Human cellular protein nucleoporin hNup98 interacts with influenza A virus NS2/nuclear export protein and overexpression of its GLFG repeat domain can inhibit virus propagation

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The non-structural protein NS2, also called nuclear export protein, of influenza A virus contains a leucine-rich nuclear-export signal that could guide viral ribonucleoproteins to cross the nuclear pore complex (NPC) and complete directional nucleocytoplasmic trafficking. In this study, human nucleoporin 98 (hNup98), an NPC protein, was identified as an NS2-binding protein by using yeast two-hybrid screening of a human cDNA library. Interaction between NS2 and hNup98 was confirmed in yeast and mammalian cells. Mapping tests further demonstrated that aa 22–53 in the N-terminal region of NS2 and the glycine-leucine-phenylalanine-glycine (GLFG) repeat domain (aa 1–511) of hNup98 are crucial for the interaction of these two proteins. Confocal microscopy showed that hNup98 could specifically recruit NS2 to the nucleoli and that this process was inhibited by leptomycin B, a specific inhibitor of human chromosomal region maintenance 1 protein. NS2 recruitment to the nucleoli was through the N-terminal GLFG repeat domain of hNup98, but not through the C-terminal domain. Moreover, influenza virus infection downregulated Nup98 levels significantly in 293T and Madin–Darby canine kidney cells. Overexpression of the GLFG repeat domain of hNup98 apparently inhibited virus propagation. Together, these findings reveal the interaction between hNup98 and NS2. The GLFG repeat domain of hNup98 might competitively inhibit the interaction between NS2 and endogenous hNup98, consequently inhibiting virus propagation.

INTRODUCTION

Influenza A virus is a negative-sense RNA virus whose virion genome exists as a viral ribonucleoprotein (vRNP) complex composed of viral RNA (vRNA), a trimeric polymerase complex of the proteins PB1, PB2 and PA, and nucleoprotein (NP). Unlike most other RNA viruses, which replicate in the cytoplasm, influenza virus replicates in the nucleus. During virus replication, vRNA is replicated and transcribed by the viral polymerase complex, and newly synthesized vRNA and proteins form progeny vRNP complexes in the nucleus. Progeny vRNP then pass through the nuclear pore complex (NPC) and are translocated to the assembly site for envelopment and budding (Boulo et al., 2007; Cros & Palese, 2003).

Embedded in the nuclear envelope, the NPC is the only path for nucleocytoplasmic transport of substances. It is composed of approximately 30 different proteins called nucleoporins, which play different roles in the overall NPC structure and function. Most nucleoporins have regions rich in Phe-Gly (FG) repeats. The active nucleocytoplasmic transport of large proteins (>50 kDa) can only be accomplished through the interaction of NPC proteins with transport receptors. Transport receptor proteins can specifically recognize the FG functional domain of nucleoporins and slide together over the NPC channel with their cargo by transient and specific binding to nucleoporins (Lim et al., 2008; Pemberton & Paschal, 2005; Tran & Wente, 2006).

At present, the distribution and function of the different nucleoporins are still under investigation. Recent work has demonstrated that different mRNA export receptors can interact selectively with different nucleoporins to form distinct export strategies (Terry & Wente, 2007). In addition, there are indications that viruses interact...
selectively with different transport receptors and nucleoporins that assist in the completion of RNA export. The Rev protein of human immunodeficiency virus type 1 (HIV-1) is responsible for the export of unspliced or partially spliced viral transcripts (Neville et al., 1997; Otero et al., 1998). Rab/hRIP, human nucleoporin 98 (hNup98), hNup214, hNup42 and hNup159 are all involved in the Rev–human chromosomal region maintenance 1 (hCRM1)-mediated nuclear export of HIV-1 RNA, whilst interactions between NSP1, hNup116, hNup153 and Rev have not been observed (Fritz et al., 1995; Stutz et al., 1996). Overexpression of the FG repeat domain in several nucleoporins can effectively inhibit the replication of HIV virus (Floer & Blobel, 1999; Zolotukhin & Felber, 1999). Moreover, the Rex protein of human T-cell leukemia virus type 1 (Bogerd et al., 1998), the EB2 protein of Epstein–Barr virus (Boyle et al., 1999) and the ICP27 protein of herpes simplex virus type 1 (Chen et al., 2002) have been reported to interact with NPC proteins. Interactions of influenza non-structural protein NS2 with human nucleoporin Rab/hRIP1 and with yeast nucleoporins yRip1, yNup1, yNup100 and yNup116 have been verified in a yeast two-hybrid system (O’Neill et al., 1998). However, the mechanism of influenza virus vRNP nuclear export is still unclear.

Influenza virus vRNPs are macromolecules, so their nuclear export is completed through active transport (Nayak et al., 2004). Current reports indicate that nuclear export of progeny vRNPs depends on the hCRM1-mediated nuclear-export pathway. Influenza virus NS2 protein serves as an adaptor. The leucine-rich nuclear-export signal (NES) at its N terminus (aa 1–53) could be recognized specifically by hCRM1, whilst its C terminus (aa 54–121) could interact with the M1 protein. The M1 protein then binds to vRNP by interacting with NP to form the hCRM1–NS2–M1–vRNP nuclear-export complex (Akarsu et al., 2003; O’Neill et al., 1998; Yasuda et al., 1993). Translocation of some complexes from the nuclear interior across the nuclear envelope and into the cytoplasm is regulated by the transient interaction between transporter molecules and NPC components (Terry & Wente, 2009; Weis, 2002). However, the specific NPC proteins that contribute to the export of influenza virus vRNPs remain unidentified.

Identification of the interactions between host-cell factors and influenza NS2 protein will be helpful in determining the nuclear-export mechanism of influenza virus vRNPs to develop effective antiviral drugs (Carmody & Wente, 2009; Kutay & Güttinger, 2005; Turner & Sullivan, 2008). In this study, we confirmed the interaction between influenza NS2 and hNup98 in both yeast and mammalian cells. Localization of the two proteins revealed that hNup98 could recruit NS2 specifically to the nucleoli. Furthermore, the domains involved in the NS2–hNup98 interaction were also identified. The truncated segment of hNup98 (aa 1–511), crucial to the interaction of the two proteins, could apparently inhibit the propagation of influenza virus.

These results of this study could provide new clues for further studies of the mechanism of influenza vRNP export.

**RESULTS**

**Cellular nucleoporin hNup98 interacts with H5N1 influenza virus NS2 protein**

To identify the host proteins that interact with influenza A virus NS2 protein, a yeast two-hybrid screen of a human cDNA library was performed. Several positive clones were obtained from approximately $5 \times 10^6$ clones. Candidate cDNAs were isolated from the positive clones, amplified in *Escherichia coli* DH5α and sent to Invitrogen for sequencing. Nucleotide sequencing revealed that one of these positive clones was hNup98.

Interaction of NS2 with hNup98 was confirmed by co-transformation of plasmids pGBK7-NS2 and pGADT7-hNup98 into *Saccharomyces cerevisiae* strain AH109. An α-galactosidase qualitative assay was used to measure the differences in the relative strength of binding between the interacting proteins. As shown in Fig. 1(a), AH109 co-transformed with pGBK7-53 and pGADT7-T was used as a positive control. AH109 co-transformed, respectively, with pGBK7T and pGADT7, with pGBK7T-NS2 and pGADT7, and with pGBK7T and pGADT7-hNup98 were used as negative controls. AH109 co-transformed with pGBK7T-NS2 and pGADT7-hNup98 grew and turned blue on synthetic defined (SD) agar medium without Ade, His, Leu and Trp (SD/–Ade/–His/–Leu/–Trp/X-α-Gal), whereas the negative control grew only on SD/–Leu/–Trp medium. These results were further validated via a β-galactosidase assay using o-nitrophenyl-β-galactoside (ONPG) as substrate. The results were consistent with those of the α-galactosidase assay.

To verify further the interaction between NS2 and hNup98, a mammalian two-hybrid assay was performed in HeLa cells. HeLa cells were co-transfected with plasmids pBIND-NS2, pACT-hNup98 and reporter plasmid pG5luc containing the firefly luciferase gene. Fig. 1(b) shows that co-expression of NS2 and hNup98 resulted in a high ratio of firefly: *Renilla* luciferase activity. These results showed that NS2 interacts with hNup98 in HeLa cells.

**Determination of the interaction domains involved in the interaction of NS2 and hNup98 by a yeast two-hybrid assay**

To understand better the interaction between NS2 and hNup98, the domains essential for the interaction of the two proteins were analysed. NS2 consists of 121 aa, comprising an N-terminal domain (aa 1–53) and a C-terminal domain (aa 54–121) (Fig. 2a). The N-terminal domain contains an NES comprising 10 aa (aa 12–21), whilst the C-terminal structure is chiefly responsible for
M1 binding. As shown in Fig. 2(b), hNup98 consists of 920 aa, with a GLFG-rich region in the N-terminal domain (aa 1–511). Based on the domain composition of the two proteins, six truncated mutants of NS2 protein, (NS21–53, NS254–121, NS21–11, NS212–21, NS222–121 and NS222–53) and two truncated mutants of hNup98 protein (hNup981–511 and hNup98512–920) were constructed. The yeast two-hybrid assay revealed that NS222–53 was the crucial domain for NS2–hNup98 interaction. Of the six mutants of NS2, NS21–53, NS222–121 and NS222–53 showed strong interactions with hNup98, whereas NS2 12–21 showed weak interaction with hNup98, and NS2 54–121 and NS2 1–11 showed no binding activity. Only mutant hNup981–511 could bind to NS2, whilst hNup98512–920 had no apparent interaction with NS2 (Fig. 2c). These results indicated that the N-terminal region (NS222–53) of the NS2 protein and the GLFG repeat domain of hNup981–511 are crucial for interaction of the two proteins.

**NS2 is recruited to the nucleoli by hNup98**

To examine the subcellular localization of hNup98 and NS2 in mammalian cells, enhanced yellow fluorescent protein (EYFP)–hNup98 and enhanced cyan fluorescent protein (ECFP)–NS2 fusion proteins were transiently expressed and detected in HeLa cells. Initially, HeLa cells were transfected individually with pEYFP-C1-hNup98 or pECFP-C1-NS2, which were detected using fluorescence microscopy at 24 h post-transfection. hNup98 showed a nuclear rim and a nuclear punctate pattern distribution (Fig. 3a). Based on size and number, these punctate subnuclear structures could have been nucleoli. To confirm this assumption, ECFP–fibrillarin (a nucleolar marker) and EYFP–hNup98 were co-expressed in HeLa cells. As shown in Fig. 3(d), fluorescence microscopy showed co-localization of EYFP–hNup98 and ECFP–fibrillarin partially within the nucleolus, demonstrating that the punctate structures in the nucleus were indeed nucleoli.

The subcellular localization of NPC protein hNup153 when it was co-expressed with fibrillarin in HeLa cells was also determined. hNup153 was observed exclusively at the nuclear rim (Fig. 3e), suggesting that localization in the nucleoli was specific to hNup98. As shown in Fig. 3(b), fluorescence microscopy showed co-localization of EYFP–hNup98 and ECFP–fibrillarin partially within the nucleolus, demonstrating that the punctate structures in the nucleus were indeed nucleoli.

**Co-localization of NS2 and hNup98 in the nucleoli is inhibited by leptomycin B (LMB)**

The next step was the determination of whether the interaction between NS2 and hNup98 was mediated by the
hCRM1 protein, which has been reported as an important host factor that interacts with NS2 protein to mediate nuclear export of influenza vRNPs. After transfection with pSG5-CRM1-GFP, subcellular localization of the hCRM1 protein was observed throughout both the cytoplasm and the nucleus, with an apparent circular distribution at the nuclear rim (Fig. 4a). hCRM1 and hNup98 exhibited nuclear rim and nuclear punctate patterns of co-localization (Fig. 4b). To confirm the relationship among the three proteins, NS2, hCRM1 and hNup98 were co-expressed in HeLa cells. The results demonstrate that the three proteins co-localized in the nucleoli (Fig. 4c).

The introduction of LMB, a specific inhibitor of the transport receptor hCRM1, can result in nuclear retention of influenza vRNPs (Iwatsuki-Horimoto et al., 2004). To examine the effect of LMB on the co-localization of NS2 and hNup98, the two proteins were co-expressed in HeLa cells in the presence of LMB. As shown in Fig. 4(d), the nuclear punctate pattern co-localization disappeared, indicating that LMB had an apparent effect on the co-localization of NS2 and hNup98. When NS2 was expressed alone, LMB treatment did not affect the distribution of NS2 (Figs 3b and 4e). However, when hNup98 was expressed alone or when hNup98 and NS2 were co-expressed in the presence of LMB, the punctate distribution of hNup98 in the nucleus became substantially smaller and more diffuse, both in the nucleus and cytoplasm (Figs 3a and 4d, f). These results implied that NS2, hCRM1 and hNup98 co-localized in the nucleoli and that LMB could inhibit nucleolar distribution of hNup98 and, further, interrupt nucleolar co-localization of NS2 and hNup98.

GLFG repeats may play an important role in the co-localization of hNup98 and NS2

As hNup98 exhibits nucleolar localization in HeLa cells, two truncated mutants of the hNup98 protein, hNup981–511 and hNup98512–920, were constructed to determine the domain crucial for nucleolar co-localization of NS2 and hNup98. HeLa cells were transfected with pEYFP-C1-hNup981–511 alone or co-transfected with pEYFP-C1-hNup981–511 and
pECFP-C1-NS2. As shown in Fig. 5(a), hNup981–511 expressed alone was localized exclusively in the nucleoli. As expected, NS2 displayed significant co-localization at these small punctate structures (Fig. 5b). hNup98512–920 alone did not show a nuclear punctate distribution (Fig. 5c). Significant nucleolar co-localization of NS2 and hNup98512–920 was also not observed (Fig. 5d).

**Downregulation of Nup98 in cells following infection by H5N1 virus**

The levels of Nup98 protein in virus-infected cells were measured, because hNup98 is an interferon-inducible NPC protein. Human 293T and Madin–Darby canine kidney (MDCK) cells were infected with influenza H5N1 virus. Influenza virus-infected cells from the immunoblot assay are shown in Fig. 6. The levels of endogenous Nup98 protein in both 293T and MDCK cells were apparently downregulated at about 4 h post-infection (p.i.) in cells infected with H5N1 virus. β-Actin levels were unaffected in virus-infected MDCK cells, but were decreased in 293T cells, at 8 h p.i. Nup98 protein levels remained stable at an extremely low level until at least 24 h p.i. These results implied that the level of the Nup98 protein is downregulated remarkably by H5N1 influenza A virus infection, which is in accordance with a recent work indicating that Nup98 levels are downregulated by infection with influenza virus strain A/WSN/33 (H1N1) (Satterly *et al.*, 2007).

**Inhibition of H5N1 virus propagation by the truncated mutant of hNup98**

The relative strength of the interaction between truncated segment hNup981–511 and NS2 in comparison with the interaction between full-length hNup98 and NS2 was determined. In the mammalian two-hybrid system, there was a stronger interaction between hNup981–511 and NS2 than between hNup98 and NS2 (Fig. 7a).

These results suggested that determining the effects of overexpression of truncated mutant hNup981–511 on virus propagation should also be investigated. Empty vector pEGFP-N1 was used as a negative control. The expression of GFP and GFP-tagged protein (GFP–hNup981–511) in 293T cells was detected using immunoblot analysis. Stable high levels of β-actin protein in GFP–hNup981–511-overexpressed...
cells indicated that hNup98<sup>1–511</sup> overexpression had no significant influence on cell conditions (Fig. 7b). The effect of overexpression of truncated hNup98 on cell viability by MTT assay revealed that there was no significant difference between GFP- and GFP–hNup98<sup>1–511</sup>-overexpressing 293T cells (P=0.29) (Fig. 7c). At 24 h post-transfection, 293T cells were infected with H5N1 virus and the supernatant was collected at 48 h p.i. Virus titres (expressed as TCID<sub>50</sub>) were calculated. As shown in Fig. 7(d), overexpression of truncated hNup98<sup>1–511</sup> inhibited virus propagation. These results suggested that the truncated hNup98<sup>1–511</sup> fragment might function as a competitive inhibitor of the interaction

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**Fig. 4.** Effect of LMB on the subcellular co-localization of NS2 and hNup98 in HeLa cells. (a) EGFP–hCRM1 expressed in HeLa cells. (b) EGFP–hCRM1 and EYFP–hNup98 co-expressed in HeLa cells. (c) ECFP–NS2, EGFP–hCRM1 and EYFP–hNup98 co-expressed in HeLa cells. (d) HeLa cells were co-transfected with plasmids pEYFP-C1-hNup98 and pECFP-C1-NS2, and treated with 11 nM LMB for 3 h at 24 h post-transfection. (e) HeLa cells were transfected with plasmid pECFP-C1-NS2 and treated with 11 nM LMB for 3 h at 24 h post-transfection. (f) HeLa cells were transfected with plasmid pEYFP-hNup98 and treated with 11 nM LMB for 3 h at 24 h post-transfection. Cells were then fixed, permeabilized and stained with DAPI. Bars, 10 μm.
between NS2 and endogenous hNup98, thereby impairing virus propagation.

**DISCUSSION**

Influenza NS2 protein is thought to be required for nuclear export of vRNPs (O’Neill et al., 1998). Using a yeast two-hybrid system, hNup98 was screened from a human cDNA library as a potential cellular protein that interacts with the H5N1 virus NS2 protein. As an important component of the NPC, the 98 kDa nucleoporin is localized at the nucleoplasmic side of the NPC (Griffis et al., 2003; Hodel et al., 2002). Moreover, hNup98 interacts with both faces of the nuclear pore, which is consistent with its nucleocytoplasmic shuttling activity (Griffis et al., 2002). It is a GLFG-rich nucleoporin and functions as one of several docking sites that mediate nucleocytoplasmic transport of specific protein or RNA cargoes (Powers et al., 1997; Radu et al., 1995a). In addition, hNup98 is an interferon-inducible NPC protein (Enninga et al., 2002) that is a preferred target for many viruses, probably as an approach to overcome host defence mechanisms (Chakraborty et al., 2009; Gustin, 2003; Kiss et al., 2003; Park et al., 2007; Ricour et al., 2009). Satterly et al. (2007) reported that influenza virus can selectively inhibit nuclear export of several host-cell mRNAs by targeting hNup98. Via genome-wide small interfering RNA screening, hNup98 was recently identified in several studies as a cellular factor involved in the influenza virus life cycle (Brass et al., 2009; Hao et al., 2008; Karlas et al., 2010).

This study discovered an interaction between influenza NS2 protein and the hNup98 protein. Recent works have identified the NS2 protein as an adaptor molecule that plays an important role in the nuclear export of influenza virus vRNPs (Boulo et al., 2007; Iwatsuki-Horimoto et al., 2004; Lommer & Luo, 2002). There have also been reports that different mRNA nuclear-export receptors specifically recognize different nucleoporins to form distinct NPC nuclear-export strategies (Radu et al., 1995b; Terry & Wente, 2007). Data from the present study further demonstrate that the hNup98 GLFG repeat domain is
crucial for NS2 binding. FG repeat domains, the hallmark of FG nucleoporins, are the most important motif in nucleocytoplasmic traffic (Bayliss et al., 2002; Terry & Wente, 2009). In a previous study, the GLFG repeat domain of Nup98, together with the peptide repeat domains of other nucleoporins, have been suggested to form an array of sites for mediated docking of transport substrates and bidirectional translocation (Radu et al., 1995a). Further studies are needed to determine the role of NS2–hNup98 interaction in the nuclear export of influenza virus vRNPs.

NS222–53, a crucial region of the NS2 protein, provided sufficient interaction between NS2 and hNup98, whereas another truncated mutant with 10 aa that encoded the leucine-rich NES of the NS2 protein (NS212–21) had only a weak interaction with hNup98. This indicates that NS2 interacts with hNup98 independent of its NES motif. Neumann et al. (2000) also found that altering the NS2 NES abolished vRNP nuclear export without affecting the interaction with hCRM1. Hence, both the NES and its adjacent sequences may be important for the specific recognition of NS2 by transport receptor.

The results of subcellular localization suggested that overexpressed hNup98 could recruit NS2 to the nucleoli compared with endogenous proteins (Fig. 3b, c). This might be attributed to the endogeneous hNup98 level in the nucleoli being too low. In fact, definitive detection of a nuclear punctate pattern distribution of endogenous hNup98 using standard immunofluorescence is difficult (Griffis et al., 2003; Oka et al., 2010; Pritchard et al., 1999). This might be because of low endogenous nucleolar hNup98 expression or lack of recognition of hNup98 in the nucleoli by anti-Nup98 antibodies. Thus, NS2 might also be recruited to the nucleoli by endogenous hNup98, although it is difficult to observe. In

Fig. 7. Effect of the hNup98 GLFG repeat domain on virus propagation. (a) HeLa cells were co-transfected with plasmids pG5luc, and with various plasmid combinations of pBIND-NS2 and pACT-hNup98 or its mutants and assessed for relative luciferase activity. The length of each bar represents relative luciferase activity, calculated as the firefly:Renilla luciferase-activity ratio. Results are shown as means ± s.e for three experiments. (b) GFP-tagged protein (GFP–hNup981–511) was detected by immunoblot analysis using rabbit anti-GFP antibody. The positions of protein markers (kDa) are shown on the left. The expected molecular masses for GFP protein and GFP–hNup981–511 are 27 and 83 kDa, respectively. β-Actin was detected by immunoblotting with anti-β-actin antibody. NT, Non-transfected cells. (c) MTT assay to determine the growth of GFP- and GFP–hNup981–511-overexpressing cells. Results are shown as means ± s.e for three experiments and were evaluated by using Student’s t-test (P=0.29). (d) Competitive inhibition of H5N1 virus propagation by overexpression of the hNup98 GLFG repeat domain. 293T cells were transfected with pEGFP-N1 or pEGFP-N1-hNup981–511. At 24 h post-transfection, cells were infected with influenza virus A/Chicken/Henan/12/2004 at an m.o.i. of 0.1. Cell supernatants were collected at 48 h p.i. and the virus titre for each supernatant was expressed as TCID50. Results are shown as means ± s.e for three experiments.
addition, there is a report that Nup98 functions as a co-factor for CRM1-mediated nuclear export in conjunction with RanBP3 and that there is nucleolar co-localization of Nup98 and CRM1 (Oka et al., 2010). Thus, together with the co-localization of NS2, hCRM1 and hNup98 in the nucleoli, these data indicate that the nucleolus might be the import site for hNup98–hCRM1–NS2 complex assembly. Further studies are necessary to achieve a better understanding of the functional significance of the nucleolar co-localization of NS2 and Nup98.

Immunoblot assays showed that the levels of endogenous Nup98 protein in 293T and MDCK cells were downregulated at about 4 h after infection with H5N1 virus and that the levels of β-actin protein were also downregulated at about 8 h p.i. This may be caused by a general host shut-off mechanism induced by influenza virus infection or by degradation triggered by the virus PA protein, which has been implicated in proteolytic activity (Sanz-Ezquerro et al., 1995). These possibilities should be examined in future studies.

Overexpression of the GLFG repeat segment hNup981–511, the essential domain for NS2–hNup98 interaction, inhibited virus propagation, implying that hNup98 plays an important role during virus infection. The dominant-negative effect of FG repeat domains on nucleocytoplasmic traffic has also been reported (Stutz et al., 1996; Zolotukhin & Felber, 1999). Downregulation of hNup98 protein levels in infected cells might be because the virus only requires a limited level of hNup98 for successful infection. As shown in Fig. 6, the hNup98 protein remained at extremely low levels until at least 24 h p.i. As hNup98 is an interferon-inducible host protein, considerable levels of hNup98 may be crucial for nuclear export of host-cell mRNAs. Thus, the export of host mRNAs might be blocked in virus-infected cells, even though the export of virus vRNPs is unaffected. Ultimately, the downregulation of hNup98 evoked by influenza virus infection might be a strategy to evade or antagonize host antiviral responses.

In summary, this study confirmed the interaction between NS2 and hNup98 in both yeast and mammalian cells, and identified the domains crucial for NS2–hNup98 interaction. Confocal microscopy revealed that NS2 can be recruited specifically to the nucleoli by hNup98. Overexpression of the GLFG repeat domain of hNup98 inhibited virus propagation. These results provide a novel basis for a further understanding of the mechanism of nuclear export of influenza A virus vRNPs.

**METHODS**

**Virus.** Highly pathogenic influenza virus strain A/Henan/12/2004 (H5N1) was isolated and preserved in the laboratory (Zheng et al., 2009). All tests associated with highly pathogenic avian influenza H5N1 virus were performed in a Biosafety Level 3 laboratory at the Wuhan Institute of Virology, Chinese Academy of Sciences, PR China.

**Plasmid construction.** For bait construction for the yeast two-hybrid screening, the NS2 ORF of influenza A (H5N1) virus was cloned into the NdeI/SalI sites of pGBK7 (Clontech) by PCR. Truncated regions corresponding to NS21–53, NS254–121, NS21–11, NS212–21, NS22–121 and NS22–53 were constructed by PCR using pGBK7-NS2 as template with corresponding primers. The ORF of hNup98 was cloned into the NdeI/EcoRI sites of pGADT7 (Clontech) via PCR using pACT2-hNup98 as template. Deletion mutants of hNup98 were cloned into the SalI/EcoRI sites of pBIND vector (Promega) using PCR, whilst hNup98 and deletion mutant hNup981–511 were cloned into the same sites of pACT vector (Promega). For fluorescent confocal microscopy, the ORFs of NS2 and fibrillarin were cloned into the HindIII/SalI sites of pECFP-C1 (Clontech) via PCR. Full-length hNup98, hNup153 and deletion mutants hNup985–511 and hNup981–511 were cloned into the EcoRI/BamHI sites of pEYFP-C1 (Clontech). Plasmid pSG5-CRM1-GFP was kindly provided by Professor Maarten Fornerod (NCI, Amsterdam, The Netherlands). To generate a plasmid expressing EGFP–hNup981–511, the deletion mutant of hNup981–511 was cloned into the Xhol/EcoRI sites of pEGFP-N1 (Clontech) via PCR using pGADT7-hNup981–511 as template. All constructs were sequenced in full.

**Yeast two-hybrid screening.** The Matchmaker Gal4 Two-Hybrid system 3 and Matchmaker Human Kidney Pretransformed cDNA library were purchased from Clontech. All procedures for growing yeast and transformation were according to the protocol provided by the manufacturer. pGBK7-NS2 plasmid was transformed into S. cerevisiae strain AH109 to obtain bait strain AH109 (pGBK7-NS2). Screening and identification of positive clones were performed according to a method reported previously (Huang et al., 2009). To perform qualitative and quantitative two-hybrid tests between two proteins, α- and β-galactosidase (liquid culture assay using ONPG as substrate) assays were carried out according to the Clontech Yeast Protocols Handbook and repeated at least three times. 2-β-Galactosidase and ONPG were both purchased from Clontech.

**Cell culture and transfection.** 293T, HeLa and MDCK cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco) supplemented with 10% FBS (Gibco). Cells were maintained in a 37 °C humidified atmosphere containing 5% CO₂. For transfection, cells were seeded on dishes. After overnight growth to reach 70% confluence, the cells were transfected with the corresponding plasmids using transfection reagent Lipofectamine 2000 (Invitrogen) in non-serum medium according to the manufacturer’s directions. After 5 h incubation, the medium was replaced with fresh complete medium.

**Mammalian two-hybrid assay.** To analyse the interaction between NS2 and hNup98 in mammalian cells, HeLa cells were co-transfected with 500 ng pBIND fusion construct, 500 ng pACT fusion construct and 500 ng reporter gene construct pG5lac (Promega) per well in a 24-well plate using Lipofectamine 2000. Cells were harvested, washed twice with cold PBS and lysed with Passive Lysis Buffer (Promega) at 48 h after transfection. A luciferase assay was carried out according to the manufacturer’s specifications for the Dual-Luciferase Reporter Assay system (Promega), and the activity of Renilla luciferase was used to correct for the variation in transfection efficiency. After sequential quantification of firefly and Renilla luciferase activities in the cell lysates, the binding interaction between the two proteins was calculated as a firefly: Renilla luciferase-activity ratio to obtain relative activity. Experiments were carried out in triplicate and luciferase activity was determined by using a T20/20 Luminometer (Turner Designs).

**Fluorescent confocal microscopy.** HeLa cells seeded on glass coverslips were co-transfected with 1.5 μg pECFP-C1-NS2 and 1.5 μg pEYFP-C1-hNup98 per well in a six-well plate using Lipofectamine 2000. At 24 h after transfection, the cells were washed with PBS, fixed with 4% paraformaldehyde (pH 7.4) for 20 min, permeabilized with...
0.2 % Triton X-100 in PBS for 15 min and stained with DAPI (Sigma) for 10 min. Cell transfection with other plasmids was performed in the same way. LMB (Sigma) was added to the designated wells at a final concentration of 11 nM for 3 h at 24 h post-transfection. Fluorescent image analysis was performed on a LEICA laser-scanning confocal microscope with associated software.

**Immunoblotting analysis.** For infection studies, A/Chicken/Henan/12/2004 (H5N1) influenza virus stocks were diluted serially with PBS to an m.o.i. of 1 and adsorbed onto confluent 293T and MDCK cells for 45 min at 37 °C. Unadsorbed virus was removed by washing with serum-free DMEM, and 2 ml infection medium (minimal essential medium containing 2 % PBS) was added to the dishes. Cells were collected at the indicated times and subsequently lysed. Cell lysates were fractionated using SDS-PAGE (12 % gel), blotted onto PVDF membranes and immunoblotted with rabbit anti-Nup98 monoclonal antibody and mouse anti-β-actin polyclonal antibody (Cell Signaling Technology).

293T cells were plated into six-well plates. At 24 h after plating, cells were transfected with 3 μg pEGFP-N1 or 3 μg pEGFP-N1-hNup981–511. The cells were then collected and lysed 24 h after transfection. Cell lysates were fractionated using SDS-PAGE (12 % gel), blotted onto PVDF membranes and immunoblotted with rabbit anti-GFP polyclonal antibody and mouse anti-β-actin polyclonal antibody (Cell Signaling Technology).

**REFERENCES**


