JOURNAL OF VIROLOGY, June 1974, p. 1245-1253
Vol. 13, No. 2

Printed in U.S.A.

Virion-Bound Protein Kinase in Semliki Forest and Sindbis Viruses

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Received for publication 31 January 1974

Semliki forest virus and Sindbis virus (Alphaviruses belonging to the togavirus group) grown in BHK-21 cells possessed very low levels of virion-associated protein kinase activity. For comparison, vesicular stomatitis virus, also grown in BHK-21 cells, contained a virion-bound protein kinase which had a specific activity 80 times greater than that of the Alphaviruses. The Alphavirus protein kinase was unmasked by the nonionic detergent Nonidet P-40 but was not activated by cyclic nucleotides. Phosvitin was the best exogenous phosphate acceptor for assaying the viral enzyme in vitro. Phosphoprotein phosphatase activity was also detected in the Alphaviruses. Both in vivo and in vitro, all of the viral structural polypeptides were phosphorylated, and the phosphorylated amino acids were found to be serine and threonine. The viral nucleocapsid protein was about four times more efficient as a phosphate acceptor than were the envelope proteins. From 33 to 50% of the total protein kinase was bound to the viral nucleocapsid, and the specific activity of this enzyme was 4 to 10 times greater than that associated with the viral envelope.

Protein kinase activity has been demonstrated in a large number of enveloped animal viruses such as RNA tumor viruses (8, 26), rhabdoviruses (9, 25, 26), herpesvirus (18, 20), vaccinia virus (5, 17), and frog virus (6, 22), but not in nonenveloped viruses such as poliovirus (26), adenovirus (34), and simian virus 40 (SV40) (31). Most of the enveloped viruses referred to above also possess enzymes required for the transcription of the viral nucleic acid. Previous demonstration that protein phosphorylation stimulated the rate of RNA synthesis in lymphocytes (12) led to the suggestion that the transcription of viral nucleic acid by virus-associated transcriptase(s) may be regulated by protein phosphorylation mediated by the virus-associated protein kinase activity (25; F. Sokol, K. B. Tan, and H. F. Clark, Abstr. Annu. Meet. Amer. Soc. Microbiol., p. 258, 1973). If a major role of the virus-associated protein kinase is to regulate transcription, then one should not find protein kinase activity in viruses that contain infectious RNA, such as the Alphaviruses. We therefore tested two Alphaviruses for protein kinase activity.

The nature of the virus-associated protein kinase has not been investigated in detail. The enzyme could be virus coded, or virus induced and firmly bound to the virion, or it could be a cellular enzyme that is incorporated into the virus particle as it buds off at the cell membrane during virus maturation. Comparative studies of two different enveloped viruses, grown in the same host cell, to determine the presence of virus-associated protein kinase and, if the enzyme is present, to characterize it, could shed light on the origin of the enzyme and were undertaken with rhabdovirus and Alphaviruses.

MATERIALS AND METHODS

Viruses. Wild-type Semliki forest virus (SFV) and Sindbis virus, generously provided by E. R. Pfefferkorn, and the Indiana serotype of vesicular stomatitis virus (VSV) were used in the experiments. All of the viruses were propagated in monolayer cultures of BHK-21 cells as described previously (2, 25).

Labeling and purification of viruses. All of the SFV and Sindbis virus preparations used were labeled with [^{3}H]leucine. After virus infection, cells were maintained in medium containing 2% fetal calf serum and 1 μCi of [^{3}H]leucine (specific activity 55 Ci/mmol; Schwarz/Mann, Orangeburg, N.Y.) per ml. For preparation of ^{32}P-labeled virus, the medium used contained only one-fifth of the regular concentration of phosphate, and 20 μCi of [^{32}P]orthophosphate (carrier-free, New England Nuclear Corp., Boston, Mass.) per ml was added at 6 h postinfection. When all of the cells showed cytopathic effect (about 20 h after infection), the culture medium was collected and clarified by low-speed centrifugation. The clarified medium was made 0.5 M in NaCl, and polyethylene glycol 6000 was added to a final concentration of 10% (wt/vol) (37). After the polyethylene glycol was dis-

solved by stirring, the medium was kept at 4 C for at least 2 h. The precipitate was sedimented (3,000 × *g*, 30 min), suspended in NTE (0.13 M NaCl, 1 mM EDTA, 50 mM Tris-hydrochloride, pH 7.9) buffer and centrifuged (Spinco SW25.1 rotor, 24,000 rpm, 4 C, 6 h) in a 22-ml gradient of 20 to 30% (wt/wt) of potassium tartrate prepared in NTE buffer. The virus band was collected, diluted with NTE buffer, and centrifuged (SW25.1 rotor, 23,000 rpm, 4 C, 16 h) in a 20-ml gradient of 30 to 50% (wt/wt) sucrose prepared in NTE buffer. The virus band was collected and diluted fivefold with NTE buffer, and the virus was sedimented (SW50 L rotor, 33,000 rpm, 4 C, 2 h). The sedimented virus was resuspended and stored in NTE buffer at 4 C. VSV was purified by precipitation with zinc acetate and banding in sucrose gradient as described previously (25) and stored in NTE buffer.

Isolation of viral nucleocapsid. SFV or Sindbis virus (2.5 mg of protein/ml) was mixed with an equal volume of 2% (vol/vol) Nonidet P-40 (NP-40) (Shell Chemical Co.) and kept at room temperature for 20 min with occasional shakings (10). A 0.3-ml sample was then centrifuged (SW50 L rotor, 38,000 rpm, 4 C, 40 min) (30) over a 4-ml gradient of 15 to 30% (wt/vol) sucrose in NTE buffer.

Dissociation of virus and treatment and electrophoretic fractionation of polypeptides. Purified virus was precipitated with trichloroacetic acid, delipidized with acetone, and prepared for electrophoresis after dissolving in sodium dodecyl sulfate (SDS) and dithiothreitol as described previously (31). For RNase treatment, virus was precipitated at 0 C with 10% trichloroacetic acid, and the precipitate was collected by centrifugation, washed with acetone, and dried. The dried precipitate was resuspended in 0.13 M NaCl, 0.005 M $MgCl_2$, and 0.05 M Tris-hydrochloride, pH 7.8, containing 200 μg of pancreatic RNase per ml and incubated at 37 C for 60 min. Prior to use, the RNase solution was boiled for 3 min. The virus was then processed for electrophoresis as described above.

Polyacrylamide gel electrophoresis. Viral polypeptides were fractionated by electrophoresis in a discontinuous SDS-polyacrylamide gel system as described by Laemmli (15) with slight modifications (21). Gels cast in Plexiglas tubes (6-mm inner diameter, 12 cm long) contained a 10% separating gel, and electrophoresis was conducted at room temperature at 3 mA per gel for 4 h. Urea (6 M) was included in the separating gel because viral polypeptides migrated as sharper bands in the presence of urea than in its absence. After electrophoresis, the gel was sliced into 1-mm-thick slices, and radioactivity in the slices was determined as described previously (31).

Determination of molecular weights of SFV and Sindbis virus structural proteins. The molecular weights of viral proteins were determined by co-electrophoresing in the same gel the following proteins of known molecular weight: bovine serum albumin (68,000), ovalbumin (43,500), aldolase (40,000), chymotrypsinogen A (25,700), and RNase (13,700). The gels were stained with Coomassie brilliant blue to locate the positions of the different proteins, and the molecular weights of the viral proteins were calculated as described by Weber and Osborn (36).

Identification of phosphoamino acids. The method used was a modification of that previously described (32). Virus was partially hydrolyzed in 2 N HCl at 110 C for 5 h. After removal of HCl, the samples were mixed with nonradioactive *O*-phosphoserine and *O*-phosphothreonine and subjected to paper electrophoresis.

Assay for protein kinase activity. The complete reaction mixture (0.2 ml) contained 0.1% NP-40, 10 μmol of Tris-hydrochloride (pH 8.0), 2 μmol of $MgCl_2$, 2 μmol of DTT, 100 μg of phosphate acceptor protein, and indicated amounts of purified virus, unlabeled ATP, and [γ-^{32}P]ATP (New England Nuclear Corp., Boston, Mass.). After incubation at 37 C for 30 min, the proteins were precipitated with trichloroacetic acid, dissolved in NaOH (7), and reprecipitated with trichloroacetic acid, and acid-insoluble radioactivity was determined as described previously (31).

Protein determination. Protein concentration was determined as described previously (16) by using bovine serum albumin as standard.

Sources of materials. α-Casein, calf thymus histones (total, arginine-rich, lysine-rich), 3′:5′-cyclic nucleotides, *O*-phosphoserine, *O*-phosphothreonine, phosvitin, protamine (salmon sperm, free base), theophylline, and beef heart protein kinase were obtained from Sigma Chemical Co. (St. Louis, Mo.). Polyethylene glycol (average molecular weight, 6,000 to 7,500) was from Union Carbide Chemicals (New York). Dithiothreitol was from Calbiochem (La Jolla, Calif.). RNase (bovine pancreas, specific activity 2,650 U/mg of protein) was from Schwarz/Mann (Orangeburg, N.Y.). Bovine serum albumin (fraction V, crystallized) was from Miles Laboratories, Inc. (Kankakee, Ill.).

RESULTS

Intracellular phosphorylation of SFV and Sindbis virus. Some or all of the viral proteins in a variety of viruses have been found to be phosphoproteins (9, 19, 22, 25, 26, 31, 35). To determine whether phosphoproteins are present in SFV and Sindbis virus, the virus was purified from cells infected in the presence of [^{32}P]phosphate in the growth medium, disrupted, extracted with lipid solvent, treated with RNase, and subjected to electrophoresis in polyacrylamide gels. Purified Sindbis virus contained a nucleocapsid protein (molecular weight, 32,000) and two envelope proteins, E1 (molecular weight, 56,500) and E2 (molecular weight, 51,000) that are glycoproteins (21) (Fig. 1). All of the viral polypeptides were phosphorylated in vivo. The nature of the peak of ^{32}P-radioactivity between 36 and 42 mm of the gel (Fig. 1) is not known. On the other hand, our gels resolved only two structural polypeptides in SFV, an envelope protein (molecular weight, 50,000) and a nucleocapsid protein (molecular weight, 31,000). Both of the structural polypeptides of

SFV were also phosphorylated in vivo. Recently, it was demonstrated that SFV contains two envelope proteins (24).

Phosphorylation in vitro of SFV and Sindbis virus proteins. Preliminary experiments demonstrated that both SFV and Sindbis virus disrupted with NP-40 exhibited low levels of protein kinase activity which could be measured in an in vitro system using [γ-^{32}P]ATP as phosphate donor. To determine which of the viral structural proteins was phosphorylated, the virus was subjected to electrophoresis in polyacrylamide gel. Both the envelope and nucleocapsid proteins of SFV and Sindbis virus were phosphorylated in vitro (Fig. 2a). This phosphorylation mostly represents binding of additional phosphate groups to the viral proteins. However, since the virus contained low levels of phosphoprotein phosphatase activity (see later), some phosphate exchange between the phosphates from [γ-^{32}P]ATP and the viral protein could have occurred. In both viruses,

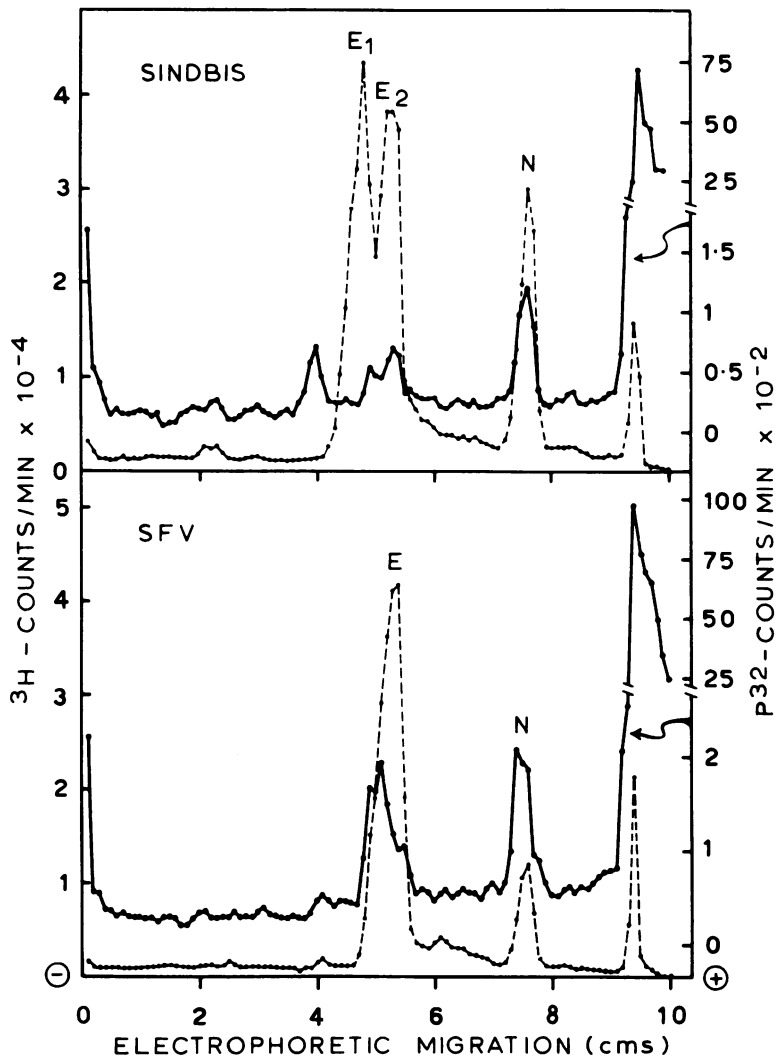

FIG. 1. *Electrophoretic pattern of viral polypeptides phosphorylated in BHK-21 cells. Virus purified from infected cells incubated in the presence of [^{3}H]leucine and [^{32}P]orthophosphate was disrupted and treated with RNase before electrophoresis in polyacrylamide gels as described in Materials and Methods. The peaks of radioactivity at 9.4 cm represent RNA and protein degradation products which comigrated with the bromophenol blue tracking dye. The estimated molecular weights of Sindbis viral polypeptides are 56,500 (E1), 51,000 (E2), and 32,000 (N), whereas those of SFV are 50,000 (E) and 31,000 (N). Abbreviations: E, envelope protein; N, nucleocapsid protein.*

the nucleocapsid protein was three to four times (calculation based on $^{32}P/^{3}H$ ratio) more efficient than the envelope protein(s) as phosphate acceptor, even though the envelope protein has a higher proportion of serine and threonine residues (11, 23). Treatment of SFV and Sindbis virus with alkali (pH 10.5), a procedure which enhanced the phosphate-accepting capacity of SV40 polypeptides (32), did not enhance the phosphorylation in vitro of these viruses. Furthermore, when NP-40-disrupted SFV or Sindbis virus was incubated in vitro with an exogenous beef heart kinase, only the E2 polypeptide of Sindbis virus accepted additional phosphate (Fig. 2b).

Identification of phosphoamino acids. SFV and Sindbis virus phosphorylated in vitro or in vivo were partially hydrolyzed in 2 N HCl at 110 C for 5 h and subjected to paper electrophoresis. Phosphothreonine and phosphoserine, predominantly the latter, were detected in both viruses phosphorylated either in vitro or in vivo (Fig. 3). The relatively large amounts of ^{32}P-radioactivity near the anode represents inorganic phosphate derived from decomposed phosphoamino acids and RNA (virus phosphorylated in vivo) and from phosphate from [γ-^{32}P]ATP (virus phosphorylated in vitro) since the samples were not treated with 0.1 N NaOH to remove contaminating, unincorporated [γ-^{32}P]ATP (7).

Properties of the virus-associated protein kinase. In our studies on the properties of the protein kinase associated with SFV and Sindbis virus, VSV-associated protein kinase was used for comparative purposes. The enzyme activity

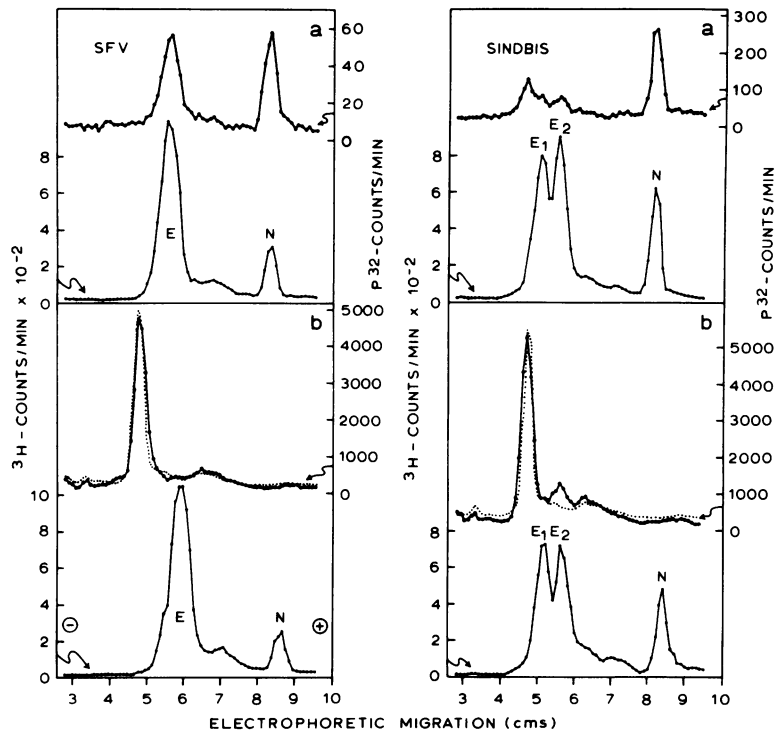

FIG. 2. *Electrophoretic pattern of virus phosphorylated in vitro by the endogenous viral or exogenous beef heart protein kinase. In panel (a), the reaction mixture contained the standard amounts of Tris-hydrochloride, $MgCl_2$, dithiothreitol, NP-40, 4 nmol of ATP, 40 μCi of [γ-^{32}P]ATP (specific activity 20 Ci/mmol), and 150 μg of SFV or Sindbis virus labeled with [^{3}H]leucine. After 30 min of incubation at 37 C, the samples were precipitated with trichloroacetic acid and analyzed by polyacrylamide gel electrophoresis. In panel (b), the reaction mixture described above contained, in addition, 2 nmol of cyclic AMP and 20 μg of beef heart protein kinase. Before the addition of virus, NP-40, and [γ-^{32}P]ATP, the mixture was incubated at 37 C for 30 min to allow for self-phosphorylation of beef heart kinase since the enzyme contains phosphate acceptor proteins (32). Virus, NP-40, and [γ-^{32}P]ATP were then added, and after a further 30-min incubation at 37 C the proteins were precipitated with trichloroacetic acid and subjected to polyacrylamide gel electrophoresis. The pattern of phosphorylated proteins in the reaction mixture containing both virus and beef heart kinase (●) was superimposed on that from a reaction mixture containing only beef heart kinase (·····) for comparison.*

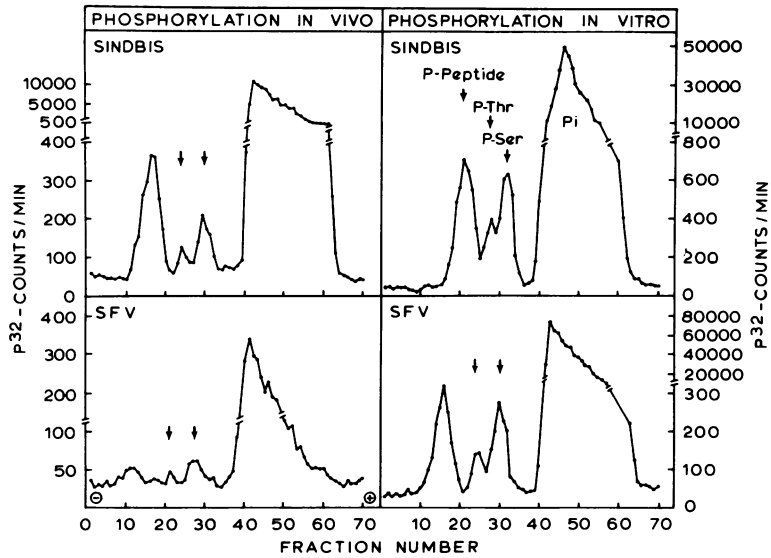

FIG. 3. *Identification of phosphoamino acids in Alphaviruses. Virus purified from cells grown in the presence of [^{32}P]orthophosphate was disrupted, treated with RNase, and precipitated with trichloroacetic acid as described in Materials and Methods. Virus phosphorylated in vitro with [γ-^{32}P]ATP was not treated with RNase before precipitation with acid. After hydrolysis with 2 N HCl at 110 C for 5 h, the samples were mixed with unlabeled marker phosphothreonine and phosphoserine and subjected to paper electrophoresis in an acetic acid (7.8%)-formic acid (2.5%) buffer (pH 1.82) for 6.5 h at 500 V, 4 C (32). The paper strips were stained to locate the phosphothreonine and phosphoserine markers and cut up into 3-mm-wide sections for determination of radioactivity. The positions of phosphothreonine and phosphoserine markers are indicated by arrows.*

of all three viruses was markedly stimulated in vitro when the viruses were partially disrupted with NP-40 (Table 1). Up to a 50-fold stimulation of the enzyme activity was observed when freshly prepared SFV or Sindbis virus, i.e., virus from sucrose gradient fractions, was assayed in vitro. Some of the viruses that were stored in NTE buffer must have undergone a spontaneous degradation resulting in a lower level of stimulation by NP-40 when compared with freshly prepared virus.

Protein kinases from animal cells and tissues are activated by cyclic nucleotides (see review, 14), but the enzymes associated with purified viruses are not (5, 8, 17, 18, 20, 22, 26). Similarly, the protein kinases associated with VSV, SFV, and Sindbis virus are not stimulated by cyclic nucleotides (Table 1). Inclusion of theophylline (2.5 mM) in the reaction mixture to inhibit phosphodiesterase activity did not affect the enzyme activity (K. B. Tan and F. Sokol, manuscript in preparation; F. Sokol and H. F. Clark, in press).

Both SFV and Sindbis virus showed very low levels of protein kinase activity when assayed in vitro in the absence of exogenous phosphate acceptors. To determine whether the viral structural proteins are poor phosphate acceptors or whether the virus contains low levels of protein kinase activity, or both, the virus was assayed for protein kinase activity in vitro in the presence of various phosphate acceptors. Phosvitin was found to be the best exogenous phosphate acceptor for the Alphaviruses as well as for VSV (Table 2). The same viruses grown in monkey kidney cells also showed a preference

TABLE 1. *Effect of NP-40 and cyclic nucleotides on protein kinase activity*

Additions[b]	[^{32}P]phosphate (pmol) incorporated per mg of viral protein[a]		
	VSV	SFV	Sindbis virus
− NP-40	42	16	26
+ NP-40	12,009	103	139
+ NP-40 + c-AMP	10,807	113	115
+ NP-40 + c-CMP	10,668	116	149
+ NP-40 + c-GMP	10,167	117	109
+ NP-40 + c-IMP	10,544	124	152
+ NP-40 + c-TMP	12,257	95	120
+ NP-40 + c-UMP	11,779	112	111

[a] Reaction mixture contained 10 μg of VSV, 20 μg of Alphavirus, 100 μg of phosvitin, 6.2 nmol of [γ-^{32}P]ATP (specific activity, 1,230 counts per min per pmol), and NP-40 and cyclic nucleotide at final concentrations of 0.1% and 10^{-5} M, respectively.

[b] c-, cyclic.

for phosvitin (K. B. Tan and F. Sokol, manuscript in preparation). Compared with SFV and Sindbis virus, the specific activity of the protein kinase associated with VSV was about 80-fold greater (Tables 1 and 2). To rule out the possibility that this great a difference might reflect the method of virus purification, VSV was precipitated with polyethylene glycol as described in Materials and Methods and banded in a sucrose gradient. The enzyme activity of polyethylene glycol- or zinc acetate-precipitated and purified virus was found to be identical.

Localization of protein kinase activity. The observation that the protein kinase activity in both SFV and Sindbis virus was activated by NP-40 treatment of the virus suggests that the enzyme was located within the virion (Table 1). To localize the enzyme activity, the viruses were disrupted with 1% NP-40 (10) and fractionated in sucrose gradients. The results (Fig. 4) show that protein kinase activity, assayed with phosvitin as substrate, cosedimented with both nucleocapsid and envelope fractions. About 50 and 33% of the total protein kinase activity was associated with the nucleocapsids of SFV and Sindbis virus, respectively. However, these values must represent minimal values because (i) the envelope fractions contained a small amount of solubilized nucleocapsid protein (Fig. 5), and (ii) the specific activity ($^{32}P/^{3}H$ ratio) of the nucleocapsid-associated enzyme was 4 (Sindbis virus) to 10 (SFV) times that of the envelope-associated enzyme in phosphorylating phosvitin.

TABLE 2. *Substrate specificity of protein kinase*

Protein added	[^{32}P]phosphate (pmol) incorporated per mg of viral protein[a]		
	VSV	SFV	Sindbis virus
None	92	8	11
Lysine-rich histones	88	13	11
Arginine-rich histones	193	11	17
Total histones	153	20	22
Protamine	145	28	16
Phosvitin	15,294	76	195
Casein	1,087	25	15
SV40[b]	418	19	24
Bovine serum albumin	130	18	11

[a] Reaction mixture contained 10 μg of VSV, 20 μg of Alphavirus, 100 μg of protein substrate, 8.1 nmol of [γ-^{32}P]ATP (specific activity, 1,250 counts per min per pmol), and NP-40 at a final concentration of 0.1%.

[b] Simian virus 40 (SV40) grown in monkey kidney cells was purified as described previously (32). Only empty capsids were used in this experiment.

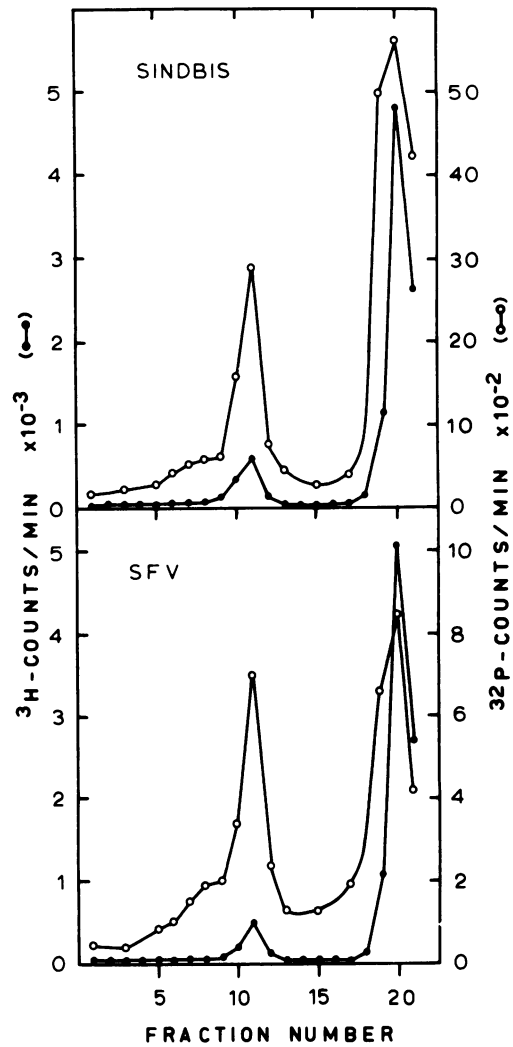

FIG. 4. *Localization of protein kinase activity in Alphaviruses. [^{3}H]leucine-labeled virus was treated with NP-40 and sedimented in a 15 to 30% sucrose gradient as described in Materials and Methods. Under these conditions, undisrupted virus particles would have been detected in about fraction 3 or 4 (30). Fractions of 0.2 ml were collected from the bottom of the tube, and a 20-μliter sample of each fraction was mixed with a water-miscible scintillation fluid (32) for determination of ^{3}H-radioactivity. From selected fractions, 70-μliter samples were assayed for protein kinase activity in a standard reaction mixture containing 100 μg of phosvitin and 2.2 nmol of [γ-^{32}P]ATP (specific activity 2,190 counts per min per pmol).*

Phosphoprotein phosphatase activity associated with purified virus. Protein kinase purified from animal cells and tissues (see review, 14) and cultured cells (33) also contain a

phosphoprotein phosphatase activity. Similarly, low levels of phosphatase activity were demonstrated in VSV, SFV, and Sindbis virus by incubating the virus with protamine previously phosphorylated by beef heart kinase in the presence of [γ-^{32}P]ATP (Fig. 6). However, unlike the phosphatase activity in purified frog virus which was activated by Mn^{2+} ions (22), the activity in VSV, SFV, and Sindbis virus was the same in the presence of either Mn^{2+} or Mg^{2+} ions.

DISCUSSION

Purified SFV and Sindbis virus analyzed on continuous SDS-polyacrylamide gels were shown to contain two polypeptides, an envelope protein and a nucleocapsid protein (2, 27). Recently, the envelope proteins of both SFV and Sindbis virus were resolved into two polypeptides by electrophoresis in discontinuous SDS-polyacrylamide gels as described by Laemmli (15). However, the envelope proteins of SFV were not separated in our discontinuous SDS-polyacrylamide gel. All of the structural polypeptides of both SFV and Sindbis virus were phosphorylated in BHK-21 cells. Similarly, all of the polypeptides of Sindbis virus grown in chicken embryo fibroblasts were also phosphorylated (35). In a cell-free system, all of the viral proteins could be further phosphorylated by an endogenous virion-bound protein kinase. The pattern of in vivo and in vitro phosphorylation was similar; i.e., the nucleocapsid protein was the best phosphate acceptor. Although the envelope proteins of both SFV and Sindbis virus could be phosphorylated by the virion-bound and cell-associated protein kinase(s), only the E2 protein of Sindbis virus could be further phosphorylated by the exogenous beef heart protein kinase under conditions where all of the viral proteins were accessible to the enzyme. The significance of this observation is not known. Therefore, the envelope proteins of SFV and Sindbis virus show differences in electrophoretic mobility and phosphate-accepting capacity.

The Alphavirus-associated protein kinase was unmasked and activated by NP-40 treatment, indicating that the enzyme is an internal component of the virus. Indeed, about 33 and 50% of the total protein kinase activity were found firmly bound to nucleocapsids obtained by NP-40 treatment of Sindbis virus and SFV, respectively. With VSV, it was shown that the protein kinase was associated mostly with the viral membrane (9), but others (J. F. Obijeski, F. Sokol, P. Madore, H. F. Clark, and D. H. L. Bishop, manuscript in preparation) found that the enzyme was associated predominantly with the viral nucleocapsids. In contrast to VSV and the Alphaviruses, which mature by budding at the cell plasma and vacuolar membranes (1, 13, 28), the envelope of intracellular vaccinia virus is not derived from the host cell membrane (4), and all of the protein kinase activity of this virus is associated with the viral core (17). From our data, it seems unlikely that a significant portion of the virion-bound protein kinase resulted from host cell contamination as the Alphavirus or VSV buds off at the cell mem-

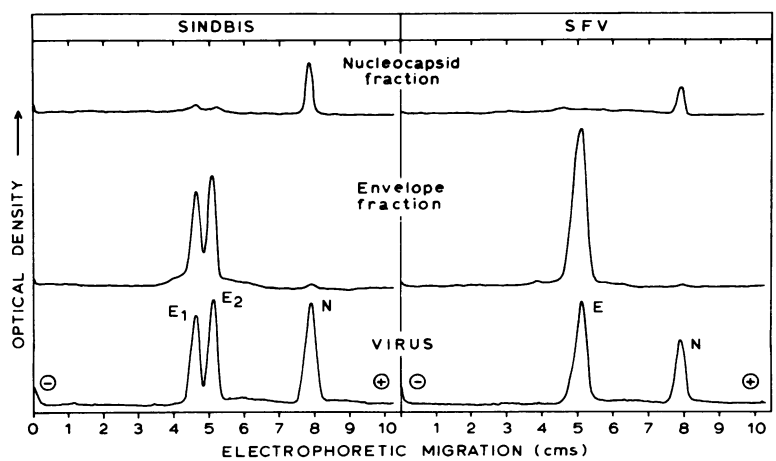

FIG. 5. *Electrophoretic pattern of virus and of subviral components obtained by treating virus with NP-40. From the remainder of the sucrose gradient fractions obtained in the experiment described in Fig. 4, 50- to 100-μliter samples of nucleocapsid (fraction 11) or envelope (fraction 20) fractions or untreated virus were solubilized with SDS and dithiothreitol and subjected to polyacrylamide gel electrophoresis. The gels were stained with Coomassie brilliant blue and scanned for absorbancy.*

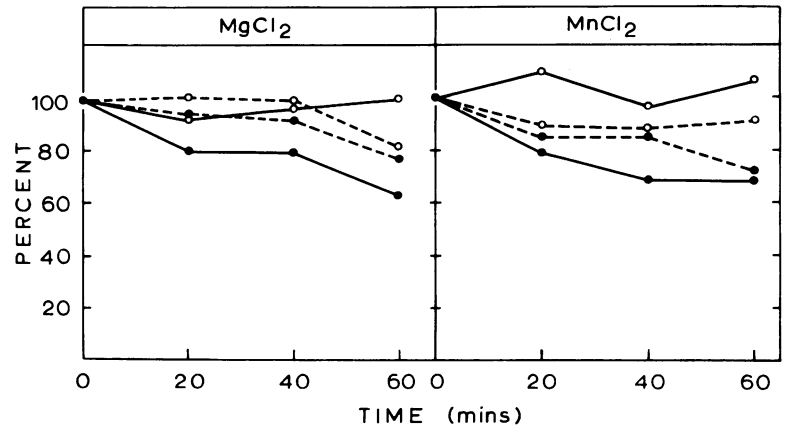

FIG. 6. *Dephosphorylation of phosphoprotein by VSV and Alphaviruses. Protamine (500 μg) was incubated in a reaction mixture containing the standard amounts of Tris-hydrochloride, $MgCl_2$, dithiothreitol, and 2 nmol of cyclic AMP, 30 μg of beef heart kinase, 12.4 nmol of ATP, and 30 μCi of [γ-^{32}P]ATP (specific activity 12.5 Ci/mmol). After 60 min of incubation at 37 C, the proteins were precipitated with trichloroacetic acid, dissolved in NaOH, and reprecipitated with trichloroacetic acid as described in Materials and Methods. The final precipitate was dissolved in 0.5 M Tris-hydrochloride, pH 7.9. The phosphorylated protamine contained 1,700 pmol of [^{32}P]phosphate per mg of protamine. A 300-μliter reaction mixture containing 15 μmol of Tris-hydrochloride (pH 8.0), 6 μmol of $MgCl_2$ or 60 nmol of $MnCl_2$, 3 μmol of dithiothreitol, 15 nmol of ATP, 0.1% NP-40, 100 μg of either VSV, SFV, or Sindbis virus, and 50 μg of [^{32}P]protamine was incubated at 37 C, and, at 0, 20, 40, and 60 min, 70-μliter samples were withdrawn and precipitated with trichloroacetic acid, and the acid-insoluble radioactivity was determined and expressed as percentage of acid-insoluble radioactivity contained in the sample not incubated at 37 C (zero time point sample). Symbols: (●——●) VSV, (●- - -●) SFV, (O- - -O) Sindbis virus, (O——O) without virus.*

brane. The envelope proteins constitute 75 to 80% of the total protein in the Alphaviruses (2, 23, 27) and about 58% in VSV (3), whereas the specific activity of the protein kinase associated with VSV grown in the same host cell as the Alphaviruses was about 80 times greater than that of the Alphaviruses. The fact that the protein kinase is firmly bound to the Alphavirus nucleocapsids even after treatment with 1% NP-40 strongly suggests that the enzyme is not derived from random adsorption of a cellular enzyme. However, it is not known whether the enzyme is cellular or virus-induced and binds specifically to viral proteins (29), or whether it is an integral component of the nucleocapsid. Free nucleocapsids extracted from rabies virus-infected cells possess protein kinase activity (25).

The virion-bound protein kinase in VSV, SFV, and Sindbis virus phosphorylated phosvitin more extensively than either protamine or histones. Similar results were obtained with viper C-type virus, rabies virus, and Sendai virus (K. B. Tan, unpublished data). In contrast, beef heart protein kinase (22, 32) and vaccinia-associated protein kinase (17) all showed a preference for histones and protamine over phosvitin as phosphate acceptor. A comparative study of the substrate specificity of virus-associated protein kinase may prove useful for classifying the enzyme.

Two possible functions have been suggested for virus-associated protein kinase, namely, (i) nongenetic reactivation of frog virus (6) and (ii) regulation of transcription of the genome of RNA tumor viruses (F. Sokol, K. B. Tan, and H. F. Clark, Abstr. Annu. Meet. Amer. Soc. Microbiol., p. 258, 1973). If a major function of the protein kinase associated with VSV is to regulate the virion-bound RNA transcriptase, then one would not expect to find protein kinase activity in the Alphaviruses which lack a virion-associated transcriptase. However, we can detect protein kinase activity in the Alphaviruses, but this enzyme activity is extremely low (80-fold less) compared with that in VSV. Because of the very low protein kinase activity in the Alphaviruses, it was concluded from preliminary experiments with Sindbis virus that it did not possess a protein kinase when the enzyme activity was measured in the absence or presence of protamine (quoted in reference 35). Whether this difference in level of protein kinase activity in the Alphaviruses and rhabdoviruses is related to their mode of replication remains to be determined.

ACKNOWLEDGMENTS

We thank M. McFalls for technical assistance, E. R. Pfefferkorn for providing stocks of Alphaviruses, and P. Madore for providing purified VSV.

This study was supported by Public Health Service research grants CA-10594 and CA-10815 from the National Cancer Institute and RR-05540 from the Division of Research Resources.

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