A peptide derived from the C-terminus of PB1 inhibits influenza virus replication by interfering with viral polymerase assembly

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Introduction
Influenza virus, a primary member of the Orthomyxoviridae family, is responsible for annual epidemics and occasional pandemics, including the 1918 Spanish influenza (by H1N1), the 1957 Asian influenza (H2N2), the 1968 Hong Kong influenza (H3N2) and the 2009 Swine influenza (reassorted H1N1) [1–3]

Efficient assembly of the influenza virus RNA-dependent RNA polymerase, a heterotrimERIC complex formed by three subunits (PA, PB1 and PB2) is critical for virus replication and pathogenicity. Therefore, interfering with the assembly of the RNA-dependent RNA polymerase complex could offer novel and effective anti-influenza therapeutics. In the present study, we show that a short peptide derived from amino acids 731–757 of PB1 (PB1731–757) can disrupt the interaction between the C-terminal part of PB1 (denoted as PB1c corresponding to PB1676–757) and the N-terminal part of PB2 (denoted as PB2n corresponding to PB21–40). We further show that PB1731–757 is capable of inhibiting viral polymerase activity and viral replication. Interestingly, we find that PB1731–757 interacts with PB1c rather than PB2n. Furthermore, mutational analyses show that the hydrophobic sites of PB1c play an essential role in the PB1c–PB1731–757 interaction. The characterization of the inhibitory effect of PB1731–757 on viral polymerase activity and viral replication could offer a potential target for anti-influenza drug development.

Structured digital abstract

PB2n physically interacts with PB1c by pull down (View interaction)

PB2n and PB1c physically interact by bimolecular fluorescence complementation (View interaction)

PB1 (731–757) physically interacts with PB1c by pull down (View interaction)

PB1 (731–757) and PB1c physically interact by bimolecular fluorescence complementation (View Interaction:

[Structured digital abstract was added on 11 February 2013 after original online publication]

Abbreviations
BIFC, bimolecular fluorescence complementation; GFP, green fluorescent protein; GST, glutathione S-transferase; IL-2, interleukin-2; IL-2Ra, interleukin-2 receptor; MBP, maltose binding protein; PolI, polymerase I; RdRp, RNA-dependent RNA polymerase; YFP, yellow fluorescent protein.
outbreaks. The resurgence of this pathogen has caused enormous economic losses and numerous human deaths, making our fight against influenza virus a long-standing challenge. Currently, the most effective methods of protecting people from influenza virus infection are vaccination and the use of antiviral drugs such as M2-ion channel inhibitors (amantadine and rimantadine) and neuraminidase inhibitors (oseltamivir and zanamivir). However, the virus has the ability to rapidly evolve either through gene mutation or gene reassortment, rendering frequent vaccine mismatches and the emergence of variants resistant to the existing anti-viral drugs [4–8]. Therefore, the development of novel therapeutics against influenza, particularly when faced with the threat of the highly pathogenic avian virus (H5N1), is urgently needed [8–10].

Given its functional essentiality for viral replication and involvement in virus pathogenicity [11–14], the RNA-dependent RNA polymerase (RdRp) complex of the influenza virus has become a potential influenza drug target [9,15–17]. The RdRp is a heterotrimeric complex composed of three subunits: PA, PB1 and PB2. The assembly of the three subunits into functional viral RdRp is one of the rate-limiting steps for influenza virus RNA synthesis and virus replication [18–20]. The polymerase subunit PB1 plays a central role in RdRp assembly [14,18,21], and thus interfaces between PB1 and PA/PB2 could be potential targets for anti-influenza drugs. In-depth analyses have found that it is the N- and C-termini of PB1 that are involved in its interactions with PA and PB2, respectively [22–24]. It has been shown previously that a peptide derived from the N-terminal 25 amino acids of PB1 (denoted as PB1_{1–25}) can induce the disruption of the PA–PB1 interaction and thus effectively inhibit influenza virus polymerase activity [15,25,26].

In the present study, we report the characterization of a new inhibitory short peptide of 27 amino acids derived from the very C-terminus of PB1, denoted as PB1_{731–757}, which can effectively inhibit influenza virus polymerase activity and virus replication. Moreover, we show that PB1_{731–757} can disrupt the association of the C-terminal part of PB1 (denoted as PB1c corresponding PB1_{676–757}) and the N-terminal part of PB2 (denoted as PB2n corresponding to PB2_{1–40}). Unexpectedly, PB1_{731–757} interacts with PB1c rather than PB2n. Further mutational analyses and computational modelling suggest that PB1_{731–757} acts as a competitor of PB2n with respect to binding to PB1c.

Results

The short peptide PB1_{731–757} is able to disrupt the interaction between PB1c and PB2n

A previous deletion study has suggested the involvement of PB1_{731–757} in the assembly of PB1c and PB2n [22,24]. In the present study, we tested whether the standalone short peptide PB1_{731–757} derived from H5N1 (A/goose/Guangdong/1/96) is able to disrupt the PB1c–PB2n interaction. We employed a disruption assay based on the bimolecular fluorescence complementation (BiFC) assay [27,28]. The H5N1-derived PB2n and PB1c were fused to the N-terminus (YN) and C-terminus (YC) of yellow fluorescent protein (YFP), respectively. When the YN-PB2n and YC-PB1c expression plasmids were co-transformed into yeast cells, it produced a relatively high YFP fluorescence intensity after induction, indicating a relatively strong interaction between YN-PB2n and YC-PB1c. As shown in Fig. 1A, the interaction between YN-PB2n and YC-PB1c was significantly disrupted when the self-competitor PB1c was expressed in the yeast cells, whereas there was no interference in the presence of an unrelated peptide PX or in the absence of the peptides (control). These results validated the feasibility of using the BiFC-based system to analyze the factors that affect the PB1c–PB2n association. The introduction of PB1_{731–757} resulted in a reduction of ~15% of the fluorescence intensity (84.26 ± 2.75%, P < 0.05), whereas the expression levels of YN-PB2n and YC-PB1c remained constant (Fig. 1A), suggesting that PB1_{731–757} can interfere with the PB1c–PB2n interaction. Furthermore, the hydrophobic residue I750 within PB1_{731–757} sequence has been reported previously to be essential for the PB1c–PB2n interaction [24]. Therefore, we also examined the effect of the PB1_{731–757}I750N mutant on the assembly of PB1c–PB2n in the BiFC disruption assay. As expected, the presence of PB1_{731–757}I750N (86.98 ± 4.59%, P > 0.05 compared to PX) did not interfere with the interaction PB1c–PB2n as efficiently as PB1_{731–757}, indicating that the hydrophobic residue I750 is important for the inhibitory function of PB1_{731–757} (Fig. 1A).

We further performed a competitive glutathione S-transferase (GST) pull-down assay to confirm the disruptive effects of PB1_{731–757} and PB1_{731–757}I750N on the PB1c–PB2n interaction in vitro. Figure 1B shows that the pull-down efficiency of maltose binding protein (MBP)-tagged PB1c (MBP-PB1c) by GST-tagged PB2n (GST-PB2n) can be significantly affected by the presence of PB1_{731–757} compared to the presence of the
control peptide PX, whereas PB1731–757I750N showed less of an inhibitory effect. These results are consistent with observations made from the BiFC disruption assay. We concluded that the presence of PB1731–757 is capable of interfering with PB1c–PB2n assembly and that the I750 of PB1731–757 contributes to its inhibitory effect.

**PB1731–757 interacts directly with PB1c instead of PB2n**

We next examined whether PB1731–757 acts as a PB1c competitor to disrupt the interaction between PB1c and PB2n by analyzing the direct interaction between PB2n and PB1731–757. We carried out a pull-down assay using GST-tagged PB1731–757 (GST-PB1731–757) to pull down MBP-tagged PB2n (MBP-PB2n), whereas the interaction between GST-PB2n and MBP-PB1c served as a positive control. To reduce the influence of fused tag to the peptide-peptide interactions, unstructured linkers between GST or MBP and our peptides were inserted. The GST pull-down assay was previously shown to be feasible with respect to detecting interactions involving peptides [29,30]. Moreover, GST or MBP can improve the solubility and stability of the fused partner [31,32]. The input amounts of these peptides in this experiment were maintained at similar levels. Unexpectedly, we found that PB1731–757 cannot pull down PB2n, whereas the interaction between PB1c and PB2n was obvious in the pull-down assay (Fig. 2A). According to the crystal structure of the complex formed by PB1c and PB2n [24], both PB1c and PB1731–757 show amphipathic characteristics in that they are organized as hydrophobic amino acids on the one side and hydrophilic amino acids on the other side (Fig. S1). Because PB1c contains a hydrophobic patch that can apparently accommodate an α-helix, and because the hydrophobic residue I750 of PB1731–757 is critical for the inhibitory function of PB1731–757 (as shown above), we were interested in testing whether PB1731–757 interacts with PB1c directly with the PB1c–PB2n interaction as a comparison. As shown in Fig. 2B (upper panel), the binding capacity of PB1731–757 with PB1c was evident but weaker than that of PB1c and PB2n, whereas the binding capacity of PB1731–757I750N to PB1c was very weak. Because purified GST-PB2n was unstable (Fig. 2B, lower panel), the amounts of purified GST fusion proteins were used to normalize the binding capacity between PB1c or PB2n and PB1731–757. As shown in Fig. 2C, the relative binding strength between PB1c and PB1731–757 was ~5% of that of PB1c–PB2n after normalization and there was no interaction.
detected between PB2n and PB1c and PB1731–757. The relative binding strength between PB1c and PB1731–757 was much lower than that between PB1c and PB1731–757I750N (P < 0.05). These results are consistent with the results obtained from both the BiFC disruption assay and the competitive GST pull-down assay.

We then conducted the BiFC assay to confirm the interaction between PB1c and PB1731–757. The co-transformation of plasmids expressing YN-PB1c and YC-PB1731–757 (YN-PB1c/YC-PB1731–757) into yeast strain ySC8 gave a much higher YFP intensity than the controls (YN/YC-PB1731–757 and YN-PB1c/YC), whereas YN-PB1c and YC-PB1731–757I750N showed a much weaker YFP intensity, and no interaction was observed between YN-PB2n and YC-PB1731–757 (Table 1). This is agreement with the results obtained between PB2n and PB1731–757. The relative binding strength between PB1c and PB1731–757I750N was much lower than that between PB1c and PB1731–757 (P < 0.05). These results are consistent with the results obtained from both the BiFC disruption assay and the competitive GST pull-down assay.

![BiFC assay](image)

**Fig. 2.** Detection of the interaction between PB2n or PB1c and PB1731–757 using the GST pull-down assay. (A) The interactions between PB2n and PB1731–757 were detected using the GST pull-down assay. The PB1c–PB2n interaction served as a positive control. The eluted proteins were separated by SDS/PAGE and detected by western blotting with MBP antibody. The position of MBP-PB1c is indicated. (B) GST pull-down assay for the bindings between PB1c and PB1731–757 or PB1731–757I750N. The PB1c–PB2n interaction was conducted as a comparison (upper panel). The purified GST proteins were analyzed by Coomassie brilliant blue staining and protein markers are shown on the left (kDa) (lower panel). (C) The relative binding activities between PB2n or PB1c and PB1731–757 or PB1731–757I750N described in (A) and (B) were normalized by the amounts of purified GST fusion proteins noted in (B) (lower panel). The relative binding activity between PB1c and PB2n (PB1c/PB2n) was set to 100%. All data are shown as the mean ± SD (n = 3). (*P < 0.05). (D) GST pull-down assay for the interactions between PB1c and two truncations of PB1731–757 (named PB1737–757 and PB1737–752).

**Table 1.** BiFC results for the interactions between PB1731–757, PB1c and PB2n. Binding activity is estimated as the ratio of relative fluorescence intensity (RFI) of the test group compared to controls. If RFI ≥ 4.0, binding activity is strong (+++); < 4.0 and ≥ 2.5, binding activity is intermediate (+); < 2.5 and ≥ 1.3, binding activity is weak (+); < 1.3, there is no interaction (–).

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<th>Binding activity</th>
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<td>PB2n</td>
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<td>+++</td>
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<tr>
<td>PB1731–757</td>
<td>PB1731–757</td>
<td>−</td>
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<td>PB1731–757</td>
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<td>PB1731–757</td>
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from the GST pull-down assay, showing that PB1\textsubscript{731-757} interacts with PB1c instead of PB2n.

To further define the minimal sequence of PB1\textsubscript{731-757} required for interaction with PB1c, we cloned two truncations of PB1\textsubscript{731-757} onto the C-terminus of GST, denoted as GST-PB1\textsubscript{737-757} and GST-PB1\textsubscript{732-752}, and detected their PB1c binding activities using the GST pull-down assay. As shown in Fig. 2D, both deletion of the six N-terminal amino acids (PB1\textsubscript{737-757}) and the five C-terminal amino acids (PB1\textsubscript{732-752}) drastically disrupted that PB1c binding capacity, suggesting that PB1\textsubscript{731-757} is the minimal sequence required for the binding capacity of PB1c.

The full-length PB1c is critical for PB1c-PB1\textsubscript{731-757} binding

The crystal structure of PB1c-PB2n complex shows that PB1c contains three helices [24]. In addition to PB1\textsubscript{731-757}, PB1c can be split into other two \( \alpha \) helices at residue F700, named PB1\textsubscript{676-700} and PB1\textsubscript{701-730} (Figs 3 and S2). To test which helix of the three helices is responsible for the binding to PB1\textsubscript{731-757}, we generated a series of PB1c helix deletion mutants (PB1\textsubscript{676-700}, PB1\textsubscript{701-730}, PB1\textsubscript{a731-757} and PB1\textsubscript{c676-700}) and analyzed the interactions between these mutants and PB1\textsubscript{731-757} using the GST pull-down assay. As shown in Fig. 3, because the input amounts of MBP fusion proteins were on the same level, either the deletion of one helix or the deletion of the end helix of the PB1c resulted in a complete loss of PB1\textsubscript{731-757} binding capacity. These results were further confirmed using the BiFC assay (Table 1). Therefore, we concluded that full-length PB1c is required for its interaction with PB1\textsubscript{731-757}, indicating that PB1\textsubscript{731-757} interacts with the three dimensional structure formed by all three helices of PB1c.

We further aimed to identify the key residues on PB1c that are responsible for its binding to PB1\textsubscript{731-757}. Given the amphipathic characteristics of both PB1c and PB1\textsubscript{731-757} (Fig. S1), we tested the effects of several hydrophobic/hydrophilic point mutants that are spread in all three helices of PB1c on PB1c-PB1\textsubscript{731-757} binding. L695, F699 and I750 of PB1c were mutated as a result of a previous study [24], showing these residues are critical for the PB1c-PB2n interaction. We also mutated another residue M718 because it is also located in the hydrophobic region of PB1c. In addition, we mutated two hydrophilic residues (R727 and K745) on the hydrophilic side of PB1c. As shown in Table 2, the mutations of the hydrophobic residues can significantly affect the interaction of PB1c with PB1\textsubscript{731-757}: L695D and M718A substitutions completely abolished the PB1c-PB1\textsubscript{731-757} interaction, whereas the F699A and I750N/I750D mutations resulted in a much lower binding capacity. By contrast, the mutations of the hydrophilic amino acids R727 and K745 did not affect the binding. Taken these findings together, the hydrophobic residues of all three helices of PB1c appear to be essential for the PB1c-PB1\textsubscript{731-757} interaction.

The peptide PB1\textsubscript{731-757} is capable of inhibiting influenza RNA polymerase activity and virus replication

Based on above observations that PB1\textsubscript{731-757} could act as a PB2n competitor to interfere with PB1-PB2 interface assembly, we next examined whether PB1\textsubscript{731-757} can inhibit influenza polymerase activity and virus replication. Because the C-terminal region 686-698 of PB1 has also been reported to be involved in
the PB1–PB2 interaction [22], we also included a PB1676-700 peptide that is the full sequence of the first α helix of PB1c in our analyses. In addition, PB11-25 was also included as a positive control [26]. We first measured their effects on polymerase activity by transfecting GFP-tagged peptide expression plasmids into 293T cells harbouring a mini-replicon system of influenza A virus (A/WSN/33). As shown in Fig. 4A (upper panel), compared to GFP-tagged Flag and GFP-tagged PX, which showed no effects on influenza replication as reported previously [15], the over-expression of GFP-tagged PB1676-700 did not significantly affect RdRp activity, whereas GFP-tagged PB1731-757, similar to GFP-tagged PB11-25, disrupted viral polymerase activity significantly. The western blotting assay of GFP expression showed that the expression levels of these peptides, except PB11-25, were similar (Fig. 4A, lower panel). By contrast, PB1731-757I750N was less efficient in inhibiting polymerase activity than PB1731-757 (P < 0.01). Therefore, we concluded that PB1731-757 is able to inhibit viral RNA polymerase activity, which is consistent with its inhibitory effect on the assembly of PB1c–PB2n.

To further analyze whether the peptide PB1731-757 can inhibit influenza virus replication in cells, we used a reporter system based on secreted Gaussia luciferase [33] to monitor influenza virus infection in the presence of PB1731-757-GFP. We also included the peptide PB11-25 reported by Ghanem et al. as a comparison [26]. As shown in Fig. 4B, PB1731-757, similar to PB11-25, can effectively block the replication of human influenza virus A/WSN/33 (H1N1).

### Discussion

The influenza virus RdRp is responsible for viral RNA transcription and replication in the nucleus of infected cells. The efficiency of viral RNA synthesis, which is critical for virus replication and pathogenicity, is depen-

### Table 2. Effects of PB1c mutations on its interactions with PB1731-757. Binding activity is estimated as the ratio of relative fluorescence intensity (RRFI) of the test group compared to controls. If RRFI: ≥ 4.0, binding activity is strong (+++); < 4.0 and ≥ 2.5, binding activity is intermediate (++); < 2.5 and ≥ 1.3, binding activity is weak (+); < 1.3, there is no interaction (–).

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<th>Location</th>
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<tr>
<td>PB1676-700</td>
<td>WT</td>
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<tr>
<td></td>
<td>L695D</td>
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<tr>
<td></td>
<td>F699A</td>
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</tr>
<tr>
<td>PB1720-730</td>
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<tr>
<td></td>
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<td>++</td>
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<tr>
<td>PB1731-757</td>
<td>K745A</td>
<td>++</td>
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<td></td>
<td>I750N/D</td>
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![Fig. 4. The over-expression of PB1731-757 inhibits influenza virus polymerase activity and virus replication. (A) Effects of over-expressed short peptides on influenza A virus polymerase activity (upper panel). The viral polymerase activity was assayed in a mini-replicon system of A/WSN/33 (H1N1) with a Poll-driven plasmid expressing influenza virus-like RNA coding for firefly luciferase to detect viral polymerase activity. The short peptides were expressed as GFP fusions. Plasmid expressing Renilla luciferase was also included in transfection used to normalize transfection efficiency. `- PB2' indicates the omission of PB2 from the transfection mixture, which served as a negative control. PB11-25-GFP (PB11-25), which has been reported to inhibit polymerase activity, served as a positive control. The polymerase activities are expressed as activity relative to that under Flag expression (Flag), which is set as 100%. All data are shown as the mean ± SD. **P < 0.01 (Student’s t-test) (n = 3). The expression levels of GFP fusion proteins and glyceraldehyde 3-phosphate dehydrogenase were detected by western blotting with the indicated antibodies (lower panel). (B) Effect of over-expressed PB1731-757 on virus replication activity of A/WSN/33. The assay is performed in A549 cells using the Gaussia luciferase reporter system. PB11-25-GFP (PB11-25) and Flag-GFP (Flag) were used as positive and negative controls, respectively. The luciferase activity under the expression of Flag-GFP (Flag) is set as 100% after subtracting the background of uninfected cells (‘Mock’). All data are shown as the mean ± SD (n = 3).
dent on the RNP assembly and RdRp activity. Given that its functionality requires the proper assembly of the three subunits, the modulation of RdRp assembly can effectively affect its activity, and thus could prove to be a very promising avenue for the development of effective therapeutics against this highly elusive pathogen.

Previously, short peptides derived from polymerase subunits were shown to inhibit virus replication by interfering with polymerase assembly. For example, the PB1_{1-25} peptide can disrupt any interaction between PA and PB1 efficiently, and PB2_{1-12} can inhibit influenza virus polymerase activity in a strain-specific manner [15,17,26]. In the present study, we characterized another novel potent inhibitory peptide, PB1_{731-757}, derived from the C-terminus of PB1, which can disrupt the assembly of PB1c and PB2n and inhibit influenza RdRp activity, and thus virus replication, as efficiently as PB1_{1-25}.

Interestingly, the data obtained in the present study show that the inhibitory effect of PB1_{731-757} on the PB1c–PB2n interaction acts through its binding to the PB1c, from where it derives, rather than PB2n. Moreover, we find that the inhibitory binding is mainly mediated by the hydrophobic interactions between the PB1c and PB1_{731-757}. This is different from the inhibitory mechanism of the interfacial peptides PB1_{1-25} and PB2_{1-12}, which inhibit complex assembly by binding to its interaction partner PAc or PB1c, respectively [15,17,23-26,34]. Similar to the findings of the present study, an inhibitory mechanism has been reported for Ro26-4550 with respect to interfering with the interaction between interleukin-2 (IL-2) and its receptor (IL-2Rα). The Ro26-4550 was designed as a peptidomimetic of the IL-2Rα binding site on IL-2 to interfere with the assembly of IL-2 and IL-2Rα, although it actually interacts with IL-2 rather than IL-2Rα by hydrophobic interactions [35,36].

Based on the crystal structure of the PB1c and our observation that the hydrophobic region of PB1c is critical for the PB1c–PB1_{731-757} interaction, we modelled the complex structure of PB1c–PB1_{731-757} using a site-constrained docking method [37]. There is no structure for H5N1 PB1c and there are only three different residues between A/Puerto Rico/8/34 (H1N1) PB1c and A/goose/Guangdong/1/96 (H5N1) PB1c, as shown in Fig. S3A, which are away from the hydrophobic patch of PB1c. Thus, that structure of A/Puerto Rico/8/34 PB1c was used in our docking assay. As shown in Fig. S4, PB1_{731-757} can be buried in the hydrophobic groove formed by the hydrophobic residues located in three helices of PB1c. Furthermore, the side chains of hydrophobic residues on PB1_{731-757} are all towards to the hydrophobic patch of PB1c, as shown in Fig. S4. Therefore, we speculate that the previously reported PB2n-derived peptide PB2_{1-12} may also interact with PB1c in a similar way as PB1_{731-757} does because it has been also shown that hydrophobic interactions between PB1c and PB2_{1-12} are critical for their interaction [17,24]. The PB1c–PB2n complex was aligned to the PB1c–PB1_{731-757} complex with PYMOL (http://www.pymol.org/) based on the PB1c structures. Both PB1c structures aligned well and only the structure of PB1c from the PB1c–PB1_{731-757} complex was shown. Figure 5B shows that PB1_{731-757} overlapped with the first α helix of PB2n (PB2_{1-12}), whereas the directions of the two helices are different. This is consistent with the fact that PB2_{1-12} contributes most of the binding energy between PB1c and PB2n [24]. It can also be seen that the first α helix of PB2n is much closer to the hydrophobic patch of PB1c than PB1_{731-757}, which correlates with the results indicating that the relative binding activity between PB1c and PB2n is much higher than that between PB1c and PB1_{731-757} (Fig. 2C and Table 1). However, we found

Fig. 5. Structural model of PB1c–PB1_{731-757} complex and its structural alignment with the PB1c–PB2n complex. (A) An overall ribbon diagram showing the docking model of the complex: PB1c coloured red and PB1_{731-757} coloured light blue. Experimental mutants are marked on the docking model. I750 from PB1_{731-757} is coloured yellow; the hydrophobic residues L695, F699, M718 and I750 on PB1c are coloured green; and the hydrophilic residues R727 and K745 are coloured dark blue. The N- and C-termini are indicated. (B) The structural alignment between the PB1c–PB2n complex and the PB1c–PB1_{731-757} complex with PYMOL based on the PB1c structures. PB1c from PB1c–PB1_{731-757}, PB1_{731-757} and PB2n are coloured red, blue and grey, respectively. The N- and C-termini are indicated.
that the PB1c-derived PB1_{676-700} could not inhibit viral RNA polymerase activity (Fig. 4A). To explore the possible reasons for this, we analyzed the hydrophobic surface of PB1_{684-700} because the structure of PB1c started from residue E684 based on the reported three-dimensional structure of PB1c and PB2n (Fig. S5). The structure shows that the hydrophobic surface of PB1_{684-700} is different from that of PB1_{731-757}. The C-terminus of PB1_{684-700} lacks hydrophilic amino acids, whereas PB1_{731-757} has hydrophilic amino acids on both termini. As shown in Figs 2D and S1B, both hydrophilic termini of PB1_{731-757} contribute to the PB1c–PB1_{731-757} interaction. In addition, the hydrophobic area of PB1_{684-700} is smaller than that of PB1_{731-757}. These findings might explain why PB1_{676-700} cannot form a similar tertiary structure with PB1c and thus inhibit polymerase activity. Furthermore, amino acid sequence alignment of the three peptides (PB1_{676-700}, PB1_{731-757} and PB2_{1-27}) shows that PB1_{731-757} and PB2_{1-27} share a larger number of conserved amino acids, whereas PB1_{676-700} does not (Fig. S3B). We consider that additional screening of the small molecules that specifically target the hydrophobic groove of the PB1c may identify potent drugs that act against the influenza virus.

Materials and methods

Cells and plasmids

HEK293T and A549 cells were maintained as described previously [33], pPolI-NP-luc, pPolI-Gluc and cDNA of influenza A/goose/Guangdong/1/96 (H5N1) were generously provided by Professor Martin Schwemmle (University of Freiburg, Germany), Professor Yuelong Shu (Center for Disease Control and Prevention, Beijing, China) and Professor Yingfang Liu (Chinese Academy of Sciences, Beijing, China), respectively.

BiFC assay

The BiFC assay was conducted as described previously [38, 39]. Briefly, two proteins/peptides of interest were fused to the N-part (amino acids 1–154; YN) and C-part (amino acids 155–238; YC) of YFP, respectively. The plasmids expressing both fusion proteins were transformed into the cells of yeast strain ySC8. After induction in SCGR/-Leu/-Trp medium at 20 °C for 24 h, the fluorescence intensities (excitation = 485 nm, emission = 535 nm) and cell density (OD600) were detected using a microplate reader (GENios Plus, Tecan Group Ltd, Männedorf, Switzerland). The assays were conducted with three repeats, and the ratio of relative fluorescence intensity of test group to controls was estimated as the relative binding strength.

BiFC disruption assay

The peptides PB1c (corresponding to PB1_{676-757}), PB1_{731-757}, PB1_{731-757}750N derived from PB1 of avian H5N1 A/goose/Guangdong/1/96 and an unrelated peptide PX derived from Borna disease virus phosphoprotein [15] were cloned into pYes260 [40] between the Nari and Xbal sites under the Gal1 promoter without introducing fusion tags onto these peptides. Next, they were transformed individually into yeast cells with plasmids expressing the interacting pair YN-PB2n (corresponding to YN-PB2_{1-40}) and YC-PB1c. After culturing in SCGR/-Leu/-Trp/-Ura medium at 20 °C for 24 h, the fluorescence intensities and cell density of the yeast were detected as described above. The relative fluorescence intensities of yeast cells in the absence of peptide were set to 100% (control). The SD was obtained from four independent tests with three repeats of every sample in every single independent test. The expressions of YN-PB2n and YC-PB1c were analyzed by western blotting with GFP polyclonal antibody.

GST pull-down assay

PCR products of peptides were cloned into pGEX6p-1 (GE Healthcare, Milwaukee, WI, USA) or pMal-C2-TEV (New England Biolabs, Beverly, MA, USA) to express GST- or MBP-fusion proteins, respectively, in Escherichia coli BL21 (DE3). GST fusion proteins were purified first and then mixed with cell lysates containing the same amounts of MBP fusion proteins at 4 °C overnight. Then the sepharoses were washed twice with NaCl/Pi and eluted with 10 mM reduced glutathione in 50 mM Tris-HCl (pH 8.0). Eluted proteins were subjected to SDS/PAGE followed by Coomassie brilliant blue staining or western blotting with anti-MBP sera. A competitive GST pull-down assay was conducted by incubating purified GST-PB2n with cell lysates containing MBP-PB1c and competitor MBP fusion peptides at 4 °C overnight. Eluted MBP-PB1c was analyzed by western blotting.

Site-directed mutagenesis

Site mutations on PB1c were generated by site-directed mutagenesis using PfuUltra (Stratagene, La Jolla, CA, USA). Plasmid expressing YN-PB1c was used as a template. Positive clones were verified by sequencing.

Reconstitution of influenza A virus polymerase activity

293T cells were transfected using poly(ethylenimine) reagent (Sigma, St Louis, MO, USA) in six-well plates. The transfection mixture contained plasmids expressing A/WSN/33-derived PB2, PB1, PA, NP and a luciferase reporter plasmid, which are all needed for the mini-replicon system
of influenza A virus [26,41]. The plasmids expressing GFP-tagged peptides and plasmids expressing Renilla luciferase were also added to each well. The reporter plasmid pPolI-NP-luc is a polymerase 1 (PolI)-driven plasmid expressing influenza virus-like firefly luciferase RNA with a noncoding region of nucleoprotein gene of influenza A virus on both ends, which was used to detect the activity of influenza A virus RdRp as described previously [26,41]. Renilla luciferase was used as an indication of transfection efficiency. Twenty-four hours after transfection, the firefly luciferase and Renilla luciferase activities of cell lysates were measured using the Dual-Glu Luciferase Assay system (Promega, Madison, WI, USA). The firefly luciferase activity of samples containing Flag-GFP after normalization by Renilla luciferase was set to 100%. The expression of GFP-tagged proteins and glyceraldehyde 3-phosphate dehydrogenase was detected by western blotting.

**Virus infection**

Influenza virus A/WSN/33 was prepared as described previously [33]. A549 cells in 24 well-plates were transfected with 1 μg of plasmids encoding GFP-tagged peptides and 0.4 μg of plasmid pPolI-Gluc, which is a PolI-driven plasmid expressing influenza virus-like RNA coding for Gaussia luciferase as a reporter for viral polymerase activity [33]. Twelve hours after transfection, cells were infected with A/WSN/33 virus (multiplicity of infection = 0.015) for 1.5 h. Next, cells were washed in NaCl/Pi and incubated in DMEM with 1 μg·mL⁻¹ trypsin at 37 °C. Twelve hours after infection, the supernatant were collected and tested using a Gluc Assay Kit (New England Biolabs). After subtracting the background of uninfected cells (‘Mock’), the luciferase activity of samples containing Flag-GFP was set to 100%.

**PB1c–PB1731–757 complex modelling**

First, the structures of PB1c and PB1731–757 were built by taking coordinates from the complex structure of PB1c–PB2n (Protein Data Bank code: 2ZTT). Then, the three sites (I750 of PB1731–757, L695 and I750 of PB1c) were added as interface constraints to direct the docking between PB1c and PB1731–757 using PATCHDOCK [37]. The final complex model was selected according to the docking scores and an additional rule proposing that the deletion of the six N-terminal amino acids of PB1731–757 will weaken the interaction between PB1c and PB1731–757. Structural alignment between the PB1c–PB2n complex and PB1c–PB1731–757 complex was analyzed using PYMOL (version 1.5.0.4) based on the PB1c structures.

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher’s web site:

- Fig. S1. Surface view of the hydrophobic region on PB1c (A) and PB1731–757 (B).
- Fig. S2. The location of three helices on PB1c.
- Fig. S3. Amino acid sequence alignment between PB1c from A/Puerto Rico/8/34 (H1N1) and A/goose/Guangdong/1/96 (H5N1) (A), PB1676–700, PB1731–757 and PB21–27 from A/goose/Guangdong/1/96 (B).
- Fig. S4. The location of the hydrophobic residues on PB1731–757 of PB1c–PB1731–757 complex.
- Fig. S5. The hydrophobic surface analyses of PB1684–700.