Molecular Basis of mRNA Cap Recognition by Influenza B Polymerase PB2 Subunit*

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Influenza virus polymerase catalyzes the transcription of viral mRNAs by a process known as "cap-snatching," where the 5’-cap of cellular pre-mRNA is recognized by the PB2 subunit and cleaved 10–13 nucleotides downstream of the cap by the endonuclease PA subunit. Although this mechanism is common to both influenza A (FluA) and influenza B (FluB) viruses, FluB PB2 recognizes a wider range of cap structures including m7GpppGm-, m7GpppG-, and GpppG-RNA, whereas FluA PB2 utilizes methylated G-capped RNA specifically. Biophysical studies with isolated PB2 cap-binding domain (PB2cap) confirm that FluB PB2 has expanded mRNA cap recognition capability, although the affinities toward m7GTP are significantly reduced when compared with FluA PB2. The x-ray co-structures of the FluB PB2cap with bound cap analogs m7GTP and GTP reveal an inverted GTP binding mode that is distinct from the cognate m7GTP binding mode shared between FluA and FluB PB2. These results delineate the commonalities and differences in the cap-binding site between FluA and FluB PB2 and will aid structure-guided drug design efforts to identify dual inhibitors of both FluA and FluB PB2.

Both pandemics and seasonal epidemics caused by human influenza viruses are of great public health concern. The influenza A (FluA)2 and influenza B (FluB) viruses are responsible for seasonal epidemics causing mild to severe respiratory illness with the potential for serious complications in the elderly, infants, and individuals with compromised immune systems. Two subtypes of FluA are currently circulating in the human population, the H1N1 and H3N2 serotypes. Influenza A viruses are characterized by a broad host range, including humans, wild aquatic birds, domestic avian species, horses, seals, dogs, and cats. Zoonotic transmission can lead to severe pandemics when a novel virus can sustain human-to-human transmission in a population with no pre-existing immunity, as was recently the case in 2009 when a new reassortant virus created a worldwide pandemic (1). FluB has long been overshadowed by the pandemic nature of FluA. However, recent epidemiologic data have shown that FluB contributes to a higher global burden than traditionally thought. Since 2001, the two FluB lineages, Yamagata and Victoria (2), have been co-circulating every season in the human population. Between 2004 and 2011, excluding the 2009 H1N1 pandemic, 22–44% of influenza-related pediatric deaths were confirmed to be due to FluB (3).

The influenza virus genome consists of eight single-stranded RNAs packed into rod-like structures of varying size, known as the ribonucleoprotein complex (RNP) (4). Each RNP contains a unique viral RNA, multiple copies of the scaffolding nucleoprotein, and a heterotrimeric viral polymerase consisting of the PA, PB1, and PB2 subunits, which catalyzes the transcription and replication of the viral genome (5). Due to its central role in the viral cycle, extensive effort has gone into understanding the function and molecular basis of RNP and its individual components. Cusack and co-workers (6, 7) have published the structures of both FluA and FluB heterotrimers, and these structures provide insight into the mechanistic understanding of cap-snatching and RNA synthesis by influenza polymerase. Consistent with the previously described “cap-snatching” mechanism, the PB2 cap-binding domain (PB2cap) first sequesters the host pre-mRNAs by binding to their 5’ cap (8). PA, the endonuclease subunit (9, 10), in turn cleaves the captured pre-mRNA 10–13 nucleotides downstream of the cap (11–13). The PB2cap then rotates about 70° to direct the capped primer from facing toward PA into the PB1 polymerase active site, as observed by comparing FluA and FluB polymerase structures (6, 7). The cap-binding pocket of FluA PB2 in the heterotrimeric structure was empty, whereas the residual RNA density was identified in FluB PB2cap pointing toward PB1. The PB1 subunit directly interacts with both PB2 and PA subunits. It contains a conserved RNA polymerase domain for the elongation of the chimeric mRNA using the viral RNA as template.

Influenza PB2 protein has attracted increasing attention as a viable anti-influenza target due to the success of VX-787 targeting the PB2 cap-binding site of FluA specifically (14). Although the cap-binding pocket of FluA PB2 was empty in the trimeric structure, the cap binding features of FluA PB2 are well understood given the structural information provided by the FluA PB2cap-m7GTP and VX-787 co-structures (14). In contrast, little is known about FluB PB2cap in complex with its
substances. The sequence identity of PB2\textsuperscript{cap} between human FluA and FluB is approximately 30%, with most of the residues important for the structure or cap binding function conserved between the two proteins (15). Using recombinant viral RNPs, it has been shown that FluA polymerase cleaves RNA capped with m\textsuperscript{7}G preferentially, whereas FluB polymerase efficiently cleaves unmethylated GpppG RNA in addition to m\textsuperscript{7}G-capped RNA, suggesting that the FluB PB2 contains a more permissive active site and may accommodate a wider range of cap analogs (15). Most recently, a co-structure with m\textsuperscript{7}GDP was determined for FluB PB2 containing a Q325F binding site mutation (16). Surprisingly, the guanine and ribose of m\textsuperscript{7}GDP in this co-structure invert along the long axis of the base when compared with m\textsuperscript{7}GTP bound in FluA PB2. This inversion was attributed to the amino acid difference in the FluB Trp-359 position (corresponding to His-357 in FluA), which provides stacking interactions with the guanine moiety (8). However, it is unclear whether this inversion of the ligand is induced by the introduction of the active site mutation, Q325F, which could have significantly altered the ligand binding mode, as evidenced in the drastic changes in binding affinity and specificity after introduction of the active site mutation, Q325F, which could have significantly altered the ligand binding mode, as evidenced in the drastic changes in binding affinity and specificity after modification of Gln-325 (16).

Herein, we have used a combination of x-ray crystallography, differential scanning fluorometry (DSF), and isothermal titration calorimetry (ITC) to examine the interactions of wild-type FluB PB2\textsuperscript{cap} with m\textsuperscript{7}GTP and GTP. We present the first structure of the wild-type FluB PB2\textsuperscript{cap} alone and in complex with the mRNA cap analogs m\textsuperscript{7}GTP and GTP. The DSF and ITC studies demonstrate that FluA PB2\textsuperscript{cap} preferentially binds to the N7-methylated GTP, whereas FluB PB2\textsuperscript{cap} recognizes both cap analogs equally well. Structural comparisons of FluB PB2\textsuperscript{cap} with FluA PB2\textsuperscript{cap} and its interaction with the cap analogs reveal the distinct conformational state of these two ligands. FluB PB2\textsuperscript{cap} binds m\textsuperscript{7}GTP using a conserved binding mode similar to that in FluA PB2\textsuperscript{cap}, but distinct from the structure in Q325F mutated FluB PB2\textsuperscript{cap} (16). Conversely, FluB PB2\textsuperscript{cap} engages GTP by inverting the guanine and ribose moieties 180° around the long axis of the base of the ribose, similar to the reported structure of FluB PB2\textsuperscript{cap}-GDP complex. Compensatory hydrogen bonding upon binding the guanine base provides an explanation for the equivalent binding affinities toward both cap analogs. Our structural analyses represent a first step in understanding the structural specificity of FluB PB2 proteins for different cap analogs and may provide insights into the rational drug design against PB2 with broader spectrum.

**Experimental Procedures**

**Cloning, Protein Expression, and Purification**—The *Escherichia coli* codon-optimized DNA sequence encoding residues 320–484 of influenza B/Lee/1940 PB2 was synthesized by Genewiz and cloned into pTrcHis2B vector, resulting in the protein with a C-terminal His\textsubscript{6} tag. The FluB PB2\textsuperscript{cap} protein was overexpressed in *E. coli* BL21 (DE3) strain. The protein was purified by a HisTrap column (5 ml, GE Healthcare), followed by a heparin column (HiTrap Heparin HP, 5 ml, GE Healthcare) and a size-exclusion column (HiLoad 16/60 S200, GE Healthcare) using an ÄKTA avant system (GE Healthcare). Fractions containing the desired protein were combined, concentrated, and stored at −80 °C in buffer containing 20 mM Tris, pH 7.5, 300 mM NaCl, 1 mM tris(2-carboxyethyl)phosphine (TCEP). The molecular mass of the protein was confirmed by LC/MS (calculated, 20,406.6 Da; observed, 20,407 Da). FluA PB2 (318–483) from influenza A/Udorn/1972 (H3N2) was cloned and purified as described previously (8).

**Crystalization and Data Collection**—All FluB PB2\textsuperscript{cap} crystals were grown by hanging drop vapor diffusion using a protein concentration of 10 mg/ml and a 1:1 protein-to-precipitant volume ratio. Crystals of the apo form were obtained from mother liquor containing 42% PEG 200, 0.1 M Hepes, pH 7.5, at 4 °C and directly flash-cooled in liquid nitrogen. B-PB2 complexes were prepared by mixing proteins with m\textsuperscript{7}GTP or GTP at 5 mM final concentration. m\textsuperscript{7}GTP and GTP co-crystals were grown in 0.2 M ammonium acetate, 20% PEG 3350, 0.1 M Bis-Tris, pH 6.0.

Before data collection, crystals were cryoprotected in reservoir solution containing 20% glycerol and flash-cooled in liquid nitrogen.

The PB2 apo crystal data set was collected using an ADSC QUANTUM 315 detector and synchrotron radiation at beam line 5.0.2 of the Advanced Light Source (Berkeley, CA). The data sets of PB2 complexes were collected using an in-house source to 1.9 Å resolution using a Rigaku FR-E Cu-Kα rotating anode generator and R-AXIS IV detector. All three B-PB2 unit cells are isomorphous and belong to space group P2\textsubscript{1}2\textsubscript{1}2\textsubscript{1}. This crystal form contains a single copy of the PB2 protein molecule in the asymmetric unit with a calculated solvent content of 56%. Data were processed using autoPROC (Global Phasing, Ltd.). Data collection statistics are shown in Table 1.

**Structure Determination and Refinement**—The structure of the Apo FluB PB2\textsuperscript{cap} was solved at 1.8 Å resolution using the program PHASER (17) and the protein coordinates from the crystal structure of FluA-PB2 bound to m\textsuperscript{7}GTP (Protein Data Bank (PDB) accession code 2VQZ). Non-conserved residues were manually rebuilt in COOT (18) using the simulated annealing composite omit map protocol implemented in Phenix (19). The initial apo FluB-PB2 model was in turn used to solve the m\textsuperscript{7}GTP and GTP co-structures.

Subsequent rounds of model building and refinement with the Phenix program suite were carried out until convergence. The quality of all models was assessed by MolProbity (20). A summary of the final refinement statistics is provided in Table 1.

**DSF**—Thermal shift assays were performed with 7 μM of purified A- or B-PB2 proteins in PBS buffer. Prior to the thermal cycle, 0.3–1 mM compound or DMSO as a control was added to the proteins with 5X SYPRO Orange (Molecular Probes). The temperature was ramped from 25 to 95 °C in a Viia7 real-time PCR machine (Applied Biosystems). The resulting fluorescence data were analyzed, and the protein melting temperature \( T_m \) was calculated using a curve fitting to a Boltzmann function using the Protein Thermal Shift software version 1.1 (Life Technologies). The \( \Delta T_m \) of the protein for a specific ligand was calculated as the difference between the \( T_m \) of the ligand-bound and ligand-free (DMSO control) protein. Error bars indicate the standard deviation of three replicate experiments.

**ITC**—ITC was used to measure the binding affinities of FluA- or FluB-PB2\textsuperscript{cap} for cap analogs m\textsuperscript{7}GTP or GTP using a GE
Healthcare autoITC200 at 10 °C. Proteins were first extensively dialyzed against PBS buffer and then filtered, degassed to avoid bubble formation, and equilibrated to the corresponding temperature before each experiment. Protein solutions in the calorimetric cell were titrated with the appropriate ligands dissolved in dialysis buffer over the course of 19 injections until the proteins were fully saturated. The resulting binding isotherms were analyzed by nonlinear least-squares fitting of the experimental data to models corresponding to a single binding site. Analysis of the data was performed using the MicroCal Origin 7.0 software.

Results

Isolated Flu B PB2cap Exhibits Elevated Structural Flexibility in Solution—We set out to elucidate the molecular basis for the recognition of mRNA cap analogs by the cap-binding domain of FluB PB2. To facilitate the structural and biophysical characterization, we overexpressed FluB PB2 in E. coli using a construct (containing residues 320–484 of influenza B/Lee/1940 PB2) analogous to the FluA PB2 construct (containing residues 318–483 of influenza A/Udorn/1972 (H3N2) PB2) previously used in the co-crystallization with m7GTP (8). However, N-terminal His-tagged FluB-PB2 when overexpressed resulted in inclusion bodies in E. coli. Attempts to refold this protein from inclusion bodies were not successful. The solubility of the protein remained limited even when fused to N-terminal solubilization tags such as GST or maltose-binding protein (data not shown). When the His tag was moved from the N terminus to the C terminus, the same construct became soluble and protein could be purified and concentrated under high salt formulation (300 mM NaCl), yielding up to 6 mg/liter of bacterial culture. Both FluA PB2cap and FluB PB2cap were purified to over 95% purity and were monomeric in solution (Fig. 1A).

The thermal stability of FluB PB2cap was tested in comparison with FluA H3N2 PB2cap by DSF using a hydrophobic fluorophore (SYPRO Orange), which preferentially binds to exposed hydrophobic surfaces once the protein unfolds by gradually increasing the temperature. Both FluA PB2cap and FluB PB2cap show a single apparent melting temperature (Tm) during the course of thermal denaturation, which is consistent with their single domain composition. Surprisingly, the Tm for FluB PB2cap is more than 11 °C lower than FluA PB2, although their solution behavior and the apparent hydrodynamic radius are similar, as reflected by the nearly identical retention time in size-exclusion chromatography (Fig. 1, A–C). This lower melting temperature suggests that FluB PB2cap has lower thermal stability and a shorter half-life in solution than FluA PB2cap.

Structures of the FluB PB2cap Alone and Bound to mRNA Cap Analogs m7GTP or GTP—Although FluB PB2cap is much less thermostable and has resisted crystallization with m7GTP in the past (16), we were able to grow crystals of wild-type FluB PB2cap in both unliganded and liganded forms by formulating the protein under a high salt condition (300 mM NaCl) during the course of purification and crystallization. The structure of FluB PB2cap in its unliganded form was determined to 1.7 Å by molecular replacement using FluA PB2cap as the search model (Table 1). The FluB PB2cap structure is similar to those of FluA PB2cap published to date (8), with an RMSD of ~1 Å when compared with the m7GTP-bound H3N2 PB2cap (PDB entry 365).
Recognition of mRNA Cap Analogos by FluB PB2\textsuperscript{cap}

**TABLE 1**

Data collection and refinement statistics for FluB PB2\textsuperscript{cap}

|                              | Apo           | m\textsuperscript{7}GTP complex | GTP complex |
|------------------------------|---------------|----------------------------------|-------------|
| Data collection              |               |                                  |             |
| Resolution (Å)               | 43.84-1.70 (1.76-1.70)	extsuperscript{a} | 36.66-1.90 (1.97-1.90)	extsuperscript{a} | 36.80-1.90 (1.97-1.90)	extsuperscript{a} |
| Space group                  | P2\textsubscript{1}, P2\textsubscript{1}, P2\textsubscript{1} | P2\textsubscript{1}, P2\textsubscript{1}, P2\textsubscript{1} | P2\textsubscript{1}, P2\textsubscript{1}, P2\textsubscript{1} |
| Cell dimension               | 40.86, 46.88, 87.68 | 40.34, 46.71, 87.79 | 40.52, 46.93, 87.95 |
| α, β, γ (°)                  | 90, 90, 90 | 90, 90, 90 | 90, 90, 90 |
| Total reflections            | 137,358 (13,628) | 88,224 (4994) | 63,886 (5502) |
| Unique reflections           | 19,171 (1884) | 13,396 (1108) | 13,743 (1328) |
| Multiplicity                 | 7.2 (7.2) | 6.6 (4.5) | 4.6 (4.1) |
| Completeness (%)             | 99.96 (99.89) | 98.12 (82.60) | 99.70 (99.70) |
| I/0                           | 12.32 (2.14) | 11.77 (1.69) | 10.99 (1.90) |
| R<sub>free</sub>             | 0.1598 (1.014) | 0.1249 (0.8274) | 0.1273 (0.7695) |
| CC1/2                        | 0.997 (0.674) | 0.996 (0.604) | 0.994 (0.661) |
| CC*                          | 0.999 (0.897) | 0.999 (0.868) | 0.998 (0.892) |
| Refinement                   |               |                                  |             |
| R<sub>work</sub>            | 0.1784 (0.2527) | 0.1760 (0.2771) | 0.1830 (0.2603) |
| R<sub>free</sub>            | 0.2258 (0.2943) | 0.2283 (0.3120) | 0.2266 (0.3498) |
| No. of non-H atoms           | 1533          | 1558                             | 1458        |
| Macromolecules               | 1378          | 1403                             | 1328        |
| Ligands                      | 155           | 155                              | 155         |
| Water                        | 155           | 122                              | 98          |
| Protein residues             | 168           | 171                              | 163         |
| RMSD (angles)                | 0.007         | 0.008                            | 0.008       |
| Ramachandran favored (%)     | 98            | 96                               | 98          |
| Ramachandran outliers (%)    | 0             | 0                                | 0           |
| Clashscore                   | 5.09          | 4.19                             | 5.54        |
| Average B-factor             | 25.20         | 28.90                            | 28.30       |
| Macromolecules               | 23.80         | 28.10                            | 27.40       |
| Ligands                      | 33.60         | 46.80                            | 46.80       |
| Solvent                      | 37.80         | 37.70                            | 34.50       |
| PDB ID                       | 5EF9          | 5EFA                             | 5EFC        |

\textsuperscript{a} Values in parentheses are for highest-resolution shell. A single crystal was used for this structure.

\textsuperscript{b} R<sub>work</sub> and R<sub>free</sub> were calculated from the working and test reflection sets, respectively. The test set contained 5% of the total reflections available.

2VQZ (8) structure (Fig. 2A). The FluB Lee PB2 adopts the conserved PB2 cap-binding domain fold: a partially open α/β barrel consisting of five anti-parallel β sheets and three helices, closed on one end by the longest helix α1, while positioning the active site on the opposite end. Two solvent-exposed, β-hairpin structures termed the 348-loop and the 424-loop are well defined in the apo FluB PB2\textsuperscript{cap} structure and assume a similar conformation to that previously observed in FluA PB2\textsuperscript{cap} (8). This apo structure shows that the cap-binding domain in FluB PB2\textsuperscript{cap} folds autonomously and that the cap-binding site is completely formed in the absence of any ligand.

To understand the structural basis for the ability of FluB PB2\textsuperscript{cap} to bind to both methylated and unmethylated mRNA caps, we solved the co-crystal structures of m7GTP and GTP in complex with the protein to 1.9 Å (Table 1, Fig. 2, E and F). The binding modes of the cap analogs are clearly defined in the electron density maps (Fig. 2, E and F). m7GTP adopts a similar U-shaped conformation as observed in the FluA H3N2 PB2\textsuperscript{cap} structure (Fig. 3, A and B) (8). Two aromatic side chains of Trp-359 and Phe-406 provide the canonical cation–π packing interactions in a “face-to-face” configuration to the methylated guanine, which is further stabilized by three key hydrogen bonds mediated by the conserved charged residues Glu-363 and Lys-378. The 2'-hydroxyl of the ribose and the phosphate extend toward the solvent and form direct hydrogen bonds with Gln-325 and Tyr-434, respectively. The terminal γ-phosphate pivots toward the base as it forms a salt bridge with Lys-341. In contrast, the FluB PB2\textsuperscript{cap}/GTP co-crystallization reveals a previously unseen ligand binding mode, which bears no similarities to the canonical m7GTP binding pattern (Fig. 3D). The GTP molecule is positioned 180° about the long axis, with the guanine base forming a single deck-stacking interaction with Trp-359. Despite prominent differences in the overall binding modes between GTP and m7GTP, the acidic residue Glu-363 still forms analogous hydrogen bonds to guanine by forming interactions with the nitrogen at the N\textsubscript{1} and N\textsubscript{2} positions. The O\textsubscript{6} of the GTP is recognized by the solvent-exposed basic residue Arg-334 in place of the deeply buried Lys-378 in the m7GTP complex. Because of the flipping of the guanine base, the ribose ring oxygen is placed within hydrogen bond distance to the indole nitrogen in Trp-359 and the hydroxyls are no longer facing the solvent. The triphosphate reaches toward the solvent region and binds directly to the side chain hydroxyl of Tyr-434. Collectively, these unique interactions are found to position GTP much more loosely bound to the active site of PB2 than m7GTP, as evidenced by multiple well ordered, tightly bound water molecules in the ligand pocket that bridge GTP to FluB PB2\textsuperscript{cap}.

**Functional Comparisons of the PB2cap in FluB and FluA by DSF and ITC**—To monitor mRNA cap binding to the PB2 proteins, we used a qualitative DSF assay to measure the T\textsubscript{m} in the presence of m7GTP, GTP, or a DMSO control (Fig. 1, B and C). The addition of GTP (0.3 or 1 mM) enhances the thermal stabilization of both FluA PB2\textsuperscript{cap} and FluB PB2\textsuperscript{cap} to similar degrees. When m7GTP (0.3 or 1 mM) was added, FluA PB2\textsuperscript{cap} showed no further T\textsubscript{m} shift was observed for FluB PB2\textsuperscript{cap} when compared with the unmethylated GTP. These results suggest that FluB PB2\textsuperscript{cap} binds to 7-methylated GTP, whereas FluB PB2\textsuperscript{cap} shows no preference in binding to m7GTP or GTP.
To further understand the substrate preferences between FluA and FluB PB2, we measured m7GTP or GTP binding to FluA-PB2cap and FluB-PB2cap, respectively, by ITC (Fig. 4, A and B), which determines binding parameters including affinity, thermodynamics, and stoichiometry in a single experiment. Not surprisingly, the protein binds to the ligand in a 1:1 ratio, in agreement with the structural results. FluA PB2cap binds more favorably to m7GTP ($K_d = 1.1 \mu M$) than to GTP ($K_d = 5.2 \mu M$). In contrast, FluB PB2cap recognizes m7GTP ($K_d = 14.7 \mu M$) and GTP ($K_d = 14.1 \mu M$) almost equally well, although the binding affinities are lower than that of FluA PB2cap. The binding of both GTP and m7GTP to FluA PB2cap is mediated by favorable free energy change in both enthalpy and entropy, almost certainly due to the formation of numerous interactions between the ligand and protein, as well as the displacement of both shell waters around the ligand and structural waters bound in the protein binding site. The contributions of the enthalpic interactions are approximately equivalent between the two complexes. The increase in $\Delta S$ is higher in the formation of the A-PB2$m^7$GTP complex, as the cage of ordered waters surrounding the exocyclic methyl group must be removed prior to binding. In contrast, the binding of GTP and m7GTP to B-PB2 shows a different pattern. Binding of GTP to B-PB2 is driven solely by the formation of numerous enthalpic interactions between ligand and protein; the enthalpic change upon the formation of the complex is negative. This observation is unexpected and is not easily explained, but could be due to rigidification of the more flexible and less stable B-PB2 structure. Conversely, the binding of m7GTP to B-PB2 is driven by a favorable formation of enthalpic interactions and a favorable
increase in ΔS. The enthalpic contribution is diminished in the case of m^7GTP binding as the interactions between protein and ligand are less ideal in regard to both distance and angles. The general trend of increased entropy of binding of m^7GTP versus GTP is again observed, likely due to the necessity of stripping the ordered waters from the exocyclic methyl.

### Discussion

FluA and FluB are the causative agents of pandemic and seasonal influenza. Although FluB is not as prevalent as FluA, it poses a serious health threat to children and the immunocompromised population. The polymerase PB2 subunit represents a clinically validated anti-influenza drug target (14), as exemplified by the azaindole-based inhibitors that demonstrate potent efficacy against FluA by targeting the cap-binding pocket. However, this class of compounds does not have any activity against FluB, suggesting distinct features in the binding pocket of FluB PB2. A wealth of structures of FluA PB2 in both ligand-free and ligand-bound forms have been published in recent years, whereas little was known about the atomic details of the FluB PB2, which represents a significant challenge in structure-based drug design for pan-PB2 inhibitors.

FluA and FluB PB2, which are related in sequence and overall fold, demonstrate significant differences in protein stability and substrate binding selectivity. Human FluA PB2 is a highly soluble and well behaved protein with multi-milligram yield isolated from E. coli. In contrast, FluB PB2 is less stable and tends to form inclusion bodies when an N-terminal tag is attached. The solubility issue can be solved with a C-terminal His tag, although the protein requires high salt to remain in solution and is more thermally sensitive when compared with FluA PB2 (Fig. 1, B and C). The apo-FluB PB2 structure shows a well organized cap-binding pocket in the absence of any bound ligand (Fig. 2, A and D). This is reminiscent of FluA PB2 and VP39 (21–23), which remain essentially unchanged whether the cap analog is bound or not. In comparison, CBP2 undergoes a significant rearrangement of its tertiary structure in response to ligand binding and the ligand-binding site in eIF4E is not fully formed in the absence of m^7GTP (24).

The binding mode of m^7GTP in the cap-binding site is similar in both the FluB PB2 and FluA PB2 complexes and the FluA PB2-m^7GTP complex. This is in stark contrast to an earlier publication that reported an inverted m^7GDP binding mode in FluB PB2 (Fig. 3, A–C) (16). In this study, a Q325F mutant was introduced based on the corresponding residue in FluA PB2 to facilitate the co-crystallization of FluB PB2 with m^7GTP. However, this mutation may not be relevant to the wild-type protein for the following two reasons. First, the Q325F mutation significantly modulates both ligand binding affinity and specificity. In the case of m^7GDP, its affinity toward FluB PB2 is increased by over 6-fold with respect to the wild-type FluB. Hence, the ligand binding mode based on this mutant may not be relevant to the wild-type FluB PB2. Second, the site of the point mutation Phe-325 is directly adjacent to the N7-methyl group, which artificially stabilizes the inverted conformation of the guanine and ribose moieties in the FluB PB2 Q325F mutant. In our experiments, when WT FluB-PB2 was co-crystallized with m^7GTP, the binding mode was equivalent to that observed in FluA PB2, suggesting a highly conserved m^7GTP recognition mechanism.

In other cap-binding proteins, such as eIF4E, CBC, and VP39, discrimination of non-methylated cap analogs is achieved by a combination of nearly optimal interactions of the guanine by
two stacked aromatic residue side chains and the delocalization of the positive charge arising from the N7-methylation of the guanine (25). FluB polymerase PB2\textsuperscript{cap-\textsuperscript{ap}} does not distinguish methylated or non-methylated cap structures in either our binding assays (Fig. 4, A and B) or the RNA cleavage reaction (15), which is different not only from the FluA polymerase but also from other well known cap-binding proteins. Previously, computer-generated docking models of FluB PB2 in complex with cap analogs positioned the GTP in the same orientation as m\textsuperscript{7}GTP (15). The lack of discrimination of methylation at the 7-position was attributed to the more favorable stacking interaction of Trp-359 with a guanine base and comparable hydrogen bonds from Gln-325 and Glu-363. However, crystal structures reported here and by Liu et al. (16) revealed a consistent GTP or GDP binding mode where the entire guanosine inverts around the long axis of the molecule when compared with m\textsuperscript{7}GTP conformation in wild-type FluB PB2\textsuperscript{cap-\textsuperscript{ap}} (Fig. 3, B, D, and E). Our studies indicate that FluB PB2\textsuperscript{cap-\textsuperscript{ap}} uses an unusual strategy for GTP recognition. Unlike the m\textsuperscript{7}GTP-FluB PB2\textsuperscript{cap-\textsuperscript{ap}} complex, we did not observe a traditional aromatic cap sandwich. Instead, the guanosine is stabilized by a “hydrophobic sandwich” between Trp-358 on one side and Phe-365 and Phe-406 on the other. This is reminiscent of the decapping scavenger enzyme DcpS, which also binds GTP (26). Arg-334 and Trp-359 are recruited by GTP and form additional H-bond contacts to compensate for the loss in binding energy in the absence of the conserved hinge interactions to Lys-378 and ribose contacts to Gln-325, respectively. In the phosphate-binding region, both Lys-341 and Tyr-434 form electrostatic interactions to the phosphates. Taken together, FluB PB2\textsuperscript{cap-\textsuperscript{ap}} provides a unique example of m\textsuperscript{7}GTP/GTP dual recognition by varying the combination of hydrogen bonding, electrostatic, and van der Waals interactions, thus accommodating various cap substrates.

Both cap analogs bind to FluB PB2\textsuperscript{cap-\textsuperscript{ap}} more weakly than to FluA PB2\textsuperscript{cap-\textsuperscript{ap}} (Fig. 4, A and B). Although FluA PB2\textsuperscript{cap-\textsuperscript{ap}} greatly prefers methylated caps, FluB PB2\textsuperscript{cap} demonstrates a relatively relaxed specificity for both the non-methylated and methylated versions, a phenomenon that has been previously reported in the in vitro capped RNA cleavage reaction (15). The processes of mRNA capping by m\textsuperscript{7}GTP involve two separate steps of guanosine cap addition and guanosine cap methylation (27). The first step is executed by a guanylyltransferase to produce the guanosine capped nascent RNA, after which m\textsuperscript{7}GTP methylation is catalyzed “co-transcriptionally” by RNA (guanine-7-) methyltransferase. These two enzymes are encoded by separate genes in higher organisms including mammals, which permits differential regulation of guanosine capping and cap methylation (27). Further work is clearly required to delineate whether FluB PB2 can “snatch” this pool of nascent mRNA, in addition to the fully processed m\textsuperscript{7}GTP capped mRNA, to initiate the viral RNA transcription.

In summary, we report how FluB PB2 differs from FluA both in their structures and in their ability to bind cap analogs. The FluB PB2\textsuperscript{cap-\textsuperscript{ap}}-m\textsuperscript{7}GTP complex revealed that the methylated cap analog adopts a similar binding mode as seen in FluA PB2\textsuperscript{cap-\textsuperscript{ap}}-m\textsuperscript{7}GTP complex. However, in the absence of the methyl group, the guanine and ribose moieties were inverted around the long axis of the base, suggesting that the FluB PB2\textsuperscript{cap} recognition site is flexible and could accommodate various cap structures. The results presented here enable a better understanding of the mechanisms of FluB PB2 recognition of cap analogs and could facilitate the development of new PB2 antagonists that are efficacious against both influenza strains.

Author Contributions—L. X., V. H. J. L., D. E. B., and X. M. designed the study and wrote the paper. L. X. and K. U. purified all PB2 proteins used in these studies. X. M. and S. S. crystallized PB2 proteins, determined their x-ray structures, and performed ITC experiments. C. W. and M. S. performed and analyzed the DSF experiments. All authors analyzed the results and approved the final version of the manuscript.

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