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Adenovirus E4orf4 Protein Binds to Protein Phosphatase 2A, and the Complex Down Regulates E1A-Enhanced *junB* Transcription

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Adenovirus E4orf4 protein was previously shown to counteract transactivation of *junB* by cyclic AMP (cAMP) and E1A protein. It was also shown to cause hypophosphorylation of E1A and c-Fos proteins. Here we show that the E4orf4 protein associates with protein phosphatase 2A. All three subunits of the phosphatase are present in the complex, and the B subunit interacts directly with the viral protein. The complex possesses a phosphatase activity typical of protein phosphatase 2A, and the phosphatase mediates the E4orf4-induced down regulation of *junB* transcription. Thus, adenovirus E4orf4 protein recruits protein phosphatase 2A into a signal transduction pathway initiated by cAMP and E1A protein.

The reversible phosphorylation of proteins is a major mechanism by which signals are transduced in eukaryotic cells. The phosphorylation state of regulated proteins influences their conformation and alters their biological properties. The level of serine, threonine, and tyrosine phosphorylation at any instant reflects the relative activities of protein kinases and phosphatases that catalyze the interconversion process. The activities of these enzymes can be controlled by a variety of mechanisms, including their own phosphorylation state. The role of protein kinases in signal transduction is well established (reviewed in references 27, 28, and 35), but much less is known about the role of serine/threonine protein phosphatases (reviewed in references 7 and 8).

We have previously shown that the combination of cyclic AMP (cAMP) and adenovirus E1A protein synergistically induces transcription of viral genes (10). The primary effect of cAMP plus E1A protein is the induction of the cellular *junB* and c-*fos* genes (23). These two proteins associate to form the AP-1 transcription factor and then activate early adenovirus genes through AP-1 DNA-binding sites. This transcriptional activation of viral genes is balanced by an opposing down-regulatory event that causes AP-1 DNA-binding activity to return to basal levels within 5 h of the induction event (22). Down regulation is mediated by the adenovirus E4 open reading frame 4 (E4orf4) protein. As the E4 protein accumulates, it inhibits *junB* transcription while inducing hypophosphorylation of the E1A and c-*fos* proteins (22).

To gain insight into the mechanism of E4orf4 protein action, we searched for cellular proteins with which it interacts. Here, we show that the E4orf4 protein associates with protein phosphatase 2A (PP2A), whose B subunit mediates the interaction. This complex down regulates *junB* transcription that has been stimulated by cAMP plus E1A protein.

MATERIALS AND METHODS

Viruses, cells, and plasmids. Wild-type adenovirus (H5*wt* 300) has been described previously (17). For analysis of E4orf4 protein complexes, S49 cells (16) were infected at a multiplicity of 20 PFU per cell.

The E4orf4 coding region was amplified by polymerase chain reaction from adenovirus DNA, its sequence was verified, and it was cloned into the glutathione *S*-transferase (GST) fusion vector pGEX-2T to create pGEX-2T-E4orf4. A deleted derivative of this plasmid, pGEX-2T-E4orf4Δ, was created by digestion of pGEX-2T-E4orf4 with *Bgl*II and *Eco*RI followed by repair of the resulting single-stranded termini and ligation. cDNAs encoding the A (33) and B (25) subunits of PP2A were gifts of G. Walter (University of California at San Diego) and D. Pallas (Dana Farber Cancer Institute), respectively. A cDNA corresponding to the C subunit of PP2A was obtained by polymerase chain reaction amplification of HeLa cell cDNA, using as primers the sequences TCTGGTACCA GATGGACGAGAAGGTGTTC and CTCGAATTCTTACA GGAAGTAGTCTGG. The amplified product was cloned into pGEM-7Zf(+). The PP2A subunit C and E4orf4 cDNAs were also cloned into a vector containing the cytomegalovirus (CMV) immediate-early promoter (15). pCMVE1A (5) was a gift from J. Nevins (Duke University) and contains an E1A 13S cDNA expressed under the control of the CMV immediate-early promoter. pjunB-CAT contains the *junB* promoter region between −144 and +43 (27a). pTKGH, which expresses human growth hormone under control of the herpes simplex virus thymidine kinase promoter, was purchased from Nichols Institute Diagnostics, Inc. (San Juan Capistrano, Calif.).

In vitro translation. Supercoiled plasmid DNAs were transcribed and translated by using the TNT system from Promega, Inc. (Madison, Wis.).

Cellular extracts, immunoprecipitations, and GST fusion protein capture experiments. GST fusion proteins were prepared as described by Kaelin et al. (18). Cells were extracted in lysis buffer containing 50 mM Tris-HCl (pH 7.4), 250 mM NaCl, 5 mM EDTA, 0.1% Triton X-100, 0.5% Nonidet P-40, 2 μg of leupeptin per ml, and 2 μg of aprotinin per ml for 40 min on ice. Debris was removed by centrifugation for 5 min at 10,000 × *g*. Immunoprecipitations and incubation of cellular extracts with GST fusion proteins were performed at 4°C in lysis buffer. The cellular extracts were precleared with GST beads prior to incubation with the fusion proteins. The antibodies to the A, B, and C subunits of PP2A were gifts from M. Mumby (University of Texas Southwestern Medical Center) and G. Walter and R. Ruediger (University of California at San Diego). Monoclonal antibodies were prepared against the intact E4orf4 protein produced in *Escherichia coli* as a histidine fusion protein, using the method of Gentz et al. (11).

Immune complex phosphatase assays. Immune complexes

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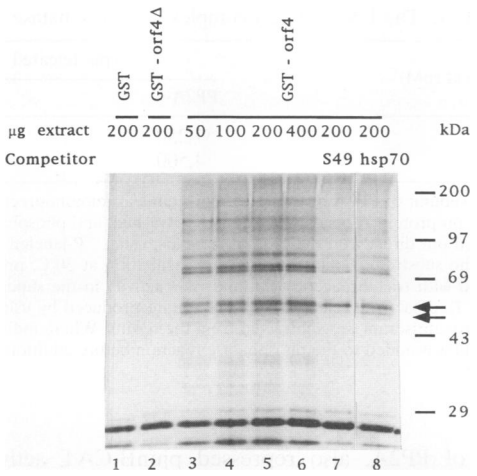

FIG. 1. Identification of adenovirus E4orf4 protein-associated cellular proteins. GST fusion proteins were prepared from bacterial extracts, captured on glutathione beads (18), and incubated with various amounts of ^{35}S-labeled extracts of uninfected mouse S49 cells. Lanes: 1, unfused GST protein; 2, GST fused to the first 47 amino acids of E4orf4 (E4orf4Δ); 3 to 8, GST fused to the intact E4orf4 protein. In lanes 7 and 8, the labeled complexes on glutathione beads were incubated for 12 h with 200 μg of unlabeled S49 cell extract (lane 7) or purified hsp70 (lane 8). The arrows mark two proteins whose binding to GST-E4orf4 was competed for by the S49 cell extract but not by hsp70.

were assayed for phosphatase activity by incubation in phosphatase buffer containing 20 mM imidazole-HCl (pH 7.63), 0.1 mM EDTA, 0.1% β-mercaptoethanol, and 1 mg of bovine serum albumin per ml, with ^{32}P-labeled phosphorylase *a* (prepared with a Gibco BRL phosphatase assay system), for 30 min at 30°C. The proteins were then precipitated by trichloroacetic acid, and the radioactivity in the supernatant fluids was measured in a beta counter. Where indicated, 5 nM okadaic acid (Boehringer Mannheim, Indianapolis, Ind.) was included in the reactions.

CAT assays. Cells were transfected with 5 μg of pTKGH, 5 μg of chloramphenicol acetyltransferase (CAT) reporter construct, 0.06 μg of CMV-E1A, and various amounts of modifier plasmids. The total amount of transfected DNA was kept constant by addition of pGEM-7Zf(+). Cells were harvested 40 h after transfection and assayed for CAT activity. All transfections were normalized to human growth hormone levels derived from the cotransfected internal control plasmid, pTKGH, whose activity was not affected by the various modifier plasmids. Where indicated, 100 nM okadaic acid was added 12 h prior to harvest of the transfected cells.

RESULTS

E4orf4 protein binds to PP2A, interacting with its B subunit. A GST-E4orf4 fusion protein was prepared and tested for its ability to interact with ^{35}S-labeled proteins from S49 cells. Several cell-specific proteins were captured by the fusion protein in a dose-dependent manner (Fig. 1, lanes 3 to 6) but not with GST alone or a GST-E4orf4 deletion product (Fig. 1, lanes 1 and 2). A displacement experiment was performed to assay binding specificity. The complex of fusion protein plus labeled cellular proteins was incubated with an unlabeled extract from the same cells (Fig. 1, lane 7) or with a nonrelevant protein (Fig. 1, lane 8). There was a general reduction in the levels of bound proteins; however, some of the labeled proteins (marked by arrows) were displaced to a greater extent by the cell extract than by the nonspecific protein. One of these polypeptides, the upper band in the doublet of 54/55-kDa proteins, marked by the upper arrow in Fig. 1, was purified by chromatography on the GST-E4orf4 matrix. It was then subjected to electrophoresis in a 10% polyacrylamide gel containing sodium dodecyl sulfate (SDS), blotted to nitrocellulose, proteolyzed, and subjected to microsequencing (1). Two stretches of amino acid sequence were obtained, SFFSEIISSIS DVK and YRDPTTVTTL, and both corresponded to the α form of the B subunit of PP2A.

PP2A designates a family of enzymes consisting of three polypeptides: a 36-kDa catalytic subunit (C), a 63-kDa regulatory subunit (A), and a second regulatory subunit which varies in size in different cell types and can be 54 kDa (B′), 55 kDa (B), 72 kDa (B″), or 74 kDa (7). E4orf4 protein was immunoprecipitated from an ^{35}S-labeled, adenovirus-infected S49 cell extract to determine whether it associates with PP2A in vivo and which subunits are present in the complex. Several cellular proteins were immunoprecipitated with E4orf4-specific antibody (Fig. 2a, lanes 2 and 8) but not with preimmune serum (Fig. 2a, lane 9). The precipitated complexes were dissociated by boiling and subjected to a second cycle of immunoprecipitation. Reprecipitation with E4orf4-specific antibody yielded a major band of E4orf4 without its associating proteins (Fig. 2a, lane 1). Reprecipitation with antibodies to the B and C subunits of PP2A (Fig. 2a, lanes 3 and 4) revealed their presence in the complex. Although the denatured A subunit of PP2A was less efficiently precipitated, antibodies to both the N terminus (Fig. 2a, lane 5) and the C terminus (Fig. 2a, lane 6) captured the same protein, which was not recognized by preimmune serum (Fig. 2a, lane 7). Thus, all three subunits of PP2A are associated with E4orf4 protein within infected S49 cells (and HeLa cells [data not shown]).

We next determined which of the three PP2A subunits directly contacts the E4orf4 protein. cDNA clones encoding each of the subunits were transcribed and translated in vitro (Fig. 2b, lanes 1, 4, and 7), and the resulting products were incubated with the GST protein or the GST-E4orf4 fusion product. The GST protein bound none of the subunits (Fig. 2b, lanes 3, 6, and 9). GST-E4orf4 protein bound most efficiently to the B subunit of PP2A (Fig. 2b, lane 8). A low level of binding was detected between the other subunits and the GST-E4orf4 fusion protein (Fig. 2b, lanes 2 and 5), probably because endogenous PP2A-B subunit in the reticulocyte lysate facilitated complex formation.

The E4orf4-PP2A complex exhibits phosphatase activity in vitro. To ascertain whether the E4orf4-PP2A complex has phosphatase activity, the complexes obtained by immunoprecipitation from infected cell extracts with E4orf4-specific antibody were incubated with ^{32}P-labeled phosphorylase *a*, and the radioactivity that was released was measured (Table 1). The E4orf4-PP2A complex exhibited phosphatase activity, as did the immune complex generated with antibody to PP2A subunit C. The E4orf4 protein-associated activity was decreased sixfold by 5 nM okadaic acid, an inhibitor of PP2A and PP1. Okadaic acid at 15 nM is required for half-maximal inhibition of PP1, while only 0.5 nM is required to achieve equivalent inhibition of PP2A (9). Thus, inhibition of the phosphatase activity by 5 nM okadaic acid is consistent with our conclusion that the E4orf4 complex possesses PP2A activity.

E4orf4 protein and the PP2A C subunit inhibit expression from the *junB* promoter. As mentioned above, expression of the E4orf4 protein within infected cells inhibits transcription of the *junB* gene, and this presumably contributes to the reduction of AP-1 DNA-binding activity that we have observed

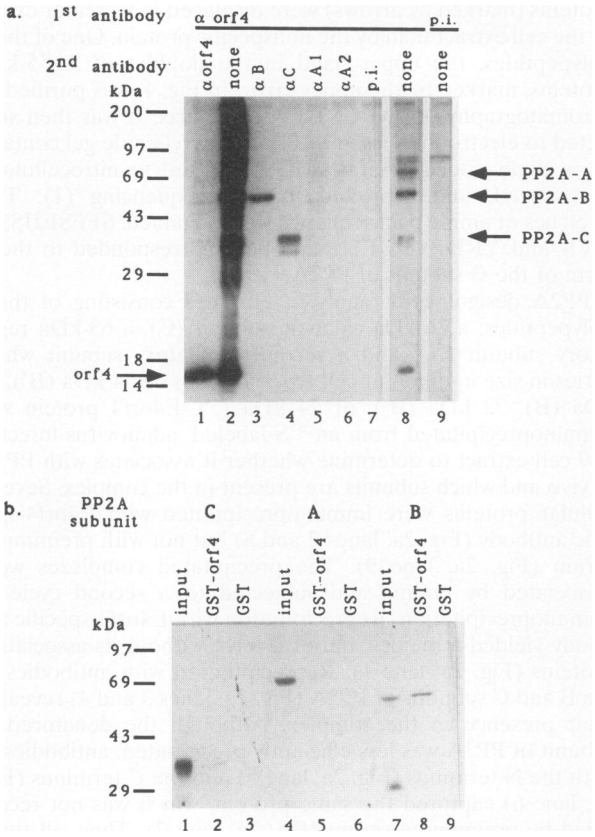

FIG. 2. Characterization of the E4orf4-PP2A complex in vivo and in vitro. (a) E4orf4 was immunoprecipitated from ^{35}S-labeled S49 cells which were infected with wild-type adenovirus type 5 at a multiplicity of 20 PFU per cell (lanes 2 and 8). A precipitation with preimmune (p.i.) serum is shown in lane 9. Lanes 8 and 9 were exposed for 1 day; lanes 1 to 7 were exposed for 10 days. The E4orf4 protein-specific immunocomplexes were washed, boiled in the presence of SDS and β-mercaptoethanol, diluted in buffer without SDS, and subjected to a second cycle of immunoprecipitation with antibodies to E4orf4 protein (lane 1), B subunit of PP2A (lane 3), C subunit of PP2A (lane 4), A subunit of PP2A, N terminus (lane 5), A subunit of PP2A, C terminus (lane 6), or preimmune serum (lane 7). (b) cDNAs encoding the A (33), B (25), and C subunits of PP2A were translated in vitro. Lanes: 1, 4, and 7, input protein; 2, 5, and 8, proteins associated with GST-E4orf4 protein; 3, 6, and 9, proteins associated with GST protein. Lanes 1, 4, and 7 contain 25% of the amount of input proteins in the other lanes.

previously (22). We next tested whether PP2A plays a role in the inhibition of *junB* transcription by E4orf4 protein. The activity of a CAT reporter fused to the *junB* promoter (pjunB-CAT) in the presence of cotransfected plasmids expressing putative regulatory proteins was assayed in HeLa cells (Fig. 3). Unlike S49 cells, HeLa cells contain a high level of endogenous cAMP, facilitating the induction of *junB* transcription by E1A without addition of exogenous cAMP (22). All transfections were normalized to growth hormone levels produced in the same cells by a control plasmid. Cotransfection of pjunB-CAT with a plasmid encoding E1A proteins (pCMV-E1A) enhanced the level of CAT activity directed by the *junB* promoter by a factor of 5 to 6 (Fig. 3, no modifier DNA). Cotransfection with pCMV-E4orf4 repressed the E1A-mediated stimulation of CAT activity in a dose-dependent fashion by a factor of 3 to 4 (Fig. 3). pCMV-PP2A-C, which expresses the catalytic

TABLE 1. The E4orf4-PP2A complex has phosphatase activity[a]

Okadaic acid (nM)	cpm released	
	PP2A-C	E4orf4
0	57,250	14,660
5	4,500	2,440

[a] The C subunit of PP2A or the E4orf4 protein was immunoprecipitated, the precipitate on protein A beads was extensively washed, and phosphatase assays were carried out directly on the protein A beads, using ^{32}P-labeled phosphorylase *a* as the substrate. Following a 30-min incubation at 30°C, proteins were precipitated with trichloroacetic acid, and radioactivity in the supernatant was quantified. Radioactivity released by a precipitate produced by using a preimmune control antiserum was subtracted from the results. Where indicated, 5 nM okadaic acid was added to the phosphatase reaction before addition of radioactive substrate.

subunit of PP2A, also repressed pjunB-CAT activity in a dose-dependent fashion, but to a lesser extent (Fig. 3a). Cotransfection with the CMV vector used to express E4orf4 and PP2A-C did not affect *junB* promoter-directed CAT activity, ruling out repression due to promoter competition. The repression did not result from generalized toxicity of E4orf4 protein or the C subunit of PP2A since expression of the internal control plasmid, driven by the thymidine kinase promoter, was not inhibited. Further, the E4orf4 protein did not inhibit the basal activity of the *junB* reporter construct; only E1A-induced expression was repressed (results not shown).

Okadaic acid, which inhibits the activity of PP2A, reversed the E4orf4-mediated repression (Fig. 3b), arguing that PP2A plays a role in the repression of *junB* promoter activity by E4orf4 protein.

DISCUSSION

Our analyses of physical interactions and transcriptional modulation lead to the conclusion that PP2A plays a role in the E4orf4-mediated down regulation of the cellular response to cAMP and E1A protein. First, immunoprecipitation experiments with antibodies to the E4orf4 protein revealed that the E4orf4 protein exists in a complex that includes all three subunits of the phosphatase within adenovirus-infected cell extracts (Fig. 2a), and this complex contains phosphatase activity that is sensitive to low levels of okadaic acid, a characteristic of PP2A (Table 1). Further, a GST-E4orf4 fusion protein was able to specifically capture the PP2A B subunit (Fig. 2b). Second, the PP2A catalytic subunit (Fig. 3a), as well as the E4orf4 protein (Fig. 3), were able to inhibit induction of the *junB* promoter by the E1A protein, and inhibition mediated by the E4orf4 protein was sensitive to okadaic acid (Fig. 3b).

E4orf4 protein mediates reduced phosphorylation of both the E1A and c-Fos proteins, and this correlates with the inhibition of *junB* transcription (22). Thus, PP2A might act directly, dephosphorylating and altering function of a transcription factor required for expression of the *junB* gene. There is precedent for such a regulatory function. Another phosphatase, PP1, has been shown to inhibit cAMP-stimulated gene expression by dephosphorylating the CREB transcription factor (13). Alternatively, PP2A might act on a factor which affects transcription indirectly, such as a kinase required to phosphorylate elements in the transcription complex. In fact, the kinetics with which E1A and c-Fos become underphosphorylated in the presence of the E4orf4 protein suggest that a kinase might be inhibited (22). Only hypophosphorylated

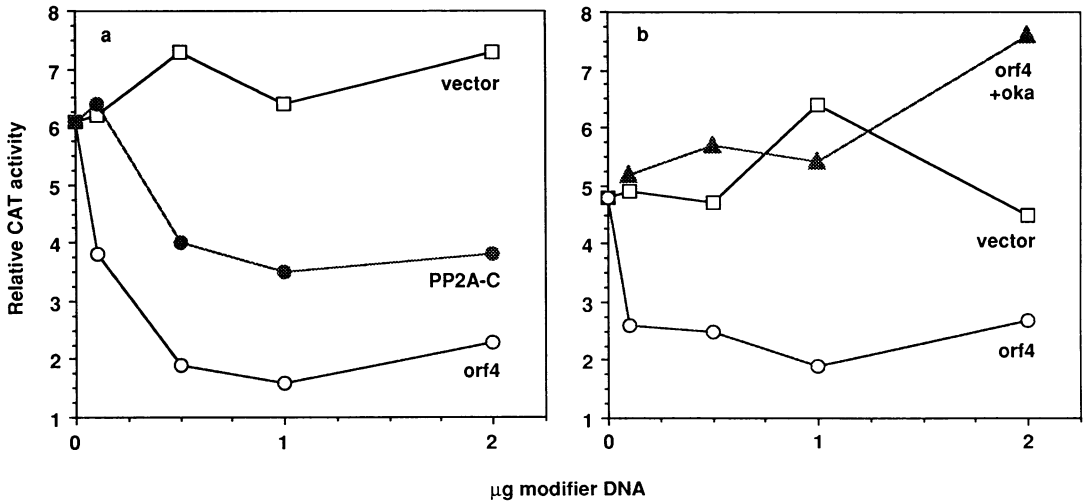

FIG. 3. Down regulation of E1A-stimulated *junB* promoter activity by E4orf4 protein and the C subunit of PP2A. The plasmids transfected into HeLa cells were 5 μg of pjunB-CAT, 0.06 μg of pCMV-E1A, and various amounts of pCMV (squares), pCMV-E4orf4 (open circles), or pCMV-PP2A-C (closed circles). Relative CAT activity is calculated by defining pjunB-CAT activity as 1. In panel b, 100 nM okadaic acid (oka) was added to cells transfected with pjunB-CAT, pCMV-E1A, and pCMV-E4orf4 (closed triangles). The plots show representative results from four experiments with similar results.

E1A protein was evident in wild-type virus-infected cells after a 10-min labeling period and a subsequent 2-h chase. In contrast, within cells infected with E4orf4 mutant viruses, hyperphosphorylated E1A protein was evident immediately after the labeling period, and it accumulated further during the chase. Thus, these results suggest that the interaction between E4orf4 and PP2A may trigger a cascade of events in which the phosphatase inhibits a kinase which is then involved in down regulation of transcription. This putative chain of events would contribute additional levels of regulation to the process. Kinases which are inactivated by PP2A in vitro include the mitogen-activated protein kinase and mitogen-activated protein kinase kinase (4, 12).

It has previously been shown that tumor antigens of polyomavirus and simian virus 40 also interact with PP2A, but in contrast to the E4orf4 protein, the tumor antigen complexes appear to contain only the A and C subunits of PP2A (24, 34). The E4orf4 protein interacts with a complex that includes all of the PP2A subunits, and several observations suggest that the E4orf4 protein does not inhibit PP2A activity. First, there is PP2A activity in E4orf4-specific immunoprecipitates (Table 1). Second, recombinant E4orf4 protein does not inhibit the ability of purified PP2A to dephosphorylate ^{32}P-labeled phosphorylase *a* (data not shown). However, as the B subunit of PP2A can either enhance or reduce dephosphorylation of various substrates (7), it may be that the adenovirus E4orf4 protein can also influence the PP2A activity differentially, depending on the substrate, and it may be necessary to assay more physiologically relevant substrates. Third, okadaic acid, which inhibits PP2A activity, can relieve the inhibitory effect of E4orf4 protein on *junB* transcription (Fig. 3b). E4orf4 protein does not alter the intracellular localization of the bulk of the phosphatase (data not shown), but it may affect the localization of specific subpopulations of PP2A, it may target the phosphatase to specific substrates, or it may enhance PP2A efficiency. Basic proteins such as protamine or histone H1 have been shown to stimulate PP2A activity without dissociating its subunits (26, 31). The E4orf4 product is a basic protein (pI = 9.5), and it may stimulate the phosphatase.

PP2A family members may play a part in several cellular events, including control of mitosis and cell cycle progression by direct or indirect regulation of p34^{cdc2} (19, 20), by dephosphorylation of p34^{cdc2} substrates (3, 30), or by other pathways (21); control of morphogenetic events of the cell cycle (14); and regulation of gene expression (2, 29). However, little is known about the regulation of PP2A within cells. Phosphorylation of tyrosine on its catalytic subunit, which inactivates the phosphatase (6), suggests involvement of PP2A in tyrosine kinase-regulated signal transduction. A mutation in the *Drosophila* gene *twins*, which is homologous to the mammalian B subunit of PP2A, leads to pattern duplication in *Drosophila* imaginal discs (32), suggesting that the B subunit recruits PP2A into a developmentally regulated signal transduction pathway. We show that adenovirus E4orf4 protein binds PP2A and recruits it into a signal transduction pathway involving E1A protein and cAMP.

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