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Phosphorylation of Simian Virus 40 Proteins in a Cell-Free System

K. B. TAN AND F. SOKOL
The Wistar Institute of Anatomy and Biology, Philadelphia, Pennsylvania 19104

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We have shown previously that all the structural proteins of simian virus 40 (SV40) are phosphoproteins. Virus phosphorylated in vivo could be further phosphorylated with exogenous cellular protein kinases in a cell-free system containing γ-^{32}P-ATP as phosphate donor. In intact infectious virus only polypeptides 1 and 2 (mol wt 49,000 and 40,800, respectively) were further phosphorylated in vitro. However, when infectious SV40 was partially disrupted, treated with nucleases, and then phosphorylated in vitro, all five structural polypeptides accepted additional phosphate groups. Similarly, all polypeptides of intact empty capsids, derived from infected cells, were further phosphorylated in vitro. Phosphorylation of empty capsids and infectious SV40 in vitro was enhanced from 4- to 11-fold after prior treatment of virus with alkali. The phosphate group was linked only to serine residues of the viral polypeptides phosphorylated both in vitro and in vivo.

Protein phosphorylation is involved not only in the regulation of cellular genetic and metabolic functions (5, 6), but also may play an important role in the regulation of viral functions in the infected cell. Viral protein phosphorylation can be accomplished by a protein kinase associated with enveloped viruses (3, 8, 10-12), by a protein kinase in the infected cell, or by both virion-bound and cellular enzymes. We have shown recently that all the structural polypeptides of simian virus 40 (SV40) are phosphorylated in vivo and that SV40 does not contain a virion-associated protein kinase (14). The extent of phosphorylation in vivo of SV40 proteins, estimated from the ratio of ^{3}H-amino acid to ^{32}P-phosphate radioactivity in the phosphorylated polypeptides, was relatively low in comparison with that of the phosphoproteins of rhabdoviruses labeled under similar conditions (11, 14). This suggested that either SV40 proteins contain less phosphate acceptor sites per polypeptide than rhabdovirus phosphoproteins or that not all the phosphate-acceptor sites of SV40 proteins are phosphorylated. The pattern of phosphorylation of SV40 proteins by an exogenous cellular protein kinase in a cell-free system, described in the present study, is compared with that obtained in vivo, and it shows that all the polypeptides are not fully phosphorylated.

MATERIALS AND METHODS

Propagation, labeling, and purification of SV40. The growth of SV40 (strain RH 911) in secondary cultures of African green monkey kidney (AGMK) cells, labeling of virus with both ^{3}H-amino acids and ^{32}P-orthophosphate, and purification of infectious virus and of empty capsids were as described previously (14). Infectious virus and empty capsids used in this study had buoyant densities in CsCl solution of 1.35 and 1.30 g/cm^{3}, respectively.

Protein kinases. Beef heart protein kinase, prepared by the method of Gilman (2), was obtained from Sigma Chemical Co. (St. Louis, Mo.). Rabbit reticulocyte protein kinase II was prepared as described by Tao et al. (15). A protein kinase from uninfected AGMK cells was prepared as follows. Cells from confluent secondary monolayers were suspended in NT buffer (0.13 M NaCl, 0.05 M Tris-hydrochloride, pH 7.9), disrupted by ultrasonic vibration, and centrifuged at 100,000 $\times$ *g* for 90 min. The soluble proteins from the supernatant fluid were precipitated with ammonium sulfate (0.32 g/ml) and the precipitate was collected, dissolved in 1 mM dithiothreitol, 10% glycerol, 0.05 M Tris-hydrochloride (pH 7.5), and dialyzed overnight against the same buffer. The sample was then adsorbed onto DEAE-cellulose and eluted with a 0.05 to 0.50 M KCl gradient. Most of the protein kinase activity was eluted with 0.16 to 0.2 M KCl. The enzyme was purified 6.3-fold, as judged from its specific activity in phosphorylating histones as substrate, and was used without further purification. Details of the purification and characterization of the AGMK kinase will be described elsewhere (K.

B. Tan and F. Sokol, manuscript in preparation).

Phosphorylation in vitro of SV40. The standard reaction mixture, similar to that in which AGMK protein kinase exhibited maximum activity (K. B. Tan and F. Sokol, manuscript in preparation), contained in 200 μliters: 10 μmol of Tris-hydrochloride (pH 8.0), 2 μmol of $MgCl_2$, 2 μmol of dithiothreitol, 4 nmol of ATP, and indicated amounts of γ-^{32}P-ATP, beef heart kinase, and SV40. After incubating at 37 C for 30 min, the sample was made 0.06 M in NaPPi and 1% in Nonident P 40 (Shell Chemical, Chicago, Ill.), layered over 1.5 ml of NT buffer saturated with KBr at 16 C (14), and centrifuged in a Spinco SW50L rotor (24,000 rpm, 3 h, 16 C). The banding of virus in KBr was necessary to separate the virus from beef heart kinase.

Polyacrylamide gel electrophoresis. The virus band from the steep KBr gradient was collected (13) and precipitated at 0 C with 10% trichloroacetic acid. The precipitate was collected by centrifugation, dissolved in 2 ml of 0.05 M Tris-hydrochloride, pH 8.0, and reprecipitated with 10% trichloroacetic acid. The final trichloroacetic acid precipitate was processed for electrophoresis in 9% polyacrylamide gel containing sodium dodecyl sulfate (SDS) and urea as described previously (14).

Acid hydrolysis of phosphorylated SV40. After phosphorylation in vitro of either empty capsids or infectious virus by beef heart kinase, the virus particles were banded in KBr and precipitated with 10% trichloroacetic acid. SV40 phosphorylated in vivo was also precipitated with trichloroacetic acid. The precipitate was resuspended in 0.3 ml of 6 N HCl and hydrolyzed in a sealed evacuated ampoule at 110 C for 14 h. HCl was removed by drying in vacuo, followed by three extractions of the residue with water and subsequent drying. The final residue was dissolved in water and mixed with 30 μg each of nonradioactive phosphoserine and phosphothreonine (Sigma Chemical Co., St. Louis, Mo.). Electrophoresis in horizontal strips of 1- by 11-inch (2.54- by 27.94-cm) Whatman no. 4 filter paper was carried out in formic acid (2.5%)-acetic acid (7.8%) buffer (pH 1.85) at 4 C and 500 V for 7 h (7, 16). After electrophoresis the paper strips were stained with ninhydrin, scanned for absorbance, and cut into 3-mm strips. Radioactivity contained in the strips was then determined (14).

Materials. Bovine spleen phosphodiesterase, bovine pancreas deoxyribonuclease I, phosvitin, calf thymus histone type 2 A, and salmon sperm protamine (free base) were purchased from Sigma Chemical Co. (St. Louis, Mo.). Purified *Escherichia coli* alkaline phosphatase was from Worthington Biochemical Corp. (Freehold, N.J.). γ-^{32}P-ATP (sp act 15–20 Ci/mmol) was from New England Nuclear Corp. (Boston, Mass.). ^{3}H-arginine (sp act 7 Ci/mmol), ^{3}H-lysine (50 Ci/mmol), ^{3}H-serine (2.2 Ci/mmol), ^{3}H-threonine (1.8 Ci/mmol), ^{3}H-tryptophan (4.6 Ci/mmol), and a ^{3}H-labeled amino acid mixture were purchased from Schwarz/Mann, Orangeburg, N.Y.

TXT, a water miscible liquid scintillation fluid, was prepared from Triton X-100 (280 ml/liter), toluene (660 ml/liter), water (60 ml/liter), 2,5-diphenyloxazole (4 g/liters), and *p*-bis (*o*-methylstyryl)-benzene (82 mg/liter).

RESULTS

Phosphorylation in vitro of proteins of infectious SV40 and of empty capsids. We were unable to demonstrate protein kinase activity in purified preparations of SV40 (14). Therefore, phosphorylation of viral proteins in vitro had to be mediated by an exogenous cellular protein kinase. Since the beef heart protein kinase used is itself a phosphate acceptor (Fig. 1), it had to be separated from the

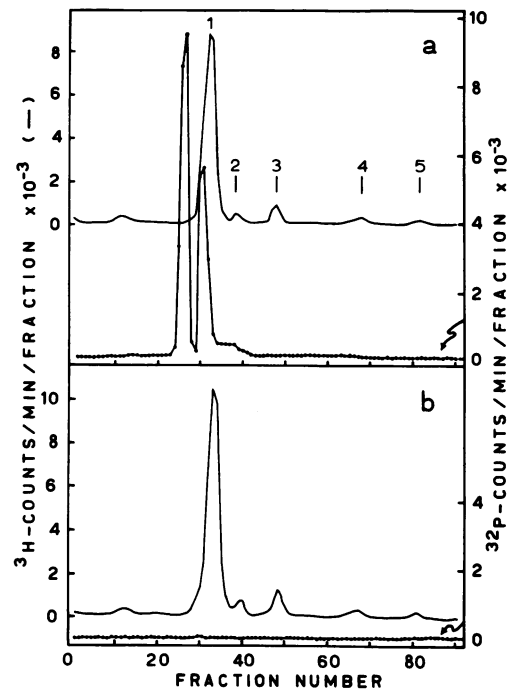

FIG. 1. *Co-electrophoresis of in vitro self-phosphorylated beef heart protein kinase with SV40. A standard reaction mixture containing 60 μg of beef heart kinase and 5 μCi of γ-^{32}P-ATP, but without SV40, was incubated at 37 C for 30 min, made 0.6 M in NaPPi, and mixed with ^{3}H-amino acid-labeled SV40. One sample (a) of the mixture was immediately precipitated with 10% trichloroacetic acid. From the other sample (b), the virus was centrifuged into a KBr solution, collected, and precipitated with 10% trichloroacetic acid (see Materials and Methods). The trichloroacetic acid precipitates from (a) and (b) were analyzed separately by electrophoresis in SDS-polyacrylamide gels. In this and all subsequent figures, electrophoresis is from left to right. The mol wt of the two beef heart protein kinase phosphoproteins were estimated (14) to be 57,000 and 52,000, respectively. Viral polypeptides are numbered 1 to 5, in order of decreasing molecular weights (14).*

phosphorylated virus (see Materials and Methods). When intact infectious SV40 was phosphorylated in vitro by this enzyme, by using γ-^{32}P-ATP as phosphate donor, only polypeptides 1 and 2 (mol wt 49,000 and 40,800, respectively) (14) were phosphorylated (Fig. 2a). The specific infectivity (PFU/mg of protein) of infectious virus remained unchanged after phosphorylation in vitro. Under similar conditions, all the polypeptides of intact empty capsids were phosphorylated in vitro (Fig. 2b). The amount of phosphate incorporated corresponded to about 47 and 206 pmol per mg of protein per 30 min for intact infectious virus and intact empty capsids, respectively (Table 1). Since detectable dephosphorylation of in vivo phosphorylated viral polypeptides occurred only after 22 h of treatment with relatively large amounts of pure alkaline phosphatase (14), the phosphorylation in vitro represents additional binding of phosphate groups rather than an exchange of preexisting protein-bound phosphate for the γ-phosphate of ATP.

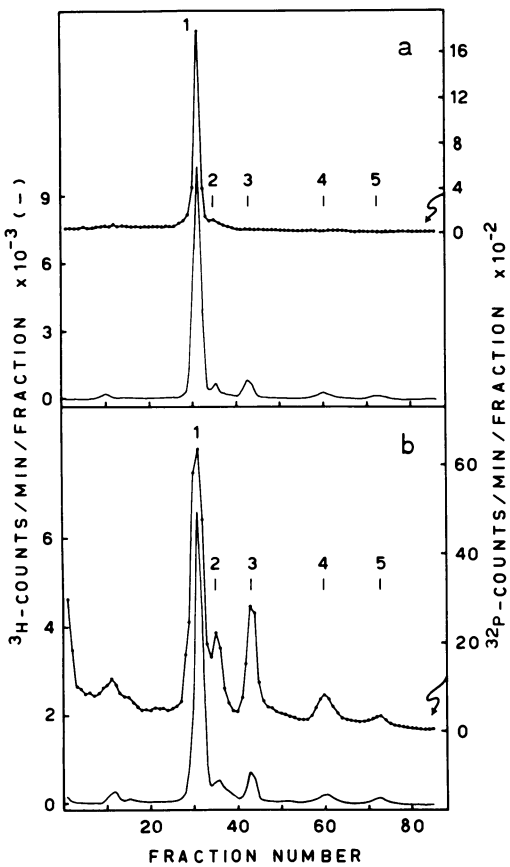

FIG. 2. *Electrophoretic patterns of in vitro phosphorylated polypeptides of infectious SV40 and empty capsids. The standard reaction mixture contained, in (a), 20 μg of beef heart kinase, 60 μg of infectious virus, and 50 μCi of γ-^{32}P-ATP, and, in (b), 40 μg of beef heart kinase, 80 μg of empty capsids, and 50 μCi of γ-^{32}P-ATP. After incubation at 37 C for 30 min, the virus was banded in KBr solution, collected, mixed with ^{3}H-amino acid-labeled marker SV40, and analyzed in SDS-polyacrylamide gels.*

TABLE 1. *Phosphorylation in vitro of untreated and alkali-treated SV40 in comparison with the phosphate-accepting capacity of histones and protamine*

Phosphate acceptor	^{32}P-phosphate incorporated (pmol/mg of acceptor protein) by[a]	
	Untreated acceptor[b]	Alkali-treated acceptor[c]
Infectious virus	47.1	539.5
Empty capsids	206.3	902.0
Histone	469.4	308.6
Protamine	1,018.0	932.4
Phosvitin	147.5	86.5
Bovine serum albumin	124.1	151.7

[a] Corrected for self phosphorylation of beef heart kinase. About 86 pmol of ^{32}P-phosphate were incorporated into 1 mg of enzyme in either untreated or alkali-treated samples.

[b] A standard reaction mixture containing 20 μg of beef heart kinase, 50 μg of the indicated protein, and 4 μCi of γ-^{32}P-ATP was incubated at 37 C for 30 min and then assayed for trichloroacetic acid-precipitable radioactivity (14).

[c] Samples were exposed to Na_2CO_3 and neutralized with HCl as described in the legend of Fig. 4 before being added to the standard reaction mixture containing beef heart kinase and γ-^{32}P-ATP.

Protein kinases prepared from rabbit reticulocytes and uninfected AGMK cells were also capable of phosphorylating all the polypeptides of empty capsids in vitro (Fig. 3).

Phosphorylation in vitro of partially disrupted SV40. We have evidence that both infectious virus and empty capsids contain the same five polypeptides, in similar proportions (14) (Table 2). The histone-like polypeptides 4 and 5 (mol wt 15,000 and 12,500, respectively), which are rich in basic amino acids and which lack tryptophan, and polypeptide 3 were previously shown to represent internal proteins (4). The inability of beef heart kinase to phosphorylate in vitro polypeptides 3, 4, and 5 may result from an inaccessibility due to their association with viral DNA and their internal location within the intact infectious virus. On the other hand, the loose surface structure of empty capsids probably allows the exogenous protein kinase to penetrate into the particle and to

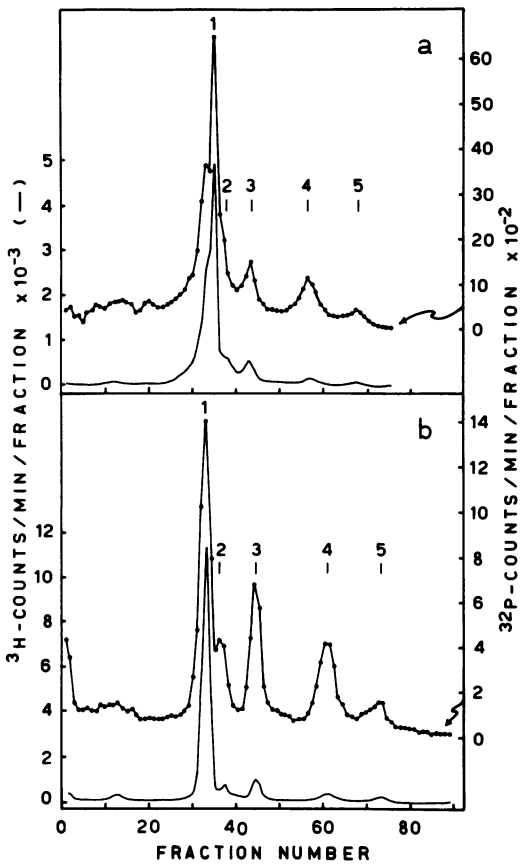

FIG. 3. *Electrophoresis of polypeptides from empty capsids phosphorylated in vitro by rabbit reticulocyte protein kinase or AGMK protein kinase. The standard reaction mixture contained, in (a), 270 μg of empty capsids, 40 μCi of γ-^{32}P-ATP, and 80 μg of rabbit reticulocyte protein kinase II, and, in (b), 350 μg of empty capsids, 50 μCi of γ-^{32}P-ATP, and 43 μg of AGMK cell protein kinase. Phosphorylation was for 30 min at 37 C. The virus was then banded in KBr solution and examined by electrophoresis after the addition of ^{3}H-amino acid-labeled SV40.*

phosphorylate all the viral polypeptides. We wanted to know whether the internal viral polypeptides can accept phosphate groups in vitro while being bound to DNA. Moreover, we wanted to determine whether altering the conformation of the virus particle by partial disruption would increase its phosphate-accepting capacity.

Both polyoma virus and SV40 can be disrupted by alkali treatment (4, 9). Exposure of SV40 to alkali solution was shown to release polypeptides 1 and 2 from the deoxynucleoprotein core consisting of viral DNA and all the other viral polypeptides (4). When infectious virus was treated with pH 10.2 buffer, neutralized, and then phosphorylated in vitro by beef heart kinase, the specific ^{32}P radioactivity of polypeptides 1, 2, and 3 was increased fourfold as compared with untreated infectious virus phosphorylated in vitro, but ^{32}P-phosphate was not incorporated into polypeptides 4 and 5 at all (Fig. 4). In vitro phosphorylation of empty capsids was also enhanced after alkali treatment as compared with untreated empty capsids (Table 1). The enhancement of phosphorylation in vitro, observed after exposure of viral proteins to pH 10.2 buffer, was not the result of the removal of pre-existing protein-bound phosphate groups followed by increased incorporation of γ-^{32}P-phosphate of ATP, because exposure of SV40 to pH 10.2 buffer did not cause dephosphorylation of viral polypeptides (14).

About 95% of the DNA contained in infectious virus could be removed by nuclease treatment of infectious virus which had been previously dialyzed against distilled water (Fig. 5). Both the "empty capsids" produced in vitro from infectious virus and empty capsids derived from infected cells have identical sedimentation rates in preformed CsCl gradients (Fig. 5) and a similar polypeptide composition (data not

TABLE 2. *Distribution of tritiated amino acids incorporated into the polypeptides of empty capsids and infectious SV40*[a]

Amino acid	Virus form	Fraction (%) of total viral protein-associated radioactivity in polypeptide:				
		1	2	3	4	5
Amino acid mixture	Virus	81.2	2.6	8.5	5.2	2.6
	Empty capsids	81.0	3.5	7.3	5.2	3.0
Lysine	Virus	83.7	2.0	5.2	6.5	2.8
	Empty capsids	83.0	1.9	4.5	7.0	3.6
Arginine	Virus	68.3	3.7	15.0	9.3	3.7
	Empty capsids	66.6	4.8	11.9	10.6	6.1
Tryptophan	Virus	72.5	6.0	21.5	0	0
	Empty capsids	76.6	6.4	17.0	0	0
Threonine[b]	Virus	85.3	2.9	6.2	3.9	1.7
Serine[b]	Virus	78.1	4.1	11.7	5.2	0.9

[a] Infected cells were labeled at 24 h postinfection with 1 μCi of either ^{3}H-amino acid mixture, ^{3}H-lysine, ^{3}H-arginine, ^{3}H-tryptophan, ^{3}H-threonine, or ^{3}H-serine per ml. Virus was harvested when cells showed an extensive cytopathic effect and was purified and analyzed by electrophoresis in SDS-polyacrylamide gels as described previously (14).

[b] Not determined for empty capsids.

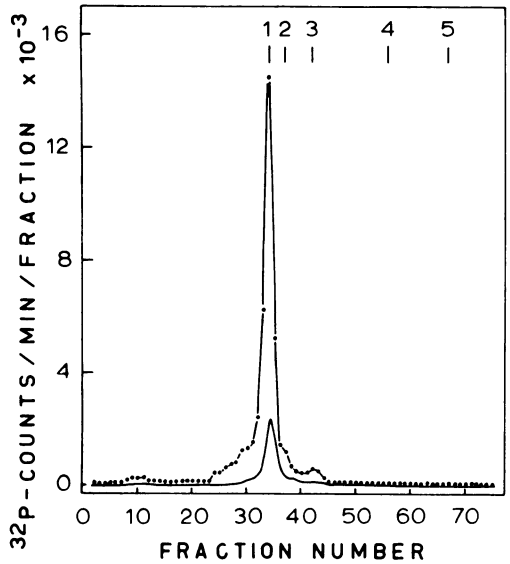

FIG. 4. *Electrophoretic pattern of SV40 polypeptides phosphorylated in vitro subsequent to alkali treatment. A sample of infectious virus (250 μg) was phosphorylated with 40 μg of beef heart kinase in a standard reaction mixture containing 40 μCi of γ-^{32}P-ATP for 30 min at 37 C (——). Another sample of virus suspended in NT buffer was treated with 0.1 M Na_2CO_3 (final pH of mixture at 22 C was 10.2) for 15 min at 37 C. The pH of the mixture was then adjusted to 8.0 by addition of 1 N HCl before the addition of beef heart kinase and γ-^{32}P-ATP and subsequent incubation at 37 C for 30 min. The virus was banded in KBr solution and examined by electrophoresis after mixing with ^{3}H-amino acid-labeled SV40 (●——●). The positions of ^{3}H-amino acid-labeled polypeptides are indicated.*

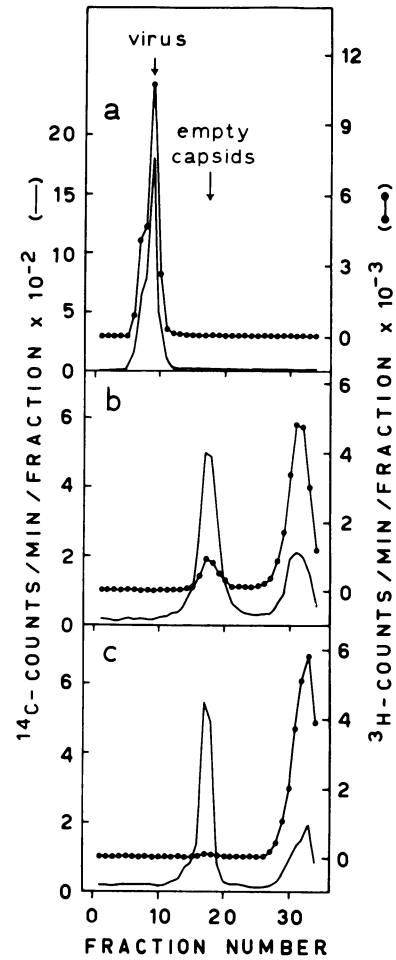

FIG. 5. *Velocity sedimentation of intact and nuclease-treated SV40 in CsCl gradients. Infectious SV40 labeled with both ^{14}C-amino acids and ^{3}H-thymidine (14) was dialyzed against 600 volumes of distilled water containing 0.1% mercaptoethanol for 4 days at 4 C. The sample was then treated with DNase (100 μg/ml) and phosphodiesterase (0.2 U/ml) in the presence of 0.13 M NaCl, 0.005 M $MgCl_2$, and 0.05 M Tris-hydrochloride (pH 8.0) for 30 min at 37 C; an equal amount of the sample not treated with nucleases served as a control. Intact, infectious virus (a), dialyzed virus not treated with nucleases (b), and dialyzed virus treated with nucleases (c) were centrifuged over preformed CsCl gradients (density 1.15 to 1.50 g/cm^3) in a Spinco SW50.1 rotor (30,000 rpm, 90 min, 10 C). Fractions (0.15 ml) were collected and their radioactivity was determined after the addition of TXT solution. The indicated position (panel a) of empty capsids, obtained from infected cells, was determined in a separate gradient. Sedimentation is from right to left. The ^{14}C radioactivity present at the top of the gradients (panels b, c) represents degraded capsids.*

shown). When "empty capsids" produced from infectious virus were phosphorylated in vitro with beef heart kinase, all the viral polypeptides could be further phosphorylated (Fig. 6). In addition, a viral polypeptide fragment (polypeptide a), resulting from cleavage by protease present in the nuclease preparations used (14), was also phosphorylated in vitro (Fig. 6). This polypeptide fragment was not phosphorylated in vivo (14). Thus, polypeptides 4 and 5 cannot accept phosphate groups when bound to viral DNA but become phosphate acceptors when detached from the viral genome.

Phosphate-accepting capacity of SV40 polypeptides. The efficiency of SV40 polypeptides as phosphate acceptor was compared with that of proteins commonly used in protein kinase assays. Phosphorylation of infectious virus and empty capsids in vitro was stimulated 11- and 4-fold, respectively, after alkali treat-

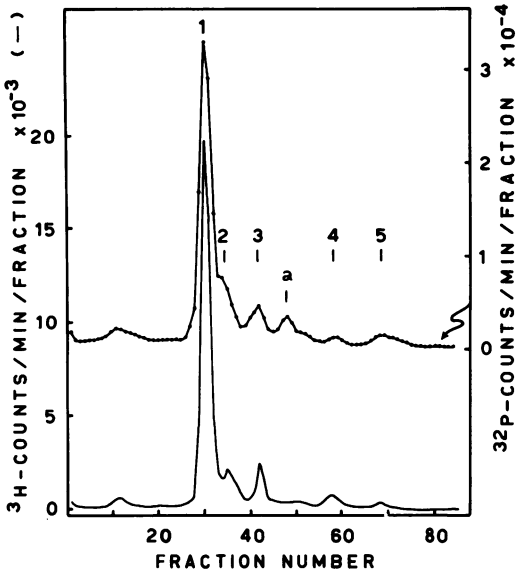

FIG. 6. *Fractionation of polypeptides of SV40 phosphorylated in vitro subsequent to nuclease treatment. Infectious SV40 (350 μg), treated as described in Fig. 5, was phosphorylated by beef heart kinase (20 μg) in a standard reaction mixture containing 100 μCi of γ-^{32}P-ATP for 30 min at 37 C and banded in KBr solution. The virus was then mixed with ^{3}H-amino acid-labeled marker SV40 (not treated with nuclease) before electrophoresis. Polypeptide a (estimated mol wt 24,500) containing ^{32}P radioactivity is a cleaved viral polypeptide fragment resulting from the presence of protease in the nuclease preparations (14).*

ment as compared with untreated virus (Table 1). Phosphorylation of both histones and protamine was, however, unaffected by prior alkali treatment. Alkali-treated empty capsids were as efficient as protamine as a phosphate acceptor. Phosphorylation of both infectious virus and empty capsids, with or without prior alkali treatment, was not increased significantly (1.6-fold increase) when cyclic AMP (5×10^{-6} M) or cyclic GMP (5×10^{-6} M) was present in the reaction mixture.

Based on the data presented in Fig. 6 and Tables 1 and 2, it was calculated that polypeptide 2 was the most efficient phosphate acceptor and that the major polypeptide 1 was the least efficient (Table 3). Much less than one picomole of phosphate was bound per picomole of polypeptide not treated with alkali.

Identification of the phosphate-acceptor amino acid residue. We have shown previously that the phosphate-protein bond of SV40 phosphorylated in vivo was cleaved by alkaline phosphatase but not by trichloroacetic acid, succinic acid, or hydroxylamine treatments, indicating the presence of phosphoserine or phosphothreonine, or both (14). Similarly, SV40 phosphorylated in vitro by beef heart kinase was also dephosphorylated by alkaline phosphatase treatment (Table 4). However, the extent of dephosphorylation of viral polypeptides phosphorylated in vitro was considerably greater than that of polypeptides phosphoryl-

TABLE 3. *Phosphate-accepting capacity of the polypeptides of empty capsids*

Polypeptide	Mol wt[a]	^{32}P-phosphate incorporated[b]	
		pmol/mg of polypeptide	pmol/pmol of polypeptide
1	49,000	86.2	0.004
2	40,800	866.9	0.035
3	29,500	348.9	0.01
4	15,000	241.4	0.004
5	12,500	168.1	0.002

[a] Estimated by the method described previously (14).

[b] Calculated from the data presented in Table 1 and in the legend to Fig. 2b for ^{32}P-phosphate incorporated by untreated empty capsids and from the data presented in Table 2 for the distribution of polypeptides labeled with ^{3}H-amino acid mixture in empty capsids.

TABLE 4. *Sensitivity of ^{32}P-phosphate bound to SV40 polypeptides to alkaline phosphatase treatment*[a]

Polypeptide	Fraction (%) of ^{32}P-phosphate retained after treatment[b]
1	65.4
2	32.3
3	39.3
4	27.3
5	21.1

[a] Empty capsids (250 μg) labeled with ^{3}H-amino acids were incubated in a standard reaction mixture containing 40 μg of beef heart kinase and 30 μCi of γ-^{32}P-ATP for 30 min at 37 C. The virus was centrifuged into a KBr gradient, collected, and precipitated with 10% trichloroacetic acid. The precipitate was dissolved in 1.0 M Tris-hydrochloride, pH 8.0, and divided into two equal samples. One sample was treated with 500 μg of alkaline phosphatase/ml for 1 h at 37 C, and the other untreated sample served as a control. The untreated and alkaline phosphatase-treated viral proteins were reprecipitated with trichloroacetic acid and subjected to electrophoresis in polyacrylamide gels.

[b] Calculated from the ratio of ^{32}P to ^{3}H radioactivity before and after treatment.

ated in vivo. The reason for this observation is not known.

To identify the phosphoamino acid(s), empty capsids phosphorylated in vitro by beef heart kinase were hydrolyzed in 6 N HCl and the hydrolysate was subjected to paper electrophoresis. Although phosphothreonine is decomposed by acid at a slower rate than phosphoserine (1), ^{32}P radioactivity was detected only in phosphoserine but not in phosphothreonine (Fig. 7). Both serine and threonine are present in similar proportions in all viral polypeptides (Table 2). Similarly, the ^{32}P-phosphate groups of infectious virus phosphorylated in vitro, alkali-treated infectious virus, and empty capsids, and of infectious virus and empty capsids phosphorylated in vivo (14) were also associated only with phosphoserine.

DISCUSSION

The observation that the polypeptides of intact infectious SV40 and empty capsids can be further phosphorylated in vitro indicates that not all of the phosphate-acceptor sites in the proteins of purified SV40 are phosphorylated. All the polypeptides of empty capsids are accessible to, and are further phosphorylated by, exogenous cellular protein kinase in vitro, whereas, in intact infectious virus, only two polypeptides (polypeptides 1 and 2) could be further phosphorylated in vitro. These observations suggest that polypeptides 1 and 2 constitute the surface proteins of SV40 virions. The results of phosphorylation of partially disrupted infectious virus in vitro indicate that polypeptides 4 and 5 could be further phosphorylated only when they are freed of viral DNA and that they bind to viral DNA more strongly than polypeptide 3. These observations are in agreement with previously reported data (4) on the localization of the polypeptides in the virion.

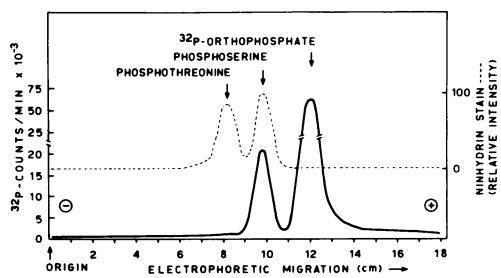

FIG. 7. *Identification of phosphoamino acid in the proteins of SV40. Empty capsids (120 μg) were phosphorylated with 50 μg of beef heart kinase in a standard reaction mixture containing 50 μCi of γ-^{32}P-ATP. The empty capsids were banded in KBr solution, hydrolyzed with 6 N HCl, mixed with marker phosphothreonine and phosphoserine, and subjected to paper electrophoresis. The position of orthophosphate was determined by electrophoresis of ^{32}P-orthophosphate in a separate paper strip.*

Since all the polypeptides of infectious SV40 were phosphorylated in vivo (14) whereas only two polypeptides could be further phosphorylated in vitro, we conclude that intracellular phosphorylation of at least polypeptides 3, 4, and 5 must have occurred prior to virus maturation. The fact that the extent of phosphorylation in vitro of infectious SV40 or empty capsids could be enhanced 4- to 11-fold after pretreatment with alkali shows that phosphate-acceptor sites were unmasked after alkali treatment.

The results of the present study suggest that mature SV40 particles exist in an "underphosphorylated" state and that they can be further phosphorylated by protein kinases. If such further phosphorylation occurs in the infected cell, it may facilitate the uncoating of parental virus. One could also speculate that the transport of newly synthesized structural proteins of SV40 from the cell cytoplasm to the nucleus may be regulated by protein phosphorylation and that the intranuclear proteins then become dephosphorylated before or during virus assembly. It would therefore be of great interest to compare the extent of phosphorylation of free viral proteins in the cell cytoplasm and in the nucleus with that of mature progeny virus. Comparative studies on the extent of phosphorylation of the proteins of parental virus before and after virus uncoating in the host cell could also shed light on the mode of SV40 uncoating.

ACKNOWLEDGMENTS

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LITERATURE CITED

1. Allerton, S. E., and G. E. Perlmann. 1965. Chemical characterization of the phosphoprotein phosvitin. J. Biol. Chem. **240**:3892-3898.
2. Gilman, A. G. 1970. A protein binding assay for adenosine 3':5'-cyclic monophosphate. Proc. Nat. Acad. Sci. U.S.A. **67**:305-312.
3. Hatanaka, M., E. Twiddy, and R. V. Gilden. 1972. Protein kinase associated with RNA tumor viruses and other budding RNA viruses. Virology **47**:536-538.
4. Huang, E. S., M. K. Estes, and J. S. Pagano. 1972. Structure and function of the polypeptides in simian virus 40. I. Existence of subviral deoxynucleoprotein complexes. J. Virol. **9**:923-929.
5. Kleinsmith, L. J., V. G. Allfrey, and A. E. Mirsky. 1966. Phosphorylation of nuclear protein early in the course of gene activation in lymphocytes. Science **154**:780-781.
6. Krebs, E. G. 1972. Protein kinases, p. 99-133. *In* B. L. Horecker and E. R. Stadtman (ed.), Current topics in cell regulation, vol. 5. Academic Press Inc., New York.

7. Lagan, T. A. 1968. Phosphorylation of proteins of the cell nucleus, p. 101-118. *In* A. San Pietro, M. Lamborg, and F. T. Kenney (ed.), Regulatory mechanisms for protein synthesis in mammalian cells. Academic Press Inc., New York.
8. Paoletti, E., and B. Moss. 1972. Protein kinase and specific phosphate acceptor proteins associated with vaccinia virus cores. J. Virol. **10**:417-424.
9. Perry, J. L., C. M. To, and R. A. Consigli. 1969. Alkaline degradation of polyoma virus. J. Gen. Virol. **4**:403-411.
10. Randall, C. C., H. W. Rogers, D. N. Downer, and G. A. Gentry. 1972. Protein kinase activity in equine herpesvirus. J. Virol. **9**:216-222.
11. Sokol, F., and H. F. Clark. 1973. Phosphoproteins, structural components of rhabdoviruses. Virology **52**:246-263.
12. Strand, M., and J. T. August. 1971. Protein kinase and phosphate acceptor proteins in Rauscher murine leukemia virus. Nature N. Biol. **233**:137-140.
13. Tan, K. B. 1972. A simple device for fractionating density gradients. Anal. Biochem. **45**:306-308.
14. Tan, K. B., and F. Sokol. 1972. Structural proteins of simian virus 40: phosphoproteins. J. Virol. **10**:985-994.
15. Tao, M., M. L. Salas, and F. Lipmann. 1970. Mechanism of activation by adenosine 3′:5′-cyclic monophosphate of a protein phosphokinase from rabbit reticulocytes. Proc. Nat. Acad. Sci. U.S.A. **67**:408-414.
16. Wunderly, C. H. 1959. Paper electrophoresis, p. 179-223. *In* M. Bier (ed.), Electrophoresis, theory, methods and applications. Academic Press Inc., New York.