Sites of In Vivo Phosphorylation of Vesicular Stomatitis Virus Matrix Protein

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We mapped the in vivo phosphorylation sites for the matrix (M) protein of the Orsay and San Juan strains of vesicular stomatitis virus, Indiana serotype, using limited proteolysis and phosphoamino acid analysis. M protein was solubilized from ^{32}P -labeled virions by using detergent and high-salt conditions, then treated with either trypsin or *Staphylococcus aureus* V8 protease, and analyzed by polyacrylamide gel electrophoresis and autoradiography to determine which fragments contained phosphate residues. The M protein fragment extending from amino acid 20 to the carboxy terminus contained approximately 70% of the control ^{32}P label, while the fragment extending from amino acid 35 to the carboxy terminus had only trace amounts of label. These data indicate that the major phosphorylation site was between amino acids 20 and 34 in the Orsay strain M protein. Phosphoamino acid analysis of M protein by thin-layer electrophoresis showed the presence of phosphothreonine and phosphoserine and that phosphothreonine continued to be released after prolonged vapor-phase acid hydrolysis. These data identify Thr-31 as the primary in vivo phosphate acceptor for M protein of the Orsay strain of vesicular stomatitis virus. The San Juan strain M protein has serine at position 32, which may also be an important phosphate acceptor. In addition, phosphorylation at Ser-2, -3, or -17 occurs to a greater extent in the San Juan strain M protein than in the Orsay strain M protein. The subcellular distribution of phosphorylated M protein was investigated to determine a probable intracellular site(s) of phosphorylation. Phosphorylated M protein was associated primarily with cellular membranes, suggesting phosphorylation by a membrane-associated kinase. Virion M protein was phosphorylated to a greater extent than membrane-bound M protein, indicating that M protein phosphorylation occurs at a late stage in virus assembly. Phosphorylation of wild-type and temperature-sensitive mutant M protein was studied in vivo at the nonpermissive temperature. The data show that phosphorylated M protein was detected only in wild-type virus-infected cells and virions, suggesting that association with nucleocapsids may be required for M protein phosphorylation or that misfolding of mutant M protein at the nonpermissive temperature prevents phosphorylation.

Vesicular stomatitis virus (VSV), an enveloped, negative-strand RNA virus, has two phosphoproteins, NS protein and M protein (6). The major phosphoprotein, NS, is associated with the viral nucleocapsid and functions together with the L protein as the RNA polymerase. The M protein plays a central role in virus assembly by binding the nucleocapsid to the cytoplasmic surface of the host cell plasma membrane during the budding process. Only a small fraction of the VSV M protein molecules are phosphorylated in vivo (1, 6). While phosphorylation of NS protein is thought to serve a regulatory function in viral transcription, no function has yet been ascribed to phosphorylation of M protein.

Virion-associated protein kinases phosphorylate VSV proteins in vitro following detergent solubilization of the envelope (6, 12, 25). Three distinct kinase activities, with different optimal reaction conditions, have been identified: two are involved in phosphorylation of NS protein, and one is involved in M protein phosphorylation (2). Using limited proteolysis and phosphoamino acid analysis of VSV M protein labeled in vitro, Beckes et al. (1) showed that 93% of the M protein phosphate mapped to serine residues between amino acids 2 and 17.

The purpose of the experiments presented here was to map the in vivo phosphorylation site(s) for the VSV M protein by using limited proteolysis and phosphoamino acid

analysis and to determine whether these sites differed from the sites of phosphorylation in vitro. Further, we studied the location of phosphorylated M protein molecules in VSV-infected cells and whether mutant M protein molecules defective in virus assembly were phosphorylated in vivo.

MATERIALS AND METHODS

Viruses. Original stocks of the Orsay strain (wtO) of VSV (Indiana serotype), the temperature-sensitive (*ts*) M protein mutant *tsO23*, and the temperature-stable revertant *ts23r1* (24, 31) were obtained from John Lenard (University of Medicine and Dentistry of New Jersey). Working stocks were prepared from isolated plaques. Radiolabeled wild-type VSV, Orsay and San Juan strains, and *ts23r1* were prepared as follows. Baby hamster kidney (BHK) cells were infected with cloned virus at a multiplicity of infection of 0.01 PFU per cell. At 1 h postinfection (p.i.), the inoculum was removed and Dulbecco's modified Eagle medium (DMEM) containing 2% fetal calf serum and 20 μCi of [^{35}S]methionine per ml or DMEM containing 10% of the normal phosphate (low-phosphate DMEM), 2% fetal calf serum, and 40 μCi of $^{32}\text{P}_i$ per ml was added to each plate. Cultures were incubated at 37°C overnight. Virus was purified by sucrose gradient centrifugation (19). The virus band was collected and dialyzed overnight against 10 mM Tris–10 mM NaCl, pH 8.1. The *ts* mutant *tsO23* was prepared similarly except that infection was done at 31°C rather than 37°C. The protein

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concentrations of purified viruses were determined by the method of Lowry et al. (18). The amount of radioactivity per microgram of protein was determined by liquid scintillation counting. Purified virus was aliquoted and stored at -70°C .

Purification of nucleocapsid-M protein complexes. Nucleocapsid-M protein complexes were purified as described previously (14). Briefly, purified virus was solubilized with Triton X-100 in low-ionic-strength buffer and then fractionated on a discontinuous sucrose gradient in the absence of detergent. The preparation was stored at 4°C and used within 24 h.

Analysis of proteolytic fragments of VSV M protein for phosphate. The San Juan strain of VSV and *ts23r1* were radiolabeled in vivo with either [^{35}S]methionine or $^{32}\text{P}_i$ as described above and then purified by sucrose gradient centrifugation. Purified virus was treated with 0.5% Triton X-100 in 10 mM Tris (pH 8.1)–250 mM NaCl, for 45 min on ice. Nucleocapsids were separated from the M and G proteins by high-speed centrifugation in a Beckman Airfuge at 30 lb/in 2 (approximately $100,000 \times g$) for 30 min. Aliquots of supernatant containing approximately 8 μg of M protein were treated with either trypsin or *Staphylococcus aureus* V8 protease at various protease/M protein ratios for 15 min at 37°C . Reactions were stopped by the addition of 2 mM phenylmethylsulfonyl fluoride (PMSF). M protein fragments were immunoprecipitated from samples as previously described (17) and then analyzed by polyacrylamide gel electrophoresis (PAGE) and autoradiography. Monoclonal antibody 23H12 was used to immunoprecipitate M protein fragments. This antibody has been previously characterized as being specific for the M protein of VSV, Indiana serotype (20). An isotype-matched monoclonal antibody that does not cross-react with the Indiana serotype of VSV was used as a negative control. Quantitation of radioactivity was determined by soft laser scanning densitometry with digital integration (Biomed Instruments, Inc., Fullerton, Calif.).

Phosphoamino acid analysis. Either purified ^{32}P -labeled virions or purified ^{32}P -labeled nucleocapsid-M protein complexes were subjected to PAGE, then electroblotted onto polyvinylidene difluoride (PVDF) membranes (13), and stained with Coomassie blue as described previously (14). M and NS protein bands were excised and subjected to vapor-phase acid hydrolysis. Membrane pieces were placed in a narrow Pyrex tube in a vial containing a layer of glass beads and 250 μl of 6 N HCl. Vials were then purged with nitrogen, vacuum sealed, and heated to 110°C for various times. Following hydrolysis, membrane pieces were soaked in 100 to 150 μl of buffer A (7.8% acetic acid, 2.5% formic acid, pH 1.9) (9) for 1 h, rinsed once, and then removed from the tubes. Samples were dried in vacuo, and radioactivity was determined by Cerenkov counting. Samples were resuspended in buffer A containing unlabeled phosphoamino acid markers. Aliquots containing 300 to 1,200 cpm were spotted onto cellulose thin-layer plates (Merck, Darmstadt, Germany), and electrophoresis was performed at pH 3.5 in 5% acetic acid–0.5% pyridine (9) for 30 min at 500 V under cooling. Following electrophoresis, the plate was dried and phosphoamino acid markers were visualized by staining with a ninhydrin solution (0.2% in acetone). The plate was subjected to autoradiography and densitometry.

Intracellular localization of phosphorylated VSV M protein. BHK cells were infected with the San Juan strain of VSV at a multiplicity of infection of 10. After a 1-h attachment period at 37°C , the inoculum was removed. Cells were incubated for 2 h in low-phosphate DMEM and then labeled for 3 h in low-phosphate DMEM containing 100 μCi of $^{32}\text{P}_i$

per ml and 2.5% fetal calf serum. Culture supernatants containing labeled virions were harvested, cellular debris was removed by low-speed centrifugation, and virions were pelleted by centrifugation at $100,000 \times g$ for 45 min at 4°C . Cells were rinsed, harvested, pelleted by low-speed centrifugation, resuspended in reticulocyte standard buffer (10 mM Tris [pH 7.4], 10 mM NaCl, 1.5 mM MgCl_2) containing 100 kallikrein units of aprotinin per ml, and homogenized. The homogenates were brought to 100 mM NaCl, nuclei were removed by low-speed centrifugation, and cytoplasmic membranes were pelleted by centrifugation at $100,000 \times g$. The supernatant (cytosol) was removed, and the labeled cellular membranes and virions (isolated from culture supernatants) were solubilized with RIPA buffer (10 mM Tris [pH 7.4], 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 1 mM PMSF) containing 10 mg of bovine serum albumin (BSA) per ml and 100 kallikrein units of aprotinin per ml. Detergents, BSA, and aprotinin were also added to cytosol samples at the same final concentration. Virion, membrane, and cytosol samples, adjusted to the same volume, were clarified by centrifugation at $10,000 \times g$ for 10 min at 4°C . M protein was immunoprecipitated, and samples were subjected to PAGE and fluorography.

Phosphorylation of *tsO23* mutant M protein. Confluent monolayers of BHK cells were infected with either wtO or *tsO23* at a multiplicity of infection of 30. Following a 1-h incubation at 39°C , the inoculum was removed and replaced with DMEM. Cells were labeled with either $^{32}\text{P}_i$ (100 $\mu\text{Ci}/\text{ml}$) or [^{35}S]methionine (25 $\mu\text{Ci}/\text{ml}$) from 2 to 4 h p.i. After the 2-h labeling period, culture supernatants were harvested, and virions were pelleted as described above, resuspended in SDS disruption buffer, boiled, and subjected to PAGE and autoradiography. Cell monolayers were solubilized with RIPA buffer and analyzed by immunoprecipitation, PAGE, and autoradiography as described above.

RESULTS

The purpose of the experiments presented here was to map the in vivo phosphorylation sites for M protein of the Orsay and San Juan strains of VSV (Indiana serotype). We used limited proteolysis of M protein with trypsin or *S. aureus* V8 protease to determine which M protein fragments contained phosphate residues. Proteolytic cleavage sites in VSV M protein were previously determined by using amino-terminal sequencing by Edman degradation (14, 27). Table 1 shows the sequences for the 50 N-terminal amino acids for *ts23r1* M protein, a temperature-stable revertant of the *ts* M protein mutant *tsO23* (24), and for the San Juan strain M protein (28). The M proteins of these two viruses differ at amino acids 21 and 32. Trypsin cleaves the M protein after Lys at positions 19 and 43 producing the 24,000- and 21,000-molecular-weight fragments (24K and 21K fragments), respectively (14, 27). V8 protease cleaves the M protein after Glu at position 21 (in the *ts23r1* strain), 34, or 50, producing 24K, 22K, or 21K fragments, respectively (14).

To determine which proteolytic fragments of VSV M protein contained phosphate residues, *ts23r1* and the San Juan strain of VSV were radiolabeled in vivo with either $^{32}\text{P}_i$ or [^{35}S]methionine and then purified by sucrose-gradient centrifugation. Purified virus was treated with Triton X-100 under high-salt conditions (250 mM NaCl) to solubilize the M protein and the transmembrane glycoprotein (G). Nucleocapsids were pelleted by high-speed centrifugation. Super-

TABLE 1. Proteolytic cleavage sites in VSV M protein^a

Protease	Cleavage site	Fragment ^b	Reference
Trypsin	19	24K	14, 27
	43	21K	27
V8 protease	21 ^c	24K	14
	34	22K	14
	50	21K	14

^a VSV M protein was subjected to limited proteolysis with either trypsin or *S. aureus* V8 protease. Proteolytic fragments of the M protein were subjected to amino-terminal sequencing by Edman degradation. The sequences for the 50 N-terminal amino acids for the M protein of *ts23r1* (24) and the San Juan strain (28) are as follows:

1 50
ts23r1 MSSLEKILGLKGKSKKLEIAPPYEDTNMEYAPSAPIKSTYFGVDE...
 San Juan G S

^b Fragments were determined from the amino acid composition.

^c *tsO23* and its temperature-stable revertants have Glu instead of Gly at position 21 (24). The 24K M protein fragment generated by V8 protease cleavage was sequenced for *ts23r3* (14) and *ts23r1* (unpublished results).

natants containing M and G proteins were treated with either trypsin or V8 protease at the indicated concentrations. M protein fragments were then immunoprecipitated from the supernatants and analyzed by PAGE and autoradiography. Figure 1 shows the results of proteolysis with trypsin for M proteins of the *ts23r1* (panel A) and the San Juan (panel B) strains. For both strains, the 24K trypsin fragment contained most of the ³²P present in the undigested M protein, while the 21K fragment contained very little of the phosphate. A small amount of NS protein was nonspecifically immunoprecipitated and is visible in ³²P-labeled samples, since virion NS protein contains approximately 120 times more phosphate per mole than virion M protein does (1). ³²P-NS protein is present in lanes 6 and 7 only (Fig. 1A and B) because these samples were not treated with trypsin. NS protein is more sensitive to trypsin than M protein is.

The ³²P/³⁵S ratios of the M protein fragments were quantitated by densitometry and are shown in Table 2. The 24K M protein fragment from *ts23r1* contained approximately 70% of the ³²P present in the undigested M protein compared with 50% for the San Juan strain, while approximately 10% of the ³²P was present in the 21K fragment of both viruses. These results indicate that a major site(s) of M protein phosphorylation is located between amino acids 20 and 43

FIG. 1. Phosphorylation of VSV M protein fragments produced by limited proteolysis with trypsin. The *ts* revertant virus *ts23r1* (A) and the San Juan strain of VSV (B) were radiolabeled in vivo with either ³²P_i or [³⁵S]methionine and then purified by sucrose gradient centrifugation. Purified virus was detergent extracted under high-salt conditions to release the M protein from nucleocapsids. Nucleocapsids were pelleted by high-speed centrifugation. Supernatants containing M and G proteins were treated with trypsin at the concentrations indicated below for 15 min at 37°C. M protein fragments were immunoprecipitated from the supernatants and analyzed by PAGE and autoradiography. Trypsin/M protein ratios (wt/wt) were 1.1 (lanes 1 and 8), 0.37 (lanes 2 and 9), 0.12 (lanes 3 and 10), 0.04 (lanes 4 and 11), and 0.01 (lanes 5 and 12). Lanes 6 and 13, no trypsin; 7 and 14, no trypsin and immunoprecipitation was done with a control antibody that does not react with VSV M protein; V, ³⁵S-labeled virions. The letters on the right indicate the positions of the VSV proteins.

TABLE 2. ^{32}P content of proteolytic fragments of VSV M protein^a

Virus strain	Protease	Fragment	Mean $^{32}\text{P}/^{35}\text{S}$ ratio $\pm$ SD (n)
<i>ts23r1</i>	Trypsin	24K	72 $\pm$ 15 (8)
		21K	9 $\pm$ 5 (5)
	V8 Protease	24K	50 $\pm$ 12 (6)
		22K	<10 (5)
		21K	11 $\pm$ 3 (6)
San Juan	Trypsin	24K	50 $\pm$ 9 (4)
		21K	6 $\pm$ 2 (3)
	V8 Protease	22K	<10 (2)
		21K	10 $\pm$ 2 (4)

^a M protein labeled with either $^{32}\text{P}_i$ or [^{35}S]methionine was subjected to limited proteolysis with either trypsin or *S. aureus* V8 protease as described in the legends to Fig. 1 and 2. M protein fragments were immunoprecipitated and analyzed by PAGE and autoradiography. The autoradiograms were quantitated by densitometry, and the $^{32}\text{P}/^{35}\text{S}$ ratios of the M protein fragments were determined as a percentage of the ratio of uncleaved M protein in the same experiment. Values represent the mean $\pm$ standard deviation for the number of experiments indicated.

and that this site contains most of the phosphate in the M protein of the *ts23r1* virus. Both viruses contain additional phosphorylation sites between amino acids 1 and 19 and carboxy terminal to amino acid 43.

Figure 2 shows the results obtained by limited proteolysis of *ts23r1* M protein with V8 protease. Similar amounts of radiolabel were present in the ^{35}S - and ^{32}P -labeled 24K M protein fragments (Fig. 2A), as expected from the results with trypsin shown in Fig. 1, although the relative $^{32}\text{P}/^{35}\text{S}$ ratios of the 24K V8 fragment were somewhat lower than those of the 24K trypsin fragment (Table 2). Little ^{32}P relative to ^{35}S was present in the 22K or 21K fragment. Figure 2B shows a darker exposure of the M protein fragments generated by V8 protease cleavage shown in panel A. Even at this darker exposure, little or no ^{32}P was seen in the 22K fragment. The small amount of ^{32}P in the 21K fragment is readily apparent since the 21K fragment is produced more efficiently than the 22K fragment by cleavage with V8 protease. These results indicate that the majority of the M protein phosphate is located between amino acids 22 and 34. Similar results were obtained following V8 protease treatment of the San Juan M protein, with the exception that no 24K fragment was produced because of the presence of Gly instead of Glu at position 21 (Table 2). Since little P-Tyr has been identified in VSV M protein (8; see below), Thr-31 is the only potential phosphate acceptor between amino acids 22 and 34 in the *ts23r1* M protein (24). Thus, the results in Fig. 2 indicate that Thr-31 is the primary *in vivo* phosphate acceptor for *ts23r1* M protein. The M protein of the San Juan strain has two potential sites of phosphorylation (Thr-31 and Ser-32) in this sequence (28). Thus, it is possible that Ser-32 also may be an important phosphate acceptor.

Phosphoamino acid analysis was done to determine whether threonine was the major phosphate acceptor for *ts23r1* M protein. ^{32}P -labeled M protein was isolated from radiolabeled virions by SDS-PAGE, followed by electroblotting onto PVDF membranes and excision of the M protein bands. The labeled M protein was subjected to acid hydrolysis, and amino acid residues were analyzed for the presence of phosphate by thin-layer electrophoresis and autoradiog-

raphy. Both the rate of release of phosphoamino acids from peptides and the rate of removal of phosphate from phosphoamino acids during acid hydrolysis vary for the different phosphoamino acids. For both reactions, the rates vary in the order phosphotyrosine (P-Tyr) is greater than phosphoserine (P-Ser) is greater than phosphothreonine (P-Thr) (10). For this reason, the M protein phosphoamino acids were analyzed after various times of acid hydrolysis.

Thin-layer electrophoresis of phosphoamino acids released from *ts23r1* virions after 2, 4, and 8 h of hydrolysis is shown in Fig. 3. Equal amounts of radioactivity from each time point were applied to the thin-layer plate. The major ^{32}P -labeled products observed after hydrolysis of M protein were P-Thr, P-Ser, P_i , and an unidentified product of unknown origin (Fig. 3, arrow), which migrated slightly faster than P-Ser. P-Ser and the unidentified product were released at early times of hydrolysis (2 to 4 h), while P-Thr was released primarily at later times (4 to 8 h). By 8 h, most of the labeled P-Ser was hydrolyzed to P_i , while the amount of labeled P-Thr was approximately the same as that at 4 h, presumably because a steady state between hydrolysis of P-Thr and release of P-Thr from peptides had been achieved (see below).

Figure 4 shows the results of acid hydrolysis of M protein isolated by SDS-PAGE of nucleocapsid-M protein complexes rather than virions for the *ts23r1* (panel A) and San Juan (panel B) strains. The major difference from the results shown in Fig. 3 is that the unidentified component was present in lesser amounts (<10% of the ^{32}P label) following detergent extraction of virions and may, therefore, represent a breakdown product of contaminating phospholipids. This unknown phosphorylated component was not present in the M protein proteolysis experiments (Fig. 1 and 2) because of extensive detergent washes during the immunoprecipitation step. After 4 to 8 h of acid hydrolysis, the ratio of P-Ser to P-Thr released was greater for the San Juan strain (Fig. 4B) than for *ts23r1* (Fig. 3 and 4A), indicating that the San Juan M protein has a higher P-Ser content.

It is important to note that even after 8 h of acid hydrolysis, less than half of the M protein radioactivity was present as P-Thr, P-Ser, or P_i . Table 3 shows the distribution of M protein radioactivity as a function of time of hydrolysis for a typical experiment. The radioactivity in P-Thr, P-Ser, and P_i was quantitated by densitometry by comparison with a standard of known radioactivity applied to the same thin-layer plate, using an autoradiogram similar to those of Fig. 3 and 4. Much of the incompletely hydrolyzed M protein remained bound to the PVDF membrane. A significant amount of the radioactivity applied to the thin-layer plate migrated toward the cathode, presumably as positively charged phosphopeptides, and was not detected by autoradiography. Although not specifically commented on, similar behavior of incompletely hydrolyzed peptides can be seen in previously published data (7, 30). The slow rate of hydrolysis of M protein phosphoamino acids shown in Table 3 is consistent with P-Thr being the major phosphoamino acid, since it is the only phosphoamino acid for which the half time for its release in 6 N HCl at 110°C is routinely longer than 4 h (4, 10). This conclusion was confirmed by the observation that after prolonged acid hydrolysis (16 to 28 h), P-Thr was the only phosphoamino acid released (Fig. 4). These data and the results shown in Fig. 1 and 2 are consistent with Thr-31 being the major phosphate acceptor for the VSV M protein, with serine residues as alternative phosphate acceptors.

The amino acid sequence surrounding Thr-31 contains

FIG. 2. (A) Phosphorylation of VSV M protein fragments produced by limited proteolysis with *S. aureus* V8 protease. Purified ^{35}S - or ^{32}P -labeled *ts23r1* was detergent extracted under high-salt conditions. Nucleocapsids were pelleted by high-speed centrifugation, and supernatants were treated with V8 protease at the indicated concentrations. M protein fragments were immunoprecipitated from the supernatants and analyzed by PAGE and autoradiography. V8 protease/M protein ratios (wt/wt) were 0.09 (lanes 1 and 8), 0.03 (lanes 2 and 9), 0.01 (lanes 3 and 10), 0.003 (lanes 4 and 11), and 0.001 (lanes 5 and 12). Lanes: 6 and 13, no protease; 7 and 14, no protease and immunoprecipitation was done with a control antibody that does not react with VSV M protein; V, ^{35}S -labeled virions. The letters on the right indicate the positions of the VSV proteins. (B) A darker exposure of the V8 protease-generated M protein fragments in panel A for better visualization of the 22K fragment.

four acidic residues at positions 28, 29, 30, and 34 (see Table 1). This suggests that M protein could be phosphorylated by protein kinases that require acidic amino acids in their substrates for recognition, such as casein kinases I and II (11). In subcellular fractionation experiments, enzymes with such specificity have been found in both cytosolic and membrane fractions (11). The subcellular distribution of phosphorylated M protein was investigated to determine the probable cellular site(s) of phosphorylation. VSV-infected cells were labeled from 3 to 6 h p.i. with either $^{32}\text{P}_i$ or ^{35}S methionine. Supernatant fluids containing virions were harvested, and infected cells were fractionated into cytosolic and membrane fractions. Following detergent solubilization of virions and cellular membranes, M protein was immunoprecipitated from all three fractions and analyzed by PAGE and autoradiography (Fig. 5). The data show that the level of phosphorylation of intracellular M protein (lanes 2 to 4) is much less than that of virion M protein (lane 1) and is detectable only in the membrane fraction (lane 2). Even

when the volume of cytosol used for immunoprecipitation was three times that used for virion and cell membrane samples (lane 4), no phosphorylated M protein was detected. Lanes 9 to 12 show immunoprecipitates of equivalent amounts of ^{35}S -labeled extracts to control for differences in amounts of M protein precipitated from each fraction. The data show that in infected cells phosphorylated M protein was primarily associated with cellular membranes.

The group III *ts* M protein mutants are defective in virus assembly (15). However, M protein is synthesized at the nonpermissive temperature, associates with membranes in the absence of nucleocapsids (23), and is released from cells in particles devoid of nucleocapsids (29). We studied the phosphorylation of wild-type and *tsO23* mutant M protein in vivo at the nonpermissive temperature to determine whether the M protein of the *ts* mutant was phosphorylated. Cells were infected with *tsO23* at 39°C in the presence of either $^{32}\text{P}_i$ or ^{35}S methionine. Control cultures were infected with wtO at the same temperature. At 4 h p.i., culture superna-

FIG. 3. Phosphoamino acid analysis of VSV M protein. The temperature-stable revertant virus *ts23r1* was labeled in vivo with $^{32}\text{P}_i$ and then purified by sucrose gradient centrifugation. Labeled virions were disrupted and subjected to PAGE. Viral proteins were electroblotted onto a PVDF membrane and stained with Coomassie blue. M and NS protein bands were excised and subjected to vapor-phase acid hydrolysis in 6 N HCl at 110°C for the indicated times. Phosphoamino acid markers were added to each sample. Samples were spotted onto a cellulose thin-layer plate and subjected to electrophoresis for 30 min at 500 V under cooling. The plate was sprayed with ninhydrin to visualize markers, and the labeled phosphoamino acids were detected by autoradiography. The arrow indicates the position of migration of an unknown phosphorylated product. NS, VSV NS protein hydrolyzed for 4 h.

tants were harvested and virions were pelleted by ultracentrifugation. Intracellular M protein was solubilized with detergents, immunoprecipitated, and subjected to PAGE and autoradiography (Fig. 6). The data show that intracellular M protein is synthesized at the nonpermissive temperature in cells infected with either mutant (lane 5) or wild-type (lane 6) virus, although to a much greater extent in wtO-infected cells. However, only wild-type M protein is phosphorylated (lanes 3 and 4). Mutant M protein migrated slightly more slowly than wild-type M protein, as previously reported, because of the substitution of Glu for Gly at position 21 (24). Virions were harvested from ^{32}P -labeled (lanes 9 to 12) and ^{35}S -labeled (lanes 13 and 14) culture supernatants at 4 h p.i. and were analyzed directly, without immunoprecipitation. Only one-fifth volume wtO virus samples (lanes 11, 12, and 14) were loaded onto the gel to prevent overexposure of the film relative to *tsO23* virus samples (lanes 9, 10, and 13), for which the entire sample was loaded. The data show that M protein was present in particles or virions from both *tsO23*-infected (lane 13) and wtO-infected (lane 14) culture supernatants, respectively, although to a much greater extent (>50-fold more) in the wtO sample. The wtO virion M protein was phosphorylated, as evident in lanes 11 and 12; however, no phosphorylated M protein was detected in the particles released from *tsO23*-infected cells (lanes 9 and 10).

DISCUSSION

The data presented here identify Thr-31 as the primary in vivo phosphorylation site for M protein of the Orsay strain of VSV. The M protein of the San Juan strain of VSV has serine at position 32 (28), which also may be an important phosphate acceptor. There are additional phosphate acceptors within the M protein sequence between residues 1 and 19 and minor phosphate acceptors carboxy terminal to

FIG. 4. Phosphoamino acid analysis of VSV M protein isolated from nucleocapsid-M protein complexes. ^{32}P -labeled purified *ts23r1* (A) or the San Juan strain of VSV (B) was extracted with Triton X-100, and the nucleocapsid-M protein complexes were isolated by sucrose gradient centrifugation and subjected to PAGE. M and NS proteins were acid hydrolyzed and analyzed by thin-layer electrophoresis as described in the legend to Fig. 3. The arrows indicate the positions of migration of an unknown phosphorylated product. NS, VSV NS protein hydrolyzed for 4 h.

amino acid 50. Trace amounts of P-Tyr were also detected as reported previously (8).

The results in vivo phosphorylation site for *ts23r1* M protein differs from the reported site for phosphorylation of VSV M protein by a virion-associated kinase in vitro (1), which involves serine residues at position 2, 3, or 17. The results from proteolysis with trypsin indicate that approximately 30% of Orsay M protein phosphorylation in vivo occurs within this region (Table 2). Although analysis with V8 protease gives a somewhat larger number (approximately 50%), the results with trypsin are more reliable, since the M protein is nearly quantitatively converted to the 24K fragment at low concentrations of trypsin (Fig. 1). In contrast, the 24K fragment resulting from cleavage with V8 protease appears to be a kinetic intermediate between intact M protein and lower-molecular-weight forms at all concentrations of V8 protease (Fig. 2). Thus, small differences in the rates of cleavage of phosphorylated versus unphosphorylated M protein could account for the lower $^{32}\text{P}/^{35}\text{S}$ ratio for the 24K fragment generated by V8 protease compared with that generated by trypsin (Table 2).

TABLE 3. Time course of acid hydrolysis of ^{32}P -labeled M protein^a

Labeled species	Radioactivity (% of total ^{32}P cpm) at:		
	2 h	4 h	8 h
P _i	11	17	19
P-Ser	5	2	<1
P-Thr	<1	2	6
Phosphopeptides			
Adsorbed ^b	47	39	34
Not detected ^c	37	40	39

^a Nucleocapsid-M protein complexes were isolated from ^{32}P -labeled ts23r1 virions and subjected to PAGE. Viral proteins were electrophoretically transferred onto PVDF membranes. M protein bands were excised and subjected to vapor-phase acid hydrolysis in 6 N HCl at 110°C for the indicated times. ^{32}P radioactivity remaining adsorbed to the membrane or released by hydrolysis was determined by Cerenkov counting. One-half of each sample (approximately 1,200 cpm) was analyzed by thin-layer electrophoresis and autoradiography. The radioactivity in each species was determined by densitometry by comparison with a known amount of ^{32}P spotted onto the same plate. The fraction of radioactivity that was not detected by autoradiography (presumably because it migrated toward the cathode) was determined by subtracting the counts per minute in P_i, P-Ser, and P-Thr from the total counts per minute applied to the plate. Data shown are from an experiment that is representative of seven separate experiments.

^b Phosphopeptides adsorbed to the PVDF membrane.

^c Phosphopeptides applied to the thin-layer plate but not detected by autoradiography.

The kinetics of hydrolysis of Orsay M protein phosphoamino acids (Table 3) are more consistent with Thr-31 as the major phosphate acceptor than with Ser-2, -3, or -17. The rate of release of free phosphoamino acids from phosphopeptides is influenced by the sequence context of the phosphoamino acid, with the half time for release of P-Ser ranging from 1 to 2 h (4), compared with >7 h for P-Thr (4, 22, 30). Superimposed on hydrolysis of the phosphoamino acid peptide bonds is hydrolysis of the phosphoester bond to release P_i. This reaction also occurs about four to five times faster for P-Ser than for P-Thr (4). By 8 h of hydrolysis,

P-Ser was almost completely hydrolyzed to P_i (Fig. 3 and 4), yet most of the M protein phosphate was still in the form of phosphopeptides, which continued to yield P-Thr upon prolonged hydrolysis. If the contribution of P-Thr hydrolysis is considered to be negligible, the amount of P_i released by 8 h of acid hydrolysis would put an upper limit on the amount of P-Ser at about 20% of the total M protein phosphate (Table 3).

Previous reports of phosphoamino acid analyses of M protein labeled in vivo showed serine as the primary phosphate acceptor (3, 7, 8). Part of the reason is that the M protein from the San Juan strain was analyzed (7, 8), which, according to our results, may have similar amounts of P-Ser and P-Thr. Another factor is that the kinetics of hydrolysis of P-Ser in M protein are similar to those of other phosphoproteins while the rate of hydrolysis of P-Thr appears to be unusually slow. This is shown by the low steady-state level of P-Thr achieved at 8 h of hydrolysis, which is about 6% of the total ^{32}P (Table 3) compared with typical values of 10 to 20% (4) and by the fact that much less than half of the P-Thr had been released by 8 h. This is presumably due to the sequence context surrounding the phosphorylation site. In two previously published reports (3, 8) hydrolysis of VSV M protein was carried out in 6 N HCl at 110°C for 1.5 h, which we have shown to be insufficient time to release significant amounts of P-Thr (Fig. 3 and 4). In one case, data are presented to account for all of the ^{32}P in labeled M protein after hydrolysis in 2 N HCl at 110°C for 5 h (Fig. 2 in reference 7). According to the data in reference 4, hydrolysis under these conditions is similar to that in 6 N HCl for 2 h. From the data presented, it can be calculated that 8% of the ^{32}P is P_i, 4% is P-Ser, 1% is P-Thr, 20% is phosphopeptide that migrates toward the cathode slowly enough to be retained, and 67% is not detected, presumably by migrating off the support during electrophoresis. Given the differences in experimental protocol, these data are in remarkably good agreement with the results obtained after 2 h of acid hydrolysis presented here (Table 3).

FIG. 5. Subcellular distribution of phosphorylated M protein. Cells infected with the San Juan strain of VSV were labeled for 3 to 6 h p.i. with either ^{32}P (lanes 1 to 7) or ^{35}S methionine (lanes 8 to 15). Supernatant fluids containing virions were collected, and infected cells were fractionated into cytosolic and membrane fractions. Following detergent solubilization of virions and plasma membranes, M protein was immunoprecipitated from the three fractions and analyzed by PAGE and autoradiography. Lanes: 1 and 9, M protein from disrupted virions; 2 and 10, M protein from membranes; 3 and 11, M protein from cytosol; 4 and 12, M protein from a threefold greater volume of cytosol; 5 and 13, disrupted virions treated with nonspecific antibody; 6 and 14, membranes treated with nonspecific antibody; 7 and 15, cytosol treated with nonspecific antibody; 8, ^{35}S -labeled virions. The letters on the right indicate the positions of the VSV proteins.

FIG. 6. Phosphorylation of *tsO23* mutant M protein. BHK cells were infected with *tsO23* at 39°C in the presence of either $^{32}\text{P}_i$ or [^{35}S]methionine. Control cultures were infected with wtO and radiolabeled at the same temperature. At 4 h p.i., intracellular M protein (lanes 1 to 8) was solubilized with detergents, immunoprecipitated, and subjected to PAGE and autoradiography. Culture supernatants were harvested, and virions were pelleted by high-speed centrifugation and analyzed directly without immunoprecipitation (lanes 9 to 14). Lanes: 1 and 2, duplicate samples of ^{32}P -M protein from *tsO23*-infected cells; 3 and 4, duplicate samples of ^{32}P -M protein from wtO-infected cells; 5, ^{35}S -M protein from *tsO23*-infected cells; 6, ^{35}S -M protein from wtO-infected cells; 7, ^{32}P -labeled *tsO23*-infected cell lysate treated with nonspecific antibody; 8, ^{35}S -labeled wtO-infected cell lysate treated with nonspecific antibody; 9 and 10, duplicate samples of ^{32}P -*tsO23* virions; 11 and 12, duplicate samples of ^{32}P -wtO virions; 13, ^{35}S -*tsO23* particles; 14, ^{35}S -wtO virions. Only one-fifth volume wtO virus samples (lanes 11, 12, and 14) was loaded onto the gel to prevent overexposure of the film relative to *tsO23* virus samples (lanes 9, 10, and 13), for which the entire sample was loaded. The letters on the right indicate the positions of the VSV proteins.

The amino acid sequence surrounding Thr-31 and Ser-32 matches the consensus sequence recognition sites for casein kinases I and II (11). Casein kinase I phosphorylates serine residues three amino acid positions carboxy terminal to Glu, and casein kinase II phosphorylates serine residues 3 amino acids N terminal to Glu (11). In the absence of available serine residues, casein kinases will phosphorylate threonine residues (11). Protein kinases with these specificities have been observed for both cytosolic and membrane subcellular fractions (11). Phosphorylated M protein was found to be primarily associated with cellular membranes (Fig. 5), suggesting that M protein is phosphorylated by a membrane-associated kinase. Alternatively, phosphorylation of M protein in the cytosol could be followed rapidly by association of M protein with membranes. Phosphorylation of cytosolic M protein has been observed in VSV-infected *Drosophila* cells (3). This difference from our results with BHK cells could be due either to phosphorylation of M protein by a different kinase in *Drosophila* cells or to cell type differences in the distribution of the M protein kinase.

The fact that phosphorylated M protein was associated primarily with cellular membranes and the fact that virion M protein was phosphorylated to a greater extent than membrane-bound M protein (Fig. 5) suggest that M protein phosphorylation occurs at a late stage in virus assembly. A role for M protein phosphorylation in virus assembly has also been suggested for members of the closely related paramyxovirus group. However, for these viruses, phosphorylated M protein is found primarily in infected cells rather than in virions (16). This suggests that dephosphorylation of paramyxovirus M protein may be associated with virus assembly.

A role for M protein phosphorylation in virus assembly was further supported by the observation that the *tsO23* M protein, which associates with membranes (23) but does not initiate budding of infectious virus (29), was not phosphorylated at the nonpermissive temperature (Fig. 6). Several lines of evidence suggest that the block in assembly of group III *ts* mutant viruses at the nonpermissive temperature is due to

failure of the mutant M protein to associate with nucleocapsids (5, 21, 26, 31). The results obtained here with *tsO23* might be due to a requirement for association with nucleocapsids for M protein phosphorylation to occur. Alternatively, the mutant M protein might have misfolded at the nonpermissive temperature such that it was no longer a substrate for its protein kinase. In either case, the lack of phosphorylation at the nonpermissive temperature does not imply that it is responsible for the block in assembly, only that there is a correlation between the two events.

Future studies will investigate the role of M protein phosphorylation in virus replication. Since only a small percentage of the M protein molecules in virions are phosphorylated (1, 6), it is clear that M protein phosphorylation is not essential for incorporation into virions. However, M protein phosphorylation may play a role in those aspects of virus assembly in which only a small percentage of M protein molecules participate, such as initiation of binding of nucleocapsids to membranes, or in the terminal events in virion release by budding.

ACKNOWLEDGMENTS

We thank John Lenard for original stocks of Orsay wild-type, *tsO23*, and revertant viruses. We also thank Mark Lively for helpful advice on sequence analysis and vapor-phase acid hydrolysis and Ivo van de Rijn for advice on phosphoamino acid analysis by thin-layer electrophoresis.

This work was supported by Public Health Service grant AI15892 from the National Institute of Allergy and Infectious Diseases. P.K. was supported by Public Health Service fellowship AI08239 from the National Institute of Allergy and Infectious Diseases. Amino acid sequence analyses were performed in the Protein Analysis Core Laboratory of the Cancer Center of Wake Forest University, supported in part by NIH grants CA-12197 and RR-04869 and a grant from the North Carolina Biotechnology Center.

REFERENCES

1. Beckes, J. D., L. C. Childers, and J. Perrault. 1989. Phosphorylation of vesicular stomatitis virus M protein: evidence for a second virion-associated protein serine kinase activity. *Virology* 169:161-171.

2. Beckes, J. D., and J. Perrault. 1991. Two distinct protein kinase activities in vesicular stomatitis virions phosphorylate the NS transcription factor. *Virology* **184**:383–386.
3. Blondel, D., S. Dezelee, and F. Wyers. 1983. Vesicular stomatitis virus growth in *Drosophila melanogaster* cells. *J. Gen. Virol.* **64**:1793–1799.
4. Bylund, D. B., and T.-S. Huang. 1976. Decomposition of phosphoserine and phosphothreonine during acid hydrolysis. *Anal. Biochem.* **73**:477–485.
5. Carroll, A. R., and R. R. Wagner. 1979. Role of the membrane (M) protein in endogenous inhibition of in vitro transcription by vesicular stomatitis virus. *J. Virol.* **29**:134–142.
6. Clinton, G. M., B. W. Burge, and A. S. Huang. 1978. Effects of phosphorylation and pH on the association of NS protein with vesicular stomatitis virus cores. *J. Virol.* **27**:340–346.
7. Clinton, G. M., B. W. Burge, and A. S. Huang. 1979. Phosphoproteins of vesicular stomatitis virus: identity and interconversion of phosphorylated forms. *Virology* **99**:84–94.
8. Clinton, G. M., and A. S. Huang. 1981. Distribution of phosphoserine, phosphothreonine and phosphotyrosine in proteins of vesicular stomatitis virus. *Virology* **108**:510–514.
9. Cooper, J. A., B. M. Sefton, and T. Hunter. 1983. Detection and quantification of phosphotyrosine in proteins. *Methods Enzymol.* **99**:387–402.
10. Duclos, B., S. Marcandier, and A. J. Cozzone. 1991. Chemical properties and separation of phosphoamino acids by thin-layer chromatography and/or electrophoresis. *Methods Enzymol.* **201**:10–21.
11. Edelman, A. M., D. K. Blumenthal, and E. G. Krebs. 1987. Protein serine/threonine kinases. *Annu. Rev. Biochem.* **56**:567–613.
12. Imblum, R. L., and R. R. Wagner. 1974. Protein kinase and phosphoproteins of vesicular stomatitis virus. *J. Virol.* **13**:113–124.
13. Kamps, M. P. 1991. Determination of phosphoamino acid composition by acid hydrolysis of protein blotted to Immobilon. *Methods Enzymol.* **201**:21–27.
14. Kaptur, P. E., R. B. Rhodes, and D. S. Lyles. 1991. Sequences of the vesicular stomatitis virus matrix protein involved in binding to nucleocapsids. *J. Virol.* **65**:1057–1065.
15. Knipe, D., H. F. Lodish, and D. Baltimore. 1977. Analysis of the defects of temperature-sensitive mutants of vesicular stomatitis virus: intracellular degradation of specific viral proteins. *J. Virol.* **21**:1140–1148.
16. Lamb, R. A., and P. W. Choppin. 1977. The synthesis of Sendai virus polypeptides in infected cells. III. Phosphorylation of polypeptides. *Virology* **81**:382–397.
17. Lefrancois, L., and D. S. Lyles. 1982. The interaction of antibody with the major surface glycoprotein of vesicular stomatitis virus. I. Analysis of neutralizing epitopes with monoclonal antibodies. *Virology* **121**:157–167.
18. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275.
19. Lyles, D. S. 1979. Glycoproteins of Sendai virus are transmembrane proteins. *Proc. Natl. Acad. Sci. USA* **76**:5621–5625.
20. Lyles, D. S., L. Puddington, and B. J. McCreedy, Jr. 1988. Vesicular stomatitis virus M protein in the nuclei of infected cells. *J. Virol.* **62**:4387–4392.
21. Mancarella, D. A., and J. Lenard. 1981. Interactions of wild-type and mutant M protein of vesicular stomatitis virus with viral nucleocapsid and envelope in intact virions. Evidence from [¹²⁵I]iodonaphthyl azide labelling and specific cross-linking. *Biochemistry* **20**:6872–6877.
22. Matsumura, S., N. Murakami, S. Yasuda, and A. Kumon. 1982. Site-specific phosphorylation of brain myosin light chains by calcium-dependent and calcium-independent myosin kinases. *Biochem. Biophys. Res. Commun.* **109**:683–688.
23. McCreedy, B. J., Jr., and D. S. Lyles. 1989. Distribution of M protein and nucleocapsid protein of vesicular stomatitis virus in infected cell plasma membranes. *Virus Res.* **14**:189–206.
24. Morita, K., R. Vanderroef, and J. Lenard. 1987. Phenotypic revertants of temperature-sensitive M protein mutants of vesicular stomatitis virus: sequence analysis and functional characterization. *J. Virol.* **61**:256–263.
25. Moyer, S. A., and D. F. Summers. 1974. Phosphorylation of vesicular stomatitis virus in vivo and in vitro. *J. Virol.* **13**:455–465.
26. Ono, K., M. E. Dubois-Dalcq, M. Schubert, and R. A. Lazzarini. 1987. A mutated membrane protein of vesicular stomatitis virus has an abnormal distribution within the infected cell and causes defective budding. *J. Virol.* **61**:1332–1341.
27. Pal, R., B. W. Grinnell, R. M. Snyder, J. R. Wiener, W. A. Volk, and R. R. Wagner. 1985. Monoclonal antibodies to the M protein of vesicular stomatitis virus (Indiana serotype) and to a cDNA M gene expression product. *J. Virol.* **55**:298–306.
28. Rose, J. K., and C. J. Gallione. 1981. Nucleotide sequences of the mRNAs encoding the vesicular stomatitis virus G and M proteins determined from cDNA clones containing the complete coding regions. *J. Virol.* **39**:519–528.
29. Schnitzer, T. J., and H. F. Lodish. 1979. Noninfectious vesicular stomatitis virus particles deficient in the viral nucleocapsid. *J. Virol.* **29**:443–447.
30. Simmerman, H. K. B., J. H. Collins, J. L. Theibert, A. D. Wegener, and L. R. Jones. 1986. Sequence analysis of phospholamban: identification of phosphorylation sites and two major structural domains. *J. Biol. Chem.* **261**:13333–13341.
31. Wilson, T., and J. Lenard. 1981. Interaction of wild type and mutant M protein of vesicular stomatitis virus with nucleocapsids *in vitro*. *Biochemistry* **20**:1349–1354.