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Nucleocytoplasmic shuttling of the human parainfluenza virus type 2 phosphoprotein

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ABSTRACT

Human parainfluenza virus type 2 phosphoprotein (P) is an essential component of viral polymerase. The P gene encodes both P and accessory V proteins by a specific gene editing mechanism. Therefore, the N-terminal 164 amino acids of P protein are common to V protein. Interestingly, while P protein is located in the cytoplasm, V protein is found mainly in the nucleus. Using deletion mutants, we show the presence of a nuclear localization signal (NLS) in the P/V common domain, and a nuclear export signal (NES) in the C-terminal P specific region. The NLS region makes a complex with importin α5 or 7. In the presence of leptomycin B, P protein is retained in the nucleus, indicating that it contains a CRM1-dependent NES. We identified the NLS (^{65}PVKPRRKK^{72}) and the NES (^{225}IIELLKGLDL^{234}) using β-galactosidase fusion proteins. Moreover, nucleocytoplasmic shuttling of P protein appears to be important for efficient viral polymerase activity.

1. Introduction

Human parainfluenza virus type 2 (hPIV2) is a member of the *Rubulavirus* genus of the family *Paramyxoviridae*. This family includes many well-known human and animal pathogens, such as Sendai virus (SeV), human parainfluenza viruses (hPIV) type 1–4, simian virus 41 (SV41), parainfluenza virus type 5 (PIV5), mumps virus (MuV), Newcastle disease virus (NDV), and measles virus (MeV), as well as important emerging viruses such as Hendra and Nipah viruses. HPIV2 has a non-segmented, single-strand, negative-sense RNA genome, and replicates in the host cell cytoplasm. Virus genome is encapsidated by nucleoprotein (NP), which is used as a template for viral polymerase complex consisting of large protein (L) and co-factor, phosphoprotein (P) (Lamb and Parks, 2007). Due to a specific RNA editing mechanism, the P gene encodes both the P and V proteins. The V mRNA is a faithful transcript of the V/P gene, whereas the P mRNA is synthesized through the cotranscriptional insertion of two pseudo-templated G nucleotides. Thus, the N-terminal 164 amino acids (aa) of the V and P proteins are common, while their C-termini are different (Ohgimoto et al., 1990). The P protein forms an oligomer via a P-oligomerization domain at aa 211–248. It also forms a complex with NP via two independent binding sites: aa 1–47 and aa 357–395, and with L via aa 278–353 (Nishio et al., 1996, 1997, 2000). All these properties are essential for virus transcription and replication. There has been very little evidence regarding the interaction of P protein with host factors, with the exception of Graf1, which binds to both P and V proteins (Ohta et al., 2016a). V protein, by contrast, interacts with several host proteins including MDA-5 (Andrejeva et al., 2004), LGP2 (Childs et al., 2012), TRAF6 (Kitagawa et al., 2013), STATs (Nishio et al., 2001, 2005a), tetherin (Ohta et al., 2016b), caspase-1 (Ohta et al., 2018), and AIP1/Alix (Nishio et al., 2007), most of which are important for innate immune responses. Although they share the same N-terminal domain, P and V proteins play distinctive roles in virus lifecycle.

P and V proteins also distribute differently in the infected cells. The P protein is organized in numerous granules with the NP protein in the cytoplasm of infected cells (Nishio et al., 1996), the V protein shows a diffuse distribution not only in the cytoplasm, but also in the nucleus (Nishio et al., 1999a). In this study, we investigated why the subcellular distribution of P and V proteins is different, despite having common N-termini. We also analyzed whether the nucleocytoplasmic shuttling of P protein is involved in hPIV2 transcription and replication by using a mini-genome system.

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2. Materials and methods

2.1. Cells and antibodies

HeLa, COS and Vero cells were grown in Eagle's minimal essential medium (MEM) supplemented with 10% fetal calf serum. BSR T7/5 (Buchholz et al., 1999) cells were cultured in Eagle's MEM supplemented with 10% fetal calf serum and 1 mg/ml G418 (Thermo Fisher Scientific, Waltham, MA, USA).

Monoclonal antibodies (MAbs) against hPIV2 P/V protein (315-1), hPIV2 P protein (335 A), hPIV2 V protein (53 V), hPIV2 NP protein (306-1), and hPIV2 L protein (L70-6) were as described previously (Nishio et al., 1997, 2000, 1999a, 1999b). Antibody to GFP (sc-8334), anti-Flag MAb, anti-β-galactosidase MAb, and anti-actin MAb were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), Sigma (St. Louis, MO, USA), Promega (Madison, WI, USA), and Wako (Osaka, Japan), respectively.

2.2. Construction of expression plasmids

Various hPIV2 P genes, and V gene cloned into pcDL-SRα, were as described previously (Nishio et al., 1996, 1997). Various point mutant P genes were generated in a manner similar to that described previously, and cloned into pcDL-SRα (Nishio et al., 1997). pDS-GFP was constructed by inserting a green fluorescent protein (GFP) gene into pcDL-SRα vector. Various V or P/V genes were inserted downstream of the GFP of pDS-GFP. To express the importin proteins in mammalian cells, cDNA expression vectors (pCI-neo, Promega) that contained importin α1, 3–7, or β cDNA fused to an N-terminal Flag epitope tag were constructed. The plasmids for transient minigenome system, pPIV2-Rluc, pTM1-P, NP, L, and Fluc, were described previously (Matsumoto et al., 2016). Various P genes of hPIV2 were amplified by PCR and subcloned into pBIND vector (Promega) for mammalian two-hybrid assay. pACT-P encoding the wild-type P of hPIV2 has been described previously (Nishio et al., 2008). Plasmid pCMV-NLSβgal was constructed by inserting an NLS fragment generated by fusion PCR into plasmid pCMVβ (Clontech, Mountain View, CA, USA). Plasmid pCMV-NLS/NESβgal was constructed by inserting an NES downstream of the NLS of pCMV-NLSβgal. pPIV2-full, containing the full-length cDNA (15654 nt; Genbank accession number ABI176531) of the hPIV2 Toshiba strain was constructed as described previously (Nishio et al., 2005a). Mutations in P gene were introduced by a standard PCR mutagenesis method.

2.3. Immunofluorescence staining

HeLa cells were grown to 60% confluence and transfected with expression plasmid coding for various P, V or β-galactosidase proteins according to manufacturer's recommendation (FuGENE 6, Promega). At 24 h post-transfection (hpt), the cells were fixed with 3% paraformaldehyde for 30 min at room temperature and rinsed twice with phosphate-buffered saline (PBS). The cells were permeabilized with PBS-0.05% Tween 20 between 20 and 30 min and washed twice with PBS. The cells were then incubated for 60 min with antibody against P or β-galactosidase and washed three times with PBS. Cells were then incubated for 60 min with fluorescein isothiocyanate-labeled secondary antibodies containing 4,6-diamidino-2-phenylindole (DAPI) and washed with PBS. Immunofluorescence-stained cells were analyzed by fluorescence microscope.

2.4. Cell extracts, immunoblotting (IB), and immunoprecipitation (IP)

For preparation of cell extracts, cells were lysed in lysis buffer (50 mM Tris-HCl [pH 7.5], 150 or 250 mM NaCl, 10 mM EDTA, 0.5% NP-40, and 4 mM phenylmethylsulfonyl fluoride). For immunoblotting, cell extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and analyzed by Western blotting technique with appropriate antibodies as described above. For immunoprecipitation, COS cells in six-well plates were transfected with pDS-V, P or various pDS-GFP-V, and various pCI-Flag-importins, by using FuGENE 6 according to the manufacturer's instructions. At 48 hpt, cells were lysed in lysis buffer. Supernatants obtained by centrifugation were incubated with MAbs and protein A-Sepharose for 2 h as described previously (Nishio et al., 1996). Polypeptides were analyzed by a Western blotting technique. Cell lysates were also subjected directly to Western blotting with MAbs to confirm protein expression.

2.5. hPIV2 Rluc mini-replicon assay

The hPIV2 Rluc mini-replicon assay was performed as described previously (Matsumoto et al., 2016). At 24 hpt, Rluc and Fluc activities were measured by using Dual-Luciferase assay system (Promega) according to the manufacturer's instructions. All obtained Rluc results were normalized by Fluc expression levels.

2.6. Mammalian two-hybrid assay

CheckMate Mammalian Two-Hybrid System (Promega) was used for the mammalian two-hybrid assay, and experiments were performed according to the manufacturer's recommendation. Plasmids for this assay were prepared as described above. HeLa cells were transfected with the indicated pBIND and pACT plasmids together with pG5luc reporter plasmid. At 48 hpt, the cells were harvested and assayed by Dual-Luciferase assay system (Promega).

2.7. Generation of recombinant hPIV2

The recombinant hPIV2 was generated as described previously (Nishio et al., 2005a, 2007; Matsumoto et al., 2016). BSR T7/5 cells were transfected with wt or P mutant pPIV2-full (2.5 μg) together with the following expression plasmids: pTM1-NP at 0.5 μg, pTM1-P at 0.25 μg and pTM1-L at 0.5 μg by using XtremeGENE HP. At three days post-transfection, viruses produced in the supernatant of the transfected cells were further amplified in Vero cells. Virus stock was prepared in Vero cells. Virus titers were measured by plaque assay using Vero cells as described previously (Nishio et al., 2005a) and shown by the plaque-forming unit (PFU).

3. Results

3.1. Identification of the region on the P/V protein for nuclear localization

To investigate the cellular localization of the P and V proteins, we constructed plasmids that expressed only the P/V common region or the P-specific region (Figs. 1A, 3 and 4). As shown in Fig. 1B, panels 3 and 4, the common region was found to be mainly located in the nuclei, and the P-specific region was found in the cytoplasm. To examine the region needed for the nuclear localization of the P/V common region, we constructed the plasmids of Fig. 1C. As shown in Fig. 1D, the region of aa 57–80 of P/V common domain (KSKPVAAGPVKPRRKKVISNTTPY) was found to be important for nuclear localization.

3.2. NLS mediates binding to importin α5 and 7

Classical nuclear localization signals (NLSs) comprise a short stretch of basic amino acids. These sequences are recognized in the cytoplasm by importin α, which forms a stable heterodimeric complex with importin β (Kohler et al., 1999). We next examined the interaction of hPIV2 V and P with six different human importin α molecules: importin α1 (karyopherin α2; gene ID 3838), importin α3 (karyopherin α4; gene ID 3840), importin α4 (karyopherin α3; gene ID 3839), importin α5 (karyopherin α1; gene ID 3836), importin α6 (karyopherin α5; gene ID

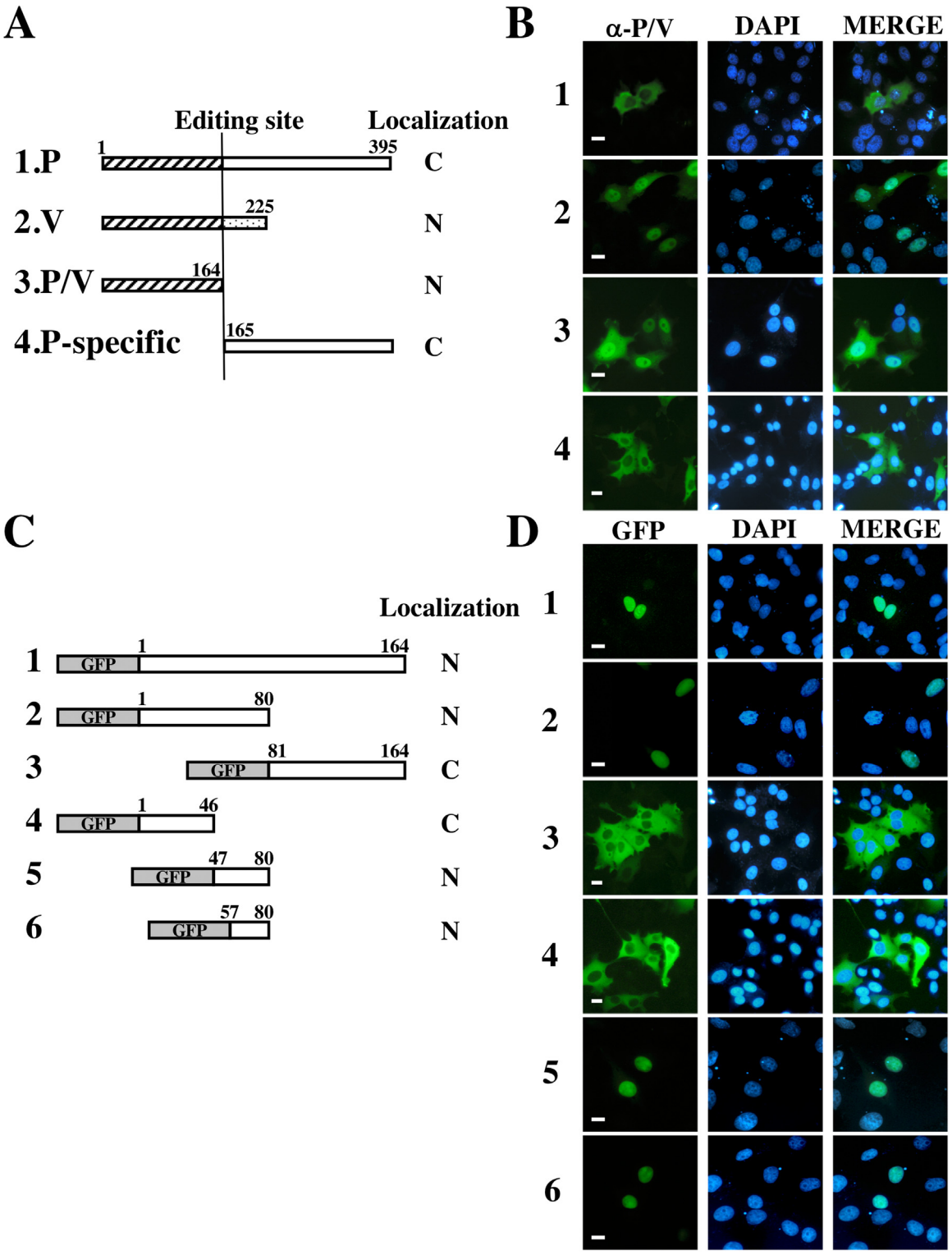

Fig. 1. Identification of a region for NLS on the P/V protein. (A) Schematic diagram of the P, V or truncated P proteins. (B) HeLa cells were transfected with plasmids encoding various proteins and immunostained as described in Materials and Methods. Numbers on the left of the figure correspond to each protein described in A. (C) Schematic diagram of the truncated P/V common regions fused to GFP at N-terminus. (D) HeLa cells were transfected with plasmids encoding various proteins and subcellular localization of GFP fusion proteins were analyzed. The numbers on the left of the figure correspond to each protein described in C. Subcellular localization in the cytoplasm (C) or in the nucleus (N) is summarized in A and C. Scale bars correspond to 10 μm.

3841) and importin α7 (karyopherin α6; gene ID 23633). The Flag-tagged importins and V or P proteins were co-expressed in COS cells. After immunoselection of cell lysates with anti-V or P, the precipitates were analyzed by Western blotting. Both V and P proteins were found to bind to importin α5 and 7 (Fig. 2A and B). To identify whether the NLS of P/V common region bound to importin α5 and 7, GFP or GFP-fused to the P/V or V-specific regions were co-expressed with importin α5 or 7 in COS cells. As expected, the region that contains the putative NLS, aa 1–80, bound with both importin α5 and 7 (Fig. 3A). We also investigated which part of importin α5 is important for the interaction

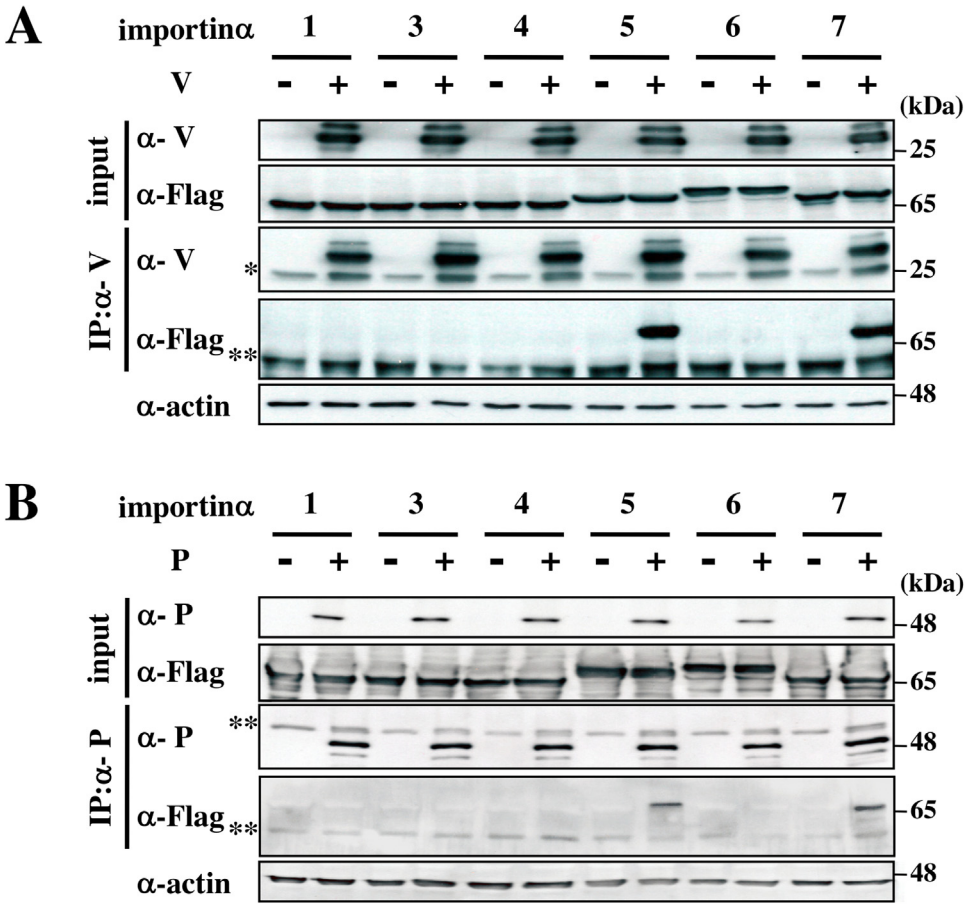

Fig. 2. hPIV2 V and P proteins complex formation with importins. (A and B) Interaction of V and P proteins with importin α1, or 3–7. COS cells were transfected with pCI-Flag-importin α1, or 3–7 plus pDS-V or P. At 48 hpt, the cell extracts were either analyzed directly by IB assay using the indicated Abs or IP with anti-V or P before IB. The single and double asterisks on the left indicate immunoglobulin light chain and heavy chain, respectively. Actin was measured as a loading control.

with the NLS on P/V common region. The N-terminal region of importin α5 is essential for binding with V (Fig. 3B). Although we furthermore investigated the interaction with importin β, V protein did not bind to it (Fig. 3C). These data suggest that proteins containing aa 57–80 prefer to use importin α5 and 7 to mediate nuclear transport.

3.3. P protein has an NES on the P-specific region

Although the P protein contains an NLS, it is nevertheless localized to the cytoplasm. This suggests the additional presence of a nuclear export signal (NES) in the C-terminal P-specific region. The putative NES appears to be N-terminal to aa 270, as C-terminally deleted PΔ270 is localized to the cytoplasm (Fig. 4, panel 1). We therefore examined mutant P proteins which are deleted for parts of aa 165–271 (Fig. 4, panels 2–5). A mutant protein deleted of aa 211–248 was found in the nucleus, suggesting that this region may contain an NES.

3.4. Identification of the NLS and NES on the P protein

Leptomycin B (LMB) is a specific inhibitor of CRM1-dependent nuclear export that inhibits interactions of CRM1 with NES. Although the P protein is cytoplasmic in untreated cells, its distribution became nuclear upon LMB treatment (Fig. 5A). P protein thus appears to contain a CRM1-dependent NES, and the complete P protein appears to enter the nucleus.

Classical NLSs consist of either one or two stretches of basic amino acids. Examples are the SV40 T antigen NLS (PKKKRRV) or the cellular nucleoplasmin protein NLS (KRPAATKKAGQAKKKK) (Kalderon et al., 1984; Robbins et al., 1991). A sequence resembling a classical motif within aa 57–80 of the P/V common region is ^{65}PVKPRRKK^{72}. CRM1-dependent NESs are characterized by a leucine-rich sequence (LxxxLxxLxL), although large hydrophobic amino acids (V, M, I) may be substituted for leucine. A sequence resembling an NES within aa 211–248 of P is ^{225}**I**IE**L**LKG**L**D**L**^{234} (similarity with LxxxLxxLxL motif is emphasized in bold). To identify whether these putative sequences are NLS or NES, chimeric proteins with these sequences fused to β-galactosidase were expressed in transfected cells in the presence or absence of LMB (Fig. 5B and C). As expected, NLS-βgal was exclusively nuclear. NLS/NES-βgal was excluded from the nucleus in the absence of LMB and its localization was sensitive to LMB. These results confirm that ^{65}PVKPRRKK^{72} is an NLS, and ^{225}IIELLKGLDL^{234} is an NES.

3.5. Determine the amino acids residues necessary for NES of P protein

To evaluate which amino acids are important for NES function, various mutant P proteins were constructed (Fig. 6A). The isoleucine-mutated (P-I225/226 A), leucine-mutated (P-L229A, P-L229/234 A, and P-L229/232/234 A) P proteins showed clearly diminished nuclear export (Fig. 6B, panels 2–5). In contrast, P-E227/L228A protein showed slightly diminished nuclear export (Fig. 6B, panel 6). Thus, these results suggest that Ile 225 and/or 226, and Leu 229, 232 and 234 are all critical for NES activity.

3.6. Nucleo-cytoplasmic shuttling of the P protein is important for polymerase activity and virus growth

To investigate the effects of NLS/NES on P protein function, various P proteins were constructed as illustrated in Fig. 7A. PΔ contains aa ^{65}PVPAHTST^{72} (derived from hPIV4 P) instead of hPIV2 NLS (^{65}PVKPRRKK^{72}). PI225/226 A, PΔ+I225/226 A, PL229/232/234 A and PΔ+L229/232/234 A (Fig. 7A, 3–6) contain mutations that destroy NES activity, as shown in the previous section.

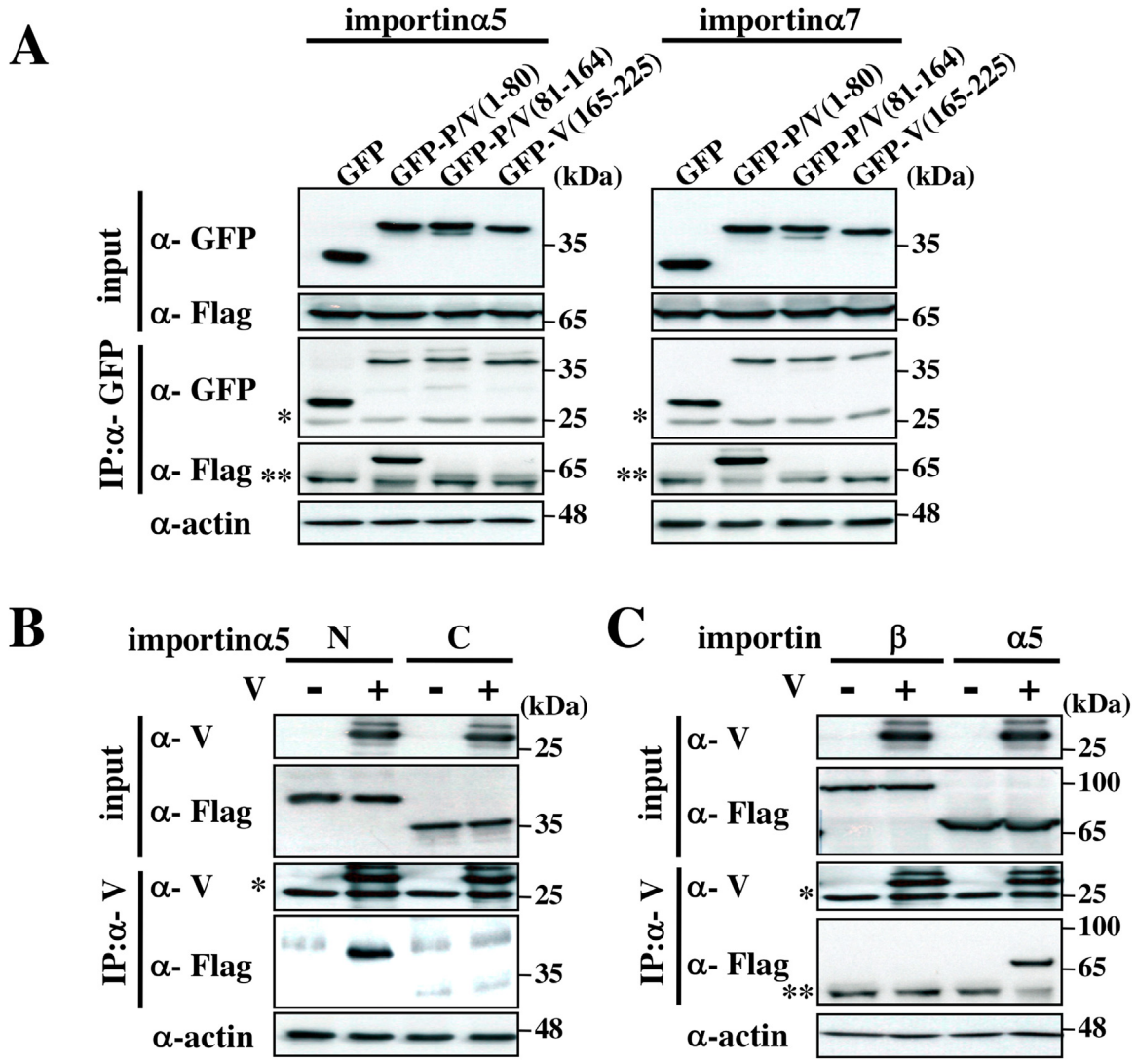

Fig. 3. Interaction of hPIV2 V with importin α5 and 7. (A) Interaction of truncated P/V proteins with importin α5 or 7. COS cells were transfected with pCI-Flag-importin α5 or 7 plus pDS-GFP or various pDS-GFP-P/V. IB and IP analyses using anti-GFP were performed. (B) Interaction of V protein with truncated importin α5. COS cells were transfected with pCI-Flag-importin α5 N-terminal or C-terminal plus pDS-V. IB and IP analyses using anti-V were performed. (C) Interaction of V protein with importin β. COS cells were transfected with pCI-Flag-importin β plus pDS-V. IB and IP analyses using anti-V were performed. The single and double asterisks on the left indicate immunoglobulin light chain and heavy chain, respectively. Actin was measured as a loading control.

The various P proteins were examined for their ability for polymerase activity using mini-replicon assay. As shown in Fig. 7B, Rluc expressions from P proteins which were able to enter the nucleus but which contained inactive NESs (lanes 3 and 5), were approximately 15–30% of those using wt P. These results are not surprising, as viral RNA synthesis occurs in the cytoplasm. However, the PΔ protein which was unable to enter the nucleus (lanes 2), were clearly less active in Rluc expression than wt P, no matter whether or not they also contained inactive NESs (lanes 4 and 6). This suggests that nucleocytoplasmic shuttling of the P protein is somehow important for hPIV2 transcription and/or replication.

The NES region (aa 225–234) partially overlaps that required for P-oligomerization (aa 211–248) (Nishio et al., 1997). We therefore also tested P-P interactions by mammalian two-hybrid analysis (Fig. 7C). The P protein interacted with all mutant P proteins like wt P protein. As they were unable to oligomerize, none of the various P protein mutants were less active than wt P.

To examine the involvement of these P mutations to virus growth, we attempted to rescue recombinant hPIV2 (rPIV2) having mutation in P gene. We could obtain rPIV2 PΔ, whose growth titer was approximately 22-fold lower than that of wt hPIV2 (Fig. 7D), indicating that the nuclear entering step of hPIV2 P may be supportive to virus growth, but not crucial. We next tried to rescue rPIV2 PL229/232/234 A that is a representative of P mutant that tends to accumulate in the nucleus (Fig. 6), but failed to obtain it (Fig. 7D). Moreover, we could not rescue rPIV2 PΔ + L229/232/234 A (Fig. 7D). These results indicate that the mutation disrupting NES function is lethal to virus rescue even in the absence of NLS.

4. Discussion

In infected cells, hPIV2 V protein (32 kDa) exhibits a characteristic nuclear localization pattern (Nishio et al., 1999a). In contrast, the 49 kDa P protein that shares a common N-terminal region with V is located in the cytoplasm, where it forms complexes with the NP and L proteins that are essential for viral RNA synthesis in the cytoplasm (Lamb and Parks, 2007). This paper reports that the hPIV2 P protein is a nucleocytoplasmic shuttling protein, which contains a typical monopartite NLS in the P/V common region (^{65}PVKPRRKK^{72}) that binds to the N-terminal regions of importin α5 and/or 7, and an NES in the C-

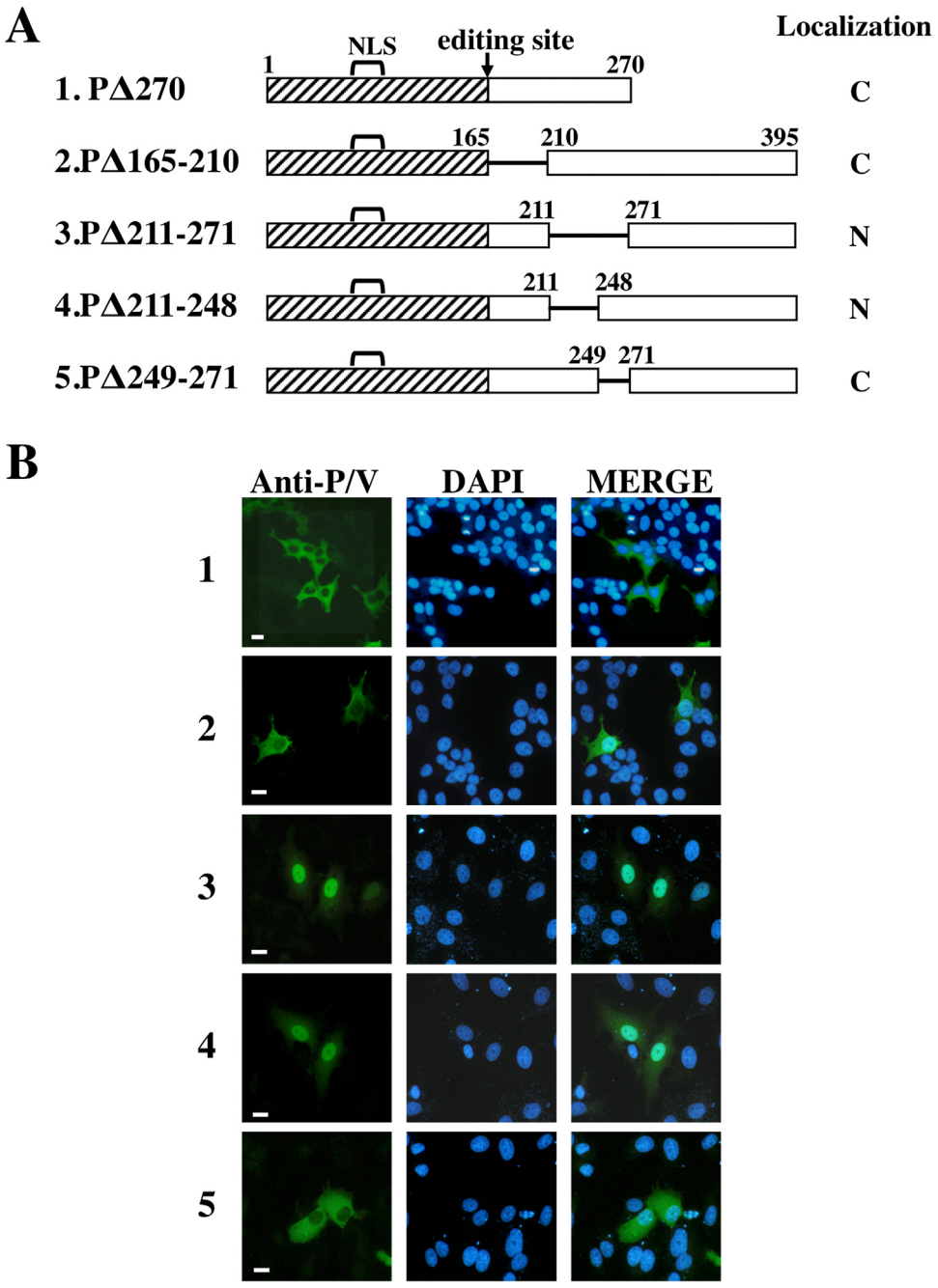

Fig. 4. Identification of a region for NES on the P protein. (A) Schematic diagram of truncated P proteins. Striped boxes, open boxes, and lines indicated the P/V common, P-specific, and deleted region, respectively. Subcellular localization in the cytoplasm (C) or in the nucleus (N) is summarized. (B) HeLa cells were transfected with plasmids encoding various proteins and immunostained as described in Materials and methods. The numbers on the left of the figure correspond to each protein described in A. Scale bars correspond to 10 μm.

terminal, P-specific region (^{225}IIxxLxxLxL^{234}). The NLS region overlaps with the RNA-binding region of the P and V proteins (Nishio et al., 2006), and the NES region overlaps with the P-oligomerization region (Nishio et al., 1997).

Transport of macromolecules into and out of the nucleus occurs via pore complexes (NPCs). NPCs allow passive diffusion of ions and small proteins (< 40 kDa), large molecules, however, use a signal-mediated transport system (Weis, 2003). The best understood system is the importin α/β-mediated import pathway. Proteins transported into the nucleus contain NLS that are recognized by importin α/β heterodimers. Importin α recognizes and binds the NLS, and importin β docks the complex to the nuclear pore and translocates it into the nucleus. The classical NLS comprises one or two short stretches of basic amino acids. Importin α is composed of a large central domain that consists of 10 tandemly-repeated armadillo (arm) motifs. Monopartite NLS has been shown to bind to the "major" binding site of importin α, which is formed by arm repeats 2–4 (near N-terminus). The downstream cluster of the bipartite NLS is also recognized by these arm repeats, whereas the upstream cluster is recognized by arm repeats 7 and 8 (near C-terminus), also called the "minor" binding site (Conti et al., 1998; Fontes et al., 2000). We identified that the NLS of these proteins, ^{65}PVKPRRKK^{72}, is 'typical monopartite NLS', and binds to the N-terminal of importin α5 and/or 7 that is the major binding site.

We previously reported that two noncontiguous regions of the V protein were required for nuclear localization and retention, namely, region I (aa 1–46 on the P/V common region) and region II (aa 175–196

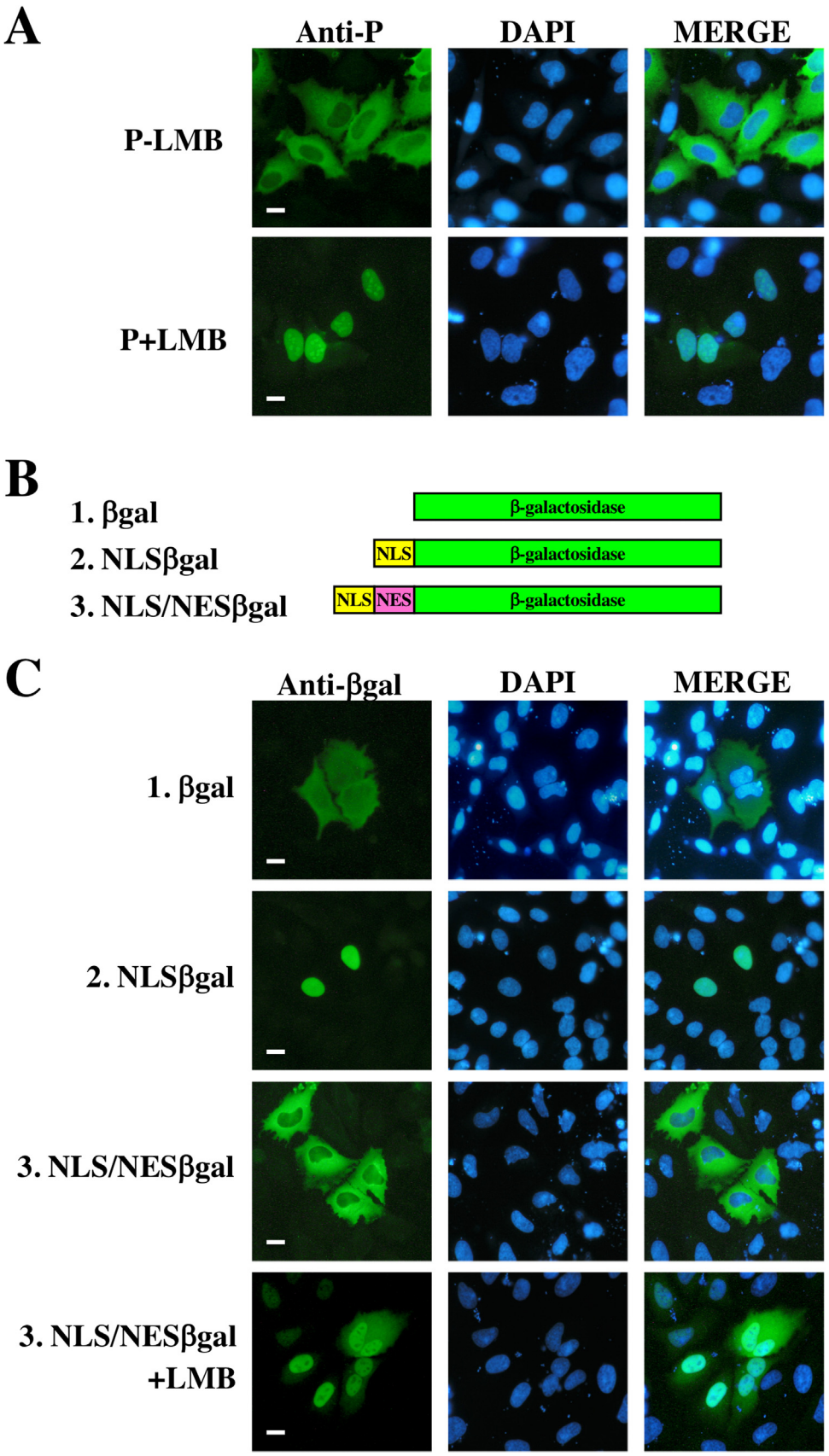

Fig. 5. Identification of NLS/NES and effects of LMB. (A) Effect of LMB on the subcellular localization of P protein. HeLa cells were transfected with pDS-P in the absent or presence of LMB at a final concentration of 2 ng/ml. After fixation, cells were permeabilized and immunostained using anti-P/V MAb. (B) Schematic diagram of the NLS βgal and NES βgal proteins. The N-terminal yellow boxes indicate the position of the putative NLS motif (PVKPRRKK). The putative NES motif (IIELLKGLDL) is shown in pink box. (C) The transfected HeLa cells were immunostained as described in Materials and Methods. Scale bars correspond to 10 μm.

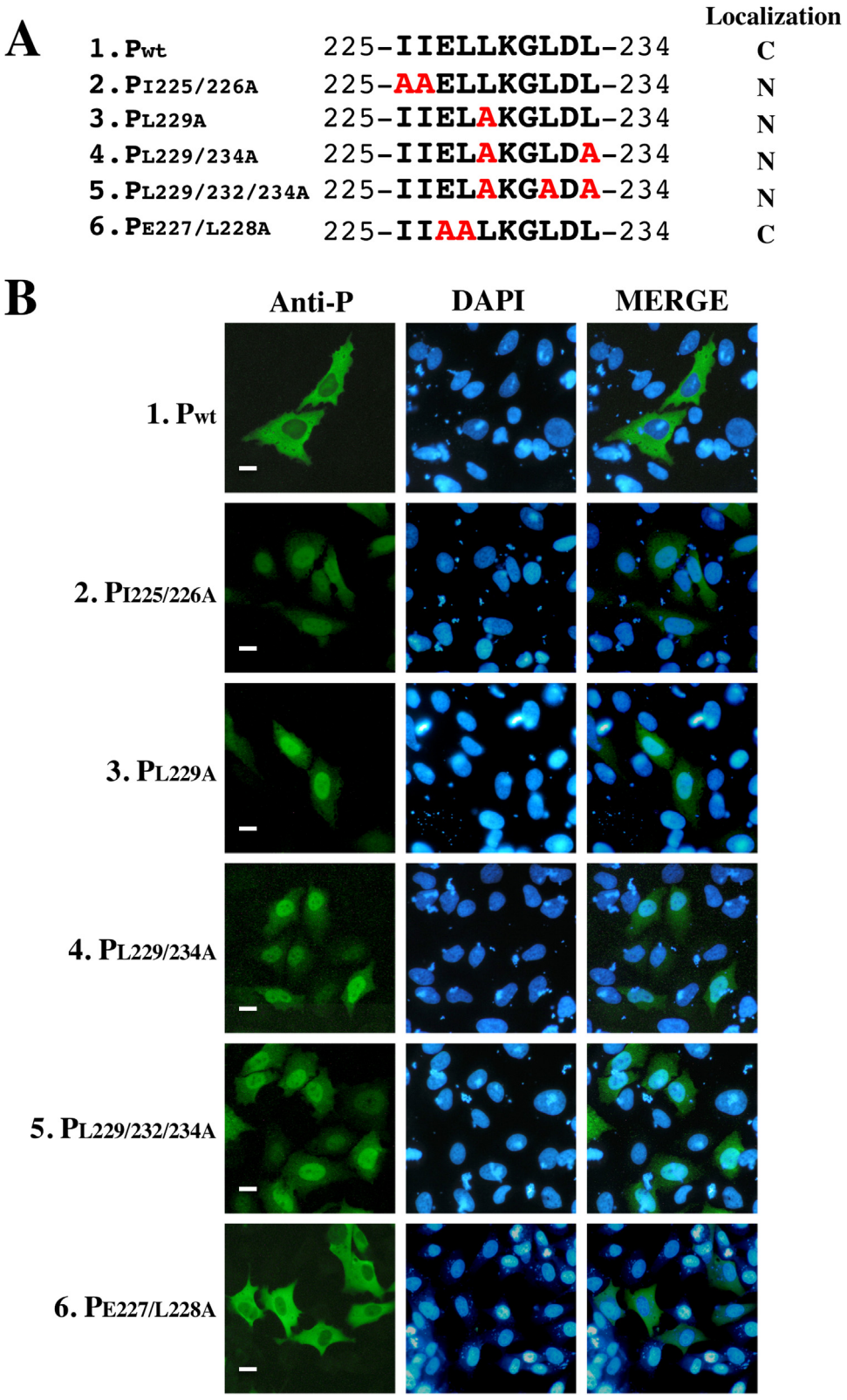

Fig. 6. Identification of amino acids required for nuclear export of the P protein. (A) Schematic diagram of the P mutants. (B) The transfected HeLa cells were immunostained as described in Materials and Methods. Scale bars correspond to 10 μm.

on the V-specific region) by using an expression system with vaccinia virus infection (Watanabe et al., 1996). Although these regions fusing with a part of MuV NP located in the nucleus, they could not be confirmed here. After that, we showed that these two regions are important for NP binding by using vaccinia virus free systems. In the present study, we demonstrated that the region containing the identified amino acids directly interacted with importin α5 and/or 7, and that the peptide alone fused to β-galactosidase resulted in its nuclear localization.

The V proteins of hPIV2, PIV5, and SV41 were detected in the nucleus, while those of MuV and hPIV4 remained in the cytoplasm (Nishio

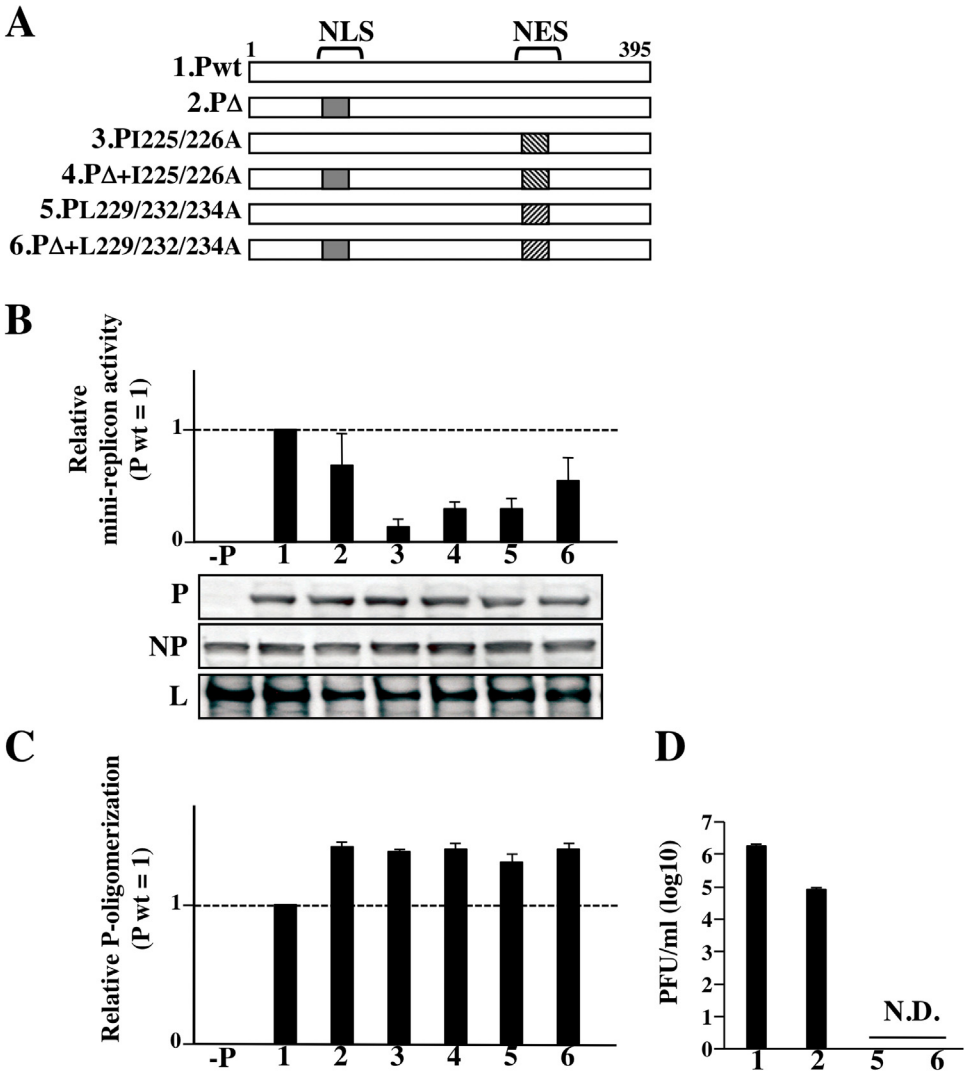

Fig. 7. Effect of P mutation in polymerase activity and virus growth. (A) Schematic diagram of the P mutants. PΔ protein is a mutant whose NLS (^{65}PVKPRRKK^{72}) is substituted with ^{65}PVPAHTST^{72} (gray boxes). Striped boxes indicate the positions of mutation residues of NES. (B) Relative Rluc activity in the mini-replicon assay with the hPIV2 polymerase complex including P mutants, as described in A. Rluc expression from minigenomes is normalized to internal control Fluc expression, and relative values are shown ($P^{wt}=1$). -P, result for the Rluc minigenome without P plasmid. Data represent means and standard deviations from triplicate experiments. Expression of P, NP and L were detected by IB using specific MAbs. (C) Mammalian two-hybrid analysis for P protein oligomerization. HeLa cells were co-transfected with pACT-P (wt) and pBIND-P mutant plasmids with luciferase reporter plasmid. After 48 h, the cells were harvested and assayed by Dual-Luciferase assay system. (D) Virus titers of recombinant hPIV2 having mutation in P gene. N.D. indicates "no detectable virus". Data represent means and standard deviations from triplicate experiments. Numbers on the bottom of B, C and D correspond to each of the P mutant proteins described in A.

et al., 2005b; Watanabe et al., 1996; Paterson et al., 1995; Ulane et al., 2003). Consistent with this, we have shown that NLS sequences similar to ^{65}PVKPRRKK^{72} are conserved in the P/V common region of SV41 (^{65}PVKPKRKK^{72}) and PIV5 (^{75}KPKKPRPK^{82}), but not in MuV or hPIV4. Moreover, the P proteins of SV41 and PIV5 have sequences similar to the NES of hPIV2 (^{225}IIELLKGLDL^{234}), namely ^{225}IIEILRGLDL^{234} and ^{208}LVTSVQSLAL^{217}, respectively. Thus, it seems likely that the P proteins of hPIV2, SV41, and PIV5 also shuttle between the nucleus and the cytoplasm. In contrast, the P and V proteins of MuV and hPIV4, however, do not appear to contain an NLS and they remain cytoplasmic. The nuclear export signal of hPIV2 P protein at aa 225–234 is canonical with a high leucine content and is a part of oligomerization domain conserved throughout rubulaviruses. The P proteins of MuV and hPIV4 also contain an NES-like sequence in the part of oligomerization domain; ^{221}IMDLLRGMDA^{230} or ^{229}ILDAIKALEV^{238}.

Interestingly, three separate mutants of the hPIV2 P protein whose NLS was exchanged with the same region of hPIV4 P, which therefore remain cytoplasmic, were found to be less active in supporting minigenome Rluc expression than wt P. The full activity of P therefore appears to require its nuclear passage, possibly because P needs to be modified in this cellular compartment for it to be fully active in the cytoplasm (e.g. by phosphorylation). Another possibility should be considered, that mutations in the NLS and NES render the structural conformation to be insufficient as the polymerase co-factor. Although we could confirm that its ability to form the P-P dimer was not affected by the mutations (Fig. 7C), which indicates that the proteins are not functionally disrupted, further study will be required to clarify this possibility.

The P proteins of paramyxoviruses are heavily phosphorylated, which are essential for viral polymerase activity (Lamb and Parks, 2007). The phosphorylation sites within P proteins of PIV5 and MuV responsible for the efficient viral RNA synthesis have been identified (Sun et al., 2011; Pickar et al., 2014). Although the mechanism of hPIV2 P phosphorylation has not been examined, it is possible that unknown factor(s) in nuclear environment may play important roles for modifying this polymerase co-factor. As rPIV2 PΔ could be rescued with low growth phenotype, it may suggest that the nuclear passage of P is supportive, but not crucial for hPIV2 lifecycle. In contrast, the mutation PL229/232/234 A, whose P protein mainly accumulates in the nucleus (Fig. 6), was lethal to virus rescue. As hPIV2 replicates in the cytoplasm, the polymerase co-factor P should be abundant in the cytoplasm. The lethality, however, could not be recovered by the additional mutation in P NLS (PΔ). These results suggest that the amino acids in P NES region may be essential for virus growth by not only its involvement in the nucleo-cytoplasmic shuttling of P protein, but also other functions that still require elucidation.

The basic life cycles of paramyxoviruses and several other negative-strand RNA virus families are thought to occur entirely in the cytoplasm. However, there is a growing list of their proteins that traffic into and/or out of the nucleus. They are often as part of the viral program to

counteract the cellular innate immune responses; e.g., NSs protein of Rift Valley fever virus, P protein of rabies virus, M protein of respiratory syncytial virus and W protein of Nipah virus (Jans and Hubner, 1996; Billecocq et al., 2004; Chelbi-Alix et al., 2006; Ghildyal et al., 2009; Shaw et al., 2005). Other paramyxovirus P proteins to date have not been reported to shuttle between the nucleus and the cytoplasm. The rabies virus P protein, which is also a co-factor of the viral RNA polymerase, however, has both an NLS and a CRM1-dependent NES, and also shuttles between the nucleus and the cytoplasm (Pasdeloup et al., 2005). Moreover, in this case, the intranuclear P protein was reported to inhibit the interaction of Stat1-containing ISGF3 transcription factor with ISRE-containing DNA, thereby inhibiting the activation of ISGs (Vidy et al., 2007). It is possible that some rubulavirus P proteins enter the nucleus to interact with multiple host factors to establish an efficient viral growth, some of which may directly contribute to the inhibition of IFN responses.

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Conflict of interest

The authors declare that they have no conflict of interest.

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