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Evidence that the C-terminal PB2-binding region of the influenza A virus PB1 protein is a discrete α-helical domain

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Abstract The influenza A virus RNA-dependent RNA polymerase is a heterotrimer composed of PB1, PB2 and PA subunits and essential for viral replication. However, little detailed structural information is available for this important enzyme. We show by circular dichroism spectroscopy that polypeptides from the C-terminus of PB1 that are capable of binding efficiently to PB2 fold into stable α-helical structures. Structure prediction analysis of this region of PB1 indicates that it likely consists of a three-helical bundle. Deletion of any of the helices abrogated transcriptional function. Thus, PB1 contains a C-terminal α-helical PB2-binding domain that is essential for nucleotide polymerization activity.

Keywords: Orthomyxovirus; Protein–protein; Drug design; Polymerase

1. Introduction

Influenza A virus is a formidable pathogen that retains the potential to cause global pandemics with mortality figures in millions, despite a vaccination programme and the availability of antiviral drugs [1]. Its genome consists of eight segments of negative sense RNA separately encapsidated into ribonucleoprotein (RNP) complexes with one copy of the viral RNA-dependent RNA polymerase and stoichiometric amounts of an RNA binding nucleoprotein, NP [2]. The RNA polymerase is a heterotrimer of the PB1, PB2 and PA subunits that both transcribes and replicates the viral genome. PB1 forms the backbone of the polymerase complex and possesses nucleotide polymerization activity [3,4]. PB2 primarily plays a role in mRNA transcription initiation [3] whilst the precise function of PA is uncertain (reviewed in [5]).

The polymerase complex is formed by a web of non-covalent protein–protein interactions which have been examined in some detail. Mapping experiments suggest a seemingly linear arrangement of the polypeptide chains in which the C-terminus of PA interacts with the N-terminus of PB1 while the C-terminus of PB1 in turn binds to the N-terminus of PB2 [4,6–9]. However, electron microscope (EM) imaging of the polymerase shows a compact, roughly globular structure [10–12] with other regions of the polymerase subunits than those listed above also contributing to trimer formation [13,14]. Mutational analysis and *trans*-dominant inhibition experiments indicate that the intermolecular contact regions of the polymerase are essential for enzymatic activity and are potentially fruitful functions to target for the design of antiviral agents [6,14–16]. Such experiments are worthwhile as existing influenza antiviral drugs suffer from problems of resistance and low efficacy and although viral polymerases often make good antiviral targets, that of influenza virus remains underexploited [17]. Rational design of influenza polymerase inhibitors is hindered by a lack of structural information on the polypeptide complex. So far, there are low resolution EM views of the polymerase trimer, in RNP form and as a free complex [10–12], a high resolution structure of a domain comprised of the C-terminal 80 amino-acids from PB2 [18] and partial proteolysis data suggesting that the N-terminal 212 amino-acids of PA represent a discrete domain [19]. Here, we extend these data by showing that the C-terminal 75 amino-acids of PB1 form an α-helical domain necessary for binding PB2 and polymerase function.

2. Materials and methods

2.1. Plasmids and antisera

Plasmids encoding glutathione transferase (GST)-PB1 fusion proteins pGEX1N683 and pGEX1N712, as well as pGEX16E6 are described elsewhere [14]. Constructional details for other plasmids used in this study are given in the supplementary information. Antisera directed against PB1, PB2 and PA have been described previously [4,20]. All materials were derived from influenza virus A/PR/8/34.

2.2. Protein expression and binding assays

GST fusion proteins were expressed and purified by affinity chromatography on glutathione sepharose (GE Healthcare) as previously described [14]. To obtain non-fused PB1 polypeptides, fractions eluted from gluthathione sepharose columns were adjusted to contain 150 mM NaCl and 2.5 mM $CaCl_2$, and incubated overnight with 10 U/mg of thrombin protease (Sigma) at room temperature. The digested samples were fractionated by gel filtration on Sephacryl S-200 (Pharmacia) equilibrated in 5 mM Tris–Cl, pH 7.6, 50 mM KCl. Frac-

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Abbreviations: RNP, ribonucleoprotein; EM, electron microscope; GST, glutathione-*S*-transferase; CAT, chloramphenicol acetyl transferase; WT, wild-type

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tions containing PB1 derived polypeptides were then passed over a glutathione-sepharose column equilibrated in the same buffer to remove residual GST protein before storage at 4 °C. Protein concentrations were determined by the Bradford method [21] or by quantitative amino-acid analysis.

Radiolabelled polymerase proteins were expressed in *Xenopus* oocytes by microinjection of the appropriate in vitro transcribed mRNA as previously described [4,14,20]. In vitro translation reactions in rabbit reticulocyte lysate were carried out as described [14]. 'Pull down' and co-immunoprecipitation binding assays were performed as previously described [4,14].

2.3. Circular dichroism spectroscopy

Measurements were recorded on an ISA Jobin Yvon CD6 instrument fitted with a thermostatically controlled cuvette holder and a 1 mm path length cuvette. Protein samples were in 5 mM Tris–Cl, pH 7.6, 50 mM KCl. Wavelength scans were recorded from 195 to 250 nm in 0.5 nm increments, with a 1 s integration time, and were averaged over five repetitions.

2.4. Virus infections and mammalian cell-based assays

293 T and CV1 cells were maintained as previously described [14,22]. Influenza virus RNPs were reconstituted by transfection of plasmids expressing the three polymerase subunits, NP and a model virus genome segment encoding chloramphenicol acetyl transferase (CAT) as previously described [22] or by infection of cells with recombinant vaccinia viruses expressing influenza virus RNP proteins and transfection of in vitro transcribed CAT minigenome RNA [14]. RNA synthesized by these RNPs was analysed by reverse-transcriptase primer extension [22] and CAT polypeptide accumulation measured by ELISA [14].

3. Results

3.1. Delineation of C-terminal PB1 residues involved in PB2-binding

Previous studies have shown that the C-terminus of PB1 contains a PB2 binding site but the precise location of the functionally important sequences remains unclear. Initial studies showed binding by relatively large fragments of the protein containing >150 amino-acids and in one study, the ability of PB1 fragments to act as *trans*-dominant inhibitors of viral gene expression was further used to infer an interaction site lying between amino acids 506 and 659 [6,8]. Contradicting this, a subsequent study showed direct binding by the C-terminal 57 amino-acids (residues 700–757; [23]) and in agreement with this we found that a GST fusion protein bearing the last 75 amino-acids of PB1 (G683) bound PB2 as efficiently as cognate polyclonal antiserum [14]. Based on the behaviour of overlapping C-terminal deletions, Ohtsu and colleagues suggested that

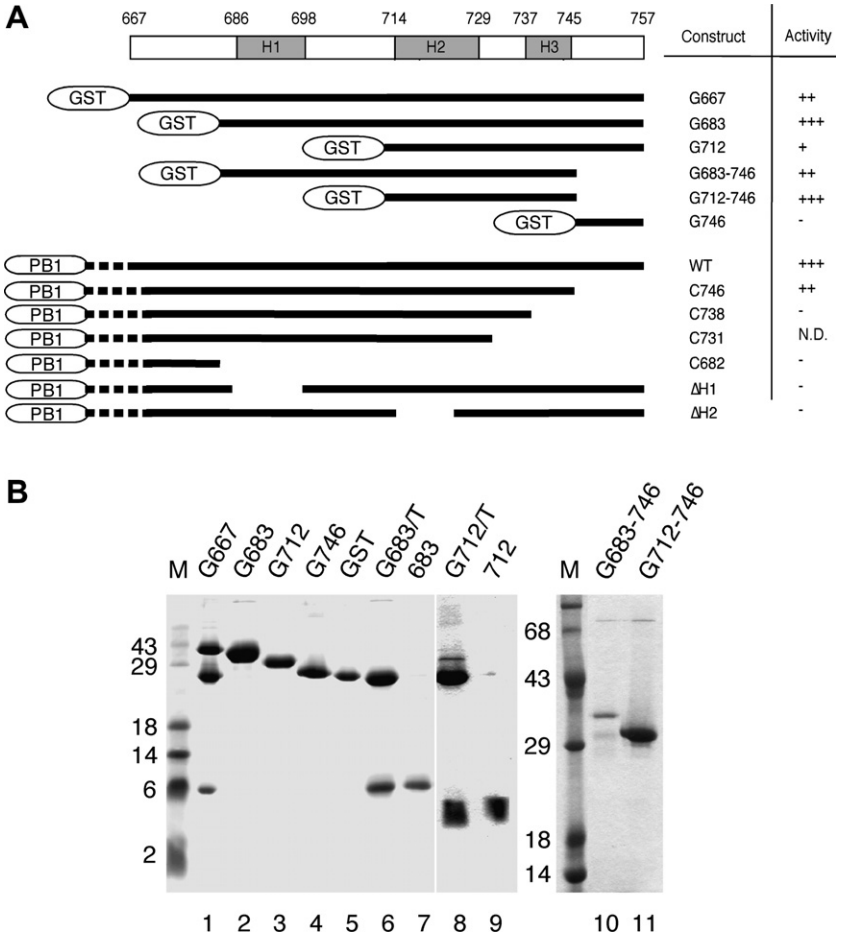

Fig. 1. PB1 C-terminal polypeptides. (A) Summary of constructs. PB1 C-terminal sequences are represented by black lines linked to GST or the rest of the polypeptide as indicated. Predicted structural elements and their amino-acids coordinates are indicated by shaded (helices) or open (loops) rectangles at the top. Qualitative summaries of the PB2-binding activity of the GST fusion proteins and the ability to support viral gene expression of the non-fused PB1 polypeptides are given on the right. N.D.: not determined. (B) SDS–PAGE and Coomassie blue staining of the indicated GST polypeptides. Thrombin digested polypeptides are denoted by/T, molecular mass standards (*M*, in kDa) are indicated on the left.