

# Human parainfluenza virus type 1 phosphoprotein is constitutively phosphorylated at Ser-120 and Ser-184

Sridhar Byrappa and Kailash C. Gupta

Department of Immunology/Microbiology, Rush Presbyterian-St. Luke's Medical Center, 1653 W. Congress Parkway, Chicago, IL 60612, USA

RNA-dependent RNA polymerases of single-stranded, negative-sense RNA viruses comprise a phosphoprotein (P) and a large protein. The constitutive phosphorylation of the P protein in these viruses is highly conserved, yet the functional significance of phosphorylation is enigmatic. To approach this problem, phosphorylation sites were determined in two closely related paramyxovirus P proteins. Sendai virus (SV) is a prototypic paramyxovirus. Previously, using a phosphopeptide mapping technique, the primary constitutive phosphorylation site of SV P protein was mapped to Ser-249. Phosphorylation at Ser-249 is dependent on the presence of Pro-250. Human parainfluenza virus type 1 (HPIV-1) P protein has 66% similarity to SV P protein and its predicted secondary structure is highly similar to that of SV P protein. However, there is no obvious conserved phosphorylation site in HPIV-1 P protein. Using the phosphopeptide mapping strategy, the constitutive phosphorylation sites of HPIV-1 P protein were mapped. The HPIV-1 P protein is primarily phosphorylated at Ser-120. Phosphorylation at Ser-120 is dependent on the presence of Pro-121. It also has a minor phosphorylation site at Ser-184. The sequence at Ser-184 does not match any consensus phosphorylation target site for the known kinases. Significantly, the P proteins from both viruses are constitutively and primarily phosphorylated at one serine and the phosphorylation of that serine is dependent on the presence of a proline on its carboxyl side.

## Introduction

Negative-sense RNA viruses possess RNA-dependent RNA polymerases that use viral ribonucleoprotein complexes (nucleocapsids) as their templates to transcribe and replicate virus RNA. Two viral proteins, the large (L) protein and the phosphoprotein (P) constitute the polymerase activity. Roles of these proteins in polymerase activity and their functional cooperation have not been dissected in-depth (Lamb & Kolakofsky, 1996). Surprisingly, amino acid sequences among these phosphoproteins are not particularly conserved (Galinski, 1991), although phosphorylation of the proteins remains stringently conserved. To gain some clues as to the functional significance of phosphorylation, we sought to define the phosphorylation sites of Sendai virus (SV) and human parainfluenza virus type 1 (HPIV-1) P proteins.

Previously, we have shown that, for biological relevance, structural and functional studies of P protein phosphorylation should be performed in virus-infected or P gene-transfected

cells (Byrappa *et al.*, 1995*b*, 1996). Cell-free phosphorylation studies of these proteins are likely to provide artifactual results (Byrappa *et al.*, 1995*b*). With that premise, we have examined the phosphorylation sites of SV and HPIV-1 P proteins from both virus-infected and P gene-transfected cells.

The SV P protein is constitutively and primarily (> 80%) phosphorylated at Ser-249 in SV-infected or P gene-transfected cells (Byrappa *et al.*, 1996). Determination of the phosphorylation site (Byrappa *et al.*, 1995*a*) was facilitated by a mutagenesis procedure that does not require any restriction site (Byrappa *et al.*, 1996). This approach facilitated deletion of a tryptic peptide or a set of tryptic peptides from the P protein. Recently, another study confirmed SV P protein phosphorylation at Ser-249 by mass spectrometric analysis of the purified P protein from insect cells supplied by us (Jonscher & Yates, 1997). Pro-250 was found to be essential for Ser-249 phosphorylation, indicating that P protein phosphorylation occurs by a proline-dependent kinase (Byrappa *et al.*, 1996). This was an unusual finding as no negative-sense RNA virus P protein has been shown to be phosphorylated by this class of kinases.

HPIV-1 is closely related to SV. It causes acute upper

**Author for correspondence:** Kailash Gupta.

Fax +1 312 942 2808. e-mail kgupta@rush.rpslmc.edu

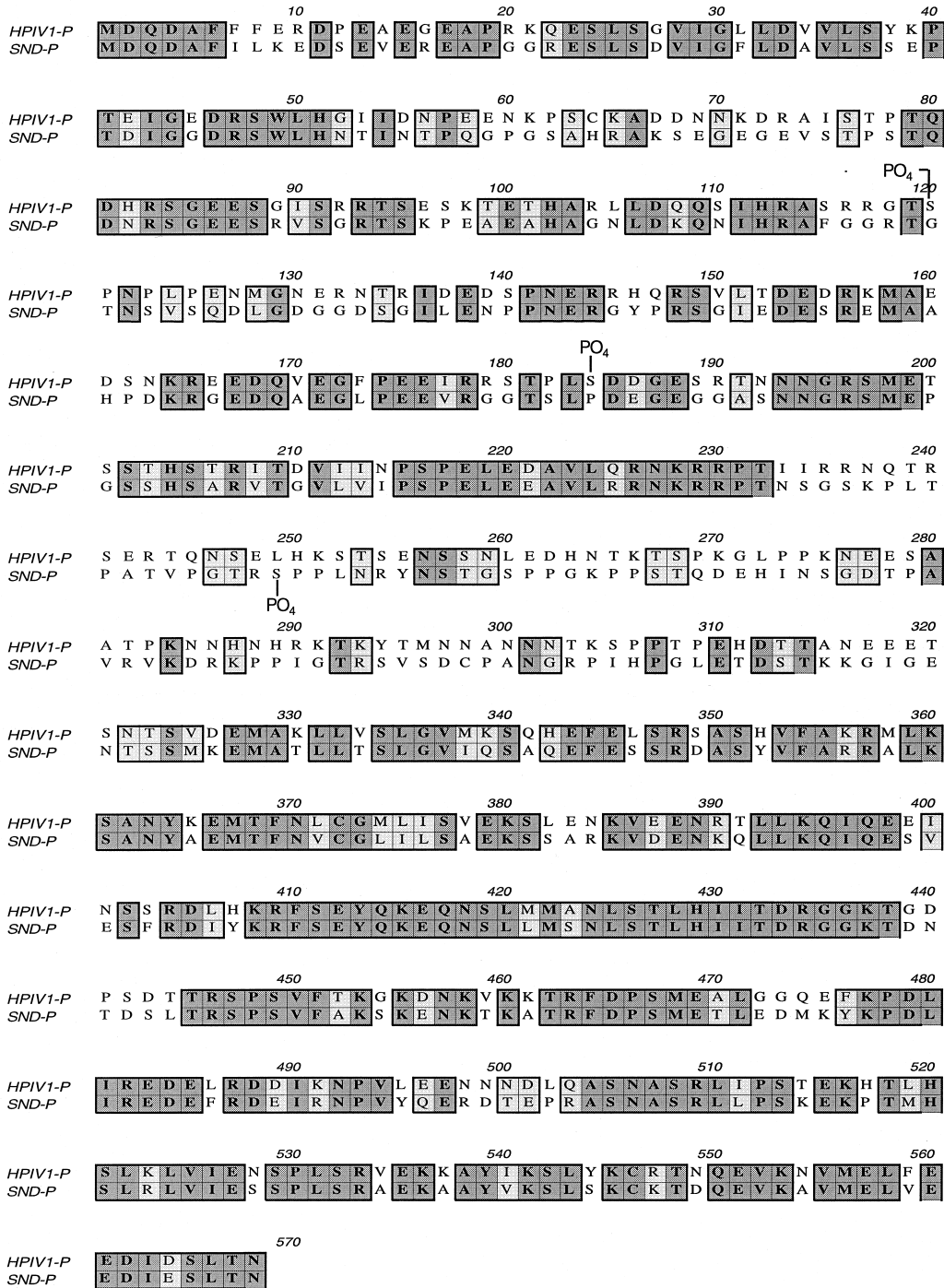


Fig. 1. CLUSTALW alignment of HPIV-1 and SND (Sendai virus) P proteins using MacVector software. Identical and similar amino acids are boxed. Identical amino acids are shaded in dark grey, while similar amino acids are shaded light grey. Non-homologous regions are not boxed. Positions of constitutively phosphorylated serines are shown. Note that phosphorylation occurs in the non-homologous region.

respiratory tract diseases in children and infants (Vainionpää & Hyypiä, 1994; Wendt & Hertz, 1995). HPIV-1 and SV P proteins have 54% aa identity and 66% similarity. Both proteins are 568 aa long and they contain two regions of high similarity at residues 1–232 and 324–568 (Fig. 1). However,

HPIV-1 P protein does not contain a region that is similar to the phosphorylation region of SV P protein. Thus, it was important to determine the constitutive phosphorylation site of HPIV-1 P protein.

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## Methods

■ **Recombinant plasmids and mutagenesis.** The full-length HPIV-1 (strain C35) P gene cloned in vector pTF1 was kindly provided to us by Allen Portner (Takahashi *et al.*, 1992). The recombinant plasmid vector, pTF1-P, carrying the P gene behind the T7 RNA polymerase promoter, was used for all site-specific deletion and point mutant constructions. Standard techniques were used for DNA manipulations (Sambrook *et al.*, 1989; Ausubel *et al.*, 1995). Deletion and point mutants were generated using a PCR-based mutagenesis technique developed in our laboratory (Byrappa *et al.*, 1995a). Briefly, deletions and site-specific changes were created using PCR amplifications of the pTF1-P with Vent polymerase (New England Biolabs). Two primers (one mutagenic and one complementary for point mutations or both complementary for deletion mutations) were used to amplify the entire plasmid. The amplified product was purified from agarose gels using GeneClean II (BIO101), self-ligated and used to transform competent *E. coli* HB101. Six to eight independent colonies were grown in 3 ml cultures and plasmid DNA was purified. Purified DNA was sequenced for the mutation authenticity and used for transfection.

■ **Cells culture, virus infection and DNA transfections.** CV1 cells grown in 35 mm dishes in DMEM containing 10% heat-inactivated foetal bovine serum were used for HPIV-1 (strain C35) infection or for transfection studies. For virus infection, near-confluent monolayers were infected with HPIV-1 at 5 p.f.u. per cell. The P gene and its mutants were expressed using the vaccinia virus/T7 RNA polymerase transfection system (Fuerst *et al.*, 1986). For transfection, CV1 monolayers were infected with vaccinia virus vTF-7 (at 5 p.f.u. per cell) 30 min prior to transfection with plasmid DNA (4 µg) emulsified with 10 µl Lipofectin (BRL). The Lipofectin-mediated transfection procedure was adapted from the supplier's protocol.

■ **Metabolic labelling and immunoprecipitations.** Twelve to fourteen hours after transfection or HPIV-1 infection, cells were transferred to methionine-free DMEM for 1 h and then labelled for 6–8 h in the presence of 100 µCi/ml Tran<sup>35</sup>S-label (ICN). For labelling with <sup>32</sup>P, cells were transferred to phosphate-free MEM for 30 min and then labelled with carrier-free [<sup>32</sup>P]orthophosphate (0.6 mCi/ml) for 6–8 h. At the end of the labelling period, cells were washed twice with PBS and lysed in 0.5 ml radioimmunoprecipitation assay (RIPA) buffer containing 20 mM Tris-HCl (pH 7.5), 2 mM EDTA, 0.15 M NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 1 mM PMSF, 1 µg/ml leupeptin and 1 µg/ml aprotinin. Phosphatase inhibitors, sodium orthovanadate (100 µM) and sodium fluoride (50 mM) were added to RIPA buffer just before cell lysis to prevent any dephosphorylation of the P protein (Wang *et al.*, 1995). After cell lysis nuclei and cellular debris were removed by centrifugation at 28 000 g for 15 min. Cell lysate (50 µl) was used for immunoprecipitation with 1 µl (1:100 dilution) guinea-pig antiserum raised against the HPIV-1 P protein (generously provided by J. Curran, University of Geneva Medical School, Geneva, Switzerland). Under these immunoprecipitation conditions, the antibodies were in excess of the antigen and the level of immunoprecipitated P protein was in a linear range (data not shown). Immunocomplexes were adsorbed to 15 µl Pansorbin (Calbiochem) and pelleted. The bacterial pellet was washed three times with RIPA buffer, suspended in 15 µl 2 × Laemmli loading buffer, heated to 100 °C for 3 min, and resolved in a 12.5% SDS-polyacrylamide gel (Laemmli, 1970). Gels were fixed, dried and exposed to X-ray films. Radioactivity in protein bands was quantified by a PhosphorImager (Molecular Dynamics). Relative levels of

phosphorylation in various mutants were determined by comparing the level of <sup>35</sup>S incorporation with <sup>32</sup>P incorporation in parallel experiments. For deletion mutants that lacked one or more methionines, the level of <sup>35</sup>S incorporation was normalized to that of the wild-type. The averages from two independent experiments were quantified.

■ **Preparation of virions.** To recover virus from HPIV-1-infected cells, labelled cells were treated with 0.5 mM EDTA containing PBS solution (lacking calcium and magnesium). Cells were collected by low-speed centrifugation (1500 g for 5 min), resuspended in water and subjected to three freeze-thaw cycles. Cellular debris was removed by low-speed centrifugation. The supernatant was centrifuged at 190 000 g in SW50.1 tubes (Beckman) for 2 h to pellet virus (Gorman *et al.*, 1991). The virus pellet was resuspended in RIPA buffer and the P protein was immunoprecipitated and analysed as described above.

■ **Phosphoamino acid analysis and tryptic peptide mapping.** Procedures to analyse phosphoamino acids and tryptic phosphopeptides have been reported in detail for the SV P protein (Byrappa *et al.*, 1995b). Essentially similar methods were followed to analyse deletion and point mutants of the HPIV-1 P protein. Briefly, the <sup>32</sup>P-labelled P protein was immunoprecipitated as described above (from 200–400 µl lysate) and resolved in a 10% SDS-polyacrylamide gel. The protein was blotted onto a nitrocellulose membrane (Schleicher and Schuell) for tryptic peptide analysis or onto a PVDF (Millipore) membrane for phosphoamino acid analysis (Boyle *et al.*, 1991; Kamps, 1991). After autoradiography, the P band from the membrane was sliced out and processed for analysis.

For phosphoamino acid analysis, PVDF membrane slices were digested with 5.7 M HCl at 100 °C (Boyle *et al.*, 1991). The digest was lyophilized and resuspended in 10–20 µl H<sub>2</sub>O, mixed with 1 µg each of phosphoserine, phosphotyrosine and phosphothreonine. Samples were spotted on a TLC plate (Kodak) and electrophoresed in a pH 2.5 buffer [5.9% glacial acetic acid, 0.8% formic acid (88%), 0.3% pyridine and 0.3 mM EDTA] (Jelinek & Weber, 1993).

For phosphopeptide analysis, nitrocellulose membrane pieces were washed with H<sub>2</sub>O and soaked in 0.5% polyvinylpyrrolidone (average molecular mass 360 000; Sigma) in 100 mM acetic acid for 30 min. The membrane was extensively washed with H<sub>2</sub>O and followed by a wash with 50 mM ammonium bicarbonate. After washing, the membrane was subjected to digestion with 25 µg/ml trypsin (TPCK-treated; Sigma) in 50 mM ammonium bicarbonate for 18–24 h. Trypsin digestion released all peptides from the nitrocellulose membrane into the solution (Byrappa *et al.*, 1996). The digest was lyophilized, dissolved into 5 µl buffer pH 1.9 (formic acid:acetic acid:water at 2.5:7.8:89.7) and resolved by two-dimensional peptide analysis on a TLC plate. The plate was subjected to electrophoresis in the first dimension in pH 1.9 buffer and chromatography in the second dimension in phospho-chromatography buffer (n-butanol:pyridine:acetic acid:water at 37.5:25:7.5:30) (Boyle *et al.*, 1991). Mixed peptide maps were produced to establish the identity of each phosphopeptide unambiguously. TLC plates were autoradiographed and the radioactivity in each phosphopeptide was measured using a PhosphorImager. Total radioactivity associated with the major tryptic peptides (TP1, TP2 and TP3) was taken as 100%.

## Results

### Phosphoamino acid and phosphopeptide analysis of P protein

To determine the biological relevance of the HPIV-1 P protein from transfected cells, we analysed the phosphopeptides of P proteins from both HPIV-1-infected and P gene-

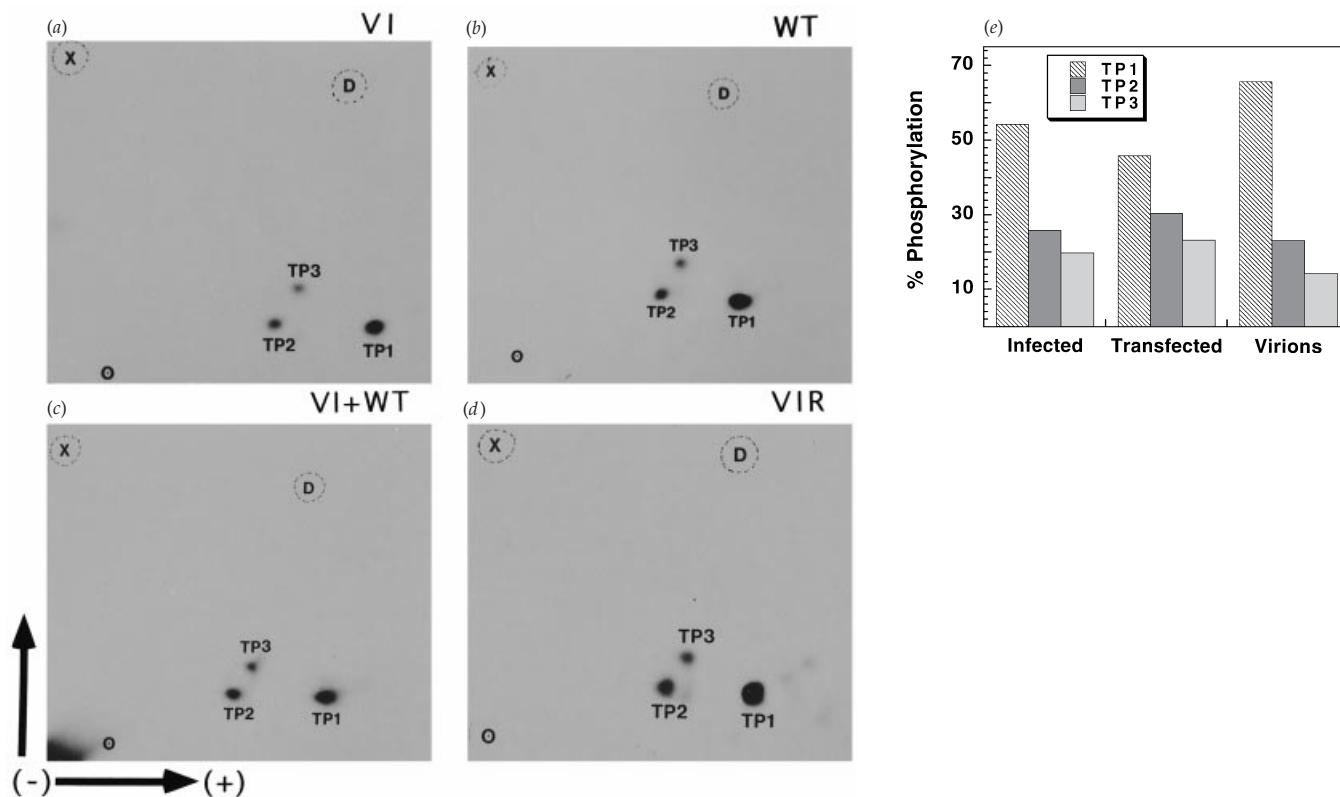
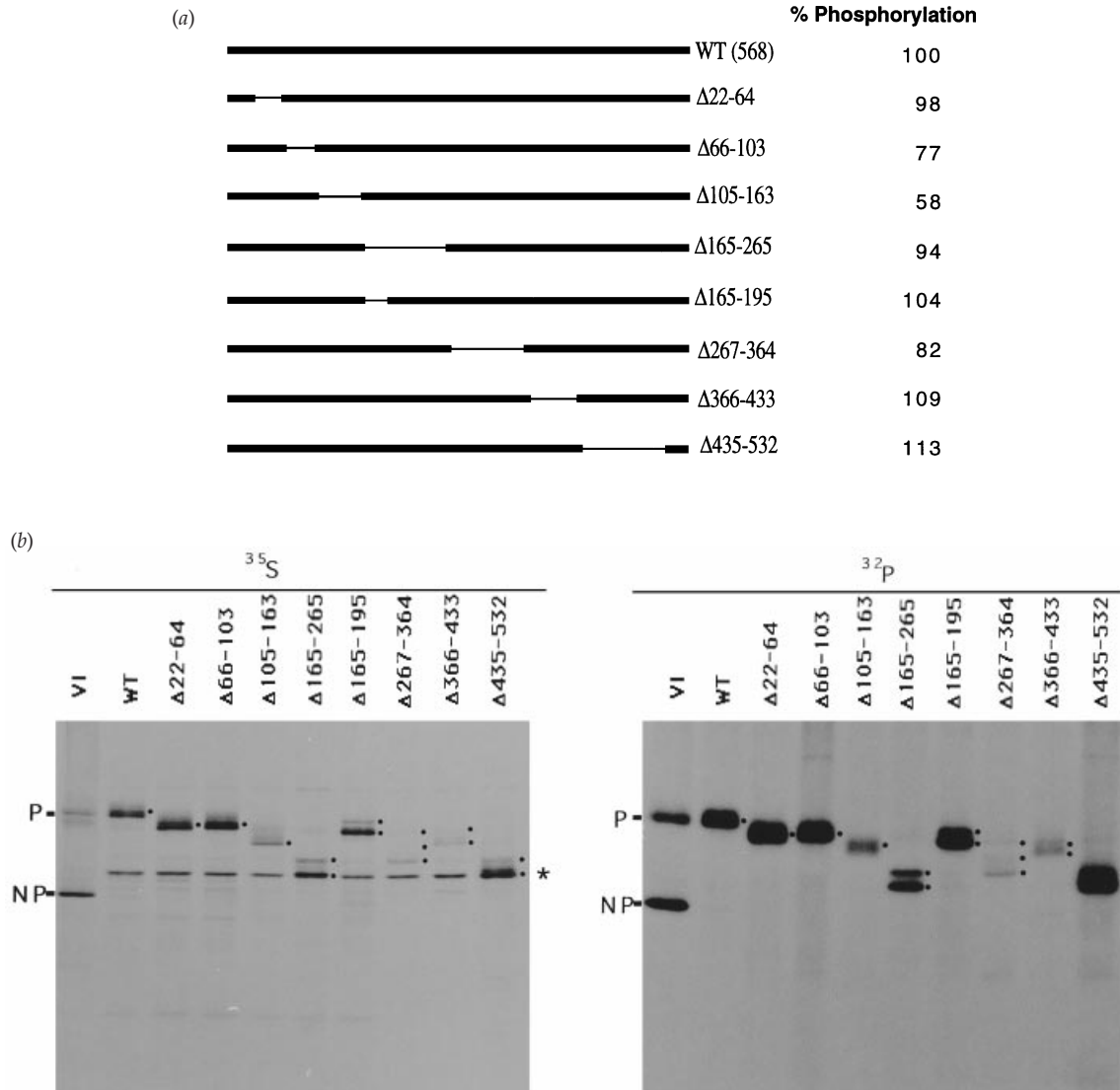


Fig. 2. Tryptic phosphopeptide maps of HPIV-1 P protein. Maps produced from: (a) virus-infected cells (VI); (b) P gene-transfected cells (WT); (c) mixed phosphopeptides of P protein from virus-infected and transfected cells (VI+WT); (d) virions (VIR). Phosphopeptides were resolved on a TLC plate by two-dimensional electrophoresis-chromatography analysis. The origin is indicated by an 'O' in the left-hand corner of the peptide map. Marker dyes dinitrophenyl-lysine and xylene cyanol used to track movement of phosphopeptides are shown by 'D' and 'X', respectively. Arrows at the bottom left-hand corner of the figure show directions of first-dimension electrophoresis and second-dimension chromatography. (e) Relative radioactivities in TP1, TP2 and TP3 (% phosphorylation, where TP1, TP2 and TP3 together constitute 100%). Note that the relative phosphorylation levels of the three phosphopeptides from virus-infected cells, P gene-transfected cells and purified virions are similar.

transfected cells. On SDS-PAGE,  $^{35}\text{S}$ - or  $^{32}\text{P}$ -labelled P protein from HPIV-1 P gene-transfected cells resolved as two closely migrating and about equally phosphorylated species (Fig. 3*b*). The reason for the occurrence of two bands is not known as yet. However, the resolution of the two species was not sufficient for their independent analysis. Therefore, both species were analysed together.

Analysis of the  $^{32}\text{P}$ -labelled HPIV-1 P protein from virus-infected cells, transfected cells and virions revealed that it was phosphorylated solely at serine residue(s) (data not shown). Two-dimensional tryptic peptide analysis of the wild-type HPIV-1 P protein from virus-infected cells yielded three phosphopeptides, TP1, TP2 and TP3, designated according to the decreasing radioactivity associated with them as determined with a PhosphorImager (Fig. 2*a, e*). TP1 is the major phosphopeptide in infected cells, accounting for approximately 54% of the total radioactivity present in the three phosphopeptides; TP2 and TP3 accounted for 26 and 20% radioactivity, respectively (Fig. 2*e*). Similarly, analysis of P protein from transfected cells showed a phosphopeptide pattern

identical to the P protein from virus-infected cells (Fig. 2*b*); TP1 is the major phosphopeptide, accounting for 46% phosphorylation of the phosphopeptides, while TP2 and TP3 accounted for 31 and 23% phosphorylation, respectively (Fig. 2*b, e*). Analysis of a mixture of phosphopeptides from transfected and infected cells (1:1 radioactivity) showed that the phosphopeptides were identical as only three peptides were detected in the autoradiograph (Fig. 2*c*). Analysis of the P protein from virions also revealed a similar phosphopeptide map (Fig. 2*d*). In virions, the major radioactivity was also associated with TP1 (65%), whereas TP2 and TP3 had 20 and 15% radioactivities, respectively (Fig. 2*e*). Analysis of the P protein labelled at various times (16, 36 and 48 h) after virus infection did not show any significant differences in their tryptic maps. Furthermore, a chase of label for 16 and 40 h in DMEM did not change the tryptic phosphopeptide map and the relative labelling of the phosphopeptides remained unchanged. However, as expected, total radioactivity associated with the P protein declined with the duration of chase (data not presented).



**Fig. 3.** Diagram illustrating P gene deletion mutants (a) and expression and phosphorylation (b) of the deletion mutants. Deletion mutants of the P gene were transfected in CV1 cells and labelled with Tran<sup>35</sup>S-label or [<sup>32</sup>P]orthophosphate as described in Methods. Immunoprecipitated P protein was resolved in 12.5% SDS-PAGE (b). Dots on the gels represent the various species of the P protein mutants. The asterisk (<sup>35</sup>S panel) denotes a non-specific protein immunoprecipitating with the P protein which is not phosphorylated (see <sup>32</sup>P panel). Expression levels of deletion mutants were quantified by a PhosphorImager. Incorporation of <sup>32</sup>P into each mutant was normalized to incorporation of Tran<sup>35</sup>S-label from a parallel experiment. The level of phosphorylation of each mutant compared to the wild-type phosphorylation is presented in (a). VI, Virus-infected cells; NP, nucleoprotein.

These results show that the HPIV-1 P protein is constitutively and primarily phosphorylated at three tryptic peptides and that the steady-state level of phosphorylation of these peptides does not modulate during the progression of virus infection. This is also reflected in the phosphopeptide map of the virion P protein, which is essentially the same as that from infected cells. Importantly, the P protein expressed in transfected cells, in the absence of other HPIV-1 proteins, is phosphorylated in a similar manner to the P protein from infected cells and is thus suitable for our analysis of the P

protein. Since P proteins from virus-infected cells and transfected cells were phosphorylated identically, it is highly likely that cellular kinases are involved in P protein phosphorylation.

#### Analysis of deletion mutants

To determine the location of the phosphopeptides in the P protein, a series of deletion mutants was constructed. Deletion mutants spanned almost the entire P protein (aa 22–532) (Fig. 3a). As described in Methods, deletion mutants were designed

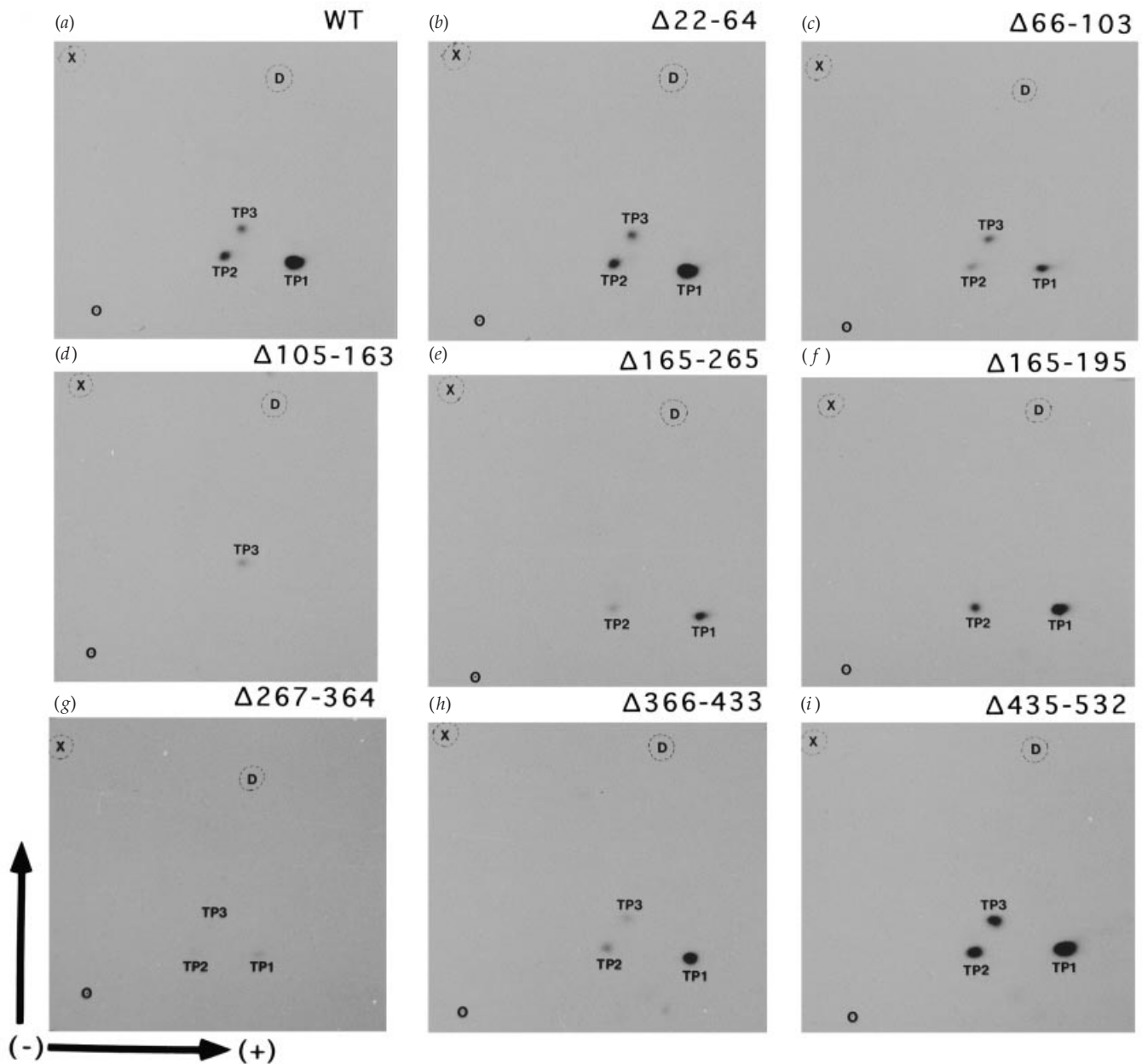
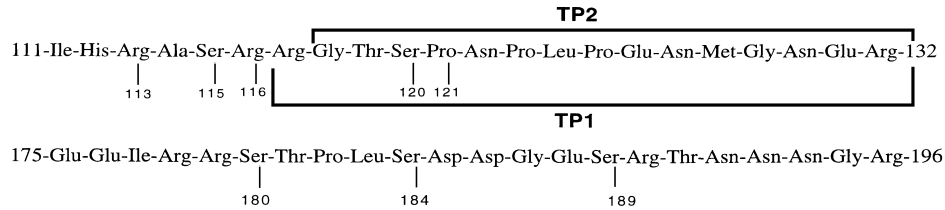


Fig. 4. Phosphopeptide maps of HPIV-1 P deletion mutants. Map of the wild-type P protein from transfected cells is shown in (a) for reference. Deletion mutant maps are: (b)  $\Delta 22-64$ ; (c)  $\Delta 66-103$ ; (d)  $\Delta 105-163$ ; (e)  $\Delta 165-265$ ; (f)  $\Delta 165-195$ ; (g)  $\Delta 267-364$ ; (h)  $\Delta 366-433$ ; (i)  $\Delta 435-532$ . Notations for marker dyes and the origin for the two-dimensional maps are as given in the legend to Fig. 2. Note the absence of TP1 and TP2 in (d) and TP3 in (e) and (f).

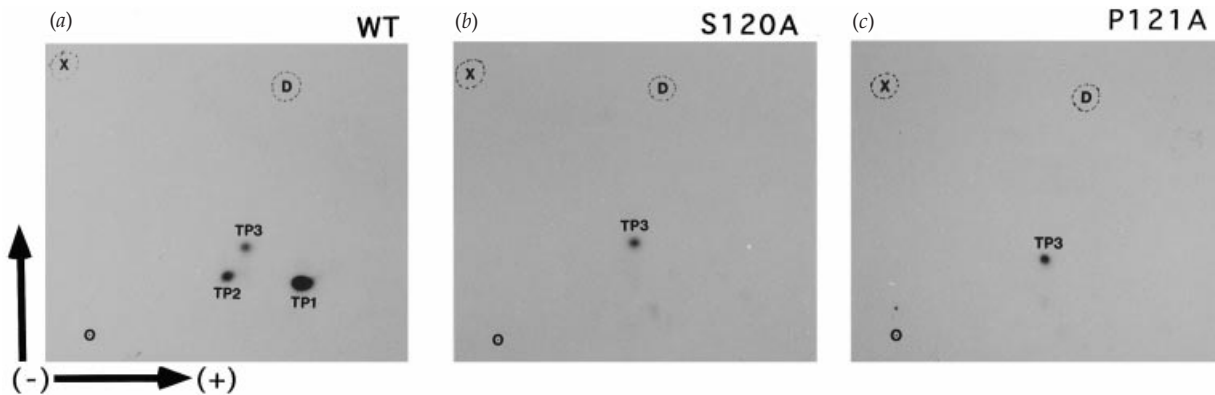
to delete one tryptic peptide or a set of tryptic peptides. This strategy would not alter the mobility of the peptides in the two-dimensional analysis except that one or more peptides will be missing from the deletion mutants. If the mutant lacked the region(s) of phosphorylation, one or more phosphopeptide would be absent from its map (Byrappa *et al.*, 1996).

Except for the deletion mutants  $\Delta 267-364$  and  $\Delta 366-433$ , mutant proteins from all deletion mutants immunoprecipitated reasonably well for analysis (Fig. 3*b*). It is possible that the

major antibody-binding domain(s) lie between aa 267–433, deletion of which compromises the immunoprecipitation of mutants  $\Delta 267-364$  and  $\Delta 366-433$ . Alternatively, these mutant proteins could be unstable. Except for the P protein from deletion mutant  $\Delta 165-265$ , the two P protein species of all the deletion mutants migrated very closely and were equally labelled (marked by dots; Fig. 3*b*). Hence, the two species were analysed together. The P protein of deletion mutants  $\Delta 165-265$  and  $\Delta 435-532$ , each with a deletion of about 100



**Fig. 5.** Amino acid sequence of the regions (aa 111–132 and 175–196) in which tryptic phosphopeptides TP1, TP2 and TP3 are localized. The positions of the serines are indicated. Positions of arginines that could generate TP1 and TP2 by partial digestion are shown. Also shown is the proline that forms a Ser-Pro motif (aa 120–121). Bracketed regions show the deduced sequence of TP1 and TP2.



**Fig. 6.** Phosphopeptide maps of HPIV-1 P point mutants. (a) Map of the wild-type for reference; (b) map of mutant S120A; (c) map of P121A. Other notation is described in the legends to Figs 2 and 4. Note the appearance of several weakly labelled phosphopeptides in (b) and (c).

aa, co-migrated with a non-specifically precipitating vaccinia virus protein band (marked by an asterisk; Fig. 3*b*). However, since this vaccinia virus protein is not phosphorylated (compare  $^{35}\text{S}$  and  $^{32}\text{P}$  panels of Fig. 3*b*), it created no problem in our analysis of these mutant proteins.

All mutant P proteins were phosphorylated, but their level of phosphorylation was 58–113% that of the wild-type (Fig. 3*a*). To get a precise measurement of phosphorylation of these mutants, the phosphorylation level was normalized to [ $^{35}\text{S}$ ]methionine/[ $^{35}\text{S}$ ]cysteine-labelled (by Tran $^{35}\text{S}$ -label) mutants in parallel experiments. Any deletion of methionine and cysteine from a deletion mutant was taken into account by the incorporation of  $^{35}\text{S}$  in that mutant. None of the mutant proteins was entirely without phosphorylation. Thus, these results were not very useful in deciphering the phosphorylation locus in the HPIV-1 P protein.

To determine which deletion mutant(s) lacked the primary phosphopeptides (TP1–TP3), each deletion P protein was subjected to two-dimensional tryptic phosphopeptide analysis (Fig. 4). Mutants  $\Delta 22$ –64 (Fig. 4*b*),  $\Delta 66$ –103 (Fig. 4*c*),  $\Delta 267$ –364 (Fig. 4*g*),  $\Delta 366$ –433 (Fig. 4*h*) and  $\Delta 435$ –532 (Fig. 4*i*) all had a phosphopeptide map similar to that of the wild-type P protein (Fig. 4*a*). However, mutant  $\Delta 105$ –163 (Fig. 4*d*) lacked phosphopeptides TP1 and TP2, while mutants

$\Delta 165$ –265 and  $\Delta 165$ –195 lacked phosphopeptide TP3 (Fig. 4*e*, *f*). These results localized the primary phosphorylation sites within aa 105–195.

#### Analysis of point mutants

Based on the premise that the HPIV-1 P protein is phosphorylated at Ser-Pro sites as in the SV P protein, we examined the amino acid sequence of the P protein between aa 105–195. There are two Ser-Pro sites at positions 120 and 140 (Figs 1 and 5). To determine whether P protein phosphorylation occurs at these Ser-Pro sites, serine residues at positions 120 and 140 were changed to alanine. The mutant P proteins were analysed for their phosphopeptide composition. Mutating Ser-120 to Ala (mutant S120A) resulted in the abrogation of two phosphopeptides, TP1 and TP2 (Fig. 6*b*). The mutant S140A (Ser-140 to Ala mutation) had a phosphopeptide map identical to that of the wild-type P protein (data not shown). Since the putative tryptic peptide containing Ser-120 has only one serine, the two phosphopeptides could originate due to a partial trypsin digestion of P. There are two adjacent arginine residues at positions 116 and 117 (Fig. 5). Partial trypsin cleavage either at Arg-116 or Arg-117 could result in the generation of two phosphopeptides. To test this idea, Arg-116 was changed to alanine (mutant R116A). This

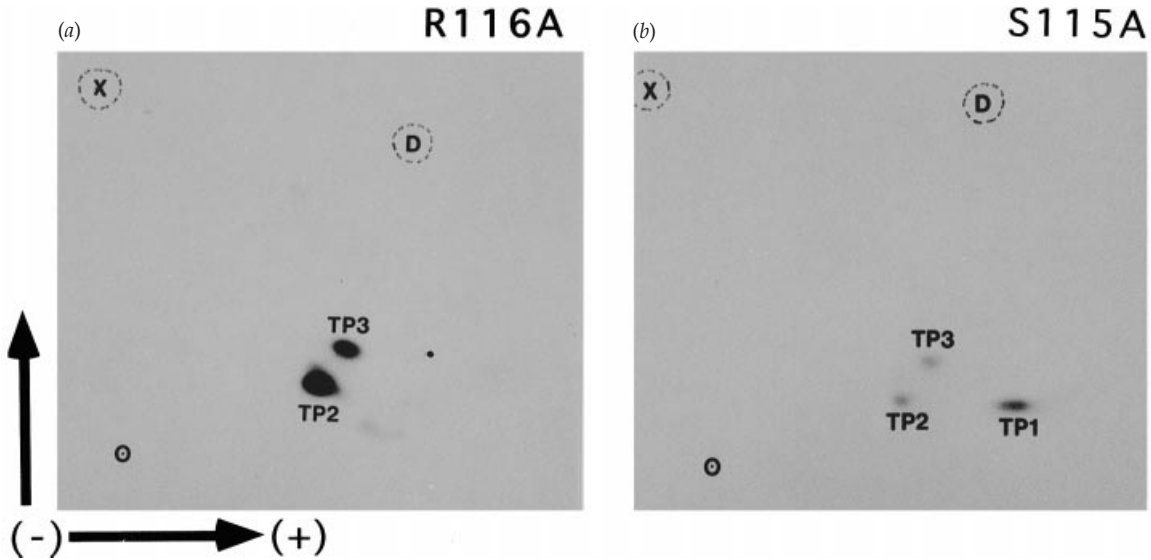


Fig. 7. Phosphopeptide maps of HPIV-1 P point mutants. (a) Map of R116A; (b) map of S115A. Other notation is described in the legend to Fig. 2. Note the disappearance of TP1 from (a) and heavier labelling of TP2 in (a) as compared to TP2 in (b).

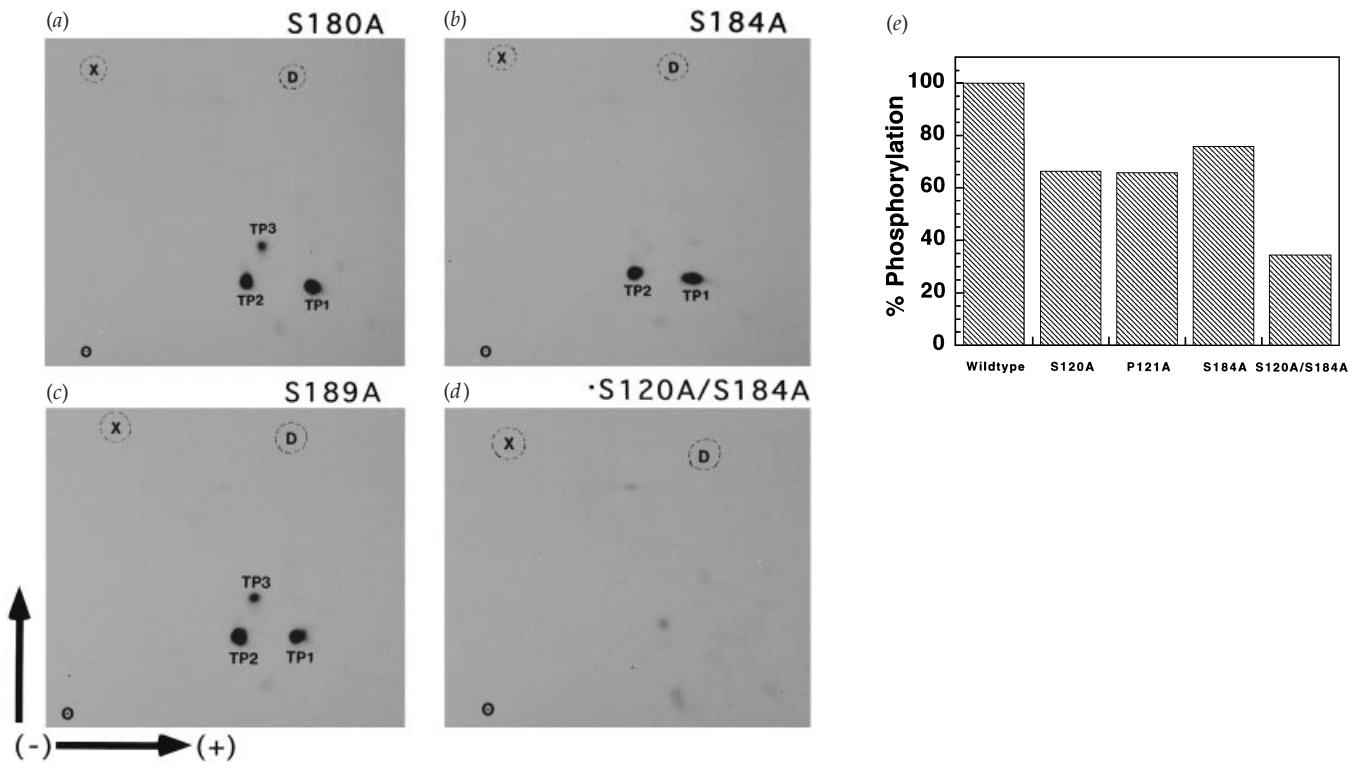


Fig. 8. Phosphopeptide maps of HPIV-1 P point mutants. (a) Map of mutant S180A; (b) map of mutant S184A; (c) map of mutant S189A; (d) map of double mutant S120A/S184A. Note the disappearance of TP3 in (b) and the appearance of weakly labelled phosphopeptides in (d). The relative labelling of these mutants as determined by a PhosphorImager is shown in (e).

mutation resulted in the abolition of TP1 in the phosphopeptide map of the mutant protein (Fig. 7a). This indicates that TP1 and TP2 are most likely the result of partial digestion of the P protein. However, there is one more serine within aa 105–163 at residue 115 (Fig. 5). The possibility existed that Ser-115 was phosphorylated and yielded TP1, but the change

of Arg-116 to alanine abrogated this phosphorylation. Alternatively, Arg-113 could be involved in the partial digestion. To eliminate these possibilities, we mutated Ser-115 to alanine. The phosphopeptide map of this mutant (S115A; Fig. 7b) was identical to that of the wild-type P showing that Ser-115 was not phosphorylated. The phosphopeptide map of a deletion

mutant  $\Delta$ 113–116 was similar to the phosphopeptide map of mutant R116A, indicating that Arg-113 is not involved in partial trypsin cleavage (data not shown). In addition, abolition of TP1 in mutant R116A resulted in a phosphopeptide map in which TP2 had the cumulative radioactivity of both TP1 and TP2, i.e. about 80% of the total radioactivity (compare Fig. 7*a* and *b*). Taken together, these results conclusively show that Ser-120 is the phosphorylated residue within aa 105–163.

To determine whether the presence of Pro-121 is necessary for Ser-120 phosphorylation, Pro-121 was changed to alanine (mutant P121A). The phosphopeptide map of this mutant was similar to that of S120A, that is both TP1 and TP2 were abrogated (Fig. 6*c*). This result indicates that Ser-120 is probably phosphorylated by a proline-dependent protein kinase.

Analysis of deletion mutants localized phosphopeptide TP3 within aa 165–195 (Fig. 4*e, f*). There are three serine residues within this region, at positions 180, 184 and 189 (Fig. 5). However, none of these serines are in the Ser-Pro sequence or other consensus target sequence for any known kinases. Therefore, to determine the phosphorylated serine, each serine was mutated to alanine and each mutant P protein was analysed for its phosphopeptide content (Fig. 8). Mutagenesis of Ser-184 to alanine (S184A) resulted in the loss of peptide TP3 (Fig. 8*b*). However, mutations of Ser-180 or Ser-189 did not result in any change in the phosphopeptide pattern of the mutant protein as compared to the wild-type (Fig. 8*a, c*). These results showed that the minor site of phosphorylation (20% phosphorylation as compared to the major site) is Ser-184.

A double mutant (S120A/S184A), which had both the constitutive phosphorylation sites changed to alanine, did not give the three phosphopeptides (Fig. 8*d*). Instead, some weakly labelled phosphopeptides were present (Fig. 8*d*). These phosphopeptides are identical to the very weakly labelled phosphopeptides in wild-type P protein (Fig. 2*d*) and point mutants S120A (Fig. 6*b*) and P121A (Fig. 6*c*). However, due to very low level of radioactivity in these phosphopeptides, we could not quantify their phosphorylation level.

The level of phosphorylation of mutant S120A or P121A was about 65% of that in the wild-type (Fig. 8*e*). Similarly, phosphorylation of S184A was about 76% of that of the wild-type P protein. When both Ser-120 and Ser-184 were mutated to alanine, the double mutant had about 35% of wild-type P protein phosphorylation. It was rather surprising to note that these point mutants carried 35–65% of radioactivity since TP1, TP2 and TP3 were the only major phosphopeptides detected in the wild-type map (Fig. 2). This is explained by the presence of several (10–12) additional weakly labelled phosphopeptides in these mutants which are detected after long exposure to an X-ray film (see Fig. 8). A similar observation was made for mutants of the SV P protein in which the primary phosphorylation site was abolished by changing Ser-249 or Pro-250 to alanine (Byrappa *et al.*, 1996). However, in the SV P mutants, alternate sites were more heavily phosphorylated.

## Discussion

The HPIV-1 P protein has 66% similarity with the SV P protein (Fig. 1). The results presented in this communication show that the similarity extends to the primary site of phosphorylation at the Ser-Pro motif. However, there is no other obvious sequence conservation of the two proteins at the loci of the Ser-Pro sites. While SV P protein has one primary phosphorylation site, HPIV-1 P protein has two, one major and one minor. Although we have not analysed the stoichiometry of HPIV-1 P protein phosphorylation, we believe that all its P protein molecules are constitutively phosphorylated at Ser-120 and only about 20% molecules are phosphorylated at Ser-184. However, there is no kinetic relationship between the phosphorylation at the two sites. Each site gets appropriately phosphorylated in the absence of other site (see Figs 4*d–f*, 6*b* and *c*, and 8*b*). In addition to the primary phosphorylation sites, the HPIV-1 P protein also has the potential for phosphorylation at several minor sites whose phosphorylation increases in the absence of primary sites. This is very similar to what has been observed for the SV P protein (Byrappa *et al.*, 1996).

The constitutive phosphorylation sites of HPIV-1 P protein (Ser-120 and Ser-184) are conserved in three clinical isolates CI-5/73, CI-14/83 and C35 (Power *et al.*, 1992). The evolutionary conservation of the constitutive phosphorylation of P proteins primarily at Ser-Pro sites indicates that this has an important role.

The Ser-Pro sequence is a consensus target for phosphorylation by proline-directed protein kinases (PDPKs) that are important in intracellular signalling. PDPKs include the mitogen-activated protein kinases, cyclin-dependent protein kinase 5 and glycogen synthase kinase 3 (Cano & Mahadevan, 1995; Pelech, 1995). The role of a putative PDPK in phosphorylation of P proteins of SV and HPIV-1 is not clear. The conservation of the Ser-Pro phosphorylation site in the SV and HPIV-1 P proteins suggests that similar, if not identical, kinase(s) phosphorylate these proteins.

SDS-PAGE of the HPIV-1 P protein from transfected cells revealed two species (Fig. 3*b*). However, this migration pattern does not appear to be due to differential phosphorylation of the P protein, as the pattern did not change in any of the deletion mutants or point mutants that removed the phosphorylation site(s) (see Fig. 3*b*). Thus, two species of P protein are most likely generated due to some other modification of the P protein.

Despite extensive work on P proteins from various negative-sense RNA viruses, the functional significance of its ubiquitous phosphorylation remains unclear. Various structural and functional domains of SV P protein have been defined. Two functionally redundant regions of aa 1–77 and 78–145 were shown to be absolutely essential for virus replication (Curran *et al.*, 1994). The N-terminal 77 aa were shown to be important for RNA encapsidation by the nucleoprotein. This

region is required for the delivery of nucleoprotein during nascent chain assembly and subsequent genome replication (Curran, 1996). Amino acids 1–77 are important for viral RNA synthesis and could substitute for residues 78–145. Residues 78–145 are needed for genome replication, but not for transcription (Curran, 1996). The nucleocapsid-binding domain of the P protein spans residues 345–412 and 479–569 (Ryan *et al.*, 1991), whereas the L protein-binding domain spans the C-terminal residues, 412–478 (Smallwood *et al.*, 1994). Interestingly, none of the defined domains encompasses the SV P protein phosphorylation site. However, a deletion mutant lacking aa 78–320, that in essence lacked the primary phosphorylation site, was found to be active in RNA synthesis (Curran *et al.*, 1994).

Phosphorylation increases the negative charge of the N-terminal half of the P protein. It has been speculated that this acidic domain in the N terminus of the P protein provides global transcriptional activation (Curran *et al.*, 1994). Perhaps the increased acidity of the region, rather than phosphorylation at specific residues is the crucial determinant in transcriptional activation. Host cell proteins have been shown to activate virus transcription and replication in several negative-sense RNA viruses. Tubulin was shown to enhance transcription in VSV, SV and measles virus (MV) (Moyer *et al.*, 1990, 1986), whereas actin was shown to enhance transcription in human parainfluenza virus 3, respiratory syncytial virus and MV (De *et al.*, 1993; Moyer *et al.*, 1990). Possibly, the acidic domains within these cellular proteins synergize with the N-terminal region of P protein to provide activation. However, conservation of the Ser-Pro phosphorylation sites in the two proteins indicates a more specific rather than the general role of phosphorylation.

Although no extensive similarity is observed at the site of phosphorylation between the two P proteins (Fig. 1), it is possible that they have a great similarity in their structures. Indeed, phosphorylation sites of both P proteins occur in regions that have a very high probability of  $\beta$ -turns. It is likely that elucidation of the higher structure of these proteins is necessary to explain the role of P protein phosphorylation.

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