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Phosphorylation of Serine Residue 89 of Human Adenovirus E1A Proteins Is Responsible for Their Characteristic Electrophoretic Mobility Shifts, and Its Mutation Affects Biological Function

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The shift in mobility on sodium dodecyl sulfate-polyacrylamide gel electrophoresis that is characteristic of the adenovirus E1A proteins is the result of posttranslational modification. In the present study, we demonstrate that phosphorylation of bacterially produced E1A in higher cell extracts occurs on serine and is responsible for the mobility shift. E1A protein expressed in *Saccharomyces cerevisiae* also undergoes the mobility shift due to serine phosphorylation. Site-directed mutagenesis was used to identify the serine residue responsible for the mobility shift. Six serine residues were altered to glycine within E1A. Substitution at serine residue 89 was shown to selectively prevent the mobility shift of both the 289R and 243R E1A proteins. We conclude that phosphorylation at serine 89 is the specific modification responsible for the mobility shift of E1A. Moreover, we demonstrate that the Ser-89-to-Gly mutation has no effect on *trans* activation or complementation of an E1A-deficient adenovirus. In contrast, the mutant protein does significantly reduce both the repression and transformation efficiency of E1A. The five other Ser-to-Gly mutations were also examined for functional effects. None affected *trans* activation, whereas repression and transformation functions were affected. One mutant affected transformation without affecting repression, suggesting that these functions are to some degree also separable. The relevance of phosphorylation to structure and activity of E1A and other nuclear oncogene proteins is discussed.

A wide variety of oncogene proteins have been described which have the ability to transform cells. One group of oncogene proteins resides in the cell nucleus (65). In addition to having the same cellular location, these nuclear oncogene proteins are posttranslationally modified. For many of these proteins (early region 1A [E1A], simian virus 40 [SV40] and polyomavirus large T antigens, *fos*, *myc*, and p53), the occurrence of posttranslational modification(s) has been identified by a distinct shift in mobility of the protein on sodium dodecyl sulfate (SDS)-polyacrylamide gels, suggesting an increase in apparent molecular weight (1, 5, 9, 20, 38, 39, 69). Significant mobility shifts (equivalent to 2 to 4 kilodaltons) have been reported for E1A (21, 47, 69) and *fos* (9). The mobility shifts of nuclear oncogene proteins suggest a significant change in protein structure which may be important to their function.

Phosphorylation has been the most documented modification of nuclear oncogene proteins; E1A (69), SV40 large T antigen (61), polyomavirus large T antigen (22), *fos* (59), *myc* (20), *myb* (2), and p53 (61) are reported to be phosphorylated on serine residues. In fact, the SDS-gel mobility shifts of the *fos* (2) and polyomavirus large T (5) proteins have been shown to be due to phosphorylation. However, the SDS-gel mobility shifts cannot be accounted for by the addition of low-molecular-weight phosphate groups alone, especially since the charge addition should increase the mobility of the protein. Therefore, if phosphorylation of serine residues is involved in the SDS-polyacrylamide gel electrophoresis (SDS-PAGE) mobility shifts, it may induce significant changes in protein structure which in turn lead to the gel mobility shifts, even under denaturing conditions.

The relationship of posttranslational phosphorylation to biological function has been investigated in some detail for SV40 large T antigen, and the reports are somewhat contradictory. SV40 large T antigen is phosphorylated on both serine and threonine residues (52). Removal of phosphates from SV40 large T antigen has been reported to have various effects on DNA binding and DNA replication (3, 19, 27, 55). Thus, it appears that phosphorylation can play a complex role in the regulation of biological activity of nuclear oncogene proteins.

The adenovirus type 5 (Ad5) E1A gene region encodes two nuclear localized oncogene proteins which are posttranslationally modified, leading to dramatic mobility shifts when examined by using SDS-polyacrylamide gels (21, 47). These two proteins (243 residues [243R] and 289 residues [289R]) are translated from alternatively spliced mRNAs and differ only by the inclusion of an extra 46 amino acids within the larger protein species. In addition to activating transcription of adenovirus promoters, the E1A proteins activate transcription from certain cellular promoters, repress transcription controlled by enhancers, and transform cells in cooperation with other oncogene proteins (4, 18, 23, 32, 49, 62). Modification of the E1A proteins yields an increase in apparent molecular mass of 2 to 4 kilodaltons on SDS-polyacrylamide gels and appears to be the result of a single posttranslational event (21).

Phosphorylation is the only posttranslational modification that is known to occur on E1A (21, 48, 57, 68, 69). Mutation of serine residue 219, a major phosphorylation site of both E1A proteins, has no detectable effect on biological function or SDS-gel mobility shift (58). Previous deletion analysis suggested that amino acid residues 23 to 120 are involved in the SDS-PAGE mobility shift and are important for E1A function (47). While this report was being prepared, Richter et al. (46) provided evidence that phosphorylations between amino acids 86 and 120 and between amino acids 224 and 289 result in this shift. However, the specific residue(s) involved

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has not been identified. In this paper, we demonstrate that the modification responsible for the gel mobility shift of E1A proteins is phosphorylation, and we map this phosphorylation event specifically to serine 89 of both E1A proteins.

Furthermore, we present evidence that an E1A mutant, with a mutation that prevents phosphorylation at serine residue 89, retains certain biological functions. However, the mutant does exhibit a reduction in both repression and transformation efficiency. This is the first report to suggest a relationship between specific phosphorylation of E1A and biological function.

In addition, other serine mutations were generated, and several affected E1A function. We discuss these results and the relevance of phosphorylation to structure, the activity of E1A, and the activity of other nuclear oncogene proteins.

MATERIALS AND METHODS

***E. coli* expression of E1A proteins and their purification.** The 243-amino-acid (243R) and 289-amino-acid (289R) E1A proteins were expressed in *Escherichia coli* by induction of pAS1-E1A412 and pAS1-E1A410, respectively (11), with nalidixic acid (42). These proteins were purified as previously described (30) and estimated to be 90% pure by Coomassie blue-stained SDS-polyacrylamide gels.

Phosphoamino acid analysis. Purified protein (6 μg) was added to 200 μl of rabbit reticulocyte lysate (Green Hectares, Oregon, Wis.) supplemented with 120 mM potassium acetate–1 mM magnesium acetate–12 mM creatine phosphate–50 mM KCl–30 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.2)–200 μCi of [γ-^{32}P]ATP (5,000 Ci/mmol, aqueous; Amersham Corp., Arlington Heights, Ill.) in a total of 500 μl. The modification reaction was incubated at 30°C for 5 h. RIPA (500 μl) (20 mM Tris hydrochloride [pH 7.5], 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 0.1% SDS) and 100 μl of preimmune rabbit antibody were added to remove nonspecific antibody-binding impurities. After 15 min at 4°C, *Staphylococcus* A cells (Immuno-Precipitin; Bethesda Research Laboratories, Inc., Gaithersburg, Md.) (washed twice in RIPA) were added and the mixture was incubated for 30 min at 4°C. The antibody-*Staphylococcus* A complex was removed by centrifugation. E1A antibody (30) (10 μl) was added to the supernatant and incubated at room temperature for 2 h. *Staphylococcus* A cells (50 μl) were added, and after 45 min at room temperature, the cells were pelleted by centrifugation. The immunoprecipitated ^{32}P-labeled E1A pellet was washed twice with RIPA and once with water, suspended in SDS sample buffer, and electrophoresed on an SDS–10% (30:1.6 acrylamide/bis) polyacrylamide gel. The ^{32}P-labeled E1A protein was located in the gel by autoradiography. The ^{32}P-labeled E1A was excised from the gel, and diced gel pieces were electroeluted in an ISCO electrophoretic concentrator (24). The concentrated ^{32}P-labeled E1A sample was ethanol precipitated, dried, and suspended in 100 μl of 0.1% SDS. A fraction (10 μl) of this sample was added to 100 μl of 6 N HCl and hydrolyzed in vacuo for 2 h at 110°C. The hydrolysate was dried and suspended in water, and 5 μg of each phosphoamino acid (Sigma Chemical Co., St. Louis, Mo.) in 0.1 N HCl–5% acetic acid was added. ^{32}P-amino acids were analyzed by high-voltage paper electrophoresis (12). Radioactive spots were identified by reference to ninhydrin-detected phosphoamino acid standards.

In vitro modification of *E. coli*-expressed E1A. Purified 243R or 289R E1A protein (4 μg) was added to 200 μl of rabbit reticulocyte lysate supplemented with 120 mM potassium acetate–1 mM magnesium acetate–12 mM creatine phosphate–50 mM KCl–30 mM HEPES (pH 7.2)–400 μCi of [γ-^{32}P]ATP (5,000 Ci/mmol, aqueous; Amersham) in a total of 500 μl. The modification mixture was incubated at room temperature for 7 h. Portions (100 μl each) of this reaction mixture were incubated with 100 μl of 200 mM Tris hydrochloride (pH 8)–10 μM $ZnCl_2$ or with this buffer containing 1.4 U (0.8 μg) of alkaline phosphatase from bovine intestinal mucosa (Sigma VII-NL; 1,890 U/mg) for 12 to 14 h at 30°C. ^{32}P-labeled E1A was immunoprecipitated as described above and electrophoresed on an SDS–10% polyacrylamide gel. The proteins in the gel were transferred to nitrocellulose which was analyzed for ^{32}P-proteins by autoradiography for 30 h at −70°C. The nitrocellulose was then processed as a Western blot (immunoblot) by using E1A antibody at a 1:10,000 dilution and ^{125}I-protein A. ^{125}I was detected by autoradiography for 3 h at −70°C.

Construction of yeast expression vector pCD192-E1A. All plasmid constructions were made by using standard recombinant DNA techniques (37). pE1A300 was prepared by B. Ferguson by cloning the 1,748-base-pair *Pvu*II fragment from pJN20 (10) which contains a complete cDNA copy of the 13S mRNA at the *Pvu*II site of pE1A100 (10). A *Bam*HI-*Hpa*I restriction fragment containing the entire 289R E1A coding sequence was isolated from pE1A300 and treated with DNA polymerase (Klenow) to create blunt ends. This fragment was inserted at the *Pvu*II site positioned immediately downstream from the CUP1 promoter in the *Saccharomyces cerevisiae* expression vector pCD192. pCD192 is a high-copy-number 2μm-based yeast-*E. coli* shuttle vector which carries the TRP1 selection marker and the CUP1 sequences for regulation and initiation of transcription. This vector was derived from pYSK105 (17) by deleting the galactokinase gene (*galK*) and replacing it by a *Pvu*II linker (New England BioLabs, Inc., Beverly, Mass.).

Expression of E1A in *S. cerevisiae*. pCD192-E1A was introduced into the yeast *trp*-BR10 strain as described previously (17). Cells were grown for 6 h in 100 ml of YNB-glucose-adenosine-histidine and induced for 1 h by the addition of 0.1 mM $CuSO_4$. Cells were pelleted by centrifugation and washed once with water. Cells were suspended in 5 ml of 50 mM Tris hydrochloride (pH 8)–0.1 mM dithiothreitol–2 mM EDTA–5% glycerol–1 mM phenylmethylsulfonyl fluoride and added to 5 ml of acid-washed glass beads. Cells were lysed by being vortexed six times for 20 s each time. Glass beads were removed by low-speed centrifugation. The supernatant was transferred to a new tube and centrifuged at 25,000 × *g* to remove cell debris. Cell lysates were stored at −70°C.

Phosphatase treatment of E1A produced in *S. cerevisiae*. E1A-containing yeast extract (30 μl) was added to 30 μl of 200 mM Tris hydrochloride (pH 8)–10 μl of $ZnCl_2$ buffer alone or containing 1 μl of calf intestine alkaline phosphatase (2,600 U/mg; Calbiochem-Behring, La Jolla, Calif.), 20 mM sodium phosphate, or both phosphate and phosphatase. These mixtures were incubated for 12 to 14 h at 30°C and analyzed by Western blot.

Construction of mammalian cell expression vectors pRE1A.13.WT and pRE1A.12.WT. pE1A300 (see above) was cut with *Bam*HI, filled in with DNA polymerase (Klenow), and recut with *Sst*I. This *Bam*HI-*Sst*I fragment was inserted between the unique *Hin*dIII (filled-in) and *Sst*I sites of mammalian expression vector pRDZB.JT (provided by J. Trill, Smith Kline & French Laboratories, King of Prussia, Pa.). This procedure placed the E1A 13S cDNA between the Rous sarcoma virus promoter and the bovine growth

hormone polyadenylation site and created pRE1A.13.WT. pRE1A.12.WT was constructed by replacing the *Cla*I-*Sst*I fragment of pRE1A.13.WT with the *Cla*I-*Sst*I fragment from pJF12 which contains the E1A 12S cDNA (47).

Oligonucleotide-directed mutagenesis. The *Sma*I-*Bam*HI fragment from pAS1E1A310 (10), containing the region coding for E1A amino acids 3 to 150, was inserted into M13 mp18 cut with *Sma*I-*Bam*HI to create mp18 E1A. *E. coli* BW313 (31) was infected with mp18 E1A, and uracil-containing single-stranded bacteriophage DNA was isolated (31). Oligonucleotides (26-mers) were synthesized with a one- or two-base change in their centers to convert a serine codon to a glycine codon. The codon corresponding to amino acid 36 was changed from AGC to GGC, that corresponding to 63 was changed from TCG to GGG, that corresponding to 69 was changed from TCT to GGT, that corresponding to 89 was changed from TCT to GGT, that corresponding to 96 was changed from TCC to GGC, and that corresponding to 111 was changed from TCT to GGT. These oligonucleotides, each containing a mutation in one of the six serine codons under study, were phosphorylated by T4 polynucleotide kinase and hybridized to single-stranded mp18 E1A, and the second strand of the duplex was completed by using Klenow or T4 DNA polymerase and ligated with T4 DNA ligase. Reaction mixtures were transformed into JM101. Mutations were confirmed by DNA sequencing (51). Mutations were inserted into pRE1A.13.WT or pRE1A.12.WT by replacing the *Bst*XI fragment in the E1A coding region with the *Bst*XI fragment from mp18 E1A carrying the mutation. The presence of the mutation in the pRE1A.13 and pRE1A.12 vectors was confirmed by sequencing.

***Xenopus* oocyte microinjection.** *Xenopus laevis* oocytes were isolated and microinjected as described previously (10, 25). A 10-nl sample of water containing 10 ng of plasmid DNA was microinjected into the nuclei of oocytes, and 10 oocytes were injected with each plasmid. After a 30-h incubation at room temperature in modified Barth medium (37), oocytes were pooled and lysed in 50 mM Tris hydrochloride (pH 8)–1 mM phenylmethylsulfonyl fluoride. The equivalent of one oocyte was analyzed by Western blot and autoradiography.

Cells and transfections. The cells used for these experiments were HeLa cells, 27-1 cells (a HeLa cell derivative provided by N. Jones containing a stable copy of the chloramphenicol acetyltransferase [CAT] gene regulated by the adenovirus E3 promoter), or REF52 cells (provided by R. Vasavada and R. Ricciardi [The Wistar Institute]; an established cell line which resembles certain primary cells with regard to transformation by *ras* oncogenes [14]). All cells were cultured at 37°C in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum. For transfections, the cells were plated at a concentration of 4×10^5 cells per 60-mm dish. After 24 h, the medium was replaced 2 to 4 h prior to the addition of DNA. Transfections were performed by the calcium phosphate method of Wigler et al. (67). The DNA was left on the cell monolayer for 15 to 18 h at 37°C and then replaced with fresh medium. In all transfections, a total of 7.2 μg of plasmid DNA was used.

***trans* Activation and repression; CAT assays.** For *trans*-activation experiments, 3.6 μg of each 289R E1A expression vector was transfected with carrier DNA into 27-1 cells to look for an increase in CAT activity as a result of activation of the E3 promoter. For repression experiments, 3.6 μg of each 243R E1A expression vector and 3.6 μg of pSV40-CAT were cotransfected into HeLa cells to look for a reduction in CAT expression. The vector pSV40-CAT (provided by R. Lee) encoding the CAT gene regulated by the SV40 early promoter is the same as pSV2CAT (16) except that *Bam*HI sites were inserted around the enhancer for other purposes. In both cases, carrier DNA alone was transfected into the appropriate cell line as a control. Following transfections, the cells were harvested as described by Gorman et al. (15) at either 42 or 66 h posttransfection. The cells were washed with phosphate-buffered saline and then removed by scraping in 40 mM Tris hydrochloride (pH 7.5)–1 mM EDTA–150 mM NaCl. The cells were pelleted by centrifugation, suspended in 50 μl of 20 mM Tris hydrochloride (pH 7.8), and disrupted by four cycles of freeze-thawing. For comparison of transfection efficiencies within each experiment, 10 μl of each cell extract was analyzed by Western blot analysis as previously described (30).

The activity of the CAT enzyme produced as a result of each transfection was analyzed by incubation of aliquots of cell extract with 0.1 μCi of [^{14}C]chloramphenicol (Amersham) and acetyl coenzyme A (Sigma) for various times at 37°C. Varying the amount of extract and repeating the assays at different time points allowed us to be sure that the results we obtained were in the linear range for detection of CAT activity. The substrate and products were then extracted with ethyl acetate and separated by thin-layer chromatography. The acetylated and nonacetylated forms of [^{14}C]chloramphenicol were located by autoradiography and quantitated by scintillation counting. Since transfection efficiencies vary, all experiments were repeated at least three times and with independent DNA preparations.

Transformation assays. A 3.6-μg sample of each 289R E1A expression vector was cotransfected with 2 μg of pT24/*neo* and carrier DNA into REF52 cells. The vector pT24/*neo*, encoding both the T24 Ha-*ras* oncogene protein and a neomycin resistance marker, were provided by R. Connors (Smith Kline & French). At 2 h after replacement of the medium, the cells were split into 6-well dishes and incubated at 37°C with G418 (400 μg/μl; 47% active; GIBCO Diagnostics, Madison, Wis.). The medium was replaced every 3 to 4 days, and the cells were stained with crystal violet after 2 weeks. The number of foci or G418-resistant colonies which were morphologically transformed was determined by phase-contrast microscopy. As a control, we used the E1A wild-type (WT) (pRE1A.13.WT) or pT24/*neo* vector alone for transfection (with carrier DNA).

d1312 complementation. A 3.6-μg portion of each E1A expression vector with carrier DNA or carrier DNA alone was transfected into HeLa cells. On day 2, the cells were infected with d1312, an E1A-deficient adenovirus mutant, at a multiplicity of infection of approximately 20 PFU per cell. Cells were labeled with 125 μCi of [^{35}S]methionine per ml for 2 h at 22 h postinfection, and the major viral coat proteins (hexon, penton base, and fiber) were visualized by SDS-PAGE and fluorography by using Amplify (Amersham).

RESULTS

In vitro phosphorylation of E1A proteins occurs on serine residues. The posttranslational modification of E1A proteins results in SDS-PAGE mobility shifts of 2 to 4 kilodaltons (69). To investigate the nature of the modification responsible for this shift, we established an in vitro system using *E. coli*-produced E1A and eucaryotic cell extracts. E1A produced in *E. coli* is used as a source of unmodified protein, since no modifications of this E1A have been detected (57, 58). Modified *E. coli*-produced E1A after microinjection into

a eucaryotic cell (47) or incubation in a mammalian cell extract appears indistinguishable by SDS-PAGE analysis from E1A examined directly from adenovirus-infected cells (35, 47). In addition, *E. coli*-produced E1A proteins are functional, as demonstrated by mammalian cell (30) and *Xenopus* oocyte (47) microinjection, in vitro transcription experiments (56), in vitro modification with cell extracts (68, 69), and protoplast fusion experiments (13). For these reasons, we chose E1A produced in *E. coli* to study E1A posttranslational processing.

E1A proteins purified from *E. coli* were modified in vitro by using [γ-^{32}P]ATP and a rabbit reticulocyte cell lysate. Under these conditions, ^{32}P was incorporated into both mobility-shifted and unshifted forms of E1A as observed by SDS-PAGE (Fig. 1A, lane 1). *E. coli* E1A protein comigrates with the lower ^{32}P-labeled E1A band (47 and data not shown). The same characteristic SDS-PAGE mobility shift was also observed when *E. coli*-produced E1A was incubated in HeLa cell extracts (data not shown). ^{32}P-labeled E1A was purified by immunoprecipitation using E1A-specific antibody and analyzed by phosphoamino acid analysis. Only [^{32}P]phosphoserine was detected in each mobility form of ^{32}P-labeled E1A (data not shown), demonstrating that all phosphate linkages were through serine. Treatment of ^{32}P-labeled protein with calf intestine alkaline phosphatase resulted in all incorporated ^{32}P being removed from the protein (Fig. 1A, lane 2). Apparently, all phosphate was incorporated in the form of phosphomonoesters (7). EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′,-tetraacetic acid] and EDTA, known nonspecific inhibitors of kinases, completely inhibited the in vitro modification of *E. coli*-produced E1A in either the reticulocyte or HeLa cell extracts (data not shown). From these results, we conclude that all posttranslationally incorporated phosphate in in vitro modified E1A is in the form of phosphoserine.

The SDS-PAGE mobility shift of in vitro modified E1A is due to a specific phosphorylation. To determine the effect of phosphorylation on the E1A mobility shift, modified E1A treated with phosphatase (see above) was analyzed by Western blot with E1A-specific antisera. After phosphatase treatment of the mobility-shifted and unshifted forms of E1A, only the unshifted precursor form of E1A was detected (Fig. 1A, lane 4). These results show that the mobility shift of modified E1A can be quantitatively reversed by the removal of phosphates.

Using purified casein kinase II (provided by R. Weinman), we were able to efficiently incorporate ^{32}P into E1A purified from *E. coli* (data not shown). Although the E1A protein was efficiently labeled, this ^{32}P-labeled E1A protein did not undergo the characteristic shift on SDS-PAGE. This result suggests that a specific phosphorylation must be responsible for the SDS-PAGE mobility shift and that this kinase cannot mimic the event.

E1A expressed in *S. cerevisiae* is phosphorylated, resulting in SDS-PAGE mobility shift. The effect of phosphatase treatment on modified E1A was also examined by using E1A expressed and modified in *S. cerevisiae*. After expression of the 289R E1A protein in *S. cerevisiae*, cell lysates were analyzed by Western blot. The majority of the E1A protein was detected in the mobility-shifted form (Fig. 1B, lane 1). After phosphatase treatment, the majority of the E1A detected was in the unshifted form (Fig. 1B, lane 2). P_i, a specific inhibitor of phosphatase (7), prevented the phosphatase effect (Fig. 1B, lane 3). These results support those obtained with the in vitro modified E1A protein (Fig. 1A) and indicate that phosphatase treatment alone can completely and selectively reverse the mobility shift.

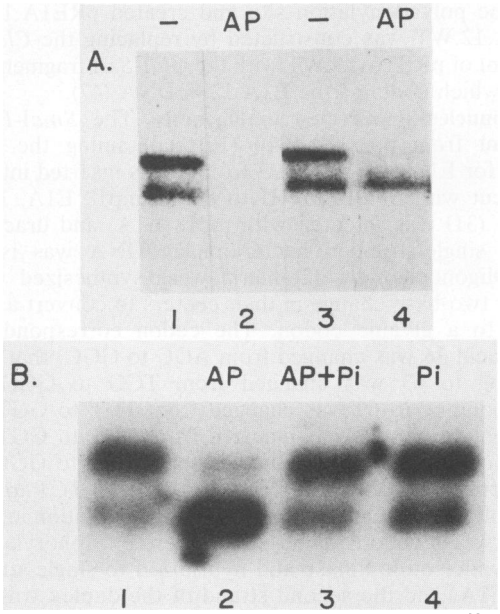

FIG. 1. Phosphatase treatment of in vitro modified ^{32}P-labeled E1A protein and in vivo modified E1A protein expressed in *S. cerevisiae*. (A) The 243R E1A protein was modified in reticulocyte lysate with added [γ-^{32}P]ATP followed by treatment with alkaline phosphatase. E1A species were immunoprecipitated and separated by using SDS-PAGE. After transfer to nitrocellulose, ^{32}P-labeled E1A species were located by autoradiography for 30 h. Then, the nitrocellulose was processed as a Western blot with E1A-specific antibody and ^{125}I-protein A, and E1A protein was detected by autoradiography for 3 h. ^{32}P radioactivity was relatively weak and did not contribute to the ^{125}I autoradiography. Lanes: 1 and 2, ^{32}P-labeled E1A protein incubated without and with phosphatase, respectively; 3 and 4, same as lanes 1 and 2, respectively, except detection was with E1A antibody and ^{125}I autoradiography. (B) Yeast cell lysates containing 289R E1A protein were incubated with and without alkaline phosphatase and analyzed by Western blot with E1A-specific antibody. Lanes: 1, no phosphatase; 2, phosphatase; 3, phosphatase and P_i; 4, P_i.

Phosphorylation of serine residue 89 causes the SDS-PAGE mobility shift. Previous deletion analysis of E1A (47) indicated that the region required for the SDS-PAGE mobility shift mapped between residues 23 and 120 of the protein. There are six serine residues in this region, located at positions 36, 63, 69, 89, 96, and 111. Presumably, one or more of these six serine residues are phosphorylated and this phosphorylation(s) causes the mobility shift.

To determine which serine residue(s) was involved in the mobility shift of E1A, expression vectors encoding E1A proteins with individual Ser-to-Gly mutations were constructed. The mutant E1A proteins were expressed in cultured mammalian cells and analyzed for the mobility shift by Western blot. Only the 289R and 243R E1A Ser-89-to-Gly mutants failed to undergo the characteristic mobility shift (Fig. 2A), whereas all the other mutants exhibited a WT mobility shift. A representative, Ser-96 to Gly, is shown for comparison in Fig. 2A. All the other Ser-to-Gly changes exhibited identical shifts (data not shown).

These results were confirmed by microinjecting the E1A expression vectors into *Xenopus* oocytes and analyzing the extracts by Western blot. Again, only the Ser-89-to-Gly

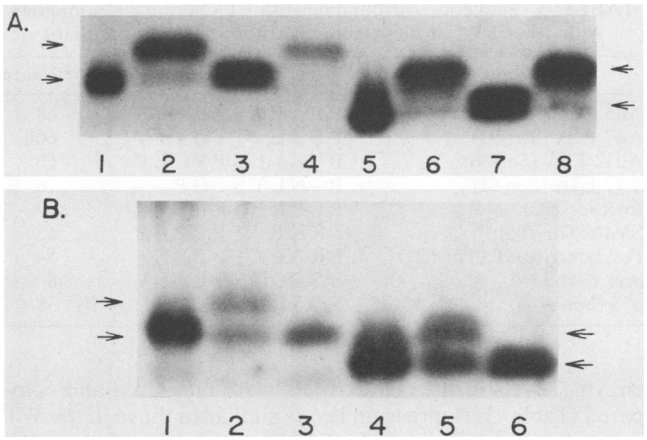

FIG. 2. Ser-89-to-Gly mutation prevents the mobility shift of E1A in HeLa cells and in *Xenopus* oocytes. (A) Vectors encoding WT or mutant E1A proteins were transfected into HeLa cells, and extracts were analyzed for mobility shift by Western blot. Lanes: 1 and 5, purified 289R or 243R E1A protein, respectively, produced in *E. coli*; 2, extract from HeLa cells transfected with pRE1A.13.WT; 3, pRE1A.13.89; 4, pRE1A.13.96; 6, pRE1A.12.WT; 7, pRE1A.12.89; 8, pRE1A.12.96. (Note that Ser-96-to-Gly 289R E1A was reproducibly detected at levels identical to that of the WT 289R E1A.) (B) Vectors encoding WT or mutant E1A proteins were microinjected into the nuclei of *Xenopus* oocytes, and extracts were analyzed for mobility shift by Western blot. Lanes: 1 and 4, purified 289R or 243R E1A protein, respectively, expressed in *E. coli*; 2, extract from *Xenopus* oocytes injected with pRE1A.13.WT; 3, pRE1A.13.89; 5, pRE1A.12.WT; 6, pRE1A.12.89.

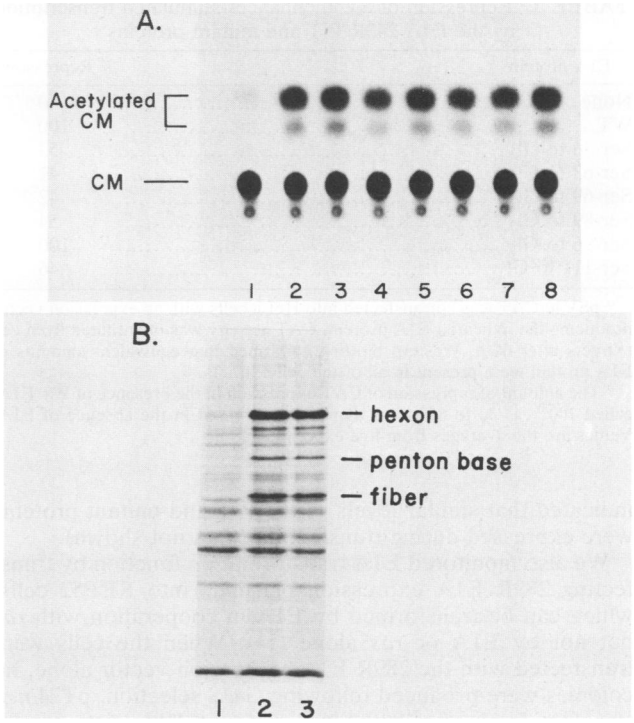

FIG. 3. E1A Ser-89-to-Gly mutant *trans* activates E3 and complements d1312 at WT level; five other serine mutants also *trans* activate E3 at WT level. (A) E1A WT and mutant expression vectors were transfected into 27-1 cells (containing an integrated E3-CAT gene cassette; provided by N. Jones). Cells were harvested at 42 h for assay of CAT activity in cell extracts. Acetylated and nonacetylated forms of [^{14}C]chloramphenicol (CM) are indicated. DNA used for transfections: lane 1, pUC18; lane 2, pRE1A.13.WT; mutant vectors: lane 3, pRE1A.13.36; lane 4, pRE1A.13.63; lane 5, pRE1A.13.69; lane 6, pRE1A.13.89; lane 7, pRE1A.13.96; lane 8, pRE1A.13.111. (B) E1A WT and mutant expression vectors were transfected into HeLa cells on day 1 and infected with approximately 20 PFU of d1312 per cell on day 2. DNA used for transfection: lane 1, pUC18; lane 2, pRE1A.13.WT; lane 3, pRE1A.13.89. The cells were incubated in the presence of [^{35}S]methionine for 2 h at 22 h postinfection, and the labeled proteins were visualized by SDS-PAGE and fluorography using Amplify. The positions of the major viral coat proteins, hexon, penton base, and fiber, are indicated. Corresponding bands cannot be seen in lane 1, where no E1A vector was used (only carrier DNA).

243R and 289R E1A proteins did not shift (Fig. 2B). A mobility shift identical to that of WT 289R and 243R E1A was observed for the other five mutants (data not shown). These results demonstrate that a Ser-to-Gly substitution at position 89 prevents the E1A mobility shift of both the 289R and 243R E1A proteins in two separate cell types known to produce functional E1A. This, together with our previous results, demonstrates that phosphorylation at serine 89 is directly responsible for the mobility shift of E1A.

Effect of Ser-89-to-Gly mutation on E1A *trans* activation. For analysis of the transcriptional activation function of the E1A Ser-89-to-Gly mutant, we transfected mammalian cell expression vectors in which the 289R WT or mutant protein is expressed under the control of the Rous sarcoma virus promoter region into 27-1 cells, HeLa derivatives containing the E1A-responsive adenovirus E3 promoter fused to the CAT gene (provided by N. Jones). Figure 3A shows that extracts from cells transfected with DNA encoding the E1A 289R WT and Ser-89-to-Gly proteins (lanes 2 and 6) contained 30 times more CAT activity than did extracts from cells containing no E1A protein (lane 1). These results were obtained in the linear range for detection of CAT activity. Western blotting indicated that similar levels of the WT and mutant proteins were expressed during the transfection (data not shown). Thus, the E1A Ser-89-to-Gly mutation had no effect on the efficiency of *trans* activation of the E3 promoter as measured in these assays.

To determine whether the Ser-89-to-Gly mutation affected overall viral growth, the E1A mutant was analyzed for complementation of the E1A-deficient adenovirus mutant d1312. This mutant is unable to replicate but can be complemented by supplying sufficient E1A to positively regulate early viral gene expression (26, 30, 43). As seen in Fig. 3B, lanes 2 and 3, plasmids expressing either the E1A WT or Ser-89-to-Gly mutant proteins efficiently complemented d1312, resulting in production of the late structural adenovirus proteins, i.e., hexon, penton base, and fiber.

Effect of Ser-89-to-Gly mutation on E1A repression and transformation. E1A has been reported to repress enhancer-stimulated transcription from the SV40 early promoter (6, 62). To determine the effect of the E1A Ser-89-to-Gly mutation on repression function, the E1A 243R WT and mutant expression vectors were cotransfected into HeLa cells with pSV40-CAT, which contains the SV40 early promoter and enhancer sequences directing CAT gene expression. The levels of CAT enzyme produced in the presence and absence of E1A protein were determined in the linear range of the CAT assay, and the results are summarized in Table 1. CAT activity was reduced approximately fivefold in the presence of WT E1A protein. In contrast, the Ser-89-to-Gly mutant only repressed SV40-CAT 2.5-fold, 50% of the repression activity of the WT. Western blotting

TABLE 1. Repression of SV40 enhancer-stimulated transcription by the E1A 243R WT and mutant proteins

E1A protein[a]	% Repression[b]
None	0
WT	100
Ser-36 to Gly	53
Ser-63 to Gly	41
Ser-69 to Gly	42
Ser-89 to Gly	54
Ser-96 to Gly	100
Ser-111 to Gly	46

[a] pSV40-CAT was cotransfected into HeLa cells with an expression vector producing the indicated E1A protein. CAT activity was quantitated from cell extracts after 66 h. Western blotting confirmed that equivalent amounts of E1A protein were present in all of the cell extracts.

[b] The amount of repression of CAT expression in the presence of WT E1A, called 100%, is 80 to 85% less than that expressed in the absence of E1A. Values are the averages from five experiments.

indicated that similar levels of the WT and mutant proteins were expressed during transfection (data not shown).

We also monitored E1A transformation function by transfecting 289R E1A expression plasmids into REF52 cells, which can be transformed by E1A in cooperation with *ras* but not by E1A or *ras* alone (14). When the cells were transfected with the 289R E1A expression vector alone, no colonies were produced following G418 selection. pT24/*neo* transfection alone resulted in very few G418-resistant colonies, none of which displayed a transformed morphology. We assume that E1A proteins are expressed at levels in REF52 cells which are similar to those in 27.1 cells and HeLa cells. The results of cotransfections of pT24/*neo* and each of the 289R E1A vectors are shown in Table 2. Cotransfection of pT24/*neo* with the Ser-89-to-Gly mutant DNA resulted repeatedly in 40% fewer transformed colonies than did cotransfection with WT E1A DNA. Thus, the Ser-89-to-Gly mutant can transform REF52 cells in cooperation with *ras*, but at significantly reduced efficiency compared with that of the WT E1A protein.

Effect of other Ser-to-Gly mutations on *trans* activation, repression, and transformation. We examined the effects of the five other Ser-to-Gly mutations at positions 36, 63, 69, 96, and 111 on the *trans*-activation function of E1A. Results of these experiments are shown in Fig. 3A. Although Ser-63 (lane 4) showed slightly less CAT activity in this transfection, all mutants reproducibly *trans* activated the E3-CAT gene at WT levels. If phosphorylation occurs on any of these serines, apparently neither the serine residue nor its phosphorylation is important for the *trans*-activation function.

The E1A Ser-to-Gly mutants were then examined for repression activity. Each E1A expression vector was cotransfected with pSV40-CAT into HeLa cells. The CAT enzyme levels in the cell extracts were measured and compared (Table 1). Repression levels similar to those in the WT were obtained only with the E1A Ser-96-to-Gly mutant. All four of the other mutants exhibited some reduction in repression activity (ranging from 50 to 60% reduction).

E1A proteins with Ser-to-Gly changes at positions 63, 69, or 96 were examined for transformation function. Plasmid DNAs were cotransfected into REF52 cells with pT24/*neo*, and the resulting morphologically transformed G418-resistant colonies were counted. As shown in Table 2, the Ser-63-to-Gly and Ser-69-to-Gly mutants transformed REF52 at significantly reduced efficiencies compared with that of the WT E1A protein (approximately 10% of WT). Surprisingly, the Ser-96-to-Gly mutant also exhibited a reduction in transformation efficiency (60% of WT), but this reduction was less severe than that observed with the Ser-63-to-Gly and Ser-69-to-Gly mutants. It is of interest that although this mutant gave a reproducibly measurable (about 40%) reduction in transformation efficiency (similar, in fact, to that of the Ser-89-to-Gly mutant), no corresponding effect on repression function could be detected.

TABLE 2. Transformation of REF52 cells by T24 *ras* and E1A 289R WT or mutant proteins

Protein(s) expressed	No. (%) of foci[a]
WT E1A	0
T24 *ras* + WT E1A	0
T24 *ras* + Ser-63 to Gly	7 (11)
T24 *ras* + Ser-69 to Gly	9 (14)
T24 *ras* + Ser-89 to Gly	40 (62)
T24 *ras* + Ser-96 to Gly	42 (64)

[a] Foci were defined as morphologically transformed G418-resistant colonies. Values are the averages from four experiments. Percentages are of foci produced by mutant E1A protein compared with that produced by WT E1A.

TABLE 3. Sequence surrounding Ad5 E1A serine 89: homology with other E1A proteins and with other oncogene proteins

Protein	Sequence	Reference
Ad5 E1A (Ser-89)	P P A P G S P E P P H	60
Ad7 E1A (Thr-91)	P E T L V T P G V V V	60
Ad12 E1A (Ser-86)	P V——L S P V C E P	60
SA7 E1A (Ser-83)	P—N L Y S P G P——	28
fos (Ser-252)	P E P K P S L E P V K	59
SV40 (Thr-706)	F K K P P T P P P E P	45
Polyomavirus T (Thr-691)	F K A P K T P P P K	54
myc (Ser-234)	S S P Q G S P E P L V	8
p53 (Ser-9)	P Q S D P S V E P P L	38

DISCUSSION

This study demonstrates that phosphatase treatment of E1A expressed in *E. coli* and modified in vitro or expressed and modified in vivo in yeast cells reverses the mobility shift of E1A while removing incorporated phosphate completely and specifically. We have also demonstrated that the phosphorylation of *E. coli*-produced E1A, which occurs in cell extracts, occurs on serine, as it does in E1A from adenovirus-infected cells (58). Replacement of serine with glycine at amino acid 89 prevented the mobility shift of E1A. This finding was identical for 289R and 243R E1A proteins in two different cell systems. Therefore, our results demonstrate that specific phosphorylation of serine residue 89 is responsible for the mobility shift of E1A.

Serine residue 89 is one of a number of residues which have been reported to be phosphorylated in E1A isolated from virus-infected cells (57). Quantitation of the event or its relationship to the gel mobility shift was not reported. Also, it has been recently reported that phosphorylation occurring somewhere between amino acid residues 86 and 120 (46) is responsible for the mobility shift. These results are consistent with our findings. However, the authors also suggested that there was another site between amino acid residues 224 and 289 which is phosphorylated and that phosphorylation at either site can independently induce the mobility shift. Our results are not consistent with this suggestion unless a lack of phosphorylation at serine residue 89 prevents phosphorylation at the putative second site. This has not been determined.

Serine residue 89 of human Ad5 E1A is not conserved among the E1A proteins from the human adenoviruses Ad7 and Ad12 or the simian adenovirus SA7 (Table 3; 60). E1A from Ad7 has a threonine at the corresponding position which potentially could be phosphorylated. However, only two of the six prolines surrounding this residue are conserved. Ad12 E1A has a serine which aligns with the Ad5 E1A serine 89, but this E1A does not exhibit the gel mobility shift (29). Thus, for Ad12 E1A, having a serine at position 89 is not sufficient to induce the mobility shift.

We propose that it is the unusual amino acid sequence and, in particular, the high proline content surrounding serine residue 89 that are likely to be responsible for the SDS-PAGE mobility shift of adenovirus E1A. Proline has been suggested to strongly influence the electrophoretic migration of several nuclear oncogene proteins, such as c-*myc* (64), *fos* (36), and p53 (38, 59), and may contribute to the anomalous apparent molecular weight on SDS-PAGE of modified (69) and unmodified (10) E1A. The phosphorylation of serine 89 may influence the SDS binding in this proline-rich region, and this may cause the mobility shift. Alternatively, the mobility shift may result from a conformational change induced in the protein by selective phosphorylation and subsequently detected by SDS-PAGE (46). In fact, these two alternatives are not mutually exclusive.

It is interesting that the nuclear oncogene products c-*myc* (8, 20, 44), *fos* (9, 59), SV40 T (45, 61), and p53 (38, 50) are also phosphorylated on serine and have proline-rich sequences surrounding specific serine residues similar to that found in E1A (Table 3). We predict that selective phosphorylation on the serines which adjoin the proline-rich sequences in these proteins results in the characteristic SDS-gel mobility shifts observed for these oncogene proteins.

We analyzed the effect of a Ser-89-to-Gly mutation on E1A biological function. Using expression vectors to introduce WT and mutant E1A proteins into mammalian cells, we could not detect any effect on the *trans* activation of the adenovirus E3 promoter or complementation of the E1A-deficient adenovirus d1312. In contrast, repression of the SV40 enhancer element and transformation of REF52 cells in cooperation with the *ras* oncogene protein are reduced 40 to 50% compared with the WT E1A repression and transformation functions (Tables 1 and 2). Therefore, a mutation of serine 89 preventing phosphorylation does alter the repression and transformation functions of the protein. However, other serine mutations in this region affect E1A function.

Serine 89 does not fall within the three strongly conserved domains previously noted among the adenovirus subgroups, but rather falls between conserved domains 1 and 2 (28, 60). Conserved domains 1 (amino acid residues 40 to 80) and 2 (residues 120 to 140) occur in both the 289R and 243R proteins and have been suggested on the basis of deletion and site-directed mutagenesis to be involved in the repression and transformation functions of E1A (33, 34, 40, 41, 53, 66, 70). In fact, it has been recently reported that deletion of amino acids 86 to 120 has no effect on transformation (46). However, other groups (53, 66, 70) have found that certain deletions or mutations in the region between domains 1 and 2 show small reductions in both repression and transformation efficiencies. Thus, our results are consistent with these findings, although we observed somewhat greater effects on repression and transformation efficiency than might be predicted from the deletion studies. Perhaps these discrepancies can be attributed to the differences in the test systems used to measure E1A activity or to the effects of point mutations as opposed to the deletion mutations used in the previous studies.

We also examined the effects on E1A function of mutations of the other serines between residues 23 and 120. Only the Ser-63-to-Gly and Ser-69-to-Gly mutations are changes located in a conserved domain thought to be important for repression and transformation functions (28, 60). The fact that these mutants resulted in a 60% reduction in repression efficiency and a 90% reduction in transformation capability (Tables 1 and 2) supports the proposed importance of this region.

Point mutations in the nonconserved regions of E1A, however, can also lead to significant changes in function. The Ser-111-to-Gly mutation, located between conserved domains 1 and 2, and the Ser-36-to-Gly mutation, located before domain 1, reproducibly reduced repression activity by 50%. Consistent with these results, Whyte et al. (66) have suggested that changes in certain amino acids outside domain 1 can reduce transformation capability.

Our data that the Ser-96-to-Gly mutant represses at WT levels but transforms at 40% reduced efficiency indicate that these functions may be separable, as has been suggested by Velcich and Ziff (63). We suggest that phosphorylation of serine residue 96, which has been observed by Tremblay et al. (57), may selectively modulate the repression function of E1A. Moreover, we suggest that the differential phosphorylation which is thought to be responsible for the numerous species of the 243R and 289R E1A proteins (21) may selectively modulate E1A function.

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