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Phosphorylation and Dephosphorylation Alter the Structure of D2 Hybrid T Antigen

ERNST A. BAUMANN† AND ROGER HAND*

McGill Cancer Centre and Department of Medicine, McGill University, Montreal, Quebec H3G 1Y6, Canada

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D2 hybrid T antigen is a protein closely related to simian virus 40 large T antigen and is synthesized in large quantities in cells infected with Ad2$^+$D2, an adenovirus-simian virus 40 hybrid. We have analyzed the effects of phosphorylation on the structure and DNA binding of this protein. On nondenaturing pore-gradient gels, the purified protein migrated with an apparent molecular weight of 135,000, with a minor band at 330,000 molecular weight. In vitro phosphorylation catalyzed by the protein kinase activity associated with the protein resulted in a structural change so that most of it migrated with an apparent molecular weight of 740,000. Treatment of the phosphorylated form of the protein with alkaline phosphatase (which removed 95% of the phosphate) caused the disappearance of the 740,000-molecular-weight form and reappearance of the smaller forms. Partial tryptic digestion showed that D2 T antigen has two major regions of phosphorylation, only one of which was phosphorylated in vitro. The region phosphorylated in vitro was responsible for the aggregation of D2 T antigen and was tentatively assigned to the N-terminal part of the protein. As shown by protein blotting onto nitrocellulose filters, it was mainly the form of 740,000 molecular weight that bound to simian virus 40 DNA. However, sucrose gradient analyses showed that only a fraction of the in vitro-phosphorylated protein bound to DNA, suggesting that aggregation alone is not sufficient for binding.

The early region of the simian virus 40 (SV40) genome specifies two proteins, large T antigen of 100,000 molecular weight (48) and small t antigen of 17,000 molecular weight (40). The genome region from map units 0.65 to 0.59 and 0.54 to 0.17 codes the amino acid sequence for large T (13, 41). This protein can stimulate or repress viral and cellular nucleic acid synthesis and is also responsible for cellular transformation (1, 19, 53). How these functions are regulated is not known. Among the possibilities, however, are that post-translational modifications of the protein cause changes in its higher order structure. Large T antigen is known to form aggregates (6, 36, 38). It undergoes several post-translational modifications: poly-ADP ribosylation (15), N-terminal acetylation (37), and phosphorylation (49). Of these, phosphorylation has attracted the greatest attention.

Large T antigen is a phosphoprotein in vivo with serine and threonine residues as phosphate acceptors (55). Tyrosine residues apparently are not phosphorylated (17, 44, 54). Two regions of the protein are phosphorylated, with a total of at least four phosphorylation sites (45, 54). An internal threonine within the last 11 amino acids at the C-terminal end is one site (44); a second is a serine residue also at the C-terminal end. The other two sites are at the N terminus of the molecule. The phosphorylated region at the N terminus extends beyond the region shared by large T antigen and small t antigen and into the region unique to large T antigen, perhaps as far as that section of the protein coded by map unit 0.44 of the SV40 genome (45, 54, 55).

Phosphorylation regulates the functions of many proteins (23). Transforming proteins from several tumor viruses are phosphoproteins (2, 7, 9, 43, 57), and there is some evidence that phosphorylation plays a role in the function of large T antigen. The turnover of phosphate on large T antigen is faster than the turnover of the protein (10), and forms of the protein with different isoelectric points have different amounts of phosphate (16).

As to how phosphorylation might alter the properties of large T antigen, there is at least preliminary evidence that it could be important in DNA binding (32). The protein binds specifically and tightly to three sites around the origin of the SV40 genome (47), and it also binds to cellular DNA though with lower affinity (35).

In the experiments presented here, we exam-

† Present address: Fachbereich Biologie, Universität Konstanz, D-7750 Konstanz, West Germany.

ined whether in vitro phosphorylation changed the aggregation and DNA-binding properties of large T antigen. We have used the D2 hybrid T antigen, a protein of 107,000 molecular weight, synthesized in cells infected with the adenovirus SV40 hybrid Ad2$^+$D2 (20). This is a fusion protein, with its N terminus (some 10% or less of its length) specified by adenovirus 2 and the remaining 90% specified by the inserted SV40 genome from map units 0.54 to 0.17. The protein differs from authentic large T antigen in that the adenovirus portion of the peptide chain replaces the amino acid sequence that authentic large T shares with small t antigen. D2 T antigen is readily purified in large amounts (50). It resembles authentic large T antigen in every way it has been tested: it is a phosphoprotein (3, 52), it binds specifically to three sites at the origin of the SV40 genome (50), it stimulates cellular DNA synthesis (51), it regulates the synthesis of SV40 early mRNA (42), it aggregates to form polymers (34), and most probably it has the same intrinsic enzyme activity, i.e., ATPase activity (14, 52). There is also a protein kinase activity associated with authentic and D2 T antigen (3, 18, 52). At least in the case of D2 T antigen, this activity appears not to be intrinsic to the protein. The present report shows that phosphorylation by this associated kinase activity causes the formation of aggregates of D2 hybrid T antigen, whereas dephosphorylation breaks down these aggregates, and that the phosphorylated form of the protein binds to SV40 DNA.

MATERIALS AND METHODS

Cells and viruses. HeLa cells and CV-1 monkey kidney cells were grown in plastic dishes in Dulbecco modified Eagle medium supplemented with calf serum (5%), gentamicin sulfate (50 μg/ml), and amphotericin B (0.5 μg/ml). Stocks of the adenovirus-SV40 hybrid Ad2$^+$D2 (obtained from John Hassell, McGill University, Montreal, Quebec, Canada) were prepared in CV-1 cells as described by Hassell et al. (20). SV40, strain 776, was grown on CV-1 cells as described by D'Alisa and Gershey (8).

Purification of D2 T antigen. Nuclear proteins were extracted from HeLa cells in suspension cultures approximately 50 h after infection with Ad2$^+$D2 as described by Baumann and Hand (3). D2 T antigen was then precipitated with ammonium sulfate (52) and further purified on Ultrogel AcA-34 and DEAE-Sephadex as described (3). The purified protein showed essentially one band after sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis.

Phosphorylation and dephosphorylation of D2 T antigen. Phosphorylation of D2 T antigen was carried out in vitro by using the protein kinase activity associated with that protein (3). From 1 to 20 μg of D2 T antigen was incubated in kinase buffer (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid, pH 7.5], 5 mM magnesium acetate, 2 mM dithiothreitol, 0.1 mM EDTA) in the presence of 0.01 to 0.1 mM [γ-^{32}P]ATP (1 to 20 Ci/mmol) or 2 mM unlabeled ATP for 30 min at 30°C. The incubation volume was 100 to 200 μl. Dephosphorylation was carried out with a high-specific-activity alkaline phosphatase from calf intestine immobilized on beaded agarose or with a soluble form of the same enzyme (Type VIIS; Sigma Chemical Co.) in phosphatase buffer (20 mM Tris-hydrochloride, pH 8.0, 5 mM $MgCl_2$, 0.01% bovine serum albumin). When the insoluble enzyme was used, a packed volume of 50 μl of beads containing 5 U of enzyme was washed twice with 1 ml of phosphatase buffer in 1.5-ml Eppendorff centrifuge tubes. The beads were then pelleted by centrifugation, and the wash buffer was discarded. D2 T antigen in phosphatase buffer was then added to a total volume of 200 to 400 μl. The tubes were kept at room temperature for 1 h on a rotating shaker. The beads then were centrifuged, and the supernatant was withdrawn for analysis by electrophoresis. For the control (no enzyme treatment), 50 μl (packed volume) of Sepharose 4B (Pharmacia) was treated identically and incubated with the same amount of D2 T antigen. The soluble enzyme was first dialyzed against phosphatase buffer, and 10 to 20 U was used in a total volume of 250 μl. The control was incubated under identical conditions without enzyme. Dephosphorylation with either enzyme resulted in removal of 95% or more of the phosphate incorporated in vitro.

SDS-polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis was carried out as described by Laemmli (25) on slab gels containing linear gradients of 5 to 15% acrylamide. Radioautography was performed on dried gels as previously described (3), using Kodak X-Omat AR film. In some experiments with [^{35}S]methionine, gels were treated before drying with En^{3}Hance (New England Nuclear Corp.) according to the instructions provided by the manufacturer. In other experiments, in which ^{32}P was used as a radioactive tracer, intensifying screens (Cronex; E. I. du Pont de Nemours & Co., Inc.) were used.

Pore-gradient (nondenaturing) gel electrophoresis. Pore-gradient electrophoresis gels were formulated according to the procedure described by Mechali et al. (31). The pH of the gel buffer was 9.6, and the acrylamide/bisacrylamide ratio was 37.5:1. The gels were formed from linear gradients of 3 to 25% acrylamide. Samples were mixed in a buffer containing 50 mM Tris-hydrochloride (pH 6.8), 5 mM KCl, and 30% glycerol and subjected to electrophoresis for 24 to 36 h at constant voltage (250 V) and at 4°C. The marker proteins were run in each gel, and their R_f's were used to estimate the molecular weights of the different forms of D2 T antigen.

Sucrose gradients. Samples of 0.2 ml were layered on top of 4.8-ml gradients of 5 to 20% sucrose in 10 mM Tris-hydrochloride (pH 8.0), 10 mM dithiothreitol, 5 mM NaCl, and 0.01% bovine serum albumin. Gradients were centrifuged in an SW50.1 or SW55 rotor (Beckman Instruments) at 45,000 rpm for 2 or 4 h at 4°C. Sedimentation markers were form I and form II SV40 DNA labeled with ^{3}H and the monomer (6S) form of ^{14}C-labeled phosphorylase *b*. Fractions were collected from the bottom of the centrifuge tubes directly into vials for scintillation counting and were counted after the addition of Aquasol. In the cases in which gradients contained D2 hybrid T antigen phos-

phorylated in the presence of [γ-^{32}P]ATP, fractions were precipitated by the addition of 1 volume of 20% trichloroacetic acid containing 10% sodium pyrophosphate. The samples were then filtered through Whatman GFC filters that had been soaked beforehand with 10% trichloroacetic acid containing 2% sodium pyrophosphate. Filters were washed extensively with 10% trichloroacetic acid containing 2% sodium pyrophosphate and were dried with ethanol and acetone. Radioactivity was then determined by liquid scintillation counting after the addition of 5 ml of Liquifluor.

Purification of SV40 DNA. CV-1 cells were infected with SV40 at 5 to 10 PFU/cell. At 24 h after infection, the cells were washed with phosphate-buffered saline and reincubated in medium containing 10 μCi of [^{3}H]thymidine per ml (50 Ci/mmol) for 16 to 24 h. After this time, SV40 DNA was extracted by the method described by Hirt (21) and purified by isopycnic centrifugation in ethidium bromide-cesium chloride gradients as described by Sebring et al. (46). Specific activity of this DNA was 2.2×10^5 cpm/μg of DNA.

In vitro labeling of SV40 DNA. SV40 DNA was labeled to high specific activity with [^{32}P]dCTP by nick translation under conditions described by Maniatis et al. (28, 29). Labeled DNA was purified on Sephadex G-50, and its integrity was determined as described by Botchan et al. (4). The specific activities obtained were 2×10^7 to 1×10^8 cpm/μg of DNA.

Partial tryptic digestion of D2 T antigen. HeLa cells in 75-cm^2 cell culture flasks were infected with Ad2$^+$D2 (10 PFU/cell). After 22 h, the cells were washed with phosphate-buffered saline and reincubated with 10 ml of methionine-free minimal essential medium containing 250 mCi of [^{35}S]methionine (100 Ci/mmol) and 5% dialyzed fetal calf serum, or washed with phosphate-free minimal essential medium and reincubated in 2 ml of that medium containing 300 mCi of ^{32}P. After a labeling period of 2 h, proteins were extracted from the cells as described by Schwyzer et al. (45). Portions of these cell extracts or D2 T antigen, purified and labeled in vitro with ^{32}P, were immunoprecipitated with hamster anti-T antiserum and protein A-Sepharose and were subjected to limited tryptic digestion essentially as described by Schwyzer et al. (45). Digestion was carried out in conical 1.5-ml centrifuge tubes containing 0.25 μg of trypsin (Type XI; Sigma Chemical Co.) added to D2 hybrid T antigen that was bound to antibody and to protein A-Sepharose beads suspended in 100 μl of extraction buffer. After the reaction was stopped by the addition of Trasylol, the beads were pelleted by centrifugation, and the supernatant was mixed with 1 volume of twice-concentrated sample buffer for electrophoresis (25). Proteins bound to protein A-Sepharose were eluted with sample buffer containing SDS. Those proteins in the supernatant and those pelleted with the protein A-Sepharose were analyzed separately on SDS-polyacrylamide gels as described above.

Transfer of proteins to nitrocellulose and DNA binding assay. D2 T antigen was subjected to electrophoresis on nondenaturing pore gradient polyacrylamide gels as described above. The gels were then sandwiched between two sheets of nitrocellulose onto which proteins were transferred by diffusion as described by Bowen et al. (5). One filter was stained with amido black 10B, and the other was used for a DNA-binding assay (5). The filter was first soaked in binding buffer for 30 min. It was then incubated with 10 ml of binding buffer containing ^{32}P-labeled SV40 DNA (2×10^6 cpm, 3×10^7 cpm/μg) for 1 h at room temperature. The filter was washed in several changes of washing buffer for 1 h. The dried filter was subjected to radioautography.

RESULTS

Characteristics of the phosphorylation reaction. Under conditions chosen for the in vitro phosphorylation reaction in which we used the kinase activity associated with the purified D2 T antigen as the catalyst, the transfer of phosphate from ATP to the purified D2 T antigen was rapid for 15 min and reached a plateau by 30 min. These kinetics were similar at concentrations of ATP between 0.01 and 2 mM. The transferred phosphate remained attached to the protein. Addition of unlabeled ATP to the phosphorylation reaction 30 min after the addition of [γ-^{32}P]ATP did not reduce the amount of radioactive phosphate on the protein for the next 30 min, and it caused only about a 40% decrease over 90 min (Fig. 1). This showed that the phosphate turnover is very slow and in fact does not take place for 30 min after the addition of unlabeled ATP.

Phosphorylation and dephosphorylation change the structure of D2 T antigen. When purified D2 T hybrid antigen that had been phosphorylated in vitro with ATP as the phosphate donor was subjected to electrophoresis on nondenaturing pore-gradient gels, it ran predominantly as a single band with an apparent molecular weight of 740,000 (Fig. 2a). Faint Coomassie blue-stained bands were also seen at the positions expected for proteins of about 330,000 and 135,000 molecular weight. If the protein was not treated with ATP, the predominant band migrated as 135,000 molecular weight, with a fainter band at 330,000 molecular weight as well. The

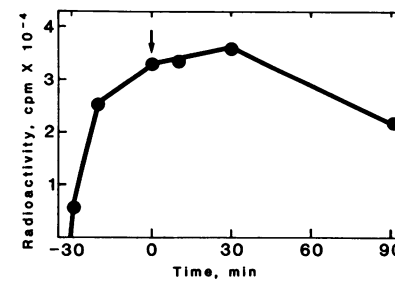

FIG. 1. Stability of phosphate incorporated into D2 hybrid T antigen. Purified D2 T antigen (8 μg) was phosphorylated in vitro with [γ-^{32}p]ATP (0.01 mM, 20 Ci/mmol). After 30 min (at time zero; arrow), nonradioactive ATP was added to a concentration of 5 mM (500-fold excess). At the times indicated on the abscissa, samples were taken to measure acid-insoluble radioactivity.

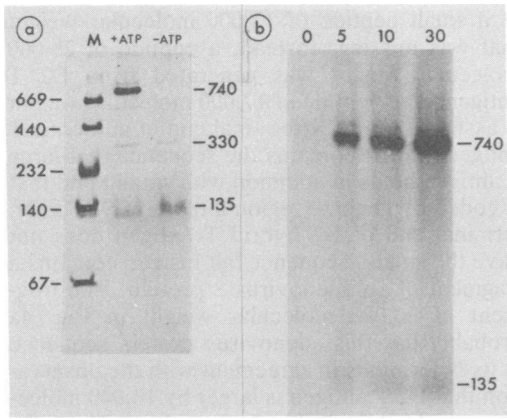

FIG. 2. Analysis of phosphorylated D2 hybrid T antigen by pore-gradient (nondenaturing) gel electrophoresis. (a) Purified D2 hybrid T antigen (10 μg) was subjected to in vitro phosphorylation conditions in the presence or absence of ATP (2 mM) and analyzed by electrophoresis on nondenaturing polyacrylamide gels. The gel was stained with Coomassie blue. The left-hand lane (M) shows the migration of marker proteins of known molecular weight: thyroglobulin, 660,000; ferritin, 440,000; catalase, 232,000; lactate dehydrogenase, 140,000; and bovine serum albumin, 67,000. The numbers on the right are the size estimates (based on migration of the marker proteins) of the three bands in the lanes containing D2 hybrid T antigen. (b) Radioautography of ^{32}P-labeled D2 hybrid T antigen. Purified D2 T antigen (4 μg) was phosphorylated in vitro with [γ-^{32}P]ATP (0.1 mM, 2 Ci/mmol) for the number of minutes indicated above each lane. The samples were then subjected to electrophoresis on nondenaturing polyacrylamide pore-gradient gels, and the radioactive protein bands were made visible by radioautography.

band of 740,000 molecular weight was not present. In repeated experiments, a very faint band could sometimes be seen at 740,000 molecular weight. AMP would not substitute for ATP; with AMP as the potential phosphate donor, there was no conversion to the form at 740,000 molecular weight (data not shown). Figure 2b, which is a radioautogram of D2 hybrid T antigen labeled in vitro with [γ-^{32}P]ATP for various periods of time, shows that the 740,000-molecular-weight form is the one that contained the radioactive phosphate.

In these nondenaturing pore-gradient gels, proteins are separated primarily according to their molecular radius (30). Therefore, a linear relationship between the logarithm of the molecular size and R_f holds only if the test protein (D2 hybrid T antigen) is globular and hydrated to approximately the same extent as the marker proteins. Since this assumption might not hold, the molecular size assignments to the three forms in Fig. 2 are tentative and are given only for convenience in identifying the bands.

When the phosphorylated protein was dephosphorylated with alkaline phosphatase, its migration on the gel changed (Fig. 3). The phosphorylated D2 T antigen showed a narrow dark band at 740,000 molecular weight and a broad band at about 135,000 molecular weight. Dephosphorylation of the same protein preparation changed its structure so that none of it migrated as the 740,000-molecular-weight form. Instead, there was a broad smear of stained material at about the position expected for the 135,000-molecular-weight form. The smear was not due to proteolysis, since the same two preparations of D2 T antigen analyzed on SDS gels each showed one band at a molecular weight of approximately 100,000 and were indistinguishable from each other (data not shown). Some of the broadness of the stained bands was probably due to a technical artifact, but it may have resulted in part from heterogeneity of structure induced by the alkaline phosphatase treatment. Despite this, the gel shows clearly that the phosphorylated form of D2 hybrid T antigen ran predominantly as a form of 740,000 molecular

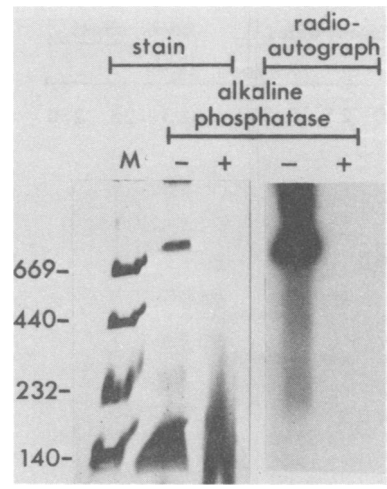

FIG. 3. Analysis of dephosphorylated D2 hybrid T antigen by pore-gradient (nondenaturing) gel electrophoresis. Purified D2 T antigen (40 μg) was phosphorylated with [γ-^{32}P]ATP (0.1 mM, 2 Ci/mmol). It was then passed through a column of Sephadex G-25 equilibrated with phosphatase buffer to remove the unreacted ATP. One half of the sample was incubated with Sepharose 4B containing no alkaline phosphatase, and the other half of the sample was incubated with agarose beads containing 5 U of alkaline phosphatase. The left-hand lane displays the same molecular weight markers as described in Fig. 2. On the left, the protein bands are stained with Coomassie blue. On the right is a radioautogram of the two lanes containing D2 hybrid T antigen.

weight, and that dephosphorylated D2 T antigen broke down to forms that migrated similarly to the form of 135,000 molecular weight. The radioautogram on the right side of Fig. 3 shows that it was only the 740,000-molecular-weight form that contained phosphate and that alkaline phosphatase completely removed the radioactive phosphate. The same result was obtained when a soluble alkaline phosphatase was used (data not shown). However, interpretation of the results on the Coomassie blue-stained gels was complicated by the appearance of the enzyme band at about the same place as D2 T antigen. (The molecular weight of alkaline phosphatase is about 100,000.)

Which site on D2 hybrid T antigen is phosphorylated by the associated kinase activity? Since T antigen is phosphorylated on several sites, it is possible that only one site or set of sites is responsible for the observed aggregation of D2 T antigen. We therefore examined whether the associated protein kinase activity phosphorylated all or only certain sites on D2 T antigen. The protein was partially digested with trypsin. The pattern of [^{35}S]methionine-containing peptides (Fig. 4a) was almost identical to that obtained with authentic T antigen (45), with the exception of a small peptide of 17,000 molecular weight that was missing. Instead, a peptide of 26,000 molecular weight was generated from D2 T antigen. The peptide of 17,000 molecular weight is assigned to the N-terminal end of authentic T antigen (45). It contains the sequence that large T antigen holds in common with small t and that is coded by genome region 0.65 to 0.59. The N-terminal end of D2 hybrid T antigen does not have the small t sequence but instead contains a fragment of an adenovirus 2 protein. The fragment of 26,000 molecular weight in Fig. 4a probably has this adenovirus protein sequence at its N terminus in agreement with the observation that D2 T antigen is larger by 10,000 molecular weight than authentic T antigen. Since the N-terminal fragment of 17,000 molecular weight from the authentic T antigen of Schwyzer et al. (45) is phosphorylated in the region unique to large T antigen, we assume that the fragment of 26,000 molecular weight that we found is also phosphorylated on the SV40 portion of D2 T antigen rather than on the adenovirus portion of the fragment. From the close agreement of the sizes of all the other fragments with those of authentic T antigen, it can be assumed that they map in the same regions; that is, the fragments

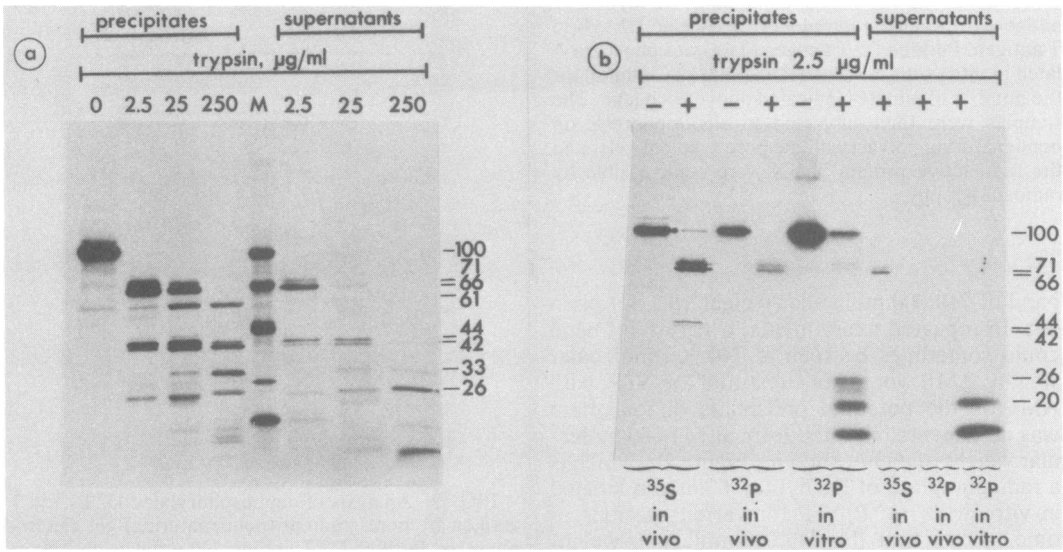

FIG. 4. Tryptic digestion of D2 hybrid T antigen. (a) [^{35}S]methionine pattern at different trypsin concentrations. An extract from Ad2$^+$D2-infected cells that had been labeled with [^{35}S]methionine was immunoprecipitated with hamster anti-T antiserum. The precipitates were bound to protein A-Sepharose and partially digested with trypsin at the concentrations indicated. The protein A-Sepharose beads were then pelleted by centrifugation. The supernatants were removed, and the proteins were eluted from the beads with SDS. The proteins eluted from the beads and those in the supernatants were analyzed by electrophoresis on SDS-polyacrylamide gradient gels and radioautography. The lane marked M contains ^{14}C-labeled molecular weight marker proteins. (b) Tryptic peptide patterns of D2 T antigen phosphorylated in vivo and in vitro. Extracts from cells infected with Ad2$^+$D2 that had been labeled in vivo with [^{35}S]methionine or $^{32}P_i$ and purified D2 T that has been labeled in vitro with [γ-^{32}P]ATP were subjected to immunoprecipitation and partial tryptic digestion with 2.5 μg of trypsin per ml. The tryptic peptides retained on the protein A-Sepharose matrix, and those released into the supernatants were then analyzed by SDS-gel electrophoresis and fluorography.

of 71,000, 66,000, 44,000, and 42,000 molecular weight all have a common N terminus at map position 0.51 and different C termini mapping between 0.17 and 0.32 map units (45).

We compared the tryptic peptide maps of D2 hybrid T antigen phosphorylated in vivo and in vitro (Fig. 4b). The in vivo phosphorylation pattern was again very similar to that of authentic T antigen (45) in that all the fragments were phosphorylated except those of 44,000 and 42,000 molecular weight, which map in the middle of the protein. In contrast to authentic T antigen, however, in vivo phosphorylation of D2 T antigen resulted in incorporation of phosphate predominantly into the two fragments of 71,000 and 66,000 molecular weight that contain the C-terminal end of the protein. In vitro phosphorylation resulted in the bulk of the phosphate being attached to four smaller fragments of 14,000 to 26,000 molecular weight. Those less than 26,000 molecular weight are most likely breakdown products of the fragment of 26,000 molecular weight since they seemed to be preferentially released into the supernatant upon digestion. This is to be expected if the fragment of 26,000 molecular weight contains part of an adenovirus protein against which there are no antibodies in the hamster tumor serum used for immunoprecipitation. These results confirm that there are at least two major regions for phosphorylation of large T antigen. In the case of D2 hybrid T antigen, one region at the N-terminal end can be phosphorylated in vitro by the associated protein kinase, whereas the other region at the C-terminal end can only be phosphorylated efficiently in vivo.

DNA binding of D2 hybrid T antigen after phosphorylation. To study the interaction of in vitro-phosphorylated D2 T antigen with DNA, protein-DNA complexes were analyzed on sucrose gradients. Phosphorylated D2 T antigen sedimented predominantly at 12S with a second peak at 5 to 6S (Fig. 5a). The 12S form probably corresponds to the 14 to 16S form and the 5 to 6S form corresponds to the 5 to 7S form of authentic T antigen described by others (11, 39). Purified D2 T antigen that had not been subjected to in vitro phosphorylation sedimented as two peaks of 12S and 6S with a shoulder on the smaller peak at about 8S (D. Stedman, E. A. Baumann, and R. Hand, unpublished data). When the phosphorylated D2 T antigen was reacted with SV40 DNA at a ratio of DNA to protein of 0.8:1 (wt/wt; origin/D2 tetramer ratio of 1:16; 33), only a small fraction of the protein (about 10% of the total counts on the gradient) was shifted to an S value greater than 12 (Fig. 5a). This fraction is presumably bound to DNA since it cosedimented with a peak of SV40 DNA that has an S value of 24, somewhat larger than the 21S found for SV40 form I DNA and compatible with SV40 DNA bound to D2 T antigen. If DNA was added in excess (DNA/protein ratio of 10:1, wt/wt; origin/D2 tetramer ratio, 1:1.25; Fig. 5b), the amount of phosphorylated D2 protein sedimenting with S values greater than 12 and cosedimenting with DNA increased to 27%. In this case, however, the S value of the DNA was not changed measurably, indicating that less protein per DNA molecule had bound. A change in the S value of DNA from 21 to 24 was observed only when the DNA/protein ratio was less than 2:1 (wt/wt; origin/D2 tetramer ratio, 1:6). A plot of the DNA/protein ratios versus the percentage of labeled D2 T antigen cosedimenting with DNA indicated that the percentage bound leveled off at ratios above 2:1 (wt/wt). This shows that the ratio used in the experiment in Fig. 5b was one in which DNA was in excess.

To investigate further the role of phosphorylation in DNA binding, we attempted to bind DNA to D2 T antigen that had been transferred to nitrocellulose after nondenaturing gel electrophoresis. For this we used the protein blotting technique described by Bowen et al. (5). Figure 6a illustrates that mock-phosphorylated D2 T antigen and phosphorylated D2 T antigen had migration patterns similar to those obtained in Fig. 2. These proteins were transferred to two sheets of nitrocellulose filter paper. One filter was stained with amido black (Fig. 6b); the other was incubated with ^{32}P-labeled SV40 DNA, and the proteins that bound DNA were made visible by radioautography (Fig. 6c). Surprisingly, it was predominantly the form at 740,000 molecular weight that exhibited DNA binding. With very prolonged exposure of the radioautogram we detected some binding of the form at 135,000 molecular weight (not visible in this figure). Though there was at least 10 times more protein in the form at 740,000 molecular weight in the phosphorylated preparation than in the untreated preparation of D2 T antigen (Fig. 6a), about the same amount of DNA was bound in each case. Since DNA might not have been in excess, this assay is not strictly quantitative. Yet, if there were a difference in DNA binding activity of a factor of 10 this should have been detectable. These results indicate that phosphorylation is not the only factor required for the protein to bind to DNA.

DISCUSSION

The results presented here show that phosphorylation of D2 hybrid T antigen is associated with a change in aggregation. The phosphorylated form sediments at 12S in sucrose gradients and migrates on nondenaturing gradient gels at 740,000 molecular weight. It almost certainly

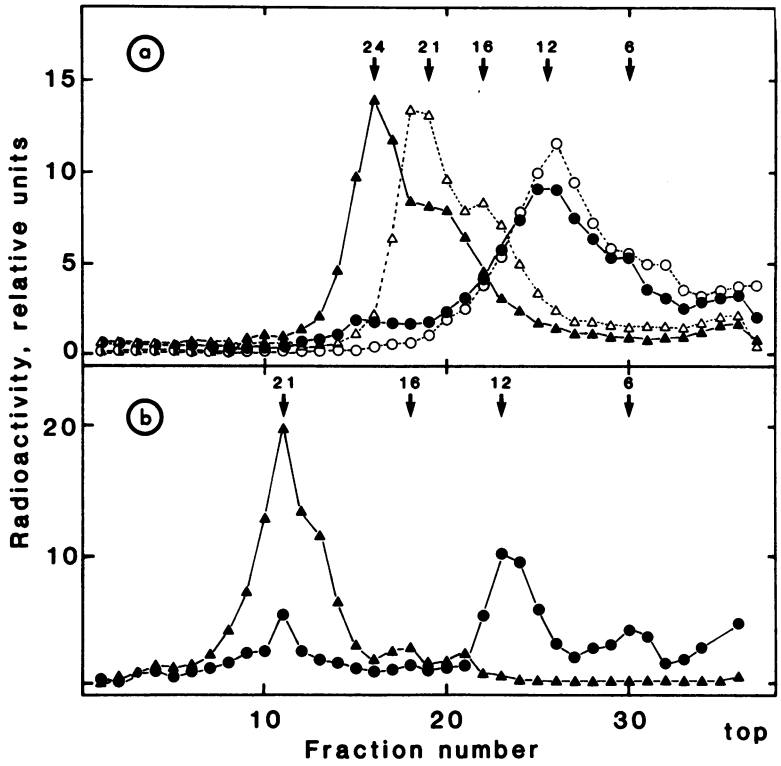

FIG. 5. Sucrose gradient analysis of in vitro-phosphorylated D2 T antigen bound to SV40 DNA. Purified D2 T antigen (4 μg) was phosphorylated in vitro with [γ-^{32}P]ATP (0.1 mM, 10 Ci/mmol). A portion of the reaction mixture was placed directly on a sucrose gradient; another portion was mixed with ^{3}H-labeled SV40 DNA, allowed to bind to the DNA under the conditions described by Tjian (50), and then placed on a sucrose gradient. ^{3}H-labeled SV40 DNA alone was placed on a third gradient. All gradients were centrifuged and analyzed as described in the text. Symbols: ●, ^{32}P radioactivity profile of in vitro-phosphorylated D2 T antigen reacted with SV40 DNA in the binding assay; ○, ^{32}P radioactivity profile of in vitro-phosphorylated D2 T antigen that was not reacted with SV40 DNA; ▲, ^{3}H radioactivity profile of SV40 DNA reacted with phosphorylated D2 T antigen; △, ^{3}H radioactivity profile of SV40 DNA that was not reacted with D2 T antigen. (a) The DNA/protein ratio (wt/wt) in the binding assay was 0.8:1, and centrifugation was in an SW50.1 rotor for 2 h at 45,000 rpm. The profiles indicated by solid circles and solid triangles are from the same gradient. The profiles indicated by open circles and open triangles are from two parallel gradients. (b) The DNA/protein ratio (wt/wt) in the binding assay was 10:1, and centrifugation was in an SW55 rotor for 4 h at 45,000 rpm. The profiles of the gradients containing DNA alone and protein alone are omitted for clarity. Relative units of radioactivity are the percentage of total counts in each fraction of the gradient. The symbols in (b) are the same as in (a). The arrows indicate S values: 21S and 16S are form I and form II SV40 DNA; 6S is ^{14}C-labeled phosphorylase *b* run in a parallel gradient. The positions of the arrows at 24S and 12S were calculated from the positions of the three markers.

represents an aggregated form of D2 T antigen, perhaps a tetramer (34). Dephosphorylation causes the molecule to disaggregate to a smaller form. This form is likely to be the monomer polypeptide chain. In vivo studies indicate that there is a 14 to 16S form of authentic T antigen that is more phosphorylated than the 5 to 6S form (11, 17). These sedimentation values agree reasonably well with the 12S we observed for the phosphorylated form of 740,000 molecular weight and the 6S which we guess represents the form of 135,000 molecular weight.

The molecular weight of the monomer protein as estimated on pore-gradient nondenaturing polyacrylamide gels, 135,000, differs from the estimate of 107,000 to 115,000 usually obtained from SDS-polyacrylamide gels (20, 50). The estimate of 135,000 molecular weight is based on the assumption that the protein is globular and hydrated to the same extent as the marker proteins. If it is not, then it might be retarded on a nondenaturing gel by virtue of its asymmetric shape or a high level of solvation. Our best estimate for the molecular weight of the D2 T antigen is 90,000. This is based on the predicted amino acid sequence coded by the SV40 genome

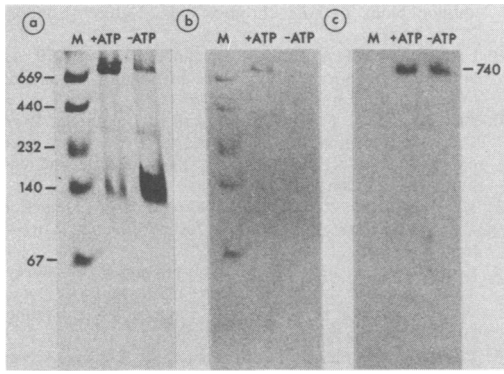

FIG. 6. DNA binding of D2 hybrid T antigen transferred to nitrocellulose. (a) D2 T antigen (20 μg) was subjected to in vitro phosphorylation conditions in the absence or presence of ATP (2 mM) and analyzed by electrophoresis on a pore-gradient (nondenaturing) polyacrylamide gel. The gel was stained with Coomassie blue after the transfer procedure described below. (b) The proteins were transferred by diffusion onto a nitrocellulose filter, and the filter was stained with amido black 10B. (c) Another filter to which proteins were transferred was incubated with ^{32}P-labeled SV40 DNA. The bound DNA was made visible by radioautography. In all three panels, the left lane shows marker proteins, the middle lane shows phosphorylated D2 T antigen, and the right lane shows mock-phosphorylated D2 T antigen.

from map units 0.54 to 0.17, plus our observation that the N-terminal tryptic peptide of D2 T antigen is larger by 9,000 molecular weight than the N-terminal peptide of authentic large T found by Schwyzer et al. (45).

The estimate of 740,000 molecular weight for the larger form is considerably greater than the value expected if the complex consisted of four monomeric subunits. At least in the electron microscope, the complexed form seems to appear as a globular structure (34). It would seem, therefore, that a high level of hydration is responsible for the aberrant migration. Alternatively, the complexed form might not be a tetramer, but rather a hexamer or even an octamer.

Genetic studies with *tsA* mutants mapping in the region between 0.35 and 0.45 map units show that T antigen produced in cells infected with these mutant viruses is partially temperature sensitive for phosphorylation (10, 17, 55). The site at the C-terminal end is not the one that is inactivated by heat in *tsA* mutants (55). Thus, the mutant protein probably cannot be phosphorylated at the sites coded by the genome around map position 0.45. Furthermore, the mutant protein is unable to aggregate at the restrictive temperature (11, 17), suggesting that the amino acid sequence in this region may be required for aggregation. Our results demonstrate directly that phosphorylation of sites in the N-terminal part of the D2 T antigen results in aggregation of the protein. The functional consequences of this aggregation are still unknown. In vivo studies suggest that the maximally phosphorylated and aggregated T antigen binds to the 48,000-molecular-weight nonviral tumor antigen (17). Whether there is a causal relationship between aggregation of T antigen and binding to the host protein, however, remains to be determined. Furthermore, a mutant of the SV40-adenovirus 7 hybrid PARA induces the synthesis of a SV40 T antigen that is not transported to the nucleus (26). The average phosphate content of this mutant protein is twofold lower than that of the T antigen induced by the wild-type virus, PARA (27). It is, therefore, conceivable that modifications such as phosphorylation and aggregation are essential for the transport of the protein into the nucleus.

The DNA-binding properties of many proteins are modulated by phosphorylation and dephosphorylation (12, 22, 24, 56). From earlier studies (39), it seemed that only the monomeric form of T antigen was able to bind to DNA. It was, therefore, surprising to find that D2 T antigen bound to DNA predominantly in the multimeric form. This can be explained if aggregation and DNA binding are independent events. Our observation that phosphorylation in vitro increased the proportion of the aggregated protein without increasing the relative amount of DNA bound suggests that the sites phosphorylated in vitro affect only the state of aggregation without changing the DNA-binding activity of D2 T antigen. Further support for this idea comes from the sucrose gradient experiments where, even in the presence of a large excess of DNA, most of the phosphorylated and aggregated D2 T antigen sedimented unbound to DNA.

Once it is bound to DNA, the T antigen probably maintains its multimeric form, probably as a tetramer (33). This would be compatible with the 24S value for a complex consisting of one molecule of form I SV40 DNA and three tetramers of D2 T antigen, as was observed at low origin/D2 tetramer ratios. These ratios correspond to those that have been shown to result in protection of all three binding sites against methylation (33). Complexes containing only one tetrameric D2 T antigen would be expected to have an S value of about 22. This is close to the value we observed at high origin/D2 tetramer ratios.

From our experiments it is still not clear how the binding of T antigen to DNA is regulated. The results indicate, however, that the difference between the binding and the nonbinding protein is not exclusively a difference in the state of aggregation. There is evidence that aged T

antigen, that is, T antigen that has been synthesized long before its isolation, binds less efficiently to DNA (35). This might be the result of a post-translational modification. We cannot exclude the possibility that phosphorylation of sites not affected by the in vitro phosphorylation does change the DNA binding properties of D2 T antigen, as suggested in a recent report (32), especially since our experiments showed a difference between the in vitro and in vivo phosphorylation patterns of the protein. Also, the changes in aggregation that we observed may not be related to those that occur in vivo. To explain exactly the relations among phosphorylation, binding, and aggregation, we will need in vivo and in vitro studies of the type presented here performed on large T antigen from several early region mutants.

Finally, we should emphasize that our studies were performed in vitro. Unequivocal demonstration that phosphorylation-dephosphorylation plays a significant role in regulating any activity of large T antigen will require in vivo studies that correlate the states of phosphorylation with functional changes in the protein and with the activities of specific kinases and phosphatases (23).

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