Short Communication

Phosphorylation of human respiratory syncytial virus P protein at threonine 108 controls its interaction with the M2-1 protein in the viral RNA polymerase complex

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The human respiratory syncytial virus (HRSV) P protein is phosphorylated, with different turnover rates, at several serine (S) and threonine (T) residues. The role of phosphothreonines in viral RNA synthesis was studied by using P protein substitution variants and the HRSV-based minigenome pM/SH. By using liquid chromatography coupled to ion-trap mass spectrometry, it was found that P protein T108 was phosphorylated by addition of a high-turnover phosphate group. This phosphorylation occurs in P protein expressed transiently and during HRSV infection. The results suggest that phosphorylation at P protein T108 affects M2-1 transcripational activities, because this modification prevents interaction between the P and M2-1 proteins. Therefore, P protein phosphorylation–dephosphorylation at T108 could distinguish the role of the P protein in viral transcription and replication.

Human respiratory syncytial virus (HRSV), a pneumovirus of the family Paramyxoviridae, causes the majority of acute respiratory-tract infections affecting babies, the immunocompromised and elderly adults (Hall, 2001). Live vaccines (Collins et al., 1999), humanized monoclonal antibodies (mAbs; Meissner et al., 1999) and specific antiviral compounds (Bitko et al., 2005; Pastey et al., 2000) are suitable prophylactic measures to control HRSV infections.

The development of specific antiviral compounds requires a molecular understanding of viral protein functions. The viral ribonucleoproteins (RNPs) are ideal targets for antiviral compounds. They are multifunctional and involved in distinctive viral processes, as they are structural virion components and the functional units for viral RNA synthesis. The RNPs include the helical nucleocapsid, containing viral (v) RNA bound to N protein, the L protein (or polymerase) and their cofactors, the phosphoproteins P and M2-1 (Collins et al., 2001).

Viral transcription and replication are distinct processes. In the first, discontinuous mRNA synthesis is driven by the gene-start (GS) and -stop (GE) signals present in each gene. In the second, continuous RNA synthesis occurs from the first nucleotide located at the vRNA 3' end to the last one at the 5' end (Lamb & Kolakofsky, 2001). The L protein displays nucleotide-polymerizing activity during viral RNA synthesis, but it requires P protein as an essential, non-catalytic cofactor. The P–L complex allows the L protein to contact the nucleocapsid (the template for viral transcription and replication) through L–P–N–RNA interactions. P–N interactions are essential to render the N protein competent for RNA encapsidation during replication (Collins et al., 2001). P–M2–1 interactions through P protein residues L101, Y102 and F109 are needed for M2-1 protein anti-termination and elongation transcriptional activities (Mason et al., 2003).

It is not clear how the viral RNA polymerase (vRdRp) is involved differentially in transcription (vRdRpT) and replication (vRdRpR) (Gubbay et al., 2001). The essential interactions established by the P protein during these processes could be the clue. As the P protein is a phosphoprotein, phosphorylation at specific site(s) may tag the P protein for different interactions. Thus, P protein phosphorylation may have a role in the distinction between vRdRpR and vRdRpT (Collins et al., 2001).

The P protein is modified by the addition of phosphate groups with different turnover rates, detected in the presence (low) or in the absence (intermediate and high) of cellular phosphatase PP2A and PP1 activities. High-turnover phosphates are added to S30, S39, S45, S54 and perhaps T46 (Asenjo et al., 2005), those with intermediate turnover modify S116, S117 and/or S119 (Asenjo et al., 2005; Navarro et al., 1991) and low-turnover phosphates modify S232 (Asenjo et al., 2005; Sánchez-Seco et al., 1995).
Phosphorylation at S237 in vitro has been suggested (Mazumder et al., 1994). Phosphorylation at these residues is not required for viral transcription or replication, either to support M2-1 transcriptional activities or for the viral growth cycle (Asenjo et al., 2005; Lu et al., 2002; Villanueva et al., 2000).

Nevertheless, in a P protein variant with all of these residues replaced by non-phosphorylatable residues (VPm30) (Fig. 1a), phosphorylation was detected at S and T residues when it was expressed transiently in HEp-2 cells and labelled with [32P]orthophosphate in the presence of 1 μM okadaic acid (OKA) to inhibit cellular phosphatases PP2A and PP1 completely (Bialojan & Takai, 1988) (Fig. 1b). This phosphorylation was about 14% of that found in wild-type P protein treated with the same conditions.

To determine the function of high-turnover P protein phosphorylated residues, P protein variants in which all T residues (except T210 and T219) were replaced by alanine (A) were constructed (Fig. 1a). Their capacities to support viral transcription and replication of the HRSV dicistronic RNA analogue pM/SH were tested (Fig. 1c, lower panel) when cotransfected in vaccinia recombinant vTF-3-infected HEp-2 cells, with pGEM3 recombinant plasmids expressing L, P and N proteins under the control of the T7 RNA polymerase promoter (Hardy & Wertz, 1998; Villanueva et al., 2000). The pM/SH dicistronic HRSV minigenome allows the analysis of M2-1 protein transcriptional activities (Hardy & Wertz, 2000; Collins et al., 1995).

All of these variants supported viral RNA synthesis, except VPT151 and VPT160. These two residues seem to be essential for P protein interactions, affecting viral transcription and replication. All P protein variants functional in viral RNA synthesis, except for T105A and T105,108A, allowed M2-1 anti-termination and elongation transcriptional activities (Fig. 1c, lower panel), indicated by rt RNA synthesis (rt RNAs are generated as a consequence of viral RNA polymerase read-through of the transcription-termination signals; rt/B is clearly visible) and by increased amounts of mRNAs. These T residues are close to those essential for P–M2-1 interaction (P protein residues L101, Y102 and F109). The result for the T105A variant differs from that obtained when the same P protein variant (Long strain) was assayed with an RSV-based minigenome luciferase reporter gene and L, N and M2-1 proteins from the HRSV A2 strain (Mason et al., 2003). This could be due to intrasubgroup heterogeneity between Long and A2 strain M2-1 proteins, as described for the P protein of these strains (Asenjo et al., 2005). As the Long and A2 strain M2-1 proteins differ only in the residue at position 179 (S in A2 and L in the Long strain), this residue may have a role in interaction with the P protein.

To analyse P protein high-turnover phosphorylated T residues, P and VPm30 proteins were expressed in HRSV-infected or -transfected HEp-2 cells, respectively, in the presence of 1 μM OKA. They were isolated from the corresponding cell extracts by two-dimensional electrophoresis. Selected spots were excised and trypsin-digested and the resulting peptides were analysed by nano-liquid chromatography coupled to ion-trap mass spectrometry (LC/MS/MS). Among others, the P protein-derived tryptic phosphopeptide 104ETIEpTFDNNEEELR117 was identified in VPm30. Fig. 2(a) displays its induced fragmentation spectrum and pT108 is clearly detected from both amino- and carboxy-terminal-end fragmentation series. T108 is included in the fragment (103TIEpTFD109) with the recognition consensus sequences (D/E–S/T) described for the cellular protein casein kinase I (Pearson & Kemp, 1991).

Due to the low amount of P protein obtained from HRSV-infected HEp-2 cells, we performed a high-sensitivity analysis based on multiple-reaction monitoring scanning within a triple-quadrupole mass spectrometer (Fig. 2b). The fragmentation spectrum from the ion eluted at 37 min was identical to that shown in Fig. 2(a) (not shown). Thus, P protein expressed transiently or after HRSV infection is modified by addition of high-turnover phosphate at T108.

To study the effect of P protein phosphorylation at T108, the P protein variants T108D, T105D and T105,108D were assayed. These variants simulate P proteins phosphorylated permanently at these residues. All variants supported viral transcription and replication, but none supported M2-1 transcriptional activities (Fig. 3a, upper panel). The expression levels of different viral proteins were similar in all cases (Fig. 3a, lower panel). These results and those obtained with the P protein variants T105A and T105,108A (simulating P proteins permanently unphosphorylated at the corresponding residues) indicate that P protein T105 and T108 are essential to support M2-1 protein transcriptional activities. The P protein residue at position 105 is probably T (it cannot be replaced by A or D), whereas T108 can be replaced by A but not by D, suggesting that both residues are involved in contact with the M2-1 protein and that phosphorylation of P protein T108 prevents it. Phosphorylation at T108 would control P protein interaction with the M2-1 protein and, therefore, the M2-1 regulatory activity on the L protein during viral transcription (Cartee et al., 2003).

Transiently expressed Long and A2 strain M2-1 proteins had an electrophoretic mobility lower than that of M2-1 protein from purified extracellular viral particles, due to their phosphorylation at T56 and S58 or at S58 and S61 (Cartee & Wertz, 2001) residues, respectively. Coexpression of M2-1 and M proteins leads to their interaction; the M2-1 protein is probably not phosphorylated in this interaction and its electrophoretic mobility increases (Cuesta et al., 2000). According to our interpretation, when M2-1 and P protein variants T105A, T105D, T108D, T105,108A and T105,108D were coexpressed, M2-1 protein phosphorylation was found, as indicated by its reduced electrophoretic mobility (Fig. 3b, upper panel). In contrast, no phosphorylation in the M2-1 protein was detected following coexpression with P protein or with its T108A variant (Fig. 3b, upper panel). When an immunoprecipitation assay was carried out with P protein.
Fig. 1. P protein variant VPm30 is phosphorylated at serine and threonine residues in the presence of 1 μM OKA. Effect on viral transcription and replication of P protein substitutions at threonine residues. (a) Scheme of Long strain P protein primary structure (line), showing the location of substituted S and T residues (numbers indicated above and below the line, respectively), in the P protein variants analysed. All P protein variant cDNAs were cloned in the corresponding pGEM3 recombinant plasmids. Substitutions were obtained as described previously (Asenjo et al., 2005). (b) P and VPm30 proteins were expressed transiently in HEp-2 cells (8 × 10⁵ cells per 35 mm² dish) infected with the vaccinia recombinant vTF-3 (m.o.i., 5 p.f.u. per cell) and transfected with 2–5 μg of the appropriate pGEM3 recombinant plasmid and Lipofectin (Invitrogen). At 20 h post-transfection, cultures were labelled with [³²P]orthophosphate [25 μCi (925 kBq) ml⁻¹] in the presence of 1 μM OKA for 4 h. P and VPm30 proteins were immunoprecipitated from cell extracts by using P protein mAb 73/P, fractionated by SDS-PAGE and visualized by autoradiography (left panel). Right panel: phosphoamino acid analysis of the VPm30 protein variant. VPm30 was blotted onto a membrane and excised for treatment with 6 M HCl; the resulting amino acids were separated by two-dimensional electrophoresis and characterized after autoradiography, as described previously (Navarro et al., 1991). Circles indicate positions of P-S, P-T and P-Y markers added before the electrophoresis, visualized with ninhydrin. (c). Upper panel: scheme of the DNA construct in the pM/SH plasmid that expresses a dicistronic HRSV RNA analogue. Sizes and polarities are shown for the different RNAs generated during transcription and replication (Hardy & Wertz, 1998). Lower panel: RNA fractionation in 3–5 % acrylamide gel containing 7 M urea. RNA was labelled with [³²P]uridine [25 μCi (925 kBq) ml⁻¹] in the presence of 10 μg actinomycin D ml⁻¹ between 16 and 48 h after transfection of HEp-2 cells (8 × 10⁵ cells per 35 mm² dish) with 1–9 μg pM/SH, together with 1–9 μg pGEM3N, 0–56 μg pL (except lane C) (Cartee et al., 2003), 0–1 μg pGEM3M2-1 and 0–77 μg pGEM3P (VP), or with the indicated VP recombinant variant plasmids. All pGEM3 recombinant plasmids contain HRSV Long strain sequences, except pM/SH and pL, which contain those of the A2 strain. At 48 h post-transfection, total RNA was isolated by using the TRizol method, following the supplier’s instructions (Invitrogen). RNAs were separated by electrophoresis in 3 % acrylamide gel containing 7 M urea. After electrophoresis, the gel was dried and autoradiographed.
Fig. 2. (a) Fragmentation spectrum (MS/MS) from doubly charged VPm30 variant-derived tryptic peptide at m/z 909-9, spanning residues 104–117 and containing a phosphothreonine in position 108 (pT residue). Main fragmentation series (carboxy-y and amino-b series) are shown. Enhanced neutral loss of phosphate group is labelled as NL and the phosphothreonine 108 is confirmed in both (y and b) fragmentation series (arrows). A similar fragmentation spectrum was obtained from P protein expressed in HRSV-infected HEp-2 cells. Both transfected and infected HEp-2 cells were treated with 1 μM OKA. (b) Chromatographic detection of phosphorylation at T108 P protein-derived tryptic peptide from HRSV-infected HEp-2 cells by multiple-reaction monitoring selection is shown. Q1 was set on parent ion at m/z 909-8 and Q3 was set on the diagnostic y8 ion at m/z 1018-4. Throughout the LC-MS/MS analysis, a chromatographic peak appeared only when these two signals were detected.

Fig. 3. P protein phosphorylation at T108 avoids M2-1 transcriptional activity by interfering with the binding between the P and M2-1 proteins. (a) Upper panel: capacities of P protein and its indicated variants to support M2-1 transcriptional activities (see Fig. 1c). Lower panel: Western blot analysis with anti-HRSV rabbit serum of cellular extracts corresponding to one-fifth of cells analysed in the upper panel. (b) P protein and the indicated variants were coexpressed in HEp-2 cells (8 × 10^5 cells per 35 mm^2 dish) infected with vTF-3 (m.o.i., 5 p.f.u. per cell) and cotransfected with appropriate total recombinant plasmid DNAs (2.5 μg). Cells were labelled with ^35 S)methionine (30 μCi (1.11 MBq) ml^-1) between 10 and 24 h post-transfection. Proteins from cell extracts before (upper panel) or after (lower panel) their immunoprecipitation with antibody 73/P were separated by SDS-PAGE and visualized by autoradiography. The electrophoretic mobility of P and those of phosphorylated (*) and non-phosphorylated M2-1 proteins are indicated. (c) Analyses as in (a) (upper panel) were performed with P and M2-1 proteins and their indicated variants.
protein mAb 73/P (Fig. 3b, lower panel) and the proteins contained in the transient coexpression cell extracts indicated above, it was found that only P protein and the variant VPT108A coimmunoprecipitated the unphosphorylated M2-1 protein. No interaction between phosphorylated M2-1 protein and the rest of the P protein variants assayed was detected. These results agree with the idea that P protein T105 and T108 contact M2-1, T108 phosphorylation being a regulator for that interaction.

We tested the M2-1 variant T56,S58A to determine whether the interaction between P and M2-1 proteins or the absence of phosphorylation in M2-1 (a consequence of that interaction) is needed for M2-1 regulation of viral RNA polymerase transcription activity. This variant has diminished transcriptional elongation and anti-termination capacities (50%) compared with those of normal M2-1 protein (Fig. 3c), as occurred for the corresponding A2 strain M2-1 protein variant (Cartee & Wertz, 2001). The M2-1 T56,S58A variant was assayed with the P protein variant T105,108A (Fig. 3c). This P protein variant does not facilitate M2-1 protein activity, although the M2-1 variant is essentially in an unphosphorylated form (Cuesta et al., 2000). It appears that P–M2-1 physical interaction, rather than the absence of M2-1 protein phosphorylation, is needed for M2-1 protein transcriptional cofactor activities, as suggested previously (Mason et al., 2003).

Our results indicate that dynamic dephosphorylation–phosphorylation at P protein T108 controls M2-1 protein incorporation into the vRdRp, mediated by P–M2-1 interaction. Although we cannot exclude the possibility that P protein substitution T108D could abolish P–M2-1 interaction independently of mimicking phosphorylation, our results support that such interaction may be regulated by P protein T108 phosphorylation. As the M2-1 protein only influences L protein transcriptional activity (Cartee et al., 2003), P protein pT108 must be included in a vRdRp complex, unable to incorporate M2-1 protein and involved in viral RNA replication (vRdRpR, replicase). In contrast, when P protein is dephosphorylated at T108, the M2-1 protein, included in the vRdRp complex, is involved in efficient viral transcription (vRdRpT, transcriptase).

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