Dengyun Sun,1,2 Pei Xu,1,2 and Biao He1*

Department of Infectious Diseases, University of Georgia, Athens, Georgia 30602,1 and Intercollege Graduate Program in Cell and Developmental Biology, Pennsylvania State University, University Park, Pennsylvania 168022

Received 24 February 2011/Accepted 14 July 2011

The P protein of parainfluenza virus 5 (PIV5) is an essential cofactor of the viral RNA-dependent RNA polymerase. Phosphorylation of the P protein can positively or negatively regulate viral gene expression, depending on the precise phosphorylation sites. Sumoylation, a process of adding small ubiquitin-like modifier (SUMO) to proteins posttranslationally, plays an important role in regulating protein function. In this study, we have found that the P protein of PIV5 was sumoylated with SUMO1 in both transfected and infected cells. The K254 residue of the P protein is within a consensus sumoylation motif. Mutation of the P protein at K254 to arginine (P-K254R) reduced PIV5 minigenome activity, as well as the sumoylation level of the P protein. Incorporation of K254R into a recombinant PIV5 (rPIV5-P-K254R) resulted in a virus that grew to a lower titer and had lower levels of viral RNA synthesis and protein expression than wild-type PIV5, suggesting that sumoylation of the P protein at K254 is important for PIV5 growth. Biochemical studies did not reveal any defect of P-K254R in its interactions with viral proteins NP and L or formation of homotetramers. We propose that sumoylation of the P protein at K254 regulates PIV5 gene expression through a host protein.

Small ubiquitin-related modifier, or small ubiquitin-like modifier (SUMO), was first identified in 1996 as a protein that is covalently linked to a target protein posttranslationally (24). Many proteins, including RanGap1, Sp3, ERK, P53, IκBα, PIAS, STAT1, MDA5, and RIG-I, have been found to be covalently linked to SUMO through a process called sumoylation (7, 22, 25, 28–30, 35, 43). Sumoylated proteins are involved in transcriptional regulation, nuclear-cytosolic transport, protein stability, stress response, signal transduction, DNA repair, and the cell cycle (1, 2, 9, 15, 36, 38). The reversible process of sumoylation is carried out similarly to ubiquitination through activation, conjugation, and ligation (9, 15). Immature SUMO is cleaved by SUMO-specific isopeptidases (sentrin-specific proteases [SENP]) to expose its C-terminal Gly-Gly motif to become mature SUMO. Activation of mature SUMO protein, carried out by the SUMO-specific E1-activating enzyme, forms a SUMO-adenylate conjugate as an intermediate. SUMO is then transferred to an E2-conjugating enzyme, such as Ube9 for SUMO1. Finally, the E2-conjugating enzyme transfers SUMO to a substrate, a reaction which E3 ligase may facilitate and for which it may determine the specificity (9). Unlike in ubiquitination, a poly-SUMO1 chain does not occur at a single site because there is no lysine residue within SUMO1 protein to serve as an acceptor for additional SUMO. Furthermore, sumoylation is not known to mediate protein degradation.

SUMO proteins are approximately 100 amino acid residues in length and 12 kDa in size. There are four SUMO isoforms encoded by the human genome, SUMO1, SUMO2, SUMO3, and SUMO4. SUMO1, SUMO2, and SUMO3 are ubiquitously expressed; however, SUMO4 is not. It is unclear whether SUMO4 can be processed to its mature form in vivo (9). The mature forms of SUMO2 and SUMO3 share 97% identity, but they have only 50% identity with SUMO1 (9). As a result, SUMO1 and SUMO2/3 are conjugated to different target proteins and serve different functions. While SUMO1 is conjugated to target protein as a monomer, SUMO2/3 can be conjugated as a monomer or a polymer (33). Among the hundreds of sumoylated proteins, most are nuclear proteins (4, 15). Viral proteins that have been found to be sumoylated are mostly from DNA viruses; examples include IE2p86 and IE1p72 of human cytomegalovirus (HCMV) (3, 14, 18) and E1, E2, and L2 capsid protein of human papillomavirus (HPV) (23, 26, 39). For RNA viruses, sumoylation of viral proteins has also been detected. Sumoylation of Gag protein of human immunodeficiency virus (HIV) may affect HIV infectivity (12). Sumoylation of CA protein of Moloney murine leukemia virus (MMLV) has been found to be important in early events during virus infection (44). The NS1 proteins of most influenza virus strains are sumoylated, which enhances NS1 stability and promotes rapid growth of virus (41). It has been reported that viral proteins can promote or reduce sumoylation of host proteins (4, 16, 37). For example, the VP35 protein of Ebola Zaire virus has been found to block type I interferon (IFN) production by increasing sumoylation of PIAS1 (protein inhibitor of activated STAT1) (6). It was reported that the L3 endoprotease of adenovirus, I7 protein of poxvirus, and S273R protein of African swine fever virus (ASFV) have sequence and functional similarity to SUMO-specific protease Ulp1 (the yeast homolog of SENP, which can process the SUMO1 precursor and remove SUMO from substrates), indicating that these viral proteases may process sumoylation and desumoylation of viral or host proteins (19, 37),...
Parainfluenza virus 5 (PIV5) is a prototypic member of the paramyxovirus family, which contains many important human and animal pathogens, including mumps virus (MuV), measles virus (MeV), Sendai virus (SeV), and the emerging Hendra virus (HeV) and Nipah virus (NiV) (17). The negative-stranded RNA genome of PIV5 is composed of seven genes and yet encodes eight known proteins (17). The viral RNA-dependent RNA polymerase consists of large (L) protein and phosphoprotein (P). The L protein has enzymatic activities capable of initiation, elongation, and termination of viral RNA synthesis, as well as addition of the 5' cap structure and 3' poly(A) sequence to the viral mRNA. The P protein is the cofactor for the polymerase, playing an essential role in regulating viral RNA synthesis (17). The P protein is heavily phosphorylated, and its phosphorylation status regulates the function of the P protein in viral RNA synthesis (8, 31, 31a, 34). In this study, we have investigated the role of P protein sumoylation in regulating viral gene expression.

MATERIALS AND METHODS

Plasmids, viruses, and cells. Human SUMO1, SUMO2, and SUMO3 genes were purchased (Open Biosystems) and cloned into pCAGGS vector. Plasmids encoding the wild-type P protein (P-WT), P-K254R, His-P, His-P-K254R, and the full-length rPIV5-P-K254R were made similarly to what has been previously described (31). The whole genome of the mutant virus rPIV5-P-K254R at a multiplicity of infection (MOI) of 3. The cells were lysed with passive lysis buffer and 1/10 of the lysis from each well were used for dual-luciferase assay (Promega). Relative luciferase activity was normalized as the ratio of Renilla luciferase (R-Luc) activity to firefly luciferase (FF-Luc) activity. An aliquot of the cell lysate from transfected cells was used for immunoblotting to detect the input amount of NP and P-WT/P-K254R. For the PIV5 transcription-only minigenome system, a mutant minigenome plasmid, pSMG-m-Rluc, carrying a deletion in the region important for PIV5 replication was used (34). The experimental process was similar to that for the PIV5 minigenome system; however, the dual-luciferase assay was performed at 2 days posttransfection.

Protein purification and CD. Circular dichroism (CD) was performed using His-P-WT and His-P-K254R purified from bacteria. Briefly, P-WT or P-K254R with 8 histidines (His) at the N terminus in a pET15b vector was transformed into BL21(DE3)E3PolS competent cells. A single colony was selected and grown in hazy ampicillin (50 ng/ml) and chloramphenicol (34 ng/ml). When the optical density at 600 nm (OD600) of the bacteria was within the range of 0.5 to 0.6, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the bacterial culture at a 1 mM final concentration to induce protein expression for 4 h at 37°C. Proteins were purified using nickel (Ni)-charged resin (Novagen) and examined by SDS-PAGE and Coomassie blue staining (50% methanol, 10% acetic acid, 0.25% Coomassie brilliant blue R250). The purified proteins were desalted and resuspended in a buffer containing 10 mM potassium phosphate (KH2PO4) and 10 mM potassium chloride (KCl) (pH 7.0). Three hundred microliters of 10 μM His-P-WT and His-P-K254R was analyzed on a Jasco-J715 spectropolarmeter using a 0.1-cm-path-length cuvette (10). Three measurements were taken for each sample, and the average of millidegrees is shown.

Gel-shift curve and plaque assay. MDBK cells in 6-well plates were infected with PIV5 or rPIV5-P-K254R at an MOI of 0.01. The supernatants were collected at 0, 1, 2, 3, 4, and 5 days postinfection and centrifuged to remove cell debris. For high-MOI infection, MDBK cells in 6-well plates were infected with PIV5 or rPIV5-P-K254R at an MOI of 3, and the supernatants were collected at 0, 12, 24, 36, and 48 h postinfection. BHK cells in 6-well plates were infected with the virus stocks in serial dilution (1:10 to 1:10^4). After 2 h, the inoculating mixture was removed and replaced with 5 ml DMEM containing 2% FBS, 10% TBL, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 1% low-melting-point agarose. The plaques were counted at 4 to 5 days postinfection. Three replicates for each time point were collected for statistical analysis.

Flow cytometry. To further compare viral protein expression levels in PIV5 and PIV5-P-K254R-infected cells, flow cytometry was performed as previously described (20, 31, 32). MDBK cells were mock infected or infected with PIV5 or rPIV5-P-K254R at an MOI of 1. Infection mixture was fixed with 0.5% formaldehyde and resuspended in 0.5 ml DMEM-FBS containing 2% FBS, 10% TBL, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 1% low-melting-point agarose. Flow cytometry was performed in MDBK, HeLa, and BSR-T7 cells at 10 h postinfection (hpi) with an MOI of 1.

RT and real-time RT-PCR. MDBK cells in 6-well plates were infected with PIV5 or rPIV5-P-K254R at an MOI of 3 or 0.5. Total RNAs from the infected cells were extracted using the RNeasy minikit (Qiagen) at different time points. One-tenth of the total RNA from each sample was used for reverse transcription (RT) using Superscript III reverse transcriptase (Invitrogen). Oligo(dT)15 was used in RT to detect viral mRNA level. BH911 annealing to the M gene of the genomic RNA was used in RT to measure the viral genome level. One percent of the cDNAs from RT were used for real-time PCR on the Step One Plus real-time PCR system as described before (34). Relative levels of viral mRNA and viral genome were determined by calculating 2^(-ΔΔCT) and normalized with the level of the input genome, defined as the viral genome level at 2 hpi. Three replicates for each sample were used for statistical analysis.

Immunoprecipitation and DSP cross-linking. To compare interactions between NP and P-WT/P-K254R, BSR-T7 cells in 6-cm plates were transfected with 1 μg NP together with 1 μg P-WT or P-K254R. After 18 to 20 h, the cells were starved and metabolically labeled with [35S]Met-Cys for 3 h. The labeled cells were lysed with WCEB and immunoprecipitated with either anti-P or anti-Flag antibody. An aliquot of the cell lysate was used for immunoblotting to show the input amount of Flag-L and P-P-K254R.

PIV5 minigenome system and dual luciferase assay. The PIV5 minigenome system used in this study had been previously described (31). Briefly, increasing amounts (0.01 to 0.16 μg/well, 24-well plate, 4 replicates for each condition) of P-WT or P-K254R were transfected together with other plasmids (0.2 μg pSMG-Rh, 0.2 μg NF, 0.3 μg L, and 1 ng FF-Luc) into BSR-T7 cells. After 20 to 22 h, the cells were lysed with passive lysis buffer and 1/10 of the lysis from each well were used for dual-luciferase assay (Promega). Relative luciferase activity was normalized as the ratio of Renilla luciferase (R-Luc) activity to firefly luciferase (FF-Luc) activity. An aliquot of the cell lysate from transfected cells was used for immunoblotting to detect the input amount of NP and P-WT/P-K254R. For the PIV5 transcription-only minigenome system, a mutant minigenome plasmid, pSMG-m-Rh, carrying a deletion in the region important for PIV5 replication was used (34). The experimental process was similar to that for the PIV5 minigenome system; however, the dual-luciferase assay was performed at 2 days posttransfection.

Immunoblotting (IB). To detect sumoylation of the P protein, SUMO1, SUMO2, or SUMO3 was cotransfected with P into BSR-T7 cells. At 24 to 24 h posttransfection, the cells were lysed with whole-cell extraction buffer (WCEB) (50 mM Tris-HCl [pH 8], 280 mM NaCl, 0.5% NP-40, 2 mM EDTA, 2 mM EGTA, and 10% glycerol) (34). The lysates were centrifuged, and the supernatants were mixed with the same volume of 2X SDS loading buffer (100 mM Tris-HCl [pH 6.8], 20% glycerol, 4% SDS, 200 mM dithiothreitol [DTT], and 0.1% bromophenol blue) (34), heated at 95°C for 5 min, and resolved by 10% SDS-PAGE. The proteins were transferred to polyvinylidene difluoride (PVDF) (Millipore) membrane. The membrane was incubated with mouse anti-P (Pk) antibody, followed by incubation with mouse anti-secondary antibody labeled with horseradish peroxidase (HRP). After washing, the PVDF membrane was incubated with ECL Advance Substrate (GE Healthcare) and scanned using a Kodak Image Station 440. To compare the interaction between L and NP, the antibody recognizing P was used for immunoprecipitation, followed by immu-
anti-NP antibody. The IP products were washed and resolved by SDS-PAGE. The gel was dried, and the proteins were visualized using a Typhoon 9700 phosphorimager (GE Healthcare) (see Fig. 8). To detect tetramer formation of P-WT or P-K254R, the transfected BSR-T7 cells were starved and labeled with [35S]Cys-Met for 3 h at 37°C. The labeled cells were incubated with 1 mM disuccinimidyltartrate (DSP) (Pierce, Rockford, IL) in phosphate-buffered saline (PBS)-0.5% NP-40 to cross-link the disulfide bond as previously described (34). After cross-linking, the cells were lysed with WCEB and immunoprecipitation was performed as described above using anti-P antibody. After washing, half of the IP products were mixed with SDS loading buffer without DTT to detect P tetramer, and half of the IP products were mixed with SDS loading buffer with 200 mM DTT to show the P band after removing the cross-linking. The mixture was resolved in a 10% SDS gel, and the proteins were visualized using a Typhoon 9700 phosphorimager (GE Healthcare).

RESULTS

The P protein is sumoylated by SUMO1 but not by SUMO2 or SUMO3. To investigate whether the P protein is sumoylated, P and SUMO1, SUMO2, or SUMO3 were cotransfected into cells and modification of the P protein was examined using immunoblotting with Pk antibody (anti-P). A slower-moving band, consistent with the size of the P protein plus 12 kDa, was detected in the cells transfected with both P and SUMO1, indicating that the P protein might be sumoylated by SUMO1 (Fig. 1A). Interestingly, there was no corresponding band in P- and SUMO2-transfected cells or in P- and SUMO3-transfected cells, suggesting that P may not be sumoylated by SUMO2 or SUMO3. Similar results were observed using Flag-tagged SUMO1, -2, and -3 (data not shown). To further confirm that the slower-moving P band was indeed sumoylated P protein, an IP-IB experiment was performed using mouse anti-P antibody for IP and rabbit anti-SUMO1 (Santa Cruz Biotechnology) for IB. The slower-moving band was recognized by both anti-P antibody and anti-SUMO1 antibody; the difference in size between the P protein band and the slower-moving band is about 12 kDa, indicating that the P protein was sumoylated by SUMO1. Additional sumoylation products were also detected in the lane containing P plus SUMO1. Since a poly-SUMO1 chain cannot form at the same site, there are likely multiple sumoylation sites within the P protein. The same results were obtained using different anti-P antibodies (data not shown), indicating that the P protein is sumoylated. Sumoylation of the P protein was also detected in PI5-infected cells using a similar IP-IB approach (Fig. 1C). Interestingly, even though the antibody used (Pk) recognizes both V and P, which precipitate with NP, no sumoylated NP or V was detected.

Identification of one consensus sumoylation site within the P protein. Most sumoylated proteins share a consensus motif, YKXD/E, with a hydrophobic amino acid residue Y at the -1 location and an acidic amino acid residue at +2; +1 can be any amino acid residue (15, 27). We have identified several potential sumoylation sites within the P protein. K254 of the P-WT protein fits the consensus motif the best. To investigate whether K254 of the P-WT protein is indeed a sumoylation site, a K254R mutation of the P protein was generated and analyzed. The K254R mutation reduced the sumoylation level of the P protein by approximately 40% in transfected cells (Fig. 2A and B), indicating that K254 is a sumoylation site of the P protein. The statistically significant reduction of sumoylation, but not a complete lack of sumoylation, of P-K254R indicates that there are other sumoylation sites within the P protein, consistent with the observation in Fig. 1.

The K254R mutation reduces PI5 minigenome activity. To examine whether sumoylation of the P-WT protein plays a role in regulating PI5 gene expression, the effect of a K254R mutation in the P protein was studied using a PI5 minigenome system. It is known that the ratio of P to NP affects the level of viral RNA synthesis and that too high a level of expression of P inhibits viral gene expression. To ensure that the maximal level of viral gene expression was detected in the...
minigenome system, a range of concentrations of P was used and expression levels of P and NP were examined. P-K254R significantly reduced minigenome activity (Fig. 3A), suggesting that sumoylation of the P-WT protein at K254 affects PIV5 gene expression. To investigate the possibility that the negative impact of the P-K254R mutation on viral gene expression may be due to a change of protein secondary structure, circular dichroism was performed to compare the secondary structures of P-K254R and the P protein. These two proteins showed identical absorption from wavelength 190 to 260 nm (Fig. 3B), suggesting that the K-to-R mutation at amino acid residue 254 did not affect the secondary structure of the P protein.

K254 of the P-WT protein is a sumoylation site in PIV5-infected cells. To examine the role of sumoylation of the P protein in virus infection, the K254R mutation was incorporated into PIV5 to generate a recombinant virus (rPIV5-P-K254R). The genome of the mutant virus rPIV5-P-K254R was sequenced, and no other amino acid residue change was detected. The sumoylation level of P-K254R in rPIV5-P-K254R-infected cells was compared with that of the P protein in PIV5-infected cells. There was a lower level of P-K254R sumoylation in rPIV5-P-K254R virus-infected cells, indicating that K254 is a sumoylation site in PIV5-infected cells (Fig. 4A and B). Interestingly, the reduction of sumoylation is modest, indicating that there is an additional sumoylation site(s) or an alternate sumoylation site is sumoylated. As in Fig. 1C, only sumoylated P was detected and no sumoylated NP or V was detected in infected cells, suggesting that NP and V are not sumoylated. Growth of rPIV5-P-K254R virus was characterized with low-MOI (0.01) (Fig. 4C) and high-MOI (3) (Fig. 4D) infection in MDBK cells. rPIV5-P-K254R grew slower and to a lower titer than PIV5, suggesting that mutation of the P protein at K254 caused a defect in growth of PIV5.

K254R mutation reduces viral protein expression. To study whether the defect in rPIV5-P-K254R growth is due to the defect in viral gene expression, levels of viral protein expression in rPIV5-P-K254R-infected cells were compared to those in PIV5-infected cells. At an MOI of 3, a lower level of viral protein expression in rPIV5-P-K254R-infected cells was found at 8 hpi, as well as at later time points, by immunoblotting and flow cytometry (Fig. 5A and B). With low-MOI infection (MOI = 1), rPIV5-P-K254R also showed a significant defect in viral protein expression at 10 hpi (data not shown). In addition, the defect of rPIV5-P-K254R in viral protein expres-
sion was also detected in HeLa and BSR-T7 cells, indicating that the phenotype is not cell line specific (Fig. 5C and D).

**FIG. 4.** Sumoylation of K254R in virus infection. (A) Sumoylation of P-K254R in infected cells. HeLa cells were mock infected or infected with PIV5 or rPIV5-P-K254R at an MOI of 5. At 24 hpi, the cells were lysed and the supernatants used for IP-IB. (B) Quantification of the sumoylation levels. Three individual experiments from panel B were performed for quantification and statistical analysis. (C) Growth rate of rPIV5-P-K254R at an MOI of 0.01. MDBK cells were infected with PIV5 or rPIV5-P-K254R, and the supernatants were collected at different time points for plaque assay. (D) Growth rate of rPIV5-P-K254R at an MOI of 3 in MDBK cells.

**FIG. 5.** Viral protein expression levels in rPIV5-P-K254R-infected cells. (A) Immunoblotting. MDBK cells were infected with PIV5 or rPIV5-P-K254R at an MOI of 3. The cells were collected at different time points and used for immunoblotting using anti-NP and Pk (anti-P/V) antibodies. β-Actin was used as a protein loading control. K254R indicates rPIV5-P-K254R virus. (B) Flow cytometry. MDBK cells were mock infected or infected with PIV5 or rPIV5-P-K254R at an MOI of 3. Flow cytometry was performed to compare viral protein expression at different time points using Pk antibody. (C and D) Flow cytometry in HeLa (C) and BSR-T7 (D) cells. Similar experiments were performed at an MOI of 1 in HeLa and BSR-T7 cells at 10 hpi.
nism of the defect in rPIV5-P-K254R growth, quantitative real-time RT-PCR (qRT-PCR) was used to compare the levels of viral mRNA and viral genome RNA in MDBK cells. With high-MOI infection (MOI = 3), rPIV5-P-K254R had a lower level of viral mRNA at 8 hpi, as well as at later time points (Fig. 6A). The genome RNA level was also reduced in rPIV5-P-K254R-infected cells at 8 hpi and at later time points (Fig. 6B). Similar results were observed with low-MOI infection (MOI = 0.5) (Fig. 6C and D), suggesting that the K254R mutation reduced viral RNA transcription and replication.

**The K254R mutation affects viral RNA transcription.** To determine which step in viral RNA synthesis was affected by the K254R mutation of the P protein, a transcription-only minigenome, which is functional only in viral transcription and not in replication, was used. P-K254R reduced the activity of the transcription-only minigenome (P < 0.01 by analysis of variance [ANOVA]) (Fig. 7), indicating that P-K254R was defective in viral mRNA transcription.

**P-K254R does not affect NP-P or P-L interaction or P tetramer formation.** The interactions of NP and P and of P and L, as well as P tetramer formation, are essential for PIV5 RNA synthesis. To study the mechanism of the defect of P-K254R, the NP and P/K254R interaction, and the L and P-WT/P-K254R interaction, were examined using coimmunoprecipitation or IP-IB in transfected cells (Fig. 8A and B). P-K254R can bind to NP and L protein, similarly to P-WT. In addition, P-K254R formed tetramers as does P-WT (Fig. 8C), indicating that there is no defect in P-NP or P-L interactions or tetramer formation.

**DISCUSSION**

Sumoylation is an important protein posttranslational modification and is involved in many essential cellular processes. In this study, we have found that PIV5 P protein, a viral protein from a nonsegmented, negative-strand RNA virus, was sumoylated by SUMO1 but not by SUMO2 or SUMO3. The mechanism of preference of SUMO1 over SUMO2/3 is not clear. Interestingly, no sumoylation of NP, L, or V was detected (Fig. 1B and 4A). Based on a consensus sumoylation motif, we have identified K254 of the P-WT protein as a sumoylation site. A K254R mutation reduced, but did not eliminate, the sumoylation level of the P protein, suggesting that there are other sumoylation sites within the P protein. Further studies need to identify these extra sumoylation sites and their function. We speculate that the other sumoylation sites are likely in the C terminus of the P protein, since the V protein, which has an N terminus of 164 amino acid residues identical to that of P, was not sumoylated. Sumoylation of the P protein at K254 plays an important role in PIV5 minigenome activity, as well as PIV5 growth, indicating that sumoylation of the P protein may regulate PIV5 gene expression. We also found that sumoylation of the P protein at K254 affected viral RNA transcription, which may lead to a reduced level of viral replication in infected cells. We propose that sumoylation of the P protein regulates viral gene expression through regulating viral RNA transcription.

Among the effects of sumoylation, transcriptional regulation is an important one. In eukaryotes, sumoylation usually has an

---

**FIG. 6.** Viral RNA levels in rPIV5-P-K254R-infected cells. (A) Viral mRNA levels after high-MOI infection. MDBK cells were infected with PIV5 or rPIV5-P-K254R at an MOI of 3. The cells were collected at different time points for RNA extraction and real-time RT-PCR. Oligo(dT) was used for RT. The relative mRNA levels were normalized with the input genome defined as the viral genome at 2 hpi. (B) Viral genome levels after high-MOI infection. The same RNA from panel A was used for RT using BH191 annealing to the M gene of the viral genome. The cDNA was used for real-time PCR analysis. The genome level at 2 hpi was used as the baseline for normalization. (C) Viral mRNA levels after low-MOI infection. MDBK cells were infected with PIV5 or rPIV5-P-K254R at an MOI of 3. The cells were collected at different time points for RNA extraction and real-time RT-PCR. Oligo(dT) was used for RT. The relative mRNA levels were normalized with the input genome defined as the viral genome at 2 hpi. (D) Viral genome levels after low-MOI infection. The same RNA from panel C was used for RT using BH191 to measure viral genome levels.
inhibitory effect on gene transcription. Mutations at the sumoylation sites of transcription factors Elk, Sp-3, STAT-1, and P300 lead to transcriptional activation (15). Although the steady-state sumoylation level is less than 5% of the given protein, most transcription factors can be significantly activated when sumoylation sites are mutated (9). SUMO can also have positive effects on transcription, for example, through activating β-catenin-activated factor Tcf-4 (42). For viruses, most studies suggest that sumoylation plays a positive role in viral gene expression. Bovine papillomavirus (BPV) E1 protein can be sumoylated at K154, and a K154R mutation results in sequestration of E1 in the cytoplasm and therefore loss of replication capacity (26). Sumoylation of human papillomavirus (HPV) E2 proteins affects its activity in both transcriptional activation and repression (40). Sumoylation of HCMV IE2p86 protein is important for IE2-mediated transactivation (14). Despite the different roles of sumoylation of host and viral proteins in transcription, the underlying mechanisms may be similar.

Sumoylation may function through modulating protein-protein interaction or altering substrate conformation (15). SUMO-dependent transcriptional repression is likely due to SUMO-dependent recruitment of downstream effector proteins. For example, only sumoylated p300 can recruit HDAC6, a transcriptional repressor (11). Interestingly, HDAC6 can also interact with P300 proteins, which also bind directly to SUMO and sumoylated proteins (15, 21). Therefore, sumoylation may be important in the complex formation of transcription factors. Similarly, one possible mechanism for the effect of viral protein sumoylation on viral transcription could be the recruitment of downstream effector proteins, of either the virus or host cells. Our study has shown that P-K254R had no defect in interacting with NP and L proteins or in homotetramer formation by the P protein. We propose that sumoylation of the P protein at K254 regulates PIV5 gene expression through interaction with an as-yet-unidentified host protein(s). However, we cannot exclude the possibility that sumoylation of the P protein at K254 also directly affects viral RNA replication. It is possible that the small portion of sumoylated P behaves differently, such as by having different affinity for its interacting partners.
ACKNOWLEDGMENTS

We appreciate helpful discussion and technical assistance from all the members of Biao He’s laboratory. We are grateful to Kaori Sakamoto for carefully reading the manuscript prior to submission.

This work was supported by grants from the National Institute of Allergy and Infectious Disease (R01AI070847, K02AI65795, and R56AI081816) to B.H.

REFERENCES