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Nuclear import of prototype foamy virus transactivator Bel1 is mediated by KPNA1, KPNA6 and KPNA7

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Abstract. Bel1, a transactivator of the prototype foamy virus (PFV), plays pivotal roles in the replication of PFV. Previous studies have demonstrated that Bel1 bears a nuclear localization signal (NLS); however, its amino acid sequence remains unclear and the corresponding adaptor importins have not yet been identified. In this study, we inserted various fragments of Bel1 into an EGFP-GST fusion protein and investigated their subcellular localization by fluorescence microscopy. We found that the ^{215}PRQKRPR^{221} fragment, which accords with the consensus sequence K(K/R)X(K/R) of the monopartite NLS, directed the nuclear translocation of Bel1. Point mutation experiments revealed that K^{218}, R^{219} and R^{221} were essential for the nuclear localization of Bel1. The results of GST pull-down assay revealed that the Bel1 peptide 215-221, which bears the NLS, interacted with the nucleocytoplasmic transport receptors, karyopherin alpha 1 (importin alpha 5) (KPNA1), karyopherin alpha 6 (importin alpha 7) (KPNA6) and karyopherin alpha 7 (importin alpha 8) (KPNA7). Finally, *in vitro* nuclear import assays demonstrated that KPNA1, KPNA6 or KPNA7, along with other necessary nuclear factors, caused Bel1 to localize to the nucleus. Thus, the findings of our study indicate that KPNA1, KPNA6 and KPNA7 are involved in Bel1 nuclear distribution.

Introduction

Foamy viruses (FVs), which comprise the *Spumaretrovirinae* in the retrovirus family, are also known as spumaretroviruses. FVs are found in primates, including humans, as well as in non-primates, including cows, cats and horses (1-5).

The prototype foamy virus (PFV) Tas protein, also known as Bel1, is a 300-amino-acid nuclear protein that is essential for virus replication (6), and can highly transactivate the PFV promoters, LTR and IP (7-9). Similar to most typical transcriptional activators, nuclear localization is required for the transactivation activity of Bel1 (10). Bel1 bears a putative nuclear localization signal (NLS) in the central highly basic region (11,12). Earlier studies have indicated that peptide 211-225 and/or 209-226 are necessary and sufficient for Bel1 nuclear localization (13-15). Later studies demonstrated that another two basic amino acids, $R^{199}H^{200}$, also regulate Bel1 nuclear localization, which suggests that Bel1 carries a bipartite NLS consisting of residues 199-200 and residues 211-223 (10,16). However, Ma *et al* further found that residues $R^{221}R^{222}R^{223}$, but not $R^{199}H^{200}$, are essential for the nuclear distribution of Bel1 (17).

Importin is a type of karyopherin (18) that transports protein molecules into the nucleus by binding to nuclear localization sequences. Importin has two subunits, karyopherin alpha (KPNA; also known as importin alpha) and karyopherin beta KPNB (also known as importin beta). Members of the KPNB family can bind and transport cargoes by themselves (19-21), or can form heterodimers with KPNA (22,23). As part of a heterodimer, KPNB mediates the interaction with nuclear pore complex (NPC), while KPNA acts as an adaptor protein to bind KPNB and the NLS on the cargo (24). The NLS-KPNA-KPNB trimer dissociates after binding to RanGTP inside the nucleus (25), with the two importin proteins being recycled to the cytoplasm for further use. Although KPNA and KPNB are used to describe importin as a whole, they actually represent larger families of proteins that share a similar structure and function. A variety of genes have been identified for both KPNA and KPNB, such as *KPNA*1-*KPNA*7 and *KPNB*1 (26). Different KPNA members show preferences for particular types of NLS cargo, although there is no absolute boundary (26,27).

In this study, we aimed to determine which adaptor importins are required for Bel1 nuclear translocation. We found that the ^{215}PRQKRPR^{221} fragment, which accords with the consensus sequence K(K/R)X(K/R) of monopartite NLS, directs the nuclear localization of Bel1. Point mutation experiments revealed that residues K^{218}, R^{219} and R^{221} were essential for the nuclear accumulation of Bel1. The results of GST pull-down assay revealed that the Bel1 NLS fragment 215-221 interacted with KPNA1, KPNA6 and KPNA7. Finally, *in vitro* nuclear import assays demonstrated that KPNA1, KPNA6 and KPNA7 caused Bel1 to localize to the nucleus. Our findings thus indicate that KPNA1, KPNA6 and KPNA7 are involved in Bel1 nuclear translocation.

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Table I. Primers used for PCR or site-directed mutagenesis PCR or RT-PCR.

Gene	Sense primer sequences (5'→3')	Antisense primer sequences (5'→3')
Bel1	TTA**GAGCTC**ATGGATTCCTACGAAAAAG	CCG**AAGCTT**TAAAACTGAATGTTCACCT
K218R	GCCTCGGCAGA**G**ACGACCCAGGAGA	TCTCCTGGGTCGT**C**TCTGCCGAGGC
K218A	CCTCGGCAG**GC**ACGACCCAGGAGA	TCTCCTGGGTCGT**GC**CTGCCGAGG
R219A	CCTCGGCAGAAA**GC**ACCCAGGAGACG	CGTCTCCTGGGT**GC**TTTCTGCCGAGG
R221A	GAAACGACCC**GC**GAGACGATCCATC	GATGGATCGTCTC**GC**GGGTCGTTTC
KPNA1	TTA**GGATCC**ATGACCACCCCAGGAAAAG	TAG**CTCGAG**TCAAAGCTGGAAACCTTCC
KPNA2	CTC**GAATTC**ATGTCCACCAACGAGAAT	TCA**CTCGAG**CTAAAAGTTAAAGGTCCC
KPNA3	ATA**GAATTC**ATGGCCGAGAACCCCAGC	GCG**CTCGAG**TTTTGTTTGAAGGTTGGC
KPNA4	TTA**GGATCCA**TGGCGGACAACGAGAAAC	GCT**CTCGAG**CTAAAACTGGAACCCTTCT
KPNA5	GCG**GAATTC**ATGGATGCCATGGCTAGT	GCG**CTCGAG**TTGAAATCCATCCATTGG
KPNA6	ATC**GAATTC**ATGGAGACCATGGCGAGC	TAT**CTCGAG**TAGCTGGAAGCCCTCCAT
KPNA7	GTC**GAATTC**ATGCCGACCTTAGATGCT	CGC**CTCGAG**TGCTAAGCATTCATAATC
KPNB1	ATA**GCGGCCGC**AATGGAGCTGATCACCAT	CCT**GGATCC**TCAAGCTTGGTTCTTCAG

Restriction sites are shown in bold and mutation sites are shown in underlined bold.

Materials and methods

Plasmids. The *Bel1* gene was amplified from the PFV full-length infectious clone, pCHFV, kindly provided by Maxine L. Linial (28). The mammalian cell expression plasmids, pC3-EGFP-X-GST, pC3-EGFP-NLS-GST, pC3-EGFP-BiNLS-GST, pC3-EGFP-Bel1-GST, pC3-EGFP-215-221)-GST and other truncated Bel1 plasmids were generated as previously described (17). The Bel1 mutants K218R, K218A, R219A and R221A were generated using a QuikChange™ site-directed mutagenesis kit (Stratagene, Palo Alto, CA, USA) using the primers listed in Table I. The coding sequences of KPNA1-KPNA7 and KPNB1 were amplified from the HeLa cDNA library by RT-PCR with the primers listed in Table I and inserted into the pCMV-Tag 2B vector (Stratagene) or the pFLAG-CMV-4 vector (Sigma-Aldrich, St. Louis, MO, USA) to express the corresponding proteins. All the new constructs were confirmed by DNA sequencing.

Cell culture and transfection, antibodies and reagents. HeLa and 293T cells (both from the Cell Bank of the Chinese Academy of Sciences, Shanghai, China) were grown in Dulbecco's modified Eagle's medium (high glucose; HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (Gibco-BRL, Grand Island, NY, USA) and Pen Strep Glutamine (PSG) (Gibco). The cells were maintained in a humidified atmosphere containing 5% CO_2 at 37˚C and transfected with polyethylenimines (PEI) (Polysciences, Inc., Warrington, PA, USA) in accordance with the manufacturer's instructions.

Anti-EGFP (sc-9996), anti-GAPDH (sc-32233), anti-GST (sc-138) and HRP-conjugated goat anti-mouse secondary antibodies (sc-2005) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-Flag (F3165) antibody and 4',6-diamidino-2-phenylindole (DAPI) were purchased from Sigma-Aldrich. FITC-conjugated affinipure goat anti-mouse secondary antibodies (115-095-003) were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA).

Immunofluorescence microscopy assay (IFA). The HeLa cells were seeded on glass coverslips. Following fixation in 4% paraformaldehyde for 10 min on ice, the cells were permeabilized in 0.2% Triton X-100 for 10 min on ice. After blocking in 3% BSA + 5% fat-free milk at 4˚C for 2 h, the cells were incubated with anti-EGFP antibodies at 4˚C for a further 2 h, and subsequently washed with 0.1% Triton X-100 in PBS 5 times at room temperature. FITC-conjugated secondary antibodies were added at 4˚C for 45 min. After the nuclei were stained with 0.2 μg/ml DAPI for 10 min at room temperature, the coverslips were observed under an Olympus IX71 fluorescence microscope (Olympus, Tokyo, Japan).

In vivo GST pull-down assay and western blot analysis. The 293T cells were transfected with the pC3-EGFP-X-GST empty vector or pC3-EGFP-Bel1-GST along with plasmids that encode Flag-KPNAs or Flag-KPNB1; at 48 h post-transfection, the cell lysates were incubated with Glutathione Sepharose 4B beads (20182003-2) (GE Healthcare, Cleveland, OH, USA) for 4 h at 4˚C. Samples from both cell lysates and Glutathione Sepharose 4B beads were mixed with loading buffer. After boiling for 20 min at 100˚C, the protein samples were resolved by SDS-PAGE and transferred onto PVDF membranes. Prior to incubation first with primary antibodies overnight at 4˚C and then with HRP-conjugated secondary antibodies for 1 h at room temperature, the membranes were blocked in 5% fat-free milk for 1.5 h at room temperature. After the membranes were treated with Luminata™ Western HRP chemiluminescence substrates (WBLUC0100; Millipore, Billerica, MA, USA), the specific protein signals were detected by exposure to X-ray films (Kodak, Xiamen, China).

In vitro nuclear import assay. *In vitro* nuclear transport assays were carried out as previously described with some modifications (29,30). Briefly, the HeLa cells (70-80% confluent), plated on glass coverslips, were washed 3 times with ice-cold transport buffer (TB) and permeabilized with digitonin (40 mg/ml) for 5 min on ice. The cells were then washed twice with

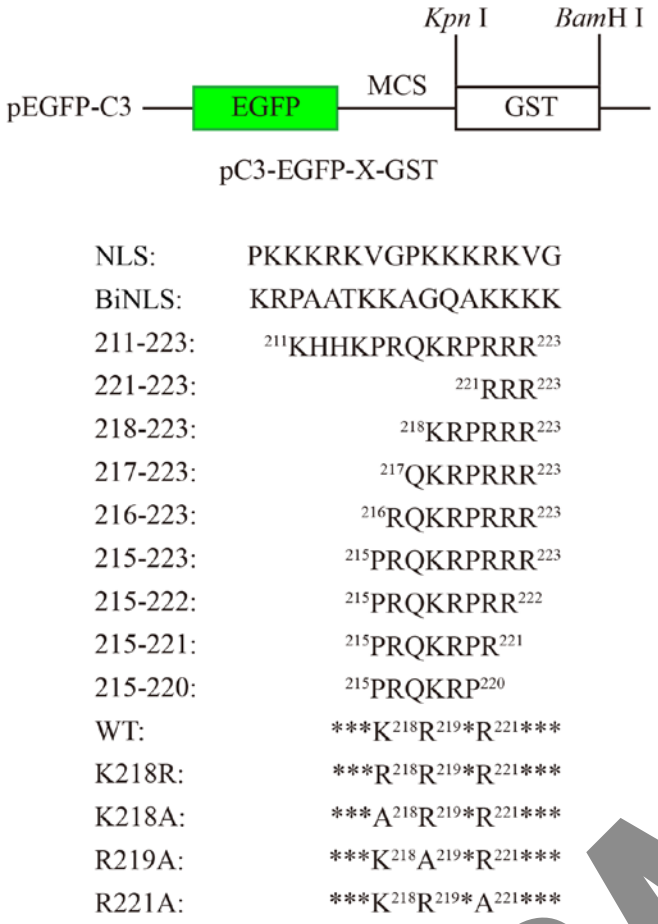

Figure 1. Schematic illustration of the pC3-EGFP-X-GST vector and the inserted amino acid sequences. The green rectangular box represents EGFP DNA; the blank rectangular box represents GST DNA; black lines represent pEGFP-C3 vector DNA. The name of the plasmid is shown underneath or on the left. MCS, multiple cloning sites; NLS, SV40 T antigen monopartite NLS; BiNLS, *Xenopus laevis* nucleoplasmin bipartite NLS; 211-223, 221-223, 218-223, 217-223, 216-223, 215-223, 215-222, 215-221 and 215-220: truncated Bel1, which are inserted at the MCS site; WT, wild-type Bel1; K218R, K218A, R219A and R221A: Bel1 mutants. Capitalized letters represent amino acid sequences; the numbers denote the amino acid position in Bel1 protein; asterisks indicate Bel1 amino acids.

ice-cold TB and soaked in TB for 10 min on ice. The complete transport solution contained import substrates (~2 μM), an adenosine triphosphate (ATP)-regenerating system (1 mM ATP, 5 mM creatine phosphate and 20 U/ml creatine phosphokinase) as a source of energy and some other soluble import factors. The import reaction was performed for 30 min at 37˚C or on ice in a humidified chamber. After the transport reaction, the cells were washed twice with ice-cold TB followed by fixation with 4% paraformaldehyde for 10 min on ice. The cells were washed 3 times first with TB and then twice with PBS. Following permeabilization with 0.2% Triton X-100 in PBS for 5 min on ice, the cells were blocked with 3% BSA + 5% fat-free milk in PBS and incubated with anti-EGFP antibodies and FITC-conjugated secondary antibodies as mentioned above. After being mounted on slides in PBS containing DAPI for 10 min on ice, the cells were visualized using an Olympus IX71 fluorescence microscope (Olympus).

Results

The NLS of Bel1 is ^{215}PRQKRPR^{221}. In order to accurately determine the NLS of Bel1, we inserted into the EGFP-GST fusion protein the truncated fragments of Bel1 that encompass the amino acid positions 211-223, 221-223, 218-223, 217-223, 216-223, 215-223, 215-222, 215-221 and 215-220 (Fig. 1) and observed their subcellular distribution by performing indirect IFA. The monopartite NLS of SV40 large T antigen (NLS) and the bipartite NLS of *Xenopus laevis* nucleoplasmin (BiNLS) were also inserted into EGFP-GST as positive controls for nuclear localization. As illustrated in Fig. 2, similar to the activity of SV40-NLS and the BiNLS, the 211-223 peptide of Bel1 enabled the nuclear localization of the fusion protein. In view of the fact that residues R^{221}R^{222}R^{223} are necessary for Bel1 nuclear distribution (10,13-17), we extended the N-terminal of the peptide segment to observe the effects. As shown in Fig. 2, the 221-223, 218-223, 217-223 and 216-223 fusion proteins still mainly distributed in the cytoplasm with little nuclear distribution, although containing the residues R^{221}R^{222}R^{223}. Until the N-terminal extended to residue P215, the fusion protein 215-223 localized to the nucleus (Fig. 2). We then shortened the C-terminal of 215-223 to continue our observation. As shown in Fig. 2, the both (215-222)- and (215-221)-containing EGFP-GST fusion proteins were distributed in the nucleus, whereas 215-220 was distributed in the cytoplasm. Taken together, these data suggest that peptide ^{215}PRQKRPR^{221} is the NLS of Bel1 and is essential for nuclear distribution.

The NLS of Bel1 is monopartite. Sequence analysis indicated that ^{215}PRQKRPR^{221} accords with the consensus sequence K(K/R) X(K/R) of monopartite NLS, comprised primarily of lysine (K) and arginine (R) residues (31), wherein the basic amino acids are critical. To confirm this result, we generated four mutations of the Bel1 protein sequence, named K218R that changed K^{218} to R^{218}, K218A that turned K^{218} into A^{218}, R219A that changed R^{219} to A^{219} and R221A that altered R^{221} to A^{221}, and then examined the subcellular distribution. As shown in Fig. 3, the K218R mutant and wild-type Bel1 (WT) were strictly localized to the nucleus in contrast to the K218A, R219A and R221A mutants that were detected predominantly in the cytoplasm. These results evidently prove that the nuclear localization sequence ^{215}PRQKRPR^{221} of Bel1 is monopartite and that residues K^{218}, R^{219} and R^{221} of Bel1 are essential for its nuclear accumulation.

Bel1 interacts with KPNA1, KPNA2, KPNA6 and KPNA7. In the conventional nuclear transport pathway, cargoes are recognized and bound by the transport receptor adaptor, importin alpha, to translocate to the nucleus (32,33). In this study, in order to determine which importins mediate the transportation of Bel1 into the nucleus, we detected the interaction between Bel1 and 7 isoforms of importin alpha (KPNA1, KPNA2, KPNA3, KPNA4, KPNA5, KPNA6 and KPNA7) and the most common importin beta protein, KPNB1, in 293T cells by *in vivo* GST pull-down assay. The results of western blot analysis revealed that Bel1 interacted with KPNA1, KPNA2, KPNA6 and KPNA7 solidly, as opposed to other isoforms of importin alpha or KPNB1 (Fig. 4). This suggests that Bel1 may use KPNA1, KPNA2, KPNA6 and KPNA7 to enter the nucleus.

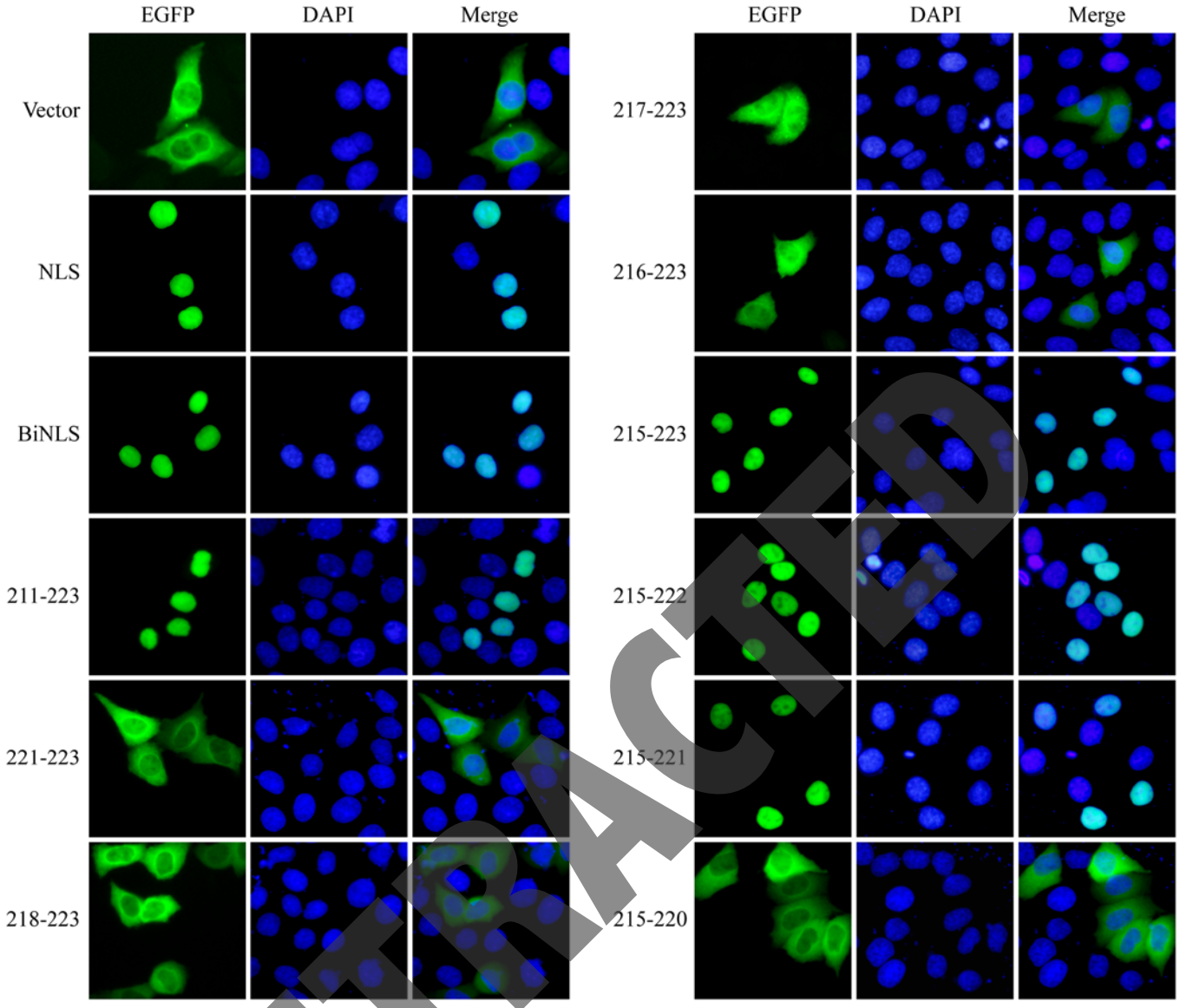

Figure 2. Subcellular distribution of Bel1 truncated mutants (x400 magnification). HeLa cells were transfected with pC3-EGFP-X-GST vector or fusion expression plasmids by polyethylenimines (PEI) and fixed 24 h post-transfection. The subcellular localization of EGFP-GST fusion proteins was visualized by indirect fluorescence microscopy assay. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Representative images are shown.

The NLS peptide of Bel1 interacts with KPNA1, KPNA6 and KPNA7 separately. Classical NLSs (cNLS) are directly recognized and bound by the adaptor protein importin alpha (34-36). To confirm this, we then determined the interrelation between truncated mutant 215-221, the NLS peptide of Bel1, and KPNA1, KPNA6 and KPNA7 in 293T cells by *in vivo* GST pull-down assay. As shown in Fig. 5, although KPNA2 bound to WT Bel1 (Fig. 4), truncated 215-221 did not interact with KPNA2. In accordance with the above findings, KPNA1, KPNA6 and KPNA7 bound solidly to the NLS sequence 215-221 of Bel1. These results further confirm that these three nuclear-import receptors are involved in the translocation of Bel1 into the nucleus.

KPNA1, KPNA6 and KPNA7 mediate the nuclear import of Bel1. To determine whether KPNA1, KPNA6 or KPNA7 can indeed mediate the nuclear import of Bel1, we finally carried out an *in vitro* nuclear import assay, but replaced the cytosol with recombinant transport receptors (Fig. 6). As a control for active transport, the SV40-NLS-containing EGFP-NLS-GST fusion protein was included in the same experiment. In contrast to KPNA2, KPNA1, KPNA6 and KPNA7 were sufficient for the nuclear accumulation of Bel1 (Fig. 6). The subcellular distribution of the positive control EGFP-NLS-GST was consistent with that previously reported (26,27): KPNA1, KPNA2 or KPNA6 were able to mediate SV40-NLS alone, while KPNA7 failed to do that. Taken together, these findings indicate that the efficient nuclear import of Bel1 in cells is mediated by KPNA1, KPNA6 and KPNA7 via the importin alpha/beta transport pathway.

Discussion

As a key positive regulator of viral gene expression, Bel1 contains a conventional NLS and is located in the nucleus to conduct its transactivational activity (11,37). Previous studies have confirmed the key role of $R^{221}R^{222}R^{223}$ in Bel1 nuclear

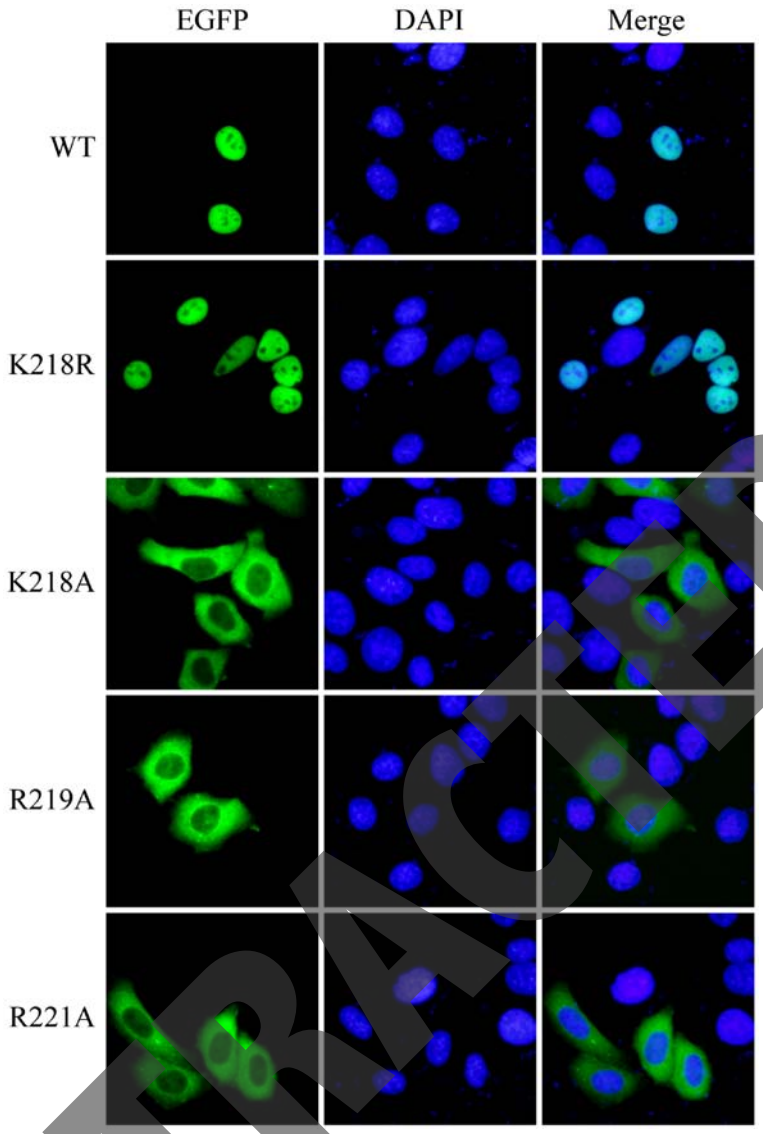

Figure 3. Subcellular distribution of Bel1 wild-type (WT) and mutants (x400 magnification). HeLa cells were transfected with pC3-EGFP-Bel1-GST plasmids WT, K218R, K218A, R219A or R221A by polyethylenimines (PEI) and fixed 24 h post-transfection. The subcellular localization of EGFP-GST fusion proteins was visualized by indirect fluorescence microscopy assay. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Representative images are shown.

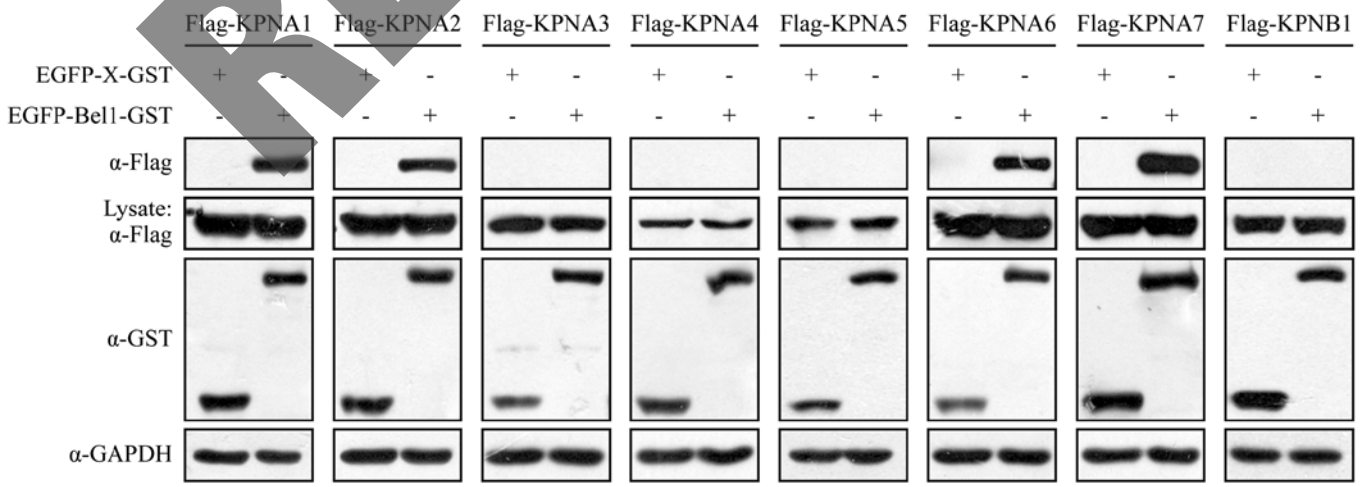

Figure 4. Interaction between Bel1 and KPNAs or KPNB1. 293T cells were transfected with pC3-EGFP-X-GST empty vector or pC3-EGFP-Bel1-GST plasmid along with plasmids that encode Flag-KPNAs or Flag-KPNB1; 48 h post-transfection, cell lysates were incubated with Glutathione Sepharose 4B beads for 4 h at 4˚C. Samples from both cell lysates and Glutathione Sepharose 4B beads were subjected to western blot analysis and probed with anti-Flag, anti-GST and anti-GAPDH antibodies. Representative results from 3 independent experiments are shown.

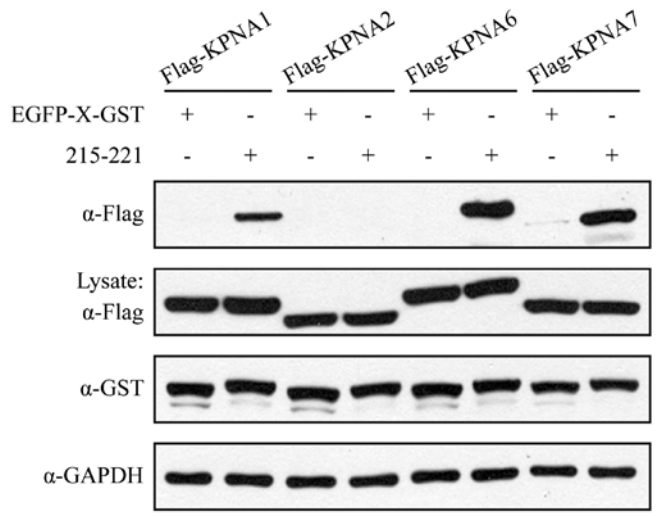

Figure 5. Interaction between 215-221 and KPNAs. 293T cells were transfected with pC3-EGFP-X-GST empty vector or the Bel1 truncated mutant 215-221, as for 215-221 in Fig. 1, along with plasmids encoding Flag-KPNAs; 48 h post-transfection, cell lysates were incubated with Glutathione Sepharose 4B beads for 4 h at 4˚C. Samples from both cell lysates and Glutathione Sepharose 4B beads were subjected to western blot analaysis and probed with anti-Flag, anti-GST and anti-GAPDH antibodies. Representative results from 3 independent experiments are shown.

localization, yet the accurate nuclear localization sequence is controversial and the adaptor-mediated Bel1 nuclear transport is unclear.

In this study, with the purpose of defining the peptide sequences that are essential for the nuclear distribution of Bel1, we introduced an EGFP-GST fusion expression system that has been widely utilized in studying the subcellular localization of retrovirus transactivators (38,39). With the Bel1 shortened mutant 211-223, we finally confirmed that ^{215}PRQKRPR^{221} is necessary and sufficient for the nuclear localization of Bel1. Furthermore, we found that residues K^{218}, R^{219} and R^{221} of Bel1 are indispensable for its nuclear accumulation by the results of mutagenesis experiments. Comprehensive analysis of the consensus sequence K(K/R)X(K/R) of monopartite NLS indicated that ^{218}KRPR^{221} is the core sequence of Bel1 NLS and the NLS of Bel1 is monopartite.

Consistent with the characteristics of the NLS sequence of mammalian cells, the residue K^{218} in the consensus sequence was replaced by the positive charge residue R, which had no change in the subcellular distribution. This suggests that the importance of the basic amino acid residues in the nuclear protein is closely related to the positive charge. That is to say, Bel1 may use a similar way to enter

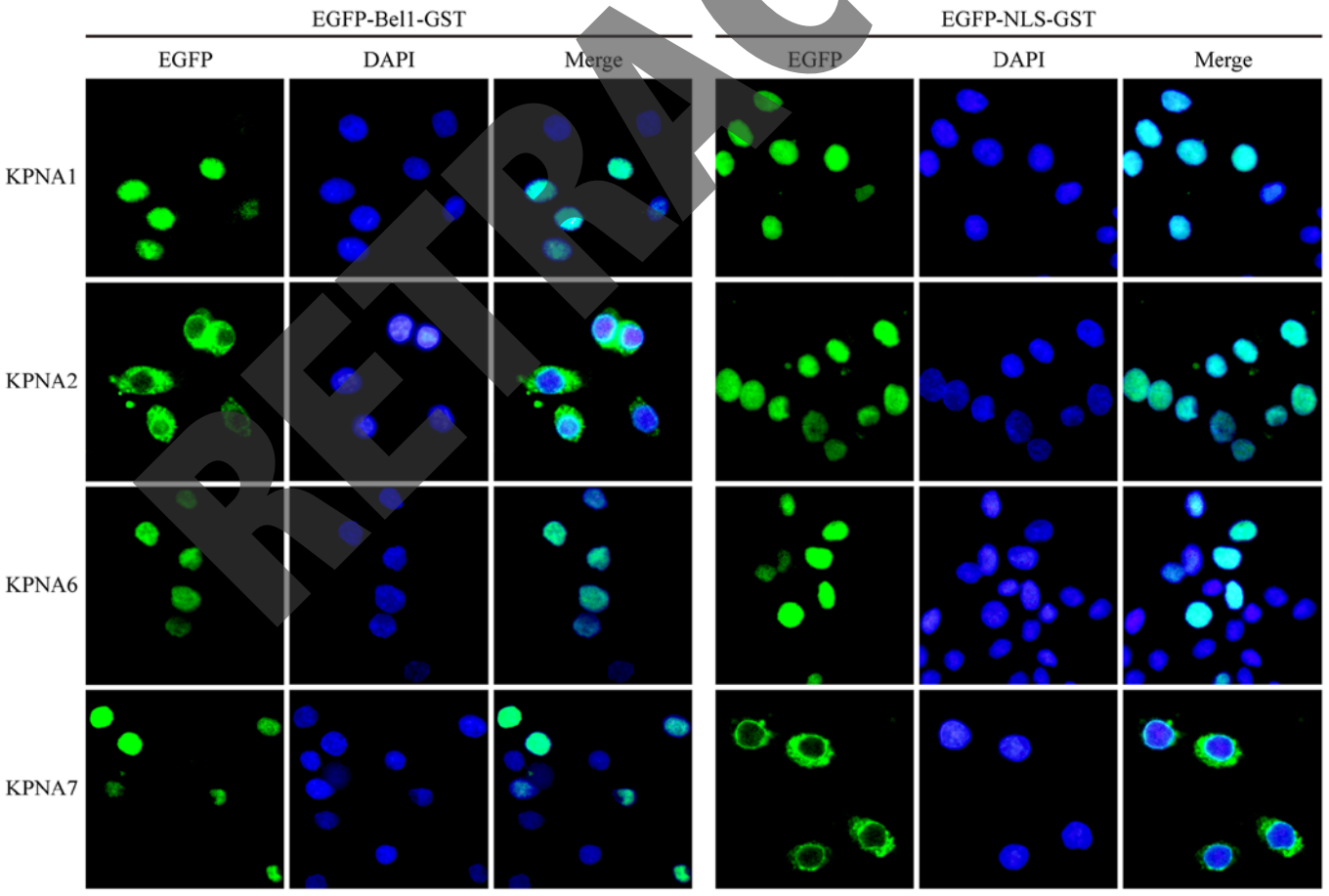

Figure 6. *In vitro* nuclear import assay using KPNAs (x400 magnification). Digitonin-permeabilized HeLa cells were washed with transport buffer (TB) and then incubated with ~2 μM either EGFP-Bel1-GST or EGFP-NLS-GST in 50 μl TB containing adenosine triphosphate (ATP) regeneration system, 2 μM His-RanGTP WT, 2 μM GST-KPNA1 or GST-KPNA2 or GST-KPNA6 or GST-KPNA7 used in combination with 2 μM GST-KPNB1. The cells were washed with TB and then fixed and stained with 4',6-diamidino-2-phenylindole (DAPI). The cells were visualized by indirect fluorescence microscopy assay. Representative images are shown.

the nucleus as a host cell transcription factor to complete its transactivational function.

NLSs are categorized into cNLSs and non-classical NLSs (ncNLS) (40). cNLSs are characterized by either monopartite (e.g., PKKKRRV from SV40 large T antigen) or bipartite (e.g., KRPAATKKAGQAKKKK from nucleoplasmin) stretches of basic amino acids (41,42). There is no consensus on whether different types of NLS have different biological functions. As RNA virus, the genome fidelity of foamy virus is lower than that of DNA genome. In the course of viral inheritance, monopartite NLS, less conserved nucleic acid sequence, may have some certain evolutionary advantages. In addition, monopartite NLS, shorter stretches of basic amino acids, may be more conducive to efficiently use of limited resources for virus.

Human KPNA isoforms are well conserved, with 26% identity and 42% conservation in their amino acid sequences (43,44). They can be divided into three subfamilies according to phylogenetic analysis: i) the α1 subfamily containing KPNA1, KPNA5 and KPNA6; ii) the α2 subfamily containing KPNA2 and KPNA7; and iii) the α3 subfamily containing KPNA3 and KPNA4. Although the α1 subfamily shares a maximum of 82.1% identity and 82% sequence conservation (26), their affinity for Bel1 differed markedly, which may due to the restriction of KPNA5 expression to the testes (45), in our GST pull-down experiments using 293T cells. The α2 subfamily is the least conserved of the KPNA subfamilies, with 55% identity and 71% conservation (24). In addition, phylogenetic analysis of the ARM repeats, responsible for identifying and combining with the NLS of cargo proteins (36), of the KPNAs shows that the KPNA7 ARM repeats is more divergent than that of KPNA2 (26). To a certain extent, this explains the different performance of KPNA2 and KPNA7. Besides, the combination between KPNA2 and Bel1 may be involved in the other amino acids apart from the NLS, and/or the peptide ^{215}PRQKRPR^{221} is not sufficient to mediate the binding between the two. It is thus revealed that KPNA2 may participate in other biological functions of Bel1 except nuclear transport.

The present study provided evidence that KPNA1, KPNA6 and KPNA7 may be 'hijacked' by PFV Bel1 for efficient nuclear import and viral replication. Given this fact, the restricted expression of KPNA isoforms may provide a mechanism for the suppression of PFV replication and disease progression.

Acknowledgements

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