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SHORT COMMUNICATION

High Phosphorylation Potential of the P Protein

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Previously we showed that the Sendai virus P protein (568 aa) in virus-infected cells and in virions was primarily and constitutively phosphorylated on serine(s) in a single tryptic phosphopeptide TP1. By two-dimensional thin-layer electrophoresis and chromatography analysis of tryptic phosphopeptides of several deletion and point mutants of the P protein, we now show that the sole phosphorylation site in TP1 is serine249. Interestingly, when serine249 was deleted or mutagenized alternate potential serine sites were more heavily phosphorylated. A similar effect was observed when the deletion was very close to serine249 (Δ208–236). Mutagenesis of proline250 to alanine abrogated phosphorylation at serine249 suggesting that proline250 is essential for the primary phosphorylation of the P protein. Conceivably, serine249 phosphorylation is mediated by a proline-directed protein kinase. This finding is unusual because a majority of the P proteins from other negative-strand RNA viruses have been shown to be phosphorylated primarily by casein kinase II. Our results demonstrate that the P protein has a strong potency to remain phosphorylated. Based on our previous and present results, we suggest that the phosphorylation sites on P are dependent on the accessibility of phosphatases rather than kinases as all potential sites are about equally competent for phosphorylation. We propose that phosphorylation is important for maintaining the structural integrity of the Sendai virus P protein.

Sendai virus, a prototypic paramyxovirus, belongs to the order of Mononegavirales. These are enveloped RNA viruses whose genome is a single strand of RNA with negative polarity. The genomic RNA is tightly covered with the nucleocapsid protein (NP) in a helical conformation. Immediately following the entry of viral helical RNP (nucleocapsid) into the cytoplasm, RNP-associated viral RNA-dependent RNA polymerase transcribes subgenomic size mRNAs (*1*). Two viral proteins, the polymerase-associated phosphoprotein (P) and the large protein (L), constitute the viral polymerase activity (*2, 3*). Over the past two decades, several studies have been carried out to define structural and functional aspects of the Sendai virus P protein. It was demonstrated by employing V8 protease digestion of the Sendai virus nucleocapsids that the carboxy terminal region of P bound to the nucleocapsid (*4*). This study was later confirmed with the observation that the carboxy terminal region contained two noncontiguous domains which were necessary in its binding to the viral nucleocapsid (*5*). Another region in the carboxy terminal portion of the P protein was found to be necessary for interaction with the L protein (*6*). Recently it was shown that the 77 amino terminal residues of P are important both for viral RNA synthesis and for binding to the cytosolic NP protein (*7, 8*). Clearly P protein elicits several functions by virtue of binding to the viral nucleocapsid, the NP, and L proteins.

The P protein in all paramyxoviruses is highly phosphorylated on a mole-per-mole basis in comparison to other viral proteins. However, the functional significance of the P protein phosphorylation is not clear. This in part may stem from the lack of precise information on the site of phosphorylation in the P protein. Two previous attempts identified the phosphorylation sites in the amino terminal half of the Sendai virus P protein but yielded ambiguous results with regard to their the precise location (*9, 10*). Previous studies have suggested that the amino terminal region of this protein which putatively contained the phosphorylation site(s) was not required for viral transcription (*11*). However, a recent cell-free transcription study has suggested a transcription enhancing role of phosphorylation in respiratory syncytial virus (RSV), another paramyxovirus (*12*). In evolutionarily related vesicular stomatitis virus (VSV), cell-free phosphorylation of the analogous P protein has similarly been implicated in enhancing the transcriptional activity of the viral polymerase (*13, 14*). However, this role of phosphorylation has been challenged by other investigators (*15, 16*). A recent study showed that the primary phophoryla-

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tion of the VSV P protein with casein kinase II was important for multimerization of the P protein (*16*). Thus, despite several studies, the role of P protein phosphorylation in both paramyxoviruses and rhabdoviruses remains unclear. As an alternate means for deciphering the functional role of the Sendai virus P protein phosphorylation, we sought to determine the precise phosphorylation site(s) in the protein from infected cells.

Our recent studies showed that the Sendai virus P protein in virus-infected cells and in purifed virions was phosphorylated primarily and constitutively on serine residue(s) in one major tryptic phosphopeptide (TP1) and that the other viral proteins are not necessary for its phosphorylation (*18*). This suggested that the P protein is phosphorylated by a cellular kinase or that the P protein is an autokinase. Further, our studies showed that the cell-free phosphorylation sites of the P protein were entirely different from those in virus-infected cells. These studies indicated that investigations on P phosphorylation must be carried out intracellularly for meaningful results. Now, we have identified the primary site of phosphorylation in the P protein by expressing its deletion and point mutants in mammalian cells. Our results show that the P protein has an strong tendency to remain phosphorylated. Disruption of its primary phosphorylation site or of the region surrounding the site causes alternate potential sites to be more heavily phosphorylated.

To identify the location of phosphoserine(s) in the P protein, we created several deletion mutants spanning 316 amino terminal residues of the protein (Fig. 1A). Due to the unavailability of an antibody that would recognize amino terminal half of the P protein, we could not use any carboxy terminal deletion mutants in our studies. The mutants were expressed by transfection in CV1 cells using vaccinia virus/T7 RNA polymerase expression system (*19*). To assess the level of P expression and its phosphorylation, cells were labeled with [^{35}S]Translabel or [^{32}P]orthophosphate in parallel experiments and P was immunoprecipitated and analyzed by SDS–PAGE as described previously by us (*18, 20*).

Although the level of P protein expression was similar for all the mutants, the level of phosphorylation varied widely in different mutants (Figs. 1B and 1C). While mutant Δ238–253 was phosphorylated as efficiently as the wildtype P protein, mutants Δ10–143 and Δ208–236 were about 40–50% more phosphorylated. In mutants Δ10–316, Δ215–316, and Δ208–253, the level of phosphorylation ranged from 20 to 50% compared to the wildtype P protein (Figs. 1B and 1C). These results created problem in our assigning any specific region of the P protein to be the site for its primary phosphorylation. Therefore, to determine the primary phosphorylation site precisely, we first localized the site of TP1 (renamed as TPi1) in P with the use of deletion mutants described above. Each deletion phosphoprotein was analyzed for its phosphoamino acid composition and TPi1 presence. All deletion mutants were phosphorylated only at serine residues (data not presented). The mutants which lacked the TPi1 peptide were Δ10–316, Δ215–316, Δ208–253, and Δ238–253, thus locating the position of TPi1 between amino acids 238–253 (Fig. 1A). Since the majority of our mutants were designed to delete precisely one or more tryptic peptides, mutant Δ238–253 represented the mutant from which two tryptic peptides with amino acid sequence n-PLTPATVPGTR/SPLNR-c were deleted. Since this mutant had only a single serine residue at position 249 missing, we surmised that the primary phosphorylation site of the P protein is at a single serine residue, serine249.

Previously we reported that the TPi1 contained greater than 80% of ^{32}P counts (*18*). Consequently, there were some additional but minor phosphopeptides present in the two-dimensional (2-D) map. We showed that these additional phosphopeptides were about equally phosphorylated in the presence of phosphatase PP1 and PP2A inhibitor, calyculin A (*18*). To get a better understanding of these minor phosphopeptides, we analyzed the wildtype P protein from virus-infected and transfected cells containing higher radioactivity and exposed the thin-layer plates for a longer time (7–10 days) to autoradiographic films. All the phosphopeptides from the P protein from infected cell were designated as TPi1–TPi11. In total, we could identify 11 phosphopeptides from virus-infected P protein, one major (TPi1) and 10 minor (TPi2–TPi11) (Fig. 2A). Interestingly, the wildtype P protein from transfected cells contained an additional minor phosphopeptide designated as TPt1 (Fig. 2B) which was consistently absent from P in virus-infected cells. Since ^{32}P counts in the minor phosphopeptides were rather low, the detectibility of these minor peptides varied. Therefore, some of the minor peptides are not readily visible in the photographs. Relative positions of all the P-specific phosphopeptides detected in both infected and transfected cells are shown in an overlay (Fig. 2C).

By comparing phosphopeptide maps of all deletion mutants (Figs. 3A–3F) with that of the wildtype P (Fig. 2B), we localized the sites of the minor peptides in the P protein (Fig. 4, Table 1). It is revealing to note that the small deletion mutants (Δ208–253 and Δ238–253) which did not contain the primary phosphopeptide TPi1 were relatively more phosphorylated at the minor phosphopeptides, TPi2, TPi3, TPi4, and TPi6 (Figs. 3D and 3F), as if to compensate for the loss of TPi1. In addition, both of these mutants contained an additional phosphopeptide not detected in other maps (shown by asterisk). On the other hand, in mutant Δ208–236 which contained TPi1, all those sites and TPi5 were also relatively more phosphorylated (Fig. 3E). In fact, in this mutant TPi5 is as heavily phosphorylated as TPi1. This is consistent with the 60% overall more phosphorylation of this mutant protein in comparison to the wildtype protein (Fig. 1C). Although the relative intensity of minor phosphopeptides

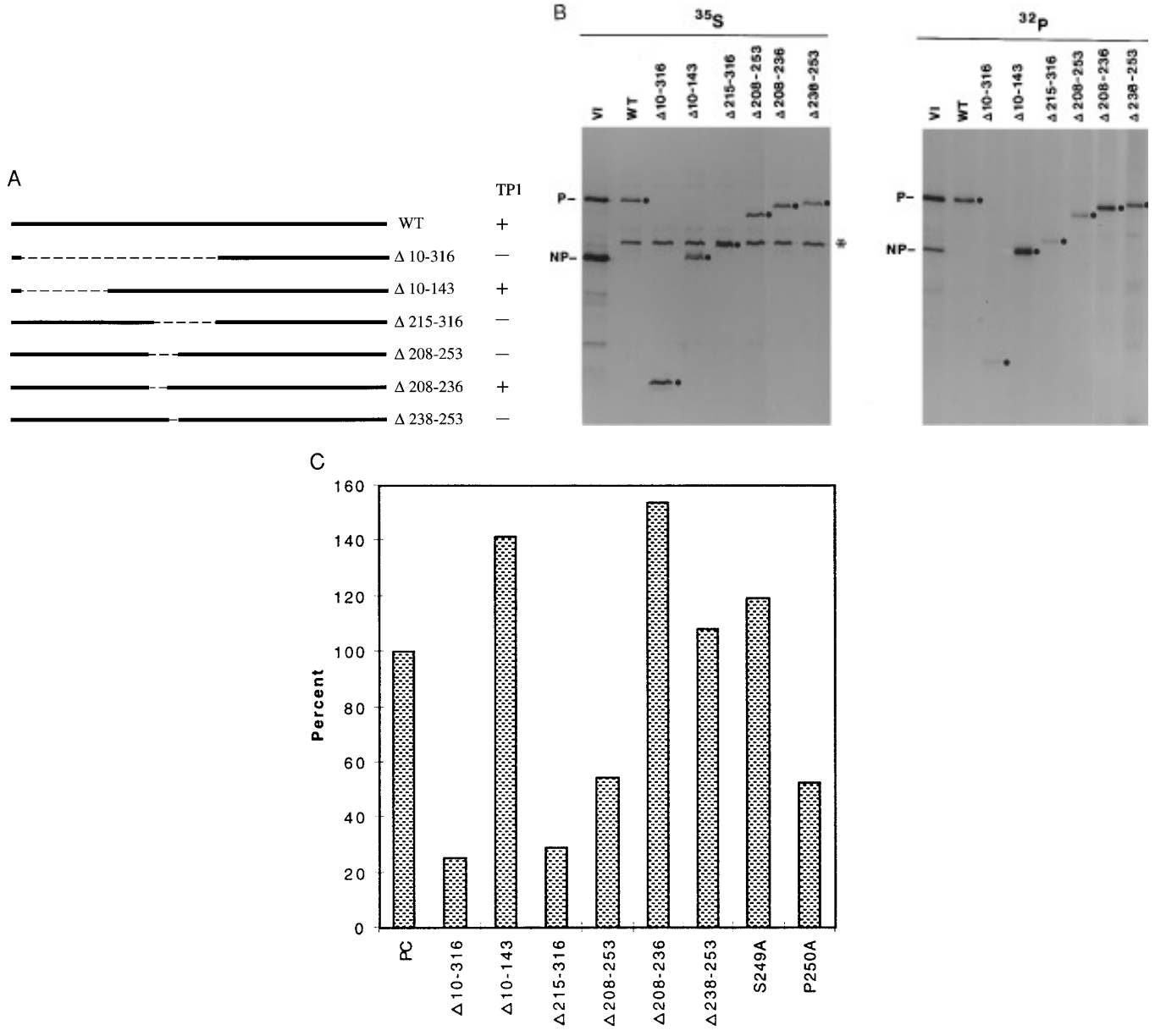

FIG. 1. Schematic presentation and expression of deletion mutants of the P protein. (A) Deletion mutants were generated by PCR amplification of the wildtype plasmid using vent polymerase (NEB) as described in detail elsewhere (*33, 34*). Broken lines represent the deleted region of the protein. Precise amino acid deletions are given at the right side of each mutant. (B) Expression of deletion mutants in CV1 cells using vaccinia virus/T7 RNA polymerase system (*18, 19*). Left panel presents the immunoprecipitated P protein from ^{35}S-labeled cells and the right panel presents ^{32}P-labeled P protein from a parallel experiment. VI and WT are the proteins from virus infected and wildtype P/C gene-transfected cells, respectively. Band corresponding to each deletion mutant is shown by a solid dot on the right side. Asterisk shows a nonspecific protein that coimmunoprecipitates with the ^{35}S-labeled P protein. Procedures for metabolic labeling of proteins and their immunoprecipitations have been described previously (*18, 20*). (C) Relative level of phosphorylation in deletion and point mutants of the P protein. Relative level of ^{32}P incorporation was determined by comparing ^{32}P incorporation to ^{35}S incorporation from parallel experiments. The level of ^{35}S of mutant Δ10−316 which lacks two methionine was adjusted to wildtype for calculation.

varied with each deletion mutant, it is obvious that TPi2–TPi6 have a greater potential for phosphorylation than the other potential sites (TPi7–TPi11). These results suggest that deletion of the primary phosphorylation site or changes in its vicinity cause conformational changes in the P protein allowing phosphorylation at alternate sites.

Based on the analysis of 2-D phosphopeptide maps of the deletion mutants, we surmised that serine249 is the sole primary phosphorylation site in the P protein. To directly test this assumption, we mutagenized serine249 to either alanine or aspartate (Fig. 5A). The mutants were analyzed for their expression and phosphorylation by labeling with [^{35}S]Translabel and [^{32}P]orthophosphate as described above. The level of expression of the mutant

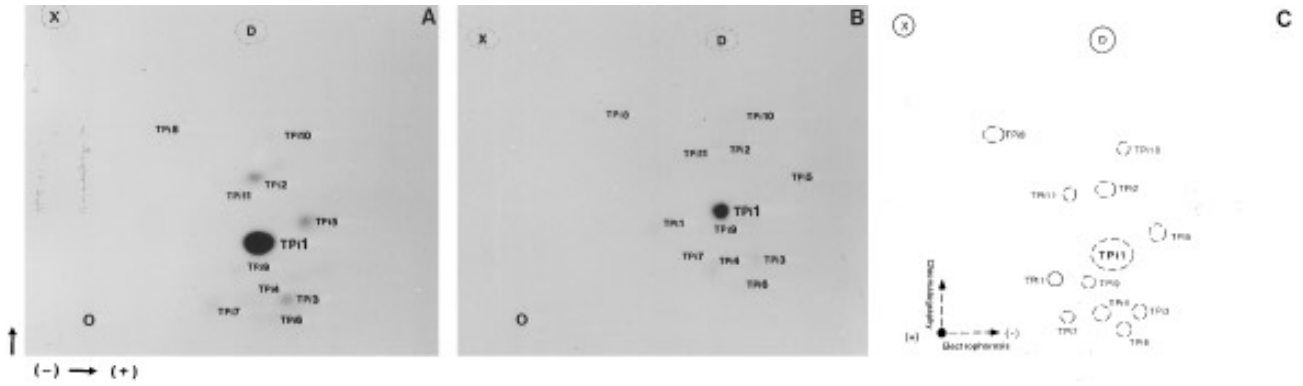

FIG. 2. Two-dimensional tryptic phosphopeptide maps of virus-infected P (A), transfected P (B), and a tracing of the all the phosphopeptides detected (C). Phosphopeptides were analyzed as described previously (*18, 35, 36*). X and D represent positions of xylene cyanol and ϵ-dinitrophenol–lysine dye markers, respectively. O is the position of sample origin. Directions of first-dimensional electrophoresis (+ → −) and second-dimensional chromatography are shown by arrows. As the minor phosphopeptides contained very low level of radioactivity, they are barely visible in the figure.

proteins was approximately equal to the wildtype P. However, the level of phosphorylation of mutant S249A was about 20% higher than the wildtype P protein (Figs. 1C and 5B). The level of phosphorylation of mutant S249D was approximately equal to the wildtype P protein (data not shown). Analysis of the mutant proteins by 2-D phosphopeptide maps showed that both mutants lacked TPi1, but other potential sites, particularly TPi2, TPi3, TPi4, and TPi6, were phosphorylated at a higher level (Fig 6; Table 1). These are the same peptides that were more heavily phosphorylated in deletion mutants Δ208–253, Δ238–253, and Δ208–236. These results further showed that whenever the primary site of phosphorylation was abolished, certain potential sites were phosphorylated at a higher level. Interestingly, mutagenesis of serine249 to aspartate to compensate for the loss of phosphoserine charge did not result in the loss of P protein phosphorylation at alternate sites.

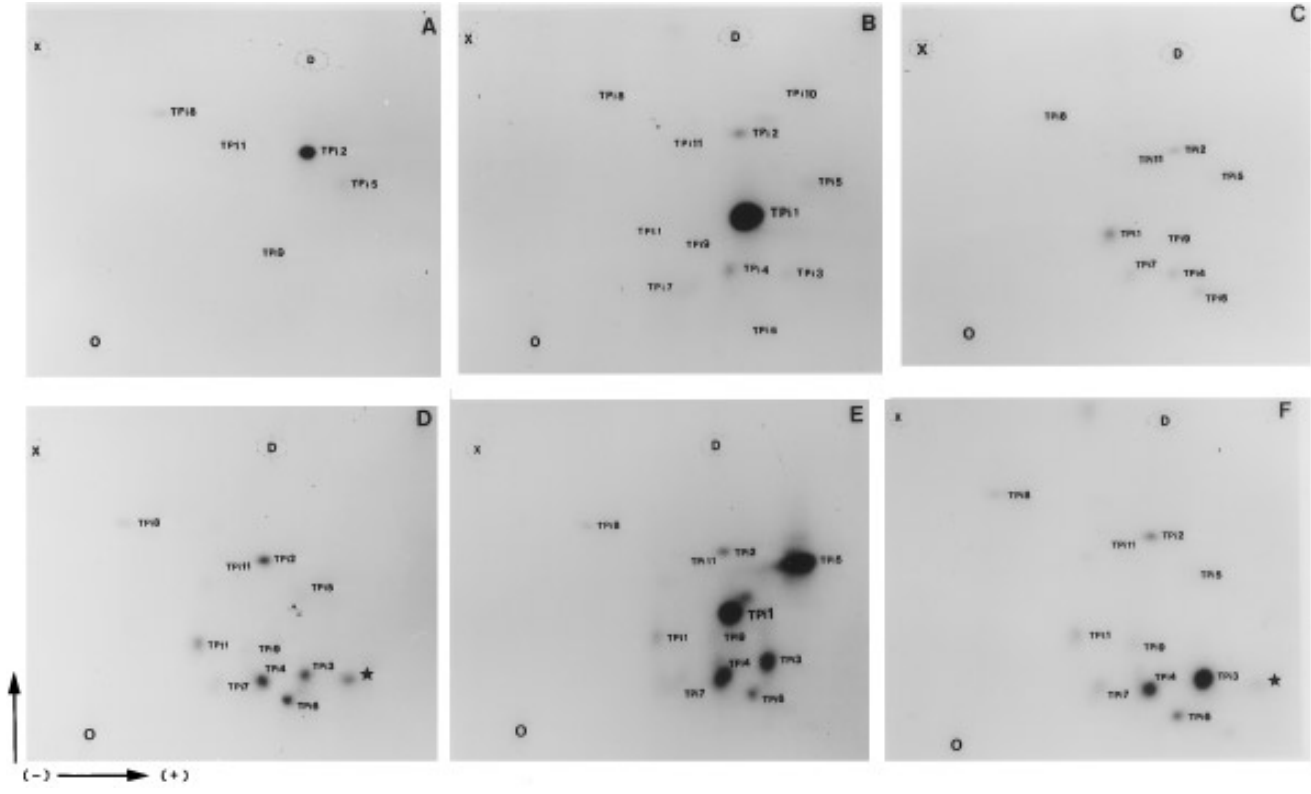

FIG. 3. Two-dimensional tryptic phosphopeptide maps of deletion mutants: (A) Δ10–316, (B) Δ10–143, (C) Δ215–316, (D) Δ208–253, (E) Δ208–236, and (F) Δ238–253. Asterisk presents the novel phosphopeptide detected in mutants Δ208–253 and Δ238–253. Other notations are as described in the legend to Fig. 2.

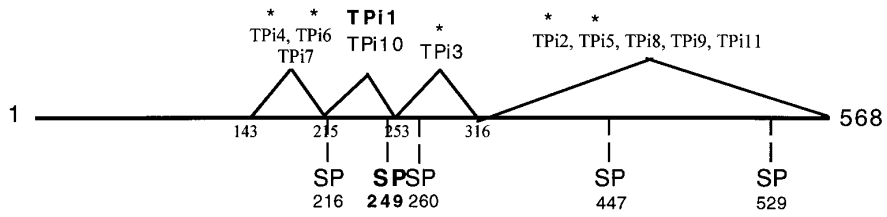

FIG. 4. Location of tryptic phosphopeptides of the P protein based on two-dimensional tryptic phosphopeptide maps of the deletion mutants. Positions of −SP− sequences in the P protein are shown. The bold SP at 249−250 aa sequence is the primary phosphorylation site. Asterisks denote the phosphopeptides that have a potential for higher phosphorylation in point and deletion mutants.

Several kinases are known to phosphorylate their substrate at specific consensus sequences (*21*). The only known consensus phosphorylation sequence present at the P phosphorylation site is −SP−. SP sequence is a substrate for several proline-directed protein kinases such as cdc/cdk family of cyclin-dependent and mitogen-activated (MAP) protein kinases (*21, 22*). To determine whether proline250 is necessary for the serine249 phosphorylation, we mutagenized the proline to alanine. On tranfection, the mutant P protein was expressed at about the wildtype P level but was phosphorylated at about 50% of the wildtype level (Fig. 5B). The two-dimensional phosphopeptide map revealed that TPi1 was absent and other potential sites, particulary the TPi4 and TPi6, were relatively more phosphorylated (Fig. 6C). It appears that mutagenesis of proline250 decreased the overall phosphorylation of the P protein. This result showed that proline250 is essential for the serine249 phosphorylation and that possibly a proline-directed protein kinase is involved in the primary phosphorylation of the P protein. However, as proline to alanine mutagenesis could also cause a conformational change (*23*), and thus serine249 may not be accessible for phosphorylation or may be readily accessible to dephosphorylation, therefore, the identity of the kinase involved in the primary phosphorylation of the Sendai virus P protein remains to be determined.

Previously, we showed, using phosphatases PP1 and PP2A inhibitor calyculin A, that the P protein has other potential phosphorylation sites. In the presence of the inhibitor, these sites were as intensely phosphorylated as the TPi1 (*18*). Apparently, all the potential sites have equal capacity for phosphorylation and thus exist in phosphorylation-competent conformation, but not all sites are equally dephosphorylated. Therefore, when serine249 or proline250 was mutated, some of the potential sites, particularly TPi2, TPi3, TPi4, and TPi6, were not efficiently dephosphorylated. We suggest that following deletion or point mutagenesis of serine249 or proline250, the conformation of the P protein is altered such that some of the potential phosphorylation site were not accessible for dephosphorylation. Thus the primary and the alternate phosphorylations of the P protein are more dependent on the accessibility of phosphatases than that of kinases. It is not clear why the P protein must maintain its phosphorylation by using several alternate phosphorylation sites. By deletion mutagenesis of the Sendai virus P protein, it was demonstrated that the amino termimal region which putatively contained the phosphorylation site was not important for the binding of P protein to the nucleocapsid (*5*). In view of our demonstration of alternate phosphorylation sites of the P protein, it is possible that the P protein used in these studies was not unphosphorylated. Recently, it was observed that deletion of amino-terminal 78−320 amino acids had no effect on the RNA synthesis ability of the P protein (*8*). Thus the role of phosphorylation in this protein still remains open.

TABLE 1

Presence and Relative Level of Phosphorylation of Tryptic Peptides

Sample	TRi1	TPi2	TPi3	TPi4	TPi5	TPi6	TPi7	TPi8	TPi9	TPi10	TPi11	TPt1
P-infected	+++	++	++	+	++	+	+	+	+	+	+	−
P-transfected	+++	+	++	++	+	+	+	+	+	+	+	++
10−316	−	+++	−	−	+	−	−	++	+	−	+	−
10−143	+++	++	++	++	+	+	+	+	+	+	+	+
215−316	−	++	−	++	+	++	++	+	+	−	+	++
208−253	−−	++	++	++	+	++	+	++	+	−−	+	++
208−236	+++	+	++	++	+++	+	+	+	+	−	+	+
238−253	−	++	+++	++	+	++	+	+	+	−	+	+
S249A	−	++	+++	+++	+	++	++	+	+	−	+	++
S249D	−	++	+++	+++	+	++	+	+	+	−	+	++
P250A	−	++	++	+++	+	++	++	+	+	−	+	++

Note. Sample represents the P protein from infected, wildtype transfected, or from various deletion or point mutant transfected CV1 cells.

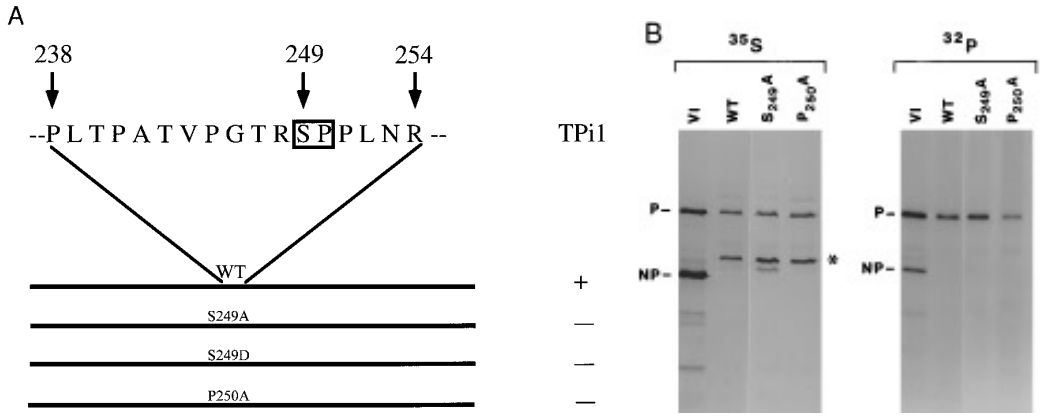

FIG. 5. Schematic presentation of point mutants and their expression in CV1 cells. Point mutants were created by PCR amplification of full-length wildtype plasmid using a mutagenic primer and a complementary primer (*34*) or using single-strand DNA (*37*). (A) Sequence of amino acids from 283 to 254 encompassing the TPi1 phosphopeptide. Presence (+) or absence (−) of TPi1 is indicated at the right side of each of the mutant. (B) Expression of point mutants in CV1 cells. In parallel experiments, cells were labeled with ^{35}S or ^{32}P and the P protein immunoprecipitated. Notations are as described in the legend to Fig. 1B.

Serine249 and most of the alternate phosphorylation sites (except TPi2 and TPi5) are localized in a region of the P protein that has a very high probability of β-turns as predicted by two different algorithms (*24, 25*). It was shown that greater than 90% of phosphates of P in nucleocapsids was susceptible to digestion by bacterial alkaline phosphatase (*9*). This finding is consistent if phosphates occurred at β-turns. However, there are only 5 −SP− sequence (at 216, 249, 260, 447, and 529) in the P protein (Fig. 4), while we could detect 11 phosphopeptides. If all the −SP− sites were phosphorylated by a proline-directed kinase (PDPK), we suggest that kinases other than PDPK are also involved in the phosphorylation of the P protein. Recently it was demonstrated that the P protein of human parainfluenza virus 3 is phosphorylated by protein kinase C isoform ζ (*26*), whereas P proteins of measles virus (*27*) and RSV (*28, 29*) have been shown to be phosphorylated by casein kinase II. Similarly VSV P protein is phosphorylated by casein kinase II (*17*). It appears, therefore, that various kinases are involved in the phosphorylation of P proteins with the ultimate goal to phosphorylate the P protein.

It is interesting to note that the P protein is the highest phosphorylated protein in Sendai virus on a mole-per-mole basis (*9, 29*). Moreover, previously it was shown that the P protein in infected cells occurs primarily in one phosphorylated isoform (*18, 30*), indicating that almost all the P protein molecules are phophorylated at serine249. However, because greater than 80% of ^{32}P radioactivity reside on serine249, the stoichiometry of the P protein phosphorylation is most likely in the order of 1.2−1.5 mole phosphate per mole of P protein. Because the level of phosphorylation of other viral protein is rather low, it is obvious, therefore, that only a few percent molecules of other protein are phosphorylated.

The Sendai virus V protein which is also encoded in the P gene has been shown to be phosphorylated (*31*). It is interesting to note that the transcriptional editing of the P mRNA to generate the V mRNA occurs at nucleotide 1053, and the frame-switching occurs at aa 317 (*32*).

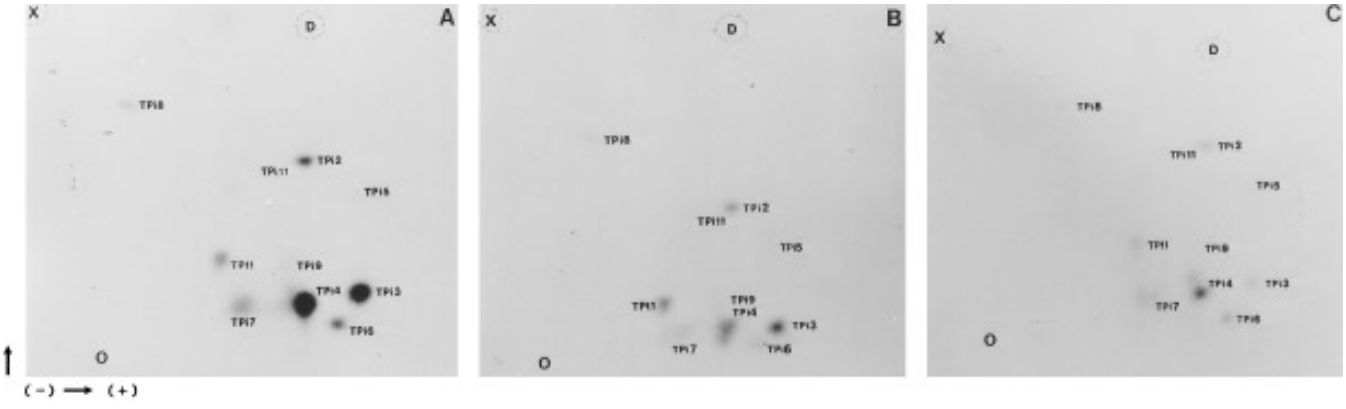

FIG. 6. Two-dimensional tryptic phosphopeptide maps of point mutants: (A) Serine249 to alanine, (B) serine249 to aspartate, and (C) proline250 to alanine. All notations have been described in the legend to Fig. 2.

Consequently the V protein retains the primary as well as most of the alternate phosphorylation sites of the P protein. It is likely, therefore, that the V protein is also primarily phosphorylated at serine249 unless the unique carboxy terminal region of the V protein has caused a global conformational change in the protein. Future experiments will determine the validity of this presumption.

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