C-Terminal phosphorylation of human respiratory syncytial virus P protein occurs mainly at serine residue 232

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To determine which human respiratory syncytial virus (HRSV) P protein serine residues are modified by cellular protein kinase(s), several mutated versions of P protein were expressed in the absence of other viral proteins. Mutations at serines 232 or 232 and 237 drastically reduced the extent of phosphorylation P protein in vivo. Serine 232 is the main site of modification and is also essential for in vitro phosphorylation by casein kinase II. Additional in vivo phosphorylation was also detected in the region containing serines 116, 117 and 119.

The P proteins of the paramyxoviruses, such as human respiratory syncytial virus (HRSV), may participate in at least three stages of the viral growth cycle: transcription and replication of viral RNA (Curran et al., 1991; Horikami et al., 1992) and particle formation. P proteins are phosphorylated and this modification may change their properties, to adapt them for these different functions.

In HRSV infection, P protein phosphorylation can be inhibited without impairing transcription or replication of the viral RNA (Villanueva et al., 1991). Also in Sendai virus infection, the P protein region (residues 78–320), containing almost all the phosphorylated amino acids, is dispensable for viral transcription and replication (Curran et al., 1993). The inhibition of HRSV particle formation, when P protein phosphorylation is abolished, suggests that P protein modification may be required in the virus assembly process (Villanueva et al., 1991). P protein serine residues phosphorylated during HRSV infection have been localized (Navarro et al., 1991; Villanueva et al., 1994) in two regions. One of these includes serine residues at positions 86, 94, 99, 116, 117, 119, 143, 156 and 161 and the other includes serine residues at positions 211, 215, 220, 232 and 237. The C-terminal region can be phosphorylated in vitro by a casein kinase II-like activity purified from HEp-2 cells (Villanueva et al., 1994). Thus, it seems that phosphorylation of P protein is in part due to the action of a cellular casein kinase II-like activity, as has been described for vesicular stomatitis virus (VSV) P protein (Barik & Banerjee, 1992). To investigate this point, cDNA for the P protein of the Long strain of HRSV was isolated from plasmid P20 (López et al., 1988) as a StuI–HpaI fragment and subcloned into the Smal site of the pSC11 vector (Chakrabarti et al., 1985). Two vaccinia virus recombinants were obtained (Chakrabarti et al., 1985; Mackett et al., 1985). One (VP) has the 5' end of the P gene close to the vaccinia 7.5 promoter. The other one (VP') has the P gene in the opposite orientation. P protein expressed by VP was phosphorylated mainly at serine residues as described for P protein synthesized during HRSV infection (Navarro et al., 1991; Villanueva et al., 1994). The phosphopeptides produced after protease treatments of the P proteins from both origins were similar (see below).

To determine which serines are modified by cellular kinases, single and multiple mutations (indicated in Fig. 1) were introduced in the P gene, using methods described by Higuchi et al. (1988). The plasmids and the pairs of internal primers, with the mutated codons in lower case letters and the numbers in parentheses indicating their positions in the P gene (López et al., 1988), were as follows.

VP1: VP plasmid, (+): (264) 5' CCTCTAGTActtTTCAAAGA 3' (283); (−): (287) 5' GTCTTCTTTGAAaagTACTA 3' (268).

VP2: VP plasmid, (+): (288) 5' CCTATACCActtGATAATCC 3' (307); (−): (311) 5' AAAGGGATTAaagTGGTA 3' (292).

VP3: VP plasmid, (+): (357) 5' GAAGAAttgaggTATttaTA 3' (376); (−): (383) 5' TTCTTCATAtaaATAcctca 3' (364).

VP4: VP plasmid, (+): (486) 5' GCAGGACCTACAtttgGCTAG3' (505); (−): (471) 5' CCTGccTGC-TACTACTAA 3' (490).

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VP5 was obtained by ligation of corresponding *NcoI*–*BclI* fragments containing the mutated regions of VP3 and VP4 plasmids to the large fragment obtained after VP digestion with *NcoI* (López et al., 1988).

VP6: plasmid VP5, (+): (434) 5' TGAAAAATTA-ctgGAAATAC 3' (453); (−): (457) 5' CCTAGTATTT-CcagTAATTT 3' (438).

VP7: plasmid VP6, (+): (300) 5' GATAATCCCTT-TctaAAACT3' (319); (−): (327) 5' CTTTGATAG-TTTtagAAAG 3' (308).

The following external primers were used for VP1 to VP7: (+): 5' (1) GGGGCAAATAATCATCATG (20) 3'; (−): 5' (622) TTTTGCCATCTTTTCACTTT (641) 3'.

VP8, VP9 and VP10: plasmid VP, (+): 5' (702) GGGAATGATgcgGACAATGA 3' (721); (−): 5' (733) TCAAGcgcTAGATCATTGTC (714) 3'.

The following external primers were used: (+): 5' cgcgcCTCGAGGAATTCA 3'; (−): 5' cgcgcCGGCCGTCAATTAGCA 3'. They begin at positions 6116 and 7106 in plasmid pSC11, respectively, and both include two CG pairs (letters in lower case) and an *XhoI* and an *EagI* site, respectively (underlined). All mutations were checked by sequencing the DNA plasmid using Sequenase version 2.0 (Maniatis et al., 1982). The corresponding vaccinia virus recombinants were named as for the plasmids.

CVI cells infected with the different vaccinia virus recombinants at an m.o.i. of 1 p.f.u./cell, were labelled, after the adsorption period, with 20 gCi/ml **[35S]**-methionine or 100 μCi/ml **[32P]**orthophosphate for 24 h. The corresponding P proteins were immunoprecipitated, using a mixture (1P, 3P, 11P, 16P, 73P) of anti-P protein monoclonal antibodies (García et al., 1993), and then were visualized after autoradiography (Fig. 2a). Densitometric analyses of samples from these experiments were used to estimate expression and phosphorylation levels for each P protein. From these data the mean value of specific activities for each mutated P protein was calculated and compared with that of the wild-type (wt) protein.

It is noteworthy that VP3 P protein, harbouring changes in serine residues at positions 116, 117 and 119, migrates in SDS-PAGE faster than VP P protein. Additional changes, like those introduced into VP5 P protein, reversed this effect. Further changes may (VP7 P protein) or may not (VP6 P protein) affect the electrophoretic behaviour of the P protein.

Since the P proteins with lower levels of phosphorylation (those of VP8 and VP10) did not show altered electrophoretic mobilities, it seems that the electrophoretic behaviour is related more to the amino acid composition of the protein than to its degree of phosphorylation. Amino acid changes in the central part of HRSV P protein related to abnormal electrophoretic behaviour have been described previously (Caravokyri & Pringle, 1992).

Fig. 2(b) shows that all mutated versions of P protein were labelled, but at different levels. VP7 and VP9 showed about 40% reduction in phosphorylation, whereas VP8 and VP10 showed reductions of 70% and 90%, respectively. These results indicate that the modification at serine 232 (VP8) accounts for 70% of P protein phosphorylation in the absence of other viral
expressed by vaccinia virus recombinants. CV1 cells were infected with the different vaccinia virus recombinants and labelled for 24 h with $[^{35}S]$methionine or $[^{32}P]$orthophosphate. In all cases, the corresponding extract produced after VP' infection (see text) was tested. The specific activities of different P protein mutants were calculated from results radiographs (a). The results (b) are expressed as a percentage of the indicated average and range values obtained from four different experiments are indicated.

Fig. 2. Phosphorylation of the P protein and its mutated versions expressed by vaccinia virus recombinants. CV1 cells were infected with the different vaccinia virus recombinants at an m.o.i, of 2.5 p.f.u./cell. The different P proteins were immunopurified from cell extracts of CV1 cells infected with the different vaccinia virus recombinants, only 30% and 9% respectively of the phosphorylation of the wt P protein. Because these variants gave the lowest levels of phosphorylation by casein kinase II-like activity, as previously described (Villanueva et al., 1994). The average and range values of different experiments are indicated.

proteins. Additional phosphorylation of VP8 may be at serine 237 (VP9), since the P protein of VP10, with mutations at serines 232 and 237, shows only one-tenth of the phosphorylation of wt P protein. However, this result like that obtained with VP7 may also indicate that the absence of serine at positions 237 and 99 may alter P protein conformation thereby reducing the accessibility of a protein kinase responsible for modifying P protein (see below). Thus, it seems that serines 232 and 237 are the main residues to be modified when P protein is expressed in vaccinia virus recombinants.

Serines 232 and 237 have an acidic residue at position n + 3, the casein kinase II consensus sequence (Pearson & Kemp, 1991). Thus, these amino acids may be essential for in vitro phosphorylation by the casein kinase II from HEp-2 cells.

To determine whether or not this is the case, the different P proteins were immunopurified from cell extracts of CV1 cells infected with the different vaccinia virus recombinants at an m.o.i. of 2.5 p.f.u./cell. The immunopurification of each P protein was made on a column that contained the anti-P protein monoclonal antibody 73/P covalently bound to Sepharose 4B (Navarro et al., 1991). Similar amounts of the different P proteins, obtained after titration in Western blot analysis with a monospecific P protein antiserum (obtained from a rabbit immunized with P protein purified by SDS–PAGE), were phosphorylated in vitro using partially purified casein kinase II-like activity, from mock-infected HEp-2 cells (Villanueva et al., 1994). The results (Fig. 3) obtained for the proteins of VP8 and VP10 were similar to those obtained for the proteins synthesized by the vaccinia virus recombinants, only 30% and 9% respectively of the phosphorylation of the wt P protein. Because these variants gave the lowest levels of phosphorylation by casein kinase II both in vivo and in vitro, it seems that serine residues 232 and 237 are essential for modification to occur under both conditions.

A reduction of 65, 35 and 50% was seen for proteins VP4, VP5 and VP6 respectively, all of which have lost the serines 156 and 161. However VP7, which contains all the mutations in VP6 together with a substitution of serine 99 showed no reduction in phosphorylation. It seems, therefore, that serine residues at positions 156 and 161 are not targets for the in vitro modification by casein kinase II. In addition, a C-terminal truncated version of P protein lacking the end 42 amino acids, obtained in a conventional in vitro transcription–translation system, was not phosphorylated by the casein kinase II-like preparation (data not shown).

The results indicate that phosphorylation at the C terminus of HRSS P protein occurs at serines 232 and 237 and is probably due to the action of a cellular casein kinase II-like protein.

Phosphorylation, outside the C-terminal region may occur, as has been described for P protein expressed in HRSS-infected cells (Navarro et al., 1991; Villanueva et al., 1994), in the central region of the molecule. To investigate this and confirm that serines 232 and 237 are
Fig. 4. Phosphopeptides produced by trypsin digestion of P proteins isolated after HRSV or vaccinia virus recombinant infections. Hep-2 cells were infected with HRSV Long strain or vaccinia virus recombinants and labelled with \[^{32}P\]orthophosphate. The corresponding P proteins were purified by affinity chromatography (Navarro et al., 1991) and characterized by SDS-PAGE. Identical amounts of P protein from different sources were treated with trypsin and the peptides that were generated were analysed. Panel (a) shows P protein from HRSV (lanes 2 and 2') or vaccinia virus recombinants (lanes 3 and 3')-infected cells or phosphorylated in vitro (lane 4) (Villanueva et al., 1994) that were treated, with 1 µg (lanes 2 and 3) or 2 µg of trypsin (lanes 2', 3' and 4). Lane 1 corresponds to undigested \[^{32}P\]-labelled P protein from HRSV-infected Hep-2 cells. (b) P proteins from VP (lane 1), VP3 (lane 2), VP4 (lane 3), VP5 (lane 4), VP6 (lane 5), VP7 (lane 6), VP8 (lane 7) and VP9 (lane 8) -infected cells were treated with 2 µg of trypsin. The positions of Mr markers (trypsinogen, lysozyme and glucagon with Mr's of 24000, 14000 and 3000, respectively) are indicated.

Phosphorylated targets, trypsin cleavage products of the protein, were analysed by electrophoresis.

Three µg of P protein was digested with 1–2 µg of trypsin for 1 h at 37 °C, in 50 µl 50 mM-NH\(_4\)HCO\(_3\), pH 8:3. Under these conditions, only a small proportion of the resulting tryptic peptides (20% of the total phosphoprotein) was large enough to be analysed in a gel of 16:5% acrylamide under the conditions of Schägger & von Jagow (1987) (46.5% w/v acrylamide–3% w/v bisacrylamide and 13.3% glycerol 6 M-urea).

The result of such an analysis is shown in Fig. 4. Fig. 4(a) shows that trypsin cleavage of P protein that was expressed during HRSV infection (lanes 2 and 2') and that of P protein from vaccinia virus recombinant-infected cells (lanes 3 and 3') generated a similar pattern although the relative proportion of the resulting phosphopeptides was different. The phosphopeptides were characterized. Peptide a is a partial tryptic digestion product, the presence of which depends on tryptic digestion conditions. Peptides b and c are located in the central region of the P protein whereas peptides d and e are present in the C-terminal region. The identification of peptides b and c was done by using an antibody raised against P protein residues 111–124 (not shown). Moreover, all the mutated forms of P protein lacking serine residues at positions 116, 117 and 119 have lost the label in peptides b and c (Fig. 4b), although it is detected by Western blotting in trypsin-digested VP3 P protein. These peptides show faster electrophoretic mobility than their wt counterparts (data not shown). A similar change in electrophoretic mobility was also observed in the P protein of VP4, and they are probably due to the introduction of an arginine at positions 117 and 156, which may result in the generation of a new target for trypsin digestion. Thus these changes in the electrophoretic behaviour of peptides b and c may result from changes in amino acid composition.

This result indicates that the residual phosphorylation in the central part of the P protein, produced in vaccinia virus recombinant-infected cells, takes place at one, two
or three of the serine residues present at positions 116, 117 and 119.

Peptides d and e were obtained by trypsin cleavage of in vitro phosphorylated P protein (Fig. 4a, lane 4), under conditions previously described by Villanueva et al. (1994), and thus should be located within the C-terminal region. Fig. 4(b), lane 7, shows that cleavage of VP8 P protein results in a minimal amount of the C-terminal phosphopeptides observed for VP9 P (lane 8), indicating that the main modification of the P protein C-terminal region is at serine 232.

In conclusion HRSV P protein is phosphorylated in two domains, in the presence or in the absence of the rest of the viral proteins. One domain is located within the central region of the P protein and includes serine residues 116, 117 and 119. The other domain is found at the C terminus of P protein and here serine 232 is the principal site of phosphorylation; serines 99 and 237 seem to be required as modulating factors of P protein phosphorylation. Phosphate label present in the central region, was quantified with respect to the total label phosphorylation. Phosphate label present in the central region of the P protein and here serine 232 is the main modification of the P protein C-terminal region. Fig. 4(b), lane 7, shows that cleavage of VP8 P protein results in a minimal amount of the C-terminal phosphopeptides observed for VP9 P (lane 8), indicating that the main modification of the P protein C-terminal region is at serine 232.

Nevertheless, the involvement of other cellular protein kinases or of the vaccinia virus-induced protein kinase B1 (Lin et al., 1992) cannot be ruled out. The participation of the latter, however, seems unlikely (Rempel & Taktman, 1992). On the other hand, the complete cycle of VSV RNA synthesis occurs in vaccinia virus-based expression systems (Pattnaik et al., 1992). Since VSV P protein must be specifically phosphorylated (first by casine kinase II and then by the L protein-associated activity) (Banerjee & Barik, 1992) in order to be functional in vitro, it appears that the vaccinia virus-induced protein kinase B1 activity is not involved in the process. The C terminus of HRSV P protein (229 GNDSDNILSLED 241) seems to be structurally similar, in its overall acidic character, to domain I of the VSV P protein (53 YQEESSDSSTYD 65), in which serines at positions 59 and 61 are phosphorylated by casein kinase II (Takacs et al., 1992). This domain has been identified as a transcriptional activator of the L protein (Takacs et al., 1991), and for in vitro transcription systems can be replaced by β-tubulin, probably its C-terminal region, or by poly-glutamic acid (Chattopadhyay & Banerjee, 1988).

The involvement of other viral proteins in the phosphorylation of the central region of the P protein might be due to their functioning as a protein kinase and/or as a chaperone, in order to assure the correct folding of the P protein. These possibilities are now being investigated.

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