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Phosphorylation of paramyxovirus phosphoprotein and its role in viral gene expression

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Abstract

Paramyxoviruses include many important human and animal pathogens such as measles virus, mumps virus, human parainfluenza viruses, and respiratory syncytial virus, as well as emerging viruses such as Nipah virus and Hendra virus. The paramyxovirus RNA-dependent RNA polymerase consists of the phosphoprotein (P) and the large protein. Both of these proteins are essential for viral RNA synthesis. The P protein is phosphorylated at multiple sites, probably by more than one host kinase. While it is thought that the phosphorylation of P is important for its role in viral RNA synthesis, the precise role of P protein phosphorylation remains an enigma. For instance, it was demonstrated that the putative CKII phosphorylation sites of the P protein of respiratory syncytial virus play a role in viral RNA synthesis using a minigenome replicon system; however, mutating these putative CKII phosphorylation sites within a viral genome had no effect on viral RNA synthesis, leading to the hypothesis that P protein phosphorylation, at least by CKII, does not play a role in viral RNA synthesis. Recently, it has been reported that the phosphorylation state of the P protein of parainfluenza virus 5, a prototypical paramyxovirus, correlates with the ability of P protein to synthesize viral RNA, indicating that P protein phosphorylation does in fact play a role in viral RNA synthesis. Furthermore, host kinases PLK1, as well as AKT1 have been found to play critical roles in paramyxovirus RNA synthesis through regulation of P protein phosphorylation status. Beyond furthering our understanding of paramyxovirus RNA replication, these recent discoveries may also result in a new paradigm in treating infections caused by these viruses, as host kinases that regulate paramyxovirus replication are investigated as potential targets of therapeutic intervention.

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Keywords

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Mononegavirales are negative-stranded, nonsegmented RNA viruses with lipid membranes [1]. Viruses in the *Paramyxoviridae* family of *Mononegavirales* include many important human and animal pathogens, such as human parainfluenza viruses (PIVs), Sendai virus (SeV), mumps virus, Newcastle disease virus, measles virus, rinderpest virus and human respiratory syncytial virus (RSV), as well as the newly discovered Nipah and Hendra viruses. There are two subfamilies, *Paramyxovirinae* and *Pneumovirinae*, within the *Paramyxoviridae* family. Until recently, *Paramyxovirinae* contained four genera, *Pneumovirinae*, *Rubulavirus*, *Respirovirus* and *Morbillivirus*. Recently, the paramyxoviruses Hendra virus and Nipah virus were isolated from Australia and Malaysia, respectively [2,3]. These zoonotic viruses cause fatal infection in humans. Their genomes are closely related, but differ substantially from the rest of the subfamily of *Paramyxovirinae*. Consequently, a new genus, *Henipavirus*, has been created to accommodate the newly discovered viruses [4].

The ssRNA genomes of the *Mononegavirales* range from approximately 11,000 to 19,000 nucleotides in length and contain a series of tandemly linked genes separated by nontranscribed sequences. For paramyxoviruses, the gene order is 3'-NP-P(V/W/C)-M-F-(SH)-HN-L-5' where genes in parentheses are not found in all species (reviewed in [1]). The viral RNA-dependent RNA polymerase that transcribes the nucleocapsid protein-encapsidated RNA into 5' capped and 3' polyadenylated mRNAs minimally consists of two proteins, phosphoprotein (P) and the large polymerase protein [5]. The viral RNA polymerase is thought to bind the genomic RNA at a single 3' entry site and to transcribe the genome by a sequential and polar process. The RNA polymerase also replicates viral RNA genomes [6–9].

While it is certain that the P proteins of all paramyxoviruses are essential for viral RNA synthesis and are heavily phosphorylated (hence, the name P protein), the precise role of P protein phosphorylation remains an enigma. It has been proposed that phosphorylation of P protein plays a critical role in viral RNA synthesis, but conclusive evidence supporting this notion is lacking. By contrast, the most recent work appears to indicate that phosphorylation of paramyxovirus P proteins does not play a role in viral RNA synthesis. Among the paramyxovirus P proteins, those of SeV and RSV are the best studied. It was first reported in the 1970s that the P protein of SeV is phosphorylated [10]. The phosphorylation sites within P protein were mapped using monoclonal antibodies [11], as well as through the use of 2D gels and chromatography analysis of tryptic phosphopeptides of wild-type and mutant P proteins [12]. While as many as 11 phosphorylation sites were detected, the serine (Ser) residue at position 249 was determined to be the major phosphorylation site [12]. Interestingly, when this primary phosphorylation site Ser249 was mutated to alanine, the level of P protein phosphorylation detected in transfected cells was not affected at all. Mutation of the adjacent proline residue at position 250 (P250) did reduce the level of P protein phosphorylation by approximately 50% [12]. Using a vaccinia virus-based minigenome system, P proteins with S249A and P250A mutations were found to have similar activities as the wild type P protein. Furthermore, when these mutations were introduced into the SeV genome using a reverse-genetics system, the resulting viruses exhibited no significant impairments in growth characteristics and pathogenicity *in vitro* (tissue culture cells) and *in vivo* (mouse) [13], indicating that the major P protein phosphorylation site is not important for viral RNA synthesis (or for any other step of the virus lifecycle). Mutating five additional phosphorylation sites besides S249 resulted in a P protein mutant whose level of phosphorylation was reduced by more than 90% in transfected cells, yet, the mutant P protein still functioned normally in the minigenome system [14]. These results appear to suggest that phosphorylation of the SeV P

protein does not play a critical role in regulating viral RNA synthesis. However, it is possible that the remaining phosphorylation sites within the P protein are important for viral RNA synthesis. Identifying these remaining phosphorylation sites may further clarify the role of P protein phosphorylation in SeV RNA synthesis.

The P protein of RSV is the most heavily phosphorylated of any paramyxovirus P protein in infected cells [15]. Two clusters of phosphorylation sites have been identified. One cluster comprises amino acid residues 116, 117 and 119, and the other cluster comprises residues 232 and 237. CKII has been found to be critical for the phosphorylation of the C-terminal (residue 232) cluster [16–19]. Using an *in vitro* transcription system reconstituted with P protein purified from bacteria, it was found that phosphorylation at position 232 by CKII was critical for viral transcription [20]. When both clusters of phosphorylation sites were mutated, the level of P protein phosphorylation was reduced by more than 90%. Interestingly, this mutant P protein was still active in synthesizing viral RNA in a vaccine virus-based minigenome system, albeit at reduced level [21,22], suggesting that phosphorylation of the P protein is not essential for P protein function, but modulates the activity of viral RNA synthesis. However, when these mutations were introduced into the RSV genome using a reverse-genetics system, expression levels of viral genes in virus-infected cells were not adversely affected by these mutations, indicating that these residues do not play a critical role in viral RNA synthesis. Interestingly, the RSV mutant viruses have normal growth characteristics in Vero cells but impaired growth in HEp-2 cells and *in vivo* (mouse) [22], suggesting that P protein may play a role in evading host innate immune responses since Vero cells are defective in interferon production due to the deletion of the interferon gene locus. Additional phosphorylation sites within the RSV P protein have been inferred based on the observation that altered P protein (with residues 116, 117, 119, 232 and 237 mutated) is still phosphorylated [22]. Further studies using mass spectrometry identified a threonine residue at position 108 (T108) as being phosphorylated. The phosphorylation of T108 is important for its interaction with M2–1, a processivity factor of viral RNA synthesis, and mutating the residue results in diminished activity in a minigenome system, suggesting that P protein may regulate viral RNA synthesis through its interaction with M2–1 [23]. However, the role of T108 in virus infection has not been investigated. Although S54 of RSV P protein is thought to be phosphorylated, it does not appear to play a role in viral RNA synthesis, but rather is thought to play a role in virus uncoating [24,25]. The P protein of human parainfluenza virus 3 is phosphorylated by PKC- ζ [26] and the Ser residue at position 333 is the likely target site [27]. However, the role of phosphorylation at Ser333 in the virus lifecycle has never been reported. Even though mutation of known phosphorylation sites reduces phosphorylation of SeV and RSV P proteins by 90%, it is possible that the remaining residues that are phosphorylated (even though they only account for ~10% of total phosphorylation) are critical for viral RNA synthesis.

Phosphorylation of proteins plays an important role in regulating protein functions. There are two major kinases that phosphorylate tyrosine residue or Ser/threonine residue in host cells. It is thought that phosphorylation of paramyxovirus proteins must be carried out by host Ser/threonine kinases owing to lack of known viral kinases [28]. At present, two host Ser/threonine kinases, CKII and PKC- ζ , have been identified as the main host kinases that phosphorylate paramyxovirus P proteins. CKII is thought to phosphorylate the P proteins of RSV [17,18] and measles virus [29]. PKC- ζ is reported to phosphorylate the P proteins of human parainfluenza virus 3 [26] and SeV [30]. The P protein of canine distemper virus is phosphorylated by both PKC- ζ and CKII [31]. While the role of CKII in phosphorylation of the P proteins has been studied most, interestingly, owing to the nature of CKII (ubiquitous expression and multiple subunits and isoforms), it has never been shown that CKII is directly involved in paramyxovirus replication in infected cells. Similarly, the role of PKC- ζ in viral RNA synthesis has not been validated using specific siRNA. The discouraging results from recombinant viruses containing phosphorylation-deficient P proteins combined with the lack of data to prove that the putative

host kinases are involved in viral RNA synthesis have led to the hypothesis that phosphorylation of P protein does not play a role in viral RNA synthesis.

Recently, Sun *et al.* found that AKT plays a critical role in replication of many paramyxoviruses by studying a prototypical paramyxovirus, PIV5 [32]. They reported that AKT, a Ser/threonine kinase, also known as PKB, interacts with P protein as well as phosphorylates P protein *in vitro*. Both AKT inhibitors and siRNA targeting AKT1 reduced PIV5 replication. Furthermore, inhibitors of AKT also reduced the replication of several other paramyxoviruses including RSV, indicating that AKT plays a critical role in paramyxovirus replication. At present, AKT binding sites and phosphorylation target sites within paramyxovirus P proteins have not been identified. Interestingly, Sun *et al.* also reported that the P protein of PIV5 has a functional binding site for PLK1, a Ser/threonine kinase that plays an essential role in cell-cycle progression [33,34]. They found that this binding site comprised of a SSP motif, the same site that was previously found to be important for virus lifecycle [35], plays an important role in regulating viral RNA genome replication. PLK1 and PIV5 P protein were found to interact, and PLK1 was shown to phosphorylate P protein *in vitro*. The amino acid residue phosphorylated by PLK1 was identified as S308 both *in vitro* and in infected cells. These results indicate that PLK1 binds to the PIV5 P protein via a SSP motif at residue 157, and phosphorylates P protein at residue S308, resulting in the downregulation of viral RNA synthesis. Intriguingly, recombinant PIV5 that overexpresses its viral genes owing to the lack of PLK1 phosphorylation causes elevated cytokine expression and rapid death of infected cells, indicating that phosphorylation of P protein by PLK1 is beneficial to virus replication (Figure 1). For the first time, a role for a kinase has been defined in the context of virus infection using a recombinant paramyxovirus.

The endeavor to understand the role of phosphorylation of paramyxovirus P proteins has had its share of confusion. The recent work on the role of phosphorylation in regulating paramyxovirus P protein function has brought new insights in understanding paramyxovirus RNA replication. The work may also result in a new paradigm for treating infections caused by these viruses. For many years, tremendous effort has been invested unsuccessfully in developing small-molecule drugs that directly target viral proteins as antiviral treatments for paramyxovirus infection. Targeting host proteins, such as AKT, which are required for nonsegmented RNA viruses/paramyxovirus replication represents a novel approach. With many anticancer drugs based on AKT inhibition already in development, it is exciting to speculate that some of these same drugs could also be readily applied to treat infections caused by paramyxoviruses.

Future perspective

It is likely that future studies of paramyxovirus P proteins will lead to identification of phosphorylation sites within the P proteins that are essential for viral gene expression and also lead to the identification of host kinases that are responsible. Targeting these critical kinases will lead to novel strategies in controlling infections of these viruses.

Executive Summary

- Recent work demonstrates that phosphorylation of a paramyxovirus phosphoprotein does play a role in viral gene expression.
- PLK1, a host kinase, downregulates gene expression of paramyxovirus parainfluenza virus 5.
- This downregulation of viral gene expression benefits the virus by avoiding activation of cytokine expression and induction of apoptosis.

- Targeting host kinase may lead to a novel strategy to control infections by these viruses.

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Figure 1. Role of polo-like kinase 1 in parainfluenza virus 5 replication

PLK1 binds to the SSP motif at residues 156–158 within the P protein, a consensus PLK1 binding site, and then phosphorylates the P protein at S308, which leads to downregulation of parainfluenza virus 5 gene expression. While the reduced viral gene expression is sufficient for efficient virus replication, it enables the virus to avoid the induction of cell death and cytokine expression, thus benefiting virus replication. P: Phosphoprotein; PLK: Polo-like kinase; S: Serine residue.