JOURNAL OF VIROLOGY, Oct. 1977, p. 401-405

Vol. 24, No. 1
Printed in U.S.A.

Phosphorylated Protein Component Present in Influenza Virions

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Received for publication 2 May 1977

The nucleoprotein of the WSN strain of influenza was found to be phosphorylated in vivo. The phosphate-protein bond was stable to hot trichloroacetic acid, RNase, DNase, succinic acid, and succinic acid-hydroxylamine, but sensitive to hydrolysis by bacterial alkaline phosphatase. This suggested that the nucleoprotein is in the form of a phosphomonoester. Acid hydrolysis of the isolated nucleoprotein followed by thin-layer electrophoresis identified the phosphorylated amino acid residue as phosphoserine.

A variety of animal viruses have been reported to contain phosphorylated proteins as components of mature virions. Among these are the three rhabdoviruses, vesicular stomatitis, rabies, and Kern Canyon (12); the enveloped DNA virus, vaccinia (9); and the unenveloped simian virus 40 (14) and adenovirus (10) virions. In addition, virion-associated protein kinases have been demonstrated in vesicular stomatitis virus (13), vaccinia virus (3, 8), herpes simplex virus (7), poxvirus (H. Silberstein et al., Fed. Proc. 31:407A, 1972), frog polyhedral cytoplasmic deoxyribovirus (11), and C-type viruses from a number of sources (4), though not all of these have been shown to possess phosphorylated protein components in vivo. We wish to report the identification of a phosphoprotein present in influenza virions (a myxovirus) and a preliminary chemical characterization of the phosphate-protein linkage.

Semiconfluent monolayers of MDCK cells were infected at multiplicities of from 10 to 30 with the WSN strain of influenza. Infection and subsequent maintenance was carried out in 15 mM *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid-buffered Dulbecco-modified Eagle medium (DME-HEPES) supplemented with 2% calf serum. At 4 h postinfection, the medium was aspirated off and replaced with 20 ml per flask of $^{31}PO_4^{2-}$-free DME-HEPES plus 2% dialyzed calf serum (Grand Island Biological Co.) containing 450 μCi of $^{32}PO_4^{2-}$ per ml (International Chemical and Nuclear Co.; carrier-free). Antibacterial and antimycoplasmal agents (streptomycin-penicillin-Fungizone and Tylocine, both from Grand Island Biological Co.) were sometimes included, with no observed effect on virus yield or level of phosphorylation. Culture supernatants were harvested at 20 h postinfection. All subsequent operations were at 4°C. A low-speed centrifugation (4,000 rpm in a Sorvall GSA rotor for 20 min) was followed by a high-speed centrifugation (28,000 rpm in a Spinco type 30 rotor for 1 h) to pellet the virus. The pellet was suspended in a small volume of flu buffer (100 mM NaCl-10 mM Tris-chloride [pH 7.6]-1 mM EDTA). Viral purification was completed by isopycnic banding in a 15 to 33% solution of sodium-potassium-tartrate in flu buffer gradient (30,000 rpm in an SW41 rotor for 4 h), collecting the viral band, repelleting after dilution with flu buffer (28,000 rpm in a Spinco type 30 rotor for 1 h), and suspending the purified influenza virions in flu buffer. At this point, either whole virus was utilized in the subsequent characterizations or the virions were phenol extracted (15), the proteins in the phenol phase were precipitated by adding 5 volumes of 95% ethanol, and the proteins were washed once with 95% ethanol and once with anhydrous ether. The ether was removed under vacuum, and the viral proteins were solubilized in sodium dodecyl sulfate (SDS) sample buffer (50 mM Tris-chloride [pH 6.8]-10% glycerol-1% SDS-1% 2-mercaptoethanol).

SDS-gel electrophoresis was performed as described previously (15), using a 5% stacking-10% running polyacrylamide gel system. WSN proteins labeled with [^{3}H]leucine were utilized as internal markers. The viral polypeptides were fixed and visualized by Coomassie brilliant blue staining. The dried gel lanes were then cut out and sliced into 1-mm thicknesses, and each slice was placed in a separate scintillation vial, solubilized in NCS-Omnifluor-toluene scintillation fluor, and counted by a dual-label method (15). ^{3}H spillover in the ^{32}P channel was estimated to be 0%; ^{32}P spillover in the ^{3}H channel was estimated to be 3%.

An initial SDS-polyacrylamide slab gel of phosphate-labeled virions demonstrated a peak of ^{32}P activity running coincident with the [^{3}H]leucine-labeled nucleoprotein (NP protein; data not shown). ^{32}P-labeled material migrating within the stacking gel was also found to be present in these untreated samples. The latter was presumed to be residual RNA, a hypothesis confirmed by the observed absence of this high-molecular-weight material in samples subjected to a hot trichloroacetic acid or RNase treatment (Fig. 1). Typically, virus harvested from five semiconfluent 150-cm^2 tissue culture flasks yielded a total of 100,000 ^{32}P cpm running in the position of NP protein. Recovery of this material was quantitative through the phenol extraction and the subsequent ethanol-ether precipitations (data not shown).

To further characterize this NP-protein-coincident, phosphorus-containing material, $^{32}PO_4^{2-}$-labeled WSN proteins recovered from the phenol phase of an RNA extraction were subjected to a variety of treaments (Table 1). Protein samples were treated as described, precipitated with 5 volumes of 95% ethanol–0.33% saturated sodium acetate, dried under vacuum, dissolved in SDS sample buffer, and run on an SDS-polyacrylamide slab gel as in Fig. 1. The recovery of ^{32}P running as NP protein relative to the recovery of [^{3}H]leucine marker running as NP protein was calculated. The ^{32}P-labeled peak was found to be refractory

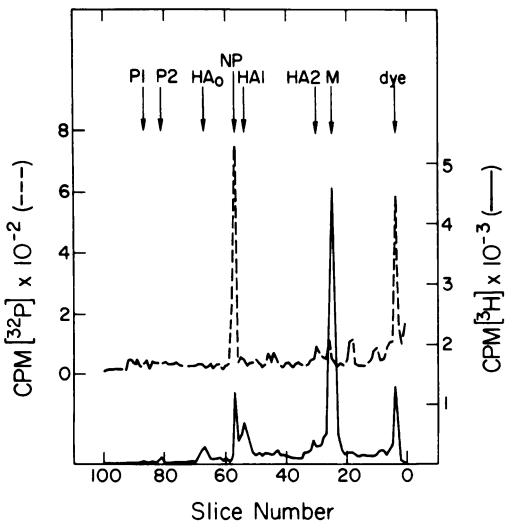

FIG. 1. *$^{32}PO_4^{2-}$-labeled WSN influenza proteins were isolated after a phenol extraction, treated with RNase-DNase as in Table 1, and subjected to SDS-polyacrylamide gel electrophoresis. [^{3}H]leucine-labeled WSN proteins, used as internal markers and run in the same lane, were prepared as in Fig. 2.*

TABLE 1. *Treatment of $^{32}PO_4^{2-}$-labeled WSN proteins*[a]

Treatment	^{3}H cpm in NP protein	^{32}P cpm in NP protein	Ratio of ^{32}P to ^{3}H
Expt 1			
None	3,660	1,280	0.350
Ethanol precipitation alone	4,300	1,300	0.302
Trichloroacetic acid 10%[b]	4,125	1,440	0.349
−BAP control[c]	4,400	1,525	0.347
+BAP[c]	3,900	0	0.000
1.0 N NaOH[b]	<100	<100	—[d]
Expt 2			
Ethanol precipitation alone	2,927	2,135	0.729
RNase-DNase[e]	1,230	925	0.752
Succinic acid[f]	1,772	1,574	0.888
Succinic acid-NH_2OH[g]	1,877	1,715	0.915

[a] $^{32}PO_4^{2-}$-labeled influenza proteins, obtained from the phenol phase and solubilized in SDS sample buffer, were mixed with SDS-disrupted [^{3}H]leucine-labeled WSN virions, and portions were placed into separate 0.6-ml test tubes. The proteins were precipitated out of the SDS buffer with ethanol, and the protein pellets were then dried and suspended in 100 μl of the appropriate incubation mixture. After the indicated incubation period, 10 μg of carrier cytochrome *c* was added per tube, the samples were re-precipitated as described in the text and run on SDS-polyacrylamide gel electrophoresis, the dried gels were sliced and counted, and the ratio of ^{32}P to ^{3}H in NP protein was determined.

[b] Incubated for 15 min at 50°C.

[c] Conditions of incubation for bacterial alkaline phosphatase (BAP) treatments are described in Fig. 2.

[d] Treatment with 1.0 N NaOH caused extensive degradation of all viral proteins.

[e] Reaction mixture consisted of 10 μg each of RNase A and DNase I (Worthington Biochemicals Corp.) in 10 mM sodium phosphate (pH 7.2)–5 mM $MgCl_2$. Incubation was carried out for 1 h at 37°C.

[f] Incubated for 1 h at 37°C in 1 M succinic acid (pH 5.5).

[g] Incubated for 1 h at 37°C in 1 M succinic acid–1 M hydroxylamine (pH 5.5).

to RNase A-DNase I incubation, hot 10% trichloroacetic acid, succinic acid, and succinic acid-hydroxylamine. Treatment with 1 N NaOH for 15 min at 50°C caused substantial degradation of all the viral polypeptides, as measured by the loss of the ^{3}H-labeled peaks, as well as a concomitant decrease in ^{32}P label associated with the NP protein. These chemical reactivities suggest that the ^{32}P label in the NP-protein peak is not due to bound RNA or DNA. The recovery of the ^{32}P label after repeated ethanol

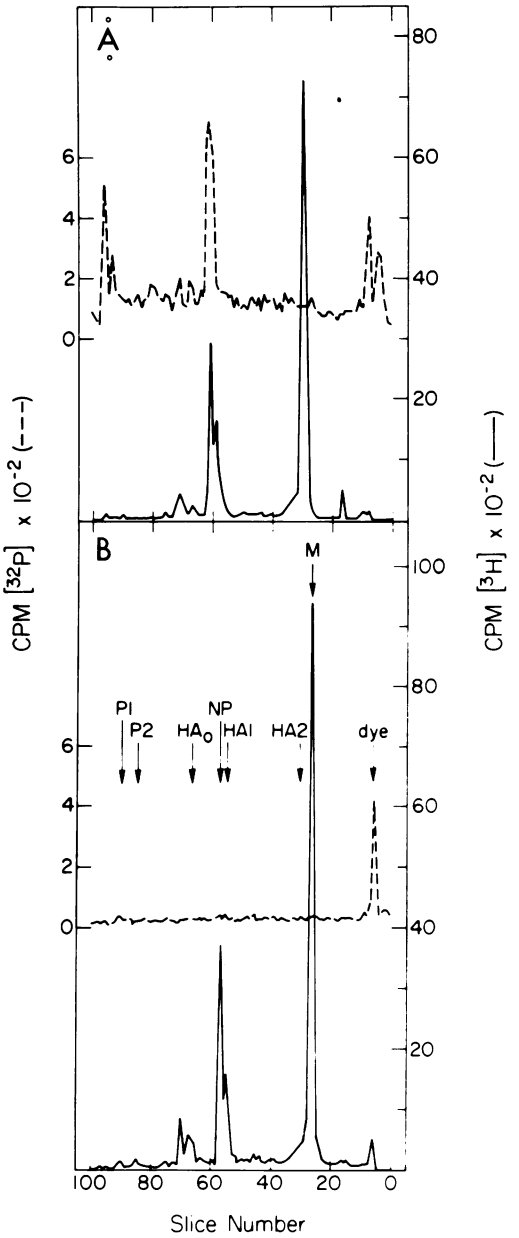

FIG. 2. *E. coli alkaline phosphatase treatment of $^{32}PO_4^{2-}$-labeled WSN influenza proteins. Phosphate-labeled viral proteins were obtained after a phenol extraction. [^{3}H]leucine-labeled WSN proteins, used as internal standards, were prepared as follows. Semiconfluent monolayers of MDCK cells were infected at a multiplicity of 2 in DME-HEPES medium-2% calf serum. At 6 h postinfection, the medium was aspirated off, the monolayers were washed once with phosphate-buffered saline, and DME-HEPES (0.1×) in unlabeled leucine containing 2% dialyzed calf serum and 2 μCi of L-[4,5-^{3}H]leucine per ml (Amersham/Searle; 60 Ci/mmol) was added. Harvest and purification of virus was as detailed for ^{32}P-labeled virions. The [^{3}H]leucine virions were then disrupted in SDS sample buffer and mixed with the ^{32}P-labeled proteins. Portions (15 μl) were placed in separate tubes, and the proteins were precipitated with 100 μl of 95% ethanol to remove excess SDS. The proteins were then suspended in 100 μl of the appropriate incubation buffer. (A) Control incubation. Labeled proteins were incubated for 90 min at 37°C in 0.1 M Tris-chloride (pH 8.0)-1.3% saturated $(NH_4)_2SO_4$. The proteins were then precipitated with ethanol and prepared for SDS-gel electrophoresis as before. (B) Alkaline phosphatase. Labeled proteins were incubated for 90 min at 37°C in 0.1 M Tris-hydrochloride (pH 8.0) containing 0.08 U of E. coli alkaline phosphatase [Worthington BAPF in 65% saturated $(NH_4)_2SO_4$; yields 1.3% saturation in incubation buffer]. The proteins were then processed as above.*

precipitation suggests that the material is not a phospholipid. Thus, the ^{32}P label appears to be in covalent linkage with the NP protein.

The insensitivity of the phosphorus-protein bond to the hot trichloroacetic acid treatment suggests that the phosphate is not in the form of a phosphoamide; its refractivity to succinic acid-hydroxylamine implies that it is not present as an acyl ester.

$^{32}PO_4^{2-}$-labeled WSN proteins were then subjected to an enzymatic digestion with *Escherichia coli* alkaline phosphatase (Worthington BAPF; RNase-free). This enzyme catalyzes the specific hydrolysis of terminal phosphomonoesters (5). The result, compared with a control incubation, is shown in Fig. 2. The bacterial enzyme does indeed appear to release the ^{32}P label from the NP protein. This strongly suggests that the phosphate is in ester linkage to the viral protein, possibly by way of a serine or threonine residue. The lability of the ^{32}P label to this enzyme is also strong evidence that it is not RNA tightly bound to the NP protein in such a way as to protect the nucleic acid from trichloroacetic acid or RNase. Bacterial alkaline phosphatase would not be expected to remove such a structure.

Determination of the precise phosphate linkage was performed on NP protein eluted from a preparative SDS-polyacrylamide gel. ^{32}P-labeled WSN proteins were run on a tube gel (0.6-cm ID by 10 cm) under conditions identical to those used in the analytical gels described previously. Immediately after electrophoresis, the gel was sliced into 2-mm thicknesses with a razor blade gel slicer, and each section was placed in a separate scintillation vial. The ^{32}P-labeled NP protein was localized by Cerenkhov counting of the slices. A tube gel run in parallel, containing [^{3}H]leucine-labeled WSN pro-

teins, confirmed this ^{32}P peak to be coincident with the ^{3}H-labeled NP protein. The ^{32}P-labeled NP protein was then eluted by shaking the gel slices for 5 h at 30°C in 50 mM Tris-chloride (pH 6.8)–1% SDS–1% 2-mercaptoethanol containing 250 μg of carrier bovine serum albumin. These conditions resulted in the elution of greater than 80% of the ^{32}P cpm. The polyacrylamide gel pieces were spun down on a tabletop centrifuge, the supernatant was removed, and the protein was precipitated from it by addition of 3.5 volumes of 95% ethanol–0.33% saturated sodium acetate. The protein pellet, collected in a Sorvall SS-34 rotor at 10,000 rpm for 20 min, was washed in ethanol-ether (1:1, vol/vol), repelleted, and dried under vacuum. The protein was then suspended in 2 N HCl and hydrolyzed under nitrogen in a sealed vial for 5 h at 100°C.

The HCl was removed by lyophilization, the hydrolysate was dissolved in a minimal volume of distilled water, and thin-layer electrophoresis was performed as described by Paoletti and Moss (8). Authentic phosphothreonine and phosphoserine markers (obtained from Sigma Chemical Co.) were run in adjacent lanes. Markers were localized by ninhydrin staining, and the hydrolysate lane was autoradiographed (Fig. 3). The major phosphoamino acid seen in the ^{32}P-labeled protein hydrolysate is phosphoserine. No phosphothreonine was detected, although the possibility of its presence at a very low concentration cannot be excluded. The release of inorganic phosphate and the presence of uncleaved phosphopolypeptides after weak-acid hydrolysis have previously been described (8).

It would therefore appear that the NP protein of influenza is phosphorylated in vivo and that this phosphate is in the form of a monoester linkage, primarily to a serine residue. This is of particular interest in that the NP protein apparently serves to package the segmented RNA genome of this virus into the nucleocapsid structures and is one of the three proteins found in flu transcription complexes isolated from infected cells (L. A. Caliguiri and H. Gerstein, Fed. Proc. 35:1737A, 1976). The NP protein has also been found in all reported subviral particles capable of synthesizing RNA in vitro (1, 2). The possibility that the level of phosphorylation of NP protein may serve a control function in transcription, translation, or packaging of the influenza virus genome presents itself. The analogy to the phosphorylation of the likewise nucleic acid-binding histones during their synthesis (6) is particularly intriguing. Experiments are presently being carried out with infected-cell extracts to explore these questions.

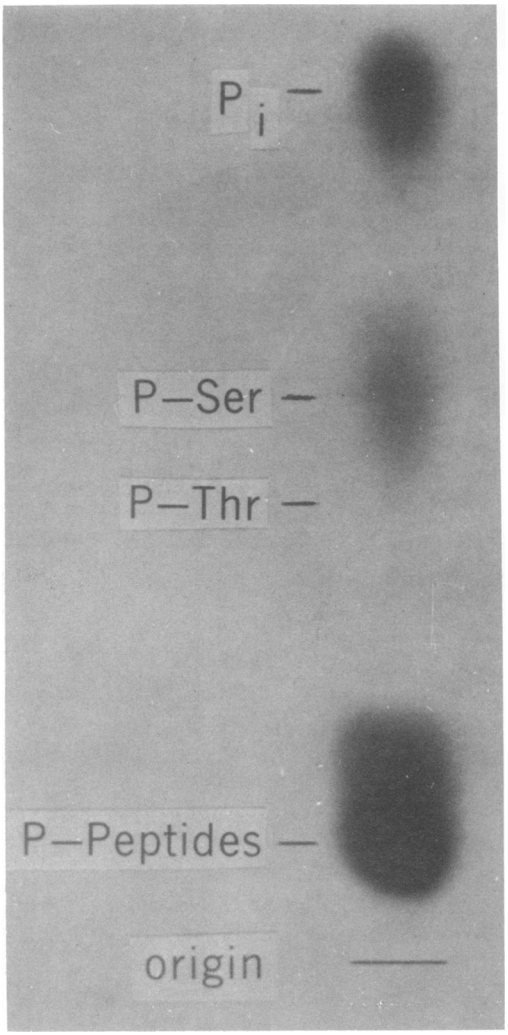

FIG. 3. *Acid hydrolysate of ^{32}P-labeled NP protein run on thin-layer electrophoresis and autographed as described in the text. The positions of authentic phosphoserine and phosphothreonine, visualized by ninhydrin staining, are indicated.*

A virion-associated protein kinase has previously been identified in crude and partially purified flu virus preparations (4). The relationship of this kinase to the phosphoprotein reported here is not known.

This research was supported by National Science Foundation grant 6B 38658 and by a grant from the California Respiratory Disease Association.

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