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The Nuclear Export Protein of H5N1 Influenza A Viruses Recruits Matrix 1 (M1) Protein to the Viral Ribonucleoprotein to Mediate Nuclear Export*

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Linda Brunotte[‡], Joe Flies[‡], Hardin Bolte[‡], Peter Reuther[‡1], Frank Vreede[§], and Martin Schwemmle[‡2]

From the [‡]Institute for Virology, University Medical Center Freiburg, Hermann-Herder-Str. 11, 79104 Freiburg, Germany and the [§]Sir William Dunn School of Pathology, University of Oxford, South Parks Rd., Oxford OX 3RE, United Kingdom

Background: NEP stimulates viral RNA synthesis and nuclear vRNP export.
Results: NEP is required to stabilize M1-vRNP binding for nuclear export. Deletion of the last three amino acids of NEP abrogates nuclear export and the polymerase-enhancing function of NEP.
Conclusion: The polymerase-enhancing function and the nuclear export function of NEP are linked functionally.
Significance: This study provides new insights into the assembly of the nuclear export complex.

In influenza A virus-infected cells, replication and transcription of the viral genome occurs in the nucleus. To be packaged into viral particles at the plasma membrane, encapsidated viral genomes must be exported from the nucleus. Intriguingly, the nuclear export protein (NEP) is involved in both processes. Although NEP stimulates viral RNA synthesis by binding to the viral polymerase, its function during nuclear export implicates interaction with viral ribonucleoprotein (vRNP)-associated M1. The observation that both interactions are mediated by the C-terminal moiety of NEP raised the question whether these two features of NEP are linked functionally. Here we provide evidence that the interaction between M1 and the vRNP depends on the NEP C terminus and its polymerase activity-enhancing property for the nuclear export of vRNPs. This suggests that these features of NEP are linked functionally. Furthermore, our data suggest that the N-terminal domain of NEP interferes with the stability of the vRNP-M1-NEP nuclear export complex, probably mediated by its highly flexible intramolecular interaction with the NEP C terminus. On the basis of our data, we propose a new model for the assembly of the nuclear export complex of Influenza A vRNPs.

Influenza A viruses belong to the family of Orthomyxoviridae and possess an eight-segmented, single-stranded RNA genome of negative polarity. In the virus particle, the genomic RNA segments are encapsidated by nucleoprotein and complexed with the heterotrimeric viral polymerase to form the viral ribonucleoproteins (vRNPs)[3] (1–3). vRNPs are surrounded by the Matrix 1 protein (M1), which is the most abundant protein in the virion, and are further associated with a few copies of the nuclear export protein (NEP) (4, 5). Following receptor binding, viral particles are internalized by endocytosis. Acidification of the endosome induces structural changes in the HA (6) and M1 proteins that eventually lead to fusion of the viral membrane with the endosome and release of vRNPs into the cytoplasm. Concurrently, M1 and probably NEP dissociate from vRNPs (7). Dissociation of M1 exposes nuclear localization signals on NP that are recognized by cellular importins that mediate the nuclear import of the incoming vRNPs (8). In the nucleus, the viral polymerase initiates transcription of viral mRNAs and replication of the viral genome. The latter requires synthesis of a full-length copy (cRNA) of the viral genomic RNA (vRNA), which is also encapsidated by the viral polymerase and NP to form the cRNP that is used as a template for vRNA synthesis (9). Because budding of progeny virions occurs at the plasma membrane, the newly replicated vRNPs have to cross the nuclear membrane to reach the cytoplasm, where they are transported in association with Rab11-positive recycling endosomes along microtubules to the viral budding sites (10–12). This requires the assembly of a nuclear export complex consisting of the vRNP, the M1 protein, and NEP, which contains two nuclear export signals (NESs) (13–16). Assembly of this complex is assumed to occur in a daisy chain manner at late time points during infection, when a subset of M1 is imported into the nucleus via its nuclear localization signals and interacts via its C-terminal domain (17) with the newly formed vRNPs, presumably by interaction with NP and the viral RNA (18–20). Because M1 is not able to interact with CRM1 itself, NEP is proposed to bridge the M1/CRM1 interaction by binding to M1 with its C terminus, whereas the two N-terminal NESs interact with CRM1 and allow access to the CRM1-dependent export pathway (Fig. 1*A*) (15, 21).

NEP is a multifunctional protein that can influence viral RNA synthesis by interacting with the PB2 and PB1 subunits of the viral polymerase (22–24). Intriguingly, the binding sites for

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[1] Recipient of a Studienstiftung des deutschen Volkes fellowship.
[2] To whom correspondence should be addressed: Institute for Virology, University Medical Center Freiburg, Hermann-Herder-Str. 11, 79104 Freiburg, Germany. Tel.: 761-203-6526; Fax: 761-203-6639; E-mail: martin.schwemmle@uniklinik-freiburg.de.
[3] The abbreviations used are: vRNP, viral ribonucleoprotein; vRNA, viral RNA; NES, nuclear export signal; hpt, hours post-transfection; LMB, leptomycin B; VLP, virus-like particle; L1, linker region 1; NP, nucleoprotein; cRNP, complementary ribonucleoprotein; PA, polymerase acidic protein; NA, neuraminidase.

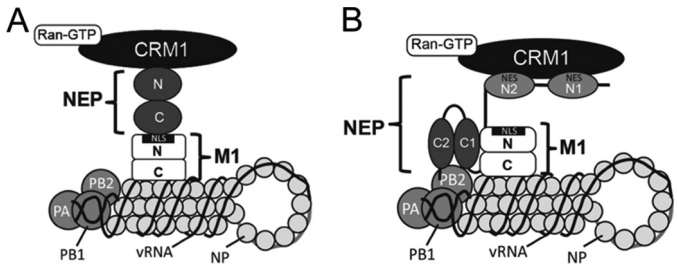

FIGURE 1. **Schematic of the nuclear export complex.** *A*, in the currently proposed daisy chain model of the nuclear export model, M1 interacts with the vRNP. NEP binds with its C-terminal (*C*) domain to vRNP-associated M1 and, with its NES-containing N terminus (*N*), establishes an interaction with the cellular export protein CRM1. *B*, refined model of the vRNP nuclear export complex. NEP interacts with the viral polymerase to provide additional binding site and support M1/vRNP association. The N-Terminus of NEP is flexible and interacts with CRM1.

M1 and PB2 on NEP have both been mapped to the C-terminal domain of NEP, comprising amino acids 50–121 (25, 26). However, experimental data are missing to elucidate whether these two interactions occur exclusively and how these two functions of NEP might be coordinated during viral replication and nuclear vRNP export. By using different NEP mutants for *in vitro* reconstitution of a vRNP-M1-NEP-containing nuclear export complex, we show here that M1 alone is not able to interact with vRNPs in the nucleus. Instead, vRNP/M1 interaction requires the C-terminal domain of NEP. We provide further evidence that the last three amino acids of NEP are equally important for the polymerase-enhancing and nuclear export functions of NEP, suggesting that these two activities of NEP are linked functionally.

EXPERIMENTAL PROCEDURES

Plasmid Construction—pCAGGS expression vectors for the avian H5N1 precursor virus (AvianPr)- and A/Thailand/1(KAN-1)/04 (KAN-1)-derived proteins PB2, PB1, PA, NP, NEP, and the mutants NEP-C and NEP_{1-118} have been described before (23, 26). Coding regions for KAN-1 M1, M2, NA, and HA proteins were amplified from the KAN-1 pHW2000 rescue plasmids (27) by PCR with Phusion polymerase (Finnzymes) using forward and reverse primers with NotI and XhoI restriction sites, respectively. PCR products were cloned into pCAGGS after NotI and XhoI digestion. To generate N-terminal Strep- and GFP-tagged proteins and the NEP mutant NEP-ΔN1, the desired coding regions were PCR-amplified using forward primers with an additional nucleotide in front of the ATG to maintain the reading frame after NotI digestion. PCR products were cloned into a modified pCAGGS vector harboring an N-terminal Strep tag or GFP tag. NEP-ΔN2 was constructed using primers specific for N2-flanking sequences and amplification of the remaining NEP and vector sequence. NEP N-GFP-C was generated by amplification of GFP-NEP-C using primers with an overhang specific for the last 20 nucleotides of the NEP N terminus. Likewise, the NEP N terminus was amplified using a reverse primer with a GFP-encoding overhang. The two fragments were fused by PCR using the abovementioned external primers and ligated into pCAGGS.

NEP-L1-FLAG and NEP-L2-FLAG were generated by insertion of the FLAG epitope, flanked by two glycine residues, into KAN-1 NEP. First, N- and C-terminal fragments of NEP were generated from pCAGGS-KAN-1 NEP using cloning primers specific for the NEP start and stop regions harboring NotI and XhoI restriction sites, respectively, together with internal primers encoding the FLAG epitope. Secondly, FLAG-tagged N- and C-terminal fragments were fused by PCR using the abovementioned start and stop primers and cloned into pCAGGS using the NotI and XhoI restriction sites. All primer sequences are available upon request.

Reconstitution of the Viral Polymerase—To reconstitute the AvianPr- or KAN-1-derived viral polymerase, HEK293T cells in 12-well plates were transfected with 50 ng each of PB2, PB1, and PA; 200 ng of NP expression plasmid; and 25 ng of a plasmid encoding a polymerase I-transcribed influenza A vRNA-like genome segment with a firefly luciferase gene in negative orientation. To normalize differences in transfection efficiency, 25 ng of a plasmid expressing a *Renilla* luciferase under the control of the polymerase II promoter was cotransfected. Cells were lysed 24 h post-transfection (hpt), and luciferase activity was measured using the Dual-Glo luciferase assay system (Promega). PB2 was omitted from the transfection mixture as a negative control. To inhibit CRM1-dependent nuclear export, 50 ng/μl leptomycin B (LMB) was added to the transfection medium at 6 hpt. A 76-nt-long short viral RNA was used for reconstitution of the NP-free RNP as described by Turrell *et al.* (28).

Primer Extension Analysis—To determine vRNA, cRNA, and mRNA synthesis by the reconstituted KAN-1-derived viral polymerase, 293T cells in 6-well plates were transfected with 125 ng each of PB2, PB1, and PA; 500 ng of NP expression plasmid; and 100 ng of a plasmid encoding a polymerase I-transcribed influenza A/WSN/33 virus vRNA on the basis of the full-length NA genome segment or a 76-nt-long short vRNA on the basis of the NP genome segment. To investigate the effect of M1 and NEP on viral RNA synthesis, 625 ng of M1-expressing plasmid and 62.5-ng of a KAN-1 NEP-expressing plasmid were cotransfected.

To purify total RNA, cells were harvested 24 hpt using 1 ml of TriFast reagent (Peqlab) per 6-well dish according to the protocol of the manufacturer. Primer extension analysis was performed using primers specific for viral genome segment-specific vRNA, cRNA, and mRNA and for cellular 5 S ribosomal RNA, as described previously (23).

Coprecipitation Analysis—293T cells in 6-well plates were transfected with 1000 ng of Strep-tagged KAN-1 M1 or 500 ng of Strep-tagged KAN-1 PB2 expression plasmids together with 1000 ng of GFP-tagged WT NEP or 2000 ng of GFP-tagged NEP mutants. Cells were lysed in radioimmune precipitation assay buffer (50 mM Tris-HCl (pH 8), 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium-deoxycholate, and 0.1% SDS). Cleared lysates where incubated with equilibrated Strep-Tactin matrix (IBA, Göttingen, Germany) for 90 min at 4 °C on a rotating wheel. Following three washes with radioimmune precipitation assay buffer, bound complexes were released by addition of 1× Laemmli SDS-PAGE buffer (63 mM Tris-HCl (pH 6.8), 10% glycerol, 0.0005% bromphenol blue, and 0.1% β-mercaptoethanol) and heated at 95 °C.

To capture the nuclear export complex, plasmids expressing KAN-1 PB2, PB1, and PA (400 ng each); 1500 ng of NP; and 300 ng of a GFP-expressing vRNA-like minigenome were cotransfected with 1000 ng of Strep-tagged M1 and 1000 ng of GFP-tagged WT NEP or 2000 ng of GFP-tagged NEP mutants. 24 hpt, cells were washed with PBS and lysed in Strep lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 1 mM DTT, and 10% protease inhibitor mix G (Promega)). Cleared lysates were mixed with equilibrated Strep-Tactin matrix and incubated at 4 °C overnight on a rotating wheel. Bound complexes were washed three times with Strep wash buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM $MgCl_2$, and 2 mM $CaCl_2$) and released by addition of 1× Laemmli SDS-PAGE buffer and heating at 95 °C. Precipitated proteins were separated on 15% polyacrylamide gels, transferred to nitrocellulose, and detected with antibodies against M1 (Abcam), NEP (Thorsten Wolff, Robert Koch Institute, Germany), and NP (Georg Kochs, University Medical Center Freiburg, Freiburg, Germany). Quantification of Western blots was performed with Image Studio software (Li-Cor).

Virus-like Particle (VLP) Assay—To investigate whether NEP or different NEP mutants supported nuclear export of vRNPs, 293T cells were transfected with plasmids expressing KAN-1- or SC35M-derived PB2, PB1, NP, M2, HA, NA, M1, and WT NEP or NEP mutant proteins together with a GFP-encoding vRNA-like template as described previously (29). The transfection medium was exchanged for infection medium (Dulbecco's modified Eagle's medium supplemented with 0.2% BSA, 2 mM L-glutamine, and 1% penicillin/streptomycin) 6 hpt. 48 hpt, VLP-containing supernatant was transferred to Madin-Darby canine kidney cells together with SC35M as a helper virus at a multiplicity of infection of 5. GFP expression was monitored 8 h post-infection by fluorescence microscopy.

RESULTS

Coexpression of NEP and M1 Abrogates Viral Replication and Transcription—To study the effect of NEP and M1 on viral polymerase activity, we reconstituted an avian H5N1 RNP (AvianPr) (23), derived from the putative avian precursor virus of the human H5N1 isolate KAN-1 (A/Thailand/1/(KAN-1)/2004) (30) in human HEK293T cells in the presence of M1 and NEP proteins. Upon transfection of 25 ng NEP expression plasmid, the viral polymerase activity increased 8-fold (Fig. 2*A*), which is consistent with the previous observation that the AvianPr polymerase is highly susceptible to the polymerase-enhancing effect of KAN-1 NEP (24, 27). However, addition of increasing amounts of M1 in the presence of NEP resulted in a dose-dependent inhibition of AvianPr polymerase activity (Fig. 2*A*, *black line*). On the other hand, M1-driven inhibition was significantly lower in the absence of NEP (Fig. 2*A*, *gray line*), suggestive of cooperation between M1 and NEP in diminishing the viral polymerase activity. Primer extension analysis confirmed that the expression of M1 alone did not affect viral RNA levels (Fig. 2*B*, *lane 3*) whereas the expression of NEP increased viral RNA synthesis (Fig. 2*B*, *lane 4*). Coexpression of M1 and NEP, however, inhibited the synthesis of all three viral RNA species (mRNA, cRNA, and vRNA) by AvianPr (Fig. 2*B*, *lane 5*). To exclude that the M1/NEP-mediated inhibition of the polymerase activity was restricted to the AvianPr strain, we also reconstituted RNPs with the KAN-1 polymerase. We found that NEP and M1 coexpression also cooperatively inhibited KAN-1 polymerase activity (Fig. 2*C*).

Turrell *et al.* (28) have demonstrated previously that NP is dispensable for transcription and replication of short viral RNAs by the viral polymerase. We used this system to investigate whether the presence of NP is required for the cooperative inhibition of polymerase activity by NEP and M1. A primer extension analysis revealed that expression of NEP is able to stimulate the synthesis of vRNA, cRNA, and mRNA in this system (Fig. 2*D*, *lane 3*). However, coexpression of NEP and M1 did not result in reduced viral RNA levels (Fig. 2*D*, *lane 5*). This suggests that M1 and NEP can only inhibit viral polymerase activity when the complete RNP, consisting of the trimeric polymerase, NP, and the viral RNA, is assembled.

Because NEP and M1 are known to mediate nuclear export of vRNPs, we speculated that the M1/NEP-mediated inhibition of viral polymerase activity is due to nuclear export of the reconstituted vRNPs. Therefore, we made use of the nuclear export inhibitor LMB, which irreversibly occupies the NES-binding pocket of CRM1 (31–33) and, thereby, impedes binding of the NEP-NES, to test this hypothesis. As shown in Fig. 2*E*, the presence of LMB, NEP, and M1 only reduced KAN-1 polymerase activity to 78% compared with 23% in the absence of LMB (Fig. 2*C*), indicating that nuclear export indeed contributes to the inhibition of viral polymerase activity. In summary, these results show that coexpression of NEP and M1 in the RNP reconstitution assay results in the reduction of viral polymerase activity. The sensitivity of this inhibition to the nuclear export inhibitor LMB further suggests that vRNPs are assembled with M1 and NEP into complexes targeted for nuclear export by CRM1.

The Polymerase-enhancing Function of NEP Is Essential for the Nuclear Export of vRNPs—To elucidate the functional domains of NEP required for the cooperative inhibition of viral polymerase activity, we made use of two truncated versions of NEP described previously. Firstly, we tested a mutant of NEP (NEP-C) that lacks the complete N terminus, including both NESs (Fig. 3*A*). NEP-C constitutively enhances the AvianPr polymerase, as reported previously (26), but cannot interact with CRM1 because of the lack of both NESs. Although coexpression of M1 reduced the polymerase-enhancing function of NEP-C 5-fold, it was not abrogated (Fig. 3*B*). This is consistent with our hypothesis that nuclear export of vRNPs is responsible for the reduction in viral polymerase activity in the presence of M1 and WT NEP. Next, we used a mutant of NEP that only lacks the last three amino acids (NEP_{1-118}) (Fig. 3*A*). We have shown previously that this deletion renders the protein unable to stimulate viral polymerase activity for unknown reasons (26). In fact, the expression of low levels of NEP_{1-118} in the RNP reconstitution assay resulted in a moderate decrease in polymerase activity to 67% (Fig. 3*C*). However, coexpression of M1 did not further reduce polymerase activity (Fig. 3*C*), indicating that these residues are not only important for the polymerase-enhancing function but might also play a role in the nuclear export function of NEP. To verify the importance of the N terminus and the last three amino acids of NEP for nuclear export, both

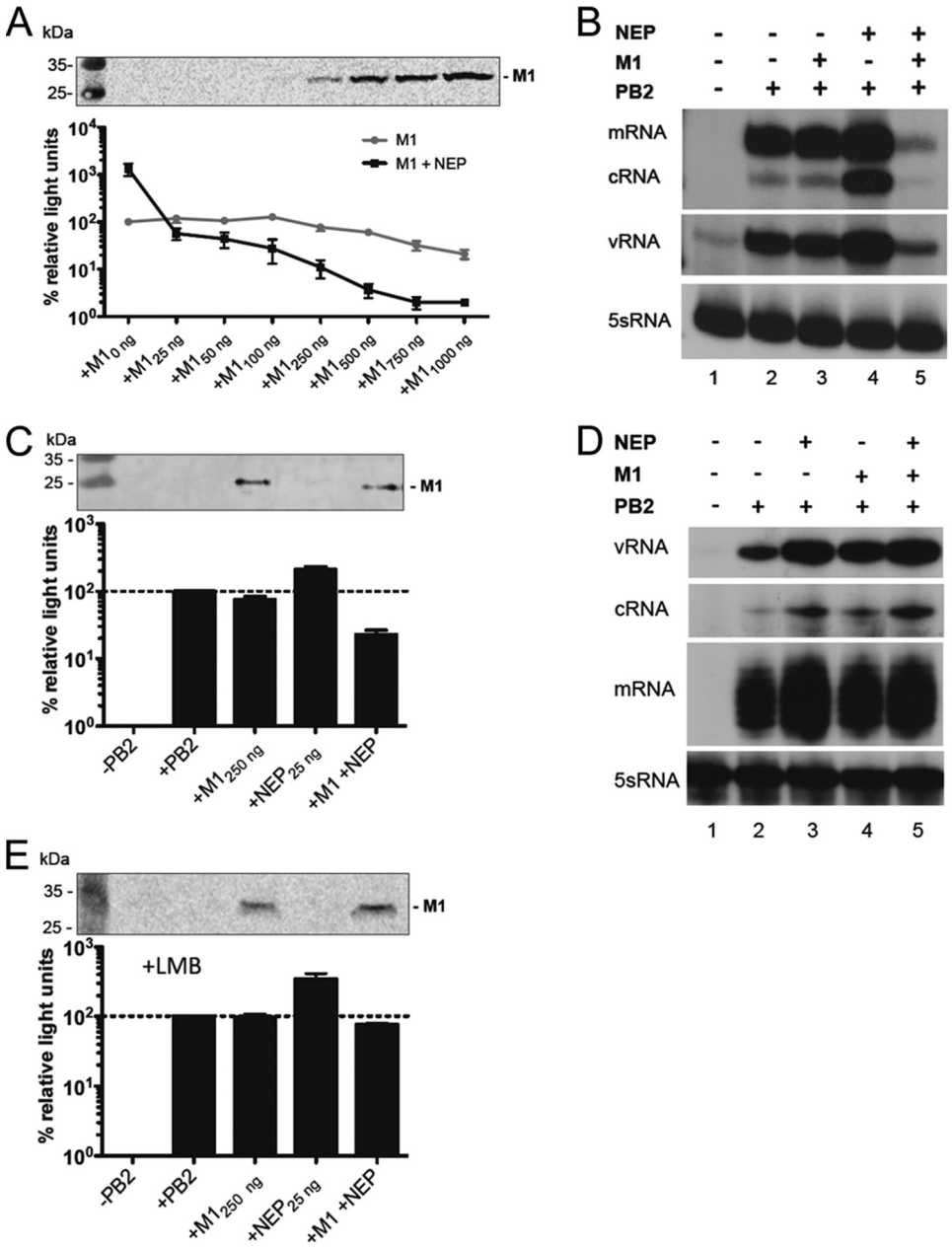

FIGURE 2. **NEP and M1 cooperatively inhibit viral polymerase activity.** *A*, analysis of the effect of M1 and NEP expression on the activity of reconstituted AvianPr- or KAN-1-derived RNPs. 293T cells were transfected with expression plasmids for the viral proteins PB1, PB2, PA, and NP together with a vRNA-like luciferase reporter plasmid under the control of the human polymerase I promoter. A polymerase II-dependent expression plasmid coding for *Renilla* luciferase was transfected as an internal control. Luciferase values were measured 24 hpt. The effect of increasing concentrations of KAN-1 M1 in the AvianPr RNP reconstitution assay is shown in the absence (*gray line*) or presence (*black line*) of 25 ng of NEP expression plasmid. M1 expression levels were determined by Western blot analysis using α-M1 antibody. *Error bars* represent mean $\pm$ S.D. from three independent experiments. *B*, primer extension analysis of viral RNA levels synthesized by reconstituted AvianPr RNPs. A polymerase I-dependent reporter plasmid coding for segment 6 from WSN was used instead of the luciferase-encoding vRNA. 625 ng of KAN-1 M1 and 62.5 ng of KAN-1 NEP expression plasmids were either transfected alone or together into a 6-well plate. Total RNA was purified 24 hpt and reverse-transcribed using radioactively labeled segment 6-specific primers to detect viral mRNA, cRNA, and vRNA. A primer against cellular 5 S ribosomal RNA was used as a control. *C*, analysis of the effect of KAN-1-derived M1 and NEP coexpression on reconstituted KAN-1 vRNPs in the absence of LMB. M1 expression levels were determined by Western blot analysis using α-M1 antibody. *Error bars* represent mean $\pm$ S.D. from three independent experiments. *D*, primer extension analysis of the effect of M1 and NEP coexpression on the activity of reconstituted NP-free RNPs. A 76-nt-long short vRNA-like template derived from segment 5 was used to allow NP-free transcription and replication. *E*, analysis of the effect of coexpression of KAN-1 M1 and NEP on reconstituted KAN-1 vRNPs as described in *C* in the presence of LMB.

NEP mutants were tested for their ability to export vRNPs in a VLP assay using a GFP-encoding vRNA (29). Polymerase activity in these experiments was controlled by monitoring GFP expression in 293T cells 24 h after transfection (Fig. 3*D*, *top row*). This showed that transfection of WT NEP led to reduced GFP expression in 293T cells compared with transfected cells with no NEP, NEP-C, or NEP_{1-118} (Fig. 3*D*, *top* row) and resembles our finding that, in the presence of M1 and WT NEP, viral polymerase activity is decreased. However, VLPs harboring GFP-vRNA were only formed in the presence of WT NEP but not in the presence of NEP-C or NEP_{1-118}, as shown by the detection of GFP in infected Madin-Darby canine kidney cells

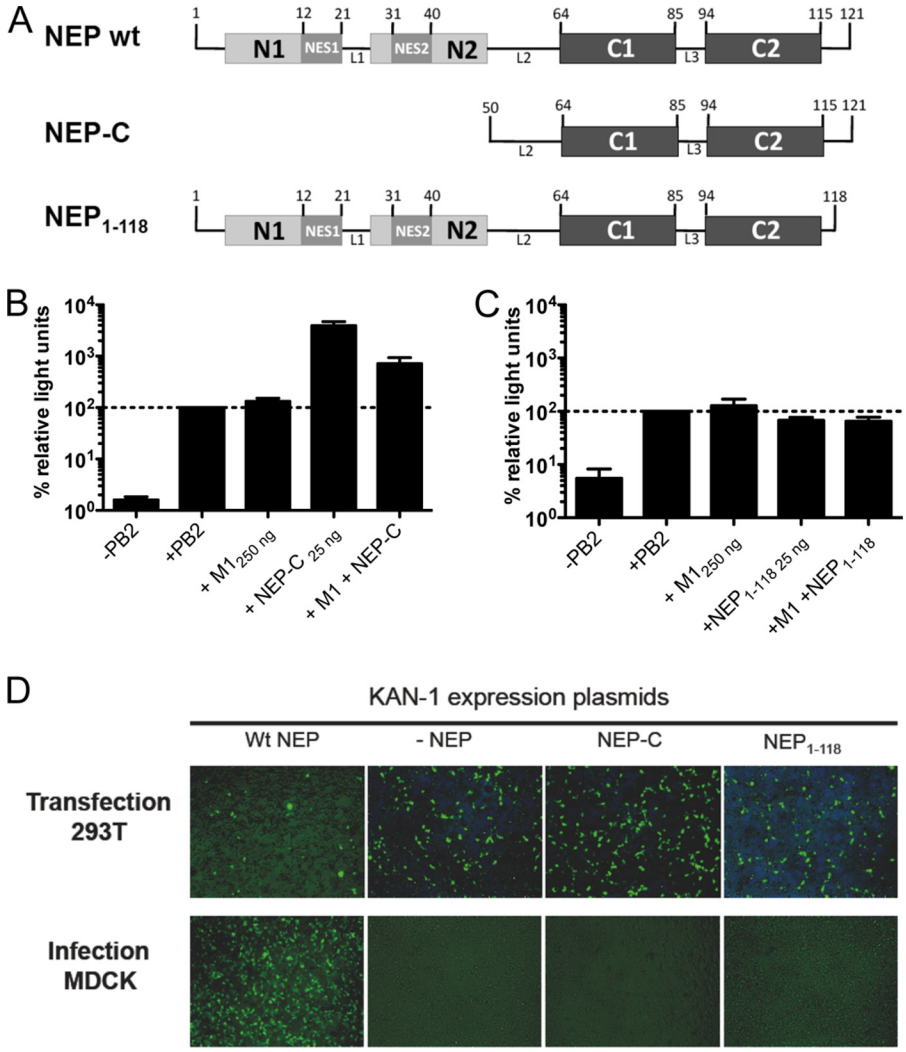

FIGURE 3. **The nuclear export function of NEP depends on its ability to enhance the activity of the viral polymerase.** *A*, schematic of KAN-1 WT NEP and the truncated NEP proteins NEP-C and NEP_{1-118}. The location of the two NESs is indicated. Predicted N-terminal α-helices are shown in *light gray*. C-terminal α helices are shown in *dark gray*. *L1-L3*, unfolded linker regions. *B*, analysis of the effect of KAN-1 M1 and NEP-C expression on the activity of the reconstituted AvianPr RNPs. *Error bars* represent mean ± S.D. from three independent experiments. *C*, analysis of the effect of KAN-1 M1 and NEP_{1-118} expression on the activity of reconstituted AvianPr RNPs. *D*, VLP analysis of nuclear export and packaging of a GFP-encoding vRNA. 293T cells were transfected with expression plasmids for KAN-1 derived PB2, PB1, PA, NP, HA, NA, M2, and M1 and WT NEP, NEP-C, or NEP_{1-118}. VLP-containing supernatants were harvested at 48 hpt and transferred to Madin-Darby canine kidney cells together with SC35M as a helper virus (multiplicity of infection of 5). As a control, the expression plasmid for NEP was omitted (−*NEP*). GFP expression was analyzed in transfected 293T cells (*top row*) and at 8 h post-infection in Madin-Darby canine kidney (*MDCK*) cells (*bottom row*).

(Fig. 3*D*, *bottom row*). These data corroborate the view that reduced viral replication is due to increased nuclear export of vRNPs. Intriguingly, coprecipitation experiments using GFP- and Strep-tagged proteins revealed that both NEP-C and NEP_{1-118} were still able to interact with PB2 (Fig. 4, *A* and *B*), indicating that neither the deletion of the N terminus (amino acids 1–49) nor removal of the last three amino acids of NEP abrogate this interaction. In contrast, NEP-C, but not NEP_{1-118}, showed reduced binding to M1 (Fig. 4, *C* and *D*). These data suggest that the ability of NEP to support nuclear export of vRNPs requires the NES-containing N terminus as well as the last three amino acids and is linked functionally to the polymerase activity-enhancing function of NEP during viral genome replication.

The Last Three Amino Acids of NEP Are Required for vRNP Export Complex Assembly—We showed that the deletion of the last three amino acids of NEP abrogates its ability to support nuclear export of vRNPs. However, this could not be explained by a lack of interaction with PB2 or M1 because neither was reduced by this deletion (Fig. 4, *A* and *B*).

To determine whether the loss of nuclear export function could instead be ascribed to a defect on the level of nuclear export complex assembly, we reconstituted KAN-1 RNPs in HEK293T cells together with Strep-tagged M1 protein and GFP-tagged NEP proteins. As shown in Fig. 4*E*, fusion of GFP to the N terminus neither abrogates the concentration-dependent activity of NEP to enhance or inhibit viral polymerase activity (Fig. 4*E*) nor its nuclear export function in the VLP assay (data not shown). After Strep-Tactin precipitation, assembly of the export complex was assessed by Western blot analysis using anti-NP antibody to detect coprecipitated RNPs.

To our surprise, despite efficient vRNP activity (Fig. 3*D*), we observed detectable vRNP coprecipitation only in the presence of the NEP-C mutant, which lacks the N-terminal domain, but

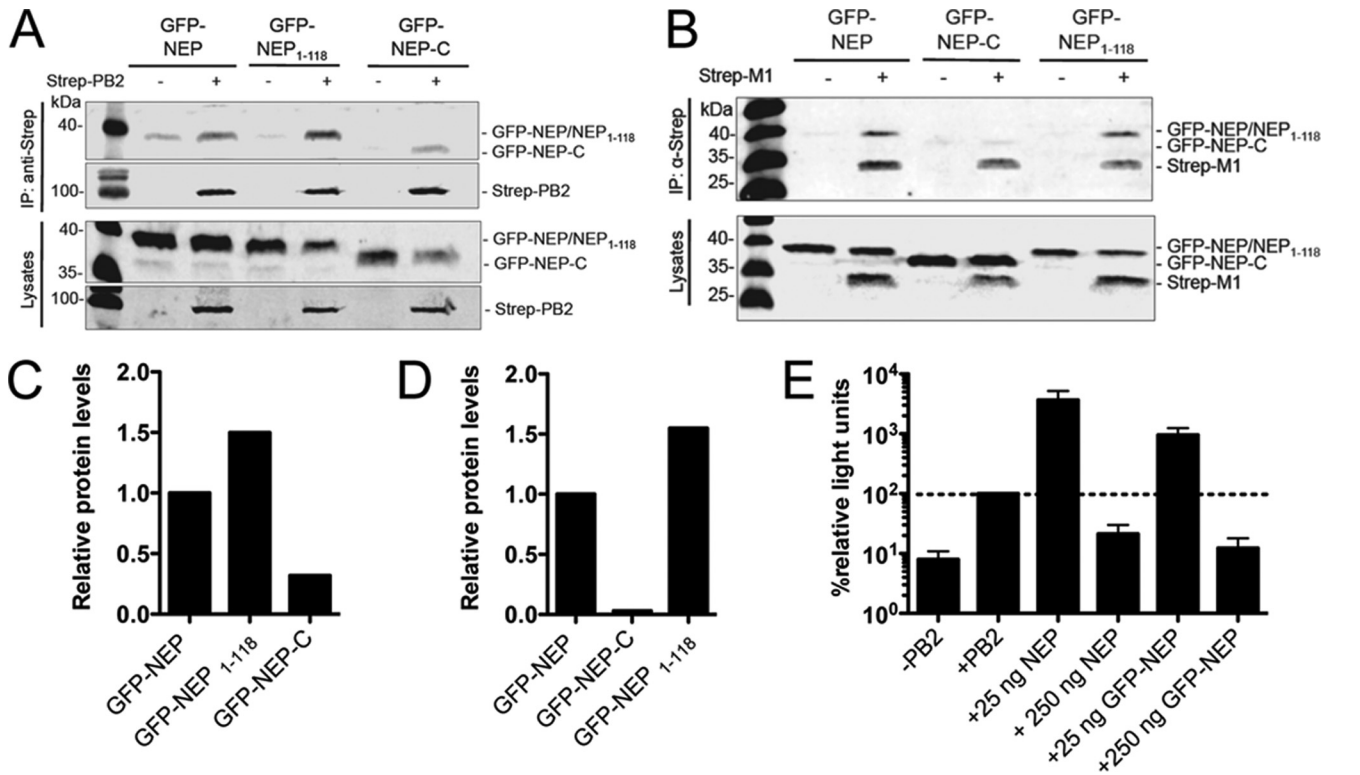

FIGURE 4. **Interaction of NEP with PB2 and M1 is not abrogated by deletion of the last three amino acids.** *A*, coprecipitation (*IP*) analysis of GFP-tagged KAN-1 NEP, NEP-C, or NEP_{1-118} proteins with Strep-tagged KAN-1 PB2. Tagged proteins were detected by Western blot analysis using α-Strep and α-NEP antibodies. *B*, quantification of *A*. *C*, coprecipitation analysis of GFP-tagged WT NEP, NEP-C or NEP_{1-118} with Strep-tagged KAN-1 derived M1. *D*, quantification of *C*. *E*, comparison of KAN-1 NEP and GFP-NEP effects on the viral polymerase in the AvianPr polymerase reconstitution assay. *Error bars* represent mean ± S.D. from three independent experiments.

not in the presence of the WT NEP or NEP_{1-118} proteins (Fig. 5*A*). This suggests that assembly of the vRNP-M1-NEP export complex in the presence of WT NEP is too unstable for detection by coprecipitation, maybe because of transient protein interactions or rapid nuclear export followed by disassembly in the cytoplasm. In contrast, the presence of the N-terminal deletion mutant NEP-C, which is unable to export assembled complexes, stabilizes the complex and allows detection in this assay.

Intriguingly, NEP-C was able to interact with M1 in the presence of the vRNP (Fig. 5*A*, *fifth lane*), whereas it did not interact with M1 alone (Fig. 4*B*, *lane 4*). Furthermore, we could not observe coprecipitation of vRNPs with Strep-tagged M1 (Fig. 5*A*, *second lane*), although M1 is known to bind to vRNPs in the viral particle (34, 35). Notably, omission of PB1 from the transfection mix abrogated NP coprecipitation, proving that the NP signal represents RNP-associated NP and not soluble NP (Fig. 5*B*, *second lane*). Taken together, these results suggest that, in the nucleus, polymerase-associated NEP is required to support the interaction between M1 and the vRNPs by providing additional binding sites for M1 to assemble a functional export complex.

Finally, we wanted to investigate the importance of the last three amino acids of NEP for the assembly of the vRNP-M1-NEP complex. However, because NEP-C, but not WT NEP, allowed purification of this complex, we deleted the last three amino acids of the NEP-C mutant, creating $NEP\text{-}C_{50-118}$. As expected, this deletion resulted in the abrogation of the polymerase-enhancing activity of NEP (Fig. 5*D*), the loss of interaction between M1 and NEP-C, and loss of vRNP coprecipitation (Fig. 5*C*, *third lane*). The latter might be due to the failure of $NEP\text{-}C_{50-118}$ to recruit M1 to the vRNP.

In summary, these results show that it is possible to purify a nuclear complex from transfected cells consisting of the vRNP, M1, and the C-terminal domain of NEP to detectable levels. Using NEP-C, we provide evidence that the last three amino acids are important to assemble the vRNP-M1-NEP nuclear export complex.

Linker Region 2 in NEP Is Involved in M1 Binding—The observation that $GFP\text{-}NEP_{1-118}$, but not $GFP\text{-}NEP\text{-}C_{50-118}$, coprecipitates with M1 (Fig. 5*A*, *fourth lane* and Fig. 5*C*, *third lane*) suggested that regions in the N-terminal domain of NEP were involved in M1 binding. Therefore, we mutated the N-terminal linker region 1 (*L1*) and 2 (*L2*) in NEP by insertion of a FLAG epitope flanked by two glycine residues (Fig. 6*A*). This leads to the elongation of the respective linker region by 10 amino acids and could lead to the disruption of potential M1 binding sites. As shown in Fig. 6*B*, mutation of the linker regions did not affect the ability of these NEP mutants to stimulate the polymerase activity of AvianPr (Fig. 6*B*). However, coexpression of NEP-L1-FLAG and M1 resulted in reduced viral polymerase activity (Fig. 6*B*), suggesting that linker region 1 is not involved in the formation of nuclear export complexes. Indeed, NEP-L1-FLAG was able to support nuclear export of vRNPs in the VLP assay (Fig. 6*C*). In contrast, FLAG tag insertion in L2 abolished the cooperative polymerase inhibition in the presence of M1 (Fig. 6*B*) and failed to export vRNPs (Fig.

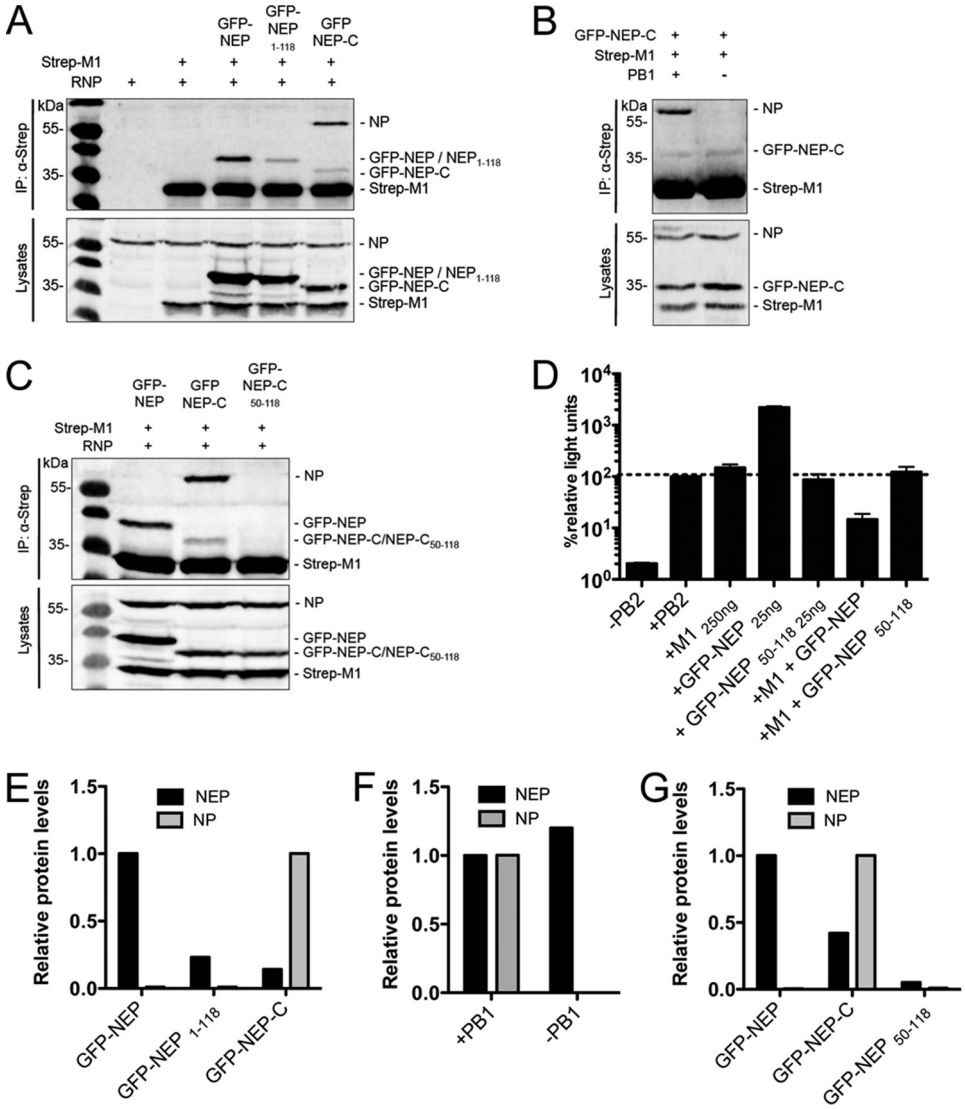

FIGURE 5. **The last three amino acids of NEP are essential for the assembly of vRNP-M1-NEP nuclear export complexes.** *A–C*, coprecipitation (*IP*) analysis of vRNP-M1-NEP complexes with Strep-tagged M1 in the presence of GFP-tagged WT NEP, NEP_{1-118}, and NEP-C (*A*); NEP-C with or without PB1 (*B*); or $NEP\text{-}C_{50-118}$ (*C*). Viral proteins were detected by Western blot analysis using antibodies against M1, NEP, and NP. *E*, analysis of the effect of NEP_{50-118} on the activity of the AvianPr polymerase. *Error bars* represent mean ± S.D. from three independent experiments. *D–G*, quantification of the Western blots in *A–C*.

6*C*). Coprecipitation experiments with Strep-tagged M1 protein revealed that NEP-L2-FLAG exhibits reduced binding to M1 when compared with WT NEP and NEP-L1-FLAG (Fig. 6, *D* and *E*), suggesting, that L2 is involved in export complex assembly and might serve as an additional M1 binding site. In summary, these data suggest that not only the last three amino acids of NEP, but also residues in L2, are important for the interaction of NEP with M1 and the assembly of a functional vRNP-M1-NEP export complex.

vRNP-M1-NEP Complexes Cannot Be Detected in the Presence of the Wild-type N-terminal Domain of NEP—Coprecipitation experiments revealed detectable levels of vRNP-M1-NEP only with the C terminus of NEP (NEP-C) but not with full-length NEP. This suggests that the N-terminal helices of NEP, which are known to negatively regulate polymerase-enhancing activity by intramolecular interaction with the C terminus (26), might be responsible for our failure to detect the vRNP-M1-NEP complex. Therefore, we constructed three additional NEP mutants by deletion of either the first N-terminal helix, N1 (GFP-NEP-ΔN1), the second N-terminal helix, N2 (GFP-NEP-ΔN2), or by increasing the distance between the N- and C-terminal domains through insertion of GFP between residues Val-49 and Met-50 (NEP N-GFP-C) (Fig. 7*A*). All three mutant proteins enhanced viral polymerase activity in the RNP reconstitution assay (Fig. 7*B*) and decreased polymerase activity, although to differing extents, when cotransfected with M1 (Fig. 7*B*). According to our hypothesis, this suggests that these NEP variants are able to support vRNP export. Indeed, as shown in Fig. 7*C*, formation of VLPs was observed with all three NEP mutants. This also demonstrates that the presence of only one NES is sufficient for nuclear export of vRNPs (Fig. 7*C*). Intriguingly, all three NEP mutants allowed detection of vRNP-M1-NEP complexes in transfected cells (Fig. 7, *D* and *E*). Taken together, these results indicate that the N-terminal helices of WT NEP interfere with the purification of the vRNP-M1-NEP export complex.

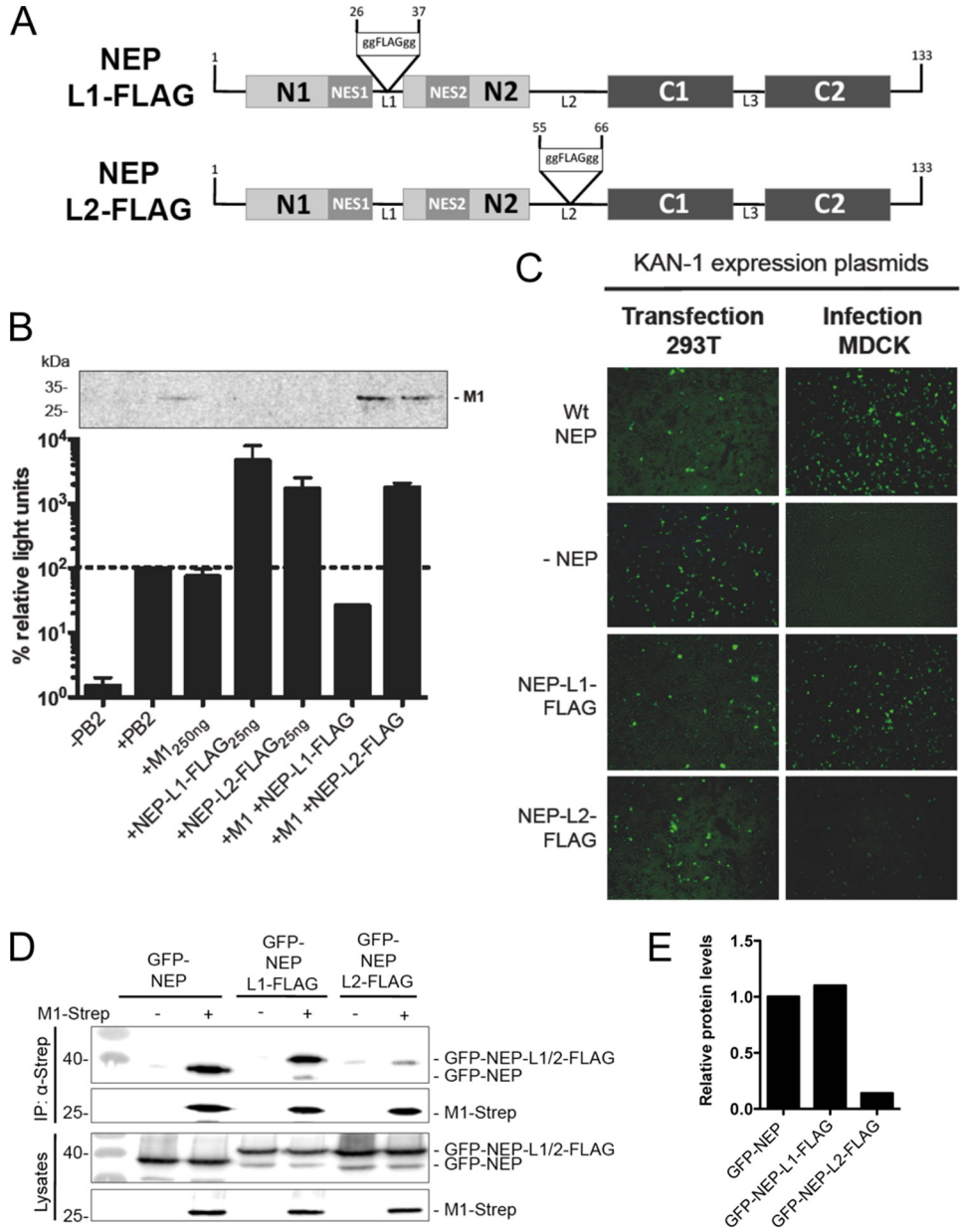

FIGURE 6. **Linker region 2 of NEP participates in export complex assembly.** *A*, schematic of sites of FLAG tag insertion in NEP. *B*, analysis of the effect of expression of NEP L1-FLAG, NEP L2-FLAG, and M1 on the activity of reconstituted AvianPr RNPs. *Error bars* represent mean ± S.D. from three independent experiments. *C*, VLP analysis as in Fig. 3*D* of reconstituted RNPs containing a GFP-expressing minigenome in the presence of NEP-L1-FLAG or NEP-L2-FLAG. *MDCK*, Madin-Darby canine kidney. *D*, coprecipitation analysis of NEP-L1-FLAG and NEP-L2-FLAG with Strep-tagged KAN-1-derived M1. *E*, quantification of *D*.

DISCUSSION

It is well established that nuclear export of vRNPs is mediated by the viral proteins M1 and NEP (reviewed in Ref. 36). However, because of the lack of suitable methods to purify vRNP-M1-NEP complexes from infected or transiently transfected cells, few details are currently known about how M1 and NEP assemble on the vRNP to form the nuclear export complex. Here we provide experimental data to demonstrate that the binding affinity of M1 to the vRNP is insufficient to establish a detectable vRNP/M1 interaction (Fig. 5*A*, *second lane*). Instead, by using a mutant NEP protein, our data show that NEP is required to enhance the binding affinity of M1 to the viral RNP. Interestingly, the C-terminal domain of NEP, which harbors the binding sites for PB2 and PB1 as well as M1 and, in addition, is responsible for the polymerase-enhancing function of NEP, is sufficient to tether M1 to the vRNP (Fig. 5*A*, *fifth lane*). Intriguingly, abrogation of the polymerase-enhancing function of NEP by deletion of the last three amino acids also resulted in the loss of the nuclear export function, suggesting that both of these activities are linked functionally (Fig. 3, *C* and *D*).

Taken together, our data argue against the established daisy chain model in which M1 binds to the vRNP by interacting with NP and viral RNA. Our data favor a refined model for the nuclear export complex (Fig. 1*B*), in which the role of NEP is extended beyond bridging the interaction between M1 and CRM1. Rather, we suggest that the C-terminal domain of NEP binds simultaneously to the viral polymerase and M1, whereas the N-terminal domain harboring the NES is not engaged in M1

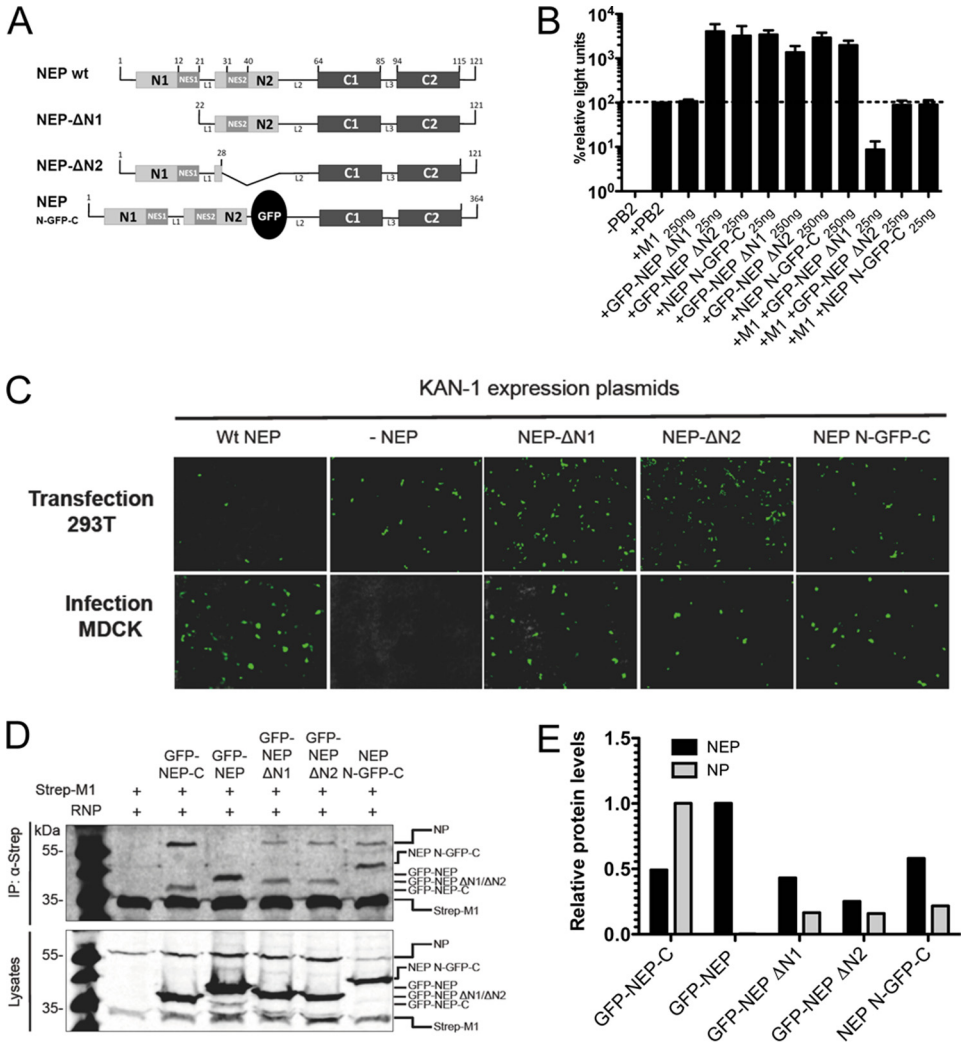

FIGURE 7. **The N-terminal α helices of NEP interfere with the assembly of stable vRNP-M1-NEP complexes.** *A*, schematic of the NEP mutants with deletions in the N terminus or the insertion of the GFP gene between the N- and C-terminal domains. *B*, analysis of the effect of expression of NEP N-terminal mutants and M1 on the activity of reconstituted AvianPr RNPs. *Error bars* represent mean ± S.D. from three independent experiments. *C*, coprecipitation analysis of vRNP-M1-NEP complexes with Strep-tagged M1 in the presence of the NEP N-terminal mutants. *MDCK*, Madin-Darby canine kidney. *D*, VLP analysis as in Fig. 3*D* of reconstituted RNPs containing a GFP-expressing minigenome in the presence of WT NEP or NEP N-terminal mutants. *IP*, immunoprecipitation. *E*, quantification of *D*.

binding and can establish the interaction with CRM1. However, because we lack a mutant of NEP without PB2 binding activity, we can, at this point, not entirely exclude that NEP binding to M1, as suggested in the daisy chain model, induces allosteric changes in the C-terminal domain of M1, leading to the enhanced vRNP/M1 interaction.

The lack of a stable vRNP/M1 interaction in our study seems to contradict results from other research groups that demonstrated binding between M1 and vRNP from purified virions (37) and direct interaction between M1 and NP proteins purified from *Escherichia coli* (38). However, in contrast to these studies, our work focuses on the interaction between M1 and reconstituted vRNPs in the nucleus of transfected cells to facilitate vRNP nuclear export. We show that association of M1 and vRNPs in this compartment requires NEP, most likely in its vRNP-bound state. Of course, this does not exclude the possibility of a more stable interaction occurring between M1 and vRNPs at later stages of infection, such as during vRNP cytoplasmic trafficking and virion assembly (36).

Although we could demonstrate that the C terminus of NEP is sufficient to stabilize the M1/vRNP interaction, we did not succeed in capturing the nuclear export complex in the presence of full-length NEP (Fig. 5*A*, *third lane*), despite very efficient nuclear export of vRNPs (Fig. 3*D*). We speculate that this could be due to rapid transport of vRNP-M1-NEP complexes across the nuclear membrane and the release of M1 and NEP during cytoplasmic dissociation. The latter is supported by the observation that vRNPs, which are specifically transported to the plasma membrane by Rab11-positive recycling endosomes, contain no detectable amounts of M1 (39). In addition, however, we speculate that the failure to detect M1-vRNP complexes with full-length NEP is due to the unusual feature of this protein to exist in open and closed conformations driven by intramolecular binding between the C- and N-terminal domains of NEP (26). Intramolecular binding requires both N-terminal α helices and is known to down-regulate the polymerase-enhancing activity of NEP (26). Such binding might also either prevent M1 association to vRNPs or enhance the

dissociation of M1 and NEP from exported vRNPs. Consistent with this hypothesis, we found that NEP mutants lacking either of the two N-terminal α helices or with GFP inserted between the N and C termini are able to support vRNP nuclear export (Fig. 7*C*) and mediate detection of a stable interaction between M1 and vRNP (Fig. 7, *D* and E). Moreover, these NEP mutants constitutively stimulate polymerase activity, also at high concentrations, in the absence of M1 (Fig. 7*B*), suggesting decreased regulatory activity of the N-terminal domain, probably because of the lack of intramolecular binding to the C terminus of NEP. This supports the hypothesis that intramolecular folding processes in NEP could influence dissociation of M1 from vRNPs following export. Interestingly, inhibition of CRM1-mediated nuclear export by treatment with LMB could not rescue vRNP/M1 association in the presence of full-length NEP (data not shown). On the basis of these data, we speculate that binding of CRM1 or other, yet unknown cellular adaptor proteins to the N-terminal NESs prevent the intramolecular interactions of NEP and, thereby, promote the assembly and rapid transport of vRNP-M1-NEP complexes across the nuclear envelope.

Remarkably, the last three amino acids of NEP seem to be a critical factor in the recruitment of M1 to vRNPs and their nuclear export. Our data also indicate that the flexible L2 of NEP contributes to stabilizing the interaction between M1 and NEP (Fig. 6). Thus, similar to other multifunctional proteins, NEP appears to possess several binding sites involved in M1 binding. Notably, the C-terminal three amino acids of NEP are required to stimulate polymerase activity but not for binding to PB2 (26). Therefore, it is tempting to speculate that binding of M1 to the C terminus of NEP abrogates its polymerase activity-enhancing function without disturbing its association to the viral polymerase, thereby initiating the nuclear export of vRNPs.

Although structurally similar to vRNPs, cRNPs (40) seem to be excluded from nuclear export because only vRNPs are detected in the cytoplasm of infected cells (41, 42). This suggests that influenza A viruses are able to discriminate between these two molecules for nuclear export. It is unlikely that this is performed by M1 alone because the binding of M1 to vRNPs is dependent on the presence of the NEP C terminus (Fig. 5*A*). Interestingly, Tchatalbachev *et al.* (42) have shown that discrimination occurs at the stage of nuclear export and is mediated by different conformations of the viral polymerase upon binding to vRNA or cRNA promoters. Our results suggest that NEP, despite tethering M1 to the vRNP, also establishes a connection between M1 and the viral polymerase. Therefore, it is therefore tempting to speculate that NEP only binds to vRNA-associated polymerases and, thereby, discriminates between cRNPs and vRNPs. However, it remains to be shown whether NEP has a preference for binding to vRNA-bound polymerases.

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The Nuclear Export Protein of H5N1 Influenza A Viruses Recruits Matrix 1 (M1) Protein to the Viral Ribonucleoprotein to Mediate Nuclear Export

Linda Brunotte, Joe Flies, Hardin Bolte, Peter Reuther, Frank Vreede and Martin Schwemmle

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