Regulated but not constitutive human respiratory syncytial virus (HRSV) P protein phosphorylation is essential for oligomerization

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Abstract Purified human respiratory syncytial virus (HRSV) P phosphoprotein from transfected HEp-2 cells is able to oligomerize forming tetramers. The bulk of constitutive P protein phosphorylation (99.8%) (serine residues 116, 117, 119, 232 and 237) can be removed without affecting protein oligomerization. However, dephosphorylated P protein, produced in bacteria, is unable to oligomerize. This difference can be explained by a transient P protein phosphorylation, detected in HEp-2 cells, that could be essential for P protein oligomerization.

Key words: Human respiratory syncytial virus; P protein; Phosphorylation; Oligomerization

1. Introduction

Human respiratory syncytial virus (HRSV) is the most important respiratory virus worldwide affecting hospitalized children, from 0 to 6 months old, and elderly people [1]. The efforts directed to control HRSV infections are opened in three fronts: vaccines (viral proteins and attenuated live virus), administration of humanized neutralizing monoclonal antibodies and developing of antiviral compounds [2–4]. This virus belongs to the paramyxovirus family, pneumovirus genera. It is an RNA negative strand (15 222 nucleotides long) enveloped virus with a capacity to encode 11 viral proteins. The order of the genes is: 3′ leader NS1-NS2-N-P-M-SH-G-F-M2-L trailer 5′. Proteins L, N, P and M2-ORF1 together with the viral RNA constitute the nucleocapsids, internal virion components surrounding the M protein, in contact with the membranous envelope in which the viral glycoproteins F, G and SH are inserted. The rest of the proteins are non-structural [1].

To determine the functions of the viral proteins that constitute the nucleocapsids should be important to obtain attenuated vaccines and to design antiviral compounds because these viral components are the functional units for viral transcription and replication and the cores of the viral particles.

We are focused on P protein from the Long strain. This protein is 241 amino acids long and is constitutively phosphorylated at serines 116, 117 and/or 119 (18.9%) in the central domain and at serines 232 (78.9%) and 237 (2%) in the C-terminal domain [5–8]. The main of this phosphorylation (98%) can be removed without affecting the protein functionality for viral transcription and replication [8,9]. However, that phosphorylation seems to be essential for the morphogenetic pathway of the virion from the formation of the nucleocapsids [9].

In other Paramyxoviruses as Sendai (SeV) and vesicular stomatitis (VSV) viruses, the corresponding P proteins have been characterized as oligomers (trimers and tetramers, respectively), oligomerization being essential for P protein functionality. For SeV, P protein phosphorylation is dispensable for oligomerization because bacterially expressed protein is a trimer [10]. In the case of VSV P protein, phosphorylation has been demonstrated to be essential for P protein tetramerization [11]. For HRSV P protein, it has been indicated that oligomers (trimers or tetramers) are formed when the protein is expressed in bacteria, precluding a role of phosphorylation in the P monomers association [12].

In this paper, it is demonstrated that P protein, transient expressed in HEp-2 cells, is a tetramer. These oligomers can be formed when 99.8% of the constitutive phosphorylation has been removed by changing the phosphorylatable serine residues by alanines. However, in contrast to other results [12], P protein expressed in bacteria is unable to oligomerize. These results suggest that the unphosphorylated P protein produced in HEp-2 cells could have a transitory phosphorylation that does not take place in bacteria. This transitory phosphorylation that occurs at different residues than those constitutively phosphorylated has been identified in HEp-2 cells by using phosphatases inhibitors. In this work, we tested the possible relation between P protein phosphorylation and oligomerization.

2. Materials and methods

2.1. P protein variants

All the P protein variants used in this paper, except VP3-10, have been previously described and characterized [7,8].

VP3-10 was obtained as VP3-8 [8], but using the large fragment *Nco*I-*Nco*I of VP10 instead of that of VP8.

The conditions used for transfection experiments, ^{32}P labelling and the immunopurification of P protein have been described [8]. When the transfected labelled cultures were treated with okadaic acid, 100 nm okadaic acid was added together with [^{32}P]orthophosphate and the cells were processed as it was indicated above.

2.2. Filtration chromatography

Sephadex G-100 and G-200 columns of 2 ml (7×0.5 cm) were packed following the manufacturer's conditions in buffer (10 mM Tris–HCl pH 7.5, 140 mM NaCl, 5 mM EDTA and 1% Triton X-100).

Immunopurified P protein in a volume of 150 μl was chromatographed in the buffer indicated. Fifty fractions of 40 μl were collected.

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P protein was detected by determining its associated radioactivity or after sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and staining. The columns were calibrated by filtration of Dextran blue, bovine serum albumin (BSA) and purified immunoglobulin G. The chromatographed proteins were detected after SDS–PAGE analyses of the different fractions and staining.

2.3. Crosslinking assays

The crosslinking conditions have been described [13]. Briefly, immunopurified P protein (0.5–1 μg, with approximately 2000 cpm ^{32}P Cerenkov) was put in buffer: 20 mM HEPES (KOH) pH 7.4, 100 mM NaCl, 0.1 mM DTT and 1.2 mM $MgCl_2$. Glutaraldehyde, freshly diluted, was added to 0.025% final concentration. At different times, 0, 15 and 30 min, aliquots were removed and 200 mM glycine pH 7.0 (final concentration) and SDS–PAGE sample buffer were added. The samples were finally analyzed in 10% SDS–PAGE.

2.4. Production of P protein in bacteria

A cDNA corresponding to Long strain P protein was obtained by PCR using as template the cDNA Long P protein gene cloned in the *Sma*I site of pGEM3 plasmid, as already described [8]. The following synthetic oligonucleotides were used in order to leave the first methionine-coded triplet upstream a canonical Shine–Dalgarno [14] sequence and to remove the extra non-coding region located after P protein C-terminal phenylalanine.

5′-G<u>GGATCC</u>*GGAGG*TGCATC**ATG**G-3′ mRNA polarity; 5′-CC<u>CTGCAG</u>**TCA**GAAATCTTCAAGTG-3′ vRNA polarity.

The underlined nucleotides corresponded to the sequences recognized by restriction enzymes *Bam*HI and *Pst*I (mRNA and vRNA polarities, respectively) used in the cloning processes, that are absent in the P protein gene. The initiation and stop codon, respectively, are indicated by bold letters. The Shine–Dalgarno sequence is indicated by italic letters.

The cDNA obtained was, after digestion with *Bam*HI and *Pst*I, cloned in pGEM3 vector digested with the restriction enzymes mentioned above.

3. Results

3.1. Immunopurified P protein is an oligomer with a molecular weight equal or higher than 1×10^5 kDa

The HRSV P protein was transient expressed in HEp-2 cells, in the vaccinia vTF-3 based system, and labelled with ^{32}P following previously described conditions [8]. From the corresponding cellular extract, the labelled P protein was immunopurified and analyzed by SDS–PAGE and autoradiography. In Fig. 1A, lane 2, it can be appreciated that the cells transfected with the plasmid pGEM3-P express a phosphorylated protein that is absent in the cell extract transfected with the plasmid pGEM3 (lane 1) and that it is immunopurified by the anti-P monoclonal antibody 73/P (lane 4). More of 90% of the radioactivity present in the immunopurified fraction corresponds to P protein and its apparent molecular mass is 33–36 kDa.

To determine if the immunopurified P protein displays some grade of oligomerization, it was chromatographed through a Sephadex G-100 superfine column. This column can fractionate globular proteins with a relative molecular mass between 4000 and 150 000. The void volume of the column was determined by filtration of Dextran blue and it is indicated by an arrow. P protein, detected by radioactivity, was found to be present in the same fractions as Dextran blue (Fig. 1A). This result indicates that P protein was an oligomer because according to its molecular mass (33–36 kDa), it must be included in the column. Its presence in the void volume indicates an apparent molecular mass of about $1–1.5\times10^5$ or higher. It is compatible with the presence of three or four P protein monomers in that fraction.

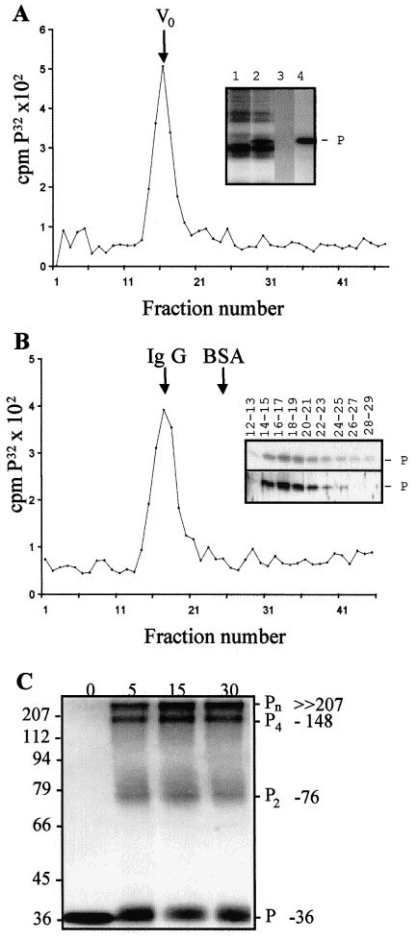

Fig. 1. P protein transiently expressed in HEp-2 cells is a tetramer. P protein was transiently expressed in HEp-2 cells with the vaccinia recombinant expression-based system by using pGEM3-P recombinant plasmid [8] (A, lanes 2 and 4 of the inset). The transfection was also done with the plasmid pGEM3 as control (A, lanes 1 and 3 of the inset). The transfected cells were labelled with [^{32}P]orthophosphate and cell extracts were prepared [8]. The cell extracts were analyzed by SDS–PAGE before (A, lanes 1 and 2 of the insert) and after (A, lanes 3 and 4 of the insert) immunochromatography through a column containing covalently bound anti-P monoclonal antibody 73/P. The immunopurified P protein was chromatographed through a Sephadex G-100 column and its presence was followed by determining radioactivity in the collected fractions. The void volume, V_o, was determined by filtration of Dextran blue (A). B: The immunopurified P protein was chromatographed through a Sephadex G-200 column and its presence was followed by determination of radioactivity in the different fractions and SDS–PAGE of the fractions containing the radioactivity peak. P protein was detected by autoradiography (B, upper part of the inset) and silver staining (B, lower part of the inset). The elution position was determined by comparing with those of purified immunoglobulin G and BSA. C: The immunopurified P protein was incubated with glutaraldehyde as is indicated in Section 2. At different times of incubation, 0, 5, 15 and 30 min, aliquots were removed and after stopping the reaction by addition of 0.2 M glycine and SDS sample buffer, analyzed in SDS–PAGE 10% acrylamide gels. The autoradiography of the resulting gel is shown, indicating the electrophoretical mobilities of molecular weight markers and the position, and relative molecular mass, determined for the different P protein oligomers.

3.2. Immunopurified P protein is a tetramer

In order to determine the presence of three or four monomers in the immunopurified P protein oligomer, immunopurified P protein was chromatographed through a Sephadex G-

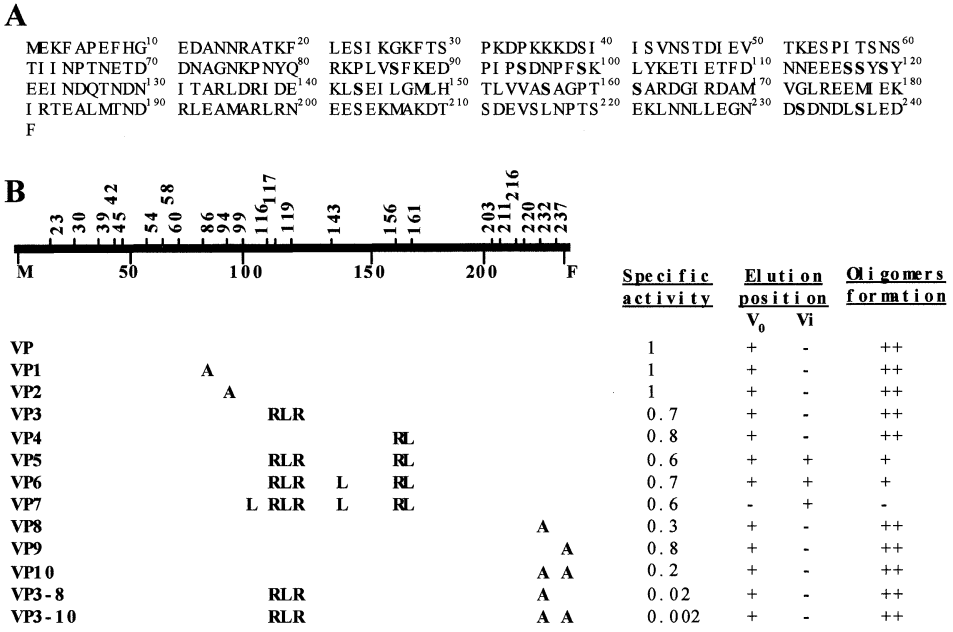

Fig. 2. P protein constitutive phosphorylation is not essential for oligomerization. A: Amino acid sequence of HRSV P protein Long strain [20], showing in bold letters the mutated serines in the different P protein variants. B: Diagram representation of P protein Long strain showing its serine residues and their substitution in the different variants VP assayed for oligomerization. The specific activity and behavior in exclusion chromatographies and crosslinking experiments of the different variants are indicated at the right part of the figure.

200 column calibrated with serum albumin and with purified immunoglobulin G. The profile of radioactivity in the column is shown in Fig. 1B and the position of the marker proteins, determined after SDS–PAGE and staining with Coomassie blue, is indicated. The presence of P protein in the different fractions was further detected by autoradiography (Fig. 1B, upper part of inset).

It can be observed that P protein appears in the same fractions as immunoglobulin G, indicating an apparent molecular mass of about 1.5×10^5. Thus, it seems that P protein is a tetramer.

In order to determine if only phosphorylated P protein is oligomerized, the gel, in which the protein present in the different fractions was characterized, was silver-stained (Fig. 1B, lower part of inset). A correlation between ^{32}P labelling and mass of P protein was found. The presence of minor amounts of P protein present in the included volume of the column indicates that not all the immunopurified P protein is in the oligomerized form and that some monomers, and perhaps some dimers, could also be present.

3.3. *Tetramerization of P protein can also be demonstrated by crosslinking of the protein*

Because in the P protein immunopurification by affinity chromatography, through Sepharose 4B with covalent bound anti-P monoclonal antibody 73/P, the protein is eluted with

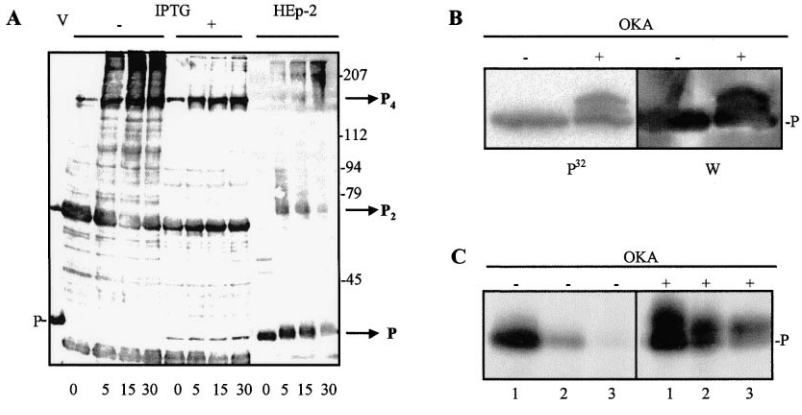

Fig. 3. P protein-regulated phosphorylation could be essential for oligomerization. A: P protein was controlled expressed in bacteria and in HEp-2 cells. The corresponding extracts were crosslinked with glutaraldehyde and, at different times, samples were removed and analyzed by Western blot with anti-P rabbit sera. The symbols − and + correspond to bacterial extract protein fraction from non-induced or induced cells, respectively. The position of the molecular weight markers and those of the different P protein oligomers are indicated at the right margin of the figure. The position of P protein from purified viral particles (lane V) is indicated at the left margin of the figure. B: Immunopurified P protein transient expressed in the presence (+) or in the absence (−) of okadaic acid (OKA) detected by autoradiography (^{32}P) and Western blot (W) with anti-P rabbit sera. C: Autoradiography of immunopurified VP (lanes 1), VP3-8 (lanes 2) and VP3-10 (lanes 3) proteins from transfected HEp-2 cells, labelled with ^{32}P in the absence (−) or in the presence (+) of okadaic acid (OKA).

0.1 M glycine pH 2.5, it was possible to argue that immunopurified P protein was denatured and that the behavior in the filtration chromatographies should be due to a loss of its hypothetical globular structure more than to its oligomerization. To clarify that point, glutaraldehyde crosslinking experiments with immunopurified P protein were done. Fig. 1C, shows that P protein, after 5 min of incubation with glutaraldehyde, generates three phosphorylated polypeptides with a higher molecular mass than that of P protein. According to the molecular mass of the stained molecular weight markers, their molecular masses correspond to 76 000, 148 000 and higher than 207 000 kDa. These results indicate that glutaraldehyde is able to covalently link two (dimer), four (tetramer) and probably eight or more (two or more tetramers) P proteins. The absence of trimers is noteworthy .

3.4. The oligomerization is independent of the bulk of P protein phosphorylation

To probe if P protein phosphorylation is essential for oligomerization, different P protein variants, containing changes at the serines phosphorylated in different combinations, listed in Fig. 2, were transiently expressed in HEp-2 cells, labelled with ^{32}P or [^{35}S]methionine and immunopurified. The specific activities of the different P protein variants, referred to as P protein, are indicated in Fig. 2, and correspond to those described previously [7,8], except for the new P protein variant VP3-10, not described before, which is mainly in the unphosphorylated form (0.02% of P protein phosphorylation; data not shown).

All these variants were chromatographed through a Sephadex G-100 column and the behavior of each of the different P proteins was inferred from the radioactivity elution profiles. For the P protein variants with lower levels of phosphorylation, VP8 (30%), VP10 (20%), VP3-8 (2%) and VP3-10 (0.2%) ([8] and this paper), the elution of corresponding P proteins was determined after SDS–PAGE analyses of the protein present in the different fractions.

In all cases, the different P proteins are present in the fractions from the excluded volume of the column, although in the cases of the P protein variants VP5, VP6 and VP7, one peak of radioactivity was also found to be present in the included volume of the column (Fig. 2).

In addition, for VP7, not a clear excluded radioactivity peak was observed. These results indicate that these protein variants, mainly VP7, oligomerized less than the normal P protein.

All P protein variants, except VP7, can crosslink with glutaraldehyde acid into tetramers (Fig. 2), more or less in the same extension than the normal P protein, but VP5 and VP6 variants showed a 40–60% decrease in the formation of oligomers (Fig. 2). These results suggest that P protein amino acid residues located between the positions 99 and 161 are important for tetramer formation, the serine at position 99 being critical.

Both kinds of experiments indicate that 99.8% of the P protein constitutive phosphorylation is not essential for P protein oligomerization.

3.5. P protein expressed in bacteria is unable to oligomerize

In order to probe if P protein phosphorylation is dispensable for the oligomerization, as the previous experiments indicate, P protein was regulated expressed in *Escherichia coli* BDE-32 transformed with a plasmid pGEM3 recombinant that contained the Long strain P protein gene, without noncoding sequences and a canonical Shine–Dalgarno sequence, as it is indicated in Section 2.

Transformed bacteria were grown in M9 media at an OD at 590 nm of 0.5 and an equal volume of the culture was or was not induced with 1 mM IPTG for 180 min. Then, bacterial extracts were prepared [15] and the proteins present in those extracts were separated by SDS–PAGE, transferred to Immobilon membranes and analyzed by Western blot with a monospecific anti-P rabbit serum. In the extract of the IPTG-induced bacteria, a protein, with the electrophoretical mobility of P protein, was detected and was absent in the non-induced bacteria extract (see below, Fig. 3). The bacterial expressed P protein was barely recognized, in immunoprecipitation assays, by anti-P 73/P and 1/P monoclonal antibodies and monospecific sera anti-P or anti-P peptides. Thus, a crosslinking experiment was carried out with the total bacterial extract. As control, an extract from HEp-2 cells in which P protein was transiently expressed was included. P protein and its oligomers were detected after Western blot with the anti-P rabbit sera.

It can be observed in Fig. 3A that in transformed *E. coli* cells, under induction (lane 0, +) conditions, there is a protein, absent under non-induction conditions (lane 0 −), recognized by anti-P sera. This protein has the same electrophoretical mobility as the P protein transiently expressed in HEp-2 cells and present in purified HRSV virions (lane V). Both proteins are recognized by the anti-P sera. After 5, 15 and 30 min of incubation with glutaraldehyde, it is clear that the P protein expressed in HEp-2 cells oligomerizes but the P protein expressed in *E. coli* does not. Thus, it seems that the P protein expressed in bacteria and the P protein variant VP3-10 produced in HEp-2 cells show different characteristics although both are in unphosphorylated form. Two possibilities, among others, can explain these results: 0.02% of the P protein phosphorylation (the level of phosphorylation of the VP3-10 variant) may be enough to allow P protein oligomerization or a regulated and dynamic phosphorylation at different residues than those constitutively phosphorylated, could take place in HEp-2 cells but not in bacteria cells and it could be responsible for oligomerization, if it is the case, the use of phosphatase inhibitors like okadaic acid [16] could indicate the existence of such a mode of regulated and not constitutive phosphorylation.

3.6. A new phosphorylated form of P protein appears in transfected HEp-2 cells treated with okadaic acid

To study if P protein phosphorylation is controlled by cellular protein phosphatases, P protein was transiently expressed in HEp-2 cells treated or untreated with okadaic acid, and labelled with [^{32}P]orthophosphate. In both cases, P protein was immunopurified.

In the presence of okadaic acid, in addition to P protein, a new phosphorylated protein with slower electrophoretical mobility than that of P protein was immunopurified (Fig. 3B). In order to assure the identity of the new protein, a Western blot analysis was developed using a monospecific anti-P rabbit serum. The phosphorylated protein with lower electrophoretical mobility was recognized by the serum (Fig. 3B). It is clear that the proteins, with the normal and the slower electrophoretical mobility, obtained in HEp-2 cells treated with okadaic

acid are both P protein because they are immunopurified and are recognized by anti-P monoclonal antibody and the monospecific P antibody, respectively. This result indicates that new phosphorylations controlled by cellular protein phosphatases PP1 and PP2A [16] could occur when P protein is transiently expressed in HEp-2 cells.

To know if this regulated phosphorylation occurs in P protein residues different to those modified in a constitutive way, phosphorylation of all P protein variants was carried out in the presence and in the absence of okadaic acid. Fig. 3C shows the results obtained for VP3-8 and VP3-10 variants. Both P protein variants increase their phosphorylation level in the presence of okadaic acid. Since those variants lack serines at positions 116, 117, 119, 232 and 237, novel residues should be modified and their dephosphorylation prevented by okadaic acid.

4. Discussion

The paramyxoviruses phosphorylated P protein is a structural component of the viral nucleocapsids required for the viral polymerase activity in order to carry out viral RNA transcription and replication. The best studied paramyxoviruses P protein is that of SeV, the mouse counterpart of human parainfluenza virus. Due to the large size of the L polymerase (about 2165 amino acids long, in HRSV A2 strain), it is though that all the functions required for RNA synthesis are carried on by this viral protein. P protein acts as an essential cofactor that stabilizes L protein, facilitating its function on viral RNA synthesis and locating L protein on the N-RNA template. Also, interactions between P protein and N protein could be important for RNA synthesis, because, as a consequence of it, N protein remains in the soluble active form required for viral RNA encapsidation. Also, P protein is responsible for the addition of new P protein molecules to the RNA-N protein template that could facilitate the elongation process during RNA synthesis [17,18].

SeV P protein is a trimer and the domain for oligomerization (amino acids 344–411) forms a region with a predicted coiled-coil structure conserved through the paramyxoviridiae family. Thus, it seems that trimerization is a general property of all the paramyxovirus P proteins [19].

For SeV P protein oligomerization, its phosphorylation is dispensable because P protein produced in bacteria is also a trimer. The formation of the trimer is essential in that case for the binding of P protein to the RNA-N protein template [17].

This general role has also been suggested for HRSV P protein because P protein produced in bacteria has been found to form aggregates determined by Sephadex G-200 column filtration [12]. This result is in contrast with that present in this paper, indicating that P protein produced in bacteria is unable to oligomerize in crosslinking assays. This assay avoids any misinterpretation of the results obtained by exclusion chromatography in which protein denaturation could promote protein aggregation. On the other hand, P protein produced in mammalian cells behaves as a tetramer, as determined by both exclusion chromatography and crosslinking experiments. The absence of trimers indicates that monomers form dimers and the dimers could oligomerize. Thus the regions that interact among dimers are not recognized for monomers to form trimers.

That indicates that pneumoviruses may be different to the rest of the paramyxovirus, as also suggested by other features, as its different gene order and its increased number of genes [1]. About the region involved in P protein oligomerization, we found that VP5 shows a 60% decrease in oligomerization capacity with respect to that of VP. VP5 contains all the mutated residues present in VP3 and VP4, two variants with similar oligomerization capacity to that of VP. Thus, it seems that the simultaneous mutations of serines 116, 117, 119, 156 and 161 alter the oligomerization domain of P protein. An additional mutation at serine 143 (variant VP6) further decreases by 20% the oligomerization capacity of P protein and the addition of a mutation in serine 99 (VP7) totally abolishes the oligomerization of P protein. Then, these results suggest that the region comprising residues 99–165 is important for P protein oligomerization.

When 99.8% of HRSV P protein constitutive phosphorylation is removed by substituting the phosphorylatable serines by alanines, it was found that the corresponding P protein variant (VP3-10) was able to oligomerize. Because of the different results obtained with unphosphorylated P protein, isolated from mammalian cells or bacteria, we postulate that a different modification like a transient phosphorylation was occurring in P protein expressed in mammalian cells. In fact, a new type of phosphorylation strongly controlled by cellular phosphatases has been detected. This phosphorylation also occurs in HRSV-infected HEp-2 cells (data not shown) and it can explain the P protein oligomerization observed in eukaryotic cells in those P protein variants devoid of their constitutive phosphorylatable serines.

The phosphatase-controlled P protein phosphorylation seems to be essential for oligomerization. In this way, it is possible to speculate that this phosphorylation produces a conformational change that could be essential for interaction among P protein monomers. After the oligomerization process, this phosphorylation could be removed, leaving P protein assembled in a tetramer form.

Now, experiments are in progress trying to get oligomerization of bacterially expressed P protein after incubating it with extracts of uninfected HEp-2 cells, as sources of kinases. Also, directed mutagenesis will be done to determine the P protein residue modified by that phosphorylation that may allow P transition from P protein monomers to P protein tetramers.

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