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ScienceDirect
Virus Research 132 (2008) 160–173
Virus Research
www.elsevier.com/locate/virusres

Residues in human respiratory syncytial virus P protein that are essential for its activity on RNA viral synthesis

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Received 22 October 2007; received in revised form 22 November 2007; accepted 22 November 2007
Available online 7 January 2008

Abstract

Human respiratory syncytial virus (HRSV) P protein, 241 amino acid long, is a structural homotetrameric phosphoprotein. Viral transcription and replication processes are dependent on functional P protein interactions inside viral ribonucleoprotein complexes (RNPs). Binding capacity to RNPs proteins and transcription and replication complementation analyses, using inactive P protein variants, have identified residues essential for functional interactions with itself, L, N and M2-1 proteins. P protein may establish some of these interactions as monomer, but efficient viral transcription and replication requires P protein oligomerization through the central region of the molecule. A structurally stable three-dimensional model has been generated in silico for this region (residues 98–158). Our analysis has indicated that P protein residues L135, D139, E140 and L142 are involved in homotetramerization. Additionally, the residues D136, S156, T160 and E179 appear to be essential for P protein activity on viral RNA synthesis and very high turnover phosphorylation at S143, T160 and T210 could regulate it. Thus, compounds targeted to those of these residues, located in the modeled three-dimensional structure, could have specific anti-HRSV effect.

Keywords: HRSV; P protein; Essential residues; Oligomerization domain; Homology model; Complementation assay

1. Introduction

Although the human respiratory syncytial virus (HRSV), a pneumovirus, produces severe respiratory infections affecting babies and toddlers, the elderly and immunocompromised adults, no vaccine or specific antiviral compounds (Bitko et al., 2005; Pastey et al., 2000) are currently available (Hall, 1994). In the Paramyxovirdae family, to which HRSV belongs, the functional units for viral transcription and replication are the structural ribonucleoproteins (RNPs). Hence, understanding HRSV RNPs interactions will be important to develop specific antiviral compounds.

Structural RNPs are made up of viral RNA bound to N protein, as a helical nucleocapsid (NC), and the viral L, P and M2-1 proteins. In transcription and replication processes, NC acts as a template for the nucleotide-polymerising L activity, that only acts bound to P protein. The P–L complex interacts with NC through P–N contacts. For viral replication, a P–N0 complex must be formed to confer encapsidation capacity to the N protein (Collins et al., 2001). For viral transcription a P–M2-1 complex, controlled by phosphorylation of P protein at T108 (Asenjo et al., 2006), allows M2-1 protein to increase elongation and antitermination activities of the L protein (Collins et al., 1995; Hardy and Wertz, 1998; Mason et al., 2003).

Some of the regions involved in the different interactions, in which P protein participates, have been determined: P–P (Castagne et al., 2004), P–L (Khattar et al., 2001), P–N0, P–N (NC) (Castagne et al., 2004; García et al., 1993; Khattar et al., 2001; Krishnamurthy and Samal, 1998) and P–M2-1 interactions (Mason et al., 2003). However, it remains unclear how P protein establishes all these different interactions required for viral transcription and replication. Because P protein is a phosphoprotein with oligomerization capacity (Asenjo and Villanueva, 2000; Llorente et al., 2006), understanding its

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doi:10.1016/j.virusres.2007.11.013

phosphorylation and oligomerization characteristics could be fundamental to gain insights into the interactions in which it participates.

HRSV Long strain P protein is phosphorylated at several serine (S) and threonine (T) residues with different turnover rates. Low turnover modification occurs at S232 (Sánchez-Seco et al., 1995) and perhaps at S237, whereas phosphorylation with intermediate turnover occurs at S116, S117 and/or S119 (Navarro et al., 1991). The phosphate added to serines at positions 30, 39, 45 and 54, and probably that at T46, displays high turnover but not as high as phosphate added to T108 (Asenjo et al., 2006; Asenjo et al., 2005). The modification of all these residues is not required for P protein oligomerization or for its participation in viral transcription and replication (Lu et al., 2002a; Villanueva et al., 2000, 1991). Nevertheless, P protein phosphorylation, at T108 controls P–M2-1 protein interaction essential for M2-1 protein incorporation into a viral polymerase complex involved in transcription (Asenjo et al., 2006). Furthermore, P protein phosphorylation at S54 also regulates its interaction with M protein. This interaction could control RNP entry into membranous vesicles (Asenjo et al., 2005).

Because in the absence of P protein residues mentioned above, still high turnover P protein phosphorylation occurred (Asenjo et al., 2006), new variants with changes that could affect P protein high turnover phosphorylations and/or impair its tetramerization have been analysed. The objective was to identify P protein residues potential regulators and/or essential for each interaction (with itself and other viral proteins), which is involved for RNA synthesis.

2. Materials and methods

2.1. Cells and viruses

HEp-2 cells were obtained from the ATCC. The origin and handling conditions for vaccinia recombinant vTF-3 have been reported (Villanueva et al., 2000).

2.2. Construction of P protein variants

All cDNAs encoding Long strain P protein variants were inserted in pGEM3 plasmid. They were constructed on the basis of VP recombinant as described (Asenjo et al., 2005). All VP nucleotide sequences encoding P protein were confirmed by automatic sequencing.

2.3. Viral transcription–replication capacities of P protein variants

The HRSV based minigenome cloned in pM/SH, the plasmids expressing L and N proteins and the assay conditions used, were described (Asenjo et al., 2006). The pM2-1 recombinant plasmid was included when the capacity to support M2-1 transcriptional activity was determined. All plasmids contain the corresponding HRSV Long strain protein cDNAs, except pL and pM/SH that contain A2 strain sequences.

2.4. Transfection, cell labeling, fractionation and immunopurification

Co-transfection experiments (Villanueva et al., 2000; Asenjo et al., 2006, 2005) and cell labeling with ^{35}S-methionine (Asenjo and Villanueva, 2000) were performed as it was indicated. Total cell extracts were obtained in 10 mM Tris–HCl pH 7.5, 0.14 M NaCl, 5 mM EDTA, 1% Triton X-100 and 1% DOC (sodium deoxycholate) or in AT buffer: 100mM Tris–HCl pH 8.0, 1 mM EDTA, 7.5 mM $(NH_4)_2SO_4$ pH 7.9, 0.025% NP40. For P and N protein immunoprecipitations, monoespecific rabbit anti-sera were used, under previously described conditions (Asenjo et al., 2006; Khattar et al., 2001). The immunoprecipitated proteins were separated by SDS-PAGE, visualized by autoradiography or Phosphoimaging, and the images were subjected to densitometric analyses using PCBAS program.

2.5. Oligomerization capacities of P protein variants

This analysis was performed following the conditions previously used (Asenjo and Villanueva, 2000).

2.6. 3D modeling of P protein tetramerization domain

Sequences related to RSV P protein were obtained from databases using Blast (Altschul et al., 1990) and aligned using ClustalW (Thompson et al., 1994) and TCOFFEE (Notredame et al., 2000) algorithms. Using the sequence-to-structure threading server Phyre, an improved version of 3D-PSSM (Kelley et al., 2000), against the SCOP database (Andreeva et al., 2004), a structural and evolutive relationship between multiple sequence alignment of P protein tetramerization domain (comprising residues 98–158) and the coordinates of Sendai virus phosphoprotein multimerization domain (PDB entry: 1EZJ; Tarbouriech et al., 2000) was found. Structural model of HRSV P tetramerization domain was then built through homology modeling procedures, based on the previously obtained alignment, using the SWISS-MODEL server (Guex and Peitsch, 1997; Peitsch, 1996; Schwede et al., 2003). The structural quality was checked using WHAT-CHECK (Hooft et al., 1996) from the WHAT IF program (Vriend, 1990). In order to perform an initial geometry optimization and correct atomic clashes, the obtained structure energy was minimized with the implementation of the GROMOS 43B1 force field in the program DeepView (Peitsch, 1996), using 500 steps of steepest descent minimization and 500 steps of conjugate-gradient minimization.

3D models for P protein variants (VPL135A-L142A and VPD139K-E140K) were performed using as template the structure previously obtained for VP protein tetramerization domain, as indicated above. Protein structures were represented using Pymol.

2.7. Molecular dynamics simulation of structural stability for normal and variant P protein tetramerization domains

Molecular dynamics (MD) simulations were performed using the PMEMD module of AMBER8 and the parm-99 parame-

ter set (Case et al., 2002). To neutralize the tetramer domain net negative charge, adequate number of Na^+ counter ions was situated around the system using a coulombic potential grid. Neutralized complexes were then immersed in a solvent box keeping a distance of 12 Å between the wall of the box and the closest atom of the solute. Counter ions and solvent molecules were added using AMBER LEAP module, and an initial relaxation of each complex was achieved by performing 10,000 steps of energy minimization and a cut-off of 10.0 Å. Subsequently and to start the MD simulations, the temperature was raised from 0 to 298 °K in a 200 ps heating phase, and velocities were reassigned at each new temperature according to a Maxwell–Boltzmann distribution. During this period, the position of protein Cα atoms in the protein were restrained with a force constant of 20 kcal mol^{-1} $Å^{-2}$. During the last 100 ps of the MD equilibration phase, the force constant was reduced stepwise until 0. To constrain all hydrogen bonds to their equilibrium values, SHAKE algorithm with an integration time step of 2 fs was used. The list of non-bonded pairs was updated every 25 steps, and the coordinates were saved every 2 ps. Periodic boundary conditions were applied and electrostatic interactions were represented using the Smooth Particle Mesh Ewald (SPME) method with a grid spacing of about 1 Å. Trajectory length was 5 ns for all measured systems. Analysis of the trajectories was performed using CARNAL module of AMBER 8. As a summarizing measure system, to monitor tetramer movements and disorganization both the modeled domains of P protein and its variants, the root mean square deviation (R.M.S.D.) values of Cα trace were measured for whole domain as well as for the more stable hydrophobic segment spanning residues L135–V153.

3. Results

3.1. P protein deletion and substitution variants

P protein deletion and substitution variants, indicated in Fig. 1 have been produced. The majority of them have affected residues at the central part of the P protein molecule. This region (between residues 100 and 180) is essential for the interactions that P protein must establish during viral RNA synthesis and it includes the trypsin resistant fragment (Navarro et al., 1991) with a predicted coiled-coil structure (Castagne et al., 2004) able to form tetramers (Llorente et al., 2006). Its residues are highly conserved, among RSV P proteins that infect different hosts (Alansari and Potgieter, 1994).

The substituted residues could be potential acceptors of high turnover phosphate groups (S or T residues) or they could affect tetramerization (VPM1, VPL135A-L142A and VPD139K-E140K). The rational of our study is to isolate P protein variants permanently phosphorylated, unable to be phosphorylated (at the substituted residue) or in monomeric form, to examine whether P protein phosphorylation or oligomerization state tags P protein for the interactions required for viral RNA synthesis. The deletion variants VPΔ203-241 and VPΔ232-241

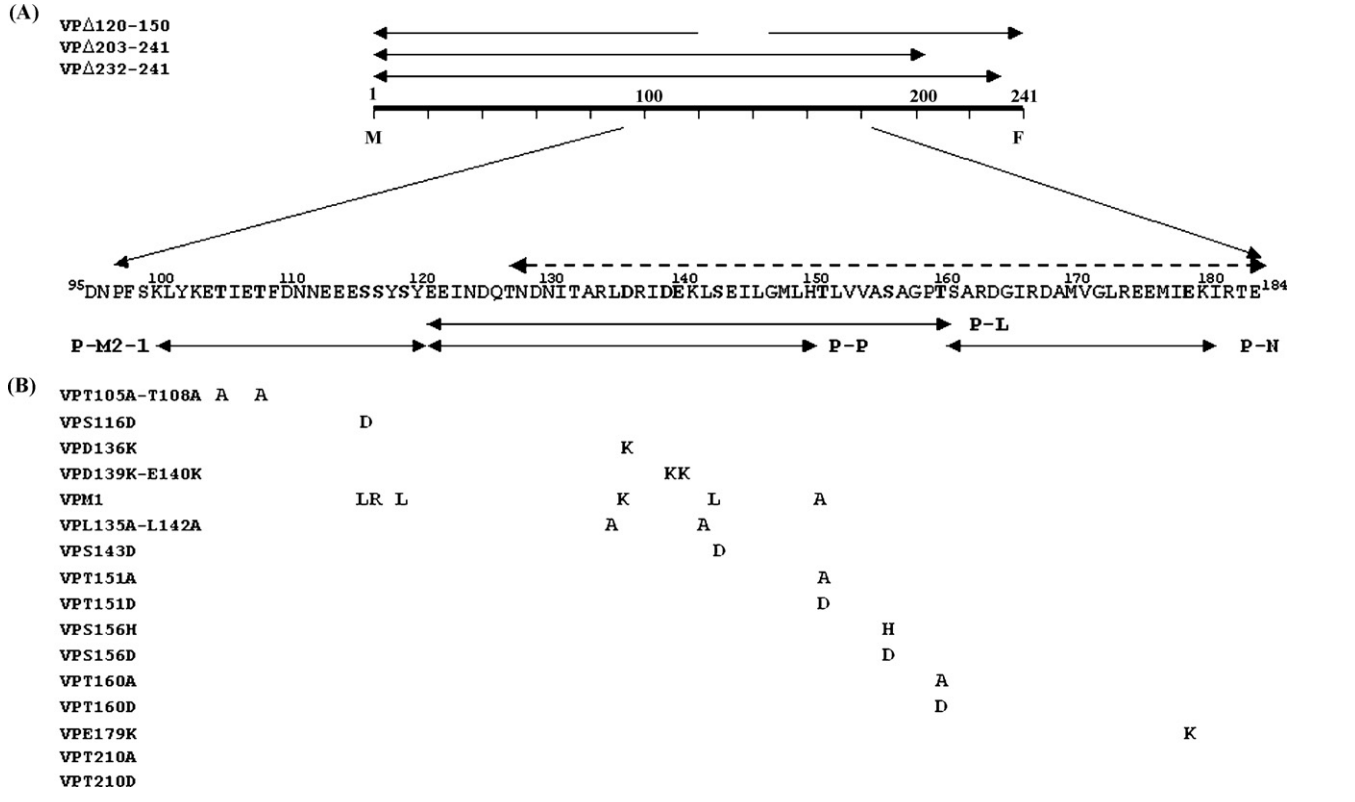


Fig. 1. Primary structure of P protein variants. (A) Schematic representation and primary structure (between residues 95 and 184) of HRSV P protein (M and F indicate the first and final residue). The regions involved in different P protein interactions, in a coil-coiled structure predicted by Lupas algorithms (Curran et al., 1995) (discontinuous line) and those deleted in VP variants, are shown. (B) The analysed P protein substitution variants, showing the substituted residues (bold letters).

were included to test mixed oligomerization with other variants. They lack residues 233–241 that are essential for the interaction with NC (García et al., 1993; Tran et al., 2007). In addition, the collection of P protein variants has been used to further study RNPs interactions, by determining P protein residues essential for each of them.

3.1.1. P protein variants activities on viral transcription and replication. Defective variants complement for viral RNA synthesis

The capacity of P protein variants to support transcription and replication of a HRSV based minigenome was analysed (Asenjo et al., 2005; Hardy and Wertz, 1998; Villanueva et al., 2000). Most of the variants were non-functional except, VPT105A-T108A, VPS116D, VPD136K and VPT160A, although VPS116D and VPT160A showed impaired transcription (Fig. 2B). None of them had synthesis and/or accumulation problems (see below).

However, complementation among variants for replication and transcription (independent or dependent of M2-1) was detected (Fig. 2C, left and right panel, respectively). Positive or negative complementations indicate that the substitutions carried out, by the analysed P protein variants, affect to different or identical, respectively, P protein interactions required for viral RNA synthesis. This allows to group P protein residues essential for each interaction with RNPs proteins. Support of M2-1 transcriptional activity was determined by complementing P protein variants with VPT105A-T108A. This variant displays normal viral RNA synthesis but does not support M2-1 transcriptional activities, because P protein T105 is essential for the interaction P–M2-1, required for M2-1 to act as a transcriptional cofactor for the L protein (Asenjo et al., 2006).

To define the P protein interactions affected in each complementation group, the following P protein physical interactions were checked with the different variants.

3.1.2. Oligomerization of the different P protein variants

The different P protein variants were transiently expressed. The corresponding cell extracts were obtained and the capacity of the P protein and its variants to form oligomers was deter-

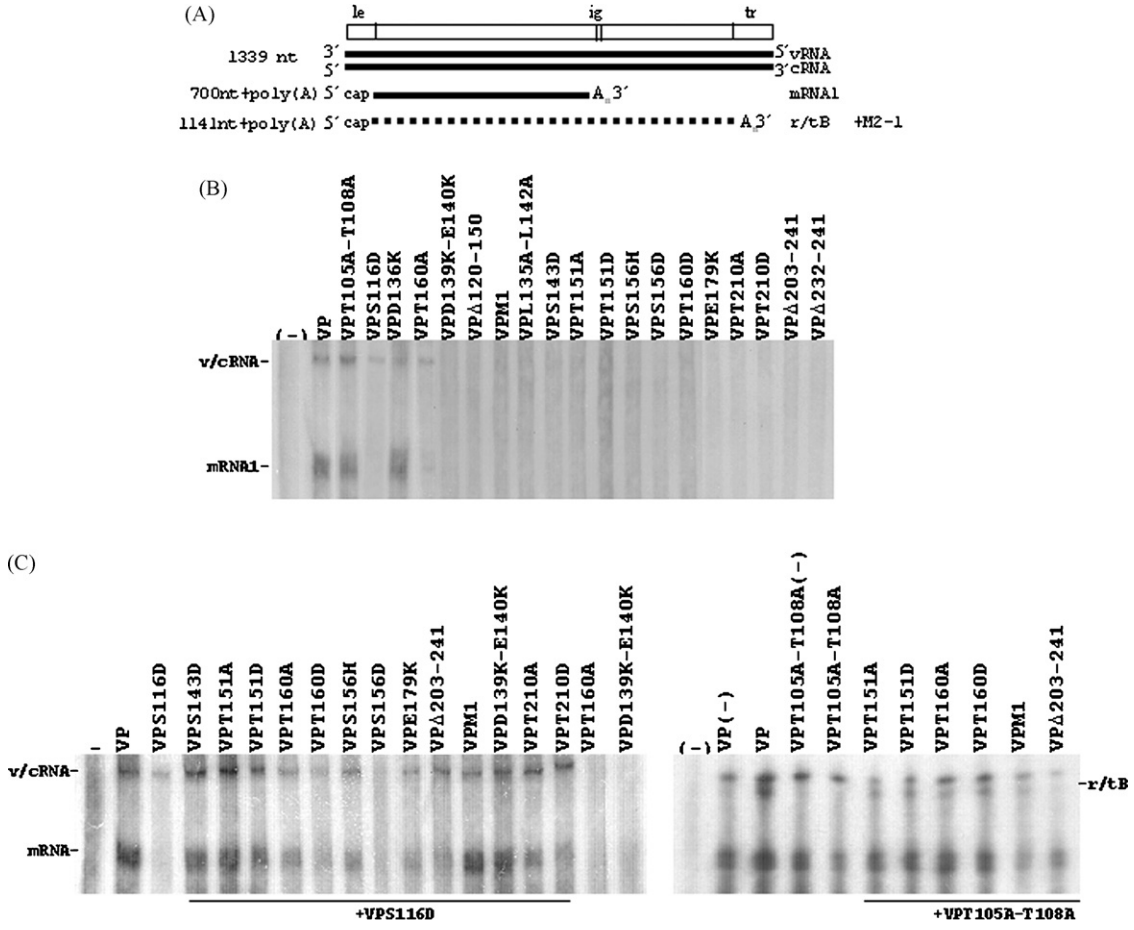


Fig. 2. Replication and transcription capacities of different P protein variants. Complementation tests (A) Schematic representation of HRSV-based mini-genome expressed by pM/SH plasmid and sizes of different RNAs produced by the viral RNA polymerase. r/t B production requires M2-1 activity (Hardy and Wertz, 1998). (B) The indicated P protein variants were transiently expressed by co-transfection, in vTF-3 infected (moi 5) HEp-2 cells (2×10^5) with plasmids pM/SH (1.9 μg), pL (0.57 μg), pN (1.9 μg) and pP (0.77 μg). Sixteen hours post-transfection the viral RNAs were labelled specifically with H_1^3 uridine (25 μCi/ml) in the presence of actinomycin D (10 μg/ml). Total RNA was obtained by the Trizol method, following the supplier's instructions and fractionated in a 3.5% acrylamide gel containing 7 M urea. The gel was dried and the corresponding autoradiography obtained. Complementation assays were carried out by assaying P protein variants two by two as it was indicated above. The total amount of pP plasmid (0.77 μg) was reached by adding 0.35 μg from each P protein variant used in the complementation test in the absence (Fig. 2C, left panel and lanes (-) right panel) or in the presence (Fig. 2C, right panel) of pM2-1 plasmid (0.11 μg).

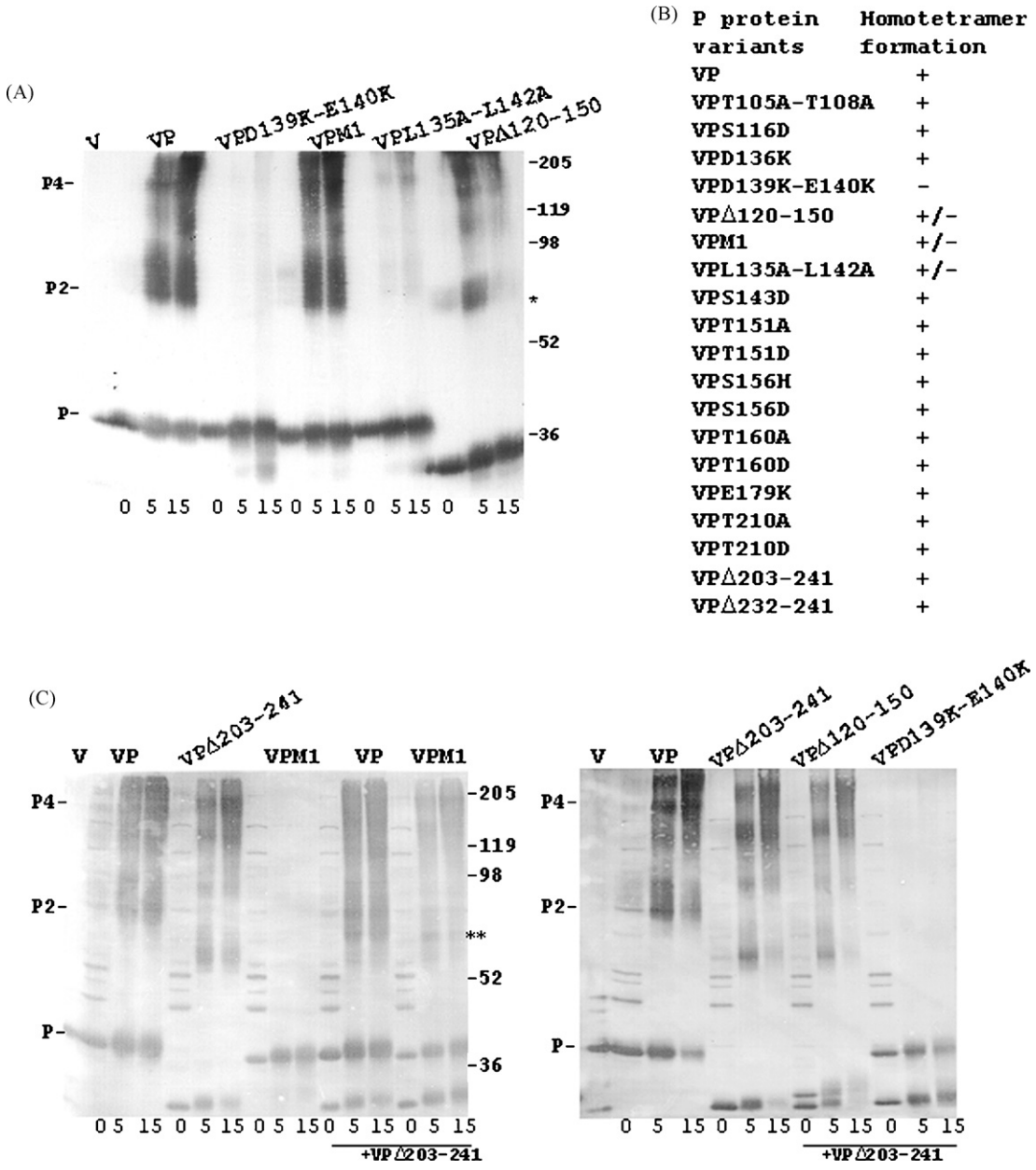


Fig. 3. Oligomerization capacities of the different P protein variants. (A) P protein and its variants were transiently expressed alone or in pairs in HEp-2 cells, infected by vaccinia recombinant vTF-3, and the corresponding soluble cellular proteins were obtained. Glutaraldehyde crosslinking experiments were performed (Asenjo et al., 2005) and at different times (min, indicated in the bottom of the panel) after glutaraldehyde addition, samples were removed and the proteins were analysed by Western blot with a RS1/P monoclonal antibody. (*) Points out VPΔ120-150 dimers. V lane corresponds to purified extracellular viral particles. A summary of the oligomerization capacity of all analysed P protein variants is shown in B. (C) The capacity of some P protein variants to form heteromers is shown with (**). Western blot was developed with monospecific rabbit anti-P protein.

mined (Asenjo and Villanueva, 2000) (Fig. 3A). All P protein variants, except VPD139K-E140K, were able to form oligomers (Fig. 3B) (Asenjo and Villanueva, 2000; Asenjo et al., 2005). VPM1 showed some difficulties in oligomerization but, surprisingly, VPΔ120-150 seems oligomerize, in some extent, despite of having lost P protein residues essential for self-interaction (Castagne et al., 2004). P protein variants VPM1 and VPL135A-L142A (not shown) but not VPD139K-E140K (Fig. 3C) form heteromers with VPΔ203-241. However, the oligomerization of VPΔ203-241 was inhibited by the presence of VPD139K-E140K, suggesting that heteromers are formed but non-crosslinked. According to this and to previous results (Castagne et al., 2004), binding of VPD139K-E140K but not of VPΔ120-150 to tagged VP protein has been detected (not shown).

3.1.3. Interaction of the different P protein variants with L protein

The different P protein variants were transiently co-expressed with L protein and S^{35}-methionine labelled. Before or after immunoprecipitation, proteins were separated and visualized (Fig. 4A). The ratio P/L was determined in all cases (Fig. 4B). The poor L protein co-immunoprecipitation with P protein,

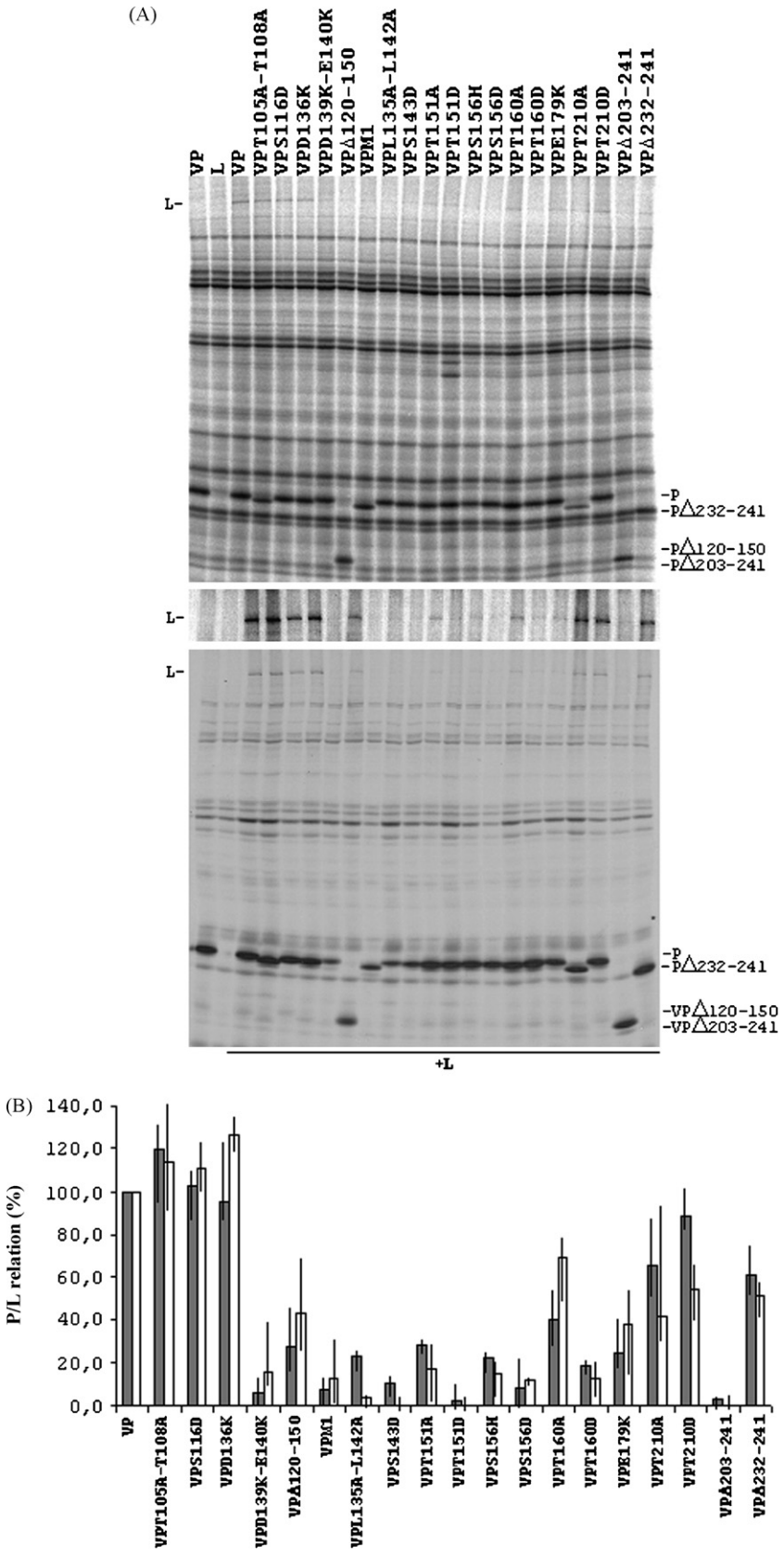


Fig. 4. Capacities of P protein and its variants to interact with L protein. P protein and its variants were transiently co-expressed with L protein in HEp-2 cells and labelled with ^{35}S-methionine (Asenjo et al., 2005). The corresponding soluble protein fractions in AT buffer were separated by SDS-PAGE, visualized by autoradiography and the ratio P/L was calculated (panel B) before (panel A, upper part, black bars) or after immunoprecipitation (panel A, lower part, white bars) with rabbit anti-P protein serum (Khattar et al., 2001). The inset corresponds to a long exposition of the gel shown in B. The P/L ratio is expressed as a percentage of the value obtained for VP in five different experiments. Value variabilities are indicated.

could indicate that their interaction was weak, at least under the experimental conditions used.

L protein co-immunoprecipitated with all the variants but at different extent (Fig. 4B, empty bars), which correlates, in most of the cases, with their capacities to increase L protein synthesis and/or its accumulation in the corresponding co-expressions (Fig. 4A upper panel). All P protein variants displayed that capacity (Fig. 4B black bars). This suggests that P protein and all its variants tested, through its interaction with L protein, protects it from degradation, as it occurs in vesicular stomatitis virus (VSV) (Canter et al., 1993).

The P protein variants with a reduced oligomerization capacity (VPD139K-E140K, VPL135A-L142A and VPM1) poorly co-immunoprecipitated L protein, suggesting that their interaction with L protein was weak and it was lost under the immunoprecipitation conditions. VPΔ120-150, without the canonical oligomerization domain, co-immunoprecipitated about 50% of L protein pulled down by normal P protein. These results indicate that P protein can interact, but poorly, with L protein as a monomer, in the absence of the oligomerization domain and that P residues out of those between positions 120 and 150, are important for this contact.

P protein variants VPΔ203-241, VPS143D, VPT151D, VPS156H, VPS156D and VPT160D also poorly interact with L protein (Fig. 4B, empty bars). Between residues 203 and 241, have not been described residues essential for the interaction between BRSV P and L proteins (Khattar et al., 2001; Krishnamurthy and Samal, 1998).

3.1.4. Interaction of the different P protein variants with N protein

The different P protein variants were transiently co-expressed with N protein and labelled with ^{35}S-methionine. Before or after immunoprecipitation, proteins were separated, visualized and quantified (Fig. 5).

Soluble N-P complexes were not detected for P protein variants VPM1, VPD139K-E140K, VPΔ203-241 and VPΔ232-241. Furthermore, the capacity of VPL135A-L142A to interact with N protein was diminished compared to that of P protein (Fig. 5B). These results suggest that in soluble protein fraction, P protein interacts with N protein, as an oligomer, because the complex formation is abolished for VPD139K-E140K, VPM1 and VPL135A-L142A, all affected in oligomerization. However, despite the absence of canonical oligomerization domain (Castagne et al., 2004), but still with oligomerization capacity, VPΔ120-150 interacts with N protein. Thus, it seems that P protein requires its nine C-terminal residues to interact with N protein but these residues do not appear to be in the correct conformation until any oligomerization has occurred. P protein variants VPD136K and VPT210A (but not VPT210D) interact with N protein but in lesser extent than that found for P protein. In contrast, no significant decrease in the interaction with N protein was found for P protein variant VPE179K, which has a substitution in a region essential for such interaction (Fig. 5B) (Khattar et al., 2000).

P protein residues 232–241 are essential for its binding to NC (Tran et al., 2007). Because in absence of these residues no interaction between P–N proteins was detected, probably P–NC complex is the only one present in the analysed protein fraction.

3.1.5. Interaction of the different P protein variants with the M2-1 protein

M2-1 protein and the different P protein variants were transiently co-expressed, labelled with ^{35}S-methionine, separated and visualised. Interactions between M2-1 and P protein variants were detected by the higher electrophoretical mobility displayed by M2-1 co-expressed with P protein, relative to that found when M2-1 protein is expressed alone. This is due to the fact that the interaction of P-M2-1 proteins, prevents phosphorylation of M2-1, responsible of its deceased electrophoretic mobility (Asenjo et al., 2006; Cuesta et al., 2000).

All P protein variants tested maintained M2-1 protein in its dephosphorylated form, including VPD139K-E140K and VPM1, suggesting that P protein can interact with M2-1 protein as a monomer (Fig. 6A and B).

3.2. Viral transcription and replication complementation studies

The results indicate that P protein, devoid of the canonical oligomerization domain, interacts with L, NC and M2-1 proteins. Nonetheless, viral RNA synthesis proceeds only when a P protein tetramer is assembled, with its monomers bound to different RNPs proteins. It seems that the tetramer formation put together, L and M2-1 on the NC.

First the capacities of different P protein variants to replicate and transcribe a HRSV based minigenoma were tested. Then, mixed co-transfections (complementation) to recover those functions were done. As result of it, P protein variants can be assigned in complementation groups. They were grouped by taking into account not only their physical interaction defects with each RNPs proteins but also the functional interactions among P protein molecules resulting in the formation of an active RNA synthesis tetramer. Simultaneous interactions on the same P protein molecule (cis) and one interaction involving several P protein molecules could be envisaged. This could explain why protein variants VPS116D, VPE179K and VPD136K, without apparent interactions defects, cannot carry out a proper RNA synthesis.

Some complementation assays are shown in Fig. 2C. Complementation groups were established, after quantification of the results (Table 1). Some P protein variants belonged to more than one group, suggesting that some analysed P protein residues are essential for more than one P protein interaction required for functional viral RNA synthesis.

P protein variant VPS156D shows a dominant negative effect (data not shown) and did not complement with any other variant. It oligomerises and interacts with N and M2-1 but not with L protein (Figs. 3, 5, 6 and 4, respectively). Likewise, the VPΔ120-150 variant was also unable to complement with any other P protein variant (not shown), despite of its capacity to interact with L, N and M2-1 proteins. Hence, every P protein molecule participating in complexes involved in transcription and replica-

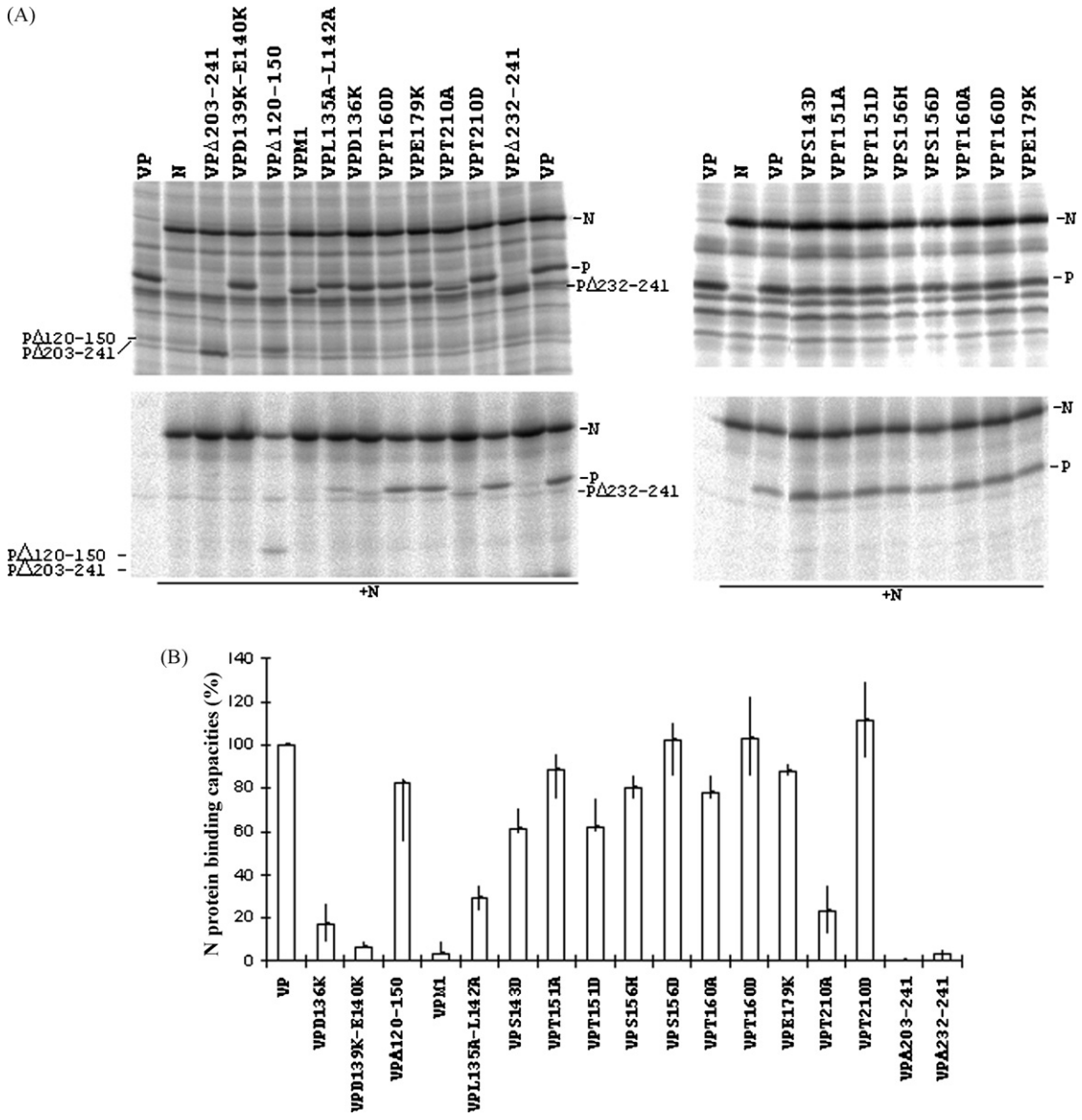


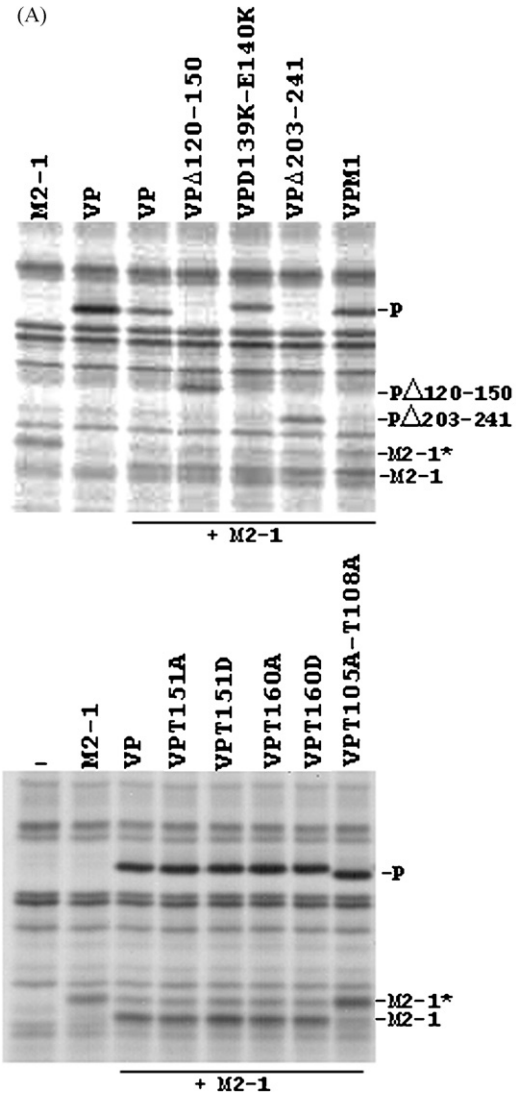
Fig. 5. Capacities of P protein and its variants to interact with N protein. (A) The P protein and its variants were transiently co-expressed with N protein. The corresponding soluble protein fractions in buffer containing Triton X-100 and DOC were immunoprecipitated by using a rabbit anti-N protein serum (Asenjo et al., 2005). The proteins before or after (upper and lower part) immunoprecipitation were separated by SDS-PAGE and visualized by autoradiography. (B) Quantification of N protein binding capacities of different P protein variants calculated as N/P ratio and in reference to that obtained for the normal P protein in five different experiments. Variability is indicated.

tion appears to require the canonical P protein oligomerization domain.

The complementation groups I and II include P protein variants mostly affected in transcription. Group I was defined by VPT105A-T108A variant and group II by VPS116D (although viral replication was also reduced by 70%). The results indicate that the monomer from the P protein tetramer that interacts with M2-1 through T105-T108 must come in contact with NC through residues 232–241. In group II, this NC interaction is not essential but in its absence only residual transcriptional activity is detected.

P protein variants VPL135A-L142A, VPD139K-E140K and VPM1, define complementation group III. All of these substitutions affect P protein oligomerization. However, while the residue substituted in VPD136K lies close to those affecting oligomerization, only its capacity to bind NC is impaired, defining the complementation group IV. The group III variants are not functional although they may oligomerise with other non-functional variants that complement them. However, VPD139K-E140K complements P protein defects affecting RNA synthesis elongation (VPT105A-T108A and VPS116D), indicating that it is able to form a functional tetramer but only with these P protein variants.

The complementation groups V and VI contain P protein variants VPS143D, VPT151A/D and VPS156H, respectively, while VPT160D belongs to both. Since these substitutions affect



(B)

P protein variants	M2-1 no phosphorylation
VP	+
VPT105A-T108A	–
VPS116D	+
VPD136K	+
VPD139K-E140K	+
VPΔ120-150	+
VPM1	+
VPL135A-L142A	+
VPS143D	+
VPT151A	+
VPT151D	+
VPS156H	+
VPS156D	+
VPT160A	+
VPT160D	+
VPE179K	+
VPT210A	+
VPT210D	+
VPΔ203-241	+
VPΔ232-241	+

Fig. 6. Capacity of P protein and its variants to maintain M2-1 protein in non-phosphorylated form. (A) P protein and its variants were transiently co-expressed with M2-1 protein. The corresponding soluble protein fractions in Triton X-100 and DOC containing buffer were separated by SDS-PAGE and visualized with the Phosphoimager. (B) Summary of obtained results.

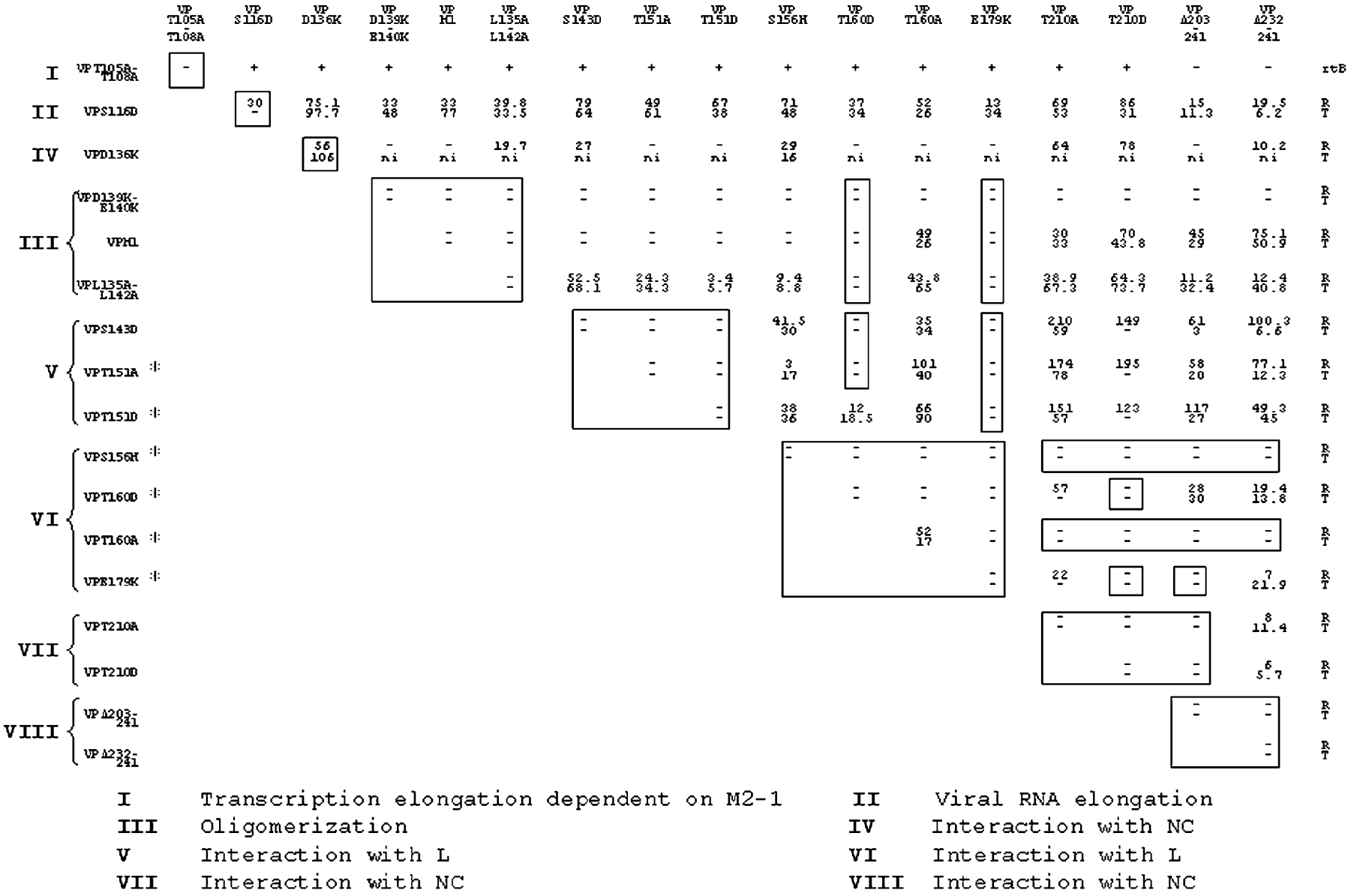
L protein binding, L protein may interact with two P protein monomers in P tetramer. Complementation group VII was defined by VPT210A/D with defects in NC binding. Complementation group VIII was defined by the variants VPΔ232-241 and VPΔ203-241 that were unable to interact with NC (Tran et al., 2007). These defects differ because there is complementation between VPT210A/D and VPΔ232-241. The VPT160A variant, without apparent defects in interaction with the NC, belonged to both complementation groups (VII and VIII) and to complementation group IV.

P protein variants VPT160D and VPE179K did not complement with groups III–V (except VPT160D with VPT151D) and VI. These results indicate that these P protein residues are important for P protein oligomerization and for its binding to L protein. By contrast, those variants complement groups VII and VIII but not to group IV, all related to NC binding.

3.3. 3D structure model of P protein region between residues 98 and 158

To locate these residues on P protein quaternary structure, a 3D model of the P protein was tried. Only the tetramerization domain was modeled (residues 98–158), based on crystallized structure of P protein from the phylogenetically related paramyxovirus SeV (Tarbouriech et al., 2000). The model was obtained through threading methods of sequence-to-structure alignment and subsequent homology modeling techniques, followed by energy minimization procedures in order to measure its in silico stability. It shows four coiled-coil alpha helices arranged in two hemi-domains separated by a non-structured small segment spanning residues 109–111 (Fig. 7A). The structural stability was maintained by two clusters of hydrophobic residues. The N-terminal cluster is composed by four L101 residues oriented towards the inner face of the tetramer and by

Table 1
Complementation tests



P protein variants were assayed two by two in the HRSV based discristronic minigenome system following the experimental conditions indicated above (Fig. 2C). The capacity to support M2-1 transcriptional activity was studied in the complementation test using, P protein variant VPT105-T108A plus each different variant, as well as the pM2-1 plasmid (0.11 μg). The different virally synthesized RNAs labelled with ^{13}H-uridine were separated in acrylamide gels containing urea, visualized by autoradiography, quantified with PCBAS program, and the obtained values were presented in reference to those found for VP protein. Replication (R, v/cRNA) and transcription (T, mRNA1) were quantified and expressed as the percentage of the values obtained for the P protein assayed in the same experiment. Average values from at least three experiments are shown. The residual activity of the P protein variants included in the complementation tests was subtracted. ni, not increase. The boxes indicate the different complementation groups. P protein variants belonging to different complementation groups (asterisks) and their defects are indicated.

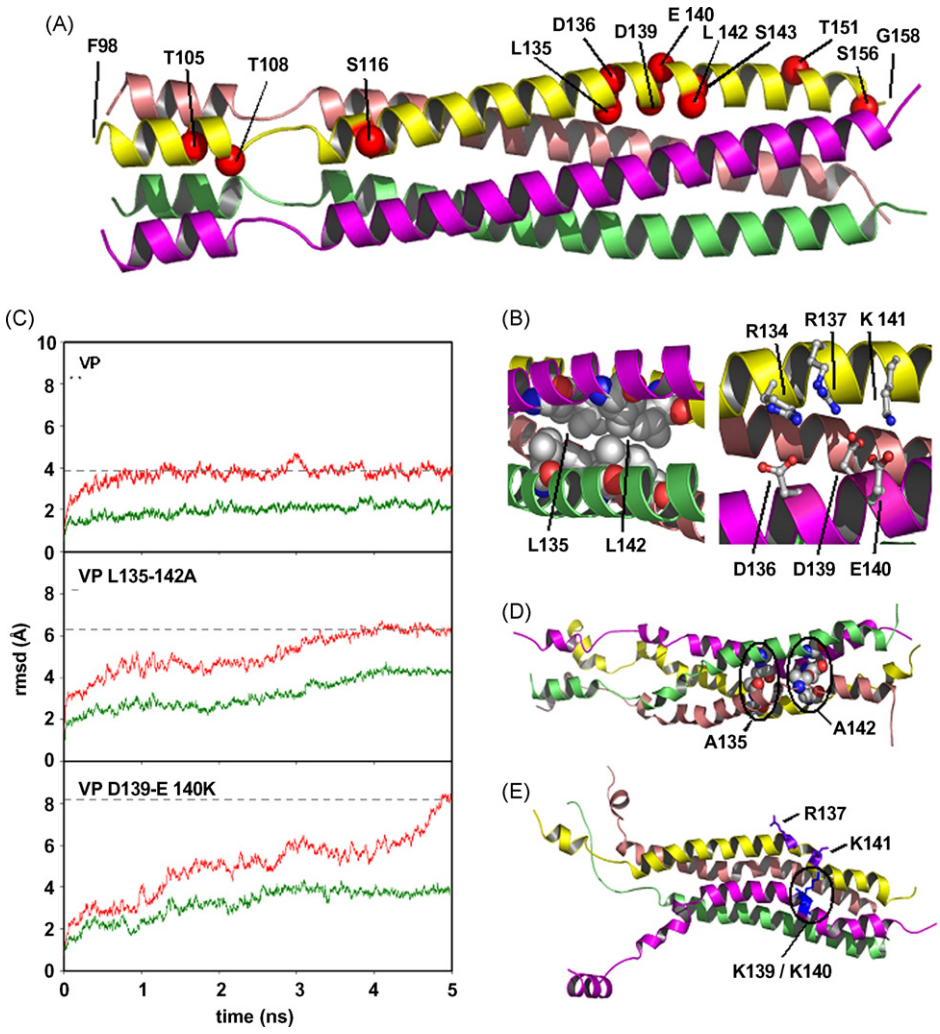


Fig. 7. 3D model structure of P protein region comprising residues 95–158. (A) Homology-based model for P protein tetrameric domain. Position of residues implicated in functionality is indicated (red spheres). (B) Location of residues L135 and L142 in the tetramer inner interface (left) and residues D136, D139 and E140 contacting to R134, R137 and K141 (right). (C) Molecular dynamics trajectories measured for P protein (upper panel), VPL135A-L142A (intermediate panel) and VPD139K-E140K (lower panel) tetramers. Red line indicates the RMSD measured for whole Cα trace while green line corresponds to the main stable segment, spanning hydrophobic cluster L135-V153. (D and E) 3D structure for VPL135A-L142A and VPD139K-E140K, respectively after 5 ns of MD simulation.

residues F98, I106 and F109. The C-terminal cluster includes contacts among residues L135, I138, L142, I145, L146, L149, L152 and V153 (Fig. 7B, left). Stability is maintained by electrostatic contacts between basic R134, R137 and K141 residues, in one monomer, and acidic D136, D139 and E140 in the contiguous one (Fig. 7B, right).

3.3.1. Molecular dynamics studies on the structural stability for the P protein and its variants

Once P protein tetrameric model had been established, MD studies were performed on its structural stability and compared to those of VP L135A-L142A and VP D139K-E140K variants displaying oligomerization problems. Thus, homology-based models were obtained for both variants and in silico stability was monitored in the three models by subjecting them to a 5 ns MD simulation in absence of structural restraints (Fig. 7C). P protein tetramer (Fig. 7C, upper panel) exhibits a structurally stable trajectory when measured at the rigid hydrophobic cluster spanning residues L135–V153 (green line), or in the whole peptide Cα trace (red line). In both cases, RMSD values were maintained within the limits of 2 and 4 Å, respectively after 5 ns of simulation, reasonable values for homology-modeled structures. In contrast, and according to the experimental lack of stable oligomerization, the trajectories of the VP L135A-L142A (Fig. 7C, intermediate panel) and VP D139K-E140K (Fig. 7C, lower panel) indicate a rapid disorganization of the whole structure including the hydrophobic core. In both cases, high values of RMSD (more than 6 and 8 Å, respectively) were obtained for the whole Cα trace after 5 ns of MD, in close agreement to their particular oligomerization properties (Fig. 7D and E).

4. Discussion

HRSV P protein, a structural tetrameric (Asenjo and Villanueva, 2000) phosphoprotein, directs RNPs task on viral RNA synthesis (Collins et al., 2001). Its pivotal role is devel-

oped through interactions with itself, L, N and M2-1 proteins (Castagne et al., 2004; Khattar et al., 2001; Tran et al., 2007, Mason et al., 2003). To understand how P protein manages these interactions, P protein variants with substitutions and deletions were constructed to determine: (1) if P protein as monomer participates in viral RNA synthesis; (2) if potential high turnover phosphorylations of P protein S and T residues, located at its central region, could control those interactions; (3) residues essential for different, simultaneous and dual P-RNPs proteins interactions, required for efficient P protein work on RNA synthesis; (4) location of some of these P protein residues on its tetramerization domain modeled three-dimensional structure.

4.1. P protein to be functional in RNA viral synthesis must be a tetramer

P protein variants unable to form tetramer (VPM1, VPL135A-L142A and VPD139K-E140K) or devoid of the canonical oligomerization domain (VPΔ120-150A) do not support viral RNA synthesis. Only those able to oligomerise (VPM1 and VPL135A-L142A) with non-functional P protein variants and bearing the canonical oligomerization domain can complement them. Thus, functional P protein for viral RNA synthesis, is a tetramer. However, the physical interactions with viral proteins involved in RNA synthesis (L, N and M2-1 proteins), can occur in the absence of the canonical P protein oligomerization domain, as described for the VSV P protein (Chen et al., 2006).

The variant VPΔ120-150 seems to form dimers in the absence of the canonical oligomerization domain suggesting that in the absence of main oligomerization domain, another oligomerization region is exposed. Its relevance in P protein function during the viral growth cycle is an open issue.

4.2. High turnover phosphorylation of some P protein residues could regulate its activity on viral RNA synthesis

Although there is no experimental evidence, in HRSV infected HEp-2 cells, for P protein modification at S143, T160 and T210, by addition of high turnover phosphate groups, this kind of modification at these residues could control its interaction with L protein and NC, respectively. Nevertheless, is a difficult task to determine if the chosen residues are phosphorylated during HRSV infection due to the very high turnover of the added phosphate. In addition, these phosphorylations occur simultaneously to those with lower and intermediate turnover (Asenjo et al., 2006). Phosphorylation at T160 could tag P protein monomers in the tetramer for binding to L or N proteins The P protein T210 is phosphorylated with very high turnover when P protein is transiently expressed (Asenjo et al., unpublished observation). It could control a P–N proteins interaction that must be formed and destroyed like that allowing L–P (RpRd) interaction along NC during viral RNA synthesis (Curran and Kolakofsky, 1999). However, T210A substitution is unrelated to RNA synthesis elongation defects defined by complementation groups I and II but related to that defined by T160A.

4.3. Complementation groups. Partial three-dimensional structure of P protein oligomerization domain (residues 98–158)

The established complementation groups confirm that efficient viral RNA synthesis requires P protein functional interactions with itself, M2-1, L and N proteins.

P protein tetramerization involved residues substituted in complementation group III, E179 and the substitution T160D.

A P protein homology model for the tetramerization domain (residues 98–158) was constructed by comparing the oligomerization domain of related Sendai virus P protein and its thermodynamic stability was tested in silico. Involved in structural stability were L135 and L142, located in a hydrophobic cluster at the inner tetrameric interface and D139 and E140 that establish electrostatic contacts with K137 and K141 in the parallel monomer. Using the same MD techniques, the modeled tetrameric structures of P protein variants VPL135A-L142A and VPD139K-E140K exhibit a rapid destabilization that correlates with their oligomerization defects (Fig. 3A).

RNA synthesis elongation, dependent on M2-1 transcriptional elongation activity requires P protein residues T105, T108 and those between residues 232 and 241 (complementation group I and VIII). These T residues, structurally located in the external face of the helical tetramerization domain, are essential for the M2-1–P proteins binding. Thus, a simultaneous interaction in at least one monomer of the P protein tetramer with M2-1 protein and NC must occur for RNA synthesis elongation depending on M2-1 protein.

RNA synthesis elongation independent of M2-1 transcriptional activity, involves substitutions S116D (complementation group II) and T160A. Based in the poor complementation of S116D with group VIII and to the belonging of VPT160A to complementation groups IV, VII and VIII; S116 and T160 may affect to P protein-NC contact. This long distance structural effect is repetitively observed (see below). In agreement with the increased RNA synthesis elongation associated with N protein encapsidation (Gubbay et al., 2001), S116D and T160A substitutions affect replication in lesser extension than transcription (Table 1). S116 is phosphorylated with low turnover (Navarro et al., 1991) and T160 could suffer high turnover phosphorylation. These modifications could serve as RNPs signal to stop or increase viral RNA synthesis, respectively. Because S116D and T160A belong to different complementation groups, it seems that at least two monomers, from the P protein tetramer, involved in transcription, must interact with NC.

Only substitutions T105A–T108A and S116D complement to D139K-E149K, suggesting that P protein participates on different complexes during transcription and replication.

P–L protein interaction is impaired by substitutions grouped in two complementation groups, V (S143D and T151A/D; VPS143A is a functional P protein variant) and VI (S156H/D and T160D) plus E179K. Thus, L protein may contact to two different monomers, perhaps as a dimer as described for Sendai L protein (Cevik et al., 2003).

In group V there are residues, whose putative high turnover phosphorylation, with impaired L binding. S143 and T151 are

oriented towards the external protein face of the P protein tetrameric domain structure. Substitutions T151A/D also belong to complementation group IV (related to NC interaction). S156H and T160D substitutions belong to complementation groups VII and VIII (related to NC interaction), and to groups III (related to P protein oligomerization) and IV, respectively.

The VPΔ203-241 variant shows L protein binding defect. Hence, the P protein residues between positions 203 and 232 appear to be necessary, at least in some molecules of the P protein tetramer, for adopting at their central regions (143–160) an adequate conformation. The reverse conformational effect has been described above and it includes T105, T108 and S116.

P protein variant VPS156D shows a dominant negative effect suggesting the sequestration of P protein, or its variants, into a very stable non-functional structure.

P protein interaction with N protein, as part of NC, involves P protein residues between positions 232 and 241 (complementation group VIII), as described previously (Tran et al., 2007), but also to P protein residues D136 (complementation group IV), the substitution T160A and T210 (complementation group VII). Only complementation group IV is affected by substitution E179K, although P protein residue E179 is closed to residues important for P–N interactions (Lu et al., 2002b). E179K substitution confers termosensitivity to the viral RNA synthesis (data not shown) a useful property for a HRSV live attenuated vaccine.

These results do not allow to conclude if the same or different RpRd are involved in viral transcription and replication. Since transcription and replication are interdependent in the minigenome system used, the existence of a tripartite complex P–L–N with RpRdR activity, as described for VSV (Qanungo et al., 2004) cannot be ruled out.

4.4. Application of this study

Many of P protein residues analysed and located in a stable three-dimensional model, like E139, D140 and S156, may be appropriated targets for in silico design of specific antiviral compounds of use in the control of HRSV infections.

5. Conclusions

1. In P protein tetramer formation and stability are involved residues: F98, L101, I106, F109, R134, L135, D136, R137, I138, D139, E140, K141, L142, I145, L146, L149, L152, V153.
2. P protein, in the absence of the oligomerization domain, interacts with M2-1, L and N proteins, suggesting that the contact residues are outside of the oligomerization domain and that P protein as monomer establishes these interactions. However, P protein substitutions L135A-L142A; D139K-E140K; S116L-S117R-S119L-S143L-D136K-T151A, S143D, T151A/D, S156H/D have a deep influence on its interactions with L and N but not with M2-1 proteins
3. Only P protein variants with oligomerization problems, able to form heterotetramers with other defective viral RNA synthesis variants, complement that activity. This, strongly, suggests that P protein involved in viral RNA synthesis, is a tetramer.
4. The variant VPD139K-E140K (able to participate in heterotetramers) only complements P protein variants affected in RNA synthesis elongation (complementation groups I and II), suggesting that P protein complexes involved in viral transcription and replication are different.
5. Although there is no experimental evidence for high turnover phosphorylation at P protein residues S143, T160 and T210 during HRSV infection, our results suggest that this type of modification, at these residues, could regulate interactions between tetrameric P protein and L, NC and perhaps N0.
6. L protein interacts with two monomers of P protein tetramer and also two monomers, one of them bound to L protein, contact with NC.
7. For functional P–M2-1 interaction, one monomer of P protein tetramer must interact with M2-1 through T105 (dependent on T108 phosphorylation state) and with the NC.
8. The central and C-terminal parts of P protein molecule are structurally related.
9. Several P protein essential residues, for its activity on viral RNA synthesis, had been located in a partial 3D model. This information can be used to synthesize specific antiviral compounds.

Acknowledgements

We are indebted to Dr. G. Wertz for providing the RSV RNA analogue replication and transcription system. We thank Dr. J. Ávila for reading of the manuscript, M. Sefton and K. Mark for editing. We are also grateful to "Biomol-Informatics SL" (http://www.biomol-informatics.com) for bioinformatics consulting. This work was supported by SAF/2002-00070 from Ministerio de Ciencia y Tecnologia (MCYT), 03/ESP31 from Instituto de Salud Carlos III (to NV) and grants SAF2004-06843 from MCYT and CIBER C03/2006/0026 from Ministerio de Sanidad (to PGP).

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