Proc. Natl. Acad. Sci. USA
Vol. 77, No. 12, pp. 7444–7447, December 1980
Microbiology

The structural polypeptides of aphthovirus are phosphoproteins

(picornavirus/foot-and-mouth disease virus/viral subunits/NaDodSO$_4$/8 M urea gel electrophoresis/phosphoamino acids)

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Communicated by Theodor O. Diener, August 29, 1980

ABSTRACT **Analysis of aphthovirus A_{12}, strain 119ab, grown in the presence of inorganic ^{32}P revealed that two of the major viral polypeptides, VP_4 and trypsin-sensitive protein VP_3, were highly phosphorylated. The other major polypeptides, VP_1 and VP_2, were also phosphorylated but to a much lesser extent. Polypeptides VP_0 and P_{56}, of which there are approximately one or two copies per aphthovirion, were also labeled with ^{32}P. Phosphoserine and phosphothreonine appeared to be the amino acids labeled with ^{32}P.**

Aphthovirus (foot-and-mouth disease virus) is an acid-sensitive member of the animal picornaviruses (1). As with other picornaviruses, the viral particles contain 60 copies each of four major proteins (VP_1, VP_2, VP_3, and VP_4), approximately 1 or 2 copies of VP_0 (2, 3), and 1 copy of a protein that is covalently linked to the 5′ end of the virion RNA (VPg), (4, 5). Picornavirus proteins are synthesized in infected cells as a large precursor polyprotein which is later processed via a series of proteolytic cleavages into functional mature forms, the structural and nonstructural proteins. The cleavages are performed by both cellular and virus-induced proteases (6–8).

The existence of processing reactions other than posttranslational proteolytic cleavages, such as chemical modification of the amino acid residues, could provide the basis for the production of conformational changes in the viral protein molecules during maturation. These changes, such as the formation of β-turns (3), may determine the appropriate recognition site(s) for the processing enzymes. Additional benefits of this type of processing can be envisioned in the assembly of a virus particle in which the macromolecular associations may be kinetically more favorable with the modified proteins. In the present study, evidence was found indicating that the structural proteins of purified aphthovirus are phosphorylated.

MATERIALS AND METHODS

Cells and Virus. Baby hamster kidney cells (BHK 21, clone 13) grown in 2-liter roller bottle cultures (9) and aphthovirus A_{12}, strain 119ab, were used in all the experiments.

Virus Growth, Labeling, and Purification. Two roller bottles containing approximately 5×10^8 cells per bottle were washed with phosphate-free medium (three changes during 1 hr at 37°C), infected with 20 plaque-forming units of virus per cell in 10 ml of medium, and incubated for 45 min at 37°C. Forty milliliters of medium containing 5 μg of actinomycin D (a gift of Merck Sharp & Dohme) was added, and the cells were incubated for an additional 45 min prior to labeling with carrier-free [^{32}P]orthophosphoric acid (0.5 mCi/ml; 1 Ci = 3.7×10^{10} becquerels; New England Nuclear). Virus was harvested 5–6 hr after infection and purified as described (10) except that, prior to harvest, the cell fluids were made 0.05% in Triton X-100 and shaken for 3 min at room temperature to improve the recovery of virus. After removal of the nuclei (3000 × *g* for 5 min), subsequent purification steps were carried out in the presence of 2% *N*-lauroylsarcosine (Sarkosyl; Sigma) (11).

Preparation and Analysis of Viral Proteins. The purified virus was pelleted, resuspended in NET buffer (0.1 M NaCl/1 mM EDTA/0.05 M Tris·HCl, pH 7.4) containing 1% NaDodSO$_4$ (NETS), and extracted twice with hot phenol/chloroform, 1:1 (vol/vol), as described (11). More than 90% of the viral RNA was recovered undegraded. The resulting organic phase was reextracted four additional times with NETS buffer to remove residual RNA, and the viral proteins were precipitated from the organic phase by addition of 4–6 vol of cold (−20°C) acetone. The protein precipitate was collected by centrifugation (9000 × *g*, 20 min, 0°C), washed three times with cold (−20°C) ethyl ether, resuspended in 150 μl of 0.01 M sodium acetate at pH 4.5, and treated for 6 hr at 37°C with pancreatic RNase A (42 μg/ml) and RNases T1 and T2 (50 units/ml). After incubation, samples (approximately 20 μg of protein) were mixed with an equal volume of 2× sample preparation buffer [2× SPB: (0.125 M Tris·HCl, pH 6.8/4% NaDodSO$_4$/1.36 M 2-mercaptoethanol/8 M urea/20% (vol/vol) glycerine/0.01% bromophenol blue)] or treated with 10% trichloroacetic acid at 85°C for 10 min and then at 0°C for 30 min. The acid-treated samples were centrifuged (10,000 × *g* for 20 min), and the precipitates were washed three times with ethyl ether and resuspended in an appropriate volume of 1× SPB. Alternatively, aliquots of the RNase-treated proteins were digested with proteinase K (250 μg/ml) in enzyme buffer (0.1 M NaCl/0.01 M Tris·HCl, pH 7.4/1 mM EDTA/0.5% NaDodSO$_4$) for 2 hr at 37°C and mixed with an equal volume of 2× SPB. In addition, 10–15 μg of [^{35}S]methionine-labeled virus or unlabeled virus was mixed with an equal volume of 2× SPB, treated with RNases as above, and run in parallel lanes as marker proteins. All samples in SPB were heated at 100°C for 10 min and analyzed by electrophoresis in 10% NaDodSO$_4$/polyacrylamide gels containing 8 M urea as described by Bachrach *et al.* (12). The gels were run at 120 V for approximately 4 hr, stained, destained, and dried onto Whatman 3MM paper. Radioactivity was detected by exposure to Kodak X-Omat R film with a DuPont Cronex Lightning Plus intensifying screen.

Detection of Phosphorylated Amino Acids. Phosphorylated amino acids were prepared and analyzed by high-voltage paper electrophoresis as described by Bitte and Kabat (13).

Preparation of 12S Viral Subunits. Highly purified ^{32}P-labeled aphthovirus was resuspended in 100 μl of 0.01 M sodium acetate at pH 4.5, heated at 55°C for 5 min, cooled, and treated with a mixture of RNases for 6 hr as described above. The sample was mixed with 200 μl of NET buffer containing

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4 M urea and 1% Triton X-100 and analyzed by centrifugation in 5–20% (wt/vol) sucrose gradients as reported by Vásquez *et al.* (14). Aliquots from each fraction of the gradient were made 10% in trichloroacetic acid and treated for 10 min at 85°C and then at 0°C for 30 min; the precipitates were collected on Whatman GF/A glass-fiber filters, dried, and assayed for radioactivity in a Beckman liquid scintillation counter. Purified ^{125}I-labeled 12S subunits (kindly provided by D. M. Moore, Plum Island Animal Disease Center) were run in a parallel gradient as an external marker; bovine IgG (7 S) and IgM (19 S) (also provided by D. M. Moore) were used as internal markers in each gradient. Fractions containing the 12S subunits (see Fig. 1, pool I) and the top fractions of the gradients (pool II) were precipitated with cold acetone, resuspended in 1× SPB, and analyzed on 10% polyacrylamide slab gels as described above.

Extraction of Protein from Gels. Sections containing the stained protein bands were excised from dried gels, swollen in water for 3–5 min, homogenized in buffer (0.01 M Tris·HCl, pH 7.4/0.5% 2-mercaptoethanol/1 mM EDTA) containing 1% $NaDodSO_4$, and shaken at 25°C overnight. The extracts were centrifuged at 15,000 × *g* for 30 min to remove the acrylamide, and the proteins were precipitated from the supernatant fluid with 4–5 vol of acetone (after addition of 30 μg of bovine gamma globulin as carrier) and analyzed on 10% polyacrylamide gels as above.

RESULTS

Detection of Phosphorylated Proteins in 12S Subunits of Aphthovirus. Highly purified ^{32}P-labeled virions were disrupted into 12S subunits, treated with a mixture of RNases, and analyzed on sucrose gradients. The ^{32}P-labeled material sedimented as a sharp peak at the 12S position of the sucrose gradient (Fig. 1*a*). Radioactivity present in the viral subunits was RNase resistant and was insoluble upon treatment with hot (85°C, 10 min) 10% trichloroacetic acid. However, treatment of the starting material with proteinase K resulted in the disappearance of the 12S peak (data not shown). The presence of RNase-resistant trichloroacetic acid-insoluble radioactivity at the top of the gradient could be due to (*i*) the presence of breakdown products (VP_1, VP_2, and VP_3) of the subunits, (*ii*) the presence of capsid protein VP_4, or (*iii*) contamination by RNA fragments. Analysis of proteins present in the 12S subunit (Fig. 1, pool I) taken from the gradient revealed the presence [as expected (15)] of VP_1, VP_2, and VP_3 in equimolar quantities as determined by staining with Coomassie brilliant blue (data not shown). However, the major phosphorylated product (Fig. 1*b*, lane 1) was VP_3; less ^{32}P was incorporated into VP_1 and VP_2. Also, a slight contamination with ^{32}P-labeled VP_4 was observed. The same analysis performed with the top fractions of the gradient (pool II) showed a small amount of ^{32}P-labeled VP_3, and the major phosphorylated polypeptide was VP_4 (Fig. 1*b*, lane 2).

Identification of Phosphorylated Structural Proteins of Aphthovirus. When aphthovirus was labeled *in vivo* with [^{35}S]methionine and purified and the viral proteins were analyzed on urea/$NaDodSO_4$/polyacrylamide gels, four major viral polypeptides were clearly resolved (Fig. 2*a*, lane 3). The positions of VP_0 and P_{56}, present in only one or two copies per virion (16), are also indicated. When the same analysis was performed with virus labeled *in vivo* with inorganic ^{32}P, only two of the major structural polypeptides, VP_3 and VP_4, were found to be highly phosphorylated (Fig. 2*a*, lanes 2 and 4); minor phosphorylated polypeptides, possibly breakdown products of VP_3, migrating between VP_3 and VP_4 and also slightly faster than VP_4 (12) were present; polypeptides VP_0,

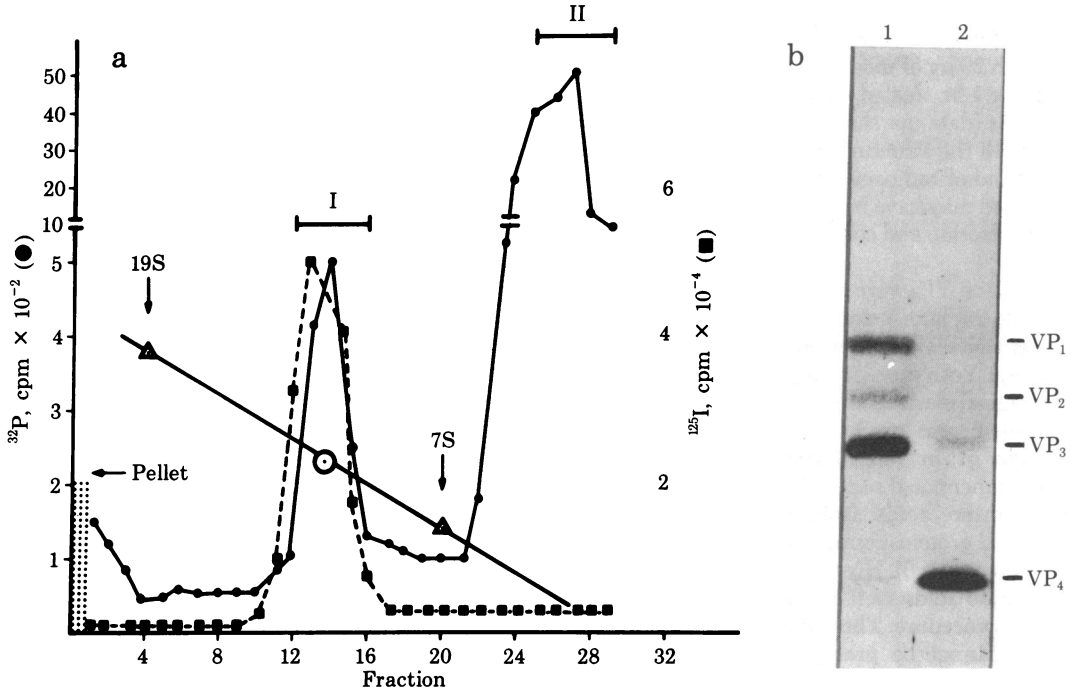

FIG. 1. Preparation and analysis of 12S viral subunits. (*a*) The 12S subunits prepared as described in *Materials and Methods* were layered on top of a 5–20% (wt/vol) sucrose gradient in NET buffer containing 4 M urea and 1% Triton X-100 and centrifuged in an SW 41 rotor at 40,000 rpm for 18 hr at 6°C. Fractions (0.4 ml) were collected from the bottom with a peristaltic pump, and 100 μl from each was treated with hot trichloroacetic acid. The acid-insoluble ^{32}P radioactivity (●) was collected on glass-fiber filters, dried, and analyzed in a liquid scintillation counter. IgG (7 S) and IgM (19 S) were used as internal markers; ^{125}I-labeled purified 12S subunits (■) were run in a parallel gradient. (*b*) Fractions 12–16 (pool I) and 25–29 (pool II) were precipitated with acetone, resuspended in 1× SPB, and subjected to electrophoresis on a 10% polyacrylamide slab gel containing 8 M urea (120 V, 4 hr). The gel was stained, dried, and exposed to X-Omat R Kodak x-ray film with an intensifying screen. Lanes: 1, ^{32}P-labeled proteins from the 12S region (pool I); 2, ^{32}P-labeled proteins from the top fractions (pool II).

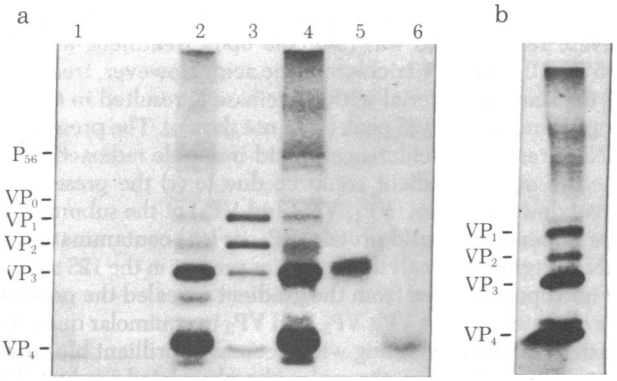

FIG. 2. ^{32}P-Labeled aphthovirus proteins. Proteins labeled *in vivo* were extracted and electrophoresed as in Fig. 1. (*a*) Lanes: 1, 20 μg of viral proteins treated with a mixture of RNases and then with proteinase K; 2, proteins treated with RNases only; 3, [^{35}S]methionine-labeled virus proteins used as markers; 4, double the amount of viral proteins treated with RNases only; 5 and 6, VP_3 and VP_4, respectively, isolated from another gel and treated with RNases. (*b*) ^{32}P-Labeled proteins treated with RNases and with hot 10% trichloroacetic acid.

VP_1, VP_2, and P_{56} were found to be relatively less labeled with ^{32}P but could be clearly seen. The intensity of staining of the labeled polypeptides with Coomassie brilliant blue was the same as in controls of stained unlabeled virus (data not shown).

The ^{32}P label present in the proteins was not removable by extensive treatment with a mixture of RNases or by treatment with hot 10% trichloroacetic acid prior to electrophoresis (Fig. 2*b*). Both procedures ensure that the RNA is degraded into acid-soluble nucleotides (5, 13, 17). However, radioactivity of the ^{32}P-labeled proteins was completely sensitive to proteinase K (Fig. 2*a*, lane 1).

To rule out possible contamination of the protein with RNA fragments or nucleotides, ^{32}P-labeled genomic RNA (6×10^6 cpm) was mixed with 20 μg of unlabeled aphthovirus proteins, incubated at 37°C for 1 hr, treated with the mixture of RNases, and analyzed on gels (data not shown). No radioactivity was found associated with the structural viral polypeptides; only a slightly labeled band of radioactivity, migrating faster than VP_4, was detected and may have been nucleotides or VP_g-pUp (5) labeled in its nucleotide and conceivably also in its protein moiety.

Polypeptides VP_3 and VP_4 were extracted from a gel similar to that shown in Fig. 2*a*, lane 4, and again treated extensively with the mixture of RNases and electrophoresed (Fig. 2*a*, lanes 5 and 6, respectively). Both proteins migrated as homogeneous bands with their original electrophoretic mobility. The recovery of VP_4 (Fig. 2*a*, lane 6) was low.

Characterization of *in Vivo* Labeled Phosphoprotein Linkages. On one-dimensional paper electrophoresis, the main phosphorylated amino acids isolated from unresolved aphthovirus structural proteins comigrated with phosphoserine and phosphothreonine (Fig. 3). However, the presence of other phosphorylated amino acids such as phosphotyrosine cannot be excluded by this procedure. The ratio of phosphoserine and phosphothreonine cannot be precisely established because prolonged treatment of the protein with 6 M HCl hydrolyzes phosphohydroxy amino acid bonds, more so in phosphoserine than in phosphothreonine (13). Because approximately 2–3 times more ^{32}P label is associated with serine than with threonine residues, serine appears to be the more abundantly labeled amino acid residue. In addition, phosphoserine was detected in purified VP_3 (data not shown), but the samples analyzed were too small to rule out the presence of other phosphorylated amino acids. The amount of VP_4 recovered (Fig. 2*a*, lane 6) was insufficient for examination of phosphorylation sites.

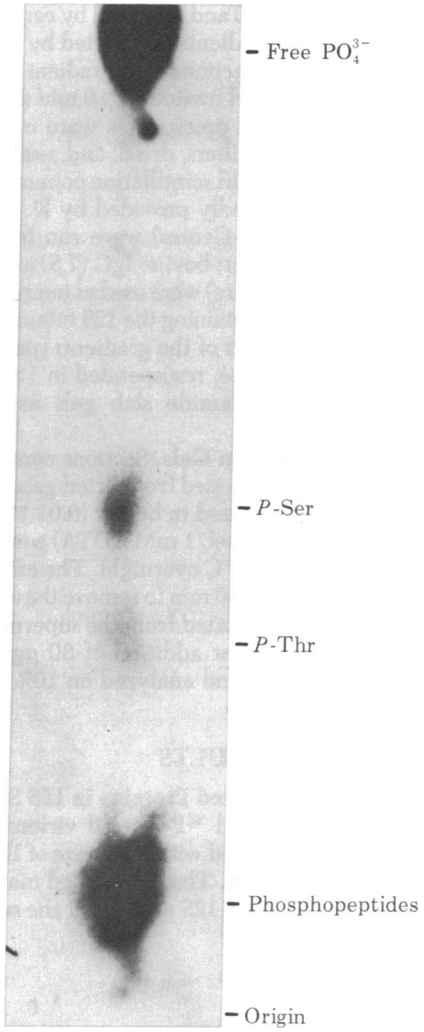

FIG. 3. Identification of protein–phosphate linkages. Samples from total virus protein were digested with 6 M HCl in the presence of 1 mg of bovine gamma globulin *in vacuo* for 5 hr at 105°C. The HCl was evaporated and the samples were resuspended in 20 μl of solvent (2.5% formic acid/7.8% acetic acid), applied to a 20 × 62 cm strip of Whatman 3MM filter paper, and electrophoresed at 3000 V for 2 hr. Unlabeled phosphoserine and phosphothreonine were used as internal markers and were identified by ninhydrin staining. The positions of free orthophosphate and phosphopeptides are also indicated. The paper strips were then exposed to Kodak X-Omat R x-ray film with an intensifying screen.

DISCUSSION

The present results demonstrate that all the structural polypeptides of aphthovirus A_{12}, strain 119ab, are phosphorylated. However, the degree of phosphorylation varies extensively: polypeptides VP_3 and VP_4 are much more highly phosphorylated than are VP_1, VP_2, VP_0, and P_{56} (Fig. 2). Photometric scanning of the autoradiogram shown in Fig. 2*b* indicates that there is 1.7 times more ^{32}P label associated with VP_4 than with VP_3. Also, VP_4 has 13 and 16 times more ^{32}P label than VP_1 and VP_2, respectively. However, in some preparations, the difference in label between VP_1 and VP_4 is more pronounced (Fig. 2*a*, lanes 2 and 4). The reason for this is obscure. Because VP_0 and P_{56} are minor structural polypeptides, their relative degrees of phosphorylation are difficult to establish. In addition, recent analyses of four strains of aphtho-

virus type O_1 have shown them to contain phosphorylated structural proteins.

During viral morphogenesis, all the major structural polypeptides originate from a single common precursor, designated P_{88} (18) or P_{93} (unpublished data). Subsequently, P_{88}/P_{93} is cleaved into VP_3 (the trypsin-sensitive protein) and P_{60}. Later, P_{60} is cleaved into VP_0 and VP_1; VP_0 is the immediate precursor of VP_2 and VP_4. Hence, if phosphorylation occurs prior to the processing of P_{88}/P_{93}, it possibly does so primarily at the ends of this precursor which are the loci of VP_3 and VP_4. Alternatively, phosphorylation may occur late in virus maturation by a virion-associated protein kinase. The fact that analysis of virus-infected cells at various times has failed to demonstrate phosphorylation of any virus-specific precursor polypeptides (data not shown) favors the latter possibility. Furthermore, the arrangement and function of the structural proteins within the virion could influence their degree of phosphorylation.

The role of protein phosphorylation in cells and viruses has been extensively reviewed (19–22). The significance of phosphorylation of the structural proteins in a picornavirus is obscure. However, the introduction or removal of phosphate groups could provide specific signals for proper cleavage of the precursors or for suitable charge interactions or conformational changes of the proteins during assembly of the three-dimensional shell of the virus.

In view of the present results, changes in mobility or isoelectric focusing of picornavirus proteins should be carefully evaluated because different degrees of phosphorylation could account for changes in protein mobility without concomitant changes in amino acid composition. It is well known that VP_3 is more basic than the other capsid proteins: (*i*) solubilization of VP_3 requires 50–70% formic acid or derivatization with organic anhydrides (presumably at basic ϵ-amino groups of surface lysine residues) (23); (*ii*) VP_3 is excluded from anion exchange resins (12); and (*iii*) VP_3 occupies the most cathodic position in isoelectric focusing (24). Hence, it is possible that the phosphorylation of VP_3 is principally at internal rather than at surface amino acid residues. Also, a unique anodic shift in VP_3 occurs in gel electrophoresis in $NaDodSO_4$/8 M urea (12) in which this detergent is de-adsorbed or loosely bound (25). This shift could be caused by conformational changes that unmask internal phosphates or by a reduction in the relative molecular bulk of the protein. On the other hand, regardless of its primary amino acid composition, VP_4 behaves as if phosphorylated in its external amino acid residues because it is the most acidic protein as demonstrated by isoelectric focusing (24).

The authors thank Mr. A. J. Franke and Ms. N. Hahn for their skillful technical assistance. Dr. J. L. La Torre, from the Consejo Nacional de Investigaciones Científicas y Técnicas of Argentina, was a visting scientist at Plum Island and is indebted to the U.S. Department of Agriculture for support and to the Junta Nacional De Carnes Argentina for travel funds.

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