CREB and Its Associated Proteins Act as Survival Factors for Human Melanoma Cells*

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cAMP response element-binding protein (CREB) and activating transcription factor 1 (ATF-1), members of the CREB/ATF family, have been implicated in cAMP- and calcium-induced transcriptional activation. We have previously demonstrated that quenching of CREB-associated proteins in metastatic melanoma cells by a dominant-negative CREB (KCREB) that is mutated within its DNA-binding domain decreased their radiation resistance, and their tumorigenic and metastatic potential in nude mice. As the induction of apoptosis by diverse exogenous signals is dependent on the elevation of intracellular Ca2+, the purpose of this study was to determine the role of CREB and its associated proteins in apoptosis using KCREB. We used thapsigargin (Tg), which inhibits endoplasmic reticulum-dependent Ca2+-ATPase and thereby increases cytosolic Ca2+, to induce apoptosis. MeWo human melanoma cells were transfected with the KCREB expression vector and subsequently analyzed for their susceptibility to Tg-induced apoptosis. Here we demonstrate that expression of KCREB in MeWo cells rendered them susceptible to Tg-induced apoptosis. Tg treatment induced phosphorylation of CREB and possibly ATF-1 transcription factors. Treatment with Tg induced CRE-negative transcription in parental cells, whereas this activation was reduced in the KCREB-transfected cells. In addition, CAT activity driven by the CRE-dependent promoter was inhibited in parental MeWo cells cotransfected with increasing concentrations of KCREB in a dose-dependent manner. We did not observe any changes in Bcl-2 or Bcl-2-related proteins (Bcl-x, Bax, and Bad) in control or KCREB-transfected cells before or after treatment with Tg. Collectively, these data indicate that CREB and its associated proteins act as survival factors for human melanoma cells, and hence contribute to the acquisition of the malignant phenotype.

The molecular basis of human malignant melanoma progression has remained largely unknown, despite the fact that the worldwide incidence of melanoma is increasing more than that of any other neoplastic disease (1). The development of malignant melanoma in humans progresses through a multistage process. The switches from melanocyte to nevi, to radial growth, and subsequently to vertical growth phase (metastatic phenotype) are associated with decreased dependence on growth factors, diminished anchorage dependence, and reduced contact inhibition (2, 3).

A large body of data concerning the molecular control of melanoma progression has come from studies using mitogens. In culture, melanocytes synergistically respond to a number of growth factors, which in combination with each other or with 12-O-tetradecanoylphorbol-13-acetate or cAMP stimulate not only proliferation but also pigmentation (4). These growth factors include several fibroblast growth factors, hepatocyte growth factor, and stem cell factor (also known as KIT ligand, MGF, and steel factor), all of which stimulate receptors tyrosine kinase. As melanocyte proliferation and differentiation are positively regulated by agents that increase cAMP (5–7), we have focused on the transcription factor CREB1 (for CRE-binding protein), which is known to be activated by cAMP, as a possible mediator of tumor growth and metastasis of human melanoma.

The 43-kDa CREB, which binds the consensus motif 5'-TGACGCTA-3' and activates transcription, was initially isolated from rat brain tissue (8, 9) and found to be ubiquitously expressed (10). CREB belongs to the leucine zipper class of proteins (10, 11). Certain leucine zipper family members can form heterodimers in specific combinations; however, the functional significance of this specific pairing is unknown.

The carboxyl terminus of CREB contains a leucine zipper that is required for dimerization and DNA binding (12). CREB also has a transactivation domain that contains several independent regions, including one identified as the kinase-inducible domain, which contains consensus phosphorylation sites for several kinases including protein kinase A (10). Indeed, one mechanism of CREB transcriptional activation is phosphorylation of Ser-133 by the catalytic subunit of cAMP-activated protein kinase A (13). Many other Ser/Thr kinases can phosphorylate CREB including protein kinase C (14); Ca2+/calmodulin-dependent protein kinases such as CaMKI, CaMKII (15), and CaMKIV (16); Ras-dependent p105 kinase (17), p90Akt (18), and Rsk 2 (19). It has been suggested that phosphorylation events alter the conformation of the transactivation domain of CREB, enhancing its interaction with the transcriptional machinery (20).

Previous studies have demonstrated that CREB expression correlates directly with the metastatic potential of murine melanoma cells (21), whereas the other CREB family transcription factor member ATF-1 is not detected in normal melanocytes but is easily found in metastatic melanoma cells (18). Whether these observations are causally related to tumorigenicity and

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1 The abbreviations used are: CREB, cAMP response element-binding protein; CRE, cAMP response element; ATF-1, activating transcription factor 1; CAT, chloramphenicol acetyltransferase; Tg, thapsigargin; St, staurosporine; KCREB, dominant-negative CREB; PBS, phosphate-buffered saline; FACS, fluorescence-activated cell sorting; TBS, Tris-buffered saline; TEM, transmission electron microscopy.
metastasis of melanoma cells was not yet clear. To study the contribution of the CREB transcription factor to tumor growth and metastasis of human melanoma cells, we have used a dominant-negative CDNA construct of CREB, KCREB, that had been mutated in the DNA-binding domain (22). When overexpressed, this protein can quench factor(s) capable of associating with CREB. CREB-associated proteins include members of the ATF and AP-1 transcription factor families (23–25). Expression of the KCREB mutant would be expected to yield proper heterodimerization with CRE-binding proteins; however, because of the mutant’s poor affinity to DNA, the associated proteins do not confer the same degree of transcriptional activity as they would in the case of the wild-type CREB. For example, previous studies using the KCREB construct in F9 teratocarcinoma and CA-77 thyroid carcinoma cells demonstrated its ability to block cAMP-induced transcription from the somatostatin promoter (22, 26).

We recently showed that the expression of the KCREB construct in metastatic MeWo melanoma cells decreased their tumorigenicity and metastatic potential in nude mice (27). The KCREB-transfected cells displayed a significant decrease in MMP-2 (72-kDa collagenase type IV) mRNA and activity that resulted in a decrease in invasiveness through the basement membrane, an important component of tumor invasion and metastasis. In addition, the cell-surface adhesion molecule MCAM/MUC18, which is involved in the metastasis of human melanoma (28–30), was down-regulated in the KCREB-transfected cells. Our results (27) indicated that, through their transcriptional activities, CREB and its associated proteins play an important role in the acquisition of the metastatic phenotype of human melanoma cells, possibly through regulation of MMP-2 and MCAM/MUC18 gene expression.

However, as CREB mediates both cAMP and Ca\textsuperscript{2+} transcriptional responses (13, 15), and as the induction of apoptosis by diverse exogenous signals depends on elevated intracellular Ca\textsuperscript{2+} (31, 32), we studied here the role of CREB and its associated proteins in apoptosis. In the present study, we used thapsigargin (Tg), which inhibits endoplasmic reticulum-dependent Ca\textsuperscript{2+}-ATPase and thereby increases cytosolic Ca\textsuperscript{2+} (33), to induce apoptosis in parental and KCREB-transfected melanoma cells. We demonstrate that expression of KCREB in MeWo melanoma cells renders them susceptible to Tg-induced apoptosis. Treatment with Tg caused phosphorylation of CREB and possibly ATF-1 transcription factors and induced CRE-dependent transcription in parental cells. However, in the KCREB-transfected cells, CREB/ATF-1 and their associated proteins were quenched, as shown by lower CAT activity driven by the CRE-dependent somatostatin promoter after treatment with Tg as compared with control cells. We did not observe any changes in protein expression of Bcl-2 and Bcl-2-related proteins (Bcl-x, Bax, and Bad) in the KCREB-transfected and control cells before and after treatment with Tg. Collectively, these results suggest that CREB and its associated proteins act as survival factors in human melanoma cells, thus providing an alternative mechanism by which dominant-negative CREB inhibits tumor growth and metastasis of human melanoma.

**EXPERIMENTAL PROCEDURES**

**Cell Lines**—The human melanoma MeWo cell line was established in culture from a lymph node metastasis of a melanoma patient (34) and was kindly provided to us by Dr. S. Ferrone (New York Medical College, New York, NY). MeWo cells are tumorigenic and metastatic in nude mice (35). Transfection of MeWo melanoma cells with the pRSV-KCREB construct and the isolation of the two clones K-2 and K-10 that express KCREB were described previously (36). Cells were maintained in culture as adherent monolayers in RPMI, supplemented with 10% fetal bovine serum (Summit, Ft. Collins, CO), sodium pyruvate, nonessential amino acids, β-glutamine, and penicillin-streptomycin (Life Technologies, Inc.). The transfected cells MeWo-Neo, K-2, and K-10 (36) were maintained in the same medium containing G418 (Life Technologies, Inc.) at 200 μg/ml. All cells were grown at 37 °C with 5% CO\textsubscript{2}.

**Antibodies**—Antibodies anti-CREB and anti-p-CREB (antibody 5522) were a gift from Dr. M. Montminy (Harvard Medical School, Boston, MA) (37). Antibody anti-CREB/ATF-1 (A1) was a gift of Dr. Michael R. Green (University of Massachusetts Medical Center, Worcester, MA) (38). Antibody to human Bcl-2 (clone 6C8) was provided by Dr. Timothy McDonnell (M. D. Anderson Cancer Center, Houston, TX). Polyclonal antibodies to Bcl-x (M1–25), Bad (K17), and Bax (N20) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA); antibody to β-actin was purchased from Sigma.

**Preparation of Nuclear and Total Cell Extracts**—Melanoma cells (5 × 10\textsuperscript{6}) were seeded onto 100-mm Petri dishes in culture medium without G418. Thapsigargin (1 μM) was added when cultures reached 70–80% cell confluence. All buffers used for the total and nuclear extracts contained protease and phosphatase inhibitors: 1 mM phenylmethylsulfonyl fluoride, 20 μM leupeptin, 0.15 unit/ml aprotinin, 1 mM Na\textsubscript{3}VO\textsubscript{4}, and 10 mM NaF. For total extract, cells were washed in cold PBS and lysed in 250 μl of Triton lysis buffer (25 mM Tris-HCl, pH 7.5 containing 150 mM NaCl, 1% Triton X-100, 5 mM EDTA) for 20 min on ice. For nuclear extract, cells were scraped off cold PBS and washed once in the same buffer. The pellet was resuspended in 400 μl of 10 mM HEPES buffer, pH 7.9 containing 1.5 mM MgCl\textsubscript{2}, 10 mM KCl, and 0.5 mM dithiothreitol. After a 10-min incubation on ice, the cytoplasmic fraction was separated by centrifugation at 14,000 rpm for 10 s at 4 °C. The nuclear pellet was resuspended in 100 μl of Triton lysis buffer and incubated for 30 min on ice. For both extracts, the soluble proteins in the lysates were separated by centrifugation at 14,000 rpm for 20 min at 4 °C. The protein content was quantified using the BCA protein assay (Pierce).

**Western Blot Analysis**—Proteins of total cell extract (40 μg) and nuclear extract (20 μg) were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to Immobilon P transfer membrane (Millipore, Bedford, MA). The membranes were washed in TBS (10 mM Tris-HCl, pH 8 containing 150 mM NaCl) and blocked with 5% nonfat milk in TBS for 2 h at room temperature. The blots were then probed overnight in TBS containing 0.1% Tween 20 and relevant antibodies with a dilution of 1/5000 except for the antibody to ATP-1 (1/5000). The membranes were then incubated with secondary antibody (anti-rabbit Ig, horseradish peroxidase-linked F(ab\textsubscript{2})\text{fragments (Amersham Pharmacia Biotech)}) with a dilution of 1/2000 in TBS containing 0.1% Tween 20 for 2 h at room temperature. In the case of Bcl-2, an anti-hamster IgG was used (Sigma) before the horseradish peroxidase-linked antibody. Immunoreactive materials were detected by enhanced chemiluminescence per manufacturer’s instructions (ECL detection system, Amersham Pharmacia Biotech).

**Plasmid Constructs**—The CRE-dependent plasmid (Somat-BgIII) was obtained from Dr. Marc R. Montminy (Harvard Medical School). The Somat-BgIII CAT construct contained the somatostatin gene promoter from -71 to +53 linked to the CAT reporter gene (39). pRSV-KCREB plasmid was kindly provided by Dr. Richard H. Good (Oregon Health Sciences, Portland, OR). pRSV-KCREB contained a full-length CREB CDNA with a single base pair substitution in the DNA-binding domain that causes a change from Arg\textsuperscript{224} to Leu (22). In parallel, a pRSV/RSV (Invitrogen, San Diego, CA) construct lacking CREB cDNA was used.

**DNA Transfection**—Melanoma cells (1.5 × 10\textsuperscript{6}) were transfected with 3 μg of the basic CAT expression vector with no promoter/enhancer sequence (pCAT-Basic), a control plasmid with SV40 promoter and enhancer (pCAT-Control) from Promega (Madison, WI) or the Somat-BgIII CAT construct using 20 μl of Lipofectin according to the manufacturer’s instructions (Life Technologies, Inc.). For cotransfection experiments, pRSV-RSV and pRSV-KCREB plasmids were added in the indicated amounts to a total concentration of 6 μg. Fifty nanograms of pRL-CMV vector, which contained the cDNA encoding Renilla luciferase (Promega) was introduced in each experiment to correct for variations of transfection efficiency. After 5 h, the transfection medium was replaced with normal growth medium. Thapsigargin (1 μM) was added to the culture medium 24 h after transfection, and the cells were incubated for an additional 24 h.

**CAT Assay**—The transfected cells were washed twice with PBS and lysed in reporter lysis buffer (Promega). After removal of cell debris by centrifugation, cell extracts were first assayed for Renilla luciferase activity using the dual-luciferase reporter assay system (Promega). Samples were normalized as the same relative luciferase activity and assayed for CAT activity (40). Briefly, cell extracts were incubated with 0.025 μCi of [14C]chloramphenicol and 0.35 μM acetyl-CoA in 25
mm Tris-HCl, pH 7.9, for 2 h at 37 °C. The nonacetylated and acetylated forms of chloramphenicol were extracted in ethyl acetate and separated by thin-layer chromatography with chloroform/methanol (95:5). The conversion of chloramphenicol to the acetylated form was quantified by scanning densitometry of an autoradiograph with a personal densitometer (Molecular Dynamics, Sunnyvale, CA). Each assay was repeated at least three times; there was less than 10% variation among individual transfections.

Detection of Apoptosis by Propidium Iodide Staining and Flow Cytometry—Cells were plated overnight in the culture medium described above without G418 and incubated in the presence of 1 μM thapsigargin (Tg) or 1 μM staurosporine (St) for 24 or 48 h. All cells (including those that had detached and those remaining adherent) were harvested in PBS containing 0.1% EDTA. Cells were washed twice with PBS; pelleted by centrifugation; resuspended in PBS containing 3 mm sodium citrate, 0.1% Triton X-100, and 50 μg/ml propidium iodide; and incubated for 2–4 h. The propidium iodide-stained cells were subjected to flow cytometric analysis on an EPICS Profile flow cytometer (Coulter Corp., Miami, FL). Results are reported as the average of at least three independent experiments.

Detection of Apoptosis by Transmission Electron Microscopy—Melanoma cells were treated by Tg or St and collected as described above. Cells were fixed in a cacodylate buffer (0.1 M) containing 3% glutaraldehyde and 2% paraformaldehyde for 1 h, washed, and treated with 0.1% tannic acid for 20 min. The samples were then incubated in 1% buffered osmium tetroxide for 1 h and stained with 1% aqueous uranyl acetate for 1 h (41). The samples were dehydrated in increasing concentrations of ethanol and then infiltrated and embedded in Spurr’s low viscosity medium. The blocks were polymerized in a 60 °C oven overnight. Thin sections were cut, stained with uranyl acetate and lead citrate, and examined using a Jeol 1200-EX transmission electron microscope (Jeol, Peabody, MA).

RESULTS

Thapsigargin Induces Apoptosis in KCREB-transfected Cells—We previously demonstrated that the transcription factor CREB and its associated proteins play an important role in the acquisition of the metastatic phenotype of human melanoma. Quenching of CREB and its associated proteins via the use of dominant-negative CREB (KCREB), which has been mutated within its DNA binding domain, led to a decrease in the tumorigenic and metastatic potential of metastatic MeWo melanoma cells in nude mice (27). As CREB mediates both cAMP and Ca2+ transcriptional responses (13, 15), and as induction of apoptosis by diverse exogenous signals is dependent on elevation of intracellular Ca2+ (31, 32), we decided to analyze if CREB and its associated proteins could be involved in the resistance of melanoma cells to apoptosis induction, hence contributing to tumor growth and metastasis. To study the involvement of CREB and its associated proteins in apoptosis, we used Tg, which inhibits endoplasmic reticulum-dependent Ca2+-ATPase and thereby increases cytosolic Ca2+ (33).

Previously, Yang et al. (36) have reported the selection and the characterization of the MeWo clones K-2 and K-10, which express the dominant-negative KCREB. We evaluated the ability of Tg to induce apoptosis in MeWo parental cells (MeWo-P), neo-transfected cells (MeWo-Neo), and the two KCREB-transfected clones K-2 and K-10 by propidium iodide staining and FACS analysis. Fig. 1A shows the results of a representative experiment after 48 h of treatment with 1 μM Tg. The cells treated with Tg showed a hypodiploid DNA content indicative of apoptosis. The percentages of hypodiploid cells were higher in K-2 and K-10 cells (46.4% and 83.3%, respectively) than in MeWo-P and MeWo-Neo cells (20.5% and 20.2%, respectively). We used St, which strongly inhibits various kinases (including tyrosine kinases) and which induces apoptosis in most nucleated mammalian cells (42), to verify that the higher percentages of hypodiploid cells observed with the two KCREB-transfected clones was specific for Tg and increase of cytosolic Ca2+. The MeWo-P, MeWo-Neo, K-2, and K-10 cells treated with St for 48 h showed comparable hypodiploid DNA contents (87.8%, 88.9%, 86.6%, and 85.6%, respectively). Fig. 1B summarizes the result of at least three independent FACS analyses of propidium iodide staining after 24 and 48 h of treatment with 1 μM Tg or 1 μM St. Only the K-2 and K-10 cells showed a hypodiploid DNA content indicative of apoptosis after 24 h of Tg treatment. The percentages of hypodiploid cells increased after 48 h of Tg treatment and were significantly higher in K-2 and K-10 cells by 2.2 and 3.3-fold, respectively, than in MeWo-P and MeWo-Neo cells. After 24 and 48 h of St treatment, the proportions of hypodiploid cells observed in MeWo-P, MeWo-Neo, K-2, and K-10 cells were not significantly different. These data demonstrate that Tg induced apoptosis in the two KCREB-transfected cells, whereas the control MeWo-P and MeWo-Neo cells were resistant to Tg-induced apoptosis. Agents that stimulate adenylylate cyclase or cAMP analogs (such as forskolin and dibutyryl-cAMP) did not cause apoptosis in MeWo-P, MeWo-Neo, K-2, or K-10 cells (data not shown), indicating that Tg-induced apoptosis was mediated via the Ca2+ and not the cAMP pathway.

Although DNA fragmentation is considered to be an excellent marker for apoptosis, there are reports of apoptosis without DNA fragmentation, particularly in studies with solid tumor cell lines. Because morphological alterations define the response, we investigated the effects of Tg and St on cellular morphology by transmission electron microscopy (TEM) to confirm the results obtained by propidium iodide-FACS analysis.
Cells were exposed to 1 mM Tg or 1 mM St for 48 h and were examined by TEM. Fig. 2A shows electron micrographs of cells (original magnification, ×1000). Cells with typical apoptotic morphology (plasma and nuclear membrane blebbing, chromatin condensation, cytoplasmic vacuolarization) were identified among KCREB-transfected K-10 and K-2 cells treated with Tg. The K-10 clone showed higher sensitivity to Tg-induced apoptosis than the K-2 clone, thus confirming the results obtained by FACS analysis after propidium iodide staining. Fig. 2B represents electron micrographs of a single cell taken from MeWo-P, MeWo-Neo, K-2, and K-10 cells after treatment with 1 mM Tg for 48 h. K-2 and K-10 Tg-treated cells showed nuclear changes consistent with apoptosis (condensation and segregation of chromatin into compact masses aligning with the inner side of the nuclear membrane). In contrast, most of the MeWo-P and MeWo-Neo cells did not display these changes (Fig. 2A and B). Treatment with St caused equivalent levels of apoptosis in all cells tested. The expression of dominant-negative KCREB did not modify the sensitivity of MeWo cells to St-induced apoptosis. Collectively, these results demonstrated that KCREB-transfected cells were more sensitive to Tg-induced apoptosis than were parental or control neo-transfected cells. The higher susceptibility of K-10 cells to Tg-induced apoptosis as compared with K-2 cells could be explained by higher KCREB activity in these cells (27, 36).

**Activation of CREB and ATF-1 by Thapsigargin**—The dominant-negative KCREB can quench any known CREB-associated proteins, including members of the ATF and AP-1 transcription factor families. Among the transcription factors that interact with KCREB, the transcription factor CREB is known to mediate transcriptional responses resulting from an increase of intracellular Ca\\textsubscript{2+} level (15). In order to understand the mechanisms by which KCREB increases sensitivity to Tg-induced apoptosis, we next investigated the activation of CREB by thapsigargin. We first analyzed the phosphorylation of CREB after treatment with 1 mM Tg in MeWo parental melanoma cells. Activation of CREB was assessed by immunoblotting using total cell extracts reacted with a-CREB antibody (5322) raised against the Ser\textsuperscript{133}-phosphorylated segment of amino acids 128–141 of CREB, recognizing only the activated form of CREB (37). In non-treated MeWo cells, a-CREB antibody detected one faint band at 38 kDa (Fig. 3, lanes 2, 4, 6, 8, and 10) that were undetectable or very weak without Tg treatment (lanes 1, 3, 5, 7, and 9). Reactivity of a-CREB antibody for these two proteins decreased after 1 h of Tg treatment (lanes 4, 6, 8, and 10). The 43-kDa protein was detected at 24 h (lane 8), whereas the 38-kDa protein (lane 10) was still observed at 48 h of Tg treatment.

To identify these two proteins, we first used a-CREB antibody to probe nuclear extracts of MeWo-P cells treated for 30 min with Tg (Fig. 4). The a-CREB antibody recognized a major protein that comigrated with the 43-kDa protein detected with a-CREB antibody (lane 2). a-CREB antibody also recognized slightly the 38-kDa protein (lane 2). ATF-1 is slightly smaller than CREB, belongs to the same protein family, and shares high homology with the CREB peptide sequence used to raise the a-CREB antiserum. These results indicate that Tg treatment of melanoma cells mediated phosphorylation of CREB. The other phos-
Regulation of a CRE-dependent Promoter by Thapsigargin—To study whether Tg also increased the transcriptional activity of CRE-binding proteins, we used the CRE-dependent plasmid (Somat-BgIII CAT), which contains the somatostatin gene promoter from −71 to +53 linked to the CAT reporter gene (39). In the following transfection experiments, cell extracts were prepared and equivalent amounts of extracts exhibiting the same Renilla luciferase activity were tested for CAT activity. We first analyzed the CAT activity driven by the CRE-dependent promoter Somat-BgIII in MeWo-P before and after treatment with 1 μM of Tg for 24 h (Fig. 5). Our preliminary studies indicated that treatment for 24 h with 1 μM Tg resulted in optimal induction of CRE-dependent transcription. This treatment had marginal effect on apoptosis of parental cells (Fig. 1B). We detected basal CAT activity before Tg treatment (Fig. 5A, lane 3), which increased after 24 h of Tg treatment by 14-fold (lane 5). The increase in the CRE-dependent transcription correlated with Tg-induced phosphorylation of CREB and ATF-1 (Fig. 3). An increase in CAT activity was also observed after 6 h of Tg treatment (data not shown). Together, these results demonstrated that Tg induced phosphorylation and increased the transcriptional activity of CREB and its associated proteins, which bind the CRE element.

Effect of KCREB on CRE-dependent Promoter Transactivation—We previously demonstrated that, unlike nuclear extracts from MeWo-P and MeWo-Neo cells, nuclear extracts from K-2 and K-10 cells fail to bind to a CRE element on a gel shift mobility assay (36). To verify that the dominant-negative KCREB inhibited the transactivation of a CRE-dependent promoter induced by Tg, we analyzed the CAT activity driven by the Somat-BglIII in one of the KCREB-transfected clones, K-2 (Fig. 5B). K-10 cells were not included in these assays because of the high rate of apoptosis induced by Tg (33%), whereas treatment of K-2 cells with 1 μM Tg for 24 h resulted in only 17.5% apoptosis (Fig. 1B). The CAT activity was low in K-2 cells before Tg treatment (lane 5). After 24 h of Tg treatment, CAT activity was observed, but the induction of the CRE-dependent promoter by Tg was significantly lower by 35% (9.5-fold activation) in the K-2 cells as compared with 14.7-fold activation in the parental MeWo cells (lanes 3 and 6). CAT activity driven by the SV40 promoter was the same in MeWo-P and K-2 cells and served as an additional internal control for transfection efficiency (lanes 1 and 4). To further characterize the ability of KCREB to inhibit the transactivation of a CRE-dependent promoter induced by Tg, we cotransfected the Somat-BgIII CAT construct into MeWo-P cells with increasing concentrations of pCS-kCREB DNA (pRc/RSV). Fig. 6 shows that CAT activity driven by the CRE-dependent promoter was inhibited by KCREB in MeWo cells treated for 24 h with Tg. The inhibition was dose-dependent and reached 50% (2-fold inhibition) when 5 μg of the expression vector encoding the dominant-negative KCREB (pRSV-KCREB) or with a control vector lacking the KCREB cDNA (pRc/RSV). Fig. 6 shows that CAT activity driven by the CRE-dependent promoter was inhibited by KCREB in MeWo cells treated for 24 h with Tg. The inhibition was dose-dependent and reached 50% (2-fold inhibition) when 5 μg of the expression vector encoding the dominant-negative KCREB was used in these cotransfection studies. Under these conditions, Tg treatment of KCREB-transfected cells did not induce significant apoptosis. These data demonstrated that the dominant-negative KCREB inhibited the transactivation of a CRE-dependent promoter. These results show that CREB and its associated proteins were involved in the resistance of MeWo cells to Tg-induced apoptosis and suggest that they act as a survival factor for human melanoma cells.

Expression of Bcl-2 and Bcl-2-related Proteins—To study for a possible mechanism by which CREB and its associated proteins might protect MeWo cells from Tg-induced apoptosis, we next analyzed the effect of KCREB on the expression of Bcl-2 and of Bcl-2-related proteins such as Bcl-x, Bax, and Bad. A previous report (43) suggested that CREB increases the expression of Bcl-2, which plays an important role in apoptosis. We analyzed the expression of Bcl-2 by Western blot analysis with a specific antibody. We did not observe any changes in the expression of Bcl-2 among MeWo-P, MeWo-Neo, and KCREB-transfected cells (Fig. 7A). Furthermore, treatment with Tg (1 μM for 24 h) did not modify Bcl-2 expression in these cells (Fig. 7B). Similar immunoblot analysis for Bcl-x, Bax, and Bad expression showed that the expression of these Bcl-2-related proteins did not change before or after treatment with Tg in control and KCREB-transfected cells (Fig. 7).
DISCUSSION

We previously showed that expression of dominant-negative CREB (KCREB) in human melanoma cells reduces their resistance to radiation (36). In addition, expression of KCREB in metastatic MeWo melanoma cells inhibits tumor growth and metastasis in nude mice (27). The mechanism by which KCREB inhibits tumor growth and metastasis of human melanoma could be explained partially by its effect on MMP-2 and MCAM/MUC18 gene transcription, as both genes are involved in the progression of human melanoma (27). In the present study, we demonstrate for the first time that CREB and its associated proteins act as negative regulators of apoptosis in diverse types of cells.

One mechanism by which CREB proteins can rescue cells from apoptosis is by up-regulation of Bcl-2 expression. Indeed, CREB proteins have been shown to function as positive regulators of the Bcl-2 gene via a direct binding of CREB and ATF family members to a CRE element within the Bcl-2 promoter (43). The CRE site in the Bcl-2 promoter appears to play a major role in the induction of Bcl-2 expression during the forces, i.e. proliferation and cell death by apoptosis, both of which are regulated by exogenous signals. The expression of the transcription factors CREB and ATF-1 changes during the progression of human melanoma. CREB expression correlates directly with the metastatic potential of murine melanoma cells (21), whereas ATF-1 is not expressed in normal melanocytes but is easily found in metastatic melanoma cells (18). As CREB and ATF-1 are implicated in cAMP and Ca\textsuperscript{2+}-induced transcriptional activation (13, 15, 38), this up-regulation in CREB and ATF-1 gene expression may confer growth advantage to melanoma cells in vivo by preventing them from undergoing apoptosis mediated through the Ca\textsuperscript{2+} pathway. Indeed, our results demonstrate that transfection of melanoma cells with dominant-negative CREB rendered them susceptible to apoptosis by thapsigargin, which inhibits endoplasmic reticulum-dependent Ca\textsuperscript{2+}-ATPase and thereby increases intracellular Ca\textsuperscript{2+} (33) but did not affect apoptosis induced by staurosporine. Our studies showed that CREB and possibly ATF-1 are involved in this process, as both were phosphorylated and activated in melanoma cells following Tg treatment.

In our studies, Tg-induced phosphorylation was higher for the 38-kDa protein, which most likely is ATF-1 and persisted for a longer time than the phosphorylation of CREB (Figs. 3 and 4), suggesting that in melanoma cells, the 38-kDa protein may play a greater role in regulating CRE-dependent genes via the Ca\textsuperscript{2+} pathway. We believe that the 38-kDa protein is ATF-1 because of its apparent molecular weight and because it was previously shown to be expressed in melanoma cells (18). In addition, we can rule out the possibility that the 38-kDa protein is an altered form of CREB, as anti-CREB antibody detected only one major band of 43-kDa protein and demonstrated a poor ability to recognize a 38-kDa protein. Indeed, although CREB and ATF-1 can mediate both cAMP- and Ca\textsuperscript{2+}-inducible gene expression, they respond differently to cAMP and Ca\textsuperscript{2+} (38). CREB has been shown to mediate cAMP and Ca\textsuperscript{2+} responses to similar levels, whereas the response of ATF-1 to the two pathways differs in magnitude (38).

Tg-induced apoptosis might be mediated through the regulation of c-fos and c-jun gene expression. Thapsigargin induces the expression of c-fos and c-jun through their 12-O-tetradecanoylphorbol-13-acetate and serum response elements, respectively (44). In addition, exposure of melanoma cells to Tg led to the induction of the zinc finger transcription factor, EGR-1, and apoptosis (45).

The involvement of CREB in apoptosis has been documented in other cell systems. For example, using the same dominant-negative form of CREB (KCREB) used in our studies, Barton et al. (46) have demonstrated that T cell development is normal in transgenic mice that express KCREB. In contrast, thymocytes and T cells from these animals displayed a profound proliferative defect characterized by markedly lower interleukin-2 production, G\textsubscript{1} cell-cycle arrest, and subsequent apoptotic death in response to a number of different activation signals including Tg (46). In addition, human monocytes infected with the influenza A virus died by apoptosis, which was associated with suppressed expression of CREB (47). Taken together, these studies and our present study suggest that CREB and its associated proteins act as negative regulators of apoptosis in diverse types of cells.
activation of mature B cells and during the rescue of immature B cells from apoptosis (43). In our studies, we did not observe any changes in the expression of Bcl-2 or Bcl-2-related proteins (Bcl-x, Bax, and Bad) in control or KCREB-transfected cells following Tg treatment, suggesting that phosphorylation of CREB or ATF-1 by Tg did not change the expression of these proteins in melanoma cells.

The therapeutic modalities to control tumor growth and metastasis of human melanoma are very limited. Based on our studies, that CREB and its associated proteins serve as survival factor for human melanoma cells, it is possible to use CRE-oligonucleotide as a transcription factor decoy to inhibit tumor growth and metastasis of human melanoma. Because the CRE cis-element TGACGTCA is palindromic, a single-stranded oligonucleotide of the CRE sequence will self-annael or form a hairpin when introduced into cells, thus acting as a decoy for the CREB/ATF family. We are currently investigating this possibility in our laboratory.

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