

Human Sorting Nexin 2 Protein Interacts With Influenza A Virus PA Protein and Has a Negative Regulatory Effect on the Virus Replication

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Abstract

Replication of the influenza A viruses occurs in the cells through the viral RdRP consisting of PB1, PB2, and PA. Several cellular proteins are involved in these processes. To identify potential host interacting proteins to the viral PA, we have carried out a yeast two-hybrid screen using a HEK293 cell cDNA library. We focused our study on human SNX2 protein, which interacts with the PA protein in yeast cells. By using the co-immunoprecipitation assays, we have demonstrated that the amino-terminal part of the PA was important for binding to the SNX2 protein. Subcellular localization of the PA and human SNX2

proteins in HeLa cells supported this interaction. Knockdown of SNX2 with siRNA transfection in the cells resulted in a significant increase in both viral transcripts and proteins, suggesting that SNX2 could be a negative factor. However, the increase of SNX2 proteins in transfected cells didn't cause a significant change in the viral RdRP activity in mini-replicon assay. This may suggest that the negative effect of SNX2 on the influenza A virus replication could be saturated with its authentic intra-cellular amount. Therefore, the regulatory mechanism for the amount of SNX2 is important to be studied in terms of influenza A virus replication.

1. Introduction

Influenza A viruses belong to the family *Orthomyxoviridae* and have a single-stranded segmented RNA genome. These viruses infect a wide range of avian and mammalian species, including humans, and mostly exist in the wild aquatic fowl reservoir [1]. They cause recurrent flu outbreaks in humans. The segmented genome structure of the influenza viruses and their ability to infect different hosts resulted in reassortment and the emergence of new virus types during co-infections [2]. The reassortment is one of the most important factors in the emergence of pandemic influenza viruses that easily spread in the absence of pre-existing immunity in humans [3]. The influenza A virus genome consists of eight single-stranded, negative-sense RNA molecules. At least 10 virus-specific proteins are encoded through this segmented genome. Viral segments 1, 3, 4, and 5 encode just one protein per segment, the PB2, PA, HA, and NP proteins, respectively. The M1 (matrix) and the M2 (ion channel) proteins are expressed on viral segment 7 by RNA splicing [4]. Similarly, the interferon-antagonist NS1 protein, and the NS2/NEP, which is involved in viral RNP export from the host cell nucleus, were expressed on segment 8 [5]. Recently, many non-structural proteins have been discovered in influenza A virus infected cells. Most of them are generated with the splicing, frame shift and truncation of the coding region of the structural proteins. These non-structural proteins are PB1 frame 2 (PB1-F2), PB1-N40 [6], PA-X [7], PA-N155, PA-N182 [8], M42 [9] and NS3 [10].

All viral proteins including the RdRP enzyme composed of PB2, PB1, and PA subunits interact with the host cell membranes or some cellular protein factors at different stages of viral replication [11]. Several host proteins, including nuclear pore complex proteins [12], G proteins [13], cytoskeletal elements [14], caspases [15], heat shock proteins [16] and some protein kinases [17, 18] interact with the viral replication processes. Additionally, the proteins of the vacuolar protein sorting pathway have been shown to interact

with the matrix proteins of several viruses including Tsg101, other ESCRT components, and proteins containing WW domains (such as Nedd4 family proteins) [19, 20]. In infected cells, many cellular proteins participate in the transport and assembly of the vRNPs that make up the genome of influenza A viruses and viral budding stages. Cytoskeletal elements, particularly microfilaments, have been proposed to be involved in the maturation of the influenza virus including bud formation and bud completion. Actin and actin-binding protein ezrin-radixin moesin (ERM) have also been found in influenza virus particles [21]. The presence of actin and actin-associated proteins in virions suggests specific functions of the actin filament during assembly and budding.

Although there are very few SNX proteins reported to be associated with influenza A viruses [22], it has been reported that several proteins belonging to the sorting nexin (SNX) protein family have interacted with some other virus replications. The SNX proteins, including the SNX2, are a large family of proteins in the cell cytoplasm that have the potential to interact with membranes through their PX (phox-homology) domains or protein-protein interactions in the membrane through protein complexes [23]. Some of the proteins in this family have been shown to contribute to protein traffic. These proteins are evolutionarily conserved from yeast to mammals and comprise a family of proteins involved in cargo recognition and sorting during retrograde transport from the early endosome to the Golgi apparatus [24, 25]. It has been reported that SNX17, a member of the Sorting Nexin family, interacts with the human papillomavirus [26] and the human T-cell leukemia-lymphoma virus (HTLV-1) [27]. The SNX3 and SNX5 proteins of the SNX family interact with the proteins of the vaccinia virus (VACV) and human cytomegalovirus (HCMV) [28, 29].

In this study, the interaction between the human SNX2 protein and the influenza A/duck/Pennsylvania-H5N2 (DkPen) PA protein was demonstrated by the yeast two-hybrid assay. The relationship of human SNX2 protein with viral PA protein in human cells and its effects on the virus replication were investigated. It was demonstrated that the amino-terminal part of the PA protein (PA Δ C) was particularly important for binding to the SNX2 protein. The human SNX2 protein was found to negatively affect avian influenza A/DkPen virus replication. These results suggested that the SNX2 protein may have a role in avian influenza A/DkPen virus pathogenesis.

2. Material And Methods

2.1. Cells and viruses

Human embryonic kidney 293 (HEK293) and HeLa cells were used in transient transfection experiments and/or viral infections. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (Gibco, USA), 100 IU/ml penicillin, 100 μ g/ml streptomycin, 2 mM glutamine, and 1.5 mg/ml sodium bicarbonate at 37°C in a humidified incubator with 5% CO₂. Avian influenza virus A/duck/Pennsylvania/10,218/84/H5N2 (DkPen) were propagated in specific pathogen-free chicken embryos. The viral titer was measured using a standard plaque-forming assay [30].

2.2. RNA extraction and first-strand cDNA preparation

For PCR amplification of SNX2 cDNA and/or quantitation of transcripts with a real-time polymerase chain reaction (qPCR), total RNA was extracted from the cells with the RNeasy mini kit (Qiagen, Germany). cDNAs were prepared from the total RNA by Moloney murine leukemia virus reverse transcriptase (ReverTra Ace, Toyobo Co. Ltd., Japan) and oligo dT as a primer for 60 min at 45 °C.

2.3. Construction of plasmid vectors

For the construction of Sorting Nexin 2 (SNX2) expression plasmids, the full length of the SNX2 cDNA consisting of 1560 bp was amplified by PCR with HEK293 cDNA and the phosphorylated specific primers, 5'-ATCATGGCGGCCGAGAGGGAA-3' and 5'-ATCTAGGCAATGGCTTTGGCTTC-3'. PCR amplification was carried out with a thermostable DNA polymerase (KOD plus, Toyobo Co., Ltd., Japan). The PCR product was purified with an agarose gel extraction kit (Qiaexll, Qiagen, Germany). In order to construct the pCHA-SNX2 plasmid encoding the HA-tagged SNX2 protein (H.SNX2), the SNX2 cDNA was cloned into a pCHA [31] plasmid digested with EcoRV (New England Biolabs, UK) and dephosphorylated with Shrimp Alkaline Phosphatase (Thermo Fisher Scientific, USA). The plasmids encoding deleted SNX2 proteins were constructed with the inverse PCR method by using the pCHA-SNX2 plasmid as a template. For SNX2 Δ N (exons 1–3 deleted), 5'-GATATCACGCGTGGTGACC-3' and 5'-ATGATTGAAGAAGAAGCAAATGG-3' were used; for SNX2 Δ M (exons 4–8 deleted), 5'-CTCTTCCCTGGATCTATCAAAG-3' and 5'-CTGCCTAGAGCAGTTAATACAC-3' were used; for SNX2 Δ C (exons 9–15 deleted), 5'-TAGATATCTTAAGTGAATTC-3' and 5'-CTCTGAACTTTCCAAGAACTG-3' phosphorylated primer pairs were used. The PCR products were purified with an agarose gel extraction kit and self-ligated.

In order to construct the bait plasmid coding influenza A/DkPen PA protein (pGBD-PA), the DNA fragment was amplified with PCR by using virus-infected cell cDNA as a template and the phosphorylated specific primers, 5'-CGGAGGATCTGGAATGGAAGACTTTGTGCGACAATG-3' and 5'-CTAGTTCTTTGTCTTTGGGATCTTC-3'. The PCR amplified gene was ligated with the pGBD-C1 [32] plasmid DNA digested with EcoRI and blunted with Klenow enzyme. For the construction of the pACT2-SNX2 yeast two-hybrid plasmid, the PCR amplified full-size SNX2 cDNA was ligated with pACT2 (Clontech, # 638822) plasmid digested with BglIII and blunted with Klenow enzyme. The nucleotide sequences of all plasmids were confirmed by DNA sequencing. The plasmids for expression of viral PA proteins in mammalian cells have been described previously [33, 34].

2.4. Yeast two-hybrid screening

Saccharomyces cerevisiae strain PJ69-4A was grown in 5 ml YPAD media at 30 °C in a shaking incubator at a speed of 200 rpm overnight. The fresh culture was transformed with pGBD-PA bait plasmid coding GAL4.BD-PA fusion by using the lithium acetate/polyethylene glycol (LiAc/PEG) protocol [35]. The transformants were selected on a synthetic defined (SD) agar medium (without Trp). One of the bait colonies was grown in YPAD media and re-transformed with a cDNA library derived from HEK293 cells (Clontech # 638826) and screened following the matchmaker two-hybrid system protocol. The positive

transformants were selected on the SD agar medium (without adenine, histidine, leucine, and tryptophan) and then tested by the β -galactosidase assay for the second screening. The plasmids carrying the cDNAs were isolated from the transformants with a yeast plasmid DNA miniprep kit (Bio Basic, Canada) according to the manufacturer's instructions, and transformed *E. coli* DH5 α and amplified. The cDNA inserts in the plasmids were sequenced and identified with *BLAST* (Basic Local Alignment Search Tool) analysis.

2.5. Transforming pACT2-SNX2 into the yeast cells and checking SNX2 and PA interaction

pACT2-SNX2 or pACT2 plasmid (control) were transformed into the *S. cerevisiae* strain PJ69-4A harboring the bait plasmid (pGBD-PA) with LiAc/PEG protocol. Double transformants were selected on SD agar medium (without Trp and Leu). A few colonies were cultured on a SD agar medium (without His, Leu, Ade, and Trp); growth profiles, were defined and tested for β -galactosidase activities.

2.6. β -galactosidase assay

Transformed yeast cells were grown in 5 ml SD medium (without Trp/Leu or Leu) at 30 °C. The cells in 500 μ l of saturated culture were recovered with centrifugation and re-suspended in 300 μ l Z-buffer (0.1 M sodium phosphate, pH 7.0, 10 mM KCl, 1 mM MgSO₄ and 0.27% β mercaptoethanol). The cells in suspensions were disintegrated with the freeze-thaw procedure in liquid nitrogen. Then, the samples were mixed with 60 μ l o-nitrophenyl- β -d-galactopyranoside (ONPG) (4 mg/ml) and incubated for 60 minutes at 37 °C. Three hundred μ l Na₂CO₃ (0.5 M) was added to stop the reactions. The supernatants were recovered by centrifugation at 15000 rpm for 5 min and the absorbance of the samples at 420 nm (OD₄₂₀) was defined.

2.7. Plasmid DNA transfection

Polyethylenimine (PEI) was used for plasmid DNA transfection into the HEK293 or HeLa cells (PA Longo *et al.* 2013). Plasmid DNA and PEI were diluted in OPTI-MEM (Gibco, USA) to a concentration of 20 ng/ μ l and 40 ng/ μ l, respectively, and mixed in equal volumes to form complexes. After incubation at room temperature for 5–10 minutes, the complexes were added to the culture media. The cultures were incubated under the standard culture conditions for 20–48 hours and used in the experiments.

2.8. Immunoblotting

The expression of the SNX2 proteins and viral proteins in transfected and/or virus-infected cells were analyzed with western blotting. The cells grown in 12-well plates were lysed in lysis buffer A (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM DTT, and 0.1% NP-40) or SDS-PAGE sample buffer. The proteins were separated using SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane. After blocking, the membrane was exposed to the specific primary antibodies (monoclonal mouse anti-HA [Santa Cruz # sc-7392]; monoclonal mouse anti-actin [Mybiosource # MBS9400413] or anti-PA polyclonal rabbit antisera overnight at 4°C and then to the horseradish peroxidase-conjugated second antibody (anti-mouse IgG-HRP [Invitrogen # 31420] and/or anti-rabbit IgG-HRP [Invitrogen # 31423]) against species-

specific immunoglobulins for 45 minutes at room temperature. The proteins were visualized with an ECL detection kit (GE Healthcare, Italy).

2.9. Co-immunoprecipitation assay

Co-immunoprecipitation assays were carried out to confirm the interaction between PA and SNX2 proteins in mammalian cells. The HEK293 cells were seeded in a 6-well plate (5×10^5 cells/well) and incubated at 37°C in a humidified incubator with 5% CO₂ for 20–24 h. The cells were co-transfected with the plasmid DNAs expressing SNX2 and viral PA proteins and incubated for 48 h. After the incubation periods, the cells were washed with PBS and treated with 1 mM DSP (dithiobis [succinimidyl propionate]) for 1 h. The cross-linking reaction was stopped by the addition of glycine at 100 mM final concentration, and then the cells were lysed in lysis buffer A. The cell lysates were clarified by centrifugation at 10000 rpm for 5 min. The lysates (250 µL) were incubated with a 5 µL monoclonal mouse anti-HA antibody (sc-7392) at 4°C for 2 h. Protein A-Sepharose (GE Healthcare, Sweden) was added to the lysates and moderately stirred overnight at 4°C. The beads were washed three times with buffer A. The proteins bound Sepharose beads were recovered by heating the samples at 95°C for 5 minutes in a SDS-PAGE sample buffer and then analyzed by western blotting.

2.10. Immunofluorescence assay

The localization of H-SNX2 and influenza PA proteins in transiently transfected HeLa cells was examined with immunofluorescence staining. The monolayers of HeLa cell on coverslips were transfected with pCAGGS-PA, pCHA-SNX2, pCHA-SNX2/pCAGGS-PA, pCHA-SNX2/pCAGGS-nPA, or pCHA-SNX2/pCAGGS-cPA plasmids. After 36–40 hours transfection, the cells were washed with PBS; fixed in 3% paraformaldehyde for 15 min at room temperature, permeabilized with 0.1% NP-40, washed twice with PBS, and then treated with 1% skim milk for 30 min. The cells were incubated with primary antibodies (mouse anti-HA and rabbit anti-PA) diluted in 1% skim milk for 60 min and washed three times with PBS. The cells were treated with 1% skim milk for 20 min once again and then stained with Alexa-488-conjugated goat anti-mouse IgG and/or Alexa-568-conjugated goat anti-rabbit IgG (at 1:300 dilutions in 1% skim milk) for 60 min. The nuclei of the cells were stained with DAPI. The coverslips were washed with PBS and mounted in a media (0.1% p-phenylenediamine and 80% glycerol) and the cells were analyzed with a laser confocal microscope (Zeiss LSM 700).

2.11. In situ proximity ligation assay (PLA)

The 50–60% confluent HeLa cells grown on coverslips were co-transfected with SNX2 and viral PA coding plasmids. After 36–40 hours of transfection, the cells were treated with primary antibodies (monoclonal mouse anti-HA and polyclonal rabbit anti-PA) as mentioned above. The assay was carried out with a Duolink PLA kit (Sigma-Aldrich # DUO92104) by following the manufacturer's instructions. Briefly, the monolayers were washed with wash buffer A (10 mM Tris, pH 7.4, 150 mM NaCl and 0.5% Tween-20) for 10 min and treated with a mixture of plus (mouse) and minus (rabbit) PLA probes for 90 min. Then, the monolayers were washed three times with wash buffer A for 10 min and subjected to ligation. After

ligation, the closed circles were amplified using a rolling-circle amplification with the DNA polymerase and complementary fluorescently labeled oligonucleotides for three hours at 37 °C. The samples were washed three times with wash buffer B (200 mM Tris, pH 7.5, 100 mM NaCl) for 10 min, rinsed once with 0.01x wash buffer B, mounted in the mount media, and visualized with the laser confocal microscope (Zeiss LSM 700).

2.12. siRNA transfection, and quantitation of viral RNAs and viral proteins

The small interfering RNA (siRNA) targeting exon 2 of the SNX2 gene (Cat. / Assay ID: Cat#1299001/HSS110066) was purchased from Life Technologies. The HeLa cells were seeded in six cm petri dishes (5×10^5 cells) and incubated at standard culture conditions for 20–24 hours. The cells were transfected with 30 pmol SNX2 gene-specific siRNA or negative control siRNA (Invitrogen # 12935–200) with lipofectamine RNAiMAX (Thermo Fisher Scientific, USA) according to the manufacturer’s protocol and incubated for 48 h. Then, the cells were sub-cultured into 12-well plates (2×10^5 cells/well) for 24 h. After the incubation period, total RNA was extracted from some monolayers for quantitation of the SNX2 transcript. Some of the monolayers were infected with influenza A/DkPen viruses at a one moi. After virus adsorption at 37 °C for 30 min, the inoculum was removed, and the cells were maintained in the maintenance medium (DMEM containing 0.2% Bovine Serum Albumin and 4 mg/ml trypsin) for six or 12 hours. The cells were lysed in SDS-PAGE sample buffer at the end of the incubation period and used for viral protein analysis with western blotting. At eight h post infection, the total RNA was extracted from some of the infected cultures for quantitation of the viral RNAs. Quantitation of SNX2 transcript and viral mRNAs/cRNA (segment 8) was carried out with qPCR. qPCR was conducted using FastStart Universal SYBR Green Master Mix (Roche, Germany). The cycle conditions included an initial denaturation step at 95°C for 10 min, followed by 45 cycles of amplification for five s at 95°C, 10 s at 55–60°C and 20 s at 72°C. The quantities of SNX2 transcript and viral RNAs were normalized by the amount of actin beta (ACTB). The oligonucleotide primers used in the real-time PCR are given in Table 1.

Table 1
The oligonucleotide primers used in qPCR experiments.

| Primer name | Sequence | Product Size |
|---------------|----------------------------|--------------|
| SNX2/846 For | 5'-GAGGATGGTGAACAAGGCTG-3' | 146 bp |
| SNX2/992 Rev | 5'-ACCAAGGCTTCAACACTGAC-3' | |
| Seg-8/542 For | 5'-TCATCGGTGGACTTGAATGG-3' | 144 bp |
| Seg-8/686 Rev | 5'-TCTGACTCAACTCTTCTCGC-3' | |
| Actin/280 For | 5'-CCACACCTTCTACAATGAGC-3' | 272 bp |
| Actin/552 Rev | 5'-TCATGAGGTAGTCAGTCAGG-3' | |

2.13. Titration of influenza A virus PA inhibitory effects on reporter SEAP expression with SNX2 proteins

The influenza A virus PA subunit of RdRP has strong inhibitory effects on PolIII polymerase activity [33, 36]. To determine the rescue effects of the SNX2 proteins on viral PA protein, the pSEAP (Clontech) reporter plasmid was used. The HEK293 cells grown in a 24-well plate (1×10^5 cells/well) were transfected with a certain amount of pSEAP and pCAGGS-PA(D) [33] and increasing amounts of the SNX2 plasmids. The cells were incubated at standard culture conditions for 24 hours, and secreted alkaline phosphatase (SEAP) activity in the medium was determined with a commercial kit (Roche Diagnostics GmbH#31420) following the instructions in the manual. The enzyme activity was measured as luminesces in the luminometer.

3. Results

3.1. Identification of interaction between human SNX2 and influenza A virus PA proteins using Y2H analysis

The yeast two-hybrid (Y2H) method is a well-established technique for detecting protein-protein interactions. In this study, using the influenza A/DkPen virus PA protein as a bait, possible PA interactor proteins encoded on the HEK293 cDNA library were screened with the Y2H method. The human SNX2 protein was identified as one of the candidate proteins that interact with viral PA protein. When the nucleotide sequence of the cDNA isolated from the positive colony was analyzed, it was determined that the peptide interacting with the PA consisted of the carboxyl-terminal region of the SNX2 protein composed of 276 amino acid residues (Fig. 1A). In order to study the interaction of the full-length SNX2 protein with the PA protein in more detail, the complete cDNA of SNX2 gene was cloned into the pACT2 plasmid and the possible interaction of this protein with PA bait in yeast cells was re-assayed. The growth profiles of the yeast cells transformed with both plasmids coding viral PA bait and human SNX2 protein on SD agar medium (without adenine, histidine, leucine and tryptophan) are given in Fig. 1B. It was defined that double transformant yeast cells expressing the GAL4 binding domain (pGBD-C1) and AD-SNX2 fusion protein (pACT2-SNX2) showed very limited growth on the SD agar medium. The yeast cells grown in a small-scale SD medium (without Trp and Leu) were further analyzed for β -galactosidase enzyme activity (Fig. 1C). The results were found in correlation with the growth profile of the colonies on the SD agar medium.

3.2. SNX2 Proteins show interactions with influenza A virus PA proteins in human cells

To investigate the interaction of the human SNX2 protein with the viral PA protein and its effect on the viral replication in mammalian cells, the plasmid vectors coding the full-length SNX2 and SNX2 proteins with a deletion in regions corresponding to specific exons were constructed. The domain structure of the

SNX2 protein and the schematic representations of the proteins encoded by plasmid vectors are given in Fig. 2A and 2B. The full-length SNX2 (variant 1) and the SNX2 proteins having deletions in some exons synthesized in HEK293 cells transiently transfected with the plasmids were analyzed by SDS-PAGE/western blotting. The proteins were found to be efficiently synthesized in transfected HEK293 cells (data not shown). The interaction between human SNX2 and viral PA protein was investigated by both co-immunoprecipitation assays and analysis of the intracellular localization of the proteins using immunofluorescence staining. The SNX2 proteins with an HA tag synthesized with viral PA proteins in HEK293 cells were precipitated with monoclonal mouse anti-HA antibodies, and SNX2 and PA proteins in the precipitates were determined by western blotting (Fig. 2C).

The results revealed that viral full-length PA protein and PA proteins with deletions (PA Δ C and PA Δ N) were co-precipitated with the SNX2 at different levels. It was observed that the SNX2 proteins with a deletion in amino-terminal (SNX2 Δ N) and inner domains (SNX2 Δ M) did not co-precipitate with the viral PA protein at all. In contrast, the SNX2 protein with a deletion in carboxyl-terminal domains (SNX2 Δ C) was efficiently precipitated with the PA protein. In addition, the SNX2 protein showed a strong interaction with PA Δ C while no co-precipitation with PA Δ N was detected. These results suggested that the amino-terminal moiety of the influenza A/DkP PA protein and some motifs located in the intermediate domains of the SNX2 protein are important for the interaction.

To support the IP results, we also examined the subcellular localization of the viral PA and human SNX2 proteins in HeLa cells. The cells were transfected alone or together with plasmids encoding SNX2 and PA proteins. Thirty-six hours after transfection, SNX2 and PA proteins were labeled with mouse monoclonal anti-HA and/or rabbit polyclonal anti-PA antibodies. Then, the proteins stained with the secondary antibodies conjugated with Alexa Fluor 488 and/or Alexa Fluor 568 were examined with a laser confocal microscope (Zeiss LSM 700, Carl Zeiss AG, Oberkochen, Germany) (Fig. 3A and 3B). The results showed that SNX2 protein encoded from the plasmid DNA in HeLa cells gave the co-localization profiles with both viral PA and PA Δ C proteins. The subcellular localization of SNX2 and PA proteins were found to confirm the co-immunoprecipitation tests.

The relationship between human SNX2 and influenza A virus PA protein encoded on plasmid DNAs in HeLa cells was also examined by the proximity ligation assay (PLA), which is a fairly new technique. After the proximity ligation reaction had taken place, fluorescent red dots were examined under the laser scanning microscope (Fig. 3C). The results revealed that SNX2 and PA proteins co-synthesized in HeLa cells were localized closer to each other than 40 nm (fluorescence dot formation), which is showed the interaction of the proteins.

3.3. Knockdown of the SNX2 gene positively affects the influenza A virus replication in the cells

RNA interference (RNAi) was used to knockdown SNX2 in HeLa cells, and the influenza A/DkPen viral replication was subsequently assessed in these cells. In the cells transfected with SNX2 siRNAs, it was observed that this gene-specific transcript level decreased by an average of 95% just before the viral

infection (Fig. 4A). In contrast, the influenza A virus replication was positively regulated in the SNX2 gene knockdown cells. It was found that the levels of cRNA and mRNA increased by 85% average in the SNX2 gene knockdown cells at the eighth hour of viral infection compared to the control cells (Fig. 4B). In correlation with the changes of viral transcripts, a significant increase was observed in the amount of PA subunit of the RdRP enzyme (Fig. 3C). These results revealed that the SNX2 protein had a negative effect on the influenza A/DkPen virus replication. The effect of over-expression of SNX2 proteins on the influenza A/DkPen virus RdRP enzyme was investigated by using the influenza mini replicon system (13). The results showed that there was no significant change in viral RdRP enzyme activities in the cells transfected with increasing amounts of SNX2 plasmids (data not shown).

3.4. The Increase of SNX2 proteins has rescue effects on the inhibitory activities of viral PA protein on host gene expression.

Influenza A virus RdRP enzyme PA subunit is known to inhibit host cell gene expression. In our previous study, it was shown that the avian influenza type influenza A/DkPen virus PA protein, in particular, more aggressively inhibits the transcription of host genes than that of human type Influenza A/WSN virus protein [33]. Based on these results, we evaluated whether SNX2 proteins neutralize the inhibitory effect of influenza A/DkPen PA protein on PolIII transcription. It was determined that full-length SNX2, SNX2 Δ C and SNX2 Δ M proteins decreased the inhibitory effects of the viral PA protein depending on the quantity of the SNX2 plasmids (Fig. 5). In contrast, SNX2 Δ N protein did not show a rescue effect on PA inhibition. These results were found important in supporting the interactions of SNX2 and PA proteins.

4. Discussion

The sorting nexin (SNX) family is a diverse group of cellular proteins characterized by the presence of a phospholipid-binding motif termed the phox homology (PX) domain [23]. These proteins are highly conserved and bind to phosphatidylinositol-3-phosphate in membranes through the PX region. SNX proteins interact with various cytoplasmic groups and membrane proteins and involve in many cellular pathways such as intracellular protein trafficking, regeneration of transmembrane proteins, transport of endosomes, and endocytosis [38, 39]. Mammalian genomes encode 33 SNXs that share the phox homology domain. SNX1, SNX2, SNX5, SNX6, and SNX32 form the membrane deformation subcomplex of the retromer. The main function of the retromer complex is selecting cargo proteins for retrograde transport from early endosomes to the Golgi apparatus. In the last few years, it has been revealed that some of the viruses utilize retromer-mediated trafficking for their replication [40]. However, no study was found that revealed a relationship between influenza A viruses and SNX proteins. In this study, we demonstrated that the human SNX2 protein interacts with the influenza A/DkPen virus PA protein by the Y2H assay (Fig. 1). Human SNX2 protein was also found to be associated with the viral PA protein in transiently transfected HEK293 cells with IP and IF analysis. The IP analyzes indicated that specifically

the amino-terminal region of the viral PA protein and some motifs close to the amino-terminal of the SNX2 protein are important in this interaction (Fig. 2). IF and PLA results correlated with IP data (Fig. 3). The results of the study to determine the relationship between PA and SNX2 in yeast and mammalian cells suggested that these two proteins interact in the cells and the SNX2 protein may interfere with the influenza A virus replication. Therefore, we investigated the effects of changes in the amount of SNX2 protein on viral replication in the cells. We found that influenza A/DkPen virus replication is upregulated both at the level of transcript and viral protein in SNX2 knockdown cells (Fig. 4). The upregulation of viral transcripts in knockdown cells suggests that the SNX 2 protein has a negative regulatory effect on influenza A/DkPen virus replication. In a previous study, it was reported that the L1 protein, which is the major component of the human papillomavirus type 16 (HPV-16) capsid, has a strong interaction with sorting nexin 17 (SNX17) and is crucial for HPV-16 virus infection [41]. Similarly, the SNX2 protein a retromer component, has interaction with HRSV structural proteins M and N and plays an important role in the traffic of HRSV structural proteins toward assembly sites [42]. It was also shown that gene knock-down of specific retromer components inhibits specific steps in the intracellular life cycle of the vaccinia virus [28], hepatitis C virus [43], and human papillomavirus [26]. Our results contradict the replication stimulating effects of SNX family proteins on some of the viruses.

We examined influenza A/DkPen virus RdRP enzyme activity in the HEK293 cells transiently transfected with increasing amounts of SNX2 plasmids by using the mini replicon assay. The increase of SNX2 proteins in the cells did not cause a significant change in viral RdRP enzyme activity. Influenza A virus RdRP enzyme is a heterotrimeric complex composed of PB2, PB1, and PA proteins [44]. The fact that the increase in SNX2 protein did not inhibit the viral RdRP enzyme in the mini replicon model suggested that the negative effect of this protein on viral replication occurs by a different mechanism associated with the PA protein.

Influenza A virus PA protein is known to inhibit host gene expression at the transcription and/or translation stage [37]. In our previous study, this inhibition was found to be much higher in the avian type virus-specific PA protein than in the human influenza A/WSN virus [33]. One of the reasons avian influenza A/DkPen viruses replicate much more slowly in mammalian cells may be the relationship of the viral PA protein with the SNX2 and some other cellular proteins. In this study, it was observed that the inhibitory effects of the viral PA protein on host Pol II dependent gene expression was partially rescued by the SNX2 protein (Fig. 5). The increase in the SNX2 Δ N protein did not affect the inhibition of the PA protein. These results support the association of the SNX2 protein with the viral PA protein, especially with some motifs close to the amino-terminal region.

In conclusion, we demonstrated with the Y2H method that human SNX2 protein, a member of the sorting nexins family, has an interaction with avian influenza A/DkPen virus PA protein. It was shown that these two proteins also interact with each other in the HEK293 cells, and the SNX2 protein has a negative regulatory function on virus replication. However, the increase in SNX2 proteins did not cause a significant change in the viral RdRP activity. This may suggest that the negative effect of the SNX2

protein on the influenza A/DkPen virus replication could be saturated with its authentic intra-cellular amount or independent of the viral RdRP enzyme.

Therefore, more knowledge is required to elucidate the action mechanism of the SNX2 protein on influenza A/DkPen virus replication at the molecular level.

Declarations

Author contributions

KT and KN conceived and designed the study. TK, EC, ER and KT performed the experiments. KT wrote the paper. All authors read and approved the manuscript and agreed to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work is appropriately investigated and resolved.

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Conflict of interest

All the authors declare no conflict of interest.

Data availability

The datasets used during the present study are available from the corresponding author upon reasonable request.

Ethical approval

In this study, neither human subjects nor animals were used.

References

1. Parrish CR, Murcia PR, Holmes EC (2015) Influenza virus reservoirs and intermediate hosts: dogs, horses, and new possibilities for influenza virus exposure of humans. *J Virol* 89:2990–2994. doi:10.1128/JVI.03146-14
2. Taubenberger JK, Kash JC (2010) Influenza virus evolution, host adaptation, and pandemic formation. *Cell Host Microbe* 7:440–451. doi:10.1016/j.chom.2010.05.009
3. Garten RJ, Davis CT, Russell CA et al (2009) Antigenic and genetic characteristics of swine-origin 2009 A(H1N1) influenza viruses circulating in humans. *Science* 325:197–201.

doi:10.1126/science.1176225

4. Lamb RA, Lai CJ, Choppin PW (1981) Sequences of mRNAs derived from genome RNA segment 7 of influenza virus: colinear and interrupted mRNAs code for overlapping proteins. *Proc Natl Acad Sci U S A* 78:4170–4174. doi:10.1073/pnas.78.7.4170
5. Lamb RA, Choppin PW, Chanock RM, Lai CJ (1980) Mapping of the two overlapping genes for polypeptides NS1 and NS2 on RNA segment 8 of influenza virus genome. *Proc Natl Acad Sci U S A* 77:1857–1861. doi:10.1073/pnas.77.4.1857
6. Chen W, Calvo PA, Malide D, Gibbs J, Schubert U, Bacik I, Basta S, O'Neill R, Schickli J, Palese P, Henklein P, Bennink JR, Yewdell JW (2001) A novel influenza A virus mitochondrial protein that induces cell death. *Nat Med* 7:1306–1312. doi:10.1038/nm1201-1306
7. Jagger BW, Wise HM, Kash JC, Walters KA, Wills NM, Xiao YL, Dunfee RL, Schwartzman LM, Ozinsky A, Bell GL, Dalton RM, Lo A, Efsthathiou S, Atkins JF, Firth AE, Taubenberger JK, Digard P (2012) An overlapping protein-coding region in influenza A virus segment 3 modulates the host response. *Science* 337:199–204. doi:10.1126/science.1222213
8. Muramoto Y, Noda T, Kawakami E, Akkina R, Kawaoka Y (2013) Identification of novel influenza A virus proteins translated from PA mRNA. *J Virol* 87:2455-62. doi: 10.1128/JVI.02656-12
9. Wise HM, Hutchinson EC, Jagger BW, Stuart AD, Kang ZH, Robb N, Schwartzman LM, Kash JC, Fodor E, Firth AE, Gog JR, Taubenberger JK, Digard P (2012) Identification of a novel splice variant form of the influenza A virus M2 ion channel with an antigenically distinct ectodomain. *PLoS Pathog* 8:e1002998. doi:10.1371/journal.ppat.1002998
10. Selman M, Dankar SK, Forbes NE, Jia JJ, Brown EG (2012) Adaptive mutation in influenza A virus non-structural gene is linked to host switching and induces a novel protein by alternative splicing. *Emerg Microbes Infect* 1:e42. doi:10.1038/emi.2012.38
11. Zhao M, Wang L, Li S (2017) Influenza A Virus-Host Protein Interactions Control Viral Pathogenesis. *Int J Mol Sci* 18. doi:10.3390/ijms18081673
12. Senbas Akyazi B, Pirincal A, Kawaguchi A, Nagata K, Turan K (2020) Interaction of influenza A virus NS2/NEP protein with the amino-terminal part of Nup214. *Turk J Biol* 44:82–92. doi:10.3906/biy-1909-49
13. Turan K, Mibayashi M, Sugiyama K, Saito S, Numajiri A, Nagata K (2004) Nuclear MxA proteins form a complex with influenza virus NP and inhibit the transcription of the engineered influenza virus genome. *Nucleic Acids Res* 32:643–652. doi:10.1093/nar/gkh192
14. Avalos RT, Yu Z, Nayak DP (1997) Association of influenza virus NP and M1 proteins with cellular cytoskeletal elements in influenza virus-infected cells. *J Virol* 71:2947–2958. doi:10.1128/JVI.71.4.2947-2958.1997
15. Zhirnov OP, Ksenofontov AL, Kuzmina SG, Klenk HD (2002) Interaction of influenza A virus M1 matrix protein with caspases. *Biochemistry* 67:534–539. doi:10.1023/a:1015542110798
16. Cao M, Wei C, Zhao L, Wang J, Jia Q, Wang X, Jin Q, Deng T (2014) DnaJA1/Hsp40 is co-opted by influenza A virus to enhance its viral RNA polymerase activity. *J Virol* 88:14078–14089.

doi:10.1128/JVI.02475-14

17. Tan SL, Katze MG (1998) Biochemical and genetic evidence for complex formation between the influenza A virus NS1 protein and the interferon-induced PKR protein kinase. *J Interferon Cytokine Res* 18:757–766. doi:10.1089/jir.1998.18.757
18. Zhang J, Li G, Ye X (2010) Cyclin T1/CDK9 interacts with influenza A virus polymerase and facilitates its association with cellular RNA polymerase II. *J Virol* 84:12619–12627. doi:10.1128/JVI.01696-10
19. Garrus JE, von Schwedler UK, Pornillos OW, Morham SG, Zavitz KH, Wang HE, Wettstein DA, Stray KM, Cote M, Rich RL, Myszka DG, Sundquist WI (2001) Tsg101 and the vacuolar protein sorting pathway are essential for HIV-1 budding. *Cell* 107:55–65. doi:10.1016/s0092-8674(01)00506-2
20. Rauch S, Martin-Serrano J (2011) Multiple interactions between the ESCRT machinery and arrestin-related proteins: implications for PPXY-dependent budding. *J Virol* 85:3546–3556. doi:10.1128/JVI.02045-10
21. Sagara J, Tsukita S, Yonemura S, Tsukita S, Kawai A (1995) Cellular actin-binding ezrin-radixin-moesin (ERM) family proteins are incorporated into the rabies virion and closely associated with viral envelope proteins in the cell. *Virology* 206:485–494. doi:10.1016/s0042-6822(95)80064-6
22. Bhowmick S, Chakravarty C, Sellathamby S, Lal SK (2017) The influenza A virus matrix protein 2 undergoes retrograde transport from the endoplasmic reticulum into the cytoplasm and bypasses cytoplasmic proteasomal degradation. *Arch Virol* 162:919–929. doi:10.1007/s00705-016-3153-8
23. Worby CA, Dixon JE (2002) Sorting out the cellular functions of sorting nexins. *Nat Rev Mol Cell Biol* 3:919–931. doi:10.1038/nrm974
24. Carlton JG, Bujny MV, Peter BJ, Oorschot VM, Rutherford A, Arkell RS, Klumperman J, McMahon HT, Cullen PJ (2005) Sorting nexin-2 is associated with tubular elements of the early endosome, but is not essential for retromer-mediated endosome-to-TGN transport. *J Cell Sci* 118:4527–4539. doi:10.1242/jcs.02568
25. Cullen PJ, Korswagen HC (2011) Sorting nexins provide diversity for retromer-dependent trafficking events. *Nat Cell Biol* 14:29–37. doi:10.1038/ncb2374
26. Bergant M, Banks L (2013) SNX17 facilitates infection with diverse papillomavirus types. *J Virol* 87:1270–1273. doi:10.1128/JVI.01991-12
27. Al-Saleem J, Dirksen WP, Martinez MP, Shkriabai N, Kvaratskhelia M, Ratner L, Green PL (2019) HTLV-1 Tax-1 interacts with SNX27 to regulate cellular localization of the HTLV-1 receptor molecule, GLUT1. *PLoS One* 14:e0214059. doi:10.1371/journal.pone.0214059
28. Hsiao JC, Chu LW, Lo YT, Lee SP, Chen TJ, Huang CY, Ping YH, Chang W (2015) Intracellular Transport of Vaccinia Virus in HeLa Cells Requires WASH-VPEF/FAM21-Retromer Complexes and Recycling Molecules Rab11 and Rab22. *J Virol* 89:8365–8382. doi:10.1128/JVI.00209-15
29. Phillips SL, Cygnar D, Thomas A, Bresnahan WA (2012) Interaction between the human cytomegalovirus tegument proteins UL94 and UL99 is essential for virus replication. *J Virol* 86:9995–10005. doi:10.1128/JVI.01078-12

30. Turan K, Nagata K, Kuru A (1996) Antiviral effect of *Sanicula europaea* L. leaves extract on influenza virus-infected cells. *Biochem Biophys Res Commun* 225:22–26. doi:10.1006/bbrc.1996.1125
31. Nagata K, Saito S, Okuwaki M, Kawase H, Furuya A, Kusano A, Hanai N, Okuda A, Kikuchi A (1998) Cellular localization and expression of template-activating factor I in different cell types. *Exp Cell Res* 240:274–281. doi:10.1006/excr.1997.3930
32. James P, Halladay J, Craig EA (1996) Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast. *Genetics* 144:1425–1436
33. CaGlavan E, Turan K (2021) Expression profiles of interferon-related genes in cells infected with influenza A viruses or transiently transfected with plasmids encoding viral RNA polymerase. *Turk J Biol* 45:88–103. doi:10.3906/biy-2005-73
34. Pham PTV, Turan K, Nagata K, Kawaguchi A (2018) Biochemical characterization of avian influenza viral polymerase containing PA or PB2 subunit from human influenza A virus. *Microbes Infect* 20:353–359. doi:10.1016/j.micinf.2018.04.003
35. Gietz RD, Schiestl RH (2007) High-efficiency yeast transformation using the LiAc/SS carrier DNA/PEG method. *Nat Protoc* 2:31–34. doi:10.1038/nprot.2007.13
36. Oishi K, Yamayoshi S, Kawaoka Y (2015) Mapping of a Region of the PA-X Protein of Influenza A Virus That Is Important for Its Shutoff Activity. *J Virol* 89:8661–8665. doi:10.1128/JVI.01132-15
37. De Vlugt C, Sikora D, Pelchat M (2018) Insight into Influenza: A Virus Cap-Snatching. *Viruses* 10. doi:10.3390/v10110641
38. Johannes L, Popoff V (2008) Tracing the retrograde route in protein trafficking. *Cell* 135:1175–1187. doi:10.1016/j.cell.2008.12.009
39. Kanzawa M, Hara S, Semba S, Yokozaki H, Hirokawa M, Itoh T (2014) Sorting Nexin 2 (SNX2): a potential marker of active thyrocytes in normal and hyperfunctioning thyroid disorders. *Appl Immunohistochem Mol Morphol* 22:302–307. doi:10.1097/PAI.0b013e31828badd3
40. Elwell C, Engel J (2018) Emerging Role of Retromer in Modulating Pathogen Growth. *Trends Microbiol* 26:769–780. doi:10.1016/j.tim.2018.04.001
41. Bergant Marusic M, Ozbun MA, Campos SK, Myers MP, Banks L (2012) Human papillomavirus L2 facilitates viral escape from late endosomes via sorting nexin 17. *Traffic* 13:455–467. doi:10.1111/j.1600-0854.2011.01320.x
42. Cle M, Desmetz C, Barthelemy J, Martin MF, Constant O, Maarifi G, Foulongne V, Bollere K, Glasson Y, De Bock F, Blaquiére M, Dehouck L, Pirot N, Tuailon E, Nisole S, Najjioullah F, Van de Perre P, Cabie A, Marchi N, Gosselet F, Simonin Y, Salinas S (2020) Zika Virus Infection Promotes Local Inflammation, Cell Adhesion Molecule Upregulation, and Leukocyte Recruitment at the Blood-Brain Barrier. *mBio* 11. doi:10.1128/mBio.01183-20
43. Yin P, Hong Z, Yang X, Chung RT, Zhang L (2016) A role for retromer in hepatitis C virus replication. *Cell Mol Life Sci* 73:869–881. doi:10.1007/s00018-015-2027-7
44. Kawaguchi A, Naito T, Nagata K (2005) Involvement of influenza virus PA subunit in assembly of functional RNA polymerase complexes. *J Virol* 79:732–744. doi:10.1128/JVI.79.2.732-744.2005

Figures

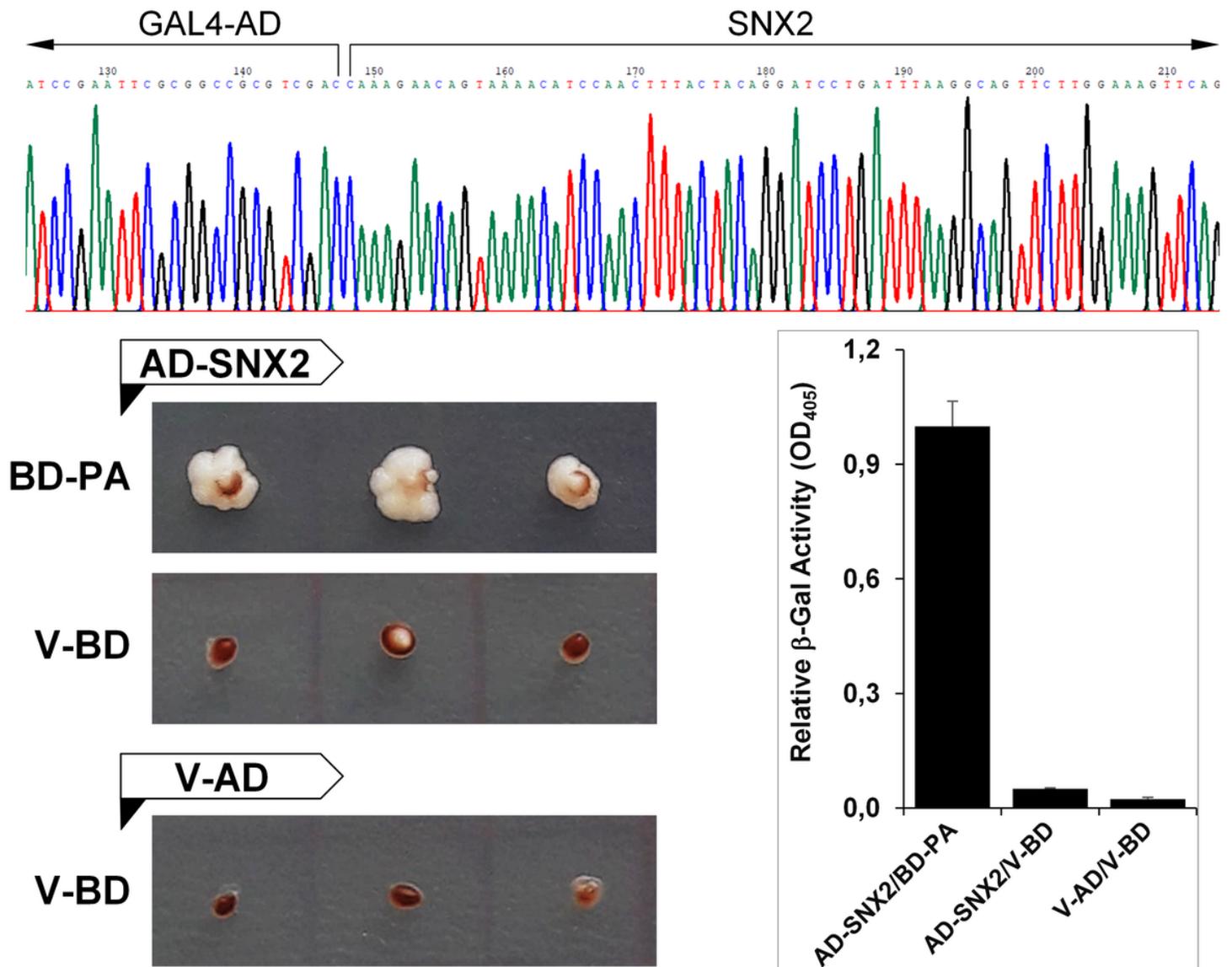


Figure 1

Human SNX2 Interacts with influenza A/DkPen PA Protein in the Yeast *S. cerevisiae* Strain PJ69-4A. A. DNA sequencing chromatogram of the cDNA isolated from the yeast cells having a positive reaction in Y2H assay. B. The growth profiles of double transformed cells with yeast two-hybrid plasmids on SD agar media. C. The β-galactosidase activity of transformed yeast cells. The cells were transformed with a plasmid coding GAL4-binding domain (V-BD) (pGBD-C1) or the viral PA bait protein fused with GAL4-BD (pGBD-PA) and then with a plasmid coding GAL4-activation domain (V-AD) (pACT2) or human SNX2 protein fused with GAL4-AD (pACT2-SNX2). To determine the growth profiles, three colonies from each sample were cultured on SD agar plates (without Ade, His, Leu, and Trp). The β-galactosidase enzyme activities of the colonies were defined as described in the Methods section.

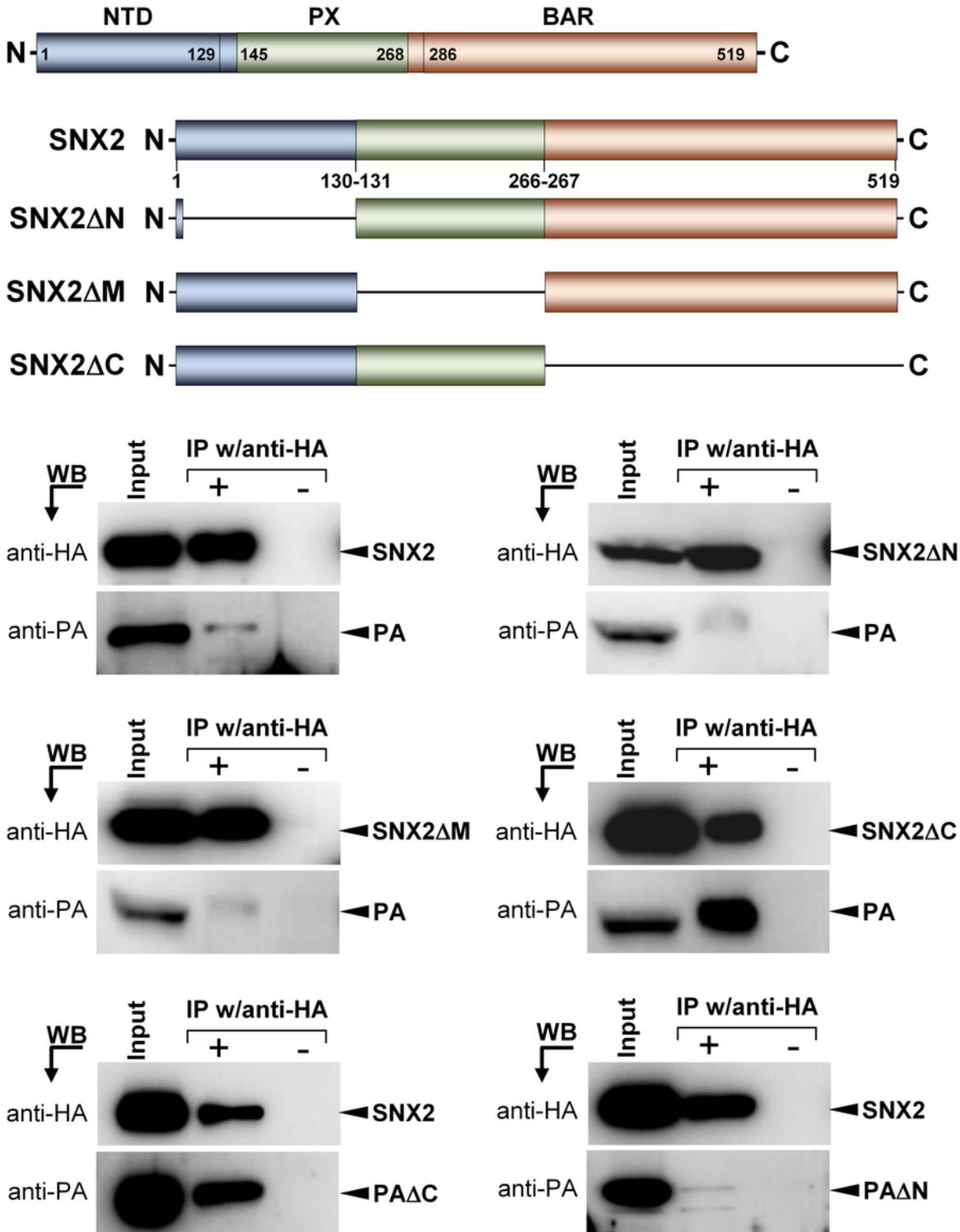


Figure 2

A. The domain structure of human SNX2 protein. B. The schematic representation of the SNX2 proteins encoded by the pCHA plasmids. Deletions in the SNX2 gene were carried out according to the exon/intron structure of the gene; Exons 1-3, exons 4-8, and exons 9-15 were deleted in the SNX2ΔN, SNX2ΔM, and SNX2ΔC genes, respectively. C. Co-immunoprecipitation of influenza A/DkPen PA proteins with human SNX2 synthesized in transiently transfected HEK293T cells. The cells were transfected with the plasmids

coding the proteins shown in the figures. Immunoprecipitation was performed with an anti-HA antibody, and the precipitate was subjected to SDS-PAGE. The proteins were detected by western blotting using either monoclonal anti-HA antibody or rabbit anti-PA. HRP conjugated goat anti-mouse IgG or anti-rabbit IgG was used as the secondary antibody for labeling the proteins.

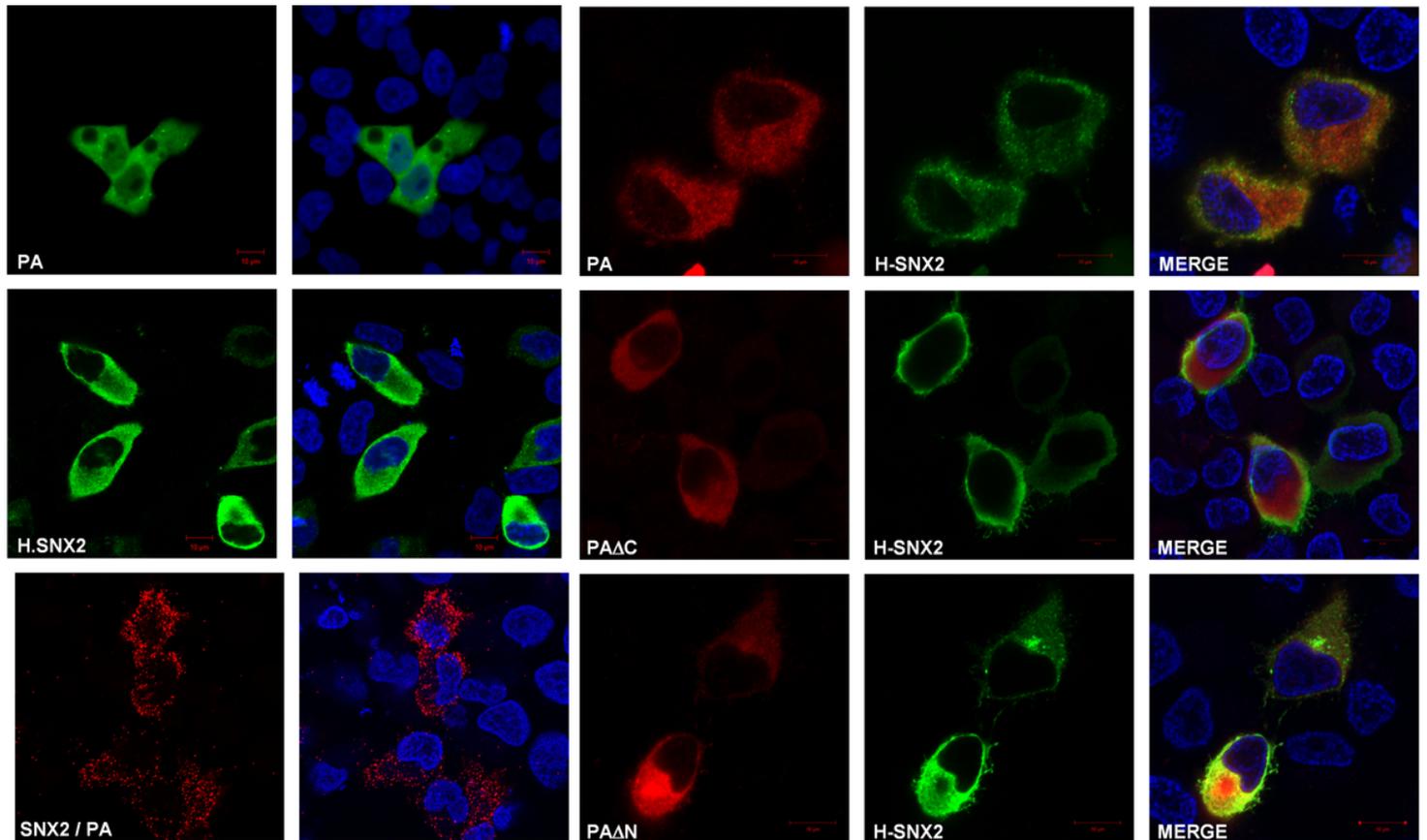


Figure 3

Subcellular detection of the PA and the SNX2 proteins in HeLa cells with immunostaining (A and B) and the PLA (C). A. HeLa cells were transfected with the plasmid encoding PA or SNX2 protein. B. The cells were co-transfected with the plasmids encoding PA and SNX2 proteins. At 36 h post-transfection, the cells were fixed, permeabilized, and labeled with mouse monoclonal anti-HA (for SNX2) and/or rabbit polyclonal anti-PA. As the secondary antibodies, monoclonal anti-mouse IgG conjugated Alexa Fluor 488 (for SNX2) and/or monoclonal anti-rabbit IgG conjugated Alexa Fluor 568 (for PA) were used. C. HeLa cells co-transfected with plasmids were treated with mouse monoclonal anti-HA (for SNX2) and rabbit polyclonal anti-PA. Then, the PLA was carried out with Duolink proximity ligation assay reagents according to the manufacturer's instructions and analyzed with the laser scanning microscope. The nuclei were counterstained by DAPI. Stained cells were examined under the laser scanning microscope.

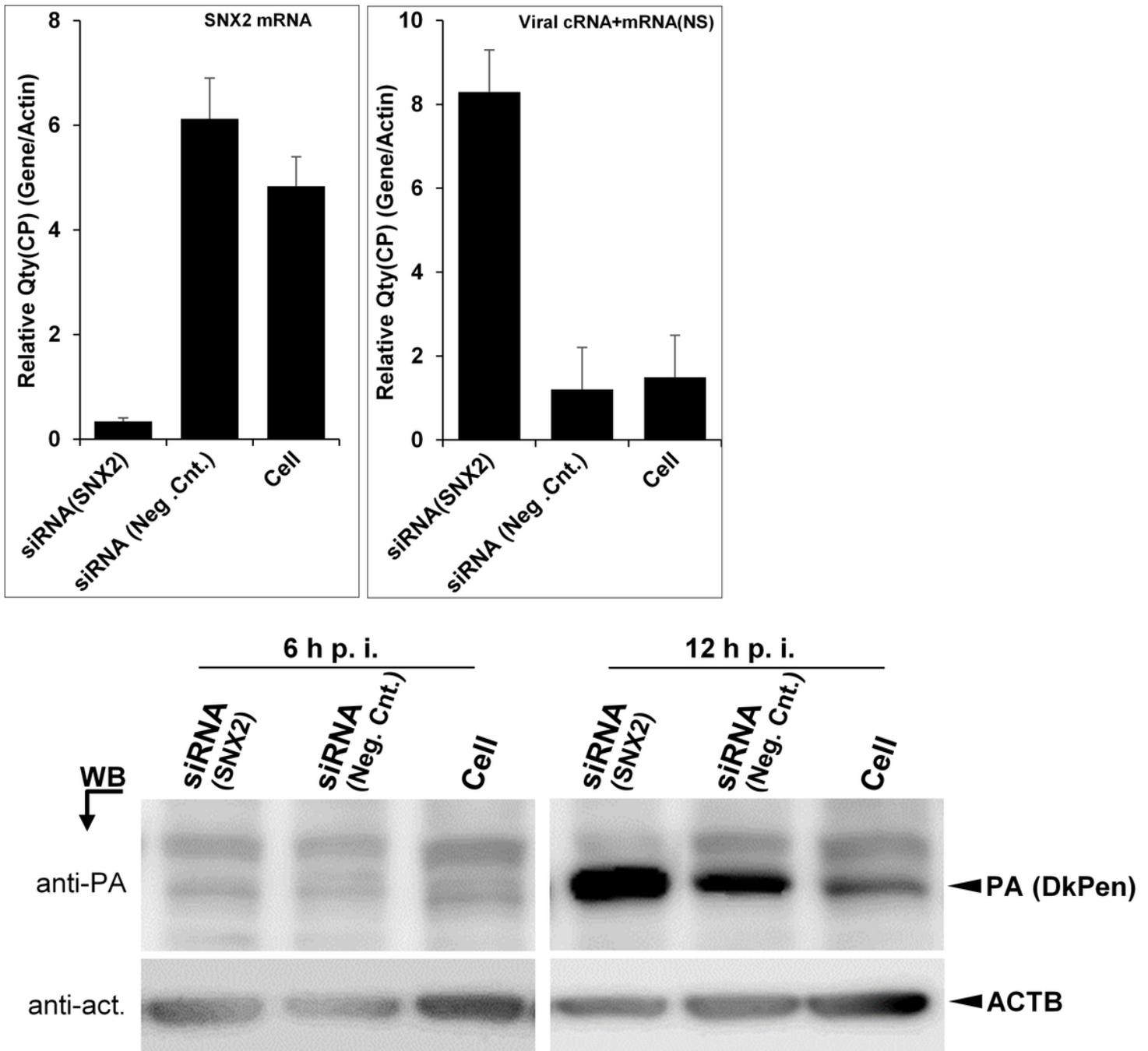


Figure 4

Influenza A/DkPen virus replication in the SNX2 gene knockdown cells. A. The endogenous SNX2 transcript level in the knockdown HeLa cells. B. The viral mRNAs and cRNA transcribed from segment 8 viral RNA levels in the virus-infected knockdown cells. The levels of SNX2 mRNA (just before infection) and viral RNAs transcribed from viral segment 8 (at 8 h p.i.) in the knockdown cells were determined with qPCR. C. Western blot analysis of viral PA protein in the virus infected knockdown cells. The proteins were labeled with rabbit polyclonal anti-PA or mouse monoclonal anti-actin, and HRP conjugated goat anti-rabbit IgG or goat anti-mouse IgG and visualized with an ECL Western blotting detection kit (GE Healthcare).

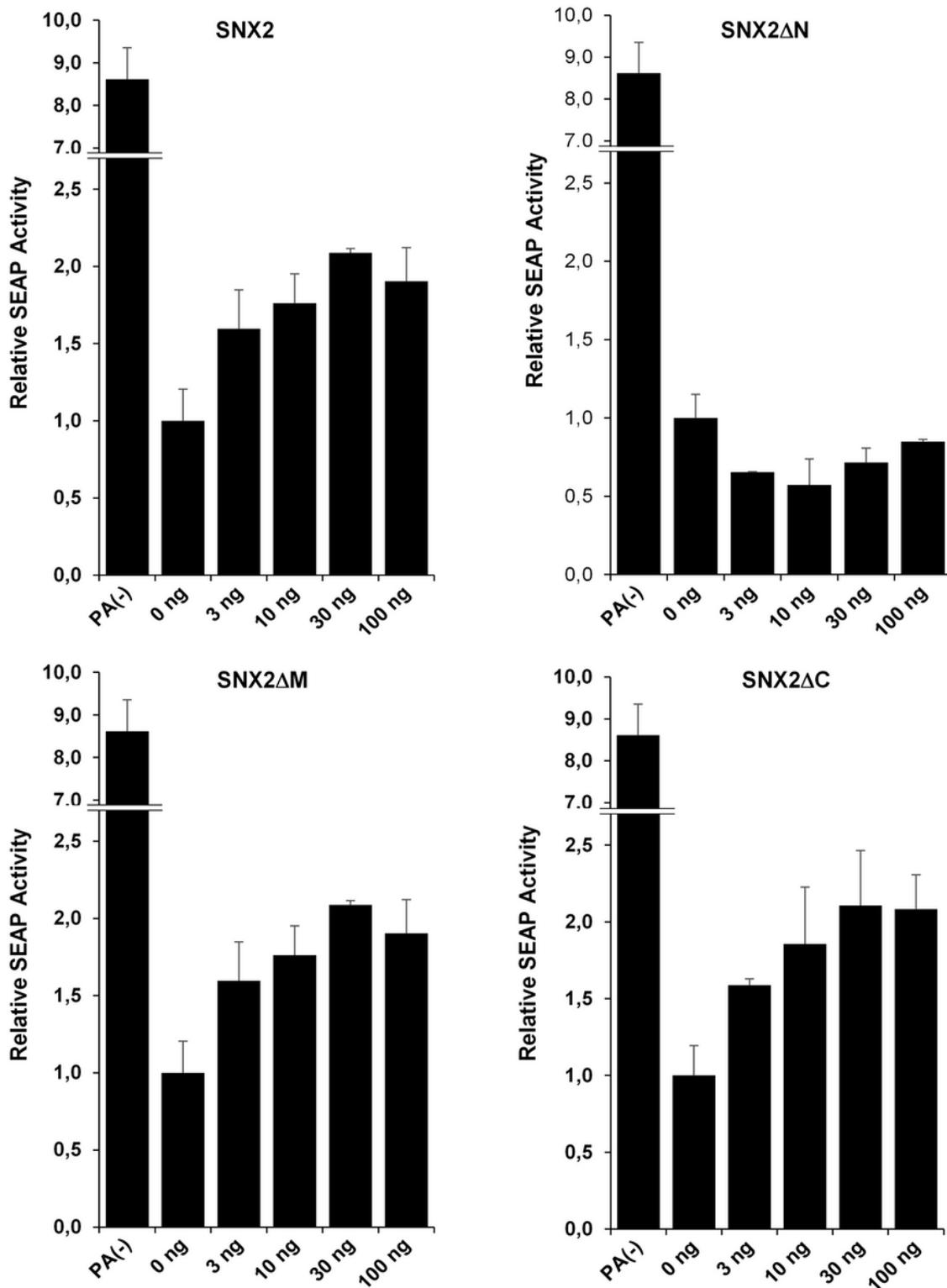


Figure 5

Rescue effect of SNX2 proteins on influenza A/DkPen PA inhibitory activity to host gene expression. The HEK293 cells were grown in a 24-well plate (5×10^4 cells/well) for 24 hours in standard culture conditions and transfected with constant amounts of pCAGGS-PA(D) (1 ng/well) and pSEAP (20 ng/well) plasmids and indicated amounts of the SNX2 plasmids in the figure. After 24 hours of transfection, the reporter SEAP activities in culture media was detected with a commercial kit.