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Special Topic **Review**

Structure-function studies of the influenza virus RNA polymerase PA subunit

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The influenza virus RNA-dependent RNA polymerase is a heterotrimeric complex (PA, PB1 and PB2) with multiple enzymatic activities for catalyzing viral RNA transcription and replication. The roles of PB1 and PB2 have been clearly defined, but PA is less well understood. The critical role of the polymerase complex in the influenza virus life cycle and high sequence conservation suggest it should be a major target for therapeutic intervention. However, until very recently, functional studies and drug discovery targeting the influenza polymerase have been hampered by the lack of three-dimensional structural information. We will review the recent progress in the structure and function of the PA subunit of influenza polymerase, and discuss prospects for the development of anti-influenza therapeutics based on available structures.

influenza, polymerase, PA subunit, crystal structure, endonuclease, protein-protein interaction

1 Introduction

The recent emergence of highly pathogenic avian influenza H5N1 viruses poses a significant global threat to human health[1]. A total of 412 human cases have been reported worldwide since 2003, with 256 fatalities (WHO, March 2009). The currently approved influenza drugs may have limited effectiveness in the event of an influenza pandemic due to the emergence of resistant strains of H5N1 subtype influenza viruses. Elucidating the underlying mechanisms of the virus life cycle is therefore crucial for the development of new approaches and drugs for anti-viral therapy.

The influenza virus contains a segmented RNA genome with eight negative-sense segments encoding 11 proteins. The influenza polymerase is a heterotrimeric ~250 kD complex of three proteins: PA, PB1 and PB2. It plays central roles in the viral life cycle and is directly responsible for RNA synthesis for both viral replication and transcription. The influenza virus polymerase can *de novo* replicate its viral genome RNA through two steps. The replication step (vRNA→cRNA→vRNA) involves synthesis of complementary RNA (cRNA) from a viral RNA (vRNA) template, followed by synthesis of vRNA from cRNA to complete the genome RNA replication process. The transcription step (vRNA→mRNA) involves viral mRNA transcription from vRNA by snatching capped primers from nascent host cell mRNA. The question of how these different RNA synthesis functions are regulated within the same large complex remains unclear. Some reports have suggested the existence of a switch in synthesis of these different RNA species, in which the polymerase may switch its conformation from a replicase state to a transcriptase. Other reports, however, found that vRNPs isolated from virus were sufficient for synthesis of both types of RNA *in vitro*. Thus it has been proposed that the polymerase complex can simultaneously synthesize both viral mRNA and cRNA.

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2 Structure and function of influenza polymerase and PA subunit

2.1 Overall structure and organization of the influenza polymerase

At the time of writing, no detailed structural model of the heterotrimeric polymerase complex has been reported, although low-resolution electron microscopy models exist for the polymerase as part of the vRNP complex with RNA and the nucleoprotein (NP), and for the isolated non-nucleocapsid polymerase (Figure 1). The polymerase heterotrimer is tightly packed with a cage-like hollow core and no obvious boundaries between domains and subunits. The models of the vRNP-associated polymerase and isolated polymerase heterotrimer have distinct conformations and the vRNP associated polymerase appears more compact than the isolated polymerase, suggesting the complex may undergo conformational changes upon activation.

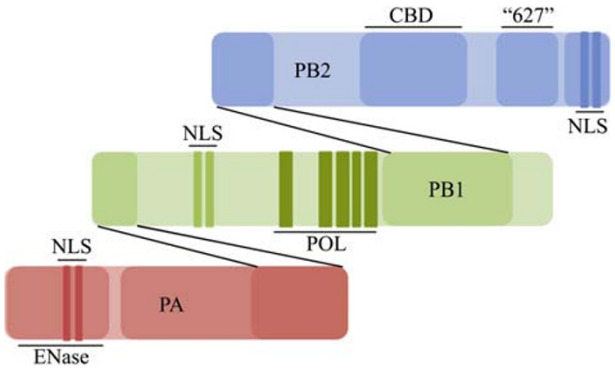

Figure 1 Organization of the influenza polymerase heterotrimer. The PA, PB1 and PB2 subunits are shown and putative functional or interaction domains are indicated. NLS, nuclear localization signal; POL, polymerase motifs; ENase, endonuclease; CBD, cap-binding domain; "627", domain containing residue 627. NLS are shown by two bands to represent bipartite signals.

PB1 is understood to be the central component of the polymerase complex and interacts directly with both PA and PB2. No direct interaction had previously been observed between PA and PB2[2], although a recent report indicates that they do interact in cells, albeit with three-fold lower affinity than for PA-PB1 or PB1-PB2. The functions of the various subunits remain controversial, but those of PB1 and PB2 have been more clearly defined[3]. PB1 is the central protein subunit for the RNA polymerase activity; it binds to the viral promoter and is responsible for viral RNA elongation and cap RNA cleavage activities[4]. The PB2 subunit is required for transcription of vRNA[3] and can bind to the methylated cap-1 structure of host RNAs for cleavage by the PB1 subunit[5]. A crystal structure was recently reported showing the basis for cap binding by PB2[6]. The role of PA in the polymerase heterotrimer, however, has only been partly outlined. It has been reported to be required for replication and for transcription of vRNA as well as endonuclease cleavage of the cap RNA primer[7–11]. It reportedly induces proteolysis of viral and host proteins[12–14] and may also be involved in virus assembly[15].

The architecture of the polymerase complex has been intensively studied with several systems in order to under stand the minimum components required for viral transcription or replication. Nakagawa and colleagues[16,17] reported that PB2 is not required for replication and PB1 alone can synthesize RNA. A PB1-PA dimeric protein complex was shown to synthesize a 53 nt long transcript directed by a vRNA promoter *de novo*, but could not efficiently synthesize RNA under a cRNA promoter[18]. Deng and colleagues[19], however, showed using a recombinant protein complex purified from 293T cells that only the heterotrimer could synthesize pppApG dinucleotides, not the PB1-PB2 dimer or the PB1-PA dimeric RNA polymerase, which is consistent with other previous reports[20,21] and also with a previous study in which PB2 was also found to be required for replication[22]. A point mutation at position 510 in PA resulting in impaired viral mRNA synthesis indicates that PA is also required for transcription[11]. It would thus appear that all three subunits are required by the influenza virus for effective transcription and replication activity.

2.2 Structure and function of the PA subunit

As described above, PA is a key protein in the influenza virus polymerase complex, although its role was only partly outlined until recently. PA can be cleaved by limited tryptic digestion into two domains: a smaller N-terminal domain of ~25 kD and a larger C-terminal of ~55 kD[23,24]. Structures for both of these domains have been reported and will be described in greater detail below, but they indicate that the two domains are separated by a long linker peptide. Guu and colleagues highlighted the importance of this linker in the PA-PB1 interface, reporting that neither the N-terminal nor C-terminal domains of PA could ensure a stable interaction with PB1 without the presence of the linker[24]. Furthermore, treatment of the PA-PB1 complex with protease showed

that its PA protein is markedly more stable than free PA, implying that the linker is protected from digestion by PB1 and forms an essential part of the subunit interface. This interface spans much of the PA sequence, with the exception of the N-terminal 154 amino acids, which is consistent with previous reports mapping the interaction domain of PA and PB1 to the C-terminal two thirds of PA[2,12]. The presence of the linker connecting the N- and C-terminal domains should afford PA with a degree of conformational flexibility, and may provide an advantage for roles in regulating polymerase functions through conformational changes in the polymerase complex[9].

Among its various putative functions, PA was reported to harbour proteolytic activity that can induce generalized proteolysis of both viral and host proteins[2,13,14,25−27]. PA can lead to degradation of the large subunit of RNA polymerase II complex in host cells[14], and has also been implicated as a novel serine protease with Ser624 at the active site[13]. This proteolytic activity has been suggested to be linked with replication activity[10,25]. Perales et al.[10,25] reported that the T157A mutation in PA results in a complete loss proteolytic activity and diminishes the cRNA synthesis ability of the polymerase, but does not influence transcription. This would suggest that protease activity in PA may be important for the polymerase to replicate. The significance of this activity remains unclear, and other studies have cast doubt on the relevance between proteolysis activity and replication[28].

The PA, PB1 and PB2 subunits of the polymerase complex have been implicated in highly pathogenic viruses, particularly K627 of the PB2 protein in mice[29] and the amino acids T515 of PA and Y436 of PB2 in mallard ducks[30]. While a PA T515A mutant gene abolished pathogenicity of the virus in wild fowl, other properties including replication, transmission and lethality via inoculation remained unaffected, suggesting the mutation may have affected an early step of the infection process. The single T515A mutation in PA does not affect pathogenicity of the influenza virus in ferrets or mice, however. RNA synthesis by the influenza virus has also been postulated to involve host factors that differ between species, with accumulating evidence to support the participation of host factors in influenza virus RNA synthesis.

2.3 The N-terminal domain of PA is a cap-dependent endonuclease

The crystal structure of the N-terminal domain of PA, which has been implicated in a diverse range of functions[7−14], such as endonuclease and protease activities, was reported in early 2009 by two groups using different constructs and virus strains. Yuan and colleagues used residues 1-256 cloned from an avian type A virus isolate (A/goose/Guangdong/1/96 (H5N1))[31], while Dias and colleagues used residues 1-209 from a human type A isolate (A/Victoria/3/1975 (H3N2))[32]. Both proteolytically stable structures were determined to 2.2 and 2.0 Å respectively. The structure from Yuan et al. was visible from residues 1−197, while the structure from Dias et al. was visible from residues 1-196; no residues were visible beyond position 197.

The PA_N structure has an α/β architecture with five mixed β-strands (β1-5) forming a twisted plane surrounded by seven α-helices (α1-7). A strongly negatively charged cavity, formed by a concentration of acidic residues, is surrounded by helices α2−α5 and strand β3 and houses a metal binding site. Structural comparison of PA_N revealed a close match with other endonucleases, pointing towards PA_N as a new member of the (P)DX_N(D/E)XK endonuclease superfamily (Figure 2). A number of catalytically important residues were identified, including P107, D108 and E119 in the (P)DX_N(D/E)XK motif. From structural analysis, the putative lysine is K134 on the basis of its close proximity to the metal binding site.

While both PA_N structures share a similar overall structure, one key difference is the identification of the metal ion(s) in the binding site: the structure by Yuan et al. included one magnesium (Mg^{2+}) ion, whereas the structure by Dias et al. featured two manganese (Mn^{2+}) ions. In each case, the metal ions are coordinated by the same residues, including H41, D108, E119 and K134. In the Mg^{2+} bound structure determined by Yuan and colleagues[31], the metal is directly coordinated by five ligands: the acidic residues E80 and D108, and three water molecules stabilized by residue H41, E119 and the carbonyl oxygens of L106 and P107. All six amino acids involved in coordinating Mg^{2+} are conserved among influenza A, B, and C viruses, with the exception of P107 that is replaced with alanine and cysteine in influenza B and C viruses, respectively. With two bound Mn^{2+} ions, as in the structure by Dias and colleagues[32],

the D108 residue serves as a bridge between the two metals. The loop from 50−74, which is absent from the structure by Yuan and colleagues, is visible in the structure by Dias and colleagues. Residue E59 from a neighbouring monomer is also involved in coordination of the second Mn^{2+} ion, occupying an equivalent position to the water molecule coordinated by H41 in the Yuan structure, although this is likely to be a crystallographic artefact (Figure 3).

Doan and colleagues previously reported that cleavage of RNA by the endonuclease is activated by the binding of divalent cations and should involve a two-metal dependent mechanism in common with other nucleases. Optimal endonuclease activity was observed for Mn^{2+} and was reduced to around 50% activity for Mg^{2+}[33]. However, given that the concentration of Mg^{2+} ions is higher than Mn^{2+} ions under physiological conditions, it may be that Mg^{2+} is the more likely cofactor in the cell. Further work is therefore needed to elucidate the mechanism of endonuclease cleavage. The influenza virus endonuclease has also been shown to cleave single-stranded DNA (ssDNA) with slightly reduced activity than for RNA, which is consistent with the observation that capped ssDNA endonuclease products can function as primers for transcription initiation by the influenza virus polymerase[34]. The endonuclease activity of the influenza virus polymerase is critical for snatching capped primers from host mRNA to initiate mRNA transcription. PA was previously reported to be involved in endonuclease activity, either via its N- or C-terminal domain[7,23], and a number of mutations in the N-terminal of PA were shown to inhibit this activity[23]. Alternative reports suggested the endonuclease activity of the influenza polymerase was located in PB1,

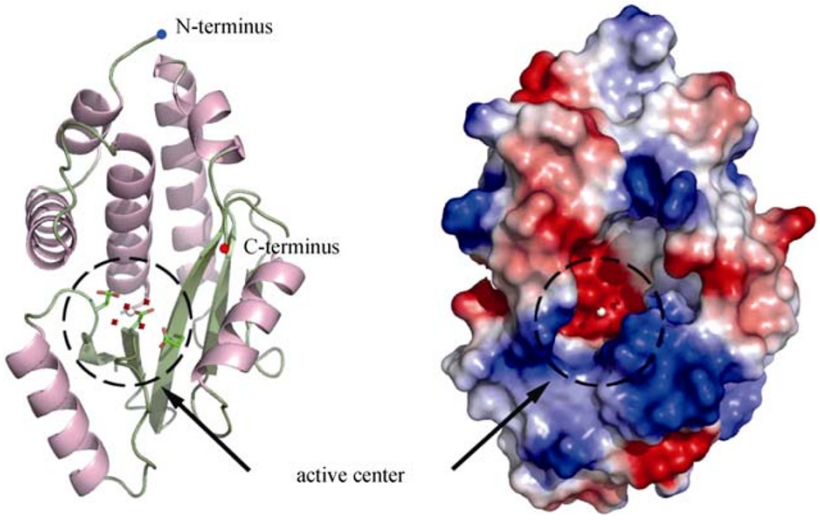

Figure 2 Structure of the PA N-terminal domain, the cap-dependent or "cap-snatching" endonuclease. Left half, crystal structure of PA_N was shown in ribbon representation and coloured by secondary structure. Right half, the potential surface of PA_N. The active center was highlighted, the residues in active center were shown as green sticks, and the metal ion and bound water molecules were shown as white and red spheres.

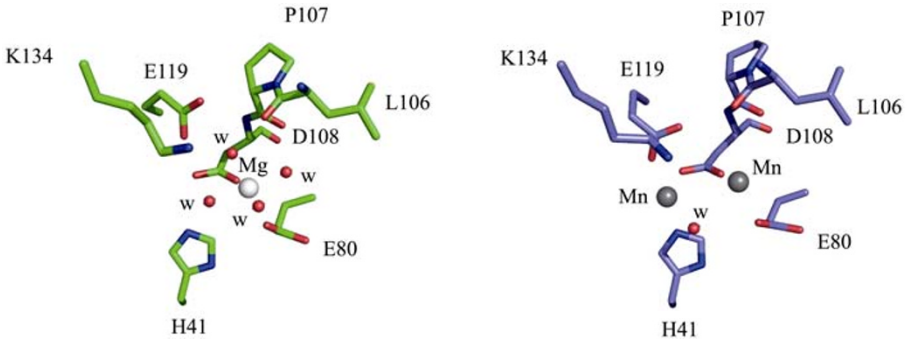

Figure 3 Comparison of the Mg^{2+} and Mn^{2+} bound endonuclease structures, respectively. In the left half, residues coordinating the metal are shown as green sticks. In the right panel, residues coordinating the metal are shown as blue sticks. Metal ions are shown as grey spheres; bound waters are shown as red spheres.

and the acidic residues E508, E519 and D522 are essential for the activity[3], while earlier studies suggested that the PB2 subunit might contain the endonuclease active site[18,35,36]. The availability of PA_N structures, therefore, clearly identifies the endonuclease active site of the influenza polymerase and help to resolve this particular controversy. Furthermore, the identification of an enzymatic active site in the polymerase complex provides another target for the design of anti-viral inhibitors. A number of compounds have been reported to inhibit the endonuclease activity of the influenza virus polymerase[37−40], but their development and optimization was presumably limited by the lack of an available structural target.

2.4 An essential subunit interaction between PA and PB1

As described above, PA and PB1 directly interact to form a stable complex which binds to the vRNA promoter. This interaction has been extensively characterized and is known to occur between the C-terminal region of PA and the N-terminal region of PB1. More specifically, the N-terminal 25-residues of PB1 bind to the C-terminal region of PA. This PB1 N-terminal peptide ($PB1_N$ hereafter) is also known to act as an inhibitor and abolishes viral replication by blocking the polymerase heterotrimer.

Using this information as a basis, two groups determined crystal structures showing the essential subunit interaction between PA and PB1. In the first study, He and colleagues used an avian type A influenza virus subtype (A/goose/Guangdong/1/96 (H5N1)) to clone PA from residues 257−716 and PB1 from residues 2−25[41]. In the second study, Obayashi and colleagues used a human avian type A influenza virus subtype (A/Puerto Rico/8/1934 (H1N1)) to clone PA from residues 239−716 and PB1 from 1−81[42]. The structures were determined to 2.9 Å (PDB code: 3CM8) and 3.2 Å (PDB code: 2ZNL) resolution, respectively.

PA_C consists of 13 α-helices, one short 3_{10} helix, nine β-strands and several loops/turns. The overall structure of PA_C resembles the head of a dragon and can be subdivided into two parts: domain I, the "brain", and domain II, the "mouth". The seven β-strands of the "brain" form a twisted plane surrounded by five α-helices and a short 3_{10} helix. The "mouth" consists of strands two β-strands and eight α-helices. A search for structural similarity indicated that PA_C bears no similarity to other structures in the Protein Data Bank (PDB) and thus has a new fold (Figure 4).

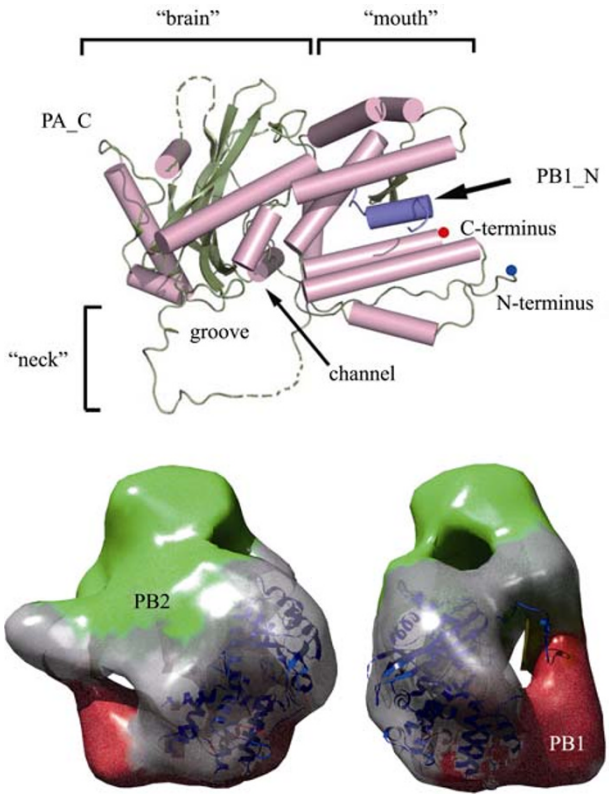

Figure 4 Structure of the PB1 interaction domain of PA. Top half, the C-terminal PB1 interaction domain of PA. The PA structure is shown in ribbon representation and coloured by secondary structure. The N-terminal PB1 peptide is coloured blue. Bottom half, front and side views of the PA_C domain docked into the electron microscopy model of the isolated polymerase heterotrimer. The PA_C structure is shown in blue ribbon representation; the PB1N peptide is shown in red ribbon representation; approximate locations of the PB1 and PB2 subunits are shown in red and green, respectively.

While He et al. and Obayashi et al. used different PB1 constructs, both PA-PB1 complex structures show the N-terminal PB1 peptide, $PB1_N$, from residues 1−15. Furthermore, both structures of the complex exhibit a similar mode of interaction. $PB1_N$ binds obliquely between the jaws of PA_C with its N-terminus pointing towards the back of the "mouth" and its C-terminus extending outwards. Residues 5 through 11 of $PB1_N$ fold into a short ordered helix. The interaction with $PB1_N$ is mediated by four helices in the "mouth" of PA_C, which form a hydrophobic core at the tip of the "mouth" and tightly interact with the peptide via hydrophobic interactions, hydrogen bonds and van der Waals forces. A short-LLFL-motif from residues 7−10 of $PB1_N$, known to be important for the interaction with PA, interacts

with the PA_C hydrophobic core formed by F411, M595, L666, W706, F710, V636 and L640. W706 also interacts with residues V3 and N4; Q408 and N412 interact with V3 and D2; and Q670 interacts with $PB1_N$ residues F9, V12, P13 and A14. Residues 620 and 621 on β8 are also located in the $PB1_N$ interaction surface. The W706A/Q670A double mutation disrupts the binding of $PB1_N$ to PA_C, as do the L666G/F710E, L666G/F710G and W706A/F710Q double mutations.

$PB1_N$ inhibits influenza A viral replication by interfering with polymerase activity[43], most likely by blocking assembly of the polymerase heterotrimer. The available crystal structures of PA_C identify a critical PB1 binding region of PA, and can thus be used as a basis to design novel anti-influenza compounds that inhibit polymerase assembly and function. Protein-protein interfaces often involve a large surface area, which can pose problems for drug discovery. In this case, however, relatively few residues drive the binding of $PB1_N$ to PA_C, implying that designing small molecule inhibitors of this interaction is feasible. Mutation of specific residues in $PB1_N$, including V3, N4, P5, L7, L8, F9 and L10, has been shown to result in loss of more than two-thirds of the binding affinity, with significant reductions in polymerase activity and virus production. D2V and A14D mutations in $PB1_N$ do not significantly influence the binding affinity to PA, but they do abolish polymerase activity and virus production, suggesting that D2 and A14 have other crucial functions. An L13P mutation in PB1 from a H7N7 virus has been associated with increased virulence[44]. In both crystal structures, a L13P substitution in $PB1_N$ would break the ordered helix and the associated structural changes may result in increased RNA synthesis by PB1. $PB1_N$ residues interacting with PA are conserved across type A, B and C influenza viruses, and PA residues shown to interact with $PB1_N$ are similarly conserved. It is expected that inhibitory peptides or compounds designed on the basis of $PB1_N$, therefore, would be effective against the majority of influenza A strains. The high conservation of the $PB1_N$ binding site on PA suggests that anti-virals targeting this site may be less susceptible to problems of resistance associated with drugs targeting the neuraminidase. Synthesized peptides designed based on the N-terminal 25 residues of PB1 show high binding affinity with PA_C *in vitro* and thus could block PA and PB1 from forming a functional complex (data not shown). This work provides a new avenue for drug discovery aimed at the influenza polymerase (Figure 5).

In addition to housing a key interaction site for PB1, the C-terminal domain of PA has been associated with other functions, including polymerase activity, viral pathogenicity and virus assembly. It reportedly interacts with the host protein hCLE via two regions, residues 493−512 and 557−574[45], and with the microchromosomal maintenance complex (MCM)[46]. The precise role of hCLE in viral replication remains unclear but, given its homology to a family of transcriptional activators, it has been shown to function as a modulator of mRNA transcription. The MCM complex, thought to be a DNA replicative helicase, is another of the host factors believed to be hijacked by the influenza virus for genome replication and may serve as a scaffold between the polymerase and nascent RNA chains. A H510A mutation in PA_C impairs nuclease activity[7] and was initially thought to be a possible location for the endonuclease active site. However, given the identification of the endonuclease site in PA_N, its role may be linked to a putative nucleotide binding motif from residues 501−509[47]. In the same region, positions 507 and 508 are associated with virus assembly defects[15] and position 515 is linked with highly pathogenic H5N1 viruses[30].

3 Prospects for therapeutic intervention

The majority of approved drugs for influenza work by targeting the neuraminidase (NA), although other targets such as the M2 proton channel also exist in the influenza virus[48]. However, there are a number of problems associated with their use as drug targets. The NA is a highly variable region of the influenza virus, and the currently licensed influenza drugs oseltamivir (Tamiflu™) and zanamivir (Relenza™) are beset by problems of resistance due to high mutation rates or selection pressures on the influenza virus, limiting their clinical effectiveness. Their efficacy also becomes significantly diminished if they are not taken within 48 hours of the onset of symptoms. Furthermore, the currently approved drugs have limited effectiveness against the H5N1 influenza virus subtypes.

From structural and functional analysis, PA has identified two significant new targets for development of inhibitors that block polymerase activity. The N-terminal domain of PA is shown to be a cap-dependent or "cap-snatching" endonuclease with a highly conserved active site[31,32]. Specific inhibitors of the polymerase cap-dependent endonuclease activity have been described

previously and can be divided into several classes of compounds: 4-substituted 2,4-dioxobutanoic acid derivatives[38]; *N*-hydroxamic acid and *N*-hydroxy- imide derivatives[37]; and a 2,6-diketopiperazine natural product of the fungus *Delitschia confertaspora* called flutimide[39]. Flutimide in particular has also been shown to inhibit the replication of influenza A virus in cell culture. One 2,4-diketobutanoic acid analog targeting PA has shown potent polymerase inhibition without any cytotoxicity[40]. The availability of high-resolution three-dimensional structures for the cap-dependent endonuclease in PA should accelerate development of anti-influenza compounds targeting this region of the polymerase. The C-terminal domain of PA mediates the interaction with the PB1 subunit, and two crystal structures have revealed the molecular basis for the interaction of PA with the N-terminal peptide of PB1 via a highly conserved region. A 15-amino acid peptide derived from the N-terminal of PB1 has been shown to bind to PA and inhibit the polymerase function, which should be achieved by blocking assembly of the polymerase complex.

It may also be possible to exploit interactions between the polymerase complex and other viral proteins or host factors in the infected cell, which are hijacked for viral replication and transcription. As described above, PA has been reported to interact with several host factors, including the microchromosomal maintenance complex (MCM)[46] and hCLE[45,49], although the precise nature and role of these interactions remains unclear and fur-

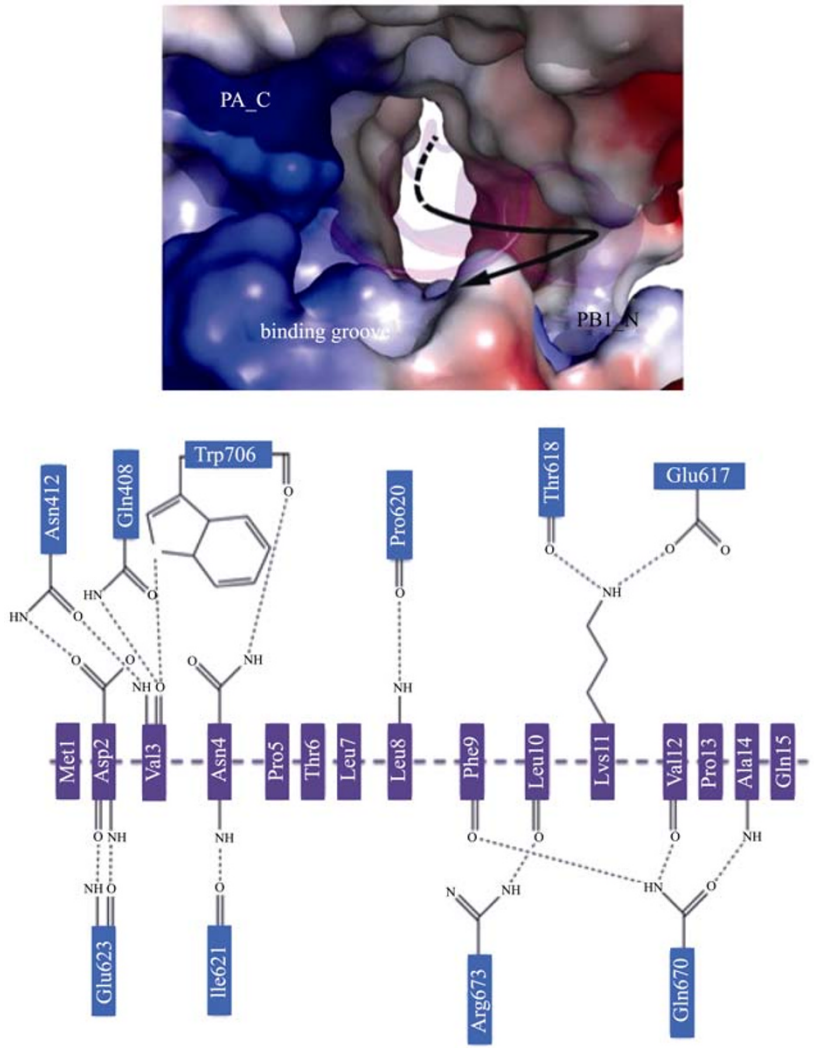

Figure 5 The $PB1_N$ binding groove in PA_C. Left half, potential surface of $PB1_N$ binding groove. The bound $PB1_N$ was shown as transparent magenta ribbon and the direction was highlighted by arrow. Right half, the schematic showing the interaction between PA_C and the $PB1_N$ peptide. Residues in the $PB1_N$ peptide are shown in magenta; residues in PA_C are shown in blue.

ther work is needed to characterize them. More recently, the influenza polymerase was reported to form part of a complex between the influenza NS1A protein and the cellular CPSF30 protein. The binding of NS1A to CPSF30 is an important mechanism employed by the virus in suppression of the host anti-viral response, in this case the production of beta-interferon (IFN-β) mRNA. A recent crystal structure of the NS1A-CPSF30 complex indicates a common binding site that may be employed by all influenza A viruses to inhibit production of anti-viral mRNA[50]. Kuo and Krug showed that the polymerase subunits and the nucleoprotein (NP) are an integral part of the complex in infected cells and help to stabilize the NS1A-CPSF30 complex[51], and furthermore showed that NS1A interacts primarily with the PA subunit and the NP.

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