FEBS Letters 585 (2011) 41–46

FEBS Letters

journal homepage: www.FEBSLetters.org

Crucial role of the influenza virus NS2 (NEP) C-terminal domain in M1 binding and nuclear export of vRNP

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ARTICLE INFO

Article history:
Received 9 June 2010
Revised 2 November 2010
Accepted 9 November 2010
Available online 16 November 2010

Edited by Ulrike Kutay

Keywords:
Influenza virus
Nuclear export
NS2
M1
vRNP

ABSTRACT

The influenza virus genome replicates in the host cell nucleus, and the progeny viral ribonucleoproteins (vRNPs) are exported to the cytoplasm prior to maturation. The influenza virus NS2 protein has a nuclear export signal (NES) and binds to M1. It is therefore postulated that vRNP is exported from the nucleus by binding to NS2 through M1. However, the significance of the association between NS2 and M1 for the nuclear export of vRNP is still poorly understood. We herein demonstrate that the C-terminal domain of NS2 (residues 81–100) is essential for M1 binding and the nuclear export of progeny vRNPs.

Structured summary:
MINT-8057301, MINT-8057317: *NS2* (uniprotkb:P03508) *binds* (MI:0407) to *M1* (uniprotkb:P03485) by *pull down* (MI:0096)

1. Introduction

The genome of type A influenza virus consists of eight single-stranded RNAs of negative polarity. The viral RNA (vRNA) exists in a complex with nucleoprotein (NP) and the three viral RNA dependent RNA polymerase subunits PA, PB1, and PB2 to form the viral ribonucleoprotein complexes (vRNP). The replication and transcription of the influenza viral genome occur in the nucleus. Newly synthesized vRNPs are exported to the cytoplasm prior to influenza virus maturation. Previous studies have demonstrated that M1 is essential for the nuclear export of vRNP because the inhibition of M1 synthesis by H-7 blocked the nuclear export of vRNPs [1,2]. There are conflicting reports on which domain of M1 binds to vRNP. Several studies have demonstrated that the N-terminal domain of M1 interacts with NP [3,4], while another study described that the C-terminal domain of M1 binds to vRNPs or to NP [5]. Previous studies also have shown that the $_{101}$RKLKR$_{105}$ sequence of M1 located in this domain mediates binding to RNA [6] and acts as a nuclear-localization signal (NLS) [7]. M1 has been reported to inhibit the re-import of vRNPs into the nucleus [8]. Therefore, it is thought that M1 plays two roles in the nuclear export of vRNPs: thus promoting the export and inhibiting the re-import of vRNP. We and others previously reported that the nuclear export of vRNP is mediated by the cellular protein CRM1/exportin1, a member of the importin β family of the nuclear transport receptors, which is inhibited by leptomycin B (LMB) [9,10]. CRM1 binds to the target protein which has a leucine-rich nuclear export signal (NES) [11–13]. M1 does not contain the leucine-rich NES, while NS2 does contain the leucine-rich NES ($_{12}$ILMRMSKMQL$_{21}$). Thus the nuclear export of vRNP is mediated by vRNP-M1-NS2 complex. It is also reported that NS2 can replace the NES function of HIV-Rev [14]. When a cell is infected with a recombinant virus that cannot express NS2 or contain mutations in the NES of NS2, vRNP nuclear export is blocked [15]. NS2$_{22–53}$ interacts with a cellular protein nucleoporin, hNup98, a cofactor for CRM1-mediated nuclear export protein [16,17]. The hNup98-CRM1-NS2 is complexed in the nucleus. NS2 appears to mediate the nuclear export of vRNP through CRM1. It has been reported that NS2 interacts with M1 in vitro [18,19]. Therefore, the progeny vRNP is thought to be transported to the cytoplasm by binding to NS2 through M1. However, NS2 may directly bind to vRNPs because NS2 regulates the transcription and replication of the virus genome in the

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doi:10.1016/j.febslet.2010.11.017

absence of M1 [20]. A previous study reported that NS2 can directly bind to NP without M1 in influenza B virus-infected cells [21]. Thus, the precise mechanism of M1 and NS2 in the nuclear export of vRNPs is not currently understood.

In the present study, we observed that NS2 binds to M1 through its C-terminal domain (residues 81–100), which is essential for the nuclear export of vRNP in infected cells. Furthermore, we characterized the NS2 binding domain on M1 using a series of mutant recombinant M1 proteins. The association between NS2 and M1 was mediated by ionic interactions. We also discuss the possibility that a factor other than NS2 may be involved in the nuclear export of vRNPs.

2. Materials and methods

2.1. Cell and antibodies

HeLa cells were grown in minimal essential medium (MEM) (SIGMA) containing 10% fetal bovine serum. The anti-M1 antibody was prepared as previously described [22]. The anti-NP monoclonal antibody (mAb61A5) preferentially immunoprecipitates the NP residing in the vRNP complex but not free NP was kindly provided by Dr. F. Momose (Kitazato University) [23]. The anti-GST antibody and anti-His antibody were purchased from GE healthcare and Santa Cruz, respectively.

2.2. Vector

The His-M1WT, His-M1ΔN, His-M1ΔM and His-M1ΔC plasmids have been described previously [24]. To construct pCAGGS-GFP, the GFP fragment was amplified from pEGFP-C1 by PCR. pCAGGS was digested with *Eco*RI and treated with the Klenow fragment. The PCR product was ligated to pCAGGS. To make the pCAGGS-GFP-NS2, pCAGGS-GFP-NS2ΔN, pCAGGS-GFP-NS2ΔC1, pGEX-NS2, pGEX-NS2ΔN, pGEX-NS2ΔC1, and pGEX-NS2ΔC2 plasmids, NS2 fragments were amplified from pET14b-NS2 by PCR using the following primer sets: NS2-up (5′-GGAATTCCATATGGATCCAAACACTGTGTCAAGCTTTCAG-3′) and NS2-low (5′-GCTTAAGCTCGAGTTAAATAAGCTGAAATGAGAAAGTTCTTATCTCTTGCT-3′), NS2ΔN (5′-GGAATTCCATATGCTCCACTCACTCCAAAACAGAAAC-3′) and NS2-low, and NS2-up and NS2ΔC1 (5′-CCCCTCGAGCTAAATCAACCATCTTATTTCTTCAAACTTCTG-3′), NS2-up and NS2ΔC2 (5′-CCCCTCGAGCTACATAAATGTTATTTGCTCAAAACTATTCTCTG-3′), and were digested with *Eco*RI and *Xho*I. The PCR fragments were inserted into the *Eco*RI- and *Xho*I-digested site of pCAGGS-GFP or pGEX6p-1, respectively. To make pCAGGS-GFP-NS2ΔM and pGEX-NS2ΔM, NS2 fragments were amplified from pET14b-NS2 by PCR using the primer sets NS2-up and NS2ΔM54 (5′-CCCCCGATATCTCCCATTCTCATCACTGCTTCTC-3′), and NS2Δ79 (5′-CCCCCGATATCGAAGAAGTGAGACACAAACTGAAGAT-3′) and NS2-low. The fragment encoding residues 1–54 of NS2 was digested with *Eco*RI and *Eco*RV, and the fragment encoding residues 80–121 of NS2 was digested with *Eco*RV and *Xho*I. These fragments were inserted into the *Eco*RI and *Xho*I site of pCAGGS-GFP or pGEX6p-1, respectively.

2.3. Purification of recombinant His-M1 and GST-NS2

His-M1 was purified in accordance with the pET-system manual (Novagen) and a previous report [24] with some modifications. Briefly, His-M1 expression *Escherichia coli* was sonicated in a binding buffer (5 mM imidazole, 0.1 M NaCl, 20 mM Tris–HCl (pH 7.9), and 1% CHAPS) and purified His-M1 was immediately used for GST-pull-down assays. GST-NS2 was purified in accordance with the glutathione sepharose 4B manual (GE healthcare) with some modifications. Briefly, the *E. coli* expressing GST-NS2 was sonicated in a NET/NP-40 buffer (50 mM Tris–HCl (pH 7.9), 0.1 M NaCl, 5 mM EDTA, and 0.1% NP-40) and the NET/NP-40 buffer was also used as wash buffer. We confirmed the purity of His-M1 and GST-NS2 by CBB staining (data not shown).

2.4. Pull-down assays

For the GST pull-down assay, GST-fused NS2 bound to 10 μl (bed volume) of glutathione sepharose beads (GE healthcare) were incubated at 4 °C for 1 h with purified His-M1 (2.0 μg) in the NET/NP-40 buffer. The beads were washed with the NET/NP-40 buffer three times at 4 °C. For the detection of GST-NS2 and His-M1, a Western blot analysis was performed using either anti-GST or anti-M1. For the His-tag pull-down assay, His-M1 bound to 10 μl (bed volume) of His-bind resin (Novagen) was incubated for 1 h at 4 °C with purified GST-NS2 (2.0 μg) in the binding buffer. The

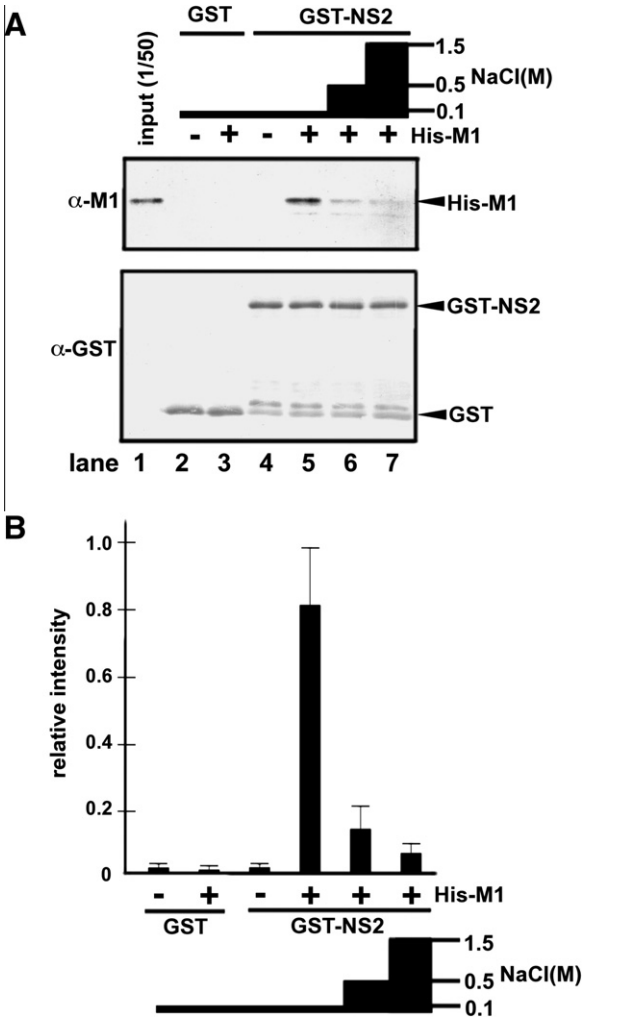

Fig. 1. Association of NS2 with M1 by ionic interactions. (A) GST pull-down assay using GST-fused NS2 (0.5 μg) and purified His-M1 (2.0 μg). The GST-fused NS2 mutants on the glutathione sepharose beads were incubated with His-M1. The beads were washed with NET/NP-40 buffer containing 0.1, 0.5, or 1.5 M NaCl. A Western blot analysis was performed using either anti-GST (lower panel) or anti-M1 (upper panel). In this assay, 1/50 of total volume of His-M1 was loaded as the input (lane 1), and 1/4 of total volume of each eluted sample was loaded (lanes 2–7). (B) The binding efficiency of GST-fused NS2 to His-M1. The relative intensity was shown as the ratios of the band intensity of His-M1 in each lane to those of GST-fused proteins bound to glutathione sepharose beads. The band intensity was measured using the image analyze software package (Image J). The data represent the means and standard deviations from three independent experiments.