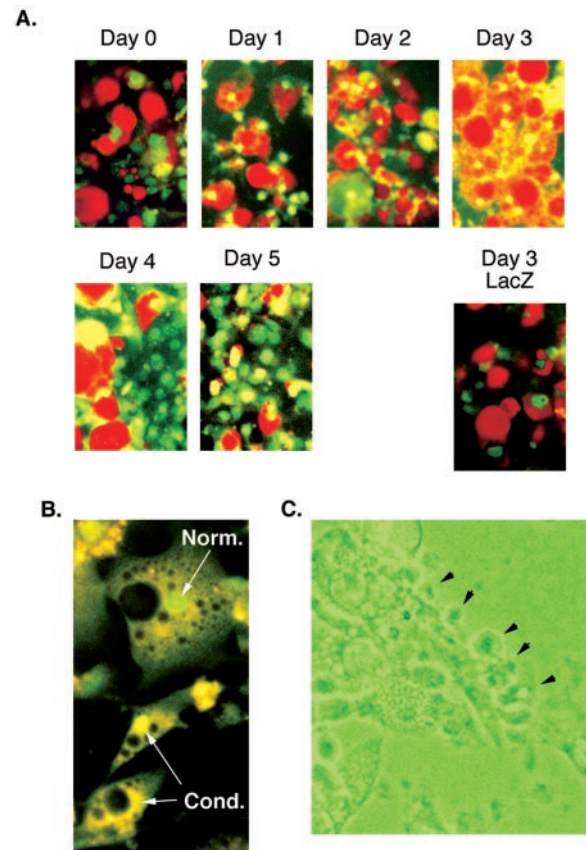


FIG. 2. KCREB expression induces adipocyte apoptosis as determined by Hoechst staining and changes in membrane structure. 3T3-L1 pre-adipocytes were stably transfected with the Ecdysone-inducible KCREB expression system as described under "Experimental Procedures." The cells were treated with a mixture of insulin, dexamethasone, and Bt_2cAMP to induce adipogenesis and cultured for 10 days until greater than 90% of the cells contained lipid vesicles. **A**, mature adipocytes were treated with $10 \mu M$ Ponasterone A to induce KCREB expression. On days 0 through 5, duplicate wells of cells were fixed in 10% formaldehyde in phosphate-buffered saline (PBS) for 10 min and stained with Oil Red O. Cells were subsequently stained with Hoechst 33342 for 15 min and rinsed three times with PBS. Representative fluorescence photomicrographs are shown of cells fixed and stained each day and visualized with a fluorescein isothiocyanate filter set. **B**, representative fluorescence photomicrograph (not Oil Red O-stained) of cells treated with $10 \mu M$ Ponasterone A for 24 h. A cell with normal (*Norm.*) nucleus and conventional adipocyte morphology is present at the top of the panel. Two cells with bright yellow, condensed nuclei and retracted, spindle-shaped morphology are present at the bottom. **C**, representative photomicrograph, taken with phase optics, showing the presence of membrane blebs on a cell treated with $10 \mu M$ Ponasterone A for 48 h.

Another hallmark of apoptotic cell death is DNA fragmentation. This parameter was measured by subjecting KCREB-expressing adipocytes to flow cytometric analysis after staining with propidium iodide. Apoptotic cells are evident on the resulting histograms as a "sub- G_0/G_1 " peak indicative of DNA fragmentation and reduced DNA content. The number of cells in the sub- G_0/G_1 peak increased from day 0 through day 3 and then declined (Fig. 3); consistent with the temporal changes in nuclear condensation shown in Fig. 2A. We also noted an increase in the number of cells in the S and G_2/M regions of the histograms on days 4–6. No lipid vesicles were evident during microscopic examination of cells in the S and G_2/M regions (data not shown). Given the uniform expression of KCREB in the cell population (18), we speculate that these cells represent undifferentiated cells that survive KCREB expression and proliferate during and after the death of mature adipocytes. It is also possible that these cells express little or no KCREB upon

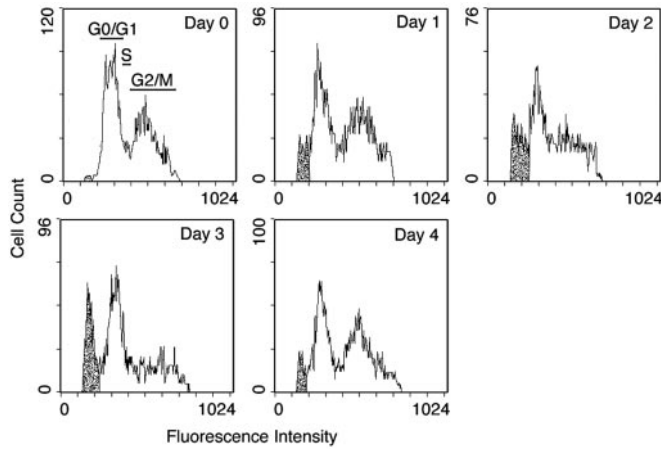


FIG. 3. KCREB expression induces adipocyte apoptosis as determined by flow cytometric analysis. Mature adipocytes stably transfected with the Ecdysone-inducible KCREB expression system were treated with $10 \mu\text{M}$ Ponasterone A to induce KCREB expression on day 0. The cells were refed on day 2 with complete medium containing $10 \mu\text{M}$ Ponasterone A. Each day, cells grown in duplicate wells were gently trypsinized, fixed, and stained with propidium iodide as described under "Experimental Procedures." Cell suspensions were subjected to flow cytometric analysis on a Becton Dickinson FACScan linked to a Macintosh G3 computer, and data were analyzed with CellQuest software. Each panel shows a representative histogram of fluorescence intensity (DNA content) versus cell count. The positions of the G_0/G_1 , S, and G_2/M regions are indicated in the day 0 panel, and the apoptotic sub- G_0/G_1 region is stippled in each panel.

exposure to Ponasterone A and, therefore, do not undergo apoptotic cell death. The specific nature of these cells is currently being investigated in our laboratory.

Given CREB's primary function in regulating gene expression, we examined the levels of expression of several apoptotic genes in untreated adipocytes, or adipocytes inducibly expressing KCREB for 24 h. For these assays, mouse Atlas 1.2 gene arrays (CLONTECH) were employed. We found that the expression of several pro-apoptotic genes were elevated in KCREB-expressing cells, most notably *Receptor Interacting Protein (RIP)*, and *Interleukin 1 β Converting Enzyme (ICE)*, or *caspase-1* (Fig. 4A). At the same time, expression of the anti-apoptotic signaling enzyme, protein kinase B/Akt (Fig. 4, *Akt*) was dramatically diminished by KCREB. Down-regulation of Akt and up-regulation of RIP and ICE by KCREB in adipocytes was confirmed by Western blot analysis (Fig. 4B). The Atlas gene arrays contain several other apoptosis-related genes, including *caspases 2, 7, and 11*, *bcl-2*, and *BAX*. However, either no change in the expression of these genes was observed, or no detectable signal was present on the autoradiograms even after prolonged exposure. Although a number of factors participate in apoptotic cell death, we have examined the contribution of Akt to adipocyte survival in preliminary experiments. We have found that ectopic expression of dominant negative Akt (Akt(K179M)) or treatment of cells with the PI3K inhibitor, LY294002, resulted in apoptosis of adipocytes as determined by FACS analysis (Fig. 5). The percentage of apoptotic cells with either of these two treatments reached ~30% to 50% within 48 h, whereas no apoptotic cells were detected in populations expressing an LacZ protein. These preliminary data suggest that one mechanism by which inhibition of CREB induces apoptosis is through decreased Akt signaling.

The ability of KCREB to induce adipocyte apoptosis suggested that constitutively active forms of CREB might block programmed cell death. To test this concept, the Ecdysone-inducible expression system was employed to express the active CREB mutant, CREB-DIEDML, which stimulates transcrip-

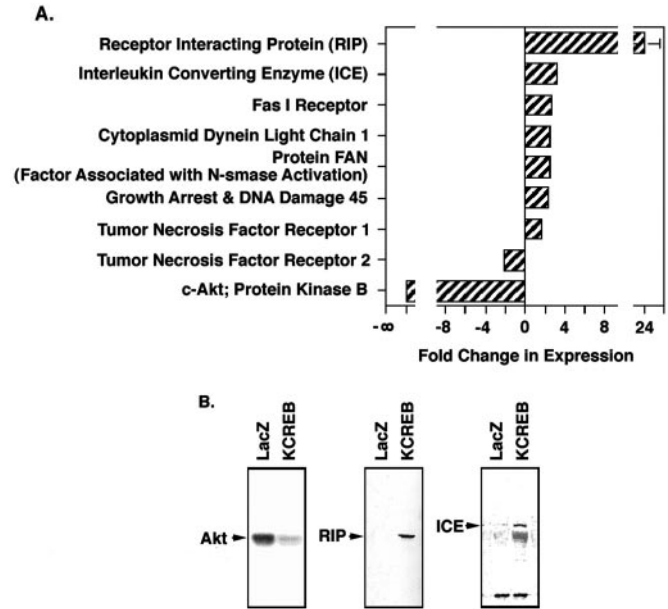


FIG. 4. KCREB expression in mature adipocytes regulates the expression of apoptosis-related genes. Mature 3T3-L1 adipocytes, stably transfected with the Ecdysone-inducible KCREB or LacZ expression systems were treated with $10 \mu\text{M}$ Ponasterone A for 24 h to induce expression of each protein. **A**, total RNA was isolated and labeled with [^{32}P]orthophosphate as described under "Experimental Procedures." Equal amounts (counts per minute) of each labeled RNA fraction were hybridized to mouse Atlas 1.2 gene arrays according to the manufacturer's specifications. Autoradiograms of the hybridized arrays were scanned into a personal computer and analyzed with Atlas Image software. The figure shows -fold change in the expression of certain apoptosis-related genes (corrected signal on KCREB blot/corrected signal LacZ blot). Data represent the averages of two blots each for KCREB and LacZ from two separate experiments. **B**, stably transfected 3T3-L1 adipocytes were treated with $10 \mu\text{M}$ Ponasterone A to induce LacZ or KCREB expression as indicated. Cells were lysed after 24 h, and $25 \mu\text{g}$ of lysates proteins was separated in 10% polyacrylamide-SDS gels and transferred to nitrocellulose. The blots were blocked and probed with antibodies to Akt, RIP, or ICE. The figure shows a representative blot of for each protein in cells expressing LacZ versus KCREB.

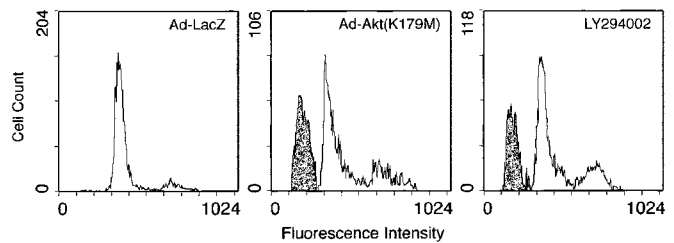


FIG. 5. Inhibition of PI3K/Akt signaling induces 3T3-L1 adipocyte apoptosis. Mature adipocytes were infected with adenoviral vectors (multiplicity of infection = 100) for LacZ or dominant negative Akt (*Akt(K179M)*) expression, or treated with $1 \mu\text{M}$ LY294002 for 48 h. The cells were gently trypsinized, fixed, and stained with propidium iodide as described under "Experimental Procedures." Cell suspensions were subjected to flow cytometric analysis on a Becton Dickinson FACScan linked to a Macintosh G3 computer, and data were analyzed with CellQuest software. Each panel shows a representative histogram of fluorescence intensity (DNA content) versus cell count. The apoptotic sub- G_0/G_1 region is stippled in each panel.

tion and associates with the transcriptional co-adaptor, CREB-binding protein (CBP) p300, in a phosphorylation-independent manner (28). First, we verified that the protein was expressed in response to Ponasterone A treatment by Western blot analysis (Fig. 6A). Simultaneously, we assessed the ability of CREB-DIEDML expression to drive transcription (luciferase production) from the CRE-containing, *somatostatin* gene promoter. We found that low doses of Ponasterone A ($0.1 \mu\text{M}$) and

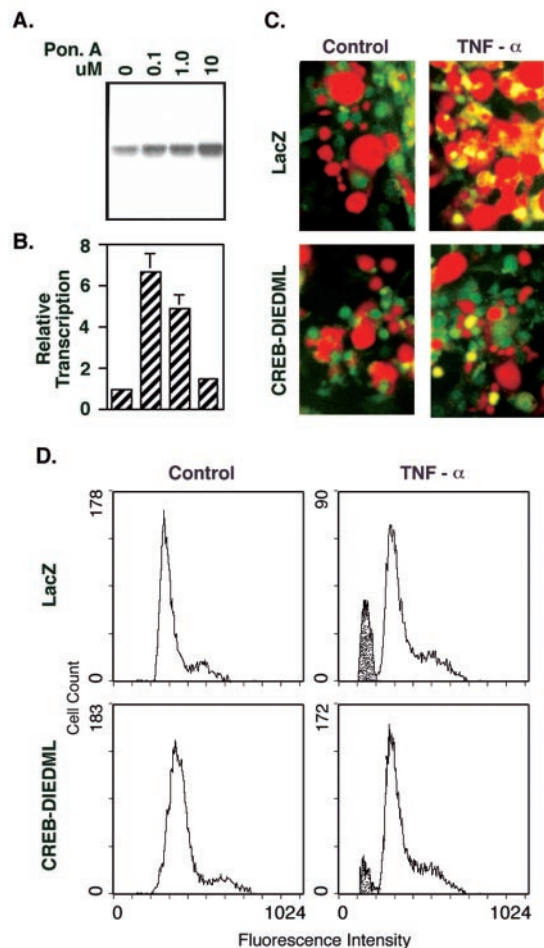


FIG. 6. CREB-DIEDML expression inhibits TNF- α -induced apoptosis of 3T3-L1 adipocytes. Stably transfected 3T3-L1 pre-adipocytes containing either an Ecdysone-inducible LacZ or CREB-DIEDML expression system were treated with insulin, dexamethasone, and Bt₂cAMP to induce adipogenesis as described under "Experimental Procedures." *A*, inducible expression of CREB-DIEDML protein (day 10 in post-induction) treated with the indicated levels of Ponasterone A (*Pon. A*) for 24 h. 25 μ g of protein from each extract was resolved on 10% polyacrylamide-SDS gels and transferred to nitrocellulose. After the blots were blocked they were incubated with antibody to total CREB, which recognizes both CREB and CREB-DIEDML. The *panel* shows a representative Western blot of induced CREB-DIEDML expression. *B*, mature adipocytes, stably transfected with the inducible CREB-DIEDML expression system, were transfected with a plasmid containing the CRE-containing *somatostatin* gene promoter linked to a luciferase reporter gene using Superfect reagent. As indicated, CREB-DIEDML expression was induced with the concentrations of Ponasterone A indicated in *A* for 24 h. Luciferase expression was measured in lysates as an index of transcriptional activity, and levels are shown relative to luciferase activity in cells not treated with Ponasterone A. Levels were corrected for transfection efficiency by co-transfecting cells with a plasmid containing the Rous sarcoma virus long terminal repeat linked to a β -galactosidase reporter. The data shown are averaged from three separate assays. *C*, mature adipocytes were treated with 0.3 μ M Ponasterone A to induce LacZ or CREB-DIEDML expression as indicated. The following day, TNF- α was added to 150 ng/ml (*Control* received no TNF- α). After 3 days, the cells were fixed and stained with Oil Red O and Hoechst 33342. Representative fluorescence photomicrographs for each treatment are shown. *D*, duplicates of the cell populations shown in *C* were gently harvested, fixed, stained with propidium iodide, and subjected to flow cytometric analysis as described under "Experimental Procedures." Representative histograms of cell number *versus* DNA content are shown for each treatment.

low levels of CREB-DIEDML elicited a 6.8-fold increase in transcription, whereas transcription levels decreased with further increases in Ponasterone A/CREB-DIEDML (Fig. 6*B*). The

decrease in transcription levels with high CREB-DIEDML expression probably reflects transcriptional squelching due to excess transactivator levels. Having optimized CREB-DIEDML expression/activity, we tested the ability of this factor to block adipocyte apoptosis induced by TNF- α . As shown in Fig. 6*C*, TNF- α produced a substantial increase in the number of control cells (ectopically expressing LacZ) with condensed nuclei as determined by Hoechst staining. However, in cells expressing CREB-DIEDML, fewer condensed nuclei were evident. These cell populations were subjected to FACS analysis to quantitate the number of apoptotic cells. No apoptotic nuclei were evident in untreated control cells (Fig. 6*D*). The percentage of apoptotic cells in the LacZ-expressing, TNF- α -treated populations averaged 30%, whereas the percentage for CREB-DIEDML-expressing, TNF- α -treated cells averaged only 5%.

DISCUSSION

In this paper we have shown that decreases in CREB activity generated by inducible, ectopic expression of KCREB and other dominant negative "CREBs" stimulate apoptosis of mature 3T3-L1 adipocytes in culture. Apoptosis was confirmed by chromatin condensation (Hoechst staining), changes in membrane morphology (retraction and blebbing), and cellular DNA degradation (subdiploid or sub-G₀/G₁ cells in FACS analysis). The ability of KCREB to induce adipocyte apoptosis appears to be due, in part, to the increased expression of certain pro-apoptotic genes, including RIP and ICE, and the decreased expression of the anti-apoptotic signaling enzyme, Akt. Although changes in the expression of most of these factors await confirmation, the decreased expression of Akt in response to KCREB was also noted by Western blot analysis. Finally, we demonstrated that ectopic expression of constitutively active forms of CREB partially inhibits the induction of adipocyte apoptosis in response to TNF- α exposure.

Other groups have shown that insulin and IGF-1 inhibit apoptosis of adipocytes and pre-adipocytes in response to TNF- α or serum deprivation (17, 29, 30). We have shown that these hormones stimulate CREB phosphorylation and transcriptional activity in several cell types, including 3T3-L1 pre-adipocytes and adipocytes through the activation of ERK and PI3K signaling systems and through the inhibition of protein phosphatase 2A (24, 25, 31, 32). Our current data implicate CREB as an important nuclear effector of the protective effects of insulin and IGF-1.

The ability of KCREB to initiate programmed cell death in adipocytes is consistent with the role of CREB in survival/apoptosis of other cell types, its regulation of survival/apoptotic genes, and its regulation by survival-associated growth factors. This is especially true of neurons. Walton *et al.* (33) have shown that CREB overexpression and prolonged phosphorylation protects neurons from okadaic acid-induced apoptosis, and Ginty and colleagues (26) have shown that nerve growth factor and other neurotrophins promote neuronal survival in part through CREB activation of bcl-2 expression and other pro-survival genes. Similarly, Pugazhenthil *et al.* (24, 25) have shown that IGF-1 prevents apoptosis of PC12 cells in culture by activating CREB, which in turn increases bcl-2 expression. Interestingly, CREB levels were reduced by caspase-mediated cleavage in neuroblastoma cells undergoing apoptosis in response to staurosporine, suggesting that apoptosis is associated with a decrease in CREB that normally protects against programmed cell death (23). Finally, addition of extracellular HIV-1 Tat protein to PC12 cells elicits a chronic down-regulation of CREB content and phosphorylation, and a progressive increase in apoptosis (34). This may account, partly, for the neuronal apoptosis and subsequent dementia frequently ob-

served in HIV-infected individuals.

CREB and proteins of the related activating transcription factor (ATF), cAMP-response element repressor/inducible cAMP-early repressor family of proteins also regulate survival of other cell types. For example, ectopic expression of KCREB in human melanoma cells decreases their resistance to radiation and renders them susceptible to thapsigargin-induced apoptosis, suggesting that CREB and the related ATF1 transcription factor may contribute to the acquisition of the malignant phenotype (35). Walker and colleagues (36) have shown that introduction of the phosphorylation-deficient, dominant negative CREBm1 into seminiferous tubules disrupts spermatogenesis through the apoptosis of spermatocyte germ cells. These data indicate that CREB and related proteins play key roles in the survival and development of numerous cell types and tissues.

An interesting observation in this study was the down-regulation of Akt/protein kinase B expression in KCREB-expressing adipocytes. Of the several apoptosis-related genes regulated by KCREB in our gene array analysis, we were able to confirm the regulation of Akt expression by Western blot analysis. The Akt serine/threonine-specific kinases are key mediators of cell survival in response to growth factors and calcium influx (37). Phosphorylation by Akt represses the activity of several pro-apoptotic molecules, including caspase-9, BAD, Forkhead transcription factors, and GSK-3 (38–41). Gagnon *et al.* (30) have shown that IGF-1 inhibits apoptosis of 3T3-L1 pre-adipocytes following serum deprivation via the activation of PI3K/Akt signaling. This pathway also contributes to adipogenesis in 3T3-L1 cells, with agents that block this signaling system inhibiting the conversion of pre-adipocytes to adipocytes (42–44). Interestingly, we have noted elevated Akt expression in gene array experiments and by Western blot, in 3T3-L1 pre-adipocytes treated with conventional differentiation-inducing agents, or inducibly expressing the constitutively active VP16-CREB or CREB-DIEDML.² We have extended these results to bovine aortic smooth muscle cells in culture, wherein KCREB decreases Akt expression and induces apoptosis, but CREB-DIEDML increases Akt expression.² Although these data are far from confirmatory, it is tempting to speculate that many of CREBs functions may be mediated via the direct regulation of Akt gene expression.

We have previously demonstrated that VP16-CREB and CREB-DIEDML induce adipogenesis in 3T3-L1 pre-adipocytes, and here we show that dominant negative KCREB stimulates adipocyte apoptosis while constitutively active CREBs protect against cell death in response to TNF- α . The development of new fat cells, and the death of mature adipocytes probably play competing roles in controlling adipose tissue mass and, in turn, in regulating insulin sensitivity. This connection is frequently manifest in obese individuals who develop insulin resistance, and ultimately type II diabetes, apparently due to the overabundance of stored fat in existing adipocytes (1). In most cases, agents that stimulate the development of new fat cells and the redistribution of fat stores vastly improves overall insulin sensitivity (45). At the other extreme, congenital and acquired lipotrophic and lipodystrophic disorders are also commonly associated with increased insulin resistance (46, 47). The conditions reflect a loss or decreased number of fat cells that leads to insulin resistance. The participation of CREB in the generation of new fat cells and its ability to block adipocyte apoptosis suggest that CREB may be a central determinant of overall insulin sensitivity. Experiments to explore the ability of constitutively active and dominant negative CREBs to modu-

late adipose tissue mass and insulin responsiveness are currently underway in our laboratories.

Our data indicate that CREB plays a central role in adipocyte survival, perhaps by regulating the expression of anti-apoptotic genes such as Akt. Our results not only extend the role of CREB in adipocyte biology but also highlight the general developmental and survival role of this factor in numerous cell and tissue types.

REFERENCES

- Spiegelman, B. M., and Flier, J. S. (1996) *Cell* **87**, 377–389
- U. S. Department of Health and Human Services (1998) *National Health and Nutrition Survey, Vol. III, 1988–1994*, Vol. 1996, Centers for Disease Control and Prevention, National Center for Health Statistics, Washington, D. C.
- McIntyre, A. M. (1998) *J. R. Soc. Health* **118**, 76–84
- Pi-Sunyer, F.-X., Laferrere, B., Aronne, L. J., and Bray, G. A. (1999) *J. Clin. Endocrinol. Metab.* **84**, 3–7
- Domingo, P., Matias-Guiu, X., Pujol, R. M., Francia, E., Lagarda, E., Sambeat, M. A., and Vazquez, G. (1999) *AIDS* **13**, 2261–2267
- Murao, S., Hirata, K., Ishida, T., and Takahara, J. (1998) *Intern. Med.* **37**, 1031–1033
- Lenhard, J. M., Furfine, E. S., Jain, R. G., Ittoop, O., Orband-Miller, L. A., Blanchard, S. G., Paulik, M. A., and Weiel, J. E. (2000) *Antiviral Res.* **47**, 121–129
- Dowell, P., Flexner, C., Kwiterovich, P. O., and Lane, M. D. (2000) *J. Biol. Chem.* **275**, 41325–41332
- Hirsch, J., and Han, P. W. (1969) *J. Lipid Res.* **10**, 77–82
- Roncari, D. A. K., Lau, D. C. W., and Kindler, S. (1981) *Metabolism* **30**, 425–427
- Engleman, J. A., Lisanti, M. P., and Scherer, P. E. (1998) *J. Biol. Chem.* **273**, 32111–32120
- Prins, J. B., Walker, N. I., Winterford, C. M., and Cameron, D. P. (1994) *Biochem. Biophys. Res. Commun.* **201**, 500–507
- Prins, J. B., Walker, N. I., Winterford, C. M., and Cameron, D. P. (1994) *Biochem. Biophys. Res. Commun.* **205**, 625–630
- Prins, J. B., and O'Rahilly, S. (1997) *Clin. Sci.* **93**, 3–11
- Prins, J. B., Niesler, C. U., Winterford, C. M., Bright, N. A., Siddle, K., O'Rahilly, S., Walker, N. I., and Cameron, D. P. (1997) *Diabetes* **46**, 1939–1944
- Qian, H., Azain, M. J., Compton, M. M., Hartzell, D. L., Hausman, G. J., and Baile, C. A. (1998) *Endocrinology* **139**, 791–794
- Qian, H., Hausman, D. B., Compton, M. M., Martin, R. J., Della-Fera, M. A., Hartzell, D. L., and Baile, C. A. (2001) *Biochem. Biophys. Res. Commun.* **284**, 1176–1183
- Reusch, J. E.-B., Colton, L. A., and Klemm, D. J. (2000) *Mol. Cell. Biol.* **20**, 1008–1020
- Klemm, D. J., Leitner, J. W., Watson, P., Nesterova, A., Reusch, J. E. B., Goalstone, M. L., and Draznin, B. (2001) *J. Biol. Chem.* **276**, 28430–28435
- Sung, J. Y., Shin, S. W., Ahn, Y. S., and Chung, K. C. (2001) *J. Biol. Chem.* **276**, 13858–13866
- Shimomura, A., Okamoto, Y., Hirata, Y., Kobayashi, M., Kawakami, K., Kiuchi, K., Wakabayashi, T., and Hagiwara, M. (1998) *J. Neurochem.* **70**, 1029–1034
- Heasley, L. E., Benedict, S., Gleavy, J., and Johnson, G. L. (1991) *Cell Regul.* **2**, 479–489
- Francois, F., Godinho, M. J., and Grimes, M. L. (2000) *FEBS Lett.* **486**, 281–284
- Pugazhenthil, S., Miller, E., Sable, C., Young, P. R., Heidenreich, K. A., Boxer, L. M., and Reusch, J. E. B. (1999) *J. Biol. Chem.* **274**, 27529–27535
- Pugazhenthil, S., Nesterova, A., Sable, C., Heidenreich, K. A., Boxer, L. M., Heasley, L. E., and Reusch, J. E. B. (2000) *J. Biol. Chem.* **275**, 10761–10766
- Riccio, A., Ahn, S., Davenport, C. M., Blendy, J. A., and Ginty, D. D. (1999) *Science* **286**, 2358–2361
- Walton, K. M., Rehfuss, R. P., Chrivia, J. C., Lochner, J. E., and Goodman, R. H. (1992) *Mol. Endocrinol.* **6**, 647–655
- Cardinaux, J. R., Notis, J. C., Zhang, Q., Vo, N., Craig, J. C., Fass, D. M., Brennan, R. G., and Goodman, R. H. (2000) *Mol. Cell. Biol.* **20**, 1546–1552
- Urso, B., Niesler, C. U., O'Rahilly, S., and Siddle, K. (2001) *Cell. Signalling* **13**, 279–285
- Gagnon, A. M., Dods, P., Roustan-Delattour, N., Chen, C. S., and Sorisky, A. (2001) *Endocrinology* **142**, 205–212
- Klemm, D. J., Roesler, W. J., Boras, T., Colton, L. A., Felder, K., and Reusch, J. E.-B. (1998) *J. Biol. Chem.* **273**, 917–923
- Reusch, J. E.-B., Hsieh, P., Klemm, D., Hoeffler, J., and Draznin, B. (1994) *Endocrinology* **135**, 2418–2422
- Walton, M., Woodgate, A. M., Muravlev, A., Xu, R., During, M. J., and Draganow, M. (1999) *J. Neurochem.* **73**, 1836–1842
- Zauli, G., Milani, D., Mirandola, P., Mazzoni, M., Secchiero, P., Miscia, S., and Capitani, S. (2001) *FASEB J.* **15**, 483–491
- Jean, D., Harbison, M., McConkey, D. J., Ronai, Z., and Bar-Eli, M. (1998) *J. Biol. Chem.* **273**, 24884–24890
- Scobey, M., Bertera, S., Someras, J., Watkins, S., Zeleznik, A., and Walker, W. (2001) *Endocrinology* **142**, 948–954
- Franke, T. F., Kaplan, D. R., and Cantley, L. C. (1997) *Cell* **88**, 435–437
- Brunet, A., Bonni, A., Zigmond, M. J., Lin, M. Z., Juo, P., Hu, L. S., Anderson, M. J., Arden, K. C., Blenis, J., and Greenberg, M. E. (1999) *Cell* **96**, 857–868
- Cardone, M. H., Roy, N., Stennicke, H. R., Salveson, G. S., Franke, T. F.,

² J. E. B. Reusch and D. J. Klemm, unpublished data.

- Stanbridge, E., Frisch, S., and Reed, J. C. (1998) *Science* **282**, 1318–1321
40. Datta, S. R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y., and Greenberg, M. E. (1997) *Cell* **91**, 231–241
41. Pap, M., and Cooper, G. M. (1998) *J. Biol. Chem.* **273**, 19929–19932
42. Xia, X., and Serrero, G. (1999) *J. Cell Physiol.* **178**, 9–16
43. Sakaue, H., Ogawa, W., Matsumoto, M., Kuroda, S., Takata, M., Sugimoto, T., Spiegelman, B. M., and Kasuga, M. (1998) *J. Biol. Chem.* **273**, 28945–28952
44. Gagnon, A. M., Chen, C. S., and Sorisky, A. (1999) *Diabetes* **48**, 691–698
45. Kletzien, R. F., Clarke, S. D., and Ulrich, R. G. (1992) *Mol. Pharmacol.* **41**, 393–398
46. Ganda, O. P. (2000) *Ann. Intern. Med.* **133**, 304–306
47. Hegele, R. A. (2000) *Curr. Atheroscler. Rep.* **2**, 397–404