New regulatory mechanisms for the intracellular localization and trafficking of influenza A virus NS1 protein revealed by comparative analysis of A/PR/8/34 and A/Sydney/5/97

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During influenza A virus infection, the NS1 protein is engaged in different functions in different intracellular compartments. In this study, we showed that the NS1 of A/PR/8/34 localized in different positions from that of A/Sydney/5/97 when transiently expressed in Madin–Darby canine kidney cells. Residue 221 of NS1 was identified to be a new key residue involved in the C-terminal nuclear localization signal (NLS) and nucleolar localization signal (NoLS) of NS1 from A/Sydney/5/97. Analysis of chimeric NS1 and further mutants showed that residues responsible for the binding between NS1 and the cleavage and polyadenylation specificity factor (CPSF) are correlated with the intracellular localization of transiently expressed NS1 proteins. Fluorescence loss in photobleaching imaging revealed that the NS1 protein with both functional NLSs and nuclear export signal (NES) was able to shuttle between the nucleus and cytoplasm. Drug inhibition experiments and fluorescence resonance energy transfer analysis suggested that NS1 was exported out of the cell nuclei via a Crm1-independent pathway. Moreover, it is likely that another cytoplasmic localization-related sequence exists in the NS1 protein other than the leucine-rich NES. These findings provide new insights into the mechanism of intracellular localization and trafficking of influenza A virus NS1 protein, which is important for understanding its function.

INTRODUCTION

The NS1 protein of influenza A virus, encoded by the eighth segment of influenza A virus genome, is a multi-functional protein that plays important roles in viral infection. NS1 has different functions in different cellular compartments. For example, in the nucleus, NS1 can bind to cleavage and polyadenylation specificity factor (CPSF) and PABII to inhibit the post-transcriptional processing of host pre-mRNA, while in the cytoplasm, NS1 can bind to the cytoplasmic enzyme protein kinase R to inhibit its activation (Chen et al., 1999; Nemeroff et al., 1998). Therefore, the intracellular localization and trafficking of the NS1 protein is important for understanding the viral infection.

Previous studies revealed that the localization of NS1 is controlled by two basic amino acid-rich nuclear localization signals (NLSs), a leucine-rich nuclear export signal (NES) and a nucleolar localization signal (NoLS) included in the C-terminal NLS (Li et al., 1998; Melén et al., 2007). The NES of NS1 can be regulated by its downstream adjacent inhibitory sequence (Li et al., 1998). Recently, it was found that one specific NS1 protein has different localization patterns in mammalian and avian cells (Volmer et al., 2010), which makes the localization-related mechanism of NS1 protein more interesting.

In the present study, we found that the NS1 of A/PR/8/34 (H1N1) localized in different positions from that of A/Sydney/5/97 (H3N2) when transiently expressed in Madin–Darby canine kidney (MDCK) cells, and then the intracellular localization and trafficking of the NS1 protein was analyzed by live-cell imaging. A novel key residue of NLS and NoLS was discovered and the contribution of different basic residues in the NLS was evaluated. Residues responsible for the interaction of NS1 and CPSF were found to be related to the localization of NS1 protein in our experiment settings. A series of experiments concluded that the leucine-rich NES of NS1 protein functions in a Crm1-independent manner. This report provides new insights into the mechanism of intracellular localization and trafficking of influenza A virus NS1 protein.

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RESULTS

NS1 of A/PR/8/34 localizes in different positions from that of A/Sydney/5/97 when transiently expressed in MDCK cells

The NS1 proteins of many influenza A virus strains such as A/Sydney/5/97 (Fig. 1a) localize in the nucleus when expressed in mammalian cells without infection (Hayman et al., 2006; Li et al., 1998; Melén et al., 2007). However, our experiment showed that NS1 of A/PR/8/34 primarily displayed a cytoplasmic distribution pattern (Fig. 1a) when expressed as a recombination protein fused to EGFP in MDCK cells, markedly different from the nuclear localization pattern of NS1 of A/Sydney/5/97 strain. Moreover, the NS1 protein of A/PR/8/34 did not show any nucleolar localization, but the NS1 protein of A/Sydney/5/97 could localize in the nucleolus (A/Sydney/5/97 NS1 co-localized with the nucleolar protein B23) in about 50% of transfected cells expressing the GFP–NS1 fusion protein.

To exclude the possibility that GFP-tag fused to the N-terminal of the NS1 protein affects intracellular localization of the NS1 protein, immunofluorescence of fixed cells transfected with pcDNA3.1-NS1-Syn or pcDNA3.1-NS1-PR/8 by using NS1-specific antibodies was performed, and the results showed the same localization patterns as GFP–NS1 fusion proteins in live cells (Fig. 1b). Moreover, for cells expressing the GFP–NS1 fusion proteins, Western blotting of the cell lysate with an antibody against GFP

Fig. 1. Influenza A virus NS1 of A/PR/8/34 localizes in different positions from that of A/Sydney/5/97. (a) MDCK cells were transiently co-transfected with EGFP–NS1 constructs and dsRed–B23 constructs simultaneously. B23, a nucleolar protein, was fused to dsRed and expressed in MDCK cells to indicate the nucleolus. (b) Immunofluorescence imaging of MDCK cells transiently expressed untagged NS1 proteins by using anti-NS1 antibody and FITC-labelled secondary antibody. (c) Western blotting assay for EGFP–NS1 fusion proteins by using anti-EGFP antibody. The cells were stained with Hoechst 33258 before imaging to indicate the nucleus. Bar, 10 μm. (d) NLS and NES amino acid sequence comparisons between influenza virus A/PR/8/34 and A/Sydney/5/97. Non-conserved residues are underlined.
showed only a band of the GFP–NS1 protein (Fig. 1c), which implied that there was no obvious degradation of the NS1 portion of the GFP–NS1 fusion proteins and results of live-cell imaging of GFP–NS1-expressed cells should show localization of intact GFP–NS1.

**Aa 221 is a key residue for the nuclear and nucleolar targeting of influenza A virus NS1 protein from A/Sydney/5/97**

Sequence alignment and analysis were performed for the two NS1 proteins from A/PR/8/34 and A/Sydney/5/97 strains (Fig. 1d). These analyses revealed that NS1 from both A/PR/8/34 and A/Sydney/5/97 contained the conserved N-terminal NLS (NLS1), NES and the regulatory sequence after the NES. However, residues 224 and 229 in the C-terminal NLS (NLS2) of the A/PR/8/34 NS1 are not basic amino acids, which are different from that of A/Sydney/5/97 or other conserved NLS2 and NoLS of NS1 proteins (Melén *et al.*, 2007). The difference of these two residues might be responsible for the cytoplasmic localization of A/PR/8/34 NS1 protein. To this end, we constructed a mutant A/PR/8/34 NS1 protein with G224R and E229K mutations and inserted it into the pEGFP–C1 expression vector. Surprisingly, this mutated NS1 (EGFP–mtNS1) EGFP fusion protein did not show exclusive nuclear localization or any nucleolar localization as expected (Fig. 2a). Therefore, other residue or residues may also be responsible for the nuclear and nucleolar localization of the NS1 protein.

Residue 221 of the NS1 protein of A/PR/8/34 was a glutamate (E) residue, while A/Sydney/5/97 possessed a lysine (K) residue at the same site. Thus, we generated two other mutated A/PR/8/34 NS1 proteins with only one mutation (E221K) and three mutations (E221K + G224R + E229K), respectively. The A/PR/8/34 NS1 mutant with E221K alone did not show any significant localization difference compared with the wild-type NS1 (wtNS1) of A/PR/8/34 (Fig. 1a and Fig. 2b). However, compared with the wild-type NS1 (wtNS1) of A/PR/8/34 that did not localize to the nucleolus, the EGFP–mtNS1 of A/PR/8/34 (E221K + G224R + E229K) localized to the nucleolus (Fig. 2c) in approximately 60% of transfected cells (78/129) expressing the triple-mutant NS1 protein. Moreover, exclusive nuclear localization of EGFP–mtNS1 of A/PR/8/34 (E221K + G224R + E229K) can be observed, although in less than 50% of transfected cells. To further confirm the necessity of 221K for nuclear and nucleolar localization of the NS1 protein, residue 221K of A/Sydney/5/97 NS1 was mutated to 221E and fluorescent microscopy of this mutant showed that EGFP–mtNS1(K221E) of A/Sydney/5/97 mainly localized in the cytoplasm, and nucleolar localization was rarely observed (Fig. 2d). These results showed that aa 221 is a key residue for the nuclear and nucleolar targeting of influenza A virus NS1 protein from A/Sydney/5/97.

**Basic amino acids in the C-terminal NLS contribute differently to the nuclear and nucleolar localization of NS1**

To further evaluate the contribution of basic amino acids in the C-terminal NLS to nuclear and nucleolar localization of NS1, single-site mutants of NLS2 of A/Sydney/5/97 NS1, including K219E, R220E, K221E, R224E, K229E, were created and the localization of these EGFP–mtNS1 fusion proteins expressed in MDCK cells was tested. The K229E mutation did not substantially change the nuclear and nucleolar localization of A/Sydney/5/97 NS1 (Fig. 1a and Fig. 2h). EGFP–mtNS1 containing single-site mutations at residue 219, 220 or 224 resulted in obviously cytoplasmic distribution of NS1 besides nuclear and nucleolar localization (Fig. 2e, f and g). Mutation K221E changed the pattern of EGFP–NS1 localization completely where the fluorescence in the cytoplasm was greater than in the nucleus (Fig. 2d). These results indicate that residue 221 of
NS1 protein is a primary determinant in the C-terminal NLS and NoLS, while residue 229 is irrelevant for localization of NS1 expressed as a recombinant protein in MDCK cells without infection in our experiment settings.

**Chimeric NS1 from both A/PR/8/34 and A/Sydney/5/97 showed that amino acid sequence 100–120 is responsible for its cytoplasmic localization**

A nuclear export inhibitory sequence adjacent to the C-terminal of the leucine-rich NES of NS1 protein has been previously reported (Li et al., 1998). Three residues (148R, 152E and 153E) are key sites for this inhibitory activity when NS1 is transiently expressed in mammalian cells (Li et al., 1998). However, for the triple-mutated A/PR/8/34 NS1 (E221K + G224R + E229K) with complete functional NLS and complete inhibitory sequence of NES (Fig. 1d), cytoplasmic localization of EGFP–mtNS1 could still be seen in about 50 % of the transfected cells (Fig. 2c). Thus, it is likely that another sequence is responsible for the cytoplasmic localization. To determine the possible sequence responsible, we created a chimeric protein containing the N-terminal of A/PR/8/34 NS1 and the C-terminal of A/Sydney/5/97 NS1 fused at different sites (Fig. 3a). The GFP-tagged chimeric NS1 proteins with the N-terminal sequence of A/PR/8/34 NS1 and the C-terminal sequence of A/Sydney/5/97 NS1 pieced together at residues 80 and 100 localized to the nucleus [Fig. 3b(i) and (ii)], similar to wildtype A/Sydney/5/97 NS1 as shown in Fig. 1(a). For the chimeric NS1 fused at residue 110, approximately 27 % of transfected cells (32/118) showed cytoplasmic localization [Fig. 3b(iii)]. However, for the chimeric NS1 proteins with an N-terminal sequence of A/PR/8/34 NS1 and C-terminal sequence of A/Sydney/5/97 NS1 pieced together at residues 120, 130 and 160, cytoplasmic localization of EGFP–NS1 could be seen in no less than 50 % of transfected cells [Fig. 3b(iv)–(vi)]. These results showed that the sequence differences between the NS1 proteins of A/PR/8/34 and A/Sydney/5/97 on residues 100–120 are probably responsible for the cytoplasmic localization.

Four residues (101, 103, 106 and 112) present in the sequence from 100 to 120 of NS1 are not conserved between NS1 of A/PR/8/34 and A/Sydney/5/97 (Fig. 4a). To further confirm the effect of the four amino acid differences, a mutant NS1 of A/Sydney/5/97 was constructed with these four residues mutated to their corresponding residues found...
in the A/PR/8/34 NS1 protein (N101E+F103S+M106I+E112A) and was inserted into the vector pEGFP–C1 for expression. Live-cell images showed that EGFP–mtNS1 of A/Sydney/5/97 with these four mutations (N101E+F103S+M106I+E112A) lost its uniform nuclear localization and showed cytoplasmic-nucleolar localization in 65 % of the transfected cells and nuclear localization in 35 % of the transfected cells [Fig. 4b(i)].

**Intracellular localization of NS1 might be related to its interaction with CPSF**

Among these four non-conserved sites in the residue (100–120) region of NS1, F103 and M106 have been reported to participate in the interaction between NS1 and CPSF (Kochs et al., 2007). Therefore, it is likely that mutation of these four residues affects the interaction between NS1 and CPSF that leads to the change in the intracellular localization of transiently expressed NS1 protein. To determine whether mutations F103S and M106I are responsible for altering the intracellular localization of the NS1 protein, the mtNS1 (F103S+M106I) of A/Sydney/5/97 strain was constructed and was inserted into the pEGFP–C1 vector. As predicted, the mtNS1 (F103S+M106I) could be localized in the cytoplasm [Fig. 4b(ii)] similar to mtNS1 (N101E+F103S+M106I+E112A). We also constructed another mutant A/Sydney/5/97 NS1, mtNS1 (184–188), which contained mutations of residues from 184 to 188 to ablate NS1’s interaction with CPSF (Das et al., 2008). When the A/Sydney/5/97 mtNS1(184–188) fused to EGFP was transiently expressed in MDCK cells, it also showed cytoplasmic localization [Fig. 4b(iii)], which was similar to mtNS1 (F103S+M106I), but different from the wild-type A/Sydney/5/97 NS1 [Fig. 4b(iv)]. These results indicated that the interaction between NS1 and CPSF might be related to the intracellular localization of influenza A virus NS1 protein.

**NS1 proteins with functional NLS and NES were able to shuttle between the nucleus and cytoplasm**

The different nuclear or cytoplasmic location patterns of NS1 and its mutants suggested that NS1 might have the ability to shuttle between different intracellular compartments. Therefore, fluorescence loss in photobleaching (FLIP) experiment was performed to analyse the dynamic behaviour of NS1 in live cells. First, an A/Sydney/5/97 NS1
with triple mutations (R148A, E152A and E153A) was constructed. These mutations ablated the function of the downstream inhibitory sequence of NS1 NES and activated the leucine-rich NES (Li et al., 1998). This mutant NS1 was inserted into the vector pEGFP–C1, pEYFP–C1 and pmCherry–C1 for live-cell imaging. Transiently expressed mtNS1 (R148A, E152A and E153A) fused to fluorescent protein, which possessed functional NLSs and NES, was found to localize in the cytoplasm. FLIP was then performed to test whether this protein was able to shuttle between the nucleus and cytoplasm (Fig. 5). It was found that cytoplasmic fluorescence decreased as the bleaching of the region of interest (ROI) in the nuclear region, while the fluorescence in a neighbouring cell remained relatively constant during the bleaching procedure. Therefore, this EYFP–mtNS1 fusion protein was able to shuttle between the nucleus and cytoplasm, although it only showed cytoplasmic localization.

**Nuclear export of NS1 protein is insensitive to leptomycin B (LMB)**

Currently, it is unknown how the influenza A virus NS1 protein is exported out of the nucleus. NS1 contains a leucine-rich NES in its effector domain. Leucine-rich NES often functions in a Crm1-mediated pathway (Fornerod et al., 1997; Fukuda et al., 1997; Neville et al., 1997). To determine whether Crm1 is necessary for the nuclear

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**Fig. 5.** Dynamic shuttling of EYFP–mtNS1 of A/Sydney/5/97 (R148A+E152A+E153A) in living cells as measured by FLIP. (a) MDCK cells expressing EYFP–mtNS1 of A/Sydney/5/97 (R148A+E152A+E153A) were imaged before and during sequential photobleaching of the indicated area in the nucleus (white circle) in the image captured before photobleaching. The unbleached cell next to the cell of interest was used as control. Bar, 10 μm. (b) Quantitative analysis for indicated areas (ROI 1 and ROI 2) of images in (a) during the FLIP procedure.
export of NS1, we added LMB, a specific inhibitor of Crm1 (Kudo et al., 1999), into the cell culture which had been previously transfected with pmCherry–mtNS1 encoding A/Sydney/5/97 NS1 with triple mutations (R148A, E152A and E153A) that localized in the cytoplasm in transfected cells (Fig. 6a). Since the mtNS1 of A/Sydney/5/97 with triple mutations (R148A, E152A and E153A) could shuttle between the nucleus and cytoplasm, mCherry–mtNS1 should relocate to the nucleus after treatment with LMB if Crm1 is indeed necessary for the nuclear export of NS1 protein. N-terminal (residues 1–51) deleted human immunodeficiency virus type-1 (HIV-1) Rev, which contains only NES but not NLS, was inserted into pmCherry–C1 to be used as the positive control of LMB treatment experiments as Crm1 is necessary for the function of NES of Rev protein (Fornerod et al., 1997; Fukuda et al., 1997; Neville et al., 1997). As shown in Fig. 6(b), mCherry–mtRev localized only in the cytoplasm without treatment with LMB. As expected, after treatment with LMB (100 ng ml$^{-1}$) for 2 h, mCherry–mtRev localized to both the nucleus and cytoplasm. However, after treatment with LMB with the same concentration for 2 h, the localization of mCherry–mtNS1 (R148A, E152A and E153A) did not change (Fig. 6a). Additional experiments showed that even after treatment with LMB for more than 12 h, mCherry–mtNS1 (R148A, E152A and E153A) still localized in the cytoplasm (Fig. 6a). Thus, nuclear export of the NS1 protein is insensitive to treatment with LMB, which suggests that the NS1 protein is exported out of the cell nuclei in a Crm1-independent pathway.

**Fluorescence resonance energy transfer (FRET) analysis of the interaction between NS1 and Crm1 NES-binding domain**

For classical Crm1-dependent leucine-rich NES, interaction between the NES and Crm1 protein is necessary to export the NES-containing cargo proteins (Fornerod et al., 1997). To form a complex with the NES-containing cargo protein, full-length Crm1 must also interact with Ran-guanosine-5'-triphosphate (GTP) through its Ran-binding domain (Noah et al., 2003). It has been proven that when the Ran-binding domain was deleted, Crm1 interacted with NES-containing cargo protein directly through its NES-binding domain (Askjaer et al., 1998). In this study, we measured whether there is an interaction between NS1 and the Crm1 NES-binding domain by FRET assay. FRET analysis was performed between the ECFP-tagged Crm1 fragment containing the NES-binding domain (ECFP–160–819Crm1) and the EYFP–mtNS1 (R148A, E152A and E153A). The NLS-deleted EYFP–mtRev protein was used as the positive control in the FRET analysis. ECFP–160–819Crm1 and the corresponding EYFP–cargo protein (mtRev or mtNS1) were co-expressed in 293T cells and FRET was monitored by an acceptor photobleaching method as described before (Li et al., 2001). The representative FRET results are shown in Fig. 7. It can be seen from Fig. 7(a) that the fluorescence intensity of ECFP–160–819Crm1 increased apparently after photobleaching of EYFP–mtRev. However, the fluorescence intensity of ECFP–160–819Crm1 did not have any apparent increase after photobleaching of EYFP–mtNS1 (Fig. 7b). The

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**Fig. 6.** Nuclear export of NS1 protein is insensitive to LMB. (a) Representative fluorescence micrographs of living cells expressing mtNS1 of A/Sydney/5/97 (R148A+E152A+E153A) fused to mCherry. (b) Representative fluorescence micrographs of living cells expressing the NES of HIV-1 Rev protein fused to mCherry. Bar, 10 μm.
FRET efficiency between ECFP–160–819Crm1 and EYFP–mtNS1 was 0.006 ± 0.014 ($n=50$), which is significantly lower than that of ECFP–160–819Crm1 and EYFP–mtRev (0.123 ± 0.059, $n=50$), which served as the positive control. These results demonstrate that there is no direct molecular interaction between the Crm1 NES-binding domain and the leucine-rich NES of the NS1 protein.

Considering that Crm1 is not responsible for the nuclear export of the NS1 protein, we further evaluated the role of the leucine-rich NES in NS1 nuclear export. It has been previously reported that I144 and L146 are key residues for the NES and mutation of either residue can lead to a malfunction of the NES (Li et al., 1998). Thus, site-directed mutagenesis of the leucine-rich NES was performed on the A/Sydney/5/97 mtNS1 (R148A + E152A + E153A). The GFP-tagged mtNS1, now with five mutations (I144A, L146A, R148A, E152A and E153A), when transiently expressed in MDCK cells, still localized in the cytoplasm (Fig. 8), although the leucine-rich NES was disabled. This result shows that the leucine-rich NES might not be the only sequence responsible for NS1 cytoplasmic localization and it is likely that other cytoplasmic localization-related sequences exist in the NS1 protein besides the leucine-rich NES of the NS1 protein.

**Other unknown sequences that contribute to cytoplasmic localization might exist in the NS1 protein**

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Further study is needed to exclude the possibility that these additional mutations also affect the nuclear import of NS1 proteins and to clarify the corresponding mechanism.

**DISCUSSION**

The present study found that NS1 of A/PR/8/34 mainly displayed a cytoplasmic distribution pattern (Fig. 1a) when it was transiently expressed in MDCK cells, different from the nuclear localization pattern of NS1 from many other strains, such as A/Sydney/5/97 (Fig. 1a) (Li et al., 1998; Melén et al., 2007). By comparing the localization-related sequences of NS1 from the two different strains, A/PR/8/34 and A/Sydney/5/97, and observing the localization and dynamics of a series of mutated and chimeric NS1 proteins fused to fluorescent proteins, some new mechanisms controlling NS1 localization patterns were discovered.

First, in addition to the key residues that have been identified previously, residue 221 was identified as a new key basic amino acid determining the nuclear and nucleolar localization of the NS1 protein of influenza A virus A/Sydney/5/97 strain. Although the A/PR/8/34 mtNS1 carrying one mutation (E221K) or two other mutations (G224R and E229K) still showed cytoplasmic localization (Fig. 2a), the localization of A/PR/8/34 NS1 changed markedly when aa 221, 224 and 229 were all mutated to basic amino acids in the same molecule (Fig. 2c). Moreover, the result that mutant NS1 of A/Sydney/5/97 with the K221E mutation mainly localized in the cytoplasm, but not in the nuclei or nucleoli further confirms the importance of residue 221 to the C-terminal NLS (Fig. 2d). However, it is worth noting that further tests are needed to determine whether this residue is important for NS1 proteins from other strains. It may also be useful to know the structural information of the interaction between NS1 proteins and the host-cell factors responsible for nuclear and nucleolar localization of NS1 proteins.

The C-terminal NLS containing basic residues and the NoLS included in the C-terminal NLS control the nuclear and nucleolar localization of the NS1 protein (Melén et al., 2007). For NS1 proteins without the C-terminal seven amino acid extension (residues 231–237), five basic amino acids (219, 220, 221, 224 and 229) are found to be the key residues of C-terminal NLS by a previous publication and our research (Melén et al., 2007). Mutational analysis of A/Sydney/5/97 NS1 to evaluate the contribution of these basic amino acids to nuclear and nucleolar localization of NS1, indicated that residue 221 of the NS1 protein is the most important one, while residue 229 is the least important.

Through sequence alignment of NS1 from several influenza A virus strains, K219, R220, K221 and R224 were found to be highly conserved, while the residue at position 229 is the least conserved (data not shown), which corresponds to the relatively less important role of K229 in the NLS and NoLS and the indispensability of the remaining four basic residues for the exclusive nuclear localization of NS1.

Construction of chimeric NS1 proteins from A/PR/8/34 and A/Sydney/5/97 showed that the amino acid sequence from 100 to 120 of NS1 played a role in its cytoplasmic localization. Further mutation studies found that F103S and M106I are responsible for NS1 cytoplasmic localization. The F103 and M106 residues are known to participate in the interaction between NS1 and CPSF (Kochs et al., 2007). The mutant A/Sydney/5/97 NS1, mtNS1 (184–188), which lost the ability to interact with CPSF (Das et al., 2008) also showed cytoplasmic localization (Fig. 4c), which further validated that the intracellular localization of NS1 might be related to its interaction with CPSF.

Crm1 is an important exportin involved in facilitating the nuclear export of a wide range of cargo molecules (Kutay & Güttinger, 2005). Leucine-rich NESs are encoded in the majority of the cargo proteins of exportin Crm1, and serve as the Crm1-binding site on the cargo proteins (Fornerod et al., 1997). LMB is a specific inhibitor of Crm1-mediated nuclear export pathway, and allows the identification of novel Crm1-dependent NES (Kudo et al., 1999). However, in several cases, the nuclear export of protein containing leucine-rich NES is insensitive to treatment with LMB, which indicated that the NES in these cases might not function through a Crm1-dependent pathway (Chen et al., 2002; Daelemans et al., 2005). In the present study, we found that nuclear export of the NS1 protein was insensitive to treatment with LMB. FRET experiments between ECFP–160–819Crm1 and EYFP–mtNS1 (R148A, E152A and E153A) showed that the NES-binding domain of Crm1 does not interact with the mtNS1 with active leucine-rich NES. This demonstrates that the leucine-rich NES in influenza A virus NS1 protein functions independently of Crm1. Structures of protein complexes composed of Ran–GTP, Crm1 and SNUPN, one cargo protein of Crm1, and that composed of only Crm1 and SNUPN have been recently resolved (Dong et al., 2009a, b; Monecke et al., 2009). It was shown that the classic leucine-rich NES.
from SNUPN forms a combined helix-extended conformation that fits into the NES-binding groove of Crm1 (Dong et al., 2009a, b). Moreover, the leucine-rich NES from SNUPN protrudes from the rest of SNUPN and is connected to the rest of SNUPN by a long loop that confers the leucine-rich NES accessibility to the NES-binding groove of Crm1 (Dong et al., 2009a, b). However, the reported leucine-rich NES from NS1 protein is part of a β-strand and appears to be inaccessible to the NES-binding groove of Crm1 (Bornholdt & Prasad, 2006). This is in agreement with our discovery that nuclear export of NS1 protein is independent of Crm1.

Additionally, it was found that site-directed mutagenesis to disable the leucine-rich NES did not alter the cytoplasmic localization pattern of A/Sydney/5/97 mtNS1. Although further experiments are needed to exclude these additional mutations also disable the NLS and affect the nuclear importing of NS1 proteins, it is possible that the leucinerich NES is not the only amino acid sequence responsible for NS1 cytoplasmic localization.

FLIP imaging performed in the current study demonstrated that the NS1 protein is dynamic inside cells and shuttles among different intracellular compartments. Based on all available information including our results, a dynamic model is proposed to explain the different localization patterns shown in this study. After being synthesized in the cytoplasm, the NS1 protein can be imported into the nucleus when both the N-terminal and C-terminal NLS are functional, and then it can be exported out of the nucleus if the nuclear export sequences are active. The trafficking of NS1 between nucleus and cytoplasm is dynamic and eventually comes into equilibrium. The distribution of NS1 inside the cells is determined by the activity of all the localization-related sequences, including the NLSs, NoLS, leucine-rich nuclear export sequence, and some other unknown nuclear export-related sequences. When all of these signals are functional, as for the A/Sydney/5/97 NS1 with triple mutations (R148A, E152A and E153A), the protein is localized in the cytoplasm of most transfected cells and the nucleolar localization of the NS1 protein can also be seen in most transfected cells (Fig. 6a). When the activity of nuclear export signal is inhibited, as for the wild-type A/Sydney/5/97 NS1, cytoplasmic localization is rarely observed and NS1 localizes to the nucleus (Fig. 1a). Furthermore, for the NS1 of A/PR/8/34, the non-functional NLS2, non-functional NoLS and loss of ability to interact with CPSF result in the cytoplasmic localization of this protein (Fig. 1a). However, the mechanism of CPSF’s involvement in the localization of NS1 is not yet clear.

Functions of localization-related signals of NS1 proteins rely on the corresponding host-cell factors. Different localization patterns might be observed in different host cells even for the same NS1 protein. For example, it was reported recently that one specific NS1 protein has different localization patterns in mammalian and avian cells (Volmer et al., 2010). Therefore, further investigation is needed to confirm whether the current finding in our report could be applied to all other host cells of influenza A viruses.

The multi-functional NS1 protein must ‘manipulate’ its different functions to benefit virus replication. Considering these different functions occur in different compartments inside cells, spatial regulation would be one of the strategies for ‘manipulation’. In this study, we focused on mechanisms of both nuclear and cytoplasmic localization of the NS1 protein. A new key residue involved in nuclear and nucleolar localization was discovered and evaluated, and the cytoplasmic localization of the NS1 protein was proven to be Crm1-independent, but likely to involve CPSF. These results provide important clues to elucidate the intracellular sorting mechanism of the NS1 protein and the localization of NS1 proteins from different strains.

**METHODS**

**Expression plasmids and DNA manipulations.** To create fluorescently tagged NS1 expressing constructs, the NS1 genes of wild-type A/Sydney/97 and wild-type A/PR/8/34 (kindly provided by W. Barclay, University of Reading, UK) were amplified by PCR and then inserted into pEGFP–C1 or pEYFP–C1 expression vectors. The mutant and chimeric NS1 genes were created by overlapping PCR and inserted into pEGFP–C1, pEYFP–C1 or pmCherry–C1 expression vector for different purposes. The fragment (478–2457) of Crm1 gene was amplified by PCR and then inserted into pECPF–C1 to create ECPF–160–819Crm1 fusion protein expressing construct. N-terminal (residues 1–51) deleted HIV-1 Rev, which only contains NES, but not NLS, was inserted into pmCherry–C1 or pEYFP–C1 for different purposes. The dsRed–B23 plasmid was kindly provided by N. Kikyo, University of Minnesota, USA. All constructs were confirmed by sequencing.

**Cells, transfection and fluorescence microscopy.** MDCK and 293T cells were maintained in RPMI 1640 medium or Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10 % FBS (Invitrogen) at 37 °C with 5 % CO2. One day before transfection, cells were plated in 35 mm glass bottom dishes. Cells were transfected with plasmids at 70–80 % confluency using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s instructions. After being incubated for about 18 h transfected cells were replaced with fresh medium for fluorescence microscopy.

Cells were imaged by an inverted wide-field fluorescence microscope (Axiovert 200; Carl Zeiss) equipped with a cooled CCD camera, Cascade 512B (Photometrics). The CCD camera was operated at 5 MHz with 16-bit digitization and controlled by MetaMorph 6.0 software (Molecular Devices).

**Immunofluorescence and Western blotting assay.** For immunofluorescence experiment, the MDCK cells transfected with pcDNA3.1-NS1-SYN or pcDNA3.1-NS1-pr8 were cultured for 18–24 h. Then, cells were fixed, permeabilized and incubated with anti-NS1 antibody (Abcam) and FITC-labelled secondary antibody (Boster) for imaging. For Western blotting, the MDCK cells transiently expressing EGF–NS1 fusion proteins or EGFP were treated with cell lysis buffer for Western and IP (Beyotime). Then, the cell lysate was transferred to a tube on ice. After incubation for 10 min on ice, the cell lysate was centrifuged at 12000 × g for 5 min and the supernatants were collected for Western blotting with anti-EGF antibody (Abcam).
FLIP. FLIP experiments were performed using a Leica TCS SP2 laser scanning spectral confocal microscope equipped with a cooled CCD camera. The prepared cell culture dishes were placed in a temperature-controlled incubator at 37 °C and detection was carried out by using a ×100 oil objective with a 514 nm excitation beam. Circular bleaching regions were specified in the nucleus. An image was taken of the whole cell before bleaching, followed by bleaching with full laser power at 514 nm for 20 s and capturing of another image. This sequence was repeated for approximately 2 min. Non-bleached cells next to the cell of interest were used as controls.

FRET. FRET experiments were performed using a Leica TCS SP2 laser scanning spectral confocal microscope equipped with a cooled CCD camera. All measurements were obtained using a ×100 oil objective. An argon laser line at 458 nm was used to excite ECFP and a 514 nm line was used to excite EYFP. FRET measurement was performed with a FRET acceptor photobleaching programme of the Leica confocal software according to the manufacturer’s instructions. In the photobleaching procedure, cells were bleached using a 514 nm laser beam at 100 % intensity. Depending on the experiment, we selected a whole cell as an ROI for bleaching. The bleaching time was 20 s. The FRET efficiency (E) was calculated by normalizing the difference of whole cell as an ROI for bleaching. The bleaching time was 20 s. The FRET efficiency (E) was calculated by normalizing the difference of the donor post- and pre-bleach intensity by the post-bleach intensity according to the equation: E = (I_{post-bleach} – I_{pre-bleach})/I_{post-bleach}. Statistical analysis was performed using the software SPSS (version 13.0; SPSS). P-values of <0.01 were considered statistically significant.

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