The bulk of the phosphorylation of human respiratory syncytial virus phosphoprotein is not essential but modulates viral RNA transcription and replication

Nieves Villanueva,1 Richard Hardy,2 Ana Asenjo,1 Qingzhong Yu2 and Gail Wertz2

1Centro Nacional de Microbiología, Instituto de Salud Carlos III, Carretera Majadahonda-Pozuelo Km2, Majadahonda, Madrid 28220, Spain
2Department of Microbiology, BBRB 17/Rm 366, University of Alabama Medical School, Birmingham, AL 3594-2170, USA

The ability of variants of the human respiratory syncytial virus (HRSV) phosphoprotein (P protein) to support RNA transcription and replication has been studied by using HRSV-based subgenomic replicons. The serine residues normally phosphorylated in P during HRSV infection have been replaced by other residues. The results indicate that the bulk of phosphorylation of P (98%) is not essential for viral RNA transcription or replication but that phosphorylation can modulate these processes.

Human respiratory syncytial virus (HRSV), a pneumovirus from the family Paramyxoviridae, is implicated in the majority of lower respiratory tract infections requiring hospitalization of young children. Respiratory infections due to HRSV are also a health problem in the elderly and in immunocompromised individuals (Collins et al., 1995). Vaccine development has been problematic and the young age of the population in most need of protection presents an extra challenge.

An alternative means of controlling HRSV infection is to develop specific antiviral compounds, a goal that requires knowledge of the function(s) of specific viral proteins. We have focused on the function of phosphorylation of the phosphoprotein (P).

The P protein (241 amino acids) is found in infected cells and is also a component of the virus ribonucleocapsid (Huang et al., 1985; Lambert et al., 1988), which is the functional template for virus transcription and replication. The P protein of HRSV is mainly phosphorylated in vivo on serines 116, 117, 119 and 232, located in the central and C-terminal regions of the molecule (Navarro et al., 1991; Villanueva et al., 1994). However, phosphorylation of serine residues 116, 117 and 119 has been questioned (Mazumder & Barik, 1994). The enzymatic activity responsible for P protein modification was partially purified from HEp-2 cells and characterized as casein kinase II (CKII) (Mazumder & Barik, 1994; Mazumder et al., 1994; Villanueva et al., 1994). P protein, produced in bacteria, can be phosphorylated on serine 232 or 237, depending on the level of purification of CKII (Barik et al., 1995), and it has been suggested that each of these CKII-mediated phosphorylations is essential to render unphosphorylated P protein active for viral transcription in vitro (Barik et al., 1995). However, inhibition of P protein phosphorylation in HRSV Long strain-infected HEp-2 cells does not impair transcription or replication (Villanueva et al., 1991).

To clarify the function of P protein phosphorylation, P proteins with substitutions at the phosphorylatable serines were analysed in vivo for their ability to support transcription and replication of subgenomic HRSV RNA replicons in a vaccinia virus–T7 expression system (Yu et al., 1995; Hardy & Wertz, 1998).

The P gene of the HRSV Long strain, contained in plasmid P20 (López et al., 1988), was subcloned under the control of the T7 promoter into the Smal site of pGEM3 (Promega) as a Stul–HpaI fragment. Mutant P genes constructed previously in vaccinia virus recombinants (Sánchez-Seco et al., 1995) were subcloned into T7 expression plasmids. The pGEM3 expression plasmids were named in the same manner as the corresponding vaccinia virus recombinants from which they were derived: VP (Long strain P protein), VP3, in which the serines at positions 116, 117 and 119 were changed to leucine, arginine and leucine (S116L, S117R, S119L), VP8, in which serine 232 was changed to alanine (S232A), and VP9, in which serine 237 was changed to alanine (S237A). VP3-6 (S116L, S117R, S119L, S232A) was prepared by substitution of the P gene NcoI fragment from plasmid VP3 for that present in plasmid VP8. The nucleotide sequences of the P genes of all recombinant plasmids were determined by sequence analysis.

The levels of phosphorylation of all P protein variants were analysed in HEp-2 cells transfected with wild-type or mutant plasmids (García et al., 1993) by using the vaccinia virus–T7 RNA polymerase expression system (Fuerst et al., 1987). The transfected cultures were labelled for 9–24 h post-transfection with 100 µCi [32P]orthophosphate or 50 µCi [35S]methionine,
cells with the N, P, L and M replication and synthesis of a single mRNA when expressed in by using a subgenomic replicon of HRSV that directs both other phosphoproteins (Roach, 1991).

The subgenomic replicon RNA, WT5, contained the HRSV leader sequence, the start of the NS1 gene fused to the end of the L gene and the trailer sequence of HRSV A2 strain. Thus, this replicon contains all the signals required for its own replication and for the transcription of a single mRNA (Hardy & Wertz, 1998) (Fig. 2a).

for determination of the levels of P protein phosphorylation (Fig. 1c) was performed by using a phosphorimeter. The relative activities obtained from the transfected cultures were similar to those found when the different P protein variants were expressed from the corresponding vaccinia virus recombinants, as reported previously (Sánchez-Seco et al., 1995).

Previous analyses of P protein phosphopeptides revealed the phosphorylation sites in the P protein and its variants VP3, VP8 and VP9 (Sánchez-Seco et al., 1995) and suggested that modification of serines 116, 117 and 119 (mutated in VP3) and 232 (mutated in VP8) accounted for 20 and 80%, respectively, of P protein phosphorylation, phosphorylation of serine 237 (mutated in VP9) being negligible. However, VP9 showed a 20% reduction in its phosphorylation level; this could be due to a conformational change that affects the overall P protein phosphorylation, as proposed previously (Sánchez-Seco et al., 1995).

Unexpectedly, the mutant VP3-8 showed only 2% of the wild-type level of phosphorylation. Thus, it seems that 98% of P protein phosphorylation is due to modification of serine residues 116, 117, 119 (19.6%) and 232 (78.4%), but there is an additional 2% residual phosphorylation. Therefore, we conclude that P protein phosphorylation shows a hierarchy for its modification at different residues, as has been reported for other phosphoproteins (Roach, 1991).

The function of the mutant P proteins was first examined by using a subgenomic replicon of HRSV that directs both replication and synthesis of a single mRNA when expressed in cells with the N, P, L and M proteins (Hardy & Wertz, 1998). The subgenomic replicon RNA, WT5, contained the HRSV leader sequence, the start of the NS1 gene fused to the end of the L gene and the trailer sequence of HRSV A2 strain. Thus,

cellular extracts were prepared and the P protein was immunoprecipitated (Villanueva et al., 1991). The results of these analyses are shown in Fig. 1(a, b). Quantitative analysis to determine the levels of P protein phosphorylation (Fig. 1c) was performed by using a phosphorimeter. The relative activities obtained from the transfected cultures were similar to those found when the different P protein variants were expressed from the corresponding vaccinia virus recombinants, as reported previously (Sánchez-Seco et al., 1995).

![Fig. 1. Phosphorylation level of P protein variants. P protein variants were expressed and labelled with [32P]orthophosphate (a) or [35S]methionine (b) by transfection of the appropriate plasmids encoding the variant P proteins into cells infected with the vaccinia virus recombinant T7. P protein variants were immunoprecipitated from the corresponding cellular extracts, separated by SDS–PAGE and visualized by autoradiography. In (a), V corresponds to extracellular viral particles, the structural viral proteins of which are indicated on the left. (c) Relative activities were calculated by densitometry and related to that of Long strain P protein (VP).](image)

![Fig. 2. Transcription and replication capacities of P protein variants determined by using the HRSV RNA analogue expressed by plasmid pWT5. (a) Diagram of plasmid pWT5 and the RNAs synthesized from it (Hardy & Wertz, 1998). T7φ, T7 RNA polymerase transcription termination signal; HφV, hepatitis delta ribozyme sequence; le, leader; tr, trailer; A, poly(A) tail. (b) Cells were infected with T7 and transfected with plasmids pWT5, pN, pM2ORF1 and pL, all containing genes from strain A2. The P protein used in each case is indicated. The transfected cells were exposed to [3H]uridine in the presence of actinomycin D. Total RNA was analysed by agarose–urea gel electrophoresis.](image)
HEp-2 cells were infected with vTF7-3 and transfected with 5 µg pWT5, 5 µg pN, 1.5 µg pL, 0.1 µg pM2ORF1 and 2 µg pP, all derived from the A2 strain (Yu et al., 1995; Hardy & Wertz, 1998), or 2 µg plasmid DNA expressing the Long strain P protein (VP) and the mutants VP3, VP8 and VP9 derived from the Long strain P protein. Cells were exposed to [3H]uridine in the presence of actinomycin D. Total RNA was analysed by agarose–urea gel electrophoresis.

The products of RNA synthesis from replicon WT5 are shown diagrammatically in Fig. 2(a) and were identified, as described previously, by annealing of specific oligonucleotides followed by RNase digestion (Hardy & Wertz, 1998). These products, as indicated in Fig. 2(a), are the products of genomic positive- and negative-strand RNA replication (rep), the single mRNA species (mRNA) and a product that resulted from the polymerase failing to terminate at the end of the mRNA and reading through into the trailer to generate a readthrough product (r/t), which migrates faster than the replication product because it lacks the 44 nucleotide leader RNA.

All the labelled RNAs species were synthesized by the viral polymerase formed by the L and P proteins, as none of them appeared when P protein was omitted (Fig. 2b, no P). Additionally, the P protein from the Long strain formed functional nucleocapsids with the N, L and M₂ proteins of the A2 strain, as shown by the finding that it was able to support transcription and replication from the WT5 replicon to a similar extent to the wild-type A2 strain P protein (Fig. 2, A2 and VP). The P protein mutants VP3 and VP8 were also able to replicate and transcribe the HRSV RNA analogue. We also tested the behaviour of the variant VP9 (S237A), since this P protein variant has been reported to have a dominant-negative effect on P protein function (Mazumder et al., 1994), although in our hands we did not find any modification of this serine residue (Sánchez-Seco et al., 1995). Again, this P protein mutant was also able to transcribe and replicate the HRSV RNA analogue (Fig. 2, VP9), although with a lower efficiency than its wild-type counterpart. This could be due to the possible conformational change induced in the variant (Sánchez-Seco et al., 1995).

Because the P protein variants VP3 and VP8 are phosphorylated at serine 232 and at serines 116, 117 and 119, respectively, the experiment described above only indicates that the simultaneous presence of both phosphorylated domains of P protein is dispensable for transcription and replication. In order to determine the effect of a P protein that was not phosphorylated at either of the two domains on viral transcription and replication, another P protein mutant, VP3-8, was prepared for use in subsequent experiments.

Since the mutant P proteins were all functional when examined by using a subgenomic replicon that expressed one mRNA, we next examined the activities of VP3, VP8 and VP3-8 during RNA replication and transcription by using a subgenomic replicon encoding two mRNAs. This was done to determine whether the P protein might have a separate role in the transit of the polymerase across a gene junction. The RNA synthesis assays were performed as indicated above but using the replicon M/SH, which encodes two mRNAs (see Fig. 3a; Hardy & Wertz, 1998). The construction of the plasmid, pM/SH, that encodes this replicon has been described previously (Hardy & Wertz, 1998). This replicon contains the leader and trailer elements surrounding two transcriptional units separated by the M/SH gene junction. The ability of the mutated P proteins to support replication and transcription of the two mRNAs was examined as described above. The results obtained are shown in Fig. 3. The major RNA products were,
as shown in Fig. 3(a), the products of genomic RNA replication (rep), the two monocistronic mRNAs, mRNA1 and mRNA2, and the bicistronic products of readthrough at the end of mRNA1 into mRNA2 (r/tB) or mRNA1 into the trailer (r/tC). The abundant readthrough products occurred because of the increased processivity of the polymerase in the presence of the M protein and its failure to terminate at the ends of the genes. The identification of each RNA species was made by annealing specific oligonucleotides followed by digestion with RNase H, as described previously (Hardy & Wertz, 1998).

Each of the P proteins examined was capable of supporting replication and transcription. However, the degree of phosphorylation appeared to modulate the efficiency of RNA synthesis. The wild-type A2 and Long strain P proteins functioned with approximately equal efficiency for replication and transcription. The VP3 and VP8 proteins, the phosphorylation levels of which were reduced relative to the wild-type by 19.6% and 78.4%, respectively, supported efficient HRSV RNA synthesis that was not substantially different from that observed with VP. The mutant P protein VP3-8, in which serines at positions 116, 117, 119 and 232 were substituted, still supported replication and transcription. However, a decrease in RNA synthesis was observed, taking into account that RNA from equivalent numbers of cells was analysed. In all cases, both mRNA1 and mRNA2 and the products of readthrough transcription (dicistronic mRNAs produced because of the failure of the polymerase to terminate transcription at the upstream end of the gene) were made, as expected from the presence of the M protein, which has previously been characterized as a transcription elongation factor that results in the synthesis of more dicistronic readthrough RNA products (Collins et al., 1996; Hardy & Wertz, 1998).

The experiments shown in Fig. 3 were also carried out in the absence of the M protein. The results obtained were qualitatively the same as those obtained in the presence of M protein, in that all the mutants could support replication and transcription although to slightly varying degrees (data not shown). However, since the mRNA levels were reduced in the absence of M protein, in that all the mutants could support replication and transcription. These results suggest that the bulk of P protein phosphorylation (98%), which occurs at residues 116, 117, 119 and 232, is not essential for transcription or replication of HRSV RNA subgenomic replicons expressing one or two mRNAs. Whether or not the residual phosphorylation (2%) displayed by the P protein variant VP3-8 is essential for viral transcription and replication remains to be determined. However, the level of modification of the P protein modulates RNA transcription and replication.

The results described here are similar to those found in HRSV-infected cells treated with the inhibitor xanthate D609, although in that case, the level of P protein phosphorylation was even less than 2% of that found in the absence of the drug (Villanueva et al., 1991). These results indicate that the bulk of phosphorylation, which occurs at serines 116, 117 and 119 (19.6% and 78.4%) (Sánchez-Seco et al., 1995), is not essential for viral transcription or replication.

Taking into account that P protein phosphorylation modulates viral RNA transcription and replication, it is possible that completely dephosphorylated P protein or P protein with only 2% of its total phosphorylation level can function in HRSV-infected cells and in the vaccinia-virus based system. However, this low level of phosphorylation may not be sufficient to support transcription in an in vitro system like that used by Barik et al. (1995).

In summary, the results presented in this paper indicate that the bulk of P protein phosphorylation is not essential for transcription or replication of viral RNA but that the level of phosphorylation can modulate P protein function in these processes.

This work was supported in Spain by FIS 97/0171 and in the USA by NIH Public Health Service grant AI 20181. We are grateful for the excellent technical assistance of R. Martinez.

References


Received 15 June 1999; Accepted 24 September 1999