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Cyclin-Dependent Kinases Phosphorylate the Adenovirus E1A Protein, Enhancing Its Ability To Bind pRb and Disrupt pRb-E2F Complexes

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The adenovirus E1A protein of 243 amino acids has been shown to affect a variety of cellular functions, most notably the immortalization of primary cells and the promotion of quiescent cells into S phase. The activity of E1A is derived, in part, from its association with various cellular proteins, many of which play important roles in regulating cell cycle progression. E1A is known to have multiple sites of phosphorylation. It has been suggested that cell cycle-dependent phosphorylation may also control some of E1A's functions. We find now that immune complexes of cyclin-dependent kinases such as cdk4, cdk2, and cdc2 are all capable of phosphorylating E1A in vitro. Additionally, the sites on E1A phosphorylated by these kinases in vitro are similar to the E1A sites phosphorylated in vivo. We have also found that a phosphorylated E1A is far more efficient than an unphosphorylated E1A in associating with pRB and in disrupting E2F/DP-pRB complexes as well. On the basis of our findings and the differences in timing and expression levels of the various cyclins regulating cdks, we suggest that E1A functions at different control points in the cell cycle and that phosphorylation controls, to some extent, its biological functions.

The primary role of the adenovirus E1A proteins is to advance viral replication once the virus has infected human quiescent cells. In this endeavor, E1A exerts its effect by activating transcription from other adenovirus early genes (96) and, more importantly, by inducing cellular DNA synthesis and cell cycle progression (77). In other situations, E1A can immortalize primary rodent cells in vitro as well as transform these cells in combination with *ras*, adenovirus E1B, or polyoma middle T (77). E1A can also inhibit muscle differentiation (11, 110) and, under appropriate conditions, induce DNA degradation and apoptosis (88). E1A's newly discovered functions are its abilities to initiate DNA synthesis in cells which have lost cellular *ras* activity (103) and to disrupt the inhibitory activities of $p27^{Kip1}$ in transforming growth factor β (TGF-β)-treated cells, thus restoring cyclin-cdk2 activity (69). Additionally, E1A can shorten the interval between G_0 and S phases while inducing DNA synthesis in quiescent cells (103) and can promote cells into S phase though they have been blocked at different points in G_0/G_1 (18).

The biological activities of E1A can best be explained by its ability to interact with a number of cellular proteins (40, 118), most of which have been identified as key regulators of the cell cycle (97). Included in this group is the product of the retinoblastoma gene, pRb, and its structural relatives p107 and p130 (16, 31, 64, 113). All three of these proteins have growth-suppressive properties (66), and when overexpressed in certain tumor cell lines they can restrict cells from entering S phase (14, 45, 121). pRb negatively regulates cell proliferation when present in a hypophosphorylated state (111), and after pRb is phosphorylated by the concerted action of cyclin-dependent kinases (cdks) such as cyclin D-cdk4, cyclin E-cdk2, and cyclin A-cdk2 (21, 30, 46), it is thought to become functionally inactive (111). The activity of p107 is also regulated by phosphorylation through D-type cyclins (9). Not yet clear is whether phosphorylation plays a role in regulating the growth-inhibitory activities of p130. One important way in which pRb, p107, and p130 are able to restrain cell growth is by regulating the function of the transcriptional factor E2F. This factor, a heterodimer composed of an E2F and a DP family member, is heavily involved in regulating genes important in promoting cell growth (78), an act which may preclude the entry and therefore the association of critical factors such as TBP or TFIIB (42). Only after pRb is significantly phosphorylated by cdks is E2F released (61), thereby stimulating transcription from genes that are E2F dependent (61).

Other cellular proteins found to interact with E1A are p300 and the CREB-binding protein CBP (28, 67). These structurally related proteins have recently been identified as coactivators of signal-activated transcriptional factors (3, 67). p300 has also been shown to associate with the TATA-binding protein TBP (1) as well as to relieve YY1 transcriptional repression (62). To some extent, E1A requires p300 in order to induce cellular DNA synthesis in a variety of growth-arrested cells (47, 109), suggesting that p300 may be involved in controlling cell cycle progression. Like pRb, p300 shows variations in its level of phosphorylation throughout the cell cycle, appearing hyperphosphorylated as cells progress toward mitosis (68, 117). Thus, the activities of p300 may be regulated by phosphorylation. This is emphasized by the fact that the cdk2 and cdc2 kinases can use p300 as a substrate in vitro (6). Additionally, E1A has recently been shown to inhibit the phosphorylation of p300 (6), although another study has shown that E1A may also be able to increase the phosphorylation of p300 (7). The results of these studies underscore the possibility that the function of p300 may be controlled by phosphorylation and that E1A may affect this activity.

E1A itself is a phosphoprotein. In mitotic cells its level of phosphorylation appears to be at its fullest (23), suggesting that E1A may also be regulated by phosphorylation throughout the cell cycle. Indeed, it is becoming evident that most factors functioning in the cell cycle are essentially controlled by phosphorylation or dephosphorylation events. Of late, a group of

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related kinases and their regulatory subunits, known as cyclins, has been shown to be largely responsible for this type of regulation. Cyclins oscillate throughout the cell cycle and perform functions that are essential to mitosis and promotion of cells into S phase (79, 97). D-type cyclins (D1, D2, and D3) and cyclin E regulate the progression of cells through G_1 (2, 81, 87), while cyclin A functions at the onset of S phase or the G_2/M transition, as does cyclin B (20, 97). cdks such as cdk4, -5, and -6, whose levels are constant throughout the cell cycle, prefer to associate with D-type cyclins (70, 74, 115). Although cdk2 can also form complexes with these cyclins (115), it appears to have a greater preference for cyclins E and A (97). cdc2, on the other hand, seems to prefer regulation by cyclins A and B only (97). Although the precise functions of these kinases in the cell cycle are still poorly understood, it is believed that they are important in phosphorylating critical substrates that are necessary for executing the next phase of the cell cycle (79, 97).

Circumstantial evidence indicates that E1A may be phosphorylated by at least one member of the cdk family. Several in vivo studies have shown E1A in association with cdk2 and regulatory partners, cyclin E or A (32, 33, 57). Since there are at least five sites of phosphorylation which have been previously identified on E1A (23, 24), it is reasonable to hypothesize that cdk2 and other cdks may actually be phosphorylating E1A as it passes through the cell cycle. We now present evidence that cdk4, cdk2, and cdc2 and their respective cyclin partners can efficiently phosphorylate E1A in vitro. Moreover, the sites phosphorylated by these kinases are similar to those phosphorylated in vivo. A connection between phosphorylation and the activity of E1A is shown by the finding that a phosphorylated E1A is far more efficient than an unphosphorylated E1A in associating with pRB, as well as in disrupting E2F1/DP1-pRB complexes. Thus, our results imply that apart from other mechanistic considerations, cdks may play an important role in controlling the functions of E1A.

MATERIALS AND METHODS

Cell culture and transfection. HeLa cells in suspension culture were grown in S-MEM (GIBCO) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), and 5.0% horse serum. HeLa cell monolayers were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. For blocking cells in G_1/S (23), subconfluent cultures of HeLa monolayer cells, in parallel, were treated with hydroxyurea (10 mM) or left untreated and incubated in fresh medium for 48 h. Afterwards, the cells were washed accordingly and prepared for labeling (see below). HeLa monolayers were transiently transfected with 15 μg of pCMV-E1A$_{12S}$ (67) encoding full-length E1A, by using a standard calcium phosphate precipitation kit (GIBCO/BRL).

Preparation of nuclear and whole-cell extracts. Nuclear extracts capable of supporting in vitro transcription (17) and reversible phosphorylation (data not shown) were prepared from suspension cultures of HeLa cells as previously described (6). After proteins were extracted from nuclei with high-salt buffer, the supernatant was dialyzed against buffer (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.9], 100 mM KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol [DTT], 20% glycerol), aliquoted, rapidly frozen, and stored at −80°C. Protein determinations were conducted by using a Bio-Rad protein assay.

For the preparation of whole-cell extracts (16), cell monolayers were initially washed in cold phosphate-buffered saline (PBS) and then extracted in 0.5 to 1 ml of lysis buffer A (50 mM HEPES [pH 7.0], 250 mM NaCl, 0.1% Nonidet P-40, 2 mM EDTA, 0.5 mM DTT) and a cocktail of protease (aprotinin [1 μg/ml]), leupeptin [1 μg/ml], pepstatin [1 μg/ml], and phenylmethylsulfonyl fluoride [PMSF] [1 mM] and phosphatase (1 mM sodium orthovanadate, 5 mM NaF, and 20 mM sodium PP_i) inhibitors. After being incubated for 30 min at 4°C, the extract was passed through a 21-gauge needle and then clarified by centrifugation at 15,000 × *g* for 10 min. After protein determination (Bio-Rad protein assay), the lysates were aliquoted and stored at −80°C until further use.

Labeling of cells, immunoprecipitation, and immunoblotting. For in vivo labeling, cells growing on 10-cm-diameter plates were washed initially with PBS and then with DMEM without methionine or cysteine. The cells were then cultured in the same medium for 30 min at 37°C and labeled (200 μCi/ml) with a mixture of [^{35}S]methionine and [^{35}S]cysteine (Trans^{35}S-label; ICN) for 2.5 h at 37°C. After being labelled, the cells were harvested by being scraped into cold PBS and then lysed in buffer A containing a cocktail of protease and phosphatase inhibitors as described above. For in vivo labeling of phosphorylated E1A, cells transfected with pCMV-E1A$_{12S}$ for 31 h were washed and then incubated with phosphate-free media for 1 h. Cells were then labeled with [^{32}P]orthophosphoric acid (1.25 mCi ml^{-1}) in the same media for 4 h. Afterwards, the cells were lysed in buffer A and radiolabeled E1A was immunoprecipitated with the M73 monoclonal antibody, as described below.

Immunoprecipitations performed with whole-cell lysates or nuclear extracts diluted in buffer B (50 mM HEPES [pH 7.0], 250 mM NaCl, 0.1% Nonidet P-40, and 1 mM PMSF) were carried out as previously described (6), except for the following modifications. Extracts or lysates were initially precleared by incubation with either preimmune serum and protein A-Sepharose beads or beads alone for 1 h followed by a 1-min centrifugation at 15,000 × *g*. Precleared extracts or lysates were then subjected to immunoprecipitation. In experiments in which immunizing peptides were used to inhibit specific antibodies, the antisera were preincubated with a 3- to 10-fold excess of peptide antigen at room temperature for 2 h prior to performance of the immunoprecipitations. Immunoprecipitates were resolved on sodium dodecyl sulfate (SDS)-polyacrylamide gels (60) and detected by autoradiography.

To test proteins for kinase activity after their release from immune complexes, the precipitated proteins were incubated in 0.2% sodium deoxycholate for 30 min at 4°C (33). This mixture was then centrifuged, to remove protein A-Sepharose beads, and afterwards diluted 20-fold with buffer B. Released proteins in buffer B were then reimmunoprecipitated with a second antibody, and this immune complex was tested for kinase activity as described below.

For reimmunoprecipitations (33), the immune complexes were initially washed in buffer B and then disrupted in 1× Laemmli buffer (60) before being boiled for 5 min. Protein A-Sepharose beads were removed by centrifugation. The supernatant was diluted 20-fold with buffer B and then reimmunoprecipitated with a second antibody.

Western blotting (immunoblotting) was performed exactly as described elsewhere (6). After incubation with the appropriate antibody, the membranes were again incubated with a horseradish peroxidase-linked secondary antibody. Proteins were then detected by using an Amersham electrochemiluminescence detection system.

Anti-cdk2 and cdk4 antibodies and peptides were obtained from Santa Cruz Biotechnology, Inc., and from H. Zhang and D. Beach (115), as well as from C. Sherr (71). Anti-cdc2 and peptide (48) were provided by P. Howe, and a monoclonal antibody to cdc2 was obtained from Zymed Laboratories. Polyclonal anti-casein kinase II (Ab 245) (120) and anti-mitogen-activated protein kinase (anti-MAPK) (TR2) (76) antibodies were provided by D. Marshak and M. J. Weber, respectively. Anti-pRb antibody (C-15) was purchased from Santa Cruz Biotechnology. E1A-specific mouse monoclonal antibody, M73, was generously provided by E. Harlow (39).

Expression and purification of recombinant proteins. Plasmid pKHA1-T, encoding an authentic adenovirus type 2 E1A protein of 243 amino acids, was propagated in a protease-deficient *Escherichia coli* strain, CAG629. Growth conditions for optimum expression of E1A as well as its purification to near homogeneity in these cells have been described elsewhere (108). Glutathione *S*-transferase–E2F1 (GST-E2F1) and GST-DP1 fusion plasmids have been previously described (19) and were a gift from S. Hiebert. A GST-pRb fusion protein containing the large pocket of pRb (positions 379 to 928) has been described elsewhere (30) and was kindly provided by D. Beach. Procedures for the purification of GST-E2F1, GST-DP1, or GST-pRb in *E. coli* BL21(DE3) have been previously described (85, 86).

In vitro phosphorylation. Conditions for phosphorylating purified E1A in vitro were as previously described (6). Briefly, E1A (200 ng) was added to a reaction volume (20 μl) containing 25 μg of nuclear extract, 500 μM cold ATP, 5 mM $MgCl_2$, and 5 μCi of [γ-^{32}P]ATP. Phosphorylation was terminated on ice and with the addition of 10 mM EDTA (29). Immediately after, the ^{32}P-labeled E1A was immunoprecipitated as described above and electrophoresed on an SDS-polyacrylamide gel. Labeled products were then visualized by autoradiography and quantitated from the gel on a Molecular Dynamics phosphoimager.

To assess the importance of phosphorylation in E1A's interaction with pRb, GST-pRb was incubated with or without E1A (equimolar amounts) in nuclear extracts (20 μg) containing 20 mM HEPES (pH 7.9), 50 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 100 μM cold ATP, and 5 μCi of [γ-^{32}P]ATP. Phosphorylation was stopped by the addition of 10 mM EDTA (29), and if it had not been added initially, E1A was then added to the mixture containing a phosphorylated GST-pRb. Afterwards, E1A or GST-pRb was recovered with M73 or glutathione-Sepharose beads, respectively, and then resolved on an SDS–8% polyacrylamide gel. Labeled products were visualized by autoradiography.

Kinase and in vitro binding assays. Immune complexes were washed two times with buffer B and then twice with kinase buffer consisting of 20 mM HEPES (pH 7.9), 5 mM $MgCl_2$, 10% glycerol, and 1 mM DTT. The complexes were then resuspended in 15 μl of the same buffer containing 200 ng of purified E1A, 50 μM cold ATP, and 5 μCi of [γ-^{32}P]ATP. The mixtures were incubated for 30 min at 30°C, and the reactions were terminated by the addition of 2× sample buffer (60). Phosphorylated products were analyzed on an SDS-polyacrylamide gel and then visualized by autoradiography.

To test for direct binding between pRb and a phosphorylated or unphosphorylated E1A, GST-pRb or E1A was initially phosphorylated, as described above, in a reaction mixture (15 ml) containing immune complexes of cdk2, 5 mM $MgCl_2$, and 50 μM cold ATP. The reaction was terminated by the addition of 10 mM EDTA, and then the mixture was centrifuged at 10,000 × *g* for 10 min. The supernatant containing the phosphorylated product was carefully removed and incubated with GST-pRb or E1A for 2 h at 4°C. Afterwards, GST-pRb was captured with glutathione-agarose beads, as previously described (67). The beads were then extensively washed with buffer A, and the bound proteins were eluted with SDS-polyacrylamide gel electrophoresis (PAGE) loading buffer. The eluted products were resolved on an SDS–8% polyacrylamide gel, transferred to polyvinylidene difluoride membrane, and then detected by the use of M73 or anti-pRb (C-19; Santa Cruz) and electrochemiluminescence.

Immune complex kinase assays with recombinant proteins produced in insect cells. Descriptions of the baculovirus transfer vectors carrying cDNA inserts of cdk4 and cyclin D1 are published elsewhere (54), and these vectors were kindly provided by C. Sherr. Baculoviruses encoding cyclin D1 and cdk4 were produced in Sf21 cells as previously described (54). Briefly, infected Sf21 cells (10^6) were harvested 48 h after infection and lysed at 4°C in kinase buffer {50 mM HEPES [pH 7.9]; 10 mM $MgCl_2$; 1 mM DTT; 2.5 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid [EGTA]; 0.1 mM PMSF; 5 μg each of aprotinin, leupeptin, and pepstatin; 10 mM β-glycerophosphate; 0.1 mM sodium orthovanadate; and 0.1 mM NaF} (55). Lysates were clarified by centrifugation (15 min at 10,000 × *g*), and aliquots were frozen in liquid nitrogen and stored for future use. For the kinase reactions, lysates containing cyclin D1 and cdk4 (50 μg) were immunoprecipitated with anti-cdk4 or nonimmune rabbit serum. The immune complexes were washed two times in buffer A containing 0.1% Tween 20 and then twice with kinase buffer as described above. Afterwards, the beads were suspended in 15 μl of kinase buffer containing GST-pRb or E1A. The kinase reaction was initiated at 30°C by the addition of 5 μCi of [γ-^{32}P]ATP and unlabeled ATP to a final concentration of 25 μM. After incubation for 30 min, the reactions were stopped by the addition of 2× sample buffer. Eluted products from the beads were separated on an SDS–8% polyacrylamide gel. The resolved products were then detected by autoradiography.

Phosphopeptide mapping. Peptide mapping by partial proteolysis in gel slices was carried out as previously described (36). Briefly, ^{32}P-labeled E1A was resolved on an SDS–10% polyacrylamide gel. The radiolabeled bands were visualized by autoradiography and removed by excision. The gel slices were then placed into a sample well of a second SDS gel and overlayered with V8 protease (1 μg) in sample buffer. After digestion and electrophoresis, the radiolabeled phosphopeptides were visualized by autoradiography.

Mobility shift assays. Gel mobility shift assays were performed as previously described (63) with the following modifications. Assays with recombinant E2F1 contained 50 ng of GST-E2F1 and 41 ng of GST-DP1. When GST-pRB or E1A was added, GST-E2F1/DP1 was first incubated with GST-pRb (54 ng) for 30 min on ice in binding buffer and then incubated for another 30 min with phosphorylated or unphosphorylated E1A (15 ng). The binding buffer contained 20 mM HEPES (pH 7.4), 40 mM KCl, 10 mM $MgCl_2$, 0.1 mM EDTA, and 0.1% Nonidet P-40. Added to this was 0.5 μg of sonicated salmon sperm DNA and 0.5 ng of a ^{32}P-labeled E2F oligonucleotide probe (ATTTAAGTTTCGCGCCCTTTCTC AA). Typically, each of the reaction mixtures was incubated at room temperature for 30 min and afterwards the reaction products were separated on a 4% polyacrylamide gel run in 0.25× Tris-borate-EDTA (TBE) at 4°C at 180 V.

Flow cytometry. Cells were washed in PBS and afterwards stained with propidium iodide by using the CycleTEST kit (Becton Dickinson), a procedure previously described by Vindelov et al. (107). The stained cells were analyzed in a fluorescence-activated cell sorter (FACScan; Becton Dickinson), and the resulting data were then subjected to mathematical modeling by using ModFit software (Verity Software House, Topsham, Maine) to determine the relative percentages of cells in different stages of the cell cycle.

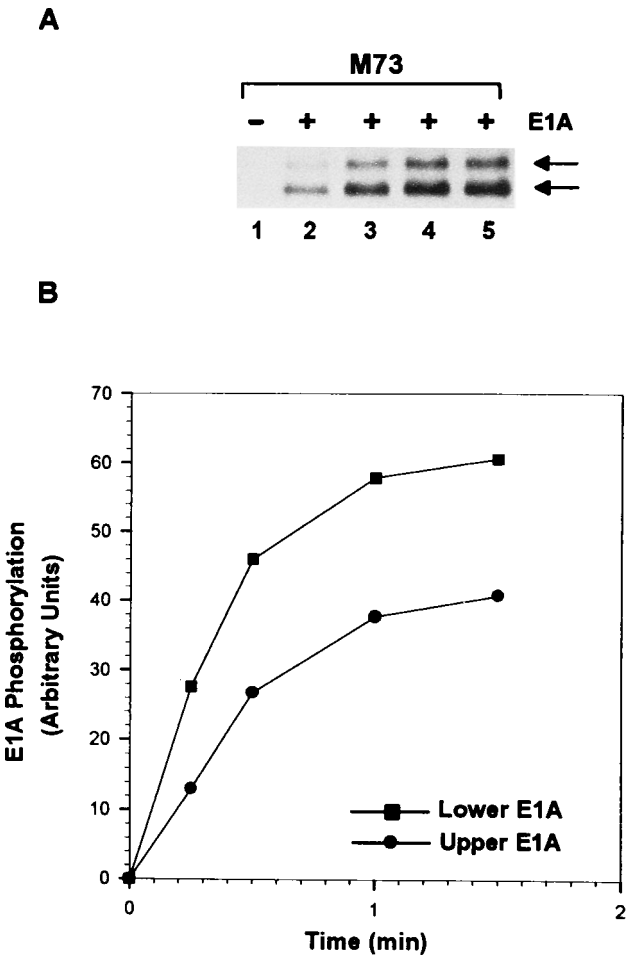


FIG. 1. Phosphorylation of E1A in nuclear extracts as a function of time. (A) Purified E1A (200 ng) was added to nuclear extracts (25 μg) of HeLa cells and then phosphorylated as described in Materials and Methods. Phosphorylation was terminated after 0.25, 0.5, 1.0, and 1.5 min (lanes 2 to 5, respectively). Afterwards, the labeled E1A was recovered by immunoprecipitation with an E1A-specific monoclonal antibody (M73). Precipitated products from each time point were resolved on an SDS–7% polyacrylamide gel and then visualized by autoradiography. As a control, a nuclear extract without the addition of E1A was phosphorylated for 1.5 min (lane 1) and then subjected to immunoprecipitation as well. The arrows indicate the two forms of E1A. (B) The amount of ^{32}P incorporated into E1A at each time point was quantitated from the gel on a Molecular Dynamics phosphoimager.

RESULTS

Time course of the phosphorylation of E1A in vitro. Studies in vivo have shown E1A to be electrophoretically heterogeneous, resolving into as many as six different forms in a high-resolution two-dimensional gel (41). In a one-dimensional gel, however, the pattern of E1A mobility is less complex, with E1A migrating as two forms differing in molecular size by about 2 kDa. One good reason for the presence of two variant forms is phosphorylation (23, 105, 106), a modification which might occur as a function of time when E1A progresses through the cell cycle. Indeed, evidence indicates that the more slowly migrating form of E1A is produced, in part, as a result of the phosphorylation of Ser-89 (75, 100). Furthermore, phosphorylation at Ser-89 may be required for phosphorylation to occur on other sites of E1A (24). Thus, to examine more closely the influence of phosphorylation on E1A, as a function of time, we incubated a purified, bacterially produced E1A protein of 243 amino acids (108) with [γ-^{32}P]ATP in a HeLa cell nuclear extract. This type of extract has been previously shown to be capable of supporting reversible phosphorylation (6, 17). After recovery by immunoprecipitation with an E1A-specific antibody (M73), the E1A products were analyzed on an SDS-polyacrylamide gel and visualized by autoradiography. As shown in Fig. 1A (lanes 2 to 5), two electrophoretic forms of E1A were readily detectable as a result of phosphorylation by the endogenous kinase(s) present in the nuclear extract. The form having the fastest mobility migrated as the primary translation product (data not shown), and it was always the more phosphorylated of the two. As expected, no phosphorylated products were visible in immune complexes from extracts without the addition of purified E1A (Fig. 1A, lane 1). To show that the electrophoretic behavior of E1A was truly induced by phosphorylation, we treated the immunoprecipitated ^{32}P-labeled

products with alkaline phosphatase and detected by Coomassie blue staining the unshifted form of E1A (8) (data not shown). We also compared the relative mobilities of in vitro- and in vivo-^{32}P-labeled E1A and found them to be indistinguishable by SDS-PAGE (data not shown), which is consistent with previous results (65).

In analyzing the incorporation of phosphate into E1A as a function of time, we discovered that the initial rate of incorporation of ^{32}P into the faster-migrating form of E1A was about 2.5 times greater than that for the more slowly migrating form (Fig. 1B). This suggests that phosphorylation of E1A is highly ordered, and perhaps phosphorylation at one site provides a recognition determinant essential for phosphorylation at another site (91). Also important to note is that the relative degree of phosphorylation observed between the two migrating forms eventually became constant, indicating that E1A had finally reached a steady-state level of phosphorylation. This observation can best be explained by the fact that the nuclear extracts used in these experiments are functional in both kinase and phosphatase activities (data not shown).

E1A is phosphorylated by cyclin-dependent kinases. Because E1A is found primarily in the nuclei of infected cells (24, 101, 119), it stands to reason that most of the phosphorylation occurring on E1A could easily result from activities of cyclin-dependent kinases. To determine whether these kinases might be responsible for phosphorylating E1A, we immunoprecipitated nuclear extracts of HeLa cells (17) using antibodies specific for cdk4, cdk2, or cdc2. The precipitates of each of these cdks were then assayed for their ability to phosphorylate purified E1A in a kinase reaction mixture containing [γ-^{32}P]ATP. As shown in Fig. 2A (lanes 1, 3, and 5), immune complexes of cdk4, cdk2, and cdc2 were clearly able to use E1A as a substrate, with E1A showing a pattern of phosphorylation similar to that observed in the experiment shown in Fig. 1A and in vivo (24, 41, 90). The kinase activity of these immune complexes was readily abolished, however, after each anti-cdk antibody had been preincubated with a competitor peptide containing the respective epitope (Fig. 2A, lanes 2, 4, and 6). This ruled out the possibility that contaminating kinase(s) was responsible for the phosphorylation of E1A. Not surprisingly, E1A was also phosphorylated by immune complexes generated by antibodies to cyclin E or A, although the kinase activity in association with the cyclin E immune complex was found to be much weaker than that of the cyclin A immune complex (data not shown). This difference in activity is most likely due to the relative amounts of cyclins A and E in the nuclear extract. The specificity of cdks in using E1A as a substrate in vitro is reinforced by the failure of immune complexes of MAP kinase to phosphorylate E1A under the same conditions (Fig. 2B, lane 1), notwithstanding that it efficiently phosphorylated myelin basic protein (lane 2), the preferred substrate for MAP kinase. Finally, we have also found that E1A can serve as a substrate for casein kinase II (CK II), an enzyme reported to phosphorylate cdc2 in HeLa cells during G_1 (94). Although immune complexes of casein kinase II were efficient in phosphorylating E1A in an in vitro kinase assay, this activity was unable to induce the characteristic shift in E1A mobility (data not shown). This result provides further evidence that a certain site(s) must be phosphorylated to effect an electrophoretic change.

The cyclin D-dependent kinase inhibitor p16^{INK4} specifically binds to and inhibits the activity of cdk4 (51). Given that there is a preponderance of p16-cdk4 complexes instead of cyclin D-cdk4 complexes in adherent HeLa cells (116), we reaffirmed E1A's ability to serve as a substrate for cyclin D-cdk4 by subjecting it to immune complexes of cdk4 recovered from insect cells expressing cyclin D1 and cdk4. As expected, E1A was efficiently phosphorylated by the immunoprecipitated enzyme (Fig. 2C, lane 3) and along with this phosphorylation came the characteristic shift in E1A electrophoretic mobility. The efficacy of immune complexes of cdk4 from insect lysate was further proven by the fact that these complexes could efficiently phosphorylate a GST-pRb fusion protein under the same conditions (Fig. 2C, lane 2). Moreover, its preference for this substrate over histone H1 was dramatically greater (data not shown), consistent with results previously observed by others (54). No kinase activity was precipitated from the baculovirus-infected cells by using the nonimmune serum (Fig. 2C, lane 1).

E1A associates with cyclin-dependent kinases. Whole-cell lysates of an adenovirus-transformed cell line (293) constitutively expressing both of the E1A proteins (38) have been used by others to show an interaction between E1A and cdk2 in association with cyclin E or A (33). 293 cells, however, do not contain a large quantity of E1A (12); therefore, interactions between E1A and other cdks may possibly have been missed. To determine whether E1A could physically target other cdks, we incubated it in nuclear extracts of HeLa cells and then recovered the E1A by immunoprecipitation with M73. Immune complexes of E1A were treated with sodium deoxycholate (see Materials and Methods), which in this case acts in releasing only those products that may have coprecipitated with E1A (4, 33). Afterwards, the released products were reimmunoprecipitated with antibodies specific for cdk2, cdc2, or E1A as a control. Immune complexes generated by each of these antibodies were then tested, in parallel, for the ability to phosphorylate E1A or histone H1. As shown in Fig. 3A, immune complexes generated by antibodies to cdk2 (lanes 3 and 4) or cdc2 (lanes 5 and 6) were clearly capable of phosphorylating E1A or histone H1 in a kinase reaction. As expected, there was no phosphorylation of E1A or histone H1 after the coprecipitated products were reimmunoprecipitated with M73 (Fig. 3A, lanes 1 and 2). Correspondent with these results was that reimmunoprecipitation of released products with antibodies to cyclin E, A, or B yielded immune complexes also able to phosphorylate histone H1 (data not shown).

Until now, E1A has not been shown conclusively to be a part of any complex containing cdc2. To further confirm that E1A can indeed affiliate itself with cdc2, we synchronized adherent HeLa cells at the G_1/S boundary, a point in the cell cycle at which a high rate of cdc2 synthesis has been previously shown to occur (112). Synchronization was achieved by treatment with hydroxyurea (23), and when the block was removed at G_1/S (0 h), the cells were immediately labeled for 3 h with [^{35}S]methionine (Fig. 3C). The lysates of these cells, with or without the addition of E1A, were immunoprecipitated with M73, and the resulting E1A immune complexes were dissociated by boiling in sample buffer (33). The supernatant of these samples was then subjected to reimmunoprecipitation by using antibodies specific for cdk2 or cdc2. Newly synthesized cdc2 was clearly recovered from the anti-E1A precipitates (Fig. 3B, lane 4), and as expected, cdc2 was not recovered from precipitates generated from extracts without the addition of E1A (Fig. 3B, lane 6). cdk2 was also recovered from the anti-E1A precipitates, although it was found in a much smaller amount than was cdc2 (Fig. 3B, lane 3). Finally, given that these cells are enriched in S phase, it was not surprising that cyclin B was virtually undetectable in E1A immune complexes, whereas cyclin A was readily detectable and in association with both cdk2 and cdc2 (data not shown). That cyclin A in HeLa cells can recognize both cdk2 and cdc2 after entry into S phase is consistent with previous results (82).

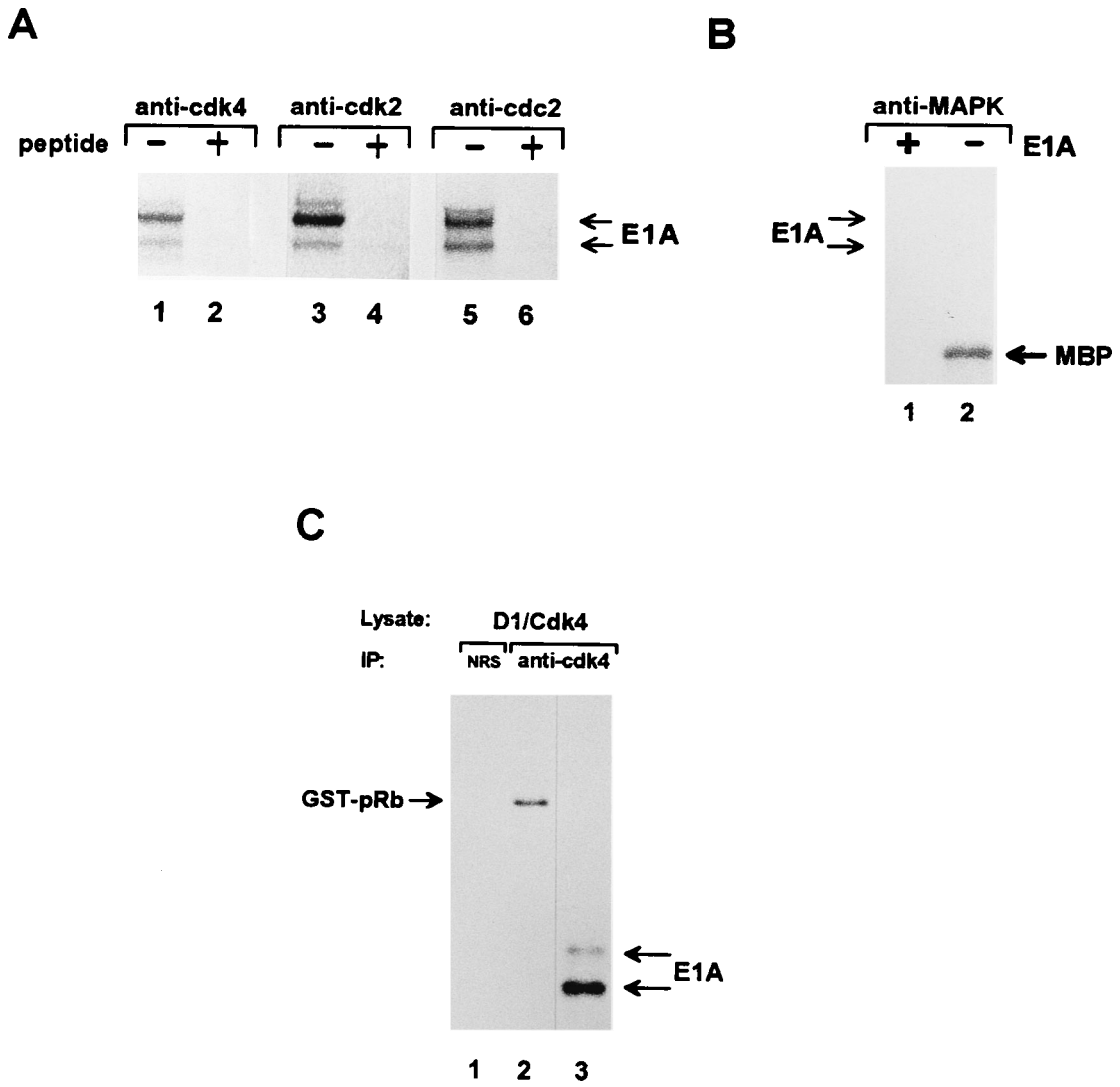
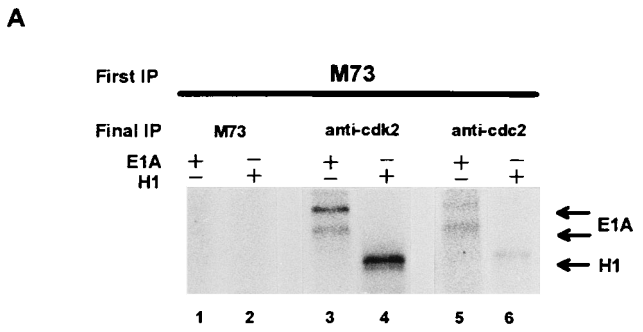
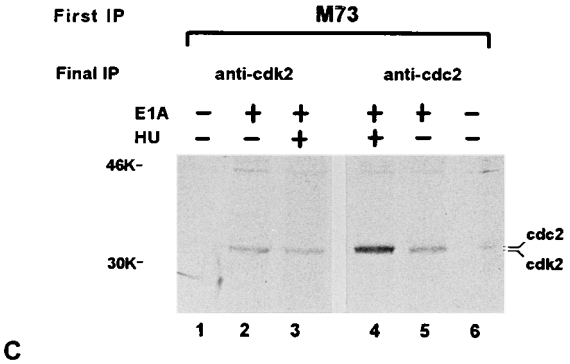


FIG. 2. Cdks can efficiently phosphorylate E1A in vitro. (A) Nuclear extracts of HeLa cells (50 μg) preincubated with (+) or without (−) an excess of antigenic peptide were subjected to immunoprecipitation with antibodies specific for cdk4 (lanes 1 and 2), cdk2 (lanes 3 and 4), or cdc2 (lanes 5 and 6). The immune complex of interest was then incubated in kinase buffer with purified E1A (200 ng) and [γ-^{32}P]ATP. Labeled products from the kinase reactions were analyzed on an SDS–8% polyacrylamide gel and viewed by autoradiography. Exposure times for lanes 1 to 4 are 4 h, and those for lanes 5 and 6 are 7 h. Arrows denote the positions of E1A. (B) Immune complexes of MAP kinase recovered from nuclear extracts of HeLa cells were separately incubated in kinase buffer with purified E1A (200 ng) or myelin basic protein (1 μg) and [γ-^{32}P]ATP. Labeled products were analyzed on an SDS–10% polyacrylamide gel and detected by autoradiography. Arrows denote the positions of E1A and myelin basic protein (MBP). (C) Lysates from insect cells infected with baculovirus encoding cdk4 and cyclin D1 were immunoprecipitated with antiserum to cdk4 or nonimmune rabbit serum (NRS). Precipitates prepared with antibodies to cdk4 were incubated with E1A (lane 3) or GST-pRb (lane 2). Those prepared from NRS were incubated with E1A (lane 1) in the presence of [γ-^{32}P]ATP and then assayed for associated-kinase activity. Products in the kinase reaction were resolved on an SDS–8% polyacrylamide gel. The autoradiography exposure time was 3 h. Arrows denote the positions of E1A and GST-pRb.

Although we did not analyze anti-E1A precipitates for the presence of cdk4 or cyclin D1 in the manner described above, we did explore the possibility of E1A associating with this cyclin-dependent kinase in other ways. Attempts to reimmunoprecipitate newly synthesized cdk4 or its primary partner cyclin D1 from E1A immune complexes derived from [^{35}S]methionine-labeled extracts with added E1A all but failed, as did attempts to detect these proteins in E1A immune complexes immunoblotted and probed sequentially with anti-cdk4 and anti-cyclin D1, D2, or D3 (data not shown). This was not entirely surprising, since earlier studies also failed to show a stable association between E1A and cyclin D1 expressed in intact cells (21).

E1A is phosphorylated at similar sites in vitro and in vivo. Our results indicate that at least four mammalian kinases can efficiently phosphorylate E1A in vitro, and two of these enzymes (cdk2 and cdc2) can stably associate with E1A as well. Since all of these kinases function in cell cycle pathways (99) significantly affected by E1A (18, 19, 103), they become excellent candidates for regulating the phosphorylation of E1A in vivo. Indeed, if any of these kinases are responsible for phosphorylating E1A in vivo, then sites phosphorylated by these enzymes in vitro should be similar. To determine this, we compared phosphopeptides of E1A labeled in vivo and in vitro after *Staphylococcus aureus* V8 protease digestion (15, 41). As shown in Fig. 4, the phosphopeptides of E1A after in vitro





C

	AS	0	3
% G1	62	83	22
% S	25	5	74
% G2/M	13	12	4

FIG. 3. E1A associates with cdks in vitro. (A) Immune complexes of E1A from nuclear extracts of HeLa cells, with or without added E1A, were disrupted by treatment with 0.2% sodium deoxycholate. The released products were then reimmunoprecipitated (Final IP) with the use of antibodies specific for E1A (lanes 1 and 2), cdk2 (lanes 3 and 4), or cdc2 (lanes 5 and 6). The resulting immune complexes were then assayed for kinase activity, by using E1A (200 ng) or histone H1 (185 ng) as the substrate. The arrows indicate the two forms of E1A and histone H1. (B) Adherent HeLa cells after release from the G_1S boundary were labeled for 3 h with [^{35}S]methionine. Lysates of these cells with or without the addition of E1A were then immunoprecipitated (IP) with the anti-E1A monoclonal antibody, M73. Afterwards, the ^{35}S-labeled products associated with the E1A immune complexes were reimmunoprecipitated with antibodies specific for cdk2 (lanes 1 to 3) or cdc2 (lanes 4 to 6). These immune complexes were then separated on an SDS–8% polyacrylamide gel and viewed by autoradiography. In parallel, an asynchronous culture of adherent HeLa cells was also labeled for 3 h with [^{35}S]methionine and then used in the same way as described above. HU denotes hydroxyuea treatment of cells. (C) The relative percentages of G_1, S, and G_2/M cells in asynchronous (AS) cultures and in cells blocked at G_1/S by hydroxyurea treatment (0 h) (0) and then released from the block (3 h) (3) were determined by flow cytometric analysis of nuclear DNA content.

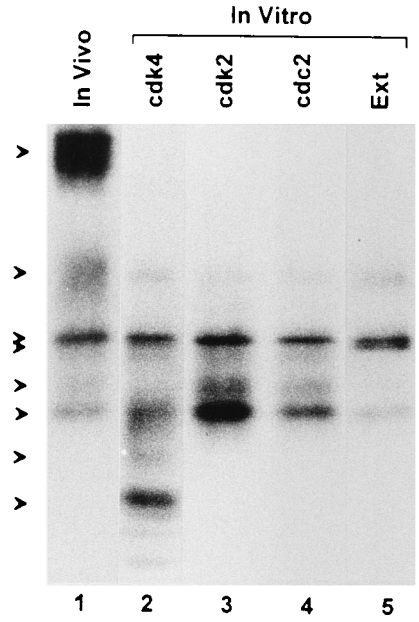


FIG. 4. Phosphopeptide comparison between in vivo- and in vitro-^{32}P-labeled E1A proteins. Purified E1A was modified in a HeLa cell nuclear extract (Ext) (lane 5) and in an in vitro kinase reaction by immune complexes of cdk4, cdk2, and cdc2 (lanes 2 to 4). HeLa cells transfected with plasmid (pCMV-$E1A_{125}$) encoding E1A were metabolically labeled with $^{32}P_i$, and cell lysates were precipitated with M73 (lane 1). Afterwards, the ^{32}P-labeled E1A proteins were resolved by SDS-PAGE, excised, and digested with V8 protease, as described in Materials and Methods. The phosphopeptides were resolved on an SDS–15% polyacrylamide gel and visualized by autoradiography.

labeling by cdk4, cdk2, or cdc2 (lanes 2 to 4) were, for the most part, a subset of the total phosphopeptides of E1A after labeling in vivo (lane 1). Equally important is that comparable results were obtained even after E1A was phosphorylated in a nuclear extract of HeLa cells (Fig. 4, lane 5), which is capable of supporting reversible phosphorylation (6, 17). Although the phosphopeptide patterns of in vitro- and in vivo-labeled E1A were remarkably similar, two important differences existed. First, one of the major peptides phosphorylated in vivo (Fig. 4, lane 1) could not be accounted for in any of the in vitro kinase reactions, which suggests that there are kinases other than cdks which can phosphorylate E1A in vivo. Second, phosphorylation of E1A by cdk4 (Fig. 4, lane 2) in vitro resulted in a major peptide, as well as one or more minor peptides, yet the occurrence of these particular peptides was not observed after E1A was labeled in vivo (lane 1). Further, these phosphopeptides did not occur after E1A was phosphorylated in vitro by cdk2 or cdc2 (Fig. 4, lanes 3 and 4), suggesting that cdk4 can phosphorylate E1A at one or more sites indistinguishable from those of cdk2 or cdc2.

Phosphorylation of E1A increases its efficiency of binding to pRb. The experiments described above indicate that kinases cdk4, cdk2, and cdc2 may be responsible, in part, for phosphorylating E1A in vivo. The functional consequences of phosphorylation by these or any other kinases, however, are still unclear. Since E1A abrogates pRb-induced growth suppression by selectively binding to unphosphorylated and phosphorylated forms of pRb (10, 26), it seemed reasonable to test the ability of unphosphorylated and phosphorylated E1A to associate with pRb in vitro. To begin, a GST-pRb fusion protein containing pRb residues 379 to 928, but which can still bind to E1A (49, 53), was incubated in a HeLa cell nuclear extract with [γ-^{32}P]ATP under conditions promoting phosphorylation. After the reaction had been stopped by the addition of EDTA (60), the modified GST-pRB protein was captured with glutathione-agarose beads and resolved on an SDS-polyacrylamide gel. As might be expected, the kinase(s) present in the nuclear extract was clearly capable of producing a ^{32}P-labeled GST-pRb (Fig. 5A, lane 1) and, moreover, one that should be useful in determining the binding ability of a phosphorylated or unphosphorylated E1A. With this in mind, we repeated the experiment except this time we added a quantity of unmodified

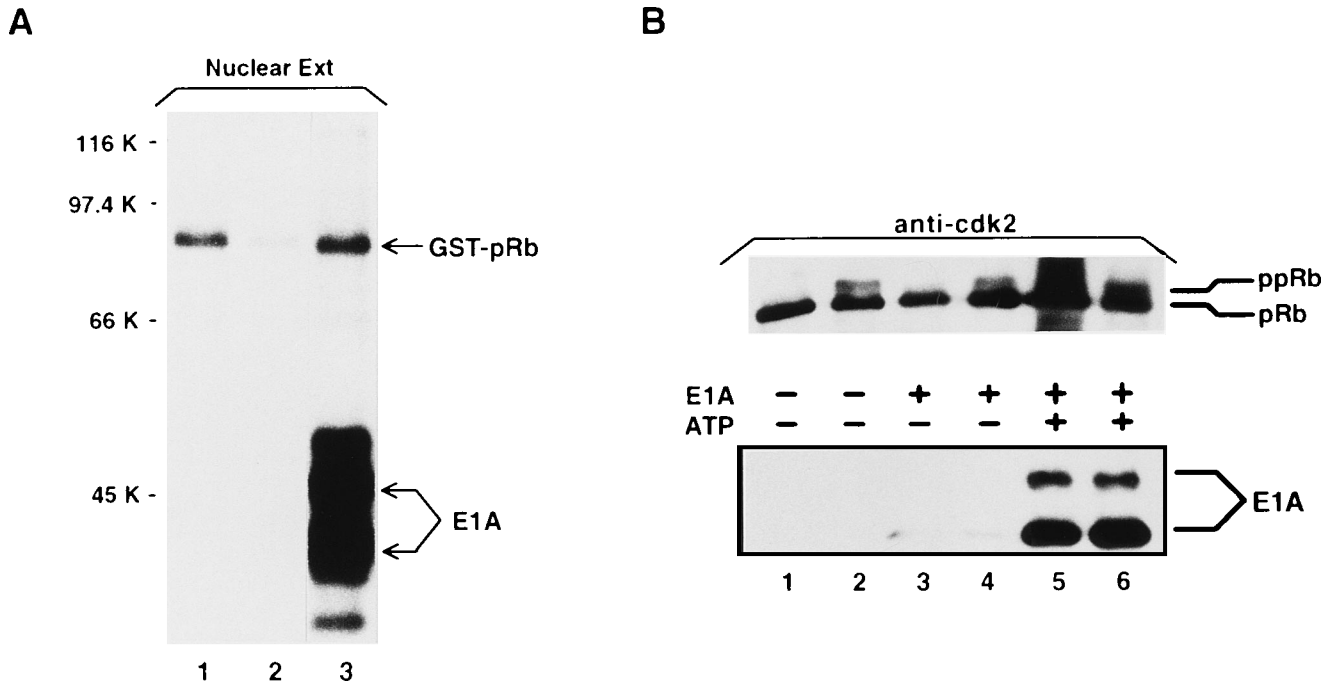


FIG. 5. Phosphorylated E1A binds more efficiently to GST-pRB. (A) Purified GST-pRB was phosphorylated with [γ-^{32}P]ATP in HeLa cell nuclear extracts (Ext) (20 μg), either alone (lanes 1 and 2) or in combination with an equimolar amount of E1A (lane 3). Phosphorylation was terminated by the addition of EDTA, with subsequent addition of unmodified E1A so that the ratio of GST-pRb to E1A was 1:1 (lane 2). E1A in combination with GST-pRb (lanes 2 and 3) was recovered from extracts by immunoprecipitation with M73, whereas GST-pRb alone was captured with glutathione-Sepharose beads (lane 1). All products were resolved electrophoretically on denaturing gels and visualized by autoradiography. (B) Purified GST-pRb was phosphorylated by immune complexes of cdk2 in a kinase reaction with cold ATP, either alone (lanes 2 and 4) or in combination with an equivalent amount of E1A (lane 6). In some cases, GST-pRb was not phosphorylated in reactions because of the absence of cold ATP. Phosphorylation was stopped by the addition of EDTA, and unmodified E1A was then added (in equivalent amounts) to reaction mixtures containing unphosphorylated (lane 3) or phosphorylated (lane 4) GST-pRB. In parallel, the phosphorylation of E1A by immune complexes of cdk2 in a kinase reaction was stopped by the addition of EDTA, and immediately afterward an equivalent amount of unmodified GST-pRB was added (lane 5). After incubation for 2 h at 4°C, glutathione-agarose beads were added to the reaction mixtures for the recovery of GST-pRB. Bound proteins were resolved on SDS-polyacrylamide gels, transferred to membrane, and probed with M73 or antibodies to pRb. Sites of antibody binding were detected by electrochemiluminescence.

E1A to the extract after the phosphorylation of GST-pRb had been terminated by the addition of EDTA. In this reaction, the molar ratio of E1A to phosphorylated GST-pRb was 1:1. The unmodified E1A protein was then recovered from this mixture by immunoprecipitation with the M73 antibody. When the immunoprecipitates of E1A were separated on denaturing gels, we were surprised to find that the E1A immune complexes contained very little of the ^{32}P-labeled GST-pRb (Fig. 5A, lane 2). Opposite results were obtained, however, after the unmodified E1A protein and GST-pRB were phosphorylated together in a nuclear extract with [γ-^{32}P]ATP under identical conditions. As shown in Fig. 5A, lane 3, the M73 antibody recovered not only a ^{32}P-labeled-E1A but also a significant amount of the ^{32}P-labeled GST-pRB protein as well. In principle, the detection of interaction between these two ^{32}P-labeled proteins, by this method, suggests that phosphorylated E1A is far more capable of forming a stable complex with pRb than unphosphorylated E1A.

To further confirm that phosphorylated E1A is directly interacting with pRb and not some higher-order complex containing pRb, we mixed phosphorylated or unphosphorylated E1A with purified GST-pRb in a 150 mM ionic-strength buffer and then performed an in vitro binding assay (67). Specifically, E1A, GST-pRB, or E1A and GST-pRb were initially phosphorylated in a kinase reaction mixture containing immune complexes of cdk2 and cold ATP. In a parallel reaction, GST-pRb was left unphosphorylated because of the absence of cold ATP. Once kinase activity had been stopped by the addition of EDTA (60), the phosphorylated or unphosphorylated product left in each of the supernatants after centrifugation was combined (in equimolar amounts) with either an unphosphorylated E1A or GST-pRb. To monitor the interaction between these proteins, GST-pRb was captured with glutathionine-agarose beads. The proteins in association with these beads were eluted, run on an SDS-polyacrylamide gel, transferred to polyvinylidene difluoride membrane, and then analyzed by immunoblotting with M73 or anti-pRb (Fig. 5B). In the experiment shown, unphosphorylated E1A (Fig. 5B, lanes 3 and 4) was far less efficient than a phosphorylated form of E1A (lanes 5 and 6) in associating with either a phosphorylated or unphosphorylated form of GST-pRb. Taken together, these results represent the first direct evidence linking the phosphorylation of E1A to its ability to efficiently interact with pRb.

Phosphorylated E1A disrupts E2F-pRb complexes more efficiently. The heterodimer E2F1/DP1 is one member in a group of closely related transcription factors (E2F) whose activity is controlled, in part, by the binding of an underphosphorylated pRb (111). In situations in which E1A is expressed, E2F1/DP1 is thought to be released by virtue of E1A's association with pRb (80). This conclusion is based primarily on gel mobility shift assays, in which complexes of E2F1/DP1-pRb bound to oligonucleotides containing E2F-binding sites (25, 43) become disrupted by E1A, as a result of its synthesis in vivo or in an in vitro translational system (5, 13). Since this means of expression guarantees, to some extent, a phosphorylated form of E1A (104) and the results presented in Fig. 5 indicate that phosphorylated E1A binds more efficiently to pRb, one would predict that a phosphorylated form of E1A might be

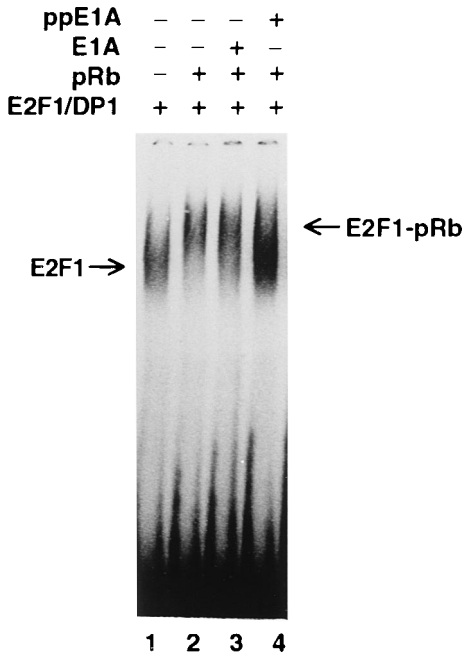


FIG. 6. Disruption of E2F-pRb complexes by phosphorylated E1A. As described in Materials and Methods, E1A (30 ng) was phosphorylated (with cold ATP) or left unphosphorylated (without ATP) in a kinase reaction mixture containing immune complexes of cdk2. Gel mobility shift assays, with a ^{32}P-labeled oligonucleotide containing the E2F consensus site, were performed with recombinant GST-E2F1 and GST-DP1 in the absence of additional proteins (lane 1) or in the presence of GST-pRB (lane 2), GST-pRb and unphosphorylated E1A (lane 3), or GST-pRB and phosphorylated E1A (lane 4). E2F1/DP1 DNA-binding activity was not observed when the reaction mixture contained a 100-fold molar excess of unlabeled E2F oligonucleotide (data not shown).

more efficient in dissociating E2F1/DP1-pRb complexes. As proof of this, we used comparable ratios of E1A to E2F1/DP1-pRb in gel mobility shift experiments in order to measure the response of DNA-bound complexes to unphosphorylated or phosphorylated E1A. After phosphorylating E1A in a kinase reaction mixture containing cold ATP and immune complexes of cdk2, we found that the complex containing purified E2F1/DP1 and pRb (Fig. 6, lane 2) could be easily disrupted by modified E1A (Fig. 6, lane 4). Unphosphorylated E1A, on the other hand, failed considerably in dissociating the complex (Fig. 6, lane 3). These results suggest that at least a part of E1A's ability to alter E2F1/DP1-pRb interaction may depend on whether it is phosphorylated.

DISCUSSION

In learning more about the functions of the E1A 243R protein, one must understand why E1A is continually phosphorylated throughout the cell cycle (23, 24). Essential to this inquiry is the identification of kinases targeting E1A and, more importantly, their effect on E1A's activities after phosphorylation. Our studies have identified at least four kinases capable of phosphorylating E1A in vitro. Three of these kinases are in the cyclin-cdk family of enzymes (cdk4, cdk2, and cdc2), which participate in coordinating cell division (50, 98). Moreover, the sites on E1A phosphorylated by these cdks in vitro correspond to the majority of E1A sites phosphorylated in vivo (Fig. 4). This strongly suggests that E1A may actually be targeted by these kinases in vivo. Since each of the kinases is active only at particular stages of the cell cycle (50, 98), this could explain the endurance of E1A's phosphorylation in the changing of cell cycle position. The coordinate activity of these kinases might also help explain why some sites on E1A appear to be differentially phosphorylated at different rates (Fig. 1). This, and the fact that others have shown that at least one site on E1A needs to be phosphorylated before phosphorylation can occur on another site (24), raises the possibility that the ordering of phosphorylation on E1A may actually be important in preparing E1A for subsequent phosphorylation by other kinases. That E1A appears to be more heavily phosphorylated in mitotic cells than in cells in G_1/S (73) certainly supports this view. Worth mentioning is that cdc2 is the enzyme generally most active in mitotic cells (50), and aside from using E1A as a substrate (73) (Fig. 2), it can also use other viral proteins in this way (34). In fact, phosphorylation by cdc2 appears to affect the DNA-binding activity of simian virus 40 large T antigen (72). Finally, although E1A may be a physiological substrate for cdc2, it is almost certainly not a physiological substrate for MAP kinase (data not shown). This is interesting, since the preferred phosphorylation sites used by this enzyme (Pro-X-Ser/Thr-Pro, where X is any amino acid) are not so different from the sites used by cdc2 (X-Ser/Thr-Pro) (83, 93, 97).

Our findings suggest that cyclin D-cdk4 complexes may play a role in regulating some of E1A's activities. For example, immune complexes of cdk4 from specially prepared extracts of HeLa cells (6, 71) can efficiently phosphorylate E1A in vitro (Fig. 2A). Further, the ability of enzymatically active cyclin D1-cdk4 complexes in insect cells to specifically phosphorylate E1A, as well (Fig. 2B), gives more evidence that cdk4 can use E1A as a substrate. These results, coupled with the fact that the majority of the E1A phosphopeptides generated by cdk4 in vitro correspond to those present in E1A-transfected cells, strongly suggest that cdk4 may play a central role in promoting the phosphorylation of E1A in vivo. Moreover, since cyclin D-dependent cdk4 activity is first detected in the mid-G_1 phase of the cell cycle (99), we believe that this may be the time at which cdk4 exerts its effect on E1A. Although our experiments failed to show an association between E1A and cdk4 complexes, this does not negate the possibility that E1A could be a putative physiologic substrate for cdk4, since in most cases enzyme-substrate intermediates rapidly dissociate once phosphorylation occurs (29, 91). In fact, stable associations between pRb and cyclins E- and A-cdk2 have not been seen, even though these kinases directly phosphorylate pRb in vivo and in vitro (45). Although D-type cyclins in association with their cdk4 partners have been shown to interact directly with pRb (21, 30), this interaction can be viewed as distinctly different, because a mutant cyclin D1 lacking the pRb-binding motif LXCXE (21) remains able to phosphorylate pRb in vivo and in vitro (46).

In addition to cyclin D-cdk4, we propose that there may be several other combinations of cyclins and kinases which phosphorylate E1A in vivo. This notion is based on the fact that stable complexes can be formed between E1A and cdk2 or cdc2 (Fig. 3), the former of which can associate with either cyclin E or A and the latter of which can associate with cyclin A or B (50, 97). Since both cyclins E and A have previously been shown to interact with E1A (33, 37, 84) and because there is evidence of E1A associating with cyclin B (67a), the combination of cyclin-cdk partners that might phosphorylate E1A in vivo could conceivably include cyclins E- and A-cdk2 and cyclins A- and B-cdc2. The fact that these cyclins and their respective partners control various stages of the cell cycle (22, 58, 82, 92) makes it tempting to speculate that these kinases may confer a property on E1A that enables it to function at different points in the cell cycle. This is supported by our recent

work showing that E1A can affect more than one pathway in G_0/G_1 when inducing cellular DNA synthesis (18, 19, 103).

Our observation that phosphorylated E1A binds more efficiently to GST-pRb in vitro (Fig. 5) and disrupts E2F1/DP1-pRb complexes in a gel retardation assay more effectively (Fig. 6) than does unphosphorylated E1A raises the question of whether phosphorylation might affect E1A's ability to activate E2F in vivo. Others have shown that E1A can liberate the transcriptional factor E2F/DP from pRb alone (5, 13) or liberate it from complexes containing pRb-related proteins p107 and p130 in association with cyclin A- or E-cdk2 (16, 35, 52, 61). In releasing E2F/DP, E1A presumably remains bound to these cellular proteins, and this, as a result, gives E2F/DP an opportunity to activate E2F/DP-responsive genes important to cell cycle progression (44, 102). Since E2F/DP's activity appears to be differentially regulated by its association with pRb or p130 (16, 95) and with p107-cyclin-cdk or p130-cyclin-cdk complexes (16, 63, 89, 114), there may be differences in the way E1A is able to effectively release E2F/DP from these proteins. In dealing with pRb, E1A may have to be phosphorylated to displace E2F/DP from the E2F/DP-pRb complex. This is plausible since others have previously reported the inability of an unmodified GST-E1A fusion protein to completely disrupt E2F/DP-pRb complexes in vitro (35). In addition, because E1A requires two of its sequence domains to make direct contact with pRb (27, 35, 52), the occurrence of phosphorylation at one or both of these domains could facilitate the interaction between these two proteins and consequently bring about the dissociation of E2F/DP. Apart from this, the activity of E1A in rescuing E2F/DP from E2F/DP-p130 complexes present in G_0 or early G_1 (16) might also be predicated on whether E1A is phosphorylated. In rescuing an active E2F/DP from p107-cyclin-cdk or p130-cyclin-cdk complexes, E1A could be acting as a competitive substrate for these kinases as they regulate the activity of E2F/DP throughout the cell cycle. Recent studies show that cyclin A-cdk2 is largely responsible for shutting off E2F1-dependent DNA binding function after phosphorylating DP1, which is in association with E2F1 (25, 59). Furthermore, evidence indicates that E2F1 can be efficiently phosphorylated by cyclin A-cdk2 as well (56). Thus, by drawing the attention of cdks from E2F/DP, E1A may be preventing the negative regulatory effect that these kinases appear to have on E2F/DP. This particular activity of E1A may also apply to other cellular proteins. For example, we have recently found that E1A can inhibit the phosphorylation of p300 as a result of interference with the activities of cdk2 and cdc2, both of which appear to be specific for p300 (6).

Finally, our results raise the interesting possibility that E1A may undergo cyclin-dependent phosphorylation in vivo and that this phosphorylation becomes a significant part of E1A's activity. If this is true, and considering that the cdks phosphorylating E1A are heightened in activity at different stages of the cell cycle (98), it is reasonable to assume that E1A can interact with a number of pathways that are essential to cell growth. Clearly, it will be important to continue the investigation of E1A's activities with phosphorylation in a temporal manner, as it traverses the cell cycle.

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