Adaptive Mutations in the Nuclear Export Protein of Human-Derived H5N1 Strains Facilitate a Polymerase Activity-Enhancing Conformation

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The nuclear export protein (NEP) (NS2) of the highly pathogenic human-derived H5N1 strain A/Thailand/1(KAN-1)/2004 with the adaptive mutation M16I greatly enhances the polymerase activity in human cells in a concentration-dependent manner. While low NEP levels enhance the polymerase activity, high levels are inhibitory. To gain insights into the underlying mechanism, we analyzed the effect of NEP deletion mutants on polymerase activity after reconstitution in human cells. This revealed that the polymerase-enhancing function of NEP resides in the C-terminal moiety and that removal of the last three amino acids completely abrogates this activity. Moreover, compared to full-length NEP, the C-terminal moiety alone exhibited significantly higher activity and seemed to be deregulated, since even the highest concentration did not result in an inhibition of polymerase activity. To determine transient interactions between the N- and C-terminal domains in cis, we fused both ends of NEP to a split click beetle luciferase and performed fragment complementation assays. With decreasing temperature, increased luciferase activity was observed, suggesting that intramolecular binding between the C- and N-terminal domains is preferentially stabilized at low temperatures. This stabilizing effect was significantly reduced with the adaptive mutation M16I or a combination of adaptive mutations (M16I, Y41C, and E75G), which further increased polymerase activity also at 34°C. We therefore propose a model in which the N-terminal moiety of NEP exerts an inhibitory function by back-folding to the C-terminal domain. In this model, adaptive mutations in NEP decrease binding between the C- and N-terminal domains, thereby allowing the protein to “open up” and become active already at a low temperature.

Zoonotic transmissions of avian influenza A viruses pose a constant threat to the human population and can cause severe pandemics associated with high numbers of fatal cases mainly due to the lack of a preexisting immunity (1–3). Because of effective species barriers, only in rare cases are avian viruses able to establish a new lineage in humans. To overcome these species barriers, changes in receptor specificity, stability, and glycosylation of the viral hemagglutinin (HA) as well as adaptation to the host immune response are required (1, 4–6). In addition, avian viruses have to overcome the poor polymerase activity in human cells (1, 7, 8). The basic underlying mechanisms for the impaired polymerase activity are unknown and are possibly due to incompatibility with cellular factors required for efficient replication (9–12). Recent evidence suggests that in the case of avian H5N1 viruses, efficient replication of genomic RNA (vRNA) in human cells is affected (13).

Several adaptive mutations in the polymerase, consisting of the subunits PA, PB1, and PB2, of avian H5N1 viruses are known to be required to overcome the impaired replication efficiency in human cells (7, 8). Although the single mutation E627K in PB2 is sufficient to increase polymerase activity to levels comparable to those of human influenza A virus strains (13–17), approximately 40% of the human-derived H5N1 strains, including A/Thailand/1(KAN-1)/04, maintained the avian 627E signature (8). This suggests that other mutations in the polymerase subunits are required to increase the polymerase activity in human cells. Indeed, several adaptive mutations in H5N1 strains were identified in all three polymerase subunits and, surprisingly, also in the nuclear export protein (NEP) (7, 8, 13).

NEP is encoded by segment 8, consists of 121 amino acids, and is translated from mRNA that represents a splice product of the NS1 coding mRNA. It has been suggested to mediate nuclear export of newly assembled viral ribonucleoproteins (vRNPs) by bridging the interaction of the viral matrix protein M1, which is associated with the vRNPs, and the nuclear export protein Crm1 (18–20). Structural investigations revealed that NEP consists of N-terminal and C-terminal domains, both harboring two α-helices (N1 and N2 and C1 and C2, respectively [Fig. 1A]) (20, 21). The two nuclear export sequences (NESs) that mediate the interaction with Crm1 are located in the first and second N-terminal α-helices of NEP (18, 22). The N-terminal domain is proposed to be highly flexible and solvent exposed (21). In contrast, the C-terminal domain that crystallizes as a homodimer comprises the M1 binding site and adopts a rigid, protease-resistant hairpin structure (20). NEP was also found to interact with the β subunit of the F$_{1}$F$_{0}$ ATPase, which appears to be important for influenza virus virion formation and budding (23).

In addition, NEP has been discovered to be a novel polymerase-associated cofactor that enhances the synthesis of cRNA and vRNA of human H1N1 strains, while mRNA transcription is sup-

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pressed (24, 25). Intriguingly, avian H5N1 polymerases seem to be especially susceptible to the polymerase activity-enhancing properties of NEPs that harbor an adaptive mutation (13). This is based on the observation that single adaptive mutations (e.g., M16I, Y41C, or E75G) in NEPs of human-derived H5N1 viruses are sufficient to greatly enhance the avian H5N1 polymerase activity in human cells (13). Remarkably, besides stimulating cRNA and vRNA synthesis, those human-adapted NEPs also strongly enhance mRNA transcription of avian H5N1 viruses.

However, the molecular mechanism for how a single adaptive mutation in NEP increases polymerase activity of avian H5N1 polymerases in human cells is unclear (8, 13). We provide experimental evidence that the N-terminal domain of NEP regulates the polymerase activity-enhancing activity that is localized in the C terminus of this protein. Furthermore, intramolecular folding studies suggest that the adaptive mutation M16I in NEP diminishes the interaction between the N and C termini, thereby allowing the exposure of the C terminus, which results in increased polymerase activity.

**MATERIALS AND METHODS**

**Plasmid construction.** The pcAGGS expression plasmids encoding the three polymerase subunits, NP and NEP (designated avNEP) of the avian H5N1 precursor virus (AvianPr) and the human-derived A/Thailand/1 (Kan-1)/04 NEP, harboring the adaptive mutation M16I (designated huNEP), are described in the work of Mänz et al. (13). To create pcAGGS-NEP-Trp, the mutations Y41C and E75G were introduced via QuikChange PCR using pcAGGS-huNEP as a template. The pcAGGS expression plasmids encoding green fluorescent protein (GFP) and RevM10 fused to huNEP were obtained by PCR cloning of the GFP and RevM10 (18, 26) coding regions into pcAGGS-Strep-NEP (13). pcAGGS expression vectors coding for the various GFP and RevM10 fusion proteins were obtained by PCR amplification of the desired sequences and subsequent cloning of the amplification products into pcAGGS-GFP-huNEP and pcAGGS-RevM10-huNEP, respectively, and digested with NotI/XhoI. For cloning of pcAGGS-GFP-P, the DNA fragment obtained after digestion of pcAGGS Flag-P (27) was cloned into pcAGGS-GFP-huNEP using the same restriction sites. pcAGGS plasmids encoding NLuc-huNEP and huNEP-CLuc were created by a two-step assembly PCR using pcAGGS-CF-KBP, pFRB-CBR-N (28), and pcAGGS-huNEP. pcAGGS-NLuc-huNEP-CLuc was generated by digestion of pcAGGS-huNEP-CLuc with EcoRV/XhoI and subsequent cloning into pcAGGS-NLuc-huNEP. In an analogous manner, pcAGGS-NLuc-avNEP-CLuc, pcAGGS-NLuc-NEP-TRP-CLuc, and pcAGGS-NLuc-avNEP-CLuc were obtained. NLuc-CLuc was generated by Xmal digestion of pcAGGS-NLuc-huNEP-CLuc and religation.

**Reconstruction of the influenza virus polymerase activity.** HEK293T cells were transiently transfected with a transfection mixture containing plasmids encoding PB1, PB2, PA, and NP of AvianPr, a polymerase I (Pol I)-driven plasmid transcribing an influenza A virus-like RNA coding for the reporter protein firefly luciferase to monitor viral polymerase activity, and expression plasmids coding for NEP derivatives. The minigenome RNA was flanked by noncoding sequences of segment 8 of H1N1a (13). The transfection mixture also contained a plasmid constitutively expressing Renilla luciferase, which served to normalize variation in transfection efficiency. The reporter activity was determined 24 h posttransfection and normalized using the Dual-Glo luciferase assay system (Promega).

**Fragment complementation assays.** HEK293T cells were transiently transfected with plasmids (1,000 ng) encoding either NEP variants flanked by the respective fragments of the click beetle luciferase or N- and C-terminal click beetle luciferase fused by a linker sequence (intramolecular binding assay). Cells were lysed 24 h posttransfection, and lysates were warmed to 37°C for 30 min. Subsequently, luciferase activity was measured after stepwise cooling down of the lysates (10 min) to a specific temperature using the Dual-Glo luciferase assay system (Promega). To determine intermolecular binding between NEPs, HEK293T cells were transiently transfected with plasmids coding for NLuc-huNEP and huNEP-CLuc (each 200 ng). Cell extract was prepared and luciferase activity was measured using the Dual-Glo luciferase assay system (Promega).

**Rev-dependent export assay.** HEK293T cells (4 × 10⁵) were transiently transfected with 700 ng of plasmid pdm128 (29) and plasmids (each 100 ng) coding for the RevM10 fusion proteins using Lipofectamine (Invitrogen) as recommended by the manufacturer; 24 h posttransfection, cell pellet were obtained in phosphate-buffered saline (PBS) by centrifugation (1,000 rpm) for 10 min at 4°C. Chloramphenicol acetyltransferase (CAT) protein levels were determined by enzyme-linked immunosorbent assay (ELISA) (CAT-ELISA; Roche).

**Primer extension analysis.** For determination of transcript levels in virus-infected or transiently transfected HEK293T cells, cells were seeded in 6-well plates. Infection was carried out with infection medium (Dulbecco’s modified Eagle medium supplemented with 0.2% bovine serum albumin [BSA], 2 mM L-glutamine, and 1% penicillin-streptomycin). At the time point postinfection indicated below or 24 h posttransfection, cells were collected in TRIzol and RNA was purified according to the manufacturer’s protocol (Invitrogen). Primer extension analysis was performed using specific primers for the NA segment (mRNA, cRNA, and vRNA) and cellular 5S RNA as described previously (13).

**Single-cycle replication assay.** HEK293T cells were cultured in 6-well plates and infected at a multiplicity of infection (MOI) of 2 in infection medium (Dulbecco’s modified Eagle medium containing 0.3% bovine serum albumin). After 1 h of incubation at 34°C, unbound viral particles were inactivated by treating the cells with PBS (pH 2) for 45 s, and the cells were subsequently washed and further incubated in 2 ml of infection medium. Viral titers at the time points indicated below were determined by plaque assay.

**FIG 1** The polymerase activity-enhancing function of NEP resides in its C terminus. (A) Diagram depicting N- and C-terminal truncation mutants of NEP fused to GFP. The black bar represents the location of the adaptive mutation M16I. (B to D) To determine the stimulatory activity of the indicated NEP variants on the polymerase activity-enhancing activity of the indicated NEP variants on the avian H5N1 polymerase, HEK293T cells were transiently transfected with expression plasmids coding for the corresponding PB2, PB1, PA, and NP proteins of avian H5N1 (AvianPr), a human polymerase I-driven VRNA-luciferase reporter plasmid, a Renilla luciferase-expressing plasmid, and the indicated concentrations of NEP expression plasmids GFP-huNEP (B), GFP-NEP-C (C), and GFP-huNEP (111) (D). Omission of PB1 (∼PB1) was used as a negative control. Luciferase reporter activity was normalized to Renilla luciferase activity to address variations in transfection efficiency. Normalized reporter activities obtained after cotransfection of an expression plasmid (25 ng) coding for GFP fused to Borna disease virus phosphoprotein P (GFP-P) were set to 100%. Levels of the GFP fusion proteins were determined by Western blotting using GFP-specific antibodies. Detection of tubulin served as a loading control. Error bars indicate the standard deviations of three independent experiments. Student’s t test was performed to determine the P value. *, P < 0.05; **, P < 0.01; ***, P < 0.001. (E and F) Polymerase reconstitution assay using segment 6 of AvianPr in the presence of NEP mutant proteins. Levels of mRNA, cRNA, and vRNA obtained after reconstitution of the AvianPr in the presence of the indicated GFP fusion constructs were determined by primer extension analysis using primers specific for segment 6. Determination of the 5S RNA levels served as an internal loading control. Omission of PB1 (∼PB1) was used as a negative control. (G) Effect of coexpression of the indicated amounts of GFP-huNEP-N and GFP-NEP-C on the polymerase-enhancing capacity of GFP-huNEP. Reporter levels obtained for the reconstituted polymerase alone were set to 100%. Error bars indicate standard deviations of three independent experiments. ns, not significant.
RESULTS AND DISCUSSION

The C-terminal moiety of NEP possesses a polymerase activity-enhancing property. To define the polymerase activity-enhancing domain of the human-adapted NEP (huNEP) of H5N1 strain A/Thailand/1(KAN-1)/04, we created GFP-NEP fusion constructs with N- and C-terminal deletions in NEP (Fig. 1A). We then analyzed their abilities to stimulate polymerase activity of the putative avian precursor virus of KAN-1, designated AvianPr (13), in human cells. Consistent with our previous findings (13), cotransfection of 25 ng of expression plasmid coding for GFP-fused huNEP harboring the adaptive mutation M16I (GFP-huNEP) increased the polymerase activity more than 10-fold, whereas transfection of 250 ng of the same expression plasmid led to a nearly complete inhibition of polymerase activity (Fig. 1B to D). In contrast, expression of GFP fused to the phosphoprotein of Borna disease virus (GFP-P) had no inhibitory effect on the avian H5N1 polymerase at high plasmid concentrations (Fig. 1B to D).

Expression of GFP fused to the N-terminal domain of huNEP consisting of amino acids 1 to 49 (GFP-huNEP-N) did not increase polymerase activity, and compared to GFP-P, we observed at the most a 2-fold decrease in viral polymerase activity upon expression at higher concentrations (Fig. 1B). In contrast, coexpression of GFP-NEP-C (GFP fused to amino acids 50 to 121 of NEP) resulted in 400-fold-higher polymerase activity (Fig. 1C). Of note, GFP-NEP-C stimulated the polymerase activity at expression levels at which GFP-huNEP abrogated polymerase activity (Fig. 1C, relative activity with 500 ng of GFP-NEP-C compared to 250 ng of GFP-huNEP).

The last three amino acids of NEP are highly conserved among influenza A viruses, and rescue of influenza A viruses coding for NEP lacking the last three amino acids failed (M. Schwemmle and L. Brunotte, unpublished data). Consistently, expression of GFP fused to huNEP lacking the last three C-terminal amino acids (GFP-huNEP_{1-118}) (25 ng of plasmid) failed to stimulate polymerase activity. Moreover, increasing amounts of GFP-huNEP_{1-118} expressing plasmid only slightly decreased the polymerase activity (3-fold compared to GFP-P [Fig. 1D]). As predicted, GFP-NEP-C lacking the C-terminal 3 amino acids was found to be inactive and failed to stimulate avian H5N1 polymerase activity in human cells (data not shown).

Together, these results indicate that the polymerase activity-enhancing function of H5N1 NEP is located in the C-terminal domain of NEP and that the integrity of the C terminus is crucial for both the polymerase activity-enhancing function of NEP and the inhibitory effect at higher protein concentrations. This is consistent with observations by others that the NEP of laboratory strain A/WSN/33 (24) or A/Puerto Rico/8/34 (30) with N-terminal deletions can still activate viral replication to a certain extent. Although the precise role of the C-terminal three amino acids remains to be shown, it is tempting to speculate that these amino acids may stabilize the interaction with other viral proteins such as the polymerase subunits.

To confirm our finding that only the C-terminal domain of NEP is sufficient to stimulate polymerase activity, we reconstituted the avian H5N1 polymerase using an authentic viral segment (segment 6) and performed primer extension analysis to visualize the levels of viral transcripts as described in reference 13. Consistent with previous findings (13), cotransfection of 75 ng of GFP-huNEP-encoding plasmids resulted in a substantial increase in vRNA and cRNA levels, while expression of mRNA was diminished (Fig. 1E and F). As expected, increasing amount of plasmid (750 ng) resulted in abrogation of viral RNA synthesis. Expression of either GFP-huNEP-N or GFP-P did not alter viral RNA levels (Fig. 1E and F). In contrast, expression of GFP-NEP-C resulted in an increase of all 3 RNA species (Fig. 1F). At the highest plasmid concentrations, however, mRNA transcript levels decreased (Fig. 1F). Together, these results confirm that NEP-C is constitutively active and sufficient to stimulate the activity of avian H5N1 polymerase.

To determine the effect of the N- and C-terminal moieties of NEP on the polymerase-enhancing function of full-length NEP, we coexpressed increasing amounts of either GFP-huNEP-N or GFP-NEP-C in the presence of GFP-huNEP. Interestingly, GFP-NEP-C did not further enhance polymerase activity in the presence of GFP-huNEP (Fig. 1G). As expected, coexpression of GFP-huNEP-N did not alter the polymerase-enhancing activity of GFP-huNEP.

Intramolecular folding of NEP is influenced by the adaptive mutation M16I. As shown in Fig. 1C, the C-terminal domain of NEP is constitutively active in the absence of the N-terminal domain. We therefore speculated that binding between the N- and C-terminal domains might regulate the polymerase activity-enhancing activity of the C-terminal moiety. To determine intramolecular interactions between the N- and C-terminal moieties, HEK293T cells were transfected with plasmids encoding avian H5N1 NEP (avNEP) or huNEP fused to both N- and C-terminal click beetle luciferase moieties (NLuc-avNEP-CLuc, NLuc-huNEP-CLuc), and cell extracts were prepared 24 h posttransfection at 4°C. To monitor temperature-dependent differences, these extracts were warmed up to 37°C for 30 min and subsequently cooled down to 34, 31, 28, 25, 22, and 19°C (20 min each temperature). At each temperature a fraction of the cell extract was used to determine the luciferase activity. The luciferase activity of each fusion protein observed at 37°C was set to 100%. As shown in Fig. 2B, with decreasing temperatures, luciferase activity for both avian (NLuc-avNEP-CLuc) and human-adapted (NLuc-huNEP-CLuc) NEPs increased. Interestingly, the temperature-dependent increase in luciferase activity was significantly delayed for NLuc-huNEP-CLuc suggesting that the M16I mutation reduces the affinity between the N- and C-terminal moieties. As expected, split click beetle luciferase moieties fused to each other via a flexible linker (NLuc-avNEP-CLuc, NLuc-huNEP-CLuc) showed a slight decrease in activity (Fig. 2B). This is consistent with the observations by others (28) that the click beetle luciferase activity itself is not affected by changes in temperature. To confirm that the signals we measured in the split-luciferase assay did result from an intramolecular and not intermolecular interaction between two NEPs, we transiently expressed both huNEP fused to the N-terminal domain of click beetle luciferase (NLuc-huNEP) and huNEP fused to the C-terminal domain of
FIG 2 Detection of intramolecular changes in NEP. (A) Diagram depicting NEP fused to split click beetle luciferase halves highlighted in black. (B) To analyze temperature-dependent intramolecular folding events, HEK293T cells were transfected with plasmids encoding either NLuc-huNEP-CLuc or NLuc-avNEP-CLuc, and 24 h posttransfection, cell lysates were prepared at 4°C. The extracts were warmed up to 37°C for 30 min and subsequently cooled down to 34, 31, 28, 25, 22, and 19°C (20 min each temperature). At each temperature, a fraction of the sample was used to determine luciferase activity. The luciferase activity observed at 37°C was set to 100%. Cell extracts containing NLuc-CLuc (NLuc and CLuc fused by linker sequence) served as a control for a temperature-stable luciferase (28). Error bars indicate standard deviations of three independent experiments. Student’s t test was performed to determine the P value. *, P < 0.05; **, P < 0.01. The model above the graph depicts the proposed temperature-dependent intramolecular interaction of NEP fused to the respective halves of a click beetle luciferase (filled halves). T, temperature. (C) Comparison of the inter- and intramolecular binding efficiencies. Luciferase activity was determined in extracts of HEK293T cells transiently transfected with plasmids expressing both NLuc-huNEP and huNEP-CLuc (intermolecular binding) or NLuc-huNEP-CLuc (intramolecular binding). Lower images show the expression levels of the respective constructs determined by Western blotting. Detection of actin served as a loading control. Error bars indicate the standard deviations of three independent experiments. (D) Analysis of the temperature-dependent affinity of the C- and N-terminal domains of NEP derived from the pandemic H1N1 isolate A/Hamburg/4/2009 (NLuc-huNEP-H1N1-CLuc) in comparison to the human-adapted H5N1 NEP (NLuc-huNEP-CLuc). Student’s t test was performed to determine the P value. *, P < 0.05. (E) Diagram depicting NEP variants fused to RevM10. The locations of the adaptive mutation M16I and the two NESs are indicated. (F and G) Crm1-dependent nuclear export activities of NEP-RevM10 fusion proteins. huNEP or avNEP (F) or the corresponding N-terminal fragments comprising amino acids 1 to 49 (G) fused to the export-inactive Rev protein (RevM10) were transiently expressed in HEK293T cells together with a plasmid that allowed the synthesis of an intron-containing CAT reporter mRNA harboring a Rev-responsive element. RevM10-NEP-C (amino acids 50 to 121), lacking the NES domains, served as the negative control. Levels of the NEP fusion proteins were analyzed by Western blotting. Detection of tubulin served as a loading control. The export activity was determined by measuring CAT protein levels and was normalized to the levels of the NEP fusion proteins. Error bars indicate standard deviations of three independent experiments.
Click beetle luciferase (huNEP-C\textsubscript{Luc}) (Fig. 2A) and compared the luciferase activity obtained with cell extract containing N\textsubscript{Luc}-huNEP-C\textsubscript{Luc} only. While the expression levels of the fusion proteins were comparable, the luciferase activity observed after expression of both N\textsubscript{Luc}-huNEP and huNEP-C\textsubscript{Luc} was significantly lower (>50-fold) than with N\textsubscript{Luc}-huNEP-C\textsubscript{Luc} only (Fig. 2C), indicating that the activity observed with the latter protein reflects indeed an intramolecular interaction.

Next, we subjected NEP derived from the pandemic H1N1 isolate A/Hamburg/4/2009 (N\textsubscript{Luc}-huNEP\textsubscript{H1N1}-C\textsubscript{Luc}) to the split click beetle luciferase assay and compared its activity to that observed with N\textsubscript{Luc}-huNEP-C\textsubscript{Luc}. As shown in Fig. 2D, the temperature-dependent changes in luminescence measured for N\textsubscript{Luc}-huNEP-C\textsubscript{Luc} and N\textsubscript{Luc}-huNEP\textsubscript{H1N1}-C\textsubscript{Luc} were similar, indicating that the intramolecular affinity of human-adapted H5N1 is comparable to that of a bona fide human NEP.

NEP is known to bind to cellular nuclear export proteins, including Crm1 (20), by virtue of its two nuclear export sequences (NESs) that are localized in the N-terminal α-helical domains N1 and N2 (18, 22). We therefore speculated that M16I-mediated changes in the kinetics of huNEP to obtain its tertiary conformation might also favor the interaction of the exposed N terminus of NEP with Crm1, resulting in export-competent protein complexes. To compare the export activities of avian and human-adapted NEPs, we fused HIV-RevM10 (18, 26), which is deficient in binding to Crm1 (31), to either avNEP (avNEP-M10), huNEP (huNEP-M10), or NEP-C (NEP-C-M10) (Fig. 2E) and coexpressed these proteins in the presence of a CAT-encoding mRNA harboring the Rev-responsive element (RRE) (29). As expected, the CAT protein levels were low in cells expressing NEP-C-M10 compared to those in huNEP-M10-expressing cells (Fig. 2F), since there is no NES located in the C-terminal part of NEP (Fig. 2E). However, compared to huNEP-M10, the CAT protein levels in cells expressing avNEP-M10 were significantly lower, despite similar levels of expression of the fusion proteins (Fig. 2F). To rule out that the adaptive mutation M16I located within the NES directly increases the affinity to Crm1, we fused RevM10 to the N terminus of either avNEP (avNEP-N-M10) or huNEP (huNEP-N-M10) (Fig. 2E) and determined the nuclear export activity. Expression of either fusion protein resulted in comparable CAT protein levels (Fig. 2G). This suggests that the adaptive mutation M16I does not increase the affinity to Crm1. Together, these results support the model that the adaptive mutation M16I causes a conformational change of NEP that allows enhanced interaction with Crm1 and likely the exposure to the C-terminal moieties. Therefore, the mutation M16I might not only increase the viral polymerase activity but also enhance nuclear export of VRNPs, a feature that might be important in the process of adaptation of avian H5N1 viruses to human cells.

**NEP with three adaptive mutations shows increased activity.** In addition to M16I, further single adaptive mutations in NEP of human-derived H5N1 isolates, including Y41C and E75G (Fig. 3A), were found to stimulate avian H5N1 polymerases in human cells (13). To test whether the combination of adaptive mutations further enhances the stimulatory activity of avian NEP, we expressed the triple mutant GFP-NEP-TRP, harboring the adaptive mutations M16I, Y41C, and E75G (Fig. 3A), in the polymerase reconstitution assay. Compared to that of GFP-huNEP, expression of GFP-NEP-TRP further increased the polymerase activity and exhibited decreased inhibitory activity at higher concentrations (Fig. 3B), suggesting that these mutations might indeed enhance the activity in an additive manner. However, the polymerase activities observed in the presence of the C terminus of NEP with (GFP-NEP-C-E75G) and without (GFP-NEP-C) the adaptive mutation E75G were comparable (Fig. 3B). This lack of increased polymerase activity with GFP-NEP-C-E75G might suggest that the adaptive mutation E75G (and most likely Y41C) functions in a manner similar to that of M16I, namely, by decreasing intramolecular interactions between the N- and C-terminal domains of NEP. We therefore fused both ends of NEP-TRP to split click beetle luciferase halves (N\textsubscript{Luc}-NEP-TRP-C\textsubscript{Luc}) and determined the relative luminescence at decreasing temperatures. This revealed that N\textsubscript{Luc}-NEP-TRP-C\textsubscript{Luc} showed no increase in luciferase activity at each temperature tested compared to N\textsubscript{Luc}-huNEP-C\textsubscript{Luc} (Fig. 3C). This indicates that the presence of these three adaptive mutations prevents detectable intramolecular folding events that would result in the close proximity of the split click beetle luciferase moieties and reconstitution of the luciferase.

**Adaptive mutations in NEP provide a replication advantage also at a lower temperature.** The relatively low temperature of the human upper respiratory tract of ca. 32°C in the upper trachea to ca. 35.5°C in the subsegmental bronchi (32) represent a major hurdle for the establishment of an infection by avian H5N1 viruses, especially since the replication machinery of avian influenza A viruses is adapted to the temperature of the avian intestinal tract of ca. 39 to 41°C (33, 34). In contrast, circulating human influenza A viruses predominantly infect the human upper respiratory airways at a characteristic mean temperature of about 34°C (32). Since the fragment complementation assay revealed that the intramolecular binding between the N- and C-terminal domains of avian NEP preferentially occurs at low temperatures (Fig. 2B), thereby possibly preventing the exposure of the polymerase activity-enhancing C-terminal domain, we reasoned that the adaptive mutation M16I increases the poor replication of avian polymerases not only at 37°C but also at the low temperature of 34°C. To demonstrate this, we first reconstructed the avian H5N1 polymerase in human 293T cells at 34°C without NEP. As expected, at the lower temperature the avian H5N1 polymerase showed only residual activity, compared to reconstitution at 37°C (Fig. 3D). Remarkably, at 34°C, in the presence of 10 ng of either huNEP or NEP-TRP the polymerase activities increased ca. 74- and 164-fold, respectively, whereas avNEP had only a minor (2-fold) stimulatory effect. At 37°C, huNEP and NEP-TRP stimulated the polymerase activity ca. 30-fold, while avian NEP increased the polymerase activity 4-fold.

These results suggest that NEP of avian H5N1 viruses harboring a human-specific adaptive mutation might increase the replication efficiency, especially at lower temperatures. To show this in the context of viral infection, HEK293T cells kept at 34°C or 37°C were infected with avian H5N1 with an NS segment coding for NEP either lacking (AvianPr) (13) or harboring (AvianPr-NS-KAN-1) (13) the adaptive mutation M16I with an MOI of 5. At 1, 2, 2.5, and 3 h postinfection, transcript levels were determined by primer extension analysis. As shown in Fig. 3E, infection with AvianPr-NS-KAN-1 resulted in increased transcript levels of mainly cRNA and mRNA compared to cells infected with AvianPr at 34°C. As shown previously (13), similar differences in transcript levels were observed after infection at 37°C, although the total viral transcript levels are significantly higher than for infections at 34°C. Consistently, determination of the single-cycle growth char-
FIG 3 Additive effects of adaptive mutations in NEP. (A) Diagram depicting NEP with three adaptive mutations (labeled) or with the C-terminal domain of NEP harboring the adaptive mutation E75G fused to split click beetle luciferase halves. (B) Effect of NEP-GFP fusion proteins harboring the single adaptive mutation M16I (GFP-huNEP), the three adaptive mutations M16I, Y41C, and E75G (GFP-NEP-TRP), and the C-terminal domain of NEP without (GFP-NEP-C) or with the adaptive mutation E75G (GFP-NEP-C-E75G) on the reconstituted avian H5N1 polymerase activity in human cells. The reporter level obtained after cotransfection of the expression plasmid coding for GFP-P was set to 100%. Amounts of expression plasmid coding for GFP fusion proteins are indicated. Levels of the GFP fusion proteins were determined by Western blotting using GFP-specific antibodies. Detection of tubulin served as a loading control. Error bars indicate standard deviations of three independent experiments. Student's t test was performed to determine the P value. *, P < 0.05; **, P < 0.01; ***, P < 0.001. (C) Comparison of the temperature-dependent changes in luciferase activity of huNEP and NEP-TRP fused to split click beetle luciferase halves. The experiments were carried out as described in the legend to Fig. 2B. Error bars represent standard deviations of three independent experiments. Student’s t test was performed to determine the P value. *, P < 0.05. (D) Comparison of the temperature-dependent stimulatory activities of huNEP, avNEP, and NEP-TRP. The avian H5N1 polymerase was reconstituted in HEK293T cells in the presence of the indicated fusion proteins at either 34°C or 37°C. Omission of PB1 was used as a negative control (background levels). Luciferase reporter activity was normalized to Renilla luciferase activity to address variations in transfection efficiency. The relative induction in reporter activity was calculated by dividing the normalized luciferase activity by the background levels. The relative induction in reporter activity at 37°C in the absence of NEP was set to 100%. Error bars indicate standard deviations of three independent experiments. Student’s t test was performed to determine the P value. *, P < 0.05. (E) HEK293T cells were infected with AvianPr or AvianPr-NS-KAN-1 at an MOI of 5. After the indicated time points postinfection (p.i.), cells were lysed and RNA levels were determined by primer extension analysis using primers specific for segment 6. Determination of the 5S RNA levels served as an internal loading control. (F) Comparison of the growth kinetics of AvianPr and AvianPr-NS-KAN-1 by single-cycle replication assay. HEK293T cells were infected at an MOI of 2 and incubated at 34°C. Viral titers at the indicated time points postinfection were determined by plaque assay. Student’s t test was performed to determine the P value. *, P < 0.05; **, P < 0.01.
characteristics revealed that 293T cells infected with AvianPr-NSKAN-1 released significantly higher numbers of infectious particles at 34°C than did 293T cells infected with AvianPr (Fig. 3F). In summary, these results support the concept that a single adaptive mutation in NEP can significantly contribute to overcome the temperature restriction avian viruses are faced with in the human upper respiratory tract.

Based on our data, we propose that the N terminus of NEP is a regulatory domain whose conformation relative to the C-terminal domain determines the protein’s activity. The C-terminal domain comprising the two α-helices C1 and C2 (Fig. 4) harbors the polymerase-activity-enhancing property of NEP. Given the rigid nature of the C terminus (20), the N terminus may act as a “lid” which opens to expose the surfaces of NEP required for cofactor activity (Fig. 4). Back-folding of the N terminus to the C terminus to a closed conformation might be also required for the inhibitory effect of NEP on the viral polymerase activity observed at higher protein concentrations. In this respect, the absence of an inhibitory effect of the NEP mutant NEP-TRP could be due to the reduced affinity between the N- and C-terminal moieties of this protein.

The fact that avian influenza viruses replicate preferentially at the high temperatures of 39 to 41°C might explain why avian NEP favors a “closed” conformation. A high temperature at the site of replication lowers the affinity between the N- and C-terminal domains, thereby increasing the polymerase-activity-enhancing activity of avian NEP. However, when avian influenza viruses cross the species barrier to humans, the temperature at the site of replication is lowered to ca. 34°C, leading to a stronger affinity between the N and C terminal. As a consequence, NEP exists in a nonstimulatory form that precludes the stimulation of the already low polymerase activity at lower temperatures by reducing the affinities between the N and C termini, thereby maintaining or even increasing the level of polymerase-stimulatory activity after species transmission. This view is supported by the observation that NEP of the pandemic 2009 H1N1 virus (13) as well as that of the laboratory strain A/WSN/33 (data not shown) strongly enhances the avian influenza polymerase activity in human cells, in contrast to avian NEP lacking adaptive mutations.

Based on its importance to stimulate viral replication, it is tempting to speculate that the conformational change of NEP is tightly regulated. Recent determination of the viral phosphoproteome revealed that NEP is phosphorylated (35) at highly conserved serine residues between the N-terminal α-helices N1 and N2 (36). This change in surface charge may also alter the intramolecular affinity and thus the polymerase activity-enhancing function of NEP. Alternatively, since the RevM10-dependent export assay suggests that the open conformation of NEP might also increase nuclear export of vRNP, phosphorylation of NEP might be required for this activity only.

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