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Nucleoprotein of influenza B virus binds to its type A counterpart and disrupts influenza A viral polymerase complex formation

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ABSTRACT

Upon co-infection with influenza B virus (FluB), influenza A virus (FluA) replication is substantially impaired. Previously, we have shown that the nucleoprotein of FluB (BNP) can inhibit FluA polymerase machinery, retarding the growth of FluA. However, the molecular mechanism underlying this inhibitory action awaited further investigation. Here, we provide evidence that BNP hinders the proper formation of FluA polymerase complex by competitively binding to the nucleoprotein of FluA. To exert this inhibitory effect, BNP must be localized in the nucleus. The interaction does not require the presence of the viral RNA but needs an intact BNP RNA-binding motif. The results highlight the novel role of BNP as an anti-influenza A viral agent and provide insights into the mechanism of intertypic interference.

1. Introduction

Having co-circulated in the human population for decades, type A and type B influenza viruses (FluA and FluB) bring about substantial health and economic losses annually. Both belonging to the *Orthomyxoviridae* family, their genomes are packaged in the form of viral ribonucleoproteins (vRNP) consisting of eight negative-sense, single-stranded viral RNA (vRNA) segments bound to nucleoprotein (NP) and a polymerase complex comprising three protein subunits: PB2, PB1 and PA [1]. A native vRNP exhibits double-helical conformation with the polymerase complex situated on one end, while the rest of the vRNA segment is decorated with NP oligomers [2,3]. Biochemical and structural analyses have confirmed that NP–NP interactions are crucial for vRNP formation and proper function of the viral polymerase [4–7]. Besides self oligomerization, NP directly interacts with PB1 and PB2 subunits of the polymerase complex, as evident from recent cryogenic electron microscopic reconstructions [2,3] and co-immunoprecipitation experiments performed on infected cell lysates [8]. Except for the additional 70 amino acids at the N-terminus of FluB's nucleoprotein recently demonstrated to contain a nuclear localization signal (NLS) [9], all protein subunits involved in vRNP formation of FluA and FluB share significant homology, and it is assumed, based on extensive research on FluA, that the vRNP of FluB also assembles and functions in a similar manner [10–12].

Although FluA and FluB co-circulate and share close phylogenic relationship, they have not produced any natural or synthetic reassortant to date [13–16]. More importantly, several reports have indicated growth impairment of FluA upon co-infection with FluB, [14,15,17,18]. Despite these long-standing observations regarding intertypic interference, the mechanism underlying this phenomenon remains elusive. Recently, we have proposed a possible mechanism that might play a role in suppressing FluA growth and limiting intertypic reassortment by demonstrating that the nucleoprotein of FluB (BNP) alone can inhibit replication of FluA. Specifically, a Madin-Darby canine kidney (MDCK) cell line stably expressing BNP moderately suppresses viral progeny production of FluA [18]. We further showed that BNP interferes with the function of FluA polymerase and that nucleoprotein of FluA (ANP) can partially alleviate this effect upon increased expression [18]. These data led us to hypothesize that BNP can competitively sequester the component of FluA polymerase(s) away from formation of a functional FluA polymerase complex. Based on partial reversion of the inhibitory effect, the most likely candidate for the BNP interaction partner is ANP.

In this study, we demonstrated that BNP could interact specifically with ANP both upon co-transfection of plasmids bearing ANP and BNP and co-infection of FluA and FluB. We showed that this intertypic protein interaction does not depend on the presence of vRNA but requires nuclear localization of both nucleoproteins to bring about the inhibitory effect on FluA. Furthermore, we found that ANP–BNP interaction could impair the interaction between ANP and FluA PB2, which is critical for activity of the FluA polymerase machinery [8]. These results led us to propose a possible molecular mechanism of BNP-mediated suppression of FluA replication.

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2. Materials and methods

2.1. Biological materials

Addition of the epitope tags on pHW2000-based plasmids encoding polymerase subunits was carried out following a site-directed mutagenesis protocol using BsmBI or BsaI restriction enzymes [19]. C-terminal fusion of GFP to ANP was described elsewhere [20]. Sequences of primers are available upon request. All tagged and fusion constructs were tested for functionality and showed no significant deviation from their wild-type counterparts in minigenome assays. The following antibodies were used according to the manufacturer's recommendations: α-ANP and α-BNP (Southern Biotechnology), α-FLAG (Cell Signalling Technology), α-Myc (Invitrogen) and α-PA (Thermo Scientific). Secondary antibodies for western blot analysis were from Santa Cruz Biotechnology. The virus strains used in this study were A/Puerto Rico/8/1934 (FluA) and B/Lee/40 (FluB). Viral genes used in pHW2000-based expression plasmids were derived from these two strains.

2.2. Co-immunoprecipitation (co-IP)

Human embryonic kidney (HEK) 293T cells were transfected with indicated plasmids (500 ng each, unless otherwise specified). Forty-eight hours post-transfection, cells were lysed with co-IP buffer [50 mM Tris–HCl pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% NP-40 supplemented with protease inhibitor cocktail (Sigma–Aldrich)]. Clarified lysates were incubated overnight at 4 °C with either α-Myc-conjugated agarose beads (Thermo Scientific) or α-ANP-conjugated agarose beads prepared according to the manufacturer's recommendation (Thermo Scientific). After three washes with co-IP buffer supplemented with 250 mM NaCl, the IP complex was eluted by boiling the beads in SDS–PAGE gel loading buffer and analyzed by Western blotting with indicated antibodies.

For Fig. 3A, the cell lysates were treated with either 40 u of RNase A (Thermo Scientific) or buffer for 15 min on ice. Co-immunoprecipitation was performed as described above using 150 μl of the treated lysates, while the rest of the lysates were used for RNA extraction (GeneJET RNA purification kit, Thermo Scientific). One-step RT-PCR (Invitrogen) with a pair of primers specific to the BNP gene was performed on the DNase I-treated extracted RNA from both conditions to confirm the absence of vRNA in the treated cell lysates.

2.3. ANP–BNP interaction in co-infected MDCK cells

MDCK cells were co-infected with FluA (MOI = 0.1) and FluB (MOI = 0.01). The unbalanced MOI ratio between the two strains was required to overcome the inhibition effect of FluB on FluA protein synthesis such that there was enough ANP for co-immunoprecipitation. Twenty-four hours post-infection, cell lysates were prepared and analyzed by co-immunoprecipitation as described above.

2.4. FluA polymerase inhibition assays

The FluA minigenome assay was performed as previously described [18]. Briefly, HEK293T cells were transfected with a set of plasmids expressing PB2, PB1, PA and NP to reconstitute the FluA polymerase complex together with an RNA Polymerase I-driven plasmid expressing negative-sense viral RNA encoding secreted neuraminidase as an assay reporter. Plasmids expressing variants of BNP were co-transfected to determine their ability to suppress expression of the reporter gene. Polymerase activity was determined by quantification of neuraminidase activity to convert 2′-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid (Sigma–Aldrich) to a fluorescent product. Values were expressed relative to FluA polymerase activity in the absence of BNP.

3. Results

3.1. BNP interacts with ANP and disrupts FluA polymerase complex formation

Previously, we found that BNP substantially inhibited FluA polymerase activity in a dose-dependent manner [18]. Interestingly, this inhibition could be partially relieved by addition of ANP [18]. Together, these data led us to postulate that BNP might inhibit FluA polymerase by competing for ANP with other components of the FluA polymerase machinery. To test this, we first asked whether BNP could bind to ANP. Total lysates prepared from HEK293T cells transfected with plasmids bearing Myc-tagged BNP and GFP-tagged ANP were analyzed by co-immunoprecipitation with an α-Myc antibody. Western blot analyses revealed that BNP-Myc could efficiently pull down ANP-GFP (Fig. 1A). BNP-Myc did not pull down other GFP-fusion proteins (data not shown). Likewise, ANP could specifically pull down BNP in co-immunoprecipitation with an α-ANP antibody (See Figs. 2 and 3). These results

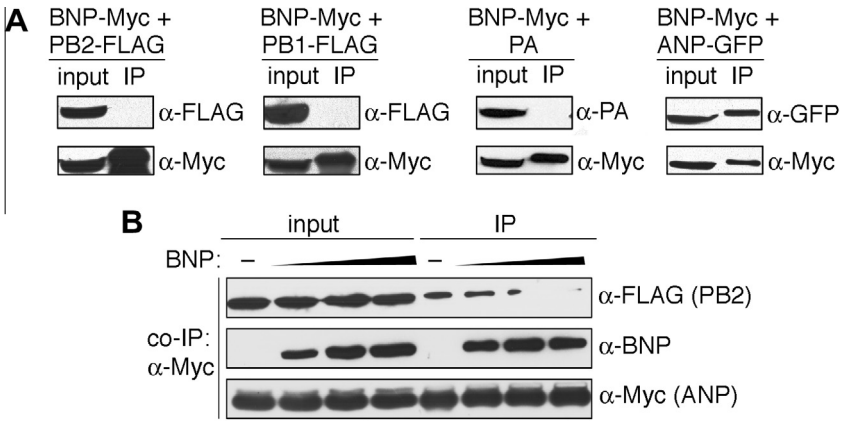

Fig. 1. BNP interacts with ANP and disrupts ANP–PB2 interaction. (A) HEK293T cell lysates prepared from transfection of indicated plasmid pairs were used for co-immunoprecipitation with α-Myc and Western blot analysis. (B) HEK293T cells were transfected with plasmids expressing ANP-Myc, PB2-FLAG, PA, and PB1 together with 0, 500, 1000 or 2000 ng of BNP-expressing plasmid. Cell lysates were used in co-immunoprecipitation with α-Myc and Western blot analysis. Input indicates 1:20 dilution of cell lysates, while IP indicates the eluted immunoprecipitate.

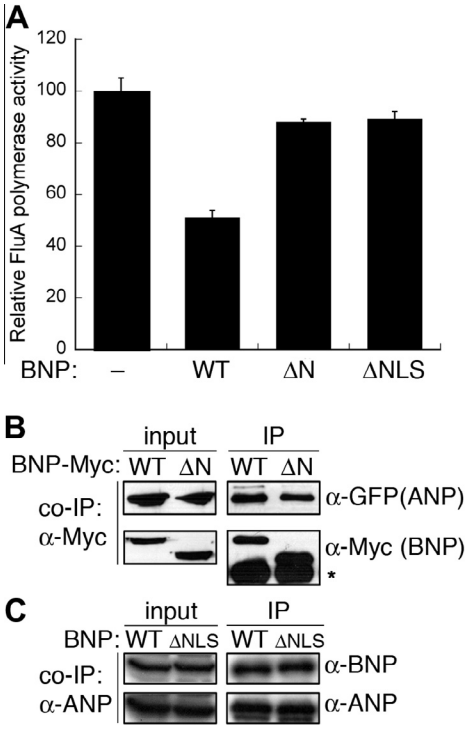

Fig. 2. Nuclear localization is required for the inhibitory action of BNP. (A) FluA polymerase activity assay was performed as described in Section 2. Values are relative to FluA polymerase activity in the presence of the pHW2000 vector (lane '–') and are average of triplicates ± S.D. (B) and (C) HEK293T cells were transfected with plasmids expressing (B) BNP-Myc (wild-type or ΔN) and GFP-tagged ANP or (C) BNP (wild-type or ΔNLS) and ANP. Cell lysates were used for co-immunoprecipitation with α-Myc or α-ANP and Western blot analysis. The asterisk marks the mouse IgG heavy chain of α-Myc, whose size is close to that of ΔN-BNP.

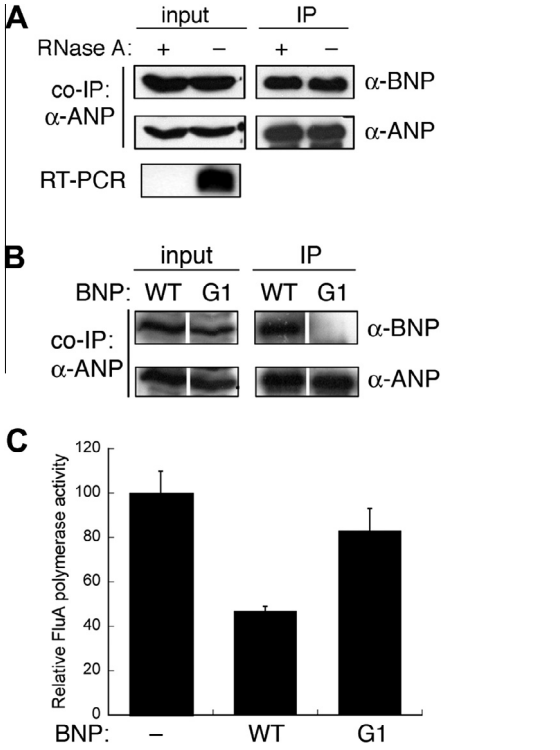

Fig. 3. vRNA is not necessary for NP intertypic interaction. (A) HEK293T cells were transfected with plasmids expressing BNP and ANP. After buffer or RNase A treatment, cell lysates were used for co-immunoprecipitation with α-ANP and Western blot analysis. The absence of an RT-PCR product in the RNase A-treated lysate was visualized on an agarose gel. (B) HEK293T cells were transfected with plasmids expressing ANP and BNP (wild-type or G1). Cell lysates were used for co-immunoprecipitation with α-ANP and Western blot analysis. (C) FluA polymerase activity assay was performed as described. Values are relative to FluA polymerase activity in the presence of the pHW2000 vector (lane '–') and are average of triplicates ± S.D.

confirm that BNP could interact with ANP, either directly or via cellular factors, in the absence of other viral proteins.

To examine if BNP could also interact with other FluA polymerase proteins, co-immunoprecipitation was performed on lysates of HEK293T cells transfected with a pair of plasmids expressing Myc-tagged BNP and a tagged FluA polymerase component (PB1-FLAG, PB2-FLAG or PA). None of the other FluA polymerase components could be pulled down by BNP-Myc (Fig. 1A). These data suggest that the ANP–BNP interaction is specific and are consistent with the observation that only ANP, but not other FluA polymerase components, could partially offset the inhibitory effect of BNP in the polymerase assay [18].

Next, we probed the mechanism by which ANP–BNP interaction could affect activity of FluA polymerase. A simple model is that this interaction prevents ANP from forming active polymerase complexes with other FluA polymerase components. In particular, the interaction between ANP and PB2 is critical for viral replication and can be manipulated by cellular anti-viral mechanisms [21,22]. To test if BNP disrupts the formation of a functional FluA polymerase complex, we monitored the interaction between ANP and PB2 in the absence or presence of BNP. Total lysates prepared from transfection of all components of the minigenome system in the absence or presence of increasing BNP-expressing plasmids were analyzed by co-immunoprecipitation using α-Myc-coupled agarose beads that capture Myc-tagged ANP. In the absence of BNP, ANP-Myc efficiently pulled down PB2-FLAG as previously observed (Fig. 1B and [8]). Notably, with increasing amounts of BNP, the amount of PB2-FLAG present in the immunoprecipitate diminished, whereas the amount of BNP correspondingly increased (Fig. 1B). Taken together, these data suggest a possible inhibitory mechanism in which BNP competes with FluA PB2 to interact with the available ANP.

3.2. Nuclear localization is a prerequisite for BNP inhibitory effect

During the influenza virus life cycle, both ANP and BNP require a nuclear phase to enable viral transcription and replication [23]. We therefore asked if nuclear localization plays an important role in the disruption of FluA polymerase function by BNP. We previously described two BNP mutants that mislocalize to the cytoplasm (NLS mutants): an N-terminal deletion mutant (here termed ΔN) lacking the first 70 amino acids encompassing the NLS and a K44A, R45A double mutant (here termed ΔNLS) carrying alanine replacements of critical basic residues within the NLS [9]. To define the role of nuclear localization in the inhibitory effect of BNP, we examined if these mutants could still inhibit FluA polymerase. Using the FluA minigenome assay, we found that, while wild-type BNP reduced FluA activity by about 50%, these two mutants had nearly no effect on expression of the FluA reporter (Fig. 2A).

To ensure that the loss of the inhibitory effect stemmed from mislocalization of these BNP mutants, we assessed the ability of these NLS mutants to interact with ANP. Co-immunoprecipitation with an α-Myc antibody (on BNP-Myc or ΔN-BNP-Myc) revealed that ANP-GFP could be pulled down by ΔN-BNP as efficiently as wild-type BNP (Fig. 2B). Similarly, co-immunoprecipitation with an α-ANP antibody exhibited comparable amounts of BNP and ΔNLS-BNP in the eluate (Fig. 2C). These data demonstrated that,

despite the ability to interact with ANP, NLS mutants did not hamper the function of FluA polymerase, implying that nuclear localization is a prerequisite for the inhibitory effect of BNP. In other words, the ANP–BNP interaction that disrupts the FluA polymerase complex requires both nucleoproteins inside the nucleus.

3.3. ANP–BNP interaction occurs independently of vRNA

The main function of both ANP and BNP is to encapsidate vRNA in the vRNP [23,24]. We thus assessed whether vRNA binding is necessary for ANP–BNP interaction. To this end, we treated cell lysates with RNase A prior to co-immunoprecipitation. Consistent with previous work demonstrating that NP-associated vRNA could be digested by RNase A [25], no BNP-encoding vRNA was detected by RT-PCR in the RNase A-treated lysate compared to the untreated lysate (Fig. 3A, bottom panel). Co-immunoprecipitation with α-ANP revealed similar amounts of pulled-down BNP from untreated lysate or lysate treated with RNase A, suggesting that the presence of vRNA does not affect ANP–BNP interaction (Fig. 3A).

To further test the role of RNA binding in intertypic interaction between nucleoproteins, we utilized a BNP mutant defective in RNA binding, 'G1' (K125A, K126A, R235A, R236A). This quadruple mutant was reported to lose RNA-binding ability and could not support FluB polymerase function [7]. Contrary to our expectation, the G1 mutant could no longer be pulled down by ANP (Fig. 3B). We then tested whether this quadruple mutant could inhibit FluA polymerase. As expected from the loss in ANP binding, the G1 mutant could not effectively inhibit FluA polymerase activity (Fig. 3C). Immunofluorescence showed that the G1 mutant still correctly localized in the nucleus, ruling out mislocalization as the cause of its inability to inhibit FluA polymerase (data not shown). This observation suggests that, although the presence of vRNA is not strictly required for the intertypic interaction between nucleoproteins, intact BNP RNA binding motifs are necessary for interaction with ANP and are required for efficient inhibition of FluA polymerase.

3.4. BNP can interact with ANP in co-infected cells

Thus far, characterization of this protein–protein interaction has been done in a transfection context. To provide evidence that the interaction has biological relevance, we attempted to detect it in the context of viral co-infection between the two strains of influenza viruses using co-immunoprecipitation of lysates prepared from infected cells. However, interference between FluA and FluB would occur upon co-infection. This resulted in diminished FluA protein synthesis that, in turn, could present a technical complication for co-immunoprecipitation (data not shown). To overcome this problem, we infected MDCK cells with FluA at a higher multiplicity of infection (MOI) than FluB (MOI_A:MOI_B is 10:1). Twenty-four hours post-infection, cell lysates were harvested and subjected to co-immunoprecipitation with α-ANP.

When FluA and FluB simultaneously infected the cells, ANP could pull down BNP, confirming the existence of this intertypic nucleoprotein interaction during co-infection (Fig. 4, lane 'A + B'). Interestingly, if FluB infection was established two hours before FluA infection, slightly less BNP could be detected in the immunoprecipitate compared to that from the concurrent infection, despite similar amounts of both ANP and BNP present in the infected cell lysate (Fig. 4, cf. lanes 'A + B' vs 'B/A'). Although the mechanism underlying this observation remains to be addressed, it raises an intriguing possibility that timing of infection, or the cellular environment during the course of viral infection, could modulate this intertypic protein–protein interaction.

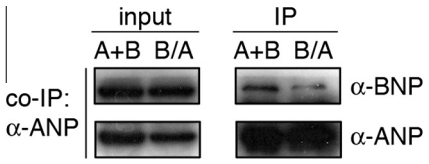

Fig. 4. BNP interacts with ANP in co-infected cells. MDCK cells were infected with A/Pueto Rico/8/34 virus simultaneously with ('A + B') or 2 h after ('B/A') the infection of B/Lee/40. Cell lysates were prepared twenty-four hours after infection, subjected to co-immunoprecipitation by α-ANP and analyzed by Western blotting.

4. Discussion

Recently, we observed a significant reduction in the number of FluA viral progeny from an MDCK cell line stably expressing BNP [18]. Using the minigenome assay, we showed that BNP expression impaired activity of the FluA polymerase and that this inhibition could be partially relieved by overexpression of ANP [18]. Based on these data, we proposed that BNP is, at least partially, responsible for the observed inhibition of FluA replication. To understand mechanistically how BNP could disable the FluA polymerase machinery, ultimately suppressing growth of co-infecting FluA, we performed co-immunoprecipitation assays to demonstrate specific interaction between ANP and BNP in both transfection and co-infection contexts. Furthermore, we presented evidence that this interaction could block the proper formation of FluA polymerase machinery by disrupting ANP–PB2 interaction.

Biochemical and structural data have implicated roles of ANP–PB2 interaction in polymerase function [2,3,8]. Recent reports demonstrated that this interaction is a target for host anti-viral responses induced by interferon [21,22]. Nuclear-localized Mx1 (or human homolog MxA) has been shown to inhibit influenza polymerase activity by interfering with formation of the polymerase machinery [22]. These results are similar to our observation that BNP competes with PB2 for ANP in a dose-dependent manner. Nonetheless, a few striking differences could be observed between the anti-influenza A mechanisms mediated by Mx1 and BNP. Mx1 can interact with both PB2 and NP, and either of these proteins can independently counteract Mx1 activity [22]. In contrast, BNP specifically interacts with ANP but cannot pull down PB2 (Fig. 1). In good agreement with the co-immunoprecipitation data, only ANP, but not PB2, can alleviate BNP-mediated inhibition of FluA polymerase [18]. Taken together, these data suggest a slightly different mode of action of BNP inhibition compared to that suggested for Mx1 [22]. In the case of BNP, our data are more consistent with the model in which BNP sequesters ANP by a non-productive interaction. This prohibits ANP from efficiently interacting with PB2 or forming a proper functional polymerase complex with other subunits.

Although mechanistic details of BNP-mediated anti-influenza A activity are still being investigated, a few important implications can be gathered from the data presented here. First, the inhibitory effect does not stem from blocking nuclear entry of ANP. It was previously shown that, in co-transfected cells, the presence of BNP does not interfere with nuclear localization of ANP [18]. Moreover, BNP itself needs to be nuclear-localized to exert the inhibitory effect; mutations that block BNP nuclear entry do not negatively affect the activity of FluA polymerase, despite efficient interaction with ANP. Therefore, this possibility can be ruled out for the BNP-mediated inhibition of FluA replication.

Second, the presence of viral RNA does not interfere with or augment binding between ANP and BNP. Although we cannot exclude the possibility that NPs still bind to small pieces of RNA carried over from the RNAse treatment, we are inclined to believe that vRNA does not affect interactions between nucleoproteins. This is

mainly because recombinant NP can oligomerize in the absence of vRNA [5–7]. Moreover, transmission electron microscopic images revealed that the gross structure of NP oligomers without RNA is not starkly different from that of RNA-bound NP oligomers [26]. Surprisingly, the BNP mutant that lost the ability to bind RNA (the G1 mutant) could no longer bind to ANP and also lost the ability to inhibit polymerase activity. This raises an intriguing possibility that, even though the physical presence of the viral RNA does not affect ANP–BNP interaction, the RNA binding motif on the nucleoprotein itself must be intact to support intertypic interaction. Either these residues are necessary in mediating the interaction directly or the wild-type amino acids are involved indirectly in structurally preserving the interaction site.

In summary, this study, for the first time to our knowledge, demonstrates the interaction between type A and type B influenza nucleoproteins and suggests that the profound effect of BNP on limiting growth of FluA may occur by inhibiting proper formation of the FluA polymerase complex. This could serve as one of the several mechanisms that may together contribute to intertypic interference [16,18,27]. The findings here shed new light on the relationship, interaction and competition between the two types of co-circulating influenza viruses.

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