Determination of phosphorylated residues from human respiratory syncytial virus P protein that are dynamically dephosphorylated by cellular phosphatases: a possible role for serine 54

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INTRODUCTION

Human respiratory syncytial virus (HRSV) is a pneumovirus of the family *Paramyxoviridae* that causes serious viral bronchiolitis and pneumonia in infants, children, the elderly and immunocompromised populations (Harrington et al., 1992; Falsey et al., 1995; Collins et al., 2001). We focused on understanding how P-protein phosphorylation acts at the molecular level, which could aid in developing live-attenuated vaccines and antiviral compounds to prevent the severe morbidity associated with HRSV infection (Dudas & Karron, 1998; Whitehead et al., 1999; Pastey et al., 2000).

This phosphorylated structural protein, the viral RNA (15 222 nt) and the N, L and M2-1 proteins form the viral ribonucleoproteins (RNP) (Yu et al., 1995; Collins et al., 1995, 1996; Hardy & Wertz, 1998). The RNP are the internal components of the virions and the functional units for the transcription and replication processes.

The P proteins of different members of the family *Paramyxoviridae* have very distinct sizes and primary and secondary structures, but all are phosphorylated (Lamb & Kolakofsky, 2001). This suggests that P-protein phosphorylation has an important role in the viral growth cycle. This role could be to differentiate the P–L complex that transcribes RNA (vRdRT or transcriptase) from that involved in RNA replication [vRdRR or replicase, according to nomenclature proposed by Gubbay et al. (2001)]. The P protein is an essential initiation factor for the viral L polymerase (Collins et al., 2001), which, through interaction with P, is bound to the viral nucleocapsid via P–N interactions (Horikami et al., 1992). Nucleocapsids are formed by v- or cRNA and by the N protein and act as templates for viral RNA synthesis. In transcription, RNA synthesis proceeds discontinuously, starting and ending at start and stop signals of each gene. Different viral mRNAs are generated, and leader, trailer and intergenic sequences (44 first and 155 last nucleotides of vRNA, respectively) are not read. In replication, RNA synthesis proceeds from the first to the last template of v- or cRNA nucleotides and a continuous copy is made. Encapsidation of nascent RNA by the N protein is concomitant with synthesis. Only those N-protein molecules that previously established the P–N complex are functionally competent for RNA encapsidation (Collins et al., 2001). The P protein also interacts with the M2-1 protein, a transcription elongation factor (Cuesta et al., 2000; Mason et al., 2003). How the P protein is committed to these different interactions is unknown, but its phosphorylation state may play a role.

P-protein phosphorylation, due mainly to casein kinase II
(CKII), takes place mainly at serine residues 116, 117, 119 and 232, when the P protein is expressed in HEp-2 cells infected by HRSV, vaccinia recombinant or by using a vaccinia-based expression system, although to a different extent (Navarro et al., 1991; Mazumder et al., 1994; Villanueva et al., 1994; Barik et al., 1995; Sánchez-Seco et al., 1995; Asenjo & Villanueva, 2000). It is not essential for either HRSV RNA transcription or replication (Villanueva et al., 1991, 2000), although inhibition of extracellular viral-particle formation was observed in its absence (Villanueva et al., 1991). Inhibition of extracellular viral-particle formation was also found in an HRSV mutant in which these P-protein serine residues were substituted (Lu et al., 2002).

Here, we show that the P protein also has high-turnover phosphorylation sites, detectable only after inhibition of cellular protein phosphatases PP1 and PP2A. The majority of P-protein residues to which these phosphates are added have been established. A large part of this phosphorylation is not essential for viral RNA synthesis or for P-protein tetramerization (Asenjo & Villanueva, 2000), although it is necessary that serine 54 is dephosphorylated to allow efficient P–M interactions that are related to the viral-particle budding process.

**METHODS**

**Cells and viruses.** HEp-2 cells were obtained from the ATCC. The HRSV Long and A2 strains were plaque-purified and passed at low multiplicity in HEp-2 cells, as described previously (Villanueva et al., 1991). The origin and handling conditions for vaccinia recombinant vTF-3 have been reported previously (Villanueva et al., 2000).

**P-protein variant construction.** All plasmids containing Long strain P-protein variant cDNAs are pGEM3 recombinants. P-protein cDNA was cloned as an HpaII–StuI fragment of the p20 plasmid (López et al., 1988) in the Smal site of the pGEM3 polylinker, to render the VP plasmid. Construction of VP3–8 and VP3–10 has been described previously (Asenjo & Villanueva, 2000; Villanueva et al., 2000). The remaining P-protein variants were obtained in VP by site-directed mutagenesis (Higuchi et al., 1988). All VP nucleotide sequences encoding the P protein were confirmed by automated sequencing.

**Construction of the pGEM3 recombinant containing SV40 small t-antigen cDNA.** Plasmid pCEP4-smt, containing SV40 small t-antigen cDNA (Alberts et al., 1994), was kindly provided by Dr Shenolikar (Department of Pharmacology, Duke University Medical Center, Durham, NC, USA). After HindIII and BamHI digestion, the fragment containing small t-antigen DNA was purified and ligated to digested pGEM3 DNA. The pGEM3 recombinant plasmid is described as smt.

**Infection, transfection, cell labelling, fractionation and P-protein purification.** HRSV infection and transfection experiments were performed as indicated previously (Villanueva et al., 1991, 1994, 2000). Cell labelling with [35S]methionine and [32P]orthophosphate was done as described previously (Asenjo & Villanueva, 2000).

For protein isolation, cells were scraped into the growth medium and isolated by low-speed centrifugation. Medium from HRSV-infected cells was removed and precipitated twice with 6% polyethylene glycol.

Pellets were washed twice with PBS and total cell extracts or soluble-and microfibrilament-protein fractions were obtained by resuspending pellets in 10 mM Tris/HCl (pH 7.5), 0.14 M NaCl, 5 mM EDTA, 1% Triton X-100 and 1% sodium deoxycholate, or by using the extraction conditions described previously (Ulloa et al., 1998), respectively.

P protein in the different fractions was purified by chromatography through Sepharose 4B containing covalently bound anti-P monoclonal antibody (mAb) 73/P or by immunoprecipitation (Villanueva et al., 1991).

**Okadaic acid (OKA) treatment of HRSV-infected or transfected HEp-2 cells.** When cytopathic effect was apparent, HRSV-infected cells were treated with 100 nM OKA in the presence of 37.5 μCi [32P]orthophosphate ml⁻¹ (24 h). Transfected HEp-2 cells were treated and labelled in the presence of 100 nM OKA at 10–24 h post-transfection.

**Formic acid and trypsin treatment of P protein.** Treatment conditions and phosphopeptide separation were as described previously (Navarro et al., 1991; Sánchez-Seco et al., 1995).

**Oligomerization, viral transcription, replication and M-interaction capacities of P-protein variants.** The conditions followed were as described by Asenjo & Villanueva (2000), Rodriguez et al. (2004) and Schmitt et al. (2002), respectively.

**RESULTS**

Inhibition of cellular protein phosphatases PP1 and PP2A reveals new P-protein phosphorylation sites in HRSV-infected and transfected HEp-2 cells

To determine whether the P protein undergoes high phosphorylation turnover at specific sites, HRSV-infected or transfected HEp-2 cells (Fig. 1a, b) were labelled with [35S]methionine or [32P]orthophosphate (upper or lower part), alone (lanes) or in the presence (+ lanes) of 100 nM OKA (Villanueva et al., 1991, 2000). PP1A and PP2A are partially and totally inhibited, respectively, at this OKA concentration (Bialojan & Takai, 1988). Extracellular viral particles (V), distinct cell-protein fractions (S, M, In) (Ulloa et al., 1998) or whole-cell extract (VP) (Asenjo & Villanueva, 2000) were isolated. P protein was immuno-purified from these samples and analysed by SDS-PAGE.

Infected-cell proteins were fractionated to improve detection of P-protein isoforms, but no major differences were observed for untreated or OKA-treated cells for P-protein cell location, phosphorylation level or in the amount of N protein that co-immunopurified with the P protein (Fig. 1a, lanes S, M and In, upper). No differences were detected either in [32P]orthophosphate-labelled P protein in transfected cells (Fig. 1b, lower) or in infected cells (Fig. 1a, lower) but, after immunochromatography, a phosphorylated protein (HP) with a slight slower mobility in SDS-PAGE than that of the P protein copurified with it in all fractions from OKA-treated cells (also visible in [35S]methionine-labelled cells; Fig. 1a, b, upper). Also, two highly phosphorylated proteins (molecular mass around 45 kDa) are found, mainly in soluble (S) and in
microfilament-bound (M) protein fractions from OKA-treated cells (Fig. 1a, + lower lanes). The slow-migrating protein (HP) may be a hyperphosphorylated form of the P protein. Thus, it appears that P protein expressed during viral infection, or in the absence of other viral proteins, is modified by the addition of phosphates that are actively removed by cellular PP1 and/or PP2A phosphatases.

Phosphorylated HP protein appears to be present in different locations in infected cells, but is not incorporated into extracellular viral particles (lanes V).

**Location of modified P-protein residues with high phosphate turnover**

The protein fraction immunopurified from whole-cell extract of HRV-infected, OKA-treated cells labelled with [32P]orthophosphate was analysed by Western blot. By using an anti-P antibody, reactivity was found with P, HP and another minor phosphorylated P-related protein showing an electrophoretic mobility close to that of P. This protein was termed PI (Fig. 2a, W and 32P). PI is better appreciated when the P protein carried the substitution T46I (see below). Thus, the HP and PI proteins may be P-protein forms with different phosphorylation levels.

To determine the phosphorylatable residues (serine, threonine or tyrosine) that are modified in the HP and PI forms, we carried out phosphoamino acid analysis of P, P plus PI, and HP proteins from HRV-infected HEp-2 cells, without (−) or with (+) OKA. P protein isolated in the absence of OKA was phosphorylated only at serine residues, concuring with previous reports (Navarro et al., 1991; Sánchez-Seco et al., 1995). In the presence of OKA, P plus PI proteins were phosphorylated at serine and threonine, whereas HP was phosphorylated at serine residues only (Fig. 2b).

To confirm that HP and PI are differently phosphorylated P proteins and to locate the new phosphorylation sites, P proteins were immunopurified from HRV-infected (I) or from transfected (T) HEp-2 cells in the absence (−) or presence (+) of OKA and 32P-labelled. Immunopurified proteins were trypsin-digested (Fig. 2c) or fractionated by SDS-PAGE, excised from the gel and treated with formic acid for peptide mapping (Fig. 2d). The phosphopeptides generated were visualized after electrophoretic separation by phosphorimaging. Under both conditions tested, the P protein showed common and uncommon phosphopeptides with the PI and HP proteins. As suggested by the Western blot analyses, this indicates that the P, HP and PI phosphoproteins are a single protein with distinct phosphorylation levels.

By contrast, the two proteins with molecular masses around 45 kDa that coimmunoprecipitated with the P protein in infected, but not in transfected, HEp-2 cells were unrelated to the P protein (data not shown). These results suggest that viral infection, but not expression of the P protein alone, may allow the high-turnover phosphorylation of these proteins with which the P protein is associated.

The size and location of the tryptic and formic acid-generated peptides have been described previously (Navarro et al., 1991; Sánchez-Seco et al., 1995; Fig. 2e and f). The tryptic phosphopeptides include P-protein residues 104–160 (Fig. 2c, bands a, b and c) and those around S232 (Fig. 2c, bands d and e). In the presence of
OKA (Fig. 2d, + lanes), some phosphopeptides, such as F3 and F5, are labelled, whereas others showed slower electrophoretic mobilities compared with those found for their counterparts in the absence of OKA (F3 being an exception) when P protein is expressed transiently (Fig. 2d, T).

Thus, it appears that the bulk of P-protein phosphorylation, controlled by cellular phosphatases, takes place at the same P-protein location (between amino acid residues 1 and 90, and 104 and 160), despite its expression by infection or transfection. Nevertheless, differences in the phosphorylation level of some peptides, such as F2, occur and could be explained by the presence or absence of the rest of the viral proteins under the two expression conditions. The vaccinia-based expression system can thus be used to determine which amino acid residue(s) is modified with high-turnover phosphates during HRSV infection.

Determination of P-protein phosphorylated residues with high turnover

**P-protein substitution variants.** To determine whether modification at serines 116, 117, 119 and/or 232, or at
...other substituted residues in our collection of P-protein variants (Fig. 3a), is due to high-turnover phosphorylation, we have analysed the behaviour of previously obtained P-protein variants (Asenjo & Villanueva, 2000) in the presence of OKA. All of the tested variants showed HP (Fig. 3b) and PI (by labelling with [35S]methionine; data not shown) isoforms in the presence of OKA. Thus, it seems that the residues whose phosphorylation generates these isoforms are different, and that their modification is independent of those residues substituted in the analysed variants. Nonetheless, isoforms from variants in which residues 116, 117 and/or 119 have been replaced showed slight differences in their electrophoretic mobility compared with that of the control protein.

Taking all the results together, it seems that the residues whose modification is responsible for the appearance of PI and HP are present between positions 1 and 90. This possibility agrees well with the observation that P-protein deletion variants containing protein residues between positions 1 and 92, 1 and 120, 1 and 170, and 1 and 203 were all able to generate their corresponding PI- and HP-like isoforms (data not shown).

A slight increase was observed in specific activity (phosphate content) of protein variants in which S116, S117 and S119 were substituted, showing a normal electrophoretic mobility compared to the counterpart protein obtained in the presence of OKA. In the rest of the variants, the increase found in phosphate content was at least twofold (data not shown).

Tryptic analysis of the variants (Fig. 3c) indicates that substitutions at S116, S117 and S119 result in the loss of the tryptic phosphopeptides from the central part of the molecule (Fig. 3c, bands a, b, c). Phosphorylation at S116, S117 and/or S119 is thus actively removed, at least in part, by cellular protein phosphatases.

**Determination of P-protein residues whose modification generates HP and PI isoforms.** Considering the high phosphorylation level of the PI and HP proteins, we have searched for P-protein NH2-terminal serines that are able to trigger a phosphorylation cascade when modified (Roach, 1991). That is the case for serine residues at positions 39 and 54. Other serines located between these residues, present between positions 16 and 60, were also substituted (Fig. 4a). The P-protein variants were transiently expressed and labelled with [35S]methionine in the presence of OKA. Cultures were processed as above, the protein was immunoprecipitated with an antibody raised against the P protein, the immunoprecipitated phospho-proteins were separated by SDS-PAGE and the labelled proteins were visualized by phosphorimaging to test for the presence or absence of the HP isoform.
In the presence of OKA (Fig. 4a, upper right part), P-protein variants VP11 and VP13 expressed HP, but in the VP12 variant in which Ser54 was replaced by Ala, only a smear was seen. A small amount of HP, with a different electrophoretic mobility, is observed when S39 was substituted by alanine (VP11). When S39 and S54 substitutions are combined in the variant VP11–12, it becomes clear that HP is generated by modification at serines 39 and 54, because in this variant, only phosphorylation at the PI and P isoforms was seen in the presence of OKA. A contribution by phosphorylation at residues 18, 23, 29 and 30 in HP formation can be ruled out, as in VP13, in which these residues are replaced by alanine, HP is found.

To analyse the P-protein phosphorylation that generates the PI isoform, T46 was the first residue that was substituted, because in the analysis of modified residues in P+PI in the presence of OKA, phosphorylation at threonine was detected and T46 is specific for the Long strain P protein. When this substitution was made (Fig. 4a, lower right part), the PI isoform was clearly identified, in addition to a wide band corresponding to isoform HP (VP14).

**Residues modified with high turnover in P protein with the T46I substitution.** To determine the residues modified in the T46I P-protein variant, this substitution was added to other P-protein variants (Fig. 4a, lower left part). The variants were transiently expressed and labelled with [35S]methionine. Labelled peptides were immunoprecipitated and characterized as indicated above. The HP isoform in P protein with the T46I substitution shows three bands (VP14); one disappears with the S39 substitution (VP15) and two with the S54 substitution (VP16). When both substitutions were combined, the three HP bands were absent (VP17). Only the prominent PI band...
remains and it can be nearly eliminated by substitution of S45 (VP18). In addition, it is observed that the S45A modification alone prevents the formation of two HP forms (data not shown); thus, two HP forms could arise from phosphorylation at S45 and S54 and at S45 and S39, respectively. To corroborate the relationship between the presence of these P isoforms and phosphate addition to those P-protein serines, we have analysed the VP19 variant with substitutions at residues S39, S45, S54 and T46. In this variant, only the P-protein isoform with normal electrophoretic mobility was detected.

The substitutions present in variants VP3–10 were added to those present in variants VP17 and VP19 in an attempt to prevent the slow and medium turnover of P-protein phosphorylation (Fig. 3, S232A, S237A and S116L, S117R, S119L, respectively). These new variants were named VP17* and VP19* and they were labelled with [35S]methionine or [32P]orthophosphate and analysed as above (Fig. 4b, upper and lower panels, respectively). Both variants showed two [32P]-labelled isoforms, P and PI. In the case of variant VP19*, PI is weakly labelled. The stronger band, corresponding to the P isoform, was eliminated when the S30A substitution was incorporated with those already present in the VP17* variant, yielding the new variant VP20*. In this way, the majority of high-turnover phosphates added to P-protein residues have been identified.

The T46I substitution in the P protein from the Long strain is sufficient to change the P-isoform pattern to that of the A2 strain P protein after inhibition of cellular phosphatases, as the same isoform phenotype was shown for Long strain P-protein variant VP14 and for A2 strain P protein, both of which bear I46 (Fig. 4c, lower part). Based on these results, we have deduced the residues modified to generate each P-protein isoform (Fig. 4c, upper part).

**Cellular PP2A phosphatase is implicated in the high-turnover phosphorylation that generates HP**

To determine which one of the cellular phosphatases inhibited by OKA (PP2A or PP1) is involved in removing the high-turnover phosphates added to P-protein residues, P protein was transiently coexpressed with a pGEM3 recombinant that expresses the SV40 small t antigen under the control of T7 RNA polymerase. This viral protein is a specific PP2A inhibitor (Alberts et al., 1994). Coexpression of the P protein and SV40 small t antigen allows the appearance of HP (Fig. 5a, b, lane 2) in a similar way to that when the protein is transiently expressed alone and not dephosphorylated because of OKA treatment (Fig. 5a, b, lane 3). Cellular PP2A phosphatase actively removes S54 and S39 phosphorylation. No phosphorylation increase was observed in residues located in the central region of the P molecule, isolated after trypsin digestion (Fig. 5c, lane 2). This suggests that the high-turnover phosphates added, mainly at S116, S117 and S119, are probably dephosphorylated by the PP1 cellular phosphatase.

As we have suggested that high (controlled)-turnover phosphorylation may be responsible for P-protein tetramerization...
(Asenjo & Villanueva, 2000), we assayed this capacity in a variant, VP21*, that lacks low-turnover (S232 and S237), medium-turnover (S116, S117 and/or S119) and high-turnover (S30, S39, S45, S46 and S54) phosphorylation residues. Protein from an extract of HEp-2 cells expressing VP21* was glutaraldehyde cross-linked as described previously (Asenjo & Villanueva, 2000) and the oligomers were characterized.

VP21* oligomerizes, as does the normal P protein (Fig. 5d). Thus, only residual high-turnover P-protein phosphorylation should be sufficient to allow homotetramer formation, or unphosphorylated P protein may be able to form tetramers.

**Viral RNA-synthesis capacity of P-protein variants with NH2-terminal substitutions**

To determine whether high-turnover P-protein phosphorylation is related to its function as a L-polymerase cofactor in viral initiation of RNA synthesis, P-protein variants were assayed in the system described by Hardy & Wertz (1998), which was previously used to test the role for other P-protein variants (Villanueva et al., 2000; Rodríguez et al., 2004). In P-protein variants with the S54A (VP12) or S54D (VP12D) substitutions (Fig. 6b), it can be observed that both variants have viral RNA polymerase cofactor activity for transcription and replication, and neither affects the intergenic read-through and elongation capacities described for the HRSV M2-1 protein. High-turnover P-protein phosphorylation at S54 is thus not involved in the P-protein function concerning the formation of P–N0 or P–L complexes with transcriptase or replicase specificities. This is also true for the VP21* variant, with substitution at all residues with detected modifications. When the M protein was coexpressed in the system, we observed inhibition of the RNA-synthesizing capacity of the viral RNP at high M-protein concentrations in all cases, as described previously (Rodríguez et al., 2004) (data not shown).

**P protein is incorporated together with M protein in membranous vesicles**

When the HRSV M protein is transiently expressed in HEp-2 cells, a small amount of the expressed protein is excreted to the growth media in membranous vesicles, as described by Schmitt et al. (2002) (Fig. 7b, lane M). P protein, expressed in the same way, is poorly excreted in these vesicles (lane VP), but if the M protein is coexpressed with the P protein, a fraction of the expressed P protein is also incorporated, together with the M protein, into the membranous vesicles (lane VP+M). It suggests an interaction between both proteins. This interaction takes place for all the P-protein substitution variants assayed, but is impaired in the VP12D variant. A decrease of 5- to 4-fold by comparison to VP and VP12, respectively, was observed (Fig. 7c).

These results suggest that phosphorylation at S54 could be the signal to regulate a novel P–M protein interaction. This P–M interaction may play a role in the formation of extracellular viral particles.

**DISCUSSION**

How paramyxovirus viral RNA polymerase, composed of L polymerase and P protein, switches RNA synthesis from transcription to the replication mode remains unsolved. The idea that the amount of N protein synthesized de novo as a P–N0 complex controls the switch of viral polymerase from transcription to replication has been shown to be incorrect for HRSV (Fearsn et al., 1997). It has been suggested that P proteins with different phosphorylation levels can differentiate transcriptase (vRdT) from replicase (vRdRR) (Collins et al., 2001). However, low-turnover phosphate groups mainly added to S232 (Navarro et al., 1991; Mazumder & Barik, 1994;
Sánchez-Seco et al., 1995) of the HRSV P protein are not essential for RNP formation or for their function in viral transcription and replication, although these modifications are related to the formation of mature extracellular viral particles (Villanueva et al., 1991, 2000; Lu et al., 2002). When these serines are replaced by unphosphorylatable residues, about 2% of the total P-protein phosphorylation remains detectable (Villanueva et al., 2000). This residual phosphorylation, decreased slightly by the substitutions S237A and S215A, varies according to HEp-2 cell passage number (A. Asenjo, unpublished results) and may be related to the existence of different P-protein species with different turnover in their added phosphates.

P protein expressed alone, or in the presence of the rest of the viral proteins, is modified by addition of high-turnover phosphate groups that are actively removed by the cellular protein phosphatases PP1 and PP2A. The phosphorylated P-protein isoforms PI and HP, with different electrophoretic mobilities, are generated by modification of serine and threonine residues mapped at the NH2-terminal (residues 1–90) and at the central (residues 104–160) regions of the P protein, under both expression conditions. The residues at the NH2 terminus (except for S30) are responsible for the slow electrophoretic mobility found for the P-protein isoforms PI and HP. Thus, the vaccinia-based expression system can be used to determine the modified P-protein residues during HRSV infection, although those modifications that occur during infection must be further checked by isolating the corresponding viral mutants, as has been the case for P-protein residues 116, 117, 118 and/or 119, and 232 (Lu et al., 2002).

To identify high-turnover phosphorylated residues, we have analysed transient expression of P-protein variants with different deletions (data not shown) and amino acid
Several P-protein NH$_2$-terminal phosphorylable residues were substituted. We found that phosphorylation at S54 and, to a lesser extent, at S39 was responsible for HP generation. These residues have consensus sequences for glycogen synthase kinase 3 and protein kinase C, respectively (Pearson & Kemp, 1991). Dephosphorylation of these residues occurs by PP2A, as HP is found when the P protein is coexpressed with SV40 small t antigen, a specific PP2A phosphatase inhibitor (Alberts et al., 1994). P-protein interaction with PP2A cellular phosphatase has been also suggested (Bitko & Barik, 1998).

Slight differences were seen in the HP-phosphorylation pattern for the T46I substitution, which is found in P protein from different RSV strains that infect mammalian and avian hosts, but is absent in the Long strain (Alansari & Potgieter, 1994). These differences were observed by comparing the A2 and Long strain P proteins. Thus, only T46I induces a decreased modification at S54 and a prominent phosphorylation of residues S30, S39 and S45. A larger amount of the PI isoform also results from S45 modification. The meaning of these differences is unknown, but may be related to the better growth of HRSV A2 strain than that of the Long strain in HEp-2 cells and BALB/c mice (Sudo et al., 1999). It is difficult to test whether phosphorylated or unphosphorylated T46 has a role in the Long strain, as its substitution generates an A2 strain P-protein phosphorylation pattern.

The high-turnover phosphorylation sites could be important for certain P-protein interactions, such as P self-association (Gao & Lenard, 1995). In this way, we have suggested that some residues with high-turnover phosphorylation were involved in P-protein tetramerization (Asenjo & Villanueva, 2000), but other reports (Mazumder et al., 1994; Castagnè et al., 2004) have indicated that unphosphorylated P protein may form oligomers. An explanation of this apparent contradiction is that our bacterially expressed P protein has a deletion of the first NH$_2$-terminal 20 residues that could be also responsible for the lack of oligomerization observed. Nevertheless, the existence of different P-protein oligomers remains open and, in some cases, phosphorylation could play a role in the formation of specific oligomers. Nonetheless, the VP21* variant, with substitutions at residues 30, 39, 45, 54, 116, 117, 119, 232 and 237, can form tetramers (Fig. 5d).

We tested whether phosphorylation at these residues is essential for P-protein interactions that are established during viral transcription and/or replication; negative results were obtained when the VP21* variant was tested in a dicistronic minireplicon replication system (Fig. 6b). Also, no effect was detected for interaction of the viral RNPs with the M protein. This interaction only takes place in the minireplicon system when a high amount of the M protein is expressed and, as a consequence, RNA synthesis by the viral RNPs is decreased (Rodríguez et al., 2004). In any case, small differences in this system cannot be ruled out. After amplification, during the growth cycle, these effects become crucial, especially if viral proteins are in limited amounts, as has been described for the N protein during HRSV infection (Tran et al., 2004).

P-protein phosphorylation remains, although in a very low proportion, in the variant containing all the substitutions previously described. As PP1 is partially inhibited in the presence of 100 nM OKA ($K_2$ 20–300 nM; Bialojan & Takai, 1988), residual high-turnover P-protein phosphorylation still occurs.

P-protein phosphorylation at S54 could decrease a novel P–M protein interaction, playing a role in the last viral-particle morphogenesis step in the budding process. This possibility explains the absence of the HP isoform in extracellular viral particles. Therefore, it can be speculated that RNPs with the P protein phosphorylated at S54 do not interact with M protein that is committed to form membranous vesicles and, as a consequence, the egress process is abolished. Alternatively, P–protein phosphorylation at S54 during adsorption or internalization at the beginning of the viral infection could be crucial for the uncoating of the viral-particle RNP and to facilitate primary transcription initiation. This interaction may be different from that described in the minireplicon system (Rodríguez et al., 2004).

The results indicated that all P-protein residues modified and characterized in this work, like the previous ones, are not related to P-protein activities concerning viral transcription and replication. P–protein phosphorylation seems to be important for regulating the formation of extracellular viral particles once functional nucleocapsids have been formed (Villanueva et al., 1991, 2000; Lu et al., 2002; this study). Among other possibilities, HRSV P-protein phosphorylation could also be involved in a process directed to stop the antiviral cell response mediated by interferon (Lu et al., 2002) or in viral cytopathogenicity (Das & Pattnaik, 2004).

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