Identification and characterization of complex dual nuclear localization signals in human bocavirus NP1

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Human bocavirus (HBoV), closely related to canine minute virus (MVC) and bovine parvovirus (BPV), is a new member of the Bocavirus genus within the Paroviridae family. The non-structural protein NP1 of HBoV is a nuclear localized protein and plays an important role in DNA replication as well as in the evasion of host innate immunity. In the current study, we provide the first evidence that NP1 possesses a non-classical nuclear localization signal (ncNLS) (amino acids 7–50). Embedded within this ncNLS is a classical bipartite nuclear localization signal (cNLS) (amino acids 14–28), capable of transporting a heterologous cytoplasmic protein β-galactosidase fusion protein (β-gal-EGFP) to the nucleus via the classical importin α/β1-mediated pathway. Amino acids 7–50 containing the cNLS and the ncNLS of NP1 or full-length NP1 interact with importin α1, importin β1 and importin β1Δ, which lacks the importin α binding domain, indicating that the nuclear import of NP1 is through both conventional importin α/β1 heterodimer- and non-classical importin β1-mediated pathways. Given that the arrangement of a cNLS embedded within an ncNLS is unusual in viral proteins, our data together reveal a novel molecular mechanism underlying the nuclear import of HBoV NP1, providing a basis for further understanding its biological function.

INTRODUCTION

Human bocavirus (HBoV), identified in 2005 by molecular screening of respiratory tract samples, is classified in the Bocavirus genus (family Paroviridae, subfamily Parovirinae) (Allander et al., 2005). Recently, additional human bocaviruses, HBoV2, HBoV3 and HBoV4, have been found mainly in human stool specimens (Arthur et al., 2009; Kapoor et al., 2009; Kapoor et al., 2010). HBoV infections have been detected worldwide mainly in respiratory samples, but also in serum, faecal and urine samples. Clinical symptoms in HBoV infected patients include cough, pharyngitis, wheezing, dyspnoea, rhinitis, acute otitis media, fever, pneumonia, diarrhoea, vomiting and nausea [reviewed by Jartti et al. (2012)]. The genome of HBoV is a single-stranded DNA, approximately 5.3 kb with still unknown terminal sequences, encoding non-structural proteins NS1, NS1-70 and NP1 (unique to bocaviruses) and structural proteins VP1 and VP2 (Allander et al., 2005; Chen et al., 2010). The functions of HBoV proteins and the pathogenesis of HBoV are poorly understood because neither an efficient in vitro culture system nor an animal model has been established so far.

Bocavirus replication occurs within the nuclei of infected cells. The NP1 of HBoV is a nuclear protein (Chen et al., 2010), playing important roles in HBoV DNA replication (Sun et al., 2009) and in the inhibition of IFN-β production (Zhang et al., 2012). Given that subcellular localization of a protein is closely related to its function (Bouyac-Bertoia et al., 2001; Grieger et al., 2006; Li et al., 2012; Vihinen-Ranta et al., 2002) and that characterization of the subcellular localization of a protein is often used as a first step towards further characterizing its function [reviewed by Simpson et al. (2001)], we attempted to understand how NP1 enter the nucleus and the consequences. Transporting of large proteins into the nucleus is usually mediated by the nuclear localization signals (NLSs). Although there is no unique consensus sequence for NLSs, most can be classified into classical NLSs (cNLSs) which contain a single (monopartite) or two short stretches of basic amino acids spaced by 10–12 residues (bipartite). The NLSs of SV40 large-T antigen (PKKKRKV) and nucleoplasmin (KRPAATKKAGQAKKKK) are the prototypical monopartite and bipartite cNLSs, respectively (Kalderon et al., 1984; Robbins et al., 1991). NLSs lacking the stretches of basic amino acids are classified into non-classical NLSs (ncNLSs). For example, the M9 sequence of the human mRNA-binding protein hnRNP A1 is a well-known ncNLS (Siomi & Dreyfuss, 1995). In general, cNLSs are mediated by importin α/β1 heterodimer while ncNLSs

Two supplementary tables are available with the online version of this paper.
are directly recognized by importin β1 or other members of the importin β family without the involvement of importin α [reviewed by Pouton et al. (2007); Sorokin et al. (2007)].

Herein, we report that the amino acids 7–50 of NP1 contain a cNLS and an ncNLS, which can transport NP1 into the nucleus via both classical importin α/β1 heterodimer and non-classical importin β1 mediated pathways. Transport of NP1 via dual nuclear import pathways may play important role in HBoV infection.

RESULTS

NP1 contains a bipartite cNLS, and both of the basic amino acid clusters are indispensable

Analysis of the full-length NP1 by Motif Scan (http://myhits.isb-sib.ch/cgi-bin/motif_scan) revealed one putative cNLS at residues 14–28 (KRKGSPERGERKKRH) which closely matches the consensus sequence (KR-X10-12-KRRK) for bipartite cNLSs that may interact with importins. To determine the functionality of the candidate cNLS, we constructed the full-length NP1, the cNLS sequence aa14–28 and the SV40NLS (120PKKKKKV) as a positive control into β-gal-EGFP, respectively (Fig. 1a). The resulting constructs were transfected into HeLa cells and the distribution of EGFP fusion proteins was monitored 24 h post-transfection. A quantitative evaluation is summarized in Table S1 (available in JGV Online). While the control vector β-gal-EGFP showed exclusive cytoplasmic localization (100 % of the observed cells; Fig. 1b, panels 1–3), the full-length NP1 displayed exclusive nuclear distribution (100 %; Fig. 1b, panels 7–9), indicating the presence of an active NLS. The fragment aa14–28 (cNLS) transported β-gal-EGFP to the nuclei more efficiently (100 %; Fig. 1b, panels 10–12) than the SV40NLS which displayed completely nuclear (3 %; Fig. 1c, panels 4–6) or mainly nuclear (9 %; arrow 2), or almost equal nucleo-cytoplasmic distribution (59 %; arrows 3), suggesting that the predicted cNLS of NP1 is a more powerful NLS than SV40NLS.

In order to determine the critical basic amino acids involved in cNLS, four cNLS mutants (termed cNLSm) were generated by replacing the basic amino acids arginine and lysine with the neutral amino acid alanine and then cloned into β-gal-EGFP (Fig. 1a). As shown in Fig. 1(c), mutation of any one of the basic amino acid clusters (cNLSm1 and cNLSm3) resulted in a significant reduction of the nuclear transport function of cNLS, as manifested in cytoplasmic distribution (72 % or 51 %, respectively, Table S1). As expected, mutation of both basic amino acid clusters completely abolished the nuclear translocation activity of cNLS as shown by the 100 % cytoplasmic distribution (Table S1) in the cells transfected with the β-gal-cNLSm4-EGFP fused plasmid (Fig. 1c, panels 10–12).

In contrast, mutation of the basic amino acids within the spacer sequence showed no alteration to cNLS function (100 %; Fig. 1c, panels 4–6). These results indicate that both basic amino acid clusters of cNLS are required for efficient nuclear import.

cNLS of NP1 is importin α/β1-dependent

The nuclear import of cNLS is mediated by the importin α/β1 heterodimer pathway. To verify if the cNLS of NP1 targets protein into the nucleus through importin α/β1 heterodimer pathway, we first performed confocal microscopy to monitor the localization of β-gal-cNLS-EGFP in the presence of the RFP-fused Bimax2 peptide (inhibitor of importin α1, α3, α6 and α7) (Kosugi et al., 2008) and then conducted a coimmunoprecipitation (Co-IP) assay to determine the interaction between cNLS and importins. As shown in Fig. 2(a), β-gal-cNLS-EGFP displayed an exclusively cytoplasmic localization in cells expressing a high level of RFP-Bimax2 (arrow 1), unlike its nuclear and cytoplasmic localization in cells expressing a low level of RFP-Bimax2 (arrow 2), indicating that RFP-Bimax2 inhibited cNLS in a dose-dependent manner. Since Bimax2 is an inhibitor specific for importin α1, α3, α6 and α7, it is reasonable to conclude that the cNLS of NP1 is likely to be an importin α/β1-dependent NLS. To further confirm interaction of cNLS-importins, we conducted a Co-IP assay. As shown in Fig. 2(b), a strong interaction between cNLS and importin α1 was observed (lane 2), whereas the importin-deficient cNLS mutant (cNLSm4) did not bind importin α1 (lane 8). An association between cNLS and importin β1 or importin β1A [amino acids 1–462 of importin β1, lacking the importin α binding domain (Kutay et al., 1997)] was barely detectable (lane 4 and lane 6). Taken together, these data indicate that the cNLS of NP1 can be recognized by importin α1 and the basic amino acids within the cNLS play important role in the recognition.

Identification of the second NLS in NP1

Although our results indicated that the cNLS of NP1 was active in the context of β-gal-cNLS-EGFP fused protein, it remained to be examined whether NP1 cNLS is required for nuclear transport of NP1. We therefore constructed β-gal-NP1cNLSm-EGFP (full-length NP1 with inactivated cNLS) and β-gal-NP1cNLSm-EGFP (NP1 without cNLS) (Fig. 3a), and then investigated their localization. Surprisingly, both NP1cNLSm and NP1cNLSm-EGFP retained a majority of the function to target β-gal-EGFP into the nuclei (Table S1; Fig. 3b, panels 1–3, panels 4–6). These results imply that NP1 may contain a NLS in addition to a cNLS.

Truncations based on full-length NP1cNLSm were subsequently made and constructed into β-gal-EGFP (Fig. 3a). When examined by confocal microscopy, the two truncations aa1–13 and aa29–219 flanking the cNLS exhibited exclusively cytoplasmic accumulation (100 %, Table S1; Fig. 3c, panels 1–3 and panels 4–6), whereas the truncation aa1–50 manifested clearly nuclear accumulation (100 %; Fig. 3c, panels 7–9), suggesting that truncation aa1–50
contains an active NLS. To narrow down the essential region containing NLS, more truncation mutants were constructed. Data from confocal microscopy showed that more than half of the observed cells transfected with the aa1–41 and aa7–13 + aa29–50 fused plasmids displayed nucleo-cytoplasmic distribution (79% or 52%, respectively, Table S1; Fig. 3c, panels 10–12, panels 19–21). However, aa7–50 fused β-gal-EGFP displayed exclusively nuclear localization (100%; Fig. 3c, panels 13–15). Furthermore, NP1 lacking aa7–50 failed to target β-gal-EGFP into the nuclei (100%; Fig. 3c, panels 16–18). Collectively, we identified a functional NLS located in aa7–50 of NP1, which is independent of the nuclear import function of the embedded cNLS (aa14–28).

Fig. 1. Identification of a cNLS in HBoV NP1. (a) Schematic representations of NP1, cNLS (basic amino acids in boldface), cNLS mutants (basic amino acids substituted by alanine in blue) and SV40NLS fused to β-gal-EGFP. Corresponding cellular protein localization is shown in the right column, where ‘N’ indicates nuclear localization, ‘C’ indicates cytoplasmic localization and ‘D’ indicates diverse intracellular distribution. (b)(c) HeLa cells were transfected with SV40NLS, NP1, cNLS, control plasmid β-gal-EGFP or the four cNLS mutants. At 24 h post-transfection, cells were fixed and the nuclei were stained with Hoechst 33258 followed by confocal microscopy. Each image is representative of the majority of cells observed in several fields. The results shown are representative of two independent experiments. Scale bar = 11 μm. Original magnification = ×600. A quantitative evaluation is given in Table S1.
Amino acids 7–50 of NP1 are a non-classical NLS

To determine the nuclear import pathway utilized by NP1cNLSm7–50, Bimax2 (inhibitor of classical NLS) and M9M (inhibitor of PY-NLS) (Cansizoglu et al., 2007) were used. NP1cNLSm7–50 completely transported β-gal-EGFP into the nuclei even in the presence of Bimax2 or M9M, implying that NP1cNLSm7-50 is not a classical NLS or PY-NLS (Fig. 4, panels 1–4, panels 9–12). Similar to NP1cNLSm7–50, full-length NP1 was also not sensitive to Bimax2 or M9M (Fig. 4, panels 5–8, panels 13–16) although the function of NP1 cNLS was inhibited by Bimax2 (Fig. 2a). These data further confirmed that the N-terminal amino acids 7–50 of NP1 indeed contain a non-classical NLS.

The N-terminal amino acids 7–50 of both NP1 and the full-length NP1 directly interact with importin α1 and importin β1

To further elucidate the mechanism employed by NP1 and aa7–50 of NP1 for nuclear import, plasmids encoding NP1cNLSm7–50 or full-length NP1 were co-transfected with constructs encoding importin α1, importin β1 or importin β1Δ into 293T cells, and the cell lysates were utilized for Co-IP assays. As shown in Fig. 5(a), aa7–50 of NP1 strongly interacted with importin α1 and importin β1 (lane 2, lane 4), as well as importin β1Δ (lane 6). Given that importin β1Δ does not contain the importin α binding domain, it is reasonable to conclude that ncNLS (NP1cNLSm7–50) can bind importin β1 without the involvement of importin α1. Similar results were found when full-length NP1 was used, showing that NP1 interacted with importin α1, importin β1 and importin β1Δ (Fig. 5b, lane 2, lane 4, lane 6). In conclusion, our data indicate that the N-terminal amino acids 7–50 of NP1 play a key role in targeting full-length NP1 through the classical importin α/β1-mediated pathway and the non-classical importin β1-dependent pathway.

DISCUSSION

In this study, we identified aa7–50(7KDKHRSY14KRK GSPERGEKRKRHRW28QTTHRSSRSPHRHSGERGSG39) of HBoV NP1 as dual NLSs containing a bipartite cNLS (14KRKGSPERGEKRKRHRW28) and an ncNLS, which can efficiently transport full-length NP1 and β-gal-EGFP into the nucleus. In particular, the bipartite cNLS is embedded in the ncNLS, which is rich in basic amino acids arginine (R), lysine (K), and neutral amino acid glycine (G). When all the basic amino acids in the embedded bipartite cNLS were substituted by the neutral amino acid alanine (A), NP1 ncNLS still exerted exclusively nuclear import function (Fig. 3c, panels 13–15), suggesting that such function is independent of the basic amino acids in the embedded bipartite cNLS. However, compared with the NP1cNLSm7-50 (100 % Table S1), only 48 % (Table S1) of the observed cells transfected with aa7–13 + aa29–50 fused β-gal-EGFP plasmid without the cNLS (NP1cNLSm7-29) displayed nuclear distribution (Fig. 3c, panels 19–21), suggesting that the non-basic amino acids in the cNLS may play a role to some extent. The arrangement of a bipartite cNLS embedded within an ncNLS in HBoV NP1 is unusual. To date, such arrangement has only been discovered in a few viral proteins, one of which is the complex NLS (aa1–51) within the ORF73 latency-associated nuclear antigen (LANA) of the Kaposi’s sarcoma-associated herpes virus (Cherezova et al., 2011). Similar to the aa7–50 of HBoV NP1, aa1–51 of LANA is a RG-rich ncNLS with an embedded KR-rich bipartite cNLS (aa24–47).

In general, nuclear import of most proteins is mediated by importin βs. The majority of importin βs, including the extensively studied importin β1, interact directly with their cargoes and sometimes via an adaptor protein importin α. In mammals, there are at least six isoforms of importin α classified into three distinct subfamilies: the Rch1 subfamily (importin α1/Rch1), the Qip1 subfamily (importin α3/Qip1 and importin α4/Qip2) and the NP1-1 subfamily (importin α5/NP1-1, importin α6 and importin α7/NP1-2) based on their sequence similarity (Goldfarb et al., 2004; Ushijima et al., 2005). Our results of nuclear import inhibition and Co-IP assays demonstrated that bipartite...
cNLS of NP1 is an importin α/β1-mediated NLS and can be recognized by importin α1, suggesting that bipartite cNLS plays a role in targeting NP1 into the nucleus through the classical import pathway. Amino acids 7–50 of NP1 and full-length NP1 both interacted with importin α1, importin β1 and importin β1Δ, indicating that aa7–50 can mediate NP1 into the nucleus through dual nuclear import pathways. NP1Δ7-50 and NP1 bound to importin β1 and importin β1Δ more strongly than to importin α1 (Fig. 5), indicating that NP1 may primarily utilize the non-classical importin β1 mediated pathway, although we cannot rule out that NP1 may also interact with other members of importin α. Many viral proteins have been shown to use either the classical importin α/β1 pathway or the non-classical importin β-dependent pathway for nuclear import. The capability of HBoV NP1 to use both classical and non-classical nuclear import pathways via the same motif likely has biological significance during HBoV infection.

Fig. 3. NP1 possesses a NLS in addition to a cNLS. (a) Schematic representations of full-length NP1 with all basic amino acids (in boldface) substituted by alanine (in blue) in cNLS (NP1cNLSm), NP1 without cNLS and seven deletion mutants fused with β-gal-EGFP. Corresponding cellular protein localization is shown in the right column, where ‘N’ indicates nuclear localization, ‘C’ indicates cytoplasmic localization and ‘D’ indicates diverse intracellular distribution. (b) HeLa cells were transfected with the indicated constructs. At 24 h post-transfection, cells were fixed and the nuclei were stained with Hoechst 33258 followed by confocal microscopy. Each image is representative of the majority of cells observed in several fields. The results shown are representative of two independent experiments. Scale bar = 11 μm. Original magnification = ×600. A quantitative evaluation is given in Table S1.
NLS regions have also been identified in other paroviruses, such as the bipartite cNLS\(^{194KK-X_{18}KKK^{216}}\) within NS1 (Nüesch & Tattersall, 1993) and cNLSs and ncNLS within capsid proteins of minute virus of mice (Lombardo\textit{et al.}, 2000; Lombardo\textit{et al.}, 2002), the cNLS\(^{6PKARRARGYK^{13}}\) within VP1 of canine parvovirus (Vihinen-Ranta\textit{et al.}, 1997, 2002) and the cNLS\(^{23PARKRL^{34}}\) within VP2 (Hoque\textit{et al.}, 1999) and separate basic region motifs within the capsid proteins of adeno-associated virus (Grieger\textit{et al.}, 2006), and the ncNLS within B19 major capsid protein VP2 (Pillet\textit{et al.}, 1999). Nuclear import pathways, the classical importin-\(\alpha\) mediated nuclear import of NP1 in infected cells through the dual nuclear import of NP1, our study suggests that the dual nuclear import of HBoV NP1 may provide information for understanding the nuclear import pathways and the functions of NP1 in BPV and MVC.

Given that the relative abundance of NP1 transcript is the highest among all HBoV transcripts (Chen\textit{et al.}, 2010) and that NP1 protein plays important roles in HBoV DNA replication and in the inhibition of IFN-\(\beta\) production, efficient transport of NP1 to the nucleus is highly likely to be essential to its function. By addressing the mechanism of nuclear import of NP1, our study suggests that the dual NLSs (aa7–50) of HBoV NP1 likely ensure an efficient nuclear transport of NP1 in infected cells through the dual nuclear import pathways, the classical importin \(\alpha/\beta\)- and the non-classical importin \(\beta\)-mediated nuclear import pathways.

**METHODS**

**Cells culture and transfection.** The human embryonic kidney 293T cells and HeLa cells were cultured in Dulbecco’s modified Eagle’s medium (Gibco) supplemented with 10% FBS (Life Technologies) at 37 °C with 5% \(\text{CO}_2\). Lipofectamine\textsuperscript{TM} 2000 (Invitrogen) and ProFection (Promega) were used for transfection in HeLa and 293T cells, respectively.

**Plasmid constructs.** All constructs were confirmed by DNA sequencing. The oligonucleotide sequences used in this study are summarized in Table S2. The coding sequence of \(\beta\)-galactosidase was amplified by PCR using pSV-\(\beta\)-galactosidase control vector (Promega) as template and cloned into the Bgl\textit{III}-Hind\textit{III} sites of pEGFP-N1 vector (Clontech) to generate pEGFP-\(\beta\)-galactosidase-N1 (termed \(\beta\)-gal-EGFP in Fig. 1). The NP1 gene was amplified from HBoV1 genomic DNA by PCR. Oligonucleotides encoding cNLS\(^{1KKRSGPRGERKKRH^{13}}\), cNLS\(_{m1}\) (K14A/R15A/K16A), cNLS\(_{m2}\) (R21A), cNLS\(_{m3}\) (R24A/K25A/R26A), cNLS\(_{m4}\) (K14A/R15A/K16A/R21A/R24A/K25A/R26A), SV40NLS\(^{1KKKRRKQV^{12}}\) and NP\(_{11-13}\) (N-terminal 13 amino acids of NP1) were synthesized, annealed and constructed into the Hind\textit{III}-Pst\textit{I} sites of \(\beta\)-gal-EGFP, respectively. To construct NP\(_{1-cNLSm}\) (full-length NP1 with inactive cNLS, K14A/R15A/K16A/R21A/R24A/K25A/R26A), two partially overlapping intermediate primers both with mutated cNLS and two terminal primers were used to amplify NP\(_{1-cNLSm}\) fragment without a stop codon. The amplified NP\(_{1-cNLSm}\) fragment was inserted into \(\beta\)-gal-EGFP. A similar method was used for constructing NP\(_{1\Delta}\) (full-length NP1 lacking cNLS and NP\(_{1\Delta,50}\) (full-length NP1 without 7–50 aa). Fragment coding NP\(_{1\Delta,29\rightarrow\Delta}\), NP\(_{1\Delta\text{CNSm1-50}}\), NP\(_{1\Delta\text{CNSm1-41}}\), NP\(_{1\Delta\text{CNSm7-50}}\) or NP\(_{1\Delta,13-29\rightarrow\Delta}\) (amino acids flanking cNLS within 7–50 aa) were also amplified and inserted into \(\beta\)-gal-EGFP. Plasmids RFP-Bimax2 and RFP-M9M were gifts from Nobuguki Nukina (RIKEN Brain Science Institute, Japan) (Kino\textit{et al.}, 2011).

For coimmunoprecipitation assays, Imp \(\alpha\)-Flag, Imp \(\beta\)-Flag and Imp \(\beta\)-Flag (lacking the Imp \(\alpha\)-interactive motif) were amplified from human cDNA with primers with a FLAG tag and a stop codon, respectively, and inserted into the Sac\textit{I}-Sal\textit{I} sites of pEGFP-N1. The annealed double-stranded oligonucleotides encoding the HA tag was

**Fig. 4.** ncNLS is not sensitive to nuclear import inhibitors Bimax2 and M9M. The cellular distribution of NP\(_{1-cNLSm7-50}\) and full-length NP1 co-transfected with RFP-Bimax2 or RFP-M9M was confirmed in HeLa cells by confocal microscopy. Each image is representative of the majority of cells observed in several fields. The results shown are representative of two independent experiments. Scale bar=11 µm. Original magnification = ×600.
lysation buffer (Beyotime Institute of Biotechnology, China) supplemented with 1 mM protease inhibitor PMSF for immunoprecipitation and immunoblotting. After centrifugation, the supernatant was incubated with corresponding antibodies plus protein G magnetic beads (Invitrogen) for 20 min. After four washes, the immunoprecipitates were eluted by boiling for 10 min in SDS protein loading buffer. Proteins were separated by SDS-PAGE and transferred onto PVDF membranes (Millipore). Immunoblotting was performed with corresponding antibodies.

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**Fig. 5.** Both NP17-50 of NP1 and full-length NP1 can interact with importin α1, importin β1 and importin β1Δ. Co-IP assays of NP17-50 (a) or full-length NP1 (b) and importins. 293T cells were co-transfected with β-gal-NP17-50-HA or full-length NP1 and Impα1-Flag, Impβ1-Flag or Impβ1Δ-Flag expression plasmid. 30 h post-transfection, cells were harvested, lysed and then the lysates were subjected to Co-IP. The results shown are representative of three independent experiments.

Fluorescence microscopy assay. HeLa cells were grown on glass-bottomed cell culture dishes (Wuxi NEST Biotechnology, China) followed by transfection with the corresponding plasmids. At 24 h post-transfection, cells were washed with PBS, fixed with 4% paraformaldehyde for 15 min, washed, permeabilized with 0.2% Triton X-100 in PBS for 15 min at room temperature and then the nuclei were stained by Hoechst 33258 (Beyotime Institute of Biotechnology, China). After three times washes (5–10 min per time), fluorescent images were taken with a confocal microscope.

Co-immunoprecipitation and immunoblot assays. Transfected 293T cells were harvested at 30 h post-transfection and lysed in cell


