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Expression of a Peptide Inhibitor of Protein Phosphatase 1 Increases Phosphorylation and Activity of CREB in NIH 3T3 Fibroblasts

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We have examined the activity and phosphorylation state of the cyclic AMP (cAMP) response element binding factor (CREB) in intact NIH 3T3 cells following microinjection of expression plasmids encoding regulatory proteins of type 1 (PP1) and 2A (PP2A) serine/threonine-specific protein phosphatases. Changes in CREB phosphorylation in the injected cells were monitored by indirect immunofluorescence using an affinity-purified antiserum (Ab5322) which specifically recognizes CREB phosphorylated at Ser-133, and changes in transcriptional activity of CREB were monitored by expression of a reporter gene regulated by cAMP. cAMP-stimulated phosphorylation in NIH 3T3 cells is normally transient, and as expected, after stimulation of cells with cell-permeable cAMP analogs, the level of phosphorylated CREB was found to initially increase and then return to a basal level within 4 h. Microinjection of an expression vector encoding a constitutively active form of inhibitor 1 (I-1), a PP1-specific inhibitor, by itself resulted in an apparent increase in phosphorylated CREB in unstimulated cells. Moreover, injection of the I-1 vector resulted in the prolonged appearance of phosphorylated CREB in cells after cAMP stimulation. In contrast, injection of a plasmid encoding simian virus 40 small t antigen, which interacts with PP2A to inhibit its activity towards several phosphoprotein substrates, had no effect on the phosphorylation state of CREB in stimulated or unstimulated NIH 3T3 cells. Consistent with these results, injection of the I-1 expression vector activated expression from a coinjected CRE-*lacZ* reporter plasmid, indicating that the increased phosphorylation of CREB also activated its transcriptional activity. These results provide further evidence for a role of a PP1 as the primary protein (Ser/Thr) phosphatase regulating the dephosphorylation of Ser-133 and thereby limiting the transcriptional activity of CREB.

Cyclic AMP (cAMP) response element binding protein (CREB) is a transcription factor that is phosphorylated in response to extracellular stimuli which elevate intracellular cAMP and activate the cAMP-regulated protein kinase (cAPK) (12). Phosphorylation of CREB on Ser-133 is associated with increased transcription activity (21, 22, 26, 39, 45). Although multiple nuclear factors involved in gene expression may be phosphorylated by cAPK, CREB phosphorylated in vitro on Ser-133 is sufficient to activate CRE-mediated gene expression when introduced into cells by microinjection (2, 15). The importance of this single phosphorylation event is underscored by data from nuclear run-on experiments which have shown that CREB-dependent transcriptional rates diminish as CREB becomes dephosphorylated, leading several recent studies of CREB inactivation to focus on the characterization of protein phosphatases which dephosphorylate the activating site at Ser-133 in CREB (27, 65).

Type 1 (PP1) and 2A (PP2A) serine/threonine protein phosphatases together account for a significant amount of Ser/Thr protein phosphatase activity in mammalian cells (17, 56). In addition to containing sequence homology, PP1 and PP2A demonstrate very similar substrate specificities in vitro with many different substrates (17). However, it is thought that in vivo, unique spectrums of substrates for these enzymes must exist (3, 6, 18, 27). One laboratory has shown that the most efficient in vitro CREB phosphatase is a heterotrimeric form of PP2A (65), while studies from our laboratories indicate that the protein phosphatase primarily responsible for in vivo inactivation of CREB is a type 1 enzyme (PP1) (3, 27).

Results of in vivo studies using the phosphatase inhibitor okadaic acid (OA), which inhibits both PP1 and PP2A, have supported a role for one or both of these enzymes in the regulation of CRE-mediated gene expression (27, 65). However, this agent inhibits both PP1 and PP2A, although at different concentrations, and thereby limits its ability to define the individual contributions of these phosphatases (54). In order to discriminate the effects of inhibiting individual phosphatases (PP1 or PP2A) in intact cells, we have utilized specific protein inhibitors of these enzymes. For PP1, several endogenous inhibitors have been characterized (17, 56). These thermostable proteins, inhibitor 1 (I-1) and the structurally related protein dopamine- and cAMP-regulated phosphoprotein of 32 kDa, as well as inhibitor 2 (I-2), specifically inhibit the activity of PP1 (10, 11, 56). We have previously shown that microinjection of purified PP1 catalytic subunit inhibited CRE-regulated transcription in several different cell lines stably transfected with a CRE-*lacZ* reporter gene (27). In accordance with this, we also found that microinjection of a plasmid encoding a constitutively active peptide form of I-1 enhanced transcription from a coinjected CRE-*lacZ* reporter gene. Furthermore, I-2 significantly inhibited the dephosphorylation of CREB phosphorylated at Ser-133 by cAPK in PC12 nuclear extracts.

Results of numerous experiments suggest that the noncatalytic subunits of PP2A play an important role in regulating its activity and/or substrate specificities (57, 63, 64). PP2A catalytic subunit (38 kDa; $PP2A_C$) can form a complex with several

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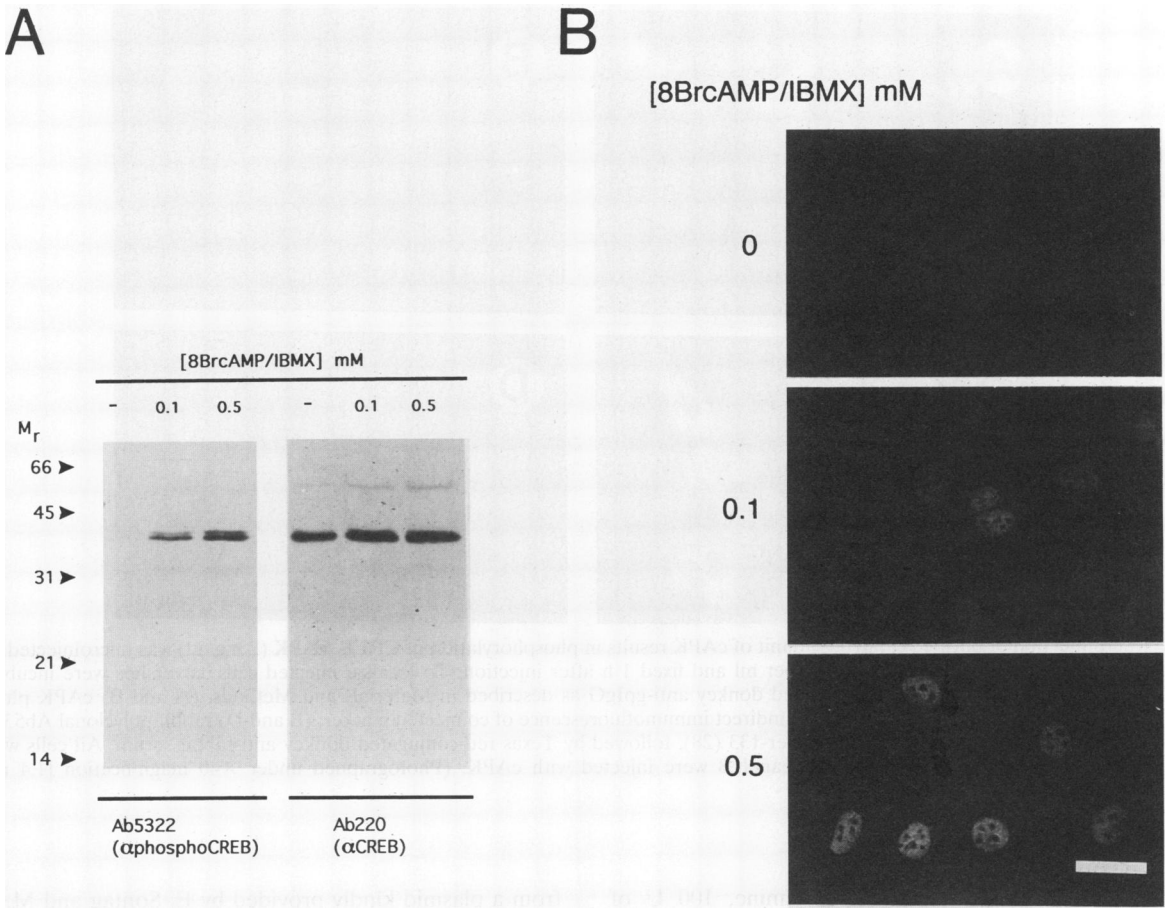

FIG. 1. Western blot and indirect immunofluorescence of phosphorylated CREB in cells treated with 8Br-cAMP and IBMX using Ab5322, which specifically recognizes CREB phosphorylated at Ser-133. NIH 3T3 cells were stimulated with 0.1 or 0.5 mM (each) 8Br-cAMP and IBMX, as indicated, for 1 h and either lysed in SDS-sample buffer, separated by SDS-polyacrylamide gel electrophoresis, and transferred to nitrocellulose for Western blotting with Ab5322 (A) or processed for phospho-CREB accumulation by indirect immunofluorescence using Ab5322 (B). (A) Duplicate lanes from either control (−) or 0.1 or 0.5 mM 8Br-cAMP and IBMX samples were incubated with either Ab5322 (anti-phospho-CREB) (left panel) or Ab220, which recognizes CREB irrespective of phosphorylation state (right panel), followed by horseradish peroxidase-conjugated goat anti-rabbit serum. Bands were visualized by chemiluminescence and exposure to X-ray film (ECL). CREB levels remain unchanged while the amount of CREB phosphorylated at Ser-133 increases with higher concentrations of 8Br-cAMP–IBMX. M_r is indicated on the left (in thousands). (B) Indirect immunofluorescence detection of phosphorylated CREB in cells treated with a cell-permeable analog of cAMP. Nuclear fluorescence reflecting levels of CREB phosphorylation assessed by indirect immunofluorescence increases in relative proportion to levels detected in the Western blots shown in panel A. (Photographed under ×63 magnification [1.4 numerical aperture]; bar represents 20 μm.)

different regulatory subunits (47). $PP2A_C$ can be purified as a dimer ($PP2A_{AC}$) consisting of $PP2A_C$ complexed to the A (63-kDa) subunit (46, 64). In cell extracts, most $PP2A_{AC}$ is found complexed with a 55-kDa B (41), or 54-kDa B′ (46), or 74-kDa B″ (64) subunit. Recent studies have shown that several viral proteins interact with $PP2A_C$ to modulate its activity (33, 47, 53, 66, 67). Simian virus 40 (SV40) small t antigen, for example, binds to and inhibits the activity of PP2A towards several substrates (53, 68). These results have led to the suggestion that the expression of small t antigen inhibits the dephosphorylation of selected substrates by PP2A, leading to altered gene expression and enhancing cell transformation (47).

In this study, we examined in more detail the effects of inhibition of either endogenous PP1 or PP2A on CREB activity. For this, we microinjected expression vectors bearing cDNAs encoding inhibitors of PP1 and PP2A, a PP1-specific inhibitor (I-1) peptide, and the PP2A-specific SV40 small t antigen and assayed their effects on CREB phosphorylation and corresponding transactivation of a cAMP-regulated reporter gene. Changes in CREB phosphorylation were monitored by indirect immunofluorescence using an antibody which recognizes CREB protein phosphorylated at Ser-133 (28). cAMP-regulated gene expression was monitored by injection of a reporter plasmid with a CRE-regulated promoter (3, 27, 51). We found that expression of the I-1 peptide caused an accumulation of CREB phosphorylated at Ser-133, resulting in an activation of CRE-regulated gene expression, while no such effects were found following expression of small t. Together, these results demonstrate that a PP1 is the most likely enzyme regulating CREB dephosphorylation in NIH 3T3 fibroblasts.

(Work performed by A. S. Alberts was done in partial fulfillment of the requirements for a Ph.D. in physiology and pharmacology from the University of California at San Diego.)

MATERIALS AND METHODS

Cell culture and microinjection. NIH 3T3 fibroblasts were grown at 37°C in 10% CO_2 in Dulbecco's modified Eagle's

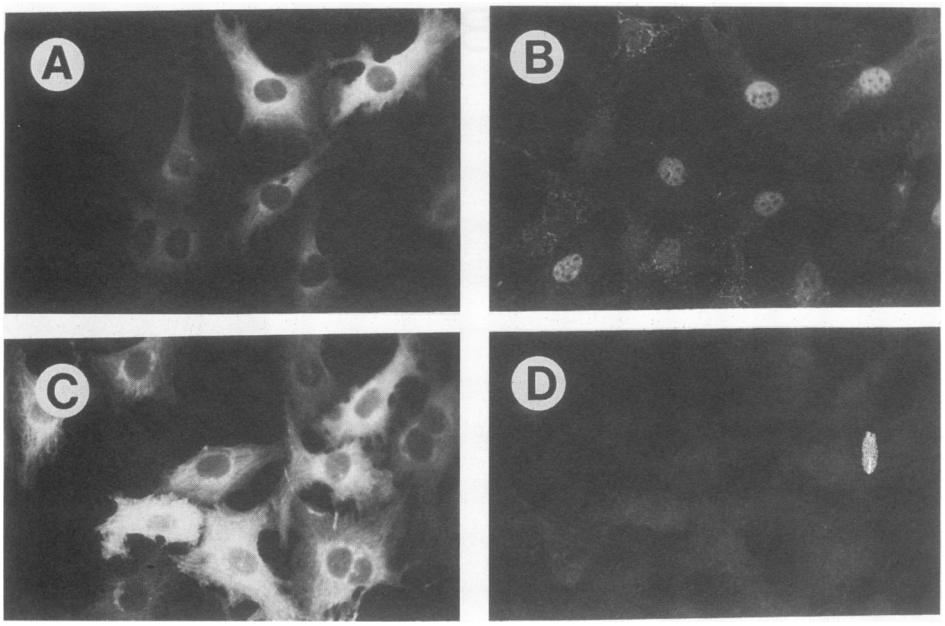

FIG. 2. Microinjection of purified catalytic subunit of cAPK results in phosphorylation of CREB. cAPK (2 mg/ml) was microinjected into NIH 3T3 cells along with 5 mg of nonspecific gpIgG per ml and fixed 1 h after injection. To localize injected cells, coverslips were incubated with 7-amino-4-methylcoumarin-3-acetic acid-conjugated donkey anti-gpIgG as described in Materials and Methods. (A and B) cAPK plus marker gpIgG; (C and D) marker gpIgG alone; (A and C) indirect immunofluorescence of coinjected marker; (B and D) rabbit polyclonal Ab5322, which specifically recognizes CREB phosphorylated at Ser-133 (28), followed by Texas red-conjugated donkey anti-rabbit serum. All cells with nuclei staining positive for CREB phosphorylation in panel B were injected with cAPK. (Photographed under ×40 magnification [1.4 numerical aperture].)

medium supplemented with 2 mM L-glutamine, 100 U of penicillin, 100 μg of streptomycin sulfate per ml, and 10% (vol/vol) fetal calf serum (Gibco). For injection into quiescent cells, cells were incubated in Dulbecco's modified Eagle's medium containing 0.05% fetal calf serum for 24 to 36 h. Cells were stimulated with the concentrations of 8-bromoadenosine-3′,5′-cyc lic monophosphate (8Br-cAMP) and/or 3-isobutyl-1-methyl-xanthine (IBMX; Sigma, St. Louis, Mo.) indicated below.

Cells were injected with the plasmids, described below, at concentrations between 0.03 and 0.2 mg/ml, where indicated, in a microinjection buffer composed of 50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.2) and 40 mM NaCl (4). Injections were done with a semiautomatic micromanipulator-injector (Eppendorf, Madison, Wis.) with needles pulled from glass capillaries on a vertical pipette puller (Kopf Industries, Tujunga, Calif.) (61, 62). Direct nuclear injection is required for the rapid expression of injected plasmid DNA (27, 61). Nonspecific guinea pig immunoglobulin G (gpIgG; 5 mg/ml) (Sigma) was coinjected with the expression plasmids for positive identification of the injected cells by indirect immunofluorescence. cAPK catalytic subunit was a gift from S. Taylor, University of California, San Diego.

Plasmids. CRE-regulated gene expression was measured by the injection of pCRE-*lacZ* reporter plasmid which contained three copies of the CRE sequence from the human vasoactive intestinal peptide gene cloned upstream of the *Escherichia coli lacZ* gene (3, 27, 42, 44). The pCMV-*lacZ* vector contains the human cytomegalovirus immediate-early gene promoter from −552 to +97 upstream from the *lacZ* gene (9). pCEP4-Sm t contains the full-length wild-type SV40 small t *Hin*dIII-*Bam*HI fragment subcloned into pCEP4 expression vector (Invitrogen) from a plasmid kindly provided by E. Sontag and M. Mumby (14). pCB6-I-1 contains a 154-bp synthetic gene encoding the sequence from 9 to 54 of rabbit skeletal muscle I-1. This represents an active fragment of I-1 (1, 31). An aspartic acid (Thr→Asp-35) was substituted in place of the threonine phosphorylated by cAPK to constitutively activate the expressed peptide.

Western blotting (immunoblotting). Duplicate plates of confluent NIH 3T3 cells, rendered quiescent by serum deprivation, were lysed in sodium dodecyl sulfate (SDS)-sample buffer following incubation with 8Br-cAMP and IBMX for 1 h at the concentrations indicated below. Whole-cell lysates from each sample (~50 μg of protein) were split and resolved on an SDS–12.5% polyacrylamide gel according to the method of Laemmli (37). Duplicate blots were probed first with antibody 5322 (Ab5322) (28) rabbit anti-phospho-CREB or Ab220 rabbit anti-CREB, which recognizes CREB irrespective of its phosphorylation state (21). The rabbit antibodies were recognized with horseradish peroxidase-conjugated donkey anti-rabbit serum (Amersham) and visualized by chemiluminescence according to the instructions of the manufacturer (ECL; Amersham) and exposed to X-ray film.

Immunofluorescence and histochemistry. Following injection, the cells were stimulated as indicated in the figure legends and fixed in 3.7% (wt/vol) formaldehyde in phosphate-buffered saline (PBS) for 5 min at ambient temperature. To identify injected cells and simultaneously the presence of phospho-CREB, coverslips were incubated with the primary rabbit antibody, anti-phospho-CREB (Ab5322) (28), diluted 1:200 in 0.5% Nonidet P-40–PBS. Following extensive rinsing in PBS, this antibody was detected with Texas red-conjugated donkey anti-rabbit IgG (Jackson Laboratories, West Grove, Pa.). Coinjected gpIgG was detected with 7-amino-4-methylcouma-

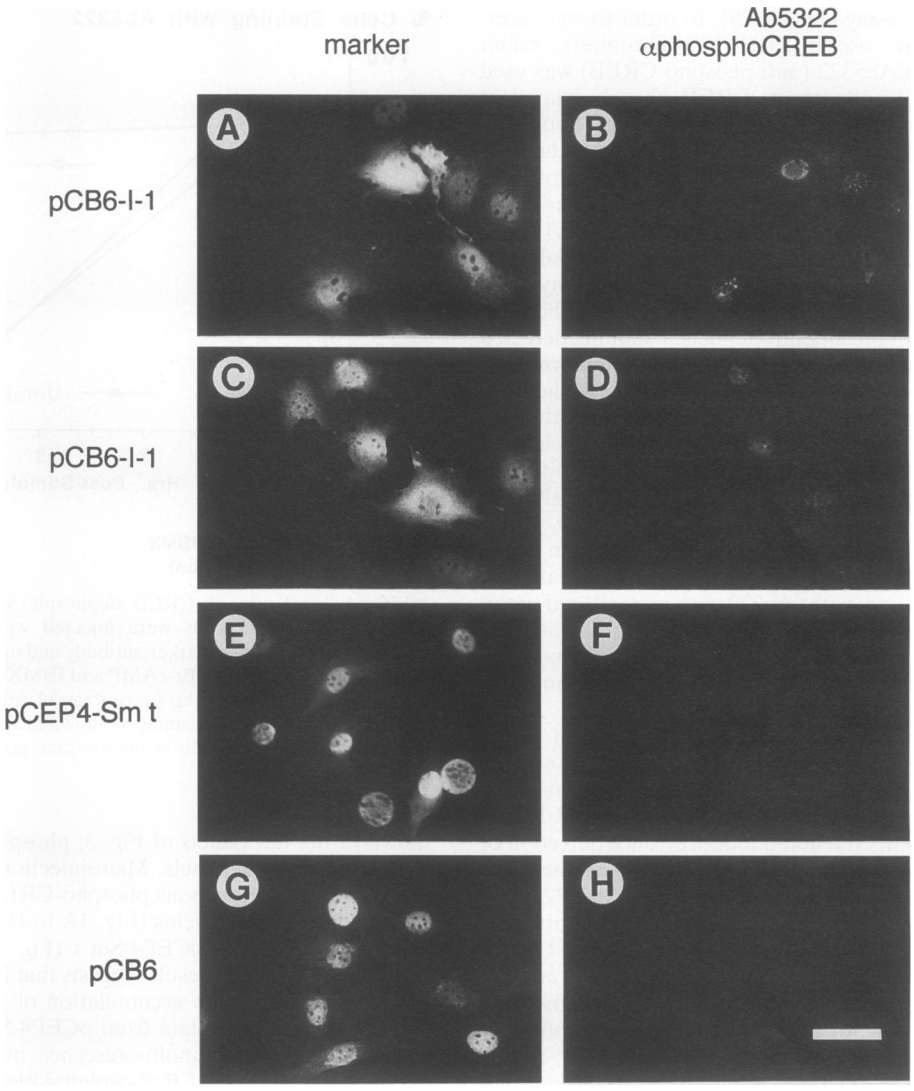

FIG. 3. Microinjection of pCB6-I-1 enhances the phosphorylation of CREB in unstimulated cells. pCB6-I-1, pCB6, and pCEP4-Sm t (0.03 mg/ml each) were microinjected into the nuclei of quiescent NIH 3T3 fibroblasts and fixed 2 h after injection as described in Materials and Methods (immunofluorescence of marker gpIgG is shown in the left column, and corresponding anti-phospho-CREB [Ab5322] immunofluorescence is in the right column). (A to D) Two representative fields of cells injected with pCB6-I-1; (E to H) cells injected with pCEP4-Sm t (E and F) or pCEP4 (G and H). (Photographed under ×40 magnification; bar represents 30 μm.)

rin-3-acetic acid-conjugated donkey anti-gpIgG (heavy plus light chains) (Jackson Laboratories) diluted 1:200 in 0.5% Nonidet P-40–PBS as indicated in the figure legends. Incubation with the anti-gpIgG antibody was performed at the same time as that with the secondary antibody for rabbit anti-phospho-CREB. For histochemical assay of β-galactosidase (β-Gal) activity, cells were washed with PBS and incubated for up to 12 h at 37°C with a mixture of 1 mg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; IBI) per ml, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, and 1 mM $MgCl_2$ in PBS. Cells expressing β-Gal contained a dark blue precipitate of the reaction product; blue cells were scored as positive and colorless injected cells were scored as negative for β-Gal expression. The blue X-Gal precipitate tends to quench immunofluorescence; thus, blue β-Gal-expressing cells which appeared negative by immunofluorescence were also scored as injected. Coverslips were then washed extensively with Tween 20-PBS and mounted in PBS containing 15% (wt/vol) polyvinyl alcohol (Gelvatol), 33% (vol/vol) glycerol, and 0.1% (wt/vol) NaN_3 on glass slides.

Fluorescence microscopy. All cells were observed and photographed with a Zeiss Axiophot epifluorescence microscope. Representative fields of cells stained with X-Gal (phase contrast) were photographed under a magnification of ×20 or ×40 (1.4 numerical aperture) as indicated in the figure legends, with Kodak Technical pan film (ASA 100). The fluorescent fields were photographed with Kodak T-Max film (ASA 800). The exposure times for each respective fluorophore were identical.

RESULTS

Characterization of antiserum which recognizes phosphorylated CREB in NIH 3T3 fibroblasts. To assess changes in CREB phosphorylation, we used an antibody which specifically recognizes CREB phosphorylated at Ser-133 in an indirect

immunofluorescence assay (21, 23, 28). In order to characterize this antibody for use in NIH 3T3 fibroblasts, rabbit polyclonal antiserum Ab5322 (anti-phospho-CREB) was used to compare levels of endogenous CREB phosphorylated in response to microinjection of purified catalytic subunit of cAPK (32, 43) or following treatment of cells with the cell-permeable cAMP analogs. As shown in Fig. 1, Western blots with Ab5322 specifically recognize a 43-kDa band in whole-cell lysates from NIH 3T3 cells treated for 1 h with either 0.1 or 0.5 mM 8Br-cAMP and IBMX (as described in Materials and Methods). The increasing levels of CREB phosphorylation resulting from treatment with 8Br-cAMP and IBMX, shown in Fig. 1A, correlate with the amount of nuclear staining detected by indirect immunofluorescence using Ab5322 in formaldehyde-fixed NIH 3T3 cells grown on glass coverslips, as shown in Fig. 1B. This induction required both reagents, as neither 0.1 mM 8Br-cAMP nor 0.1 mM IBMX alone was sufficient to significantly stimulate the accumulation of phospho-CREB detectable by anti-phospho-CREB within 1 h (data not shown). Total CREB levels were monitored by using Ab220, which recognizes CREB irrespective of its phosphorylation state. Either by Western blotting (Fig. 1A) or in situ (data not shown), CREB staining with Ab220 was not altered upon treatment of cells with 8BrcAMP and IBMX, indicating that changes in staining with Ab5322 represented changes in the phosphorylation levels of CREB at Ser-133 and not an accumulation of CREB protein.

Figure 2A and B show cells injected with 2 mg of cAPK catalytic subunit per ml along with 5 mg of marker antibody per ml and assayed 1 h later for CREB phosphorylation by indirect immunofluorescence as described in Materials and Methods. Panel A shows the immunofluorescence detection of the coinjected marker antibody, and panel B shows the same field of cAPK-injected cells counterstained with Ab5322; cytoplasmic injection of cAPK produces intensely fluorescent nuclei, demonstrating the increased phosphorylation of CREB. Cells injected with marker antibody alone (Fig. 2C and D) showed staining levels similar to those of the neighboring uninjected cells. Staining for phospho-CREB in cells following the injection of cAPK or treatment with 8Br-cAMP–IBMX reached a maximum at ~1 h and was reduced to background levels ~4 h after injection (see below). In cells maintained under serum deprivation, <5% had nuclei which stained with Ab5322. Upon stimulation with cAMP analog, typically 80 to 90% of treated cells stained with the phospho-CREB antiserum (data not shown). These data show that Ab5322 allowed for the analysis of the relatively rapid changes in the phosphorylation state of CREB that occur in individual microinjected cells by immunofluorescence.

Injection of a plasmid expressing a constitutively active form of I-1 peptide stimulates the accumulation of phosphorylated CREB. To compare the effects of inhibition of cellular PP1 and PP2A on phosphorylated CREB levels, we microinjected into fibroblasts eukaryotic expression plasmids under control of the cytomegalovirus promoter encoding either a constitutively activated form of I-1 peptide (pCB6-I-1) or encoding full-length SV40 small t antigen (pCEP4-Sm t) and monitored changes in cellular levels of phospho-CREB (7, 49). Microinjection of expression plasmids of this type into the nucleus results in high-level expression of encoded proteins within 30 to 60 min (4, 13, 34). Quiescent NIH 3T3 cells were injected with pCB6-I-1, the pCB6 expression plasmid alone (no insert), or pCEP4-Sm t, each mixed with gpIgG (5 mg/ml) as a marker, and the effects on CREB phosphorylation were analyzed by indirect immunofluorescence. Cells were fixed and stained 2 h after injection of the plasmid. Marker protein is

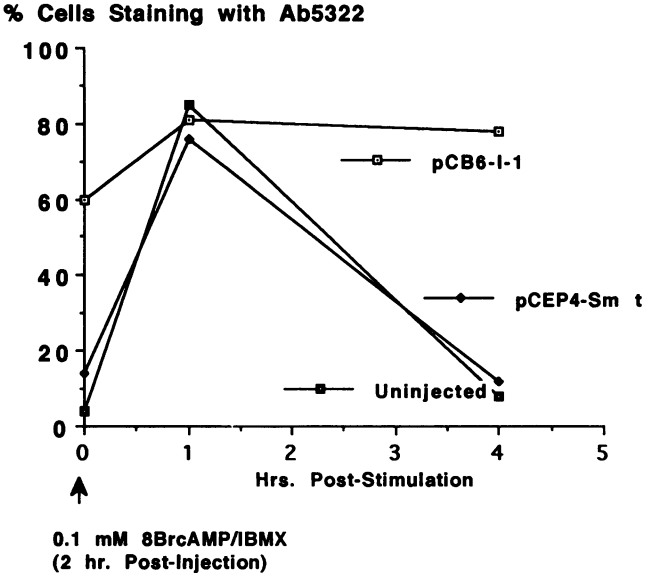

FIG. 4. Inhibition of CREB dephosphorylation by injection of pCB6-I-1. Quiescent cells were injected with either pCB6-I-1 or pCEP4-Sm t along with marker antibody and incubated for 2 h prior to stimulation with 0.1 mM 8Br-cAMP and IBMX for the indicated times. The ordinate represents the percentage of cells which demonstrated nuclear phospho-CREB staining as shown in Fig. 1B and 2. Representative fields of injected cells at the 4-h time point are shown in Fig. 5.

shown in the left panels of Fig. 3; phospho-CREB staining is shown in the right panels. Microinjection of pCB6-I-1 markedly increased endogenous phospho-CREB staining, as shown in two representative fields (Fig. 3A to D). No effect was seen in cells injected with pCEP4-Sm t (Fig. 3E and F) or pCB6 (Fig. 3G and H). This result suggests that inhibition of PP1, but not PP2A, induced the accumulation of phospho-CREB. Expression of small t protein from pCEP4-Sm t in injected cells was confirmed by immunofluorescence, indicating that the lack of effect of Sm t on CREB phosphorylation did not result from a lack of expression of Sm t protein (20). Furthermore, we have shown that pCEP4-Sm t can activate AP-1 activity when injected in REF52 cells along with mitogen-activated kinases and that pCEP4-Sm t on its own activates AP-1 in CV-1 cells, thus demonstrating the functional expression of Sm t and its apparent ability to inhibit PP2A (20). Interestingly, accumulation of phospho-CREB in pCB6-I-1-injected cells did not require further stimulation with cAMP analogs. This effect suggests that an equilibrium exists between a CREB Ser-133 kinase and an I-1-sensitive CREB phosphatase in quiescent cells, such that the inhibition of the phosphatase can by itself change the balance to higher levels of phosphorylated CREB.

The phosphorylation of CREB at Ser-133 is transient (27). Phospho-CREB staining induced by treatment of cells with both 8Br-cAMP and IBMX reaches an apparent maximum 1 h after stimulation and returns to nondetectable levels within approximately 4 h. We tested the ability of either pCB6-I-1 or pCEP4-Sm t to inhibit the normal dephosphorylation of CREB. Cells were injected with each vector, as before, incubated for 2 h to allow for expression of each respective factor, and then stimulated with 0.1 mM 8Br-cAMP and IBMX; cells were fixed and stained with Ab5322 after stimulation at various times thereafter. These injections are summarized in Fig. 4; photomicrographs of representative fields of injected cells are shown in Fig. 5. Cells injected with pCB6-I-1 plasmid and fixed

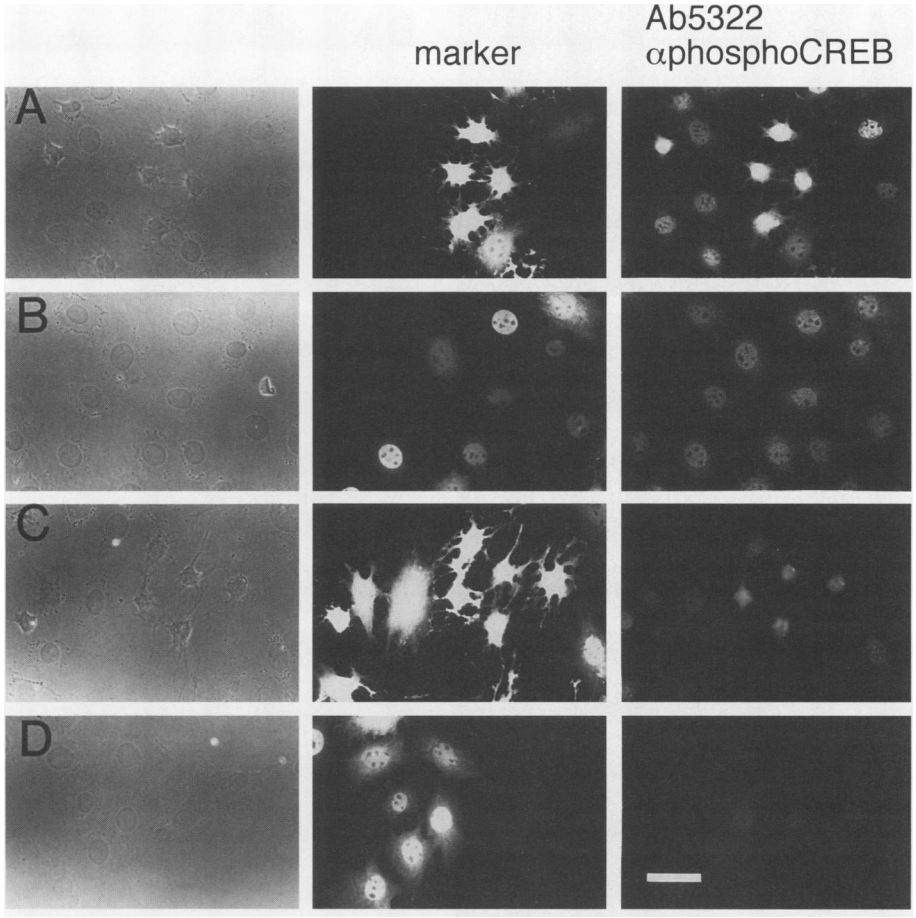

FIG. 5. Injection of pCB6-I-1 inhibits the dephosphorylation of CREB. pCB6-I-1 and pCEP4-Sm t were tested for their ability to prolong the normally transient phosphorylation of CREB at Ser-133. Cells were injected with pCB6-I-1 (row A) or pCEP4-Sm t (row B), incubated for 2 h to allow for expression from the injected plasmid, and stimulated with 0.1 mM 8Br-cAMP and IMBX for 1 h. Indirect immunofluorescence of coinjected marker antibody is shown in the middle panels; Ab5322 (anti-phospho-CREB) is shown in the right panels. (C and D) Cells injected with pCB6-I-1 (C) and pCEP-Sm t (D) stimulated for 4 h with 0.1 mM 8Br-cAMP and IBMX (fixed 6 h postinjection). Cells injected with pCB6-I-1 remained intensely stained, while injection of pCEP4-Sm t had no effect on phospho-CREB staining at 4 h after stimulation. (Photographed under ×40 magnification; bar represents 30 μm.)

at 1 h after stimulation with drugs are shown in Fig. 5, row A. For comparison, row B shows cells injected with pCEP4-Sm t after the same treatment; phospho-CREB levels in cells injected with pCEP4-Sm t and stimulated for 1 h were identical to levels observed in uninjected cells. Phospho-CREB staining is markedly brighter in pCB6-I-1-injected cells at this time point. At 4 h after injection, only cells injected with pCB6-I-1 maintained high levels of phospho-CREB staining (Fig. 5, row C), while levels of phospho-CREB staining in cells injected with pCEP4-Sm t (Fig. 5D) had returned to the intensity observed in unstimulated cells. We also noted again that even before the addition of the drugs, cells injected with pCB6-I-1 showed increased phospho-CREB staining, as shown in Fig. 3. I-1-injected cells had a stellate morphology usually observed in NIH 3T3 cells when treated with significantly higher concentrations of cAMP analogs (i.e., 1 mM 8Br-cAMP and IBMX) or following injections of high concentrations of cAPK (38). Cells injected with pCB6-I-1 exhibited phospho-CREB staining for up to 4 h after treatment with 8Br-cAMP–IBMX. These results suggest that expression of a specific inhibitor of PP1 but not PP2A led to a prolonged accumulation of CREB phosphorylated at Ser-133 by blocking the normal physiological dephosphorylation of CREB.

Expression of I-1 stimulates the activity of a CRE-*lacZ* reporter gene. The reporter plasmid pCRE-*lacZ* expresses β-Gal in response to elevations in intracellular cAMP levels. β-Gal expression is readily detected in single, microinjected cells by incubation with the chromogenic substrate X-Gal, the reaction product of which forms a blue precipitate (19, 27, 42, 50). Quiescent NIH 3T3 fibroblasts were coinjected with the expression plasmids encoding I-1 or small t along with the pCRE-*lacZ* reporter plasmid (3, 42). The cells were incubated for 5 h and then fixed and stained for the presence of the marker antibody and β-Gal; marker protein is shown on the left of Fig. 6, and X-Gal staining is shown on the right. Approximately 50% of cells injected with pCB6-I-1 were found to express β-Gal (Fig. 6A and B), while cells injected with pCB6 (Fig. 6C and D) or pCEP4-Sm t (Fig. 6E and F) showed virtually no β-Gal expression. The results of these injections (three to four separate experiments) are summarized in Table 1. These data are in agreement with our previously published results of experiments which were performed with a different cell type (REF52) using I-1 cloned into a different expression vector (27) and are consistent with the results reported above showing that only pCB6-I-1 stimulated phospho-CREB accumulation in unstimulated cells (Fig. 3). To test the specificity of

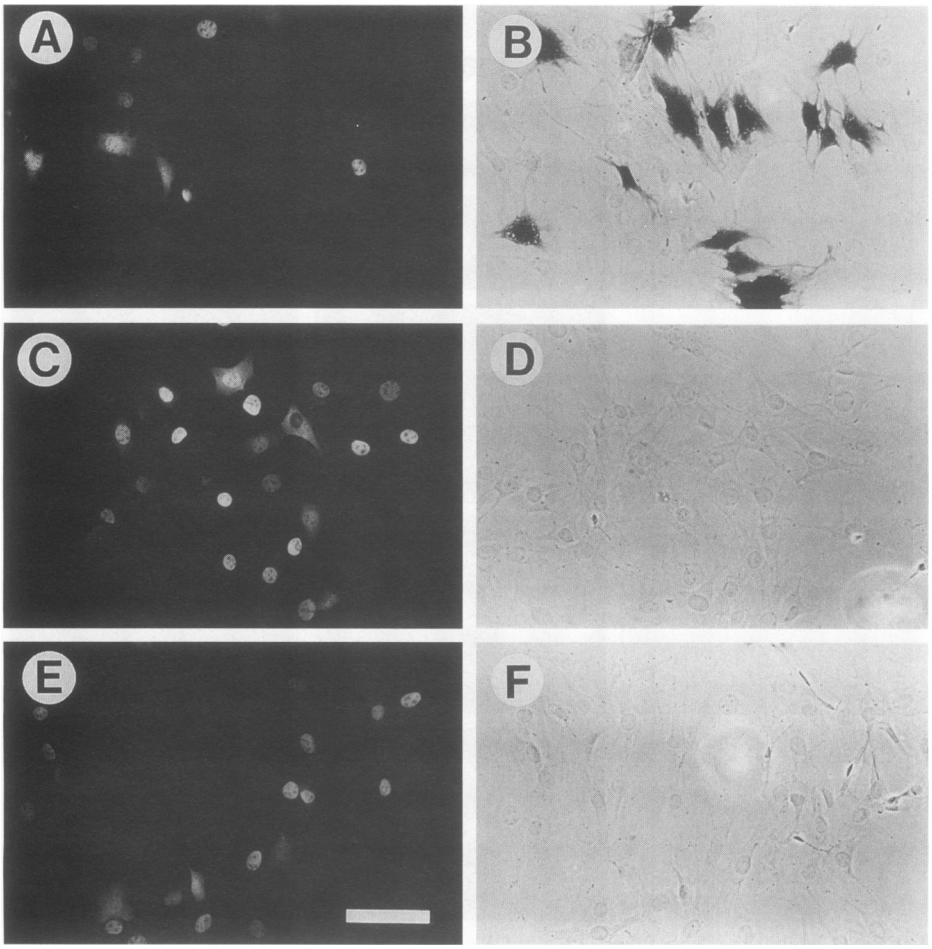

FIG. 6. pCB6-I-1 activates expression from coinjected CRE-*lacZ* reporter gene. Cells were injected with pCB6-I-1 (A and B), pCEP4-Sm t (C and D), or pCB6 (E and F) along with gpIgG as a marker. Representative fields of injected cells are shown; results from these experiments are shown in Table 1. Cells were fixed and stained 5 h after injection for the presence of marker gpIgG (left panels) and β-Gal expression (right panels) by histochemical staining with X-Gal as previously described (44). Note that immunofluorescent staining for presence of coinjected marker IgG is quenched in cells expressing the reporter β-Gal. (Photographed under ×20 magnification; bar represents 60 μm.)

these constructs, we analyzed the effects of coinjection of the respective expression vectors with a reporter plasmid not responsive to cAMP, pCMV-*lacZ* (9). Injections of the two vectors had no effect on the expression of β-Gal from a pCMV-*lacZ* reporter gene (Table 1) (4, 9), indicating that the two expression constructs did not simply interfere with reporter expression under the constitutive cytomegalovirus promoter.

DISCUSSION

Our previous studies comparing the effects of the catalytic subunits of PP1 ($PP1_C$) and PP2A ($PP2A_C$) suggested that only overexpression of $PP1_C$ could inhibit or limit CRE-dependent gene expression (27). Furthermore, we showed that CREB phosphatase activity in PC12 nuclear extracts was inhibited by the PP1-specific I-2 or by OA at concentrations characteristically required to inhibit PP1. Finally, we found that microinjection of an expression plasmid bearing PP1-specific I-1 peptide by itself activated a CRE-dependent reporter gene and potentiated the ability of submaximal concentrations of cell-permeable cAMP analogs to stimulate this reporter gene. In this study, we examined the effects of expression of specific inhibitors of endogenous PP1 or PP2A on the in vivo phosphorylation of the transcription factor CREB in NIH 3T3 cells by indirect immunofluorescence with a phospho-CREB-specific antibody. These current experiments markedly differ from our previous ones. Our prior studies utilized the microinjection of purified catalytic subunits of PP1 and PP2A. The possibility that noncatalytic regulatory subunits were affecting the biological activity of the injected enzymes existed. With this caveat in mind, we sought to compare the effects of inhibition of either enzyme in vivo. We have found that I-1 stimulates CREB phosphorylation and activates CRE-dependent gene expression, while SV40 small t had no effect on these parameters, suggesting that the apparent inhibition of PP1 but not PP2A leads to higher levels of phospho-CREB and CRE-regulated gene expression.

Regulation of intracellular protein phosphorylation normally reflects the opposing catalytic activities of protein kinases and phosphatases. OA and other potent phosphatase inhibitors have been widely employed to characterize the role of the major protein serine/threonine phosphatases, PP1 and PP2A, both of which are inhibited by these compounds in several biological systems (54). In contrast, we have taken a different approach of overexpressing specific protein inhibitors

TABLE 1. pCB6-I-1 and pCEP4-Sm t: effects on CRE-regulated gene expression

Expression plasmid[a]	Reporter plasmid	% Blue cells[b] (mean ± SD)	n[c]
pCB6-I-1	pCRE-*lacZ*	44 ± 11	580
pCB6	pCRE-*lacZ*	2 ± 2	484
pCEP4-Sm t	pCRE-*lacZ*	3.0 ± 2	505
pCB6-I-1	pCMV-*lacZ*	84 ± 5	347
pCB6	pCMV-*lacZ*	95 ± 6	226
pCEP4-Sm t	pCMV-*lacZ*	82 ± 10	434

[a] pCRE-*lacZ* (42) and pCMV-*lacZ* (9) were injected at 0.1 mg/ml each. pCB6-I-1, pCB6, and pCEP4-Sm t were each injected at 0.03 mg/ml. Cells were incubated for 5 h after injection to allow for reporter expression.
[b] Following incubation of injected cells in X-Gal as previously described (50).
[c] Total number of cells injected in three to four separate experiments.

of PP1 and PP2A to define the roles of the endogenous phosphatases in CREB dephosphorylation and regulation of gene expression. We have recently demonstrated the utility of a constitutively active (Thr→Asp-35) I-1 derivative to examine the role of PP1 in the control of gene expression (27). For PP2A, we introduced the SV40 small t antigen, which competes for the B subunit in the native heterotrimeric form of PP2A to inhibit activity towards many substrates (52, 53, 67, 68). Expression of small t has also recently been shown to increase the activity of several cytoplasmic mitogen-activated kinases and induce cell proliferation (58). Our studies show that SV40 small t has no effect on CRE-regulated gene expression, in contrast to the effects found for expression of I-1. To provide a degree of confidence that small t is capable of impacting endogenous PP2A in vivo, we examined the effect of its expression on an AP-1-regulated *lacZ* reporter construct. In these studies, we found that small t cooperates with mitogen-activated protein or extracellularly regulated kinases and other kinases, such as Raf, to stimulate AP-1-regulated gene expression (20), an effect which appears to indicate that the expressed small t is indeed inhibiting endogenous PP2A. Some of these kinases are themselves activated by phosphorylation and may subsequently be inactivated by PP2A or other phosphatases (16, 24, 25, 29, 48, 59). Overexpression of small t in cells activates several of these kinases (58). Thus, the ability of small t to activate AP-1 function may reflect altered regulation of PP2A in the regulation of mitogen-activated protein kinases.

A recent report has suggested that PP2A is responsible for inactivating CREB transcriptional activity examined in vitro in liver nuclear extracts (65). Wadzinski et al. (65) concluded that PP2A was the predominant protein (Ser/Thr) phosphatase in liver nuclei and that the heterotrimeric form of PP2A partially purified from rat liver nuclear extract was more efficient in dephosphorylating cAPK-phosphorylated CREB than the PP1 enzyme found in these nuclear extracts. These studies also demonstrated the ability of OA to synergize with dibutyryl-cAMP in the activation of a reporter gene containing a portion of the phosphoenolpyruvate carboxykinase promoter in HepG2 cells. Deletion analyses of the phosphoenolpyruvate carboxykinase promoter attributed the effects of OA and dibutyryl-cAMP to a proximal CRE (−94 to −72) in this promoter, although substantially higher induction was observed with reporters containing additional domains outside of the proximal CRE (40, 65). It will be important to determine if some of these additional effects are due to regions in the promoter that bind AP-1 (35, 40, 55, 60).

Our studies using a number of different cell lines support a primary role for PP1 in the regulation of CREB function (3, 27). As such, these data appeared consistent with the findings of Kuret et al. (36), who showed that phosphorylase phosphatase activity (representing PP1 and PP2A) measured in rat liver nuclear extracts was essentially eliminated by the PP1-specific inhibitors, I-1 and I-2. By contrast, a more recent study (65) demonstrated that 60 to 70% of the phosphorylase phosphatase measured in rat liver nuclear extracts was insensitive to I-2 and therefore probably represented PP2A. Whether this represents a difference in the preparation of the rat liver nuclear extracts, or differences in the in vitro assays used to quantitate PP1 and PP2A, remains unclear.

It will be of importance to test the potential role of other regulatory subunits in the function of PP1 and PP2A. We have previously found that regulatory subunits can influence the apparent biological activity of PP2A (5, 6). For PP1, variations in the activity of other inhibitor proteins, like I-2 and novel nuclear phosphatase inhibitors such as NIPP, may effect cellular functions such as gene expression and cell cycle progression (8, 11). There may also be tissue-specific differences in the expression of other regulatory subunits for PP1 or PP2A (30). It may also be necessary to establish the identity of subunits of native PP1 and PP2A complexes present in different tissues and subcellular compartments to further explore this possibility.

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