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Phosphate Acceptor Amino Acid Residues in Structural Proteins of Rhabdoviruses

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Partial acid hydrolysates of the [^{32}P]phosphate- or [^{3}H]serine-labeled proteins of purified vesicular stomatitis, rabies, Lagos bat, Mokola, or spring viremia of carp virions and of purified intracellular nucleocapsids of these viruses have been analyzed by paper electrophoresis for the presence of phosphorylated amino acids. Both phosphoserine and phosphothreonine, with the former predominant, were present in virion and nucleocapsid preparations that contained phosphoproteins. An exception was the fish rhabdovirus, which contained only phosphoserine. When vesicular stomatitis or rabies virus proteins were phosphorylated in a cell-free system by the virion-associated protein kinase and analyzed for the presence of phosphorylated amino acid residues, phosphoserine was again found to be more abundant than phosphothreonine. After in vitro protein phosphorylation, another phospho-compound, possibly a third phosphoamino acid, was detected in the partial acid hydrolysates of these viruses.

Two types of protein modification have been observed in rhabdoviruses. The protein of the envelope spikes is glycosylated in all rhabdoviruses which have been analyzed for the presence of glycoproteins (G protein) (3, 13, 27, 28, 31). The other type of modification involves phosphorylation in the infected cells of one or several core proteins. The results of comparative studies on the structural phosphoproteins of various animal rhabdoviruses can be summarized as follows.

(i) In viruses of the vesicular stomatitis subgroup, the core-associated minor NS component is the only phosphoprotein (26, 27). In the cell, the NS protein is also phosphorylated in the free or nucleocapsid-bound state (7, 12).

(ii) In rabies and rabies-related Mokola and Lagos bat (22, 23) viruses, the N proteins, which are directly bound to the viral RNA (28, 30, 31), are phosphorylated in vivo. The N protein of the intracellular free nucleocapsids and the N protein present in the corresponding virions are phosphorylated to a similar extent. The phosphorylation is confined to a terminal segment of the N polypeptide, which can be cleaved off by treatment of the viral nucleocapsid with trypsin (26, 28). For comparison, the N protein of vesicular stomatitis virus (VSV) is not phosphorylated and cannot be cleaved by exposure of the nucleocapsid to trypsin. While the N protein is the only phosphoprotein component of rabies virus, the rabies-related viruses contain two additional structural phosphoproteins.

(iii) Spring viremia virus of carp (SVCV) (5) exhibits a pattern of intracellular phosphorylation which resembles that of rabies-related viruses, the N protein and one additional core protein being phosphorylated in vivo. Although the N phosphoprotein of this virus does not show any antigenic relatedness to the N phosphoproteins of rabies and rabies-related viruses or to the N protein of VSV (28), the phosphorylated segment of the SVCV N protein can be removed too by treatment of the nucleocapsid with trypsin.

(iv) In Kern Canyon virus, which is antigenically not related to VSV or rabies virus (14), the envelope glycoprotein and the N protein seem to be phosphorylated in vivo to a very low extent. On the other hand, the N protein of the free intracellular nucleocapsid is not phosphorylated and, similar to the N protein of VSV, cannot be cleaved by treatment of the nucleocapsid with trypsin.

(v) All rhabdoviruses mentioned above contain a virion-bound protein kinase which can catalyze in a cell-free system the transfer of the gamma-phosphate group of ATP or dATP to viral proteins. The phosphoproteins are usually the best in vitro phosphate acceptors; they accept additional phosphate groups in vitro without losing those acquired in vivo. Among

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the viral proteins which are not phosphorylated in vivo are some which can accept phosphate in a cell-free system (7, 15, 26, 27, 32).

The purpose of the present study was to determine the nature of the phosphoamino acid residues found in rhabdovirus phosphoproteins. It will be shown that viral proteins phosphorylated in vivo or in vitro contain either phosphoserine and phosphothreonine or phosphoserine alone.

MATERIALS AND METHODS

Viruses, their propagation, labeling and purification. Rabies virus (strain ERA, clone W), plaque purified VSV (Indiana serotype), Lagos bat virus, Mokola virus, and SVCV were grown in monolayer cultures of BHK-21 cells as has been described previously (10, 26, 28, 29). Virions released into the serum-free medium containing 0.2% bovine serum albumin were purified by a procedure consisting of precipitation with zinc acetate, resuspension in EDTA solution, filtration through a Sephadex G-75 column, sedimentation by high-speed centrifugation, and banding by centrifugation in a sucrose density gradient (29). The latter step allowed the separation of infectious B particles from defective T particles. Only B particles were used in the present study. If required, the virus collected from the sucrose density gradient was diluted with a neutral buffer and resedimented by high-speed centrifugation.

In some experiments, the virus was labeled throughout the infection with 10 μCi of [^{32}P]phosphate (carrier-free; Schwarz-Mann, Orangeburg, N.Y.) per ml or with 5 μCi of [^{3}H]serine (specific activity, 3.38 Ci/mmol; Schwarz-Mann) per ml. The Eagle minimal essential medium used did not contain unlabeled serine. For labeling with [^{32}P]phosphate, the concentration of unlabeled inorganic phosphate was reduced to one-fifth of the normal level, so that the specific activity of [^{32}P]phosphate in the medium was 10 mCi/mmol.

Isolation of intracellular nucleocapsids. Free intracellular rhabdovirus nucleocapsids were isolated from disrupted cells by repeated isopycnic centrifugation in CsCl solution (25, 26, 28).

Analytical methods. The purified virus or nucleocapsid was prepared for acid hydrolysis in the following manner. Preparations labeled in vitro with [γ-^{32}P]ATP were precipitated, together with 250 μg of carrier bovine serum albumin, with 20% trichloroacetic acid in 0.05 M sodium pyrophosphate. The precipitate was washed with acetone at 0 C and dried. Preparations labeled in vivo were dissociated in sodium dodecyl sulfate solution, ethanol-precipitated, resuspended in 0.1 ml of neutral phosphate buffer, and then treated for 30 min at 20 C with 50 μg of RNase (26). The samples were reprecipitated with trichloroacetic acid, washed with acetone, and dried. Unless stated otherwise, all preparations were then resuspended in 0.3 ml of 2 N HCl and hydrolyzed at 110 C in a sealed, evacuated ampoule for 5 h. The hydrolysates were evaporated three times, redissolved after each evaporation in 0.3 ml of distilled water, and passed through a glass-wool filter. They were then mixed with 15 μg each of unlabeled phosphoserine and phosphothreonine and dried on a strip (4 by 25 mm) of Whatman no. 4 filter paper. The dried sample was added to a strip (2.5 by 28 cm) of Whatman no. 4 paper for electrophoresis at 500 mV and 4 C for 6.5 to 7.5 h in a solution containing 7.8% (vol/vol) acetic acid and 2.5% formic acid (pH 1.85) (34). The electrophoretograms were dried, stained with ninhydrin and, after marking the location of phosphoserine and phosphothreonine bands, cut into 3-mm strips for determination of radioactivity.

The purity of virus or nucleocapsid preparations was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of a sample of dissociated polypeptides, staining of the polypeptide bands by Coomassie blue, scanning of the gels for absorbance, and then cutting of the gel for determination of radioactivity (26, 31). Virus or nucleocapsid preparations which contained significant amounts of polypeptide-bound [^{32}P]phosphate in components other than the viral structural proteins were discarded.

RESULTS

Amino acid residues of rhabdoviral structural proteins phosphorylated in vivo. We have reported previously that the phosphate-amino acid residue bonds in NS or N phosphoproteins of VSV and rabies virus, respectively, are resistant to treatment with hydroxylamine or succinic acid. This indicated that the intracellularly formed phosphate-amino acid residue bonds are not acylphosphates or phosphohistidine. On the other hand, treatment of rabies virus nucleocapsid with alkaline phosphatase resulted in an extensive dephosphorylation of the N protein (26). These findings suggested that the phosphoproteins of VSV and rabies virus contain *O*-phosphoserine or *O*-phosphothreonine residues, or both. Partial acid hydrolysis and electrophoretic analysis of five [^{32}P]phosphate-labeled rhabdoviruses (Fig. 1) and of their intracellular nucleocapsids (Fig. 2) derived from the same cell cultures confirmed this indication. The NS phosphoprotein of VSV, the N phosphoprotein of rabies virus, and the phosphoproteins of Mokola and Lagos bat viruses contained both phosphothreonine and phosphoserine. Phosphoserine residues predominate, in spite of the fact that phosphothreonine is known to be more refractory to decomposition by acid than phosphoserine (1). In hydrolysates of SVCV phosphoproteins, phosphoserine was the only detectable phosphoamino acid.

In addition to the phosphoserine and phosphothreonine residues, the electrophoretograms indicate the presence of several other

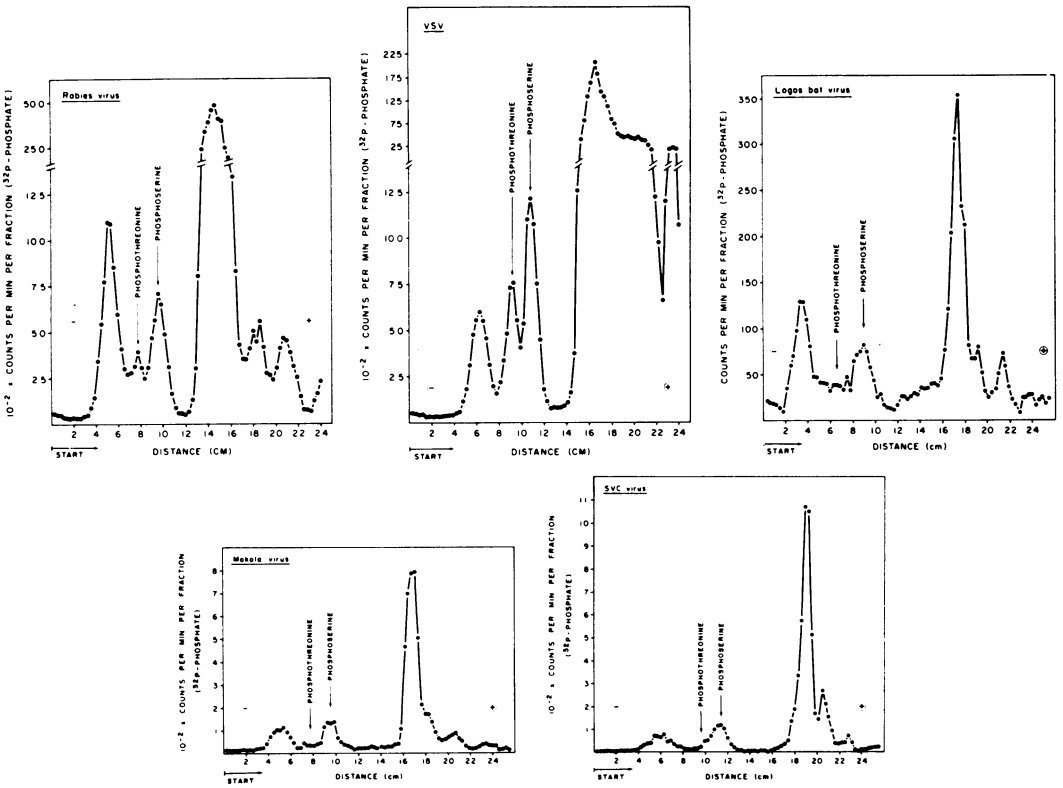

FIG. 1. *Fractionation by paper electrophoresis of partial acid hydrolysates of the protein moiety of five rhabdoviruses. The rhabdoviruses, grown in [^{32}P]phosphate-labeled BHK-21 cells, were purified, freed of lipids and RNA, hydrolyzed in acid, and electrophoresed as described in Materials and Methods. The positions of the internal phosphoserine and phosphothreonine standards, determined after staining with ninhydrin, are indicated by the arrows.*

^{32}P-labeled components bearing a net negative charge. The component migrating more slowly than phosphothreonine represents phosphoprotein hydrolysis (16, 20). This conclusion is supported by the presence of serine residues in this component (Fig. 3). The component containing the greatest amount of ^{32}P radioactivity is inorganic phosphate, since a [^{32}P]phosphate standard electrophoresed in parallel exhibited the same mobility. Two additional [^{32}P]phosphate-containing components of unknown origin, which migrated faster than the inorganic phosphate, were detected in the rhabdovirion hydrolysates. A rapidly migrating component was also detected in the hydrolysates of the [^{3}H]serine-labeled rhabdovirions (Fig. 3). The virtual absence of these compounds from electrophoretograms of [^{32}P]phosphate-labeled viral nucleocapsids, whose protein moiety is represented almost exclusively by the phosphorylated N protein molecules (26, 28), indicates that they probably do not correspond to short phosphopeptides with two or more phosphoamino acid residues.

The possibility that ^{32}P-labeled components other than phosphoamino acid residues could account for the bands observed in the electrophoretograms was examined in the following experiment.

A preparation of intracellular VSV nucleocapsid, containing an amount of ^{32}P radioactivity similar to that present in ^{32}P-labeled rabies virus nucleocapsid preparation used in these experiments (2.81×10^5 versus 2.11×10^5 counts/min), was freed of RNA and contaminating lipids and subjected to partial acid hydrolysis and subsequent electrophoresis. The VSV nucleocapsid is composed of the viral RNA and of nonphosphorylated N protein molecules (26). As expected, phosphoserine, phosphothreonine, or phosphopeptides were not detected in the chromatogram (Fig. 2). The relatively small band of inorganic [^{32}P]phosphate is obviously derived from the residual amount of

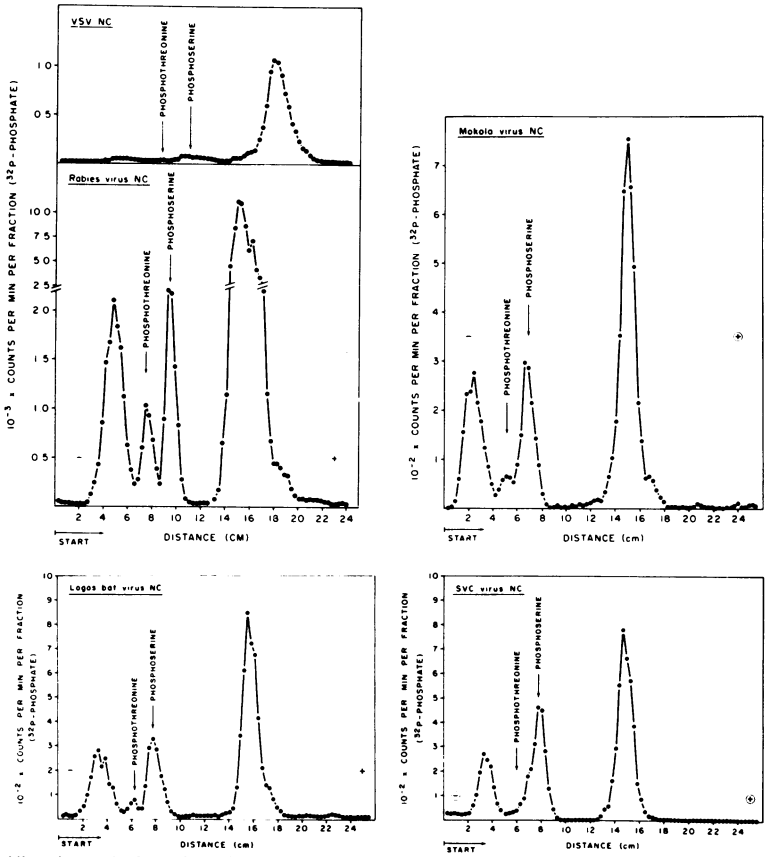

FIG. 2. *Identification of the phosphate acceptor amino acid residues in the phosphoprotein moiety of rhabdovirus nucleocapsids. Intracellular nucleocapsids were isolated from [^{32}P]phosphate-labeled BHK-21 cells, treated with lipid solvents, freed of RNA, hydrolyzed in acid, and subjected to paper electrophoresis. The VSV nucleocapsid was included as a negative control. The band of inorganic [^{32}P]phosphate present in partial acid hydrolysates of this nucleocapsid is probably derived from residual phospholipids or RNA, or both. NC, Nucleocapsid.*

viral RNA which was not removed by ribonuclease digestion. The results also indicate that only about 1% of the inorganic phosphate recovered in the acid hydrolysates of viral nucleocapsids is formed by degradation of residual viral RNA; the overwhelming majority of phosphate must be formed by decomposition of phosphorylated amino acid residues. Manipulations of the conditions of acid treatment so that free phosphoamino acids would be released but not dephosphorylated were only partially successful. Hydrolysis of virions in 6 N HCl at 110 C for 14 h resulted in complete disappearance of phosphoamino acids and phosphopeptides, the only ^{32}P-containing component still detectable in the electrophoretograms being the inorganic phosphate (not shown). Reduction of the standard time of hydrolysis (5 h) in 2 N HCl (at 110 C) to 2.5 h did not change substantially the ratio of phosphoamino acids to phosphopeptides (Fig. 3), indicating that the rate of hydrolysis of phosphopeptides and that of dephosphorylation of free phosphoamino acids are similar. Hydrolysis of phosphoproteins at 110 C for 5 h in 2 N HCl was found to be the most suitable condition for the release and preservation of free phosphoamino acids.

Amino acid residues of rhabdoviral proteins capable of accepting phosphate in vitro. Since viral phosphoproteins can accept in vitro additional phosphate groups ("superphosphorylation") and because some viral proteins which are not phosphorylated in the host cell become phosphorylated in a cell-free system (for references see the introduction), it was of interest to find out whether the phosphate-amino acid residue bonds formed in vitro are of the same nature as those formed in vivo. Proteins of purified B particles of VSV and of rabies virus were phosphorylated by the endogenous protein

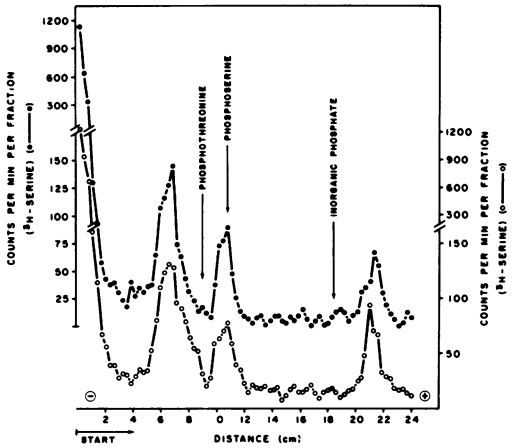

FIG. 3. *Electrophoretic fractionation of products of partial acid hydrolysis of [^3H]serine-labeled rabies virus phosphoprotein. A [^3H]serine-labeled rabies virus preparation, containing 1.7 × 10^6 counts per min per sample, was subjected to hydrolysis in 2 N HCl, at 110 C for 2.5 (○) or 5 (●) h. The products of hydrolysis carrying a net negative charge were separated by paper electrophoresis. The position of phosphothreonine and phosphoserine was determined by coelectrophoresing unlabeled phosphoamino acid standards and staining with ninhydrin. The band of inorganic phosphate was localized by coelectrophoresis of [^{32}P]phosphate.*

kinase in a reaction mixture containing [γ-^{32}P]ATP, subjected to partial acid hydrolysis, and examined by paper electrophoresis (Fig. 4). The hydrolysates of both viruses contained both [^{32}P]phosphoserine and [^{32}P]phosphothreonine, the amount of the latter being relatively very low in the VSV preparation. Inorganic phosphate and phosphopeptides were present, but ^{32}P-labeled components migrating faster than the inorganic phosphate were not. Of interest was the presence in both preparations of an unidentified ^{32}P-labeled compound, possibly a phosphoamino acid, that migrated between phosphoserine and phosphothreonine. Since in vitro phosphorylated proteins of rhabdoviruses (F. Sokol, unpublished observation) or of other viruses (16, 32) cannot be dephosphorylated by treatment with hydroxylamine or acid (at relatively low temperatures), the unknown compound cannot contain acylphosphate bonds and cannot represent phosphohistidine.

DISCUSSION

Cyclic AMP- or cyclic GMP-stimulated protein kinases, capable of catalyzing the phosphorylation of amino acid residues with a free hydroxyl group, are widely distributed in animal tissues and organisms. These enzymes are believed to mediate, via protein phosphorylation, most if not all physiological effects of cyclic nucleotides in animal cells (11). The protein kinases associated with enveloped viruses are not stimulated by cyclic nucleotides to any significant extent (4, 6, 15, 16, 18, 21, 24, 27, 32), suggesting that they represent the free catalytic subunits, devoid of regulatory subunits, of cyclic nucleotide-dependent enzymes (19) or cyclic nucleotide-independent protein kinases. The latter type of enzyme is also found in a variety of tissues of different species, and they too catalyze the formation of an ester between phosphate and the serine and threonine residues of polypeptides (11). Cyclic nucleotide-dependent protein kinases, however, are responsible for only a fraction of the total amount of serine and threonine residue phosphorylation which takes place in an animal cell, this being particularly true in the nuclei of animal cells (8).

It is known that intracellular phosphorylation of the core protein(s) of rhabdoviruses is accomplished before the envelopment of the viral nucleocapsids (26, 28) and possibly even before the assembly of the nucleocapsids (7, 12). It is not known, however, whether the enzyme catalyzing the intracellular phosphorylation of viral proteins is a cyclic AMP-dependent or cyclic nucleotide-independent protein kinase which eventually becomes associated with mature virus particles. Whether the enzymes involved in in vivo and in vitro phosphorylation of viral proteins are identical or not, they both catalyze the phosphorylation of serine residues and, in some rhabdoviruses, also of threonine residues of viral polypeptides. The partial acid hydrolysates of rhabdovirions, but not the corresponding nucleocapsids, were shown to contain as yet unidentified phosphate- and amino acid-containing compounds, the properties of which indicate that they may represent pyrophosphorylated serine or threonine. If pyrophosphorylated amino acid residues are really shown to be constituents of virion proteins, one would have to assume that pyrophosphorylation occurs after virus assembly and is catalyzed by a virus-bound enzyme.

After phosphorylation of VSV and rabies virus proteins in a cell-free system by the endogenous protein kinase, another unknown phosphorylated compound, possibly a phosphoamino acid, was detected in partial acid hydrolysates. Acylphosphate bonds (phosphorylated glutamic or aspartic acid residues) (2), phosphohistidine (9), and the -N-P- bond of phosphoarginine (17), if present in viral phosphoproteins, would have been destroyed by

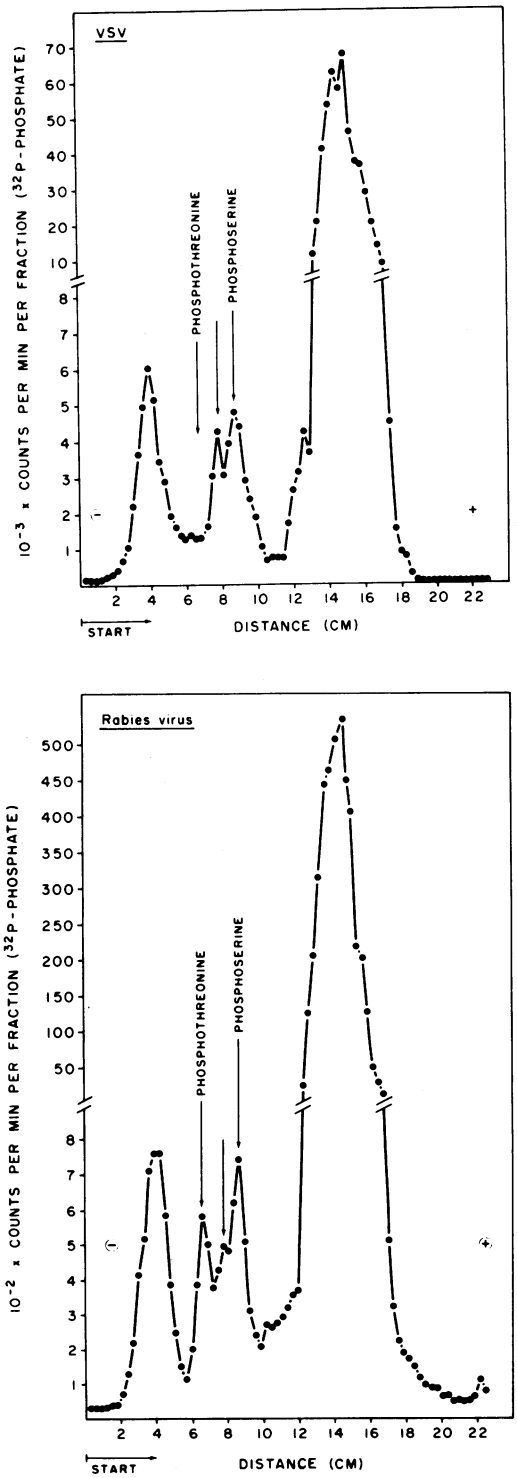

FIG. 4. *Fractionation by paper electrophoresis of products of partial acid hydrolysis of in vitro phosphorylated VSV and rabies virus proteins. The in vitro reaction mixtures contained in 0.29-ml volumes an amount of purified VSV or of rabies virus each corresponding to 110 μg of protein, 12.5 nmol of [γ-^{32}P]ATP (specific activity, 685 counts per min per pmol), 5 μmol of $MgCl_2$, 5 μmol of dithiothreitol, 31.5 μmol of Tris-hydrochloride (pH 7.9), 16.9 μmol of NaCl, 130 nmol of EDTA, and 0.25 μliters of Nonidet P-40. Incubation was at 37 C for 30 min. The phosphorylated virus proteins were hydrolyzed, and the products were fractionated by paper electrophoresis as described in Materials and Methods. No efforts were made to remove the residual ATP adhering to the protein precipitate, and therefore the majority of the ^{32}P radioactivity in the inorganic phosphate band is derived from the nucleoside triphosphate.*

heating in acid. Moreover, acid-labile phosphate-polypeptide bonds were not found in virion phosphoproteins (16, 26, 32). The unidentified phospho-compound could be phosphohydroxyproline.

Very few of the serine and threonine residues of rhabdovirus proteins are phosphorylated in vivo. Although all structural proteins of VSV and of rabies virus contain these two amino acids (26–28), only one protein of each virus becomes phosphorylated in vivo. Moreover, in vivo phosphorylation of the N protein molecules of rabies, rabies-related, and SVC viruses is confined only to a terminal segment of the polypeptide chains (see the introduction), in spite of the fact that the nonphosphorylated portions of the polypeptides also contain serine residues (F. Sokol, unpublished data). Data presented in this paper are only of qualitative character and cannot be utilized for determining the extent of in vivo phosphorylation of rhabdovirus protein. Quantitative data should be obtained by direct determination of the number of phosphate groups per polypeptide.

Analyses similar to those described in the present study were carried out with in vivo phosphorylated proteins of vaccinia virus (20), simian virus 40, togaviruses (33, 35), and with in vitro phosphorylated proteins of an RNA tumor virus (32) and of the frog polyhedral cytoplasmic DNA virus (24). Both serine and threonine residues, predominantly the latter, or only serine residues were found to be phosphorylated. One major and a few minor phosphopeptides were detected in trypsin digests of in vivo phosphorylated vaccinia virions. More than 15 phosphopeptides were found, however, when in vitro phosphorylated vaccinia virus was subjected to similar analysis (20). It would be of interest to determine by fractionation of trypsin digests the site specificity of in vivo phosphorylation of rhabdovirus core proteins.

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