LETTERS

The cap-snatching endonuclease of influenza virus polymerase resides in the PA subunit

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The influenza virus polymerase, a heterotrimer composed of three subunits, PA, PB1 and PB2, is responsible for replication and transcription of the eight separate segments of the viral RNA genome in the nuclei of infected cells. The polymerase synthesizes viral messenger RNAs using short capped primers derived from cellular transcripts by a unique 'cap-snatching' mechanism[1]. The PB2 subunit binds the 5′ cap of host pre-mRNAs[2–4], which are subsequently cleaved after 10–13 nucleotides by the viral endonuclease, hitherto thought to reside in the PB2 (ref. 5) or PB1 (ref. 2) subunits. Here we describe biochemical and structural studies showing that the amino-terminal 209 residues of the PA subunit contain the endonuclease active site. We show that this domain has intrinsic RNA and DNA endonuclease activity that is strongly activated by manganese ions, matching observations reported for the endonuclease activity of the intact trimeric polymerase[6,7]. Furthermore, this activity is inhibited by 2,4-dioxo-4-phenylbutanoic acid, a known inhibitor of the influenza endonuclease[8]. The crystal structure of the domain reveals a structural core closely resembling resolvases and type II restriction endonucleases. The active site comprises a histidine and a cluster of three acidic residues, conserved in all influenza viruses, which bind two manganese ions in a configuration similar to other two-metal-dependent endonucleases. Two active site residues have previously been shown to specifically eliminate the polymerase endonuclease activity when mutated[9]. These results will facilitate the optimisation of endonuclease inhibitors[10–12] as potential new anti-influenza drugs.

The exact role of the influenza polymerase PA subunit remains unclear, although it has been implicated in cap-binding, endonuclease activity, viral RNA binding and replication[9,13–15]. PA is separable by trypsinization[9] into a large carboxy-terminal domain—the crystal structure of which has recently been reported[16,17]—and a small N-terminal domain, which contains residues important for protein stability, promoter binding, cap-binding and endonuclease activity of the polymerase complex[9]. In particular, the importance of Asp 108 and Lys 134 for endonuclease function called into question a previous conclusion that three acidic residues of PB1 were exclusively responsible for the endonuclease activity[2]. The enzymology of the endonuclease within the context of intact viral ribonucleoprotein particles (RNPs) has been extensively studied. The endonuclease is activated by the binding of divalent cations, most effectively manganese followed by cobalt, and less efficiently magnesium, zinc and nickel, but not cadmium, calcium or trivalent ions[6]. RNPs bind four manganese ions in a highly cooperative fashion, presumably two each in the nucleotidyl-transferase and endonuclease sites[6]. It was concluded that the enzyme probably has a two-metal dependent mechanism in common with several other nucleases. Furthermore,

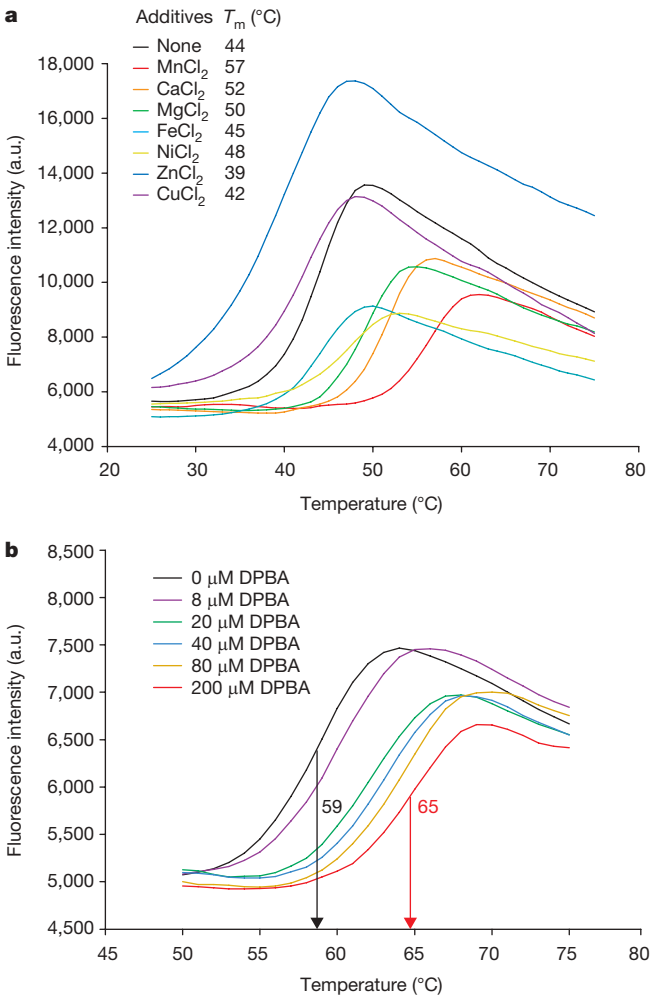

Figure 1 | Manganese ions stabilize PA-Nter. a, Assay of thermal stability using Thermofluor. Thermal shift assays were performed with different metal salts. The results obtained in the absence of metal salts are indicated with a black line and those in the presence of metal salts with coloured lines. The different melting points (T_m) extracted from the curves at pH 8.0 are given in the inset. The effect of $CoCl_2$ on protein stability at pH 7.0 was investigated but was not interpretable owing to quenching by the metal. **b,** Assay of thermal stability with DPBA. Thermofluor assays of PA-Nter were performed in the presence of $MnCl_2$ with increasing concentrations of DPBA. a.u., arbitrary units.

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the endonuclease cleaves single-stranded (ss)DNA with only slightly reduced activity compared to RNA; indeed capped ssDNA oligomers are substrates for cap-snatching[7].

To clarify the functional role of the N-terminal domain of PA we studied a proteolytically stable fragment from residues 1 to 209 (denoted PA-Nter). To optimise the stability and mono-dispersity of PA-Nter we used dynamic light scattering and thermal shift assays with a variety of buffers and additives. These experiments showed a marked increase in thermal stability (apparent melting temperature, T_m, shifts from 44 to 57 °C) and improved mono-dispersity after the addition of manganese ions and to a lesser extent calcium and magnesium ions, but not other divalent metal ions (Fig. 1a). The structural effect of manganese binding, investigated by circular dichroism, demonstrated a small, but reproducible increase in helical content after the addition of 1 mM $MnCl_2$ (Supplementary Fig. 1). Because PA-Nter has been implicated in the viral endonuclease activity[9], which is known to depend on divalent cations, notably manganese[6], we assayed the domain for endonuclease activity using both structured and unstructured ssRNA (Fig. 2) and ssDNA substrates (Supplementary Fig. 2). Using a partially structured 81-nucleotide panhandle RNA (ph-RNA) we found that PA-Nter has intrinsic RNase activity that is strictly divalent-cation-dependent (Fig. 2a, b). Consistent with previous results on RNPs[6], strong activity was observed at pH 8 with manganese, weaker activity with magnesium and none with zinc, calcium or cobalt, whereas at pH 7 activity was also observed with cobalt (Fig. 2b). After 40 min incubation highly structured RNAs such as transfer RNA and signal recognition particle (SRP) *Alu* RNA are relatively resistant to degradation, partially structured ph-RNAs and short ph-RNAs are partially degraded and unstructured U-rich RNA is completely degraded, suggesting that the enzyme is single-strand specific (Fig. 2c). The enzyme also completely degrades circular ssDNA showing that it is a nonspecific endonuclease (Supplementary Fig. 2a). The endonuclease activity on both RNA and DNA is inhibited in a dose-dependent manner by the compound 2,4-dioxo-4-phenylbutanoic acid (DPBA), a known inhibitor of influenza endonuclease[8] (Fig. 2d and Supplementary Fig. 2b). The inhibition

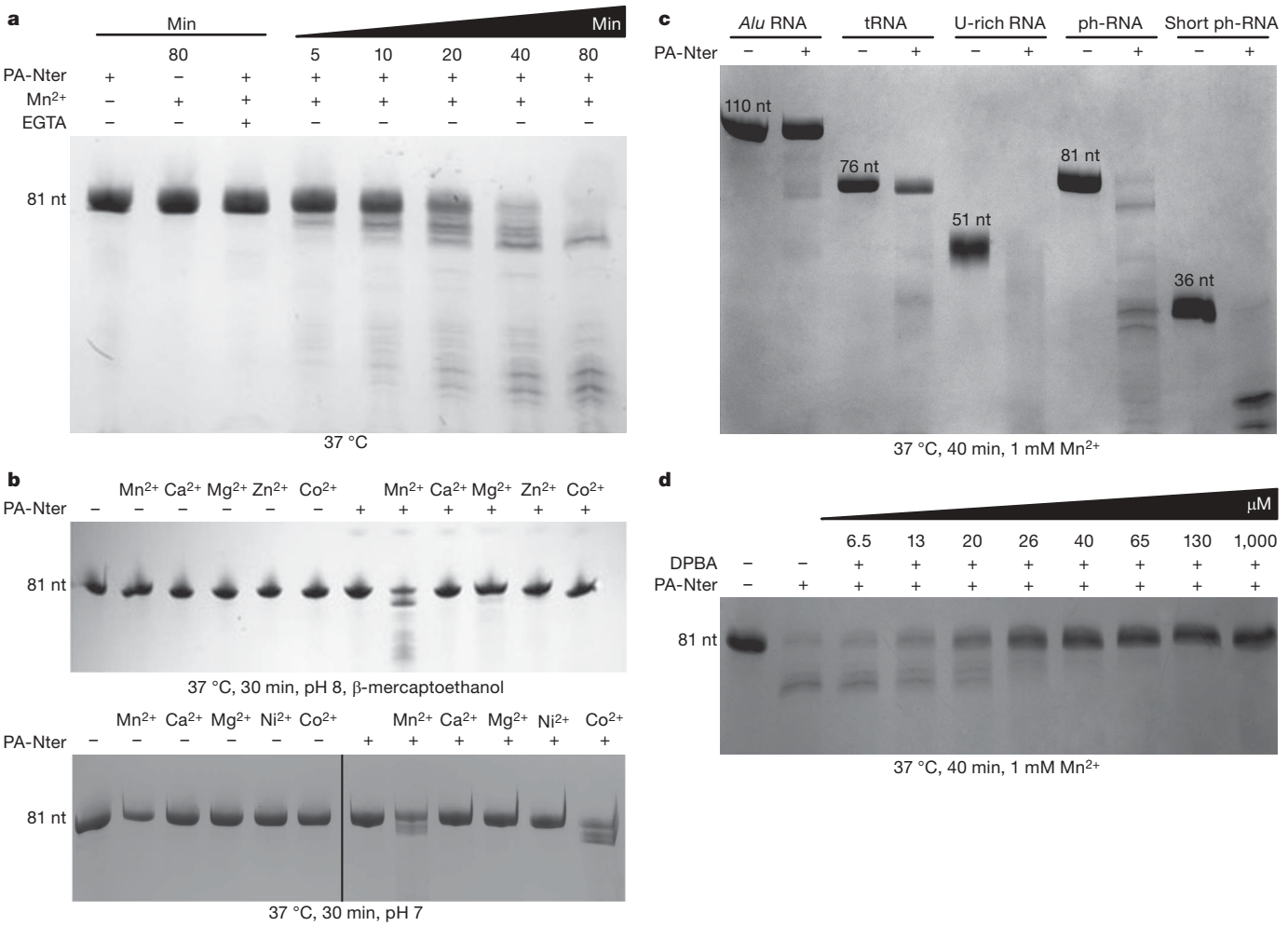

Figure 2 | Endonuclease activity of PA-Nter. **a**, A time series is shown. Purified ph-RNA was incubated with PA-Nter plus 1 mM $MnCl_2$. The incubation at 37 °C was stopped by adding 20 mM EGTA after 5, 10, 20, 40 and 80 min (lanes 4 to 8). As controls, ph-RNA was incubated for 80 min at 37 °C with PA-Nter only (lane 1), 1 mM $MnCl_2$ only (lane 2), or PA-Nter plus 1 mM $MnCl_2$ and 20 mM EGTA (lane 3). The reaction products were loaded on an 8% acrylamide and 8 M urea gel (15% for **b**, bottom panel and **c**) and stained with methylene blue. nt, nucleotides. **b**, The effect of divalent cations on PA-Nter RNase activity. In the top panel, purified ph-RNA plus PA-Nter were incubated at pH 8 in the presence of β-mercaptoethanol and 1.5 mM $MnCl_2$, $CaCl_2$, $MgCl_2$, $ZnCl_2$ or $CoCl_2$. In the bottom panel, ph-RNA and PA-Nter were incubated at pH 7 with 1.5 mM $MnCl_2$, $CaCl_2$, $MgCl_2$, $NiCl_2$ or $CoCl_2$. After 30 min the reactions were stopped by the addition of 20 mM EGTA. Controls were performed using either salts or PA-Nter alone as indicated. At pH 7, $CoCl_2$ stimulated the endonuclease stronger than $MnCl_2$, whereas at pH 8, $CoCl_2$ precipitates and thus does not activate the endonuclease[6]. **c**, PA-Nter RNase activity on different RNA substrates. SRP *Alu* RNA, tRNA, U-rich RNA, ph-RNA or short ph-RNA were incubated with 1 mM $MnCl_2$ plus PA-Nter (lanes 2, 4, 6, 8 and 10) or in the absence of PA-Nter (lanes 1, 3, 5, 7 and 9). The digestion was performed at 37 °C. After 40 min the reaction was stopped by the addition of 20 mM EGTA. **d**, The inhibition of PA-Nter endonuclease activity by DPBA. Cleavage of ph-RNA by PA-Nter was tested at 37 °C during 40 min in the presence of 1 mM $MnCl_2$ and increasing concentrations of DPBA (0, 6.5, 13, 20, 26, 40, 65, 130 and 1,000 μM). As a control, ph-RNA was incubated with 1 mM $MnCl_2$ alone (lane 1).

constant (K_i) for this compound is estimated at 26 μM, in excellent agreement with the half-maximal inhibitory concentration (IC_{50}) reported for the same compound inhibiting cleavage of capped RNA by the intact influenza virus polymerase[8]. Titrating the inhibitor to manganese-bound PA-Nter increases the thermal stability even further (apparent T_m shifts from 59 to 65 °C) (Fig. 1b), whereas the inhibitor has no effect on the stability of metal-free enzyme (data not shown).

We grew small square-plate crystals of PA-Nter only in the presence of both manganese and magnesium that diffracted to about 2 Å resolution, with three independent molecules in the asymmetric unit. The crystal structure reveals a single, folded domain with residues 1–196 visible, comprising seven α-helices and a mixed, five-stranded β-sheet (Fig. 3a). The structure-based sequence alignment among influenza A, B and C viruses (Fig. 3b) projected onto a surface representation shows a very highly conserved depression that is strongly negatively charged owing to a concentration of acidic residues (Supplementary Fig. 3a, b), suggestive of an active site. A structure similarity search gave no high scoring hits, indicating that the global fold is new. The most similar protein found is the archaeal Holliday junction resolvase Hjc from *Pyrococcus furiosus*[18]. The structural alignment of PA-Nter with Hjc superposes helix α3 and strands β1–β5 (Fig. 4a, b) encompassing a structural motif characteristic of many nucleases including resolvases and type II restriction enzymes. The motif includes catalytically important divalent metal ion binding acidic residues Asp 33 and Glu 46 of Hjc[18], on which Asp 108 and Glu 119 of PA-Nter exactly superpose. Structural alignment of PA-Nter with type II restriction endonucleases such as BamHI or EcoRV shows a similar superposition of active site elements (Fig. 4c). The catalytically important residues Glu 45, Asp 74, Asp 90 and Lys 92 of EcoRV align with His 41, Asp 108, Glu 119 and Lys 134 of PA-Nter, respectively, although the lysines are positioned differently in the primary sequence (Fig. 4d)[19]. The conserved lysine is implicated in stabilizing the attacking hydroxide nucleophile during catalysis[19]. Thus PA-Nter is a new member of the PD-(D/E)XK nuclease superfamily which encompasses a diversity of enzymes involved in various aspects of DNA metabolism[20]. In PA-Nter, the characteristic motif occurs at 107-PDLYDYK, although the separation between the two acidic residues is unusually short and the putative catalytically important lysine (Lys 134) has 'migrated' to an alternative position, as in some other members of the superfamily[20]. In this family, PA-Nter is unusual in that it is biologically functional as an RNase and has a histidine in the active site.

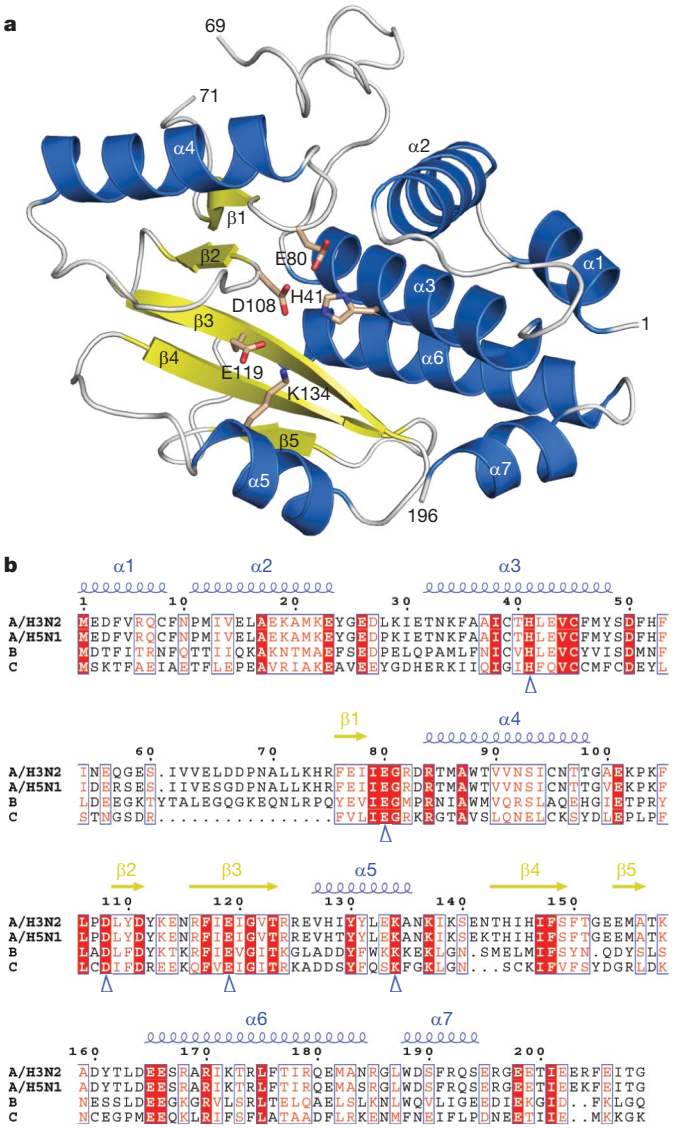

Figure 3 | Three-dimensional structure of PA-Nter. a, Ribbon diagram of the structure of influenza A/Victoria/3/1975 PA-Nter with α-helices in blue and β-strands in yellow, labelled as in **b**. The key active site residues are indicated in stick representation. **b**, Sequence alignment of PA-Nter from representative influenza strains: A/Victoria/3/1975 (human H3N2), A/Duck/Vietnam/1/2007 (avian H5N1), B/Ann Arbor/1/1966 (B) and C/Johannesburg/1/1966 (C). The secondary structure of PA-Nter of A/Victoria/3/1975 is shown over the sequence alignment in accordance with colours used for **a**. Residues in a solid red background are identical between the four sequences. The blue triangles indicate the key active site residues.

To confirm that the conserved acidic residues of PA-Nter are metal binding residues we calculated an anomalous difference map using data collected at the manganese K absorption edge. Two manganese ions were identified in each active site as adjacent anomalous peaks separated by about 3.8 Å (Fig. 4e). The stronger peak (Mn1) is coordinated by Glu 80, Asp 108 and two water molecules; the weaker site (Mn2) by His 41, Asp 108, Glu 119 and the carbonyl oxygen of Ile 120. The cited residues are absolutely conserved in all influenza virus PA sequences (except for Ile 120, which is conservatively substituted) (Fig. 3b). The two metal sites correspond closely with those observed in restriction enzymes such as EcoRV (Fig. 4f). His 41 (positioned as Glu 45 in EcoRV) from helix α3 could be important in conferring manganese specificity, because magnesium and calcium bind less readily to histidine. Manganese binding by His 41 and the resulting stabilization of helix α3 could account for the extra helical content (estimated as 8–9 residues) detected after incubating PA-Nter with manganese (Supplementary Fig. 1). In the crystal, Mn1 and Mn2 are also coordinated by Glu 59 from a loop of an adjacent molecule. Superposition of DNA complexes of BamHI or EcoRV on PA-Nter shows that the Glu 59 carboxylate group corresponds closely to the position of the scissile phosphate group (Supplementary Fig. 4). Thus our structure mimics, to some extent, a substrate or product complex. A more precise understanding of the reaction mechanism and the roles of additionally conserved residues near the active centre (for example, Arg 84, Tyr 130 and Lys 137) must await structural studies with substrate or transition state analogues.

Our structural and biochemical results combined with previous observations on the trimeric polymerase provide compelling evidence that PA-Nter is the endonuclease that cleaves host mRNAs during cap-snatching. First, the domain has intrinsic RNA and DNA endonuclease activity that is preferentially activated by manganese, in accordance with observations reported for the viral RNPs (Fig. 2b)[6,7]. Second, this activity is inhibited by a compound known to inhibit influenza endonuclease activity with a nearly identical K_i (Fig. 2d)[8]. Third, the domain contains a structural motif characteristic of the catalytic core of a broad family of nucleases, including type II endonucleases. The active site features a cluster of three acidic residues (Glu 80, Asp 108 and Glu 119) and a putative catalytic lysine (Lys 134) (Fig. 4). Fourth, these acidic residues, together with His 41,

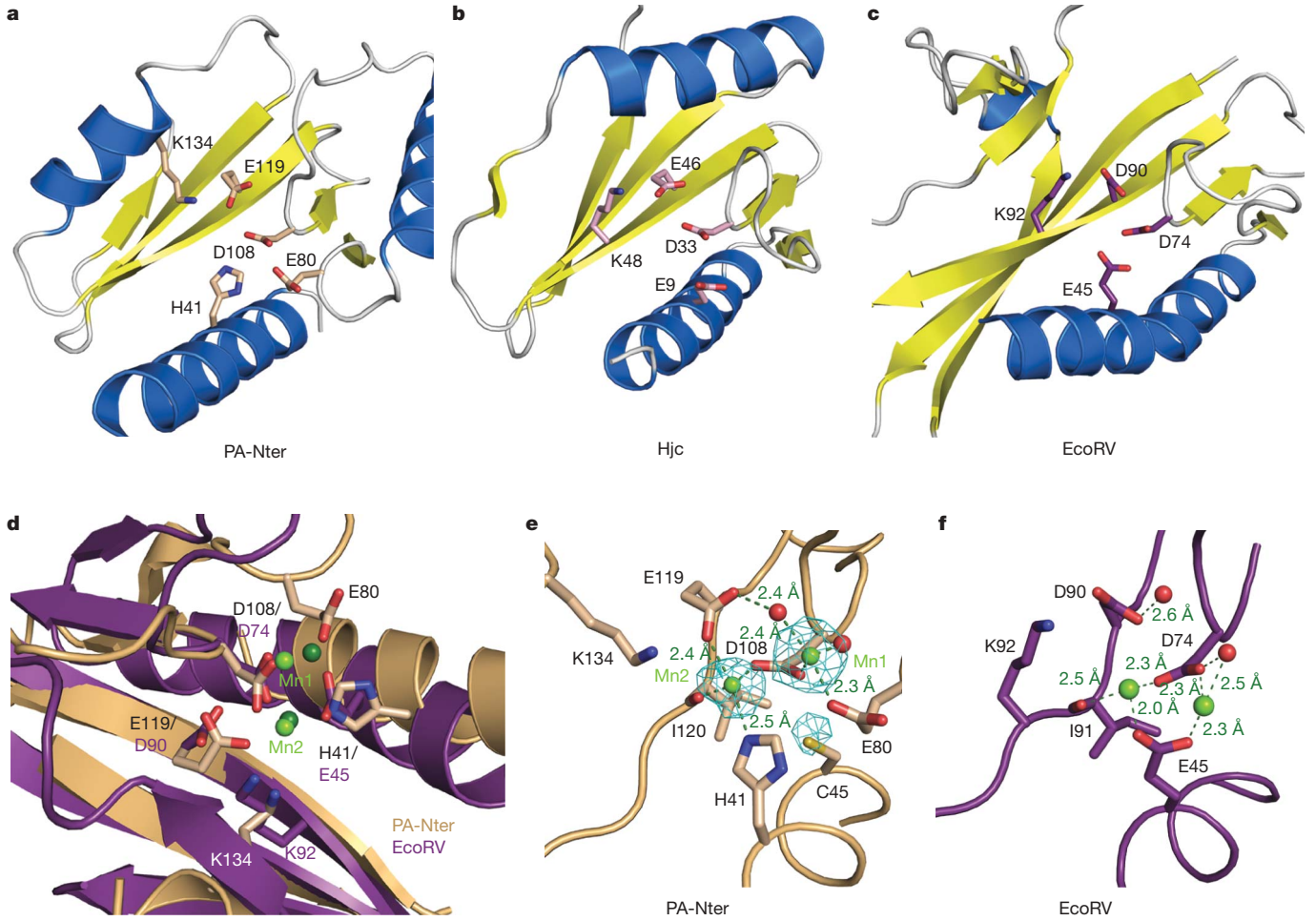

Figure 4 | Comparison of PA-Nter with other nucleases of the PD-(D/E)XK superfamily. **a**–**c**, Comparison of PA-Nter (**a**), *P. furiosus* Holliday junction resolvase Hjc (PDB accession 1GEF)[18] (**b**), and *E. coli* EcoRV restriction enzyme (PDB accession 1STX, product complex with DNA and manganese)[19] (**c**) after superposition of the conserved core active site structural motif. The root mean squared deviations are 2.9 Å for 77 aligned Cα atoms of Hjc (*Z*-score 3.3), and 2.46 (3.1) Å for 55 (72) aligned Cα atoms of EcoRV. Secondary-structure elements are as in Fig. 3a with key active sites residues in stick representation. **d**–**f**, Details of the metal-binding active sites of influenza PA-Nter (molecule A) (beige) and *E. coli* EcoRV restriction enzyme product complex (purple). **d**, Superposition of the active sites showing overlap of key active site metal binding and catalytic functional groups of the two proteins. Manganese ions are light green for PA-Nter and dark green for EcoRV. **e**, **f**, Manganese ion interactions within the active sites of PA-Nter (**e**) and the EcoRV product complex (**f**). Manganese ions and water molecules are shown as green and red spheres, respectively. In **e** the anomalous difference map contoured at 3σ, calculated using manganese K edge diffraction data and model phases, is in cyan. Peak heights are 14.1, 10.1 and 5.0σ for Mn1, Mn2 and the sulphur of Cys 45, respectively.

all absolutely conserved in influenza viruses, coordinate two manganese ions in a configuration consistent with a two-metal-dependent reaction mechanism as proposed for many nucleases (Fig. 4e)[19,21,22]. Fifth, mutations of important active site residues D108A and K134A have been shown to specifically abolish endonuclease activity of the trimeric polymerase[9]. In addition, the mutation H41A abolished all polymerase activity, consistent with a crucial role in both the stability of PA and the activity of the endonuclease[9].

Our results establish for the first time, to our knowledge, a unique role for the PA subunit of influenza virus polymerase and contradict the widely held view that the endonuclease active site is located in the PB1 subunit. This was on the basis of data showing that PB1 residues 508–522 could be cross-linked to capped RNAs by means of a thiouridine adjacent to the endonucleolytic cleavage site[2]. The cross-linked region includes acidic residues Glu 508, Glu 519 and Asp 522, which when individually mutated to alanine specifically abolished endonuclease activity, although their exact function was not determined. These data can be reconciled with our observations if the cross-linked region of PB1 is in spatial proximity to the actual endonuclease active site in PA-Nter. We note that similar cross-linking experiments[2] led to the incorrect identification of the cap-binding site on PB2 (refs 4, 23). Indeed it is very likely that other regions of the polymerase regulate the intrinsic endonuclease activity of PA-Nter, because it is known that there is a cooperative functional interaction between capped RNA binding, 3′ and 5′ end viral RNA (vRNA) binding, and endonuclease activity[24,25]. It is therefore surprising that, as with the cap-binding domain of PB2, a small independently folded domain should have so many of the functional properties of the endonuclease reported for the trimeric complex, as both cap-snatching activities were thought to be detectable only in the trimeric complex. Nonetheless, full understanding of the concerted mechanism of cap-dependent transcription will undoubtedly require high resolution structures of the intact polymerase with bound vRNA and capped RNA.

Influenza can be a serious illness and there is a continuing risk of a devastating pandemic resulting from the eventual acquisition by highly pathogenic avian strains (for example, H5N1) of the ability to be transmissible from human to human. Whereas mutations in the viral polymerase are known to have an important role in virulence and interspecies transmission[26,27], none of the systematic differences in PA-Nter between human and avian strains (Fig. 3b) has been implicated in these processes nor seems to be critically placed as to affect endonuclease function. Several specific influenza virus endonuclease inhibitors

have been described[8,10,11] including some designed to chelate metal ions bound in the active site[12]. Our results show that at least one of these inhibitors is active against PA-Nter and that it binds and stabilizes the metal-bound form of the enzyme (Fig. 1b). These observations will be helpful in developing potential new antivirals using a structure-based approach.

METHODS SUMMARY

PA-Nter, residues 1–209 from A/Victoria/3/1975 (H3N2), was expressed in *Escherichia coli* and purified by affinity and gel filtration chromatography. The influence of metal ions on thermal stability was tested by Thermofluor assays[28]. The endonuclease activity was tested by incubation at 37 °C of 13 μM PA-Nter with 10 μM of various RNA substrates: *Alu* RNA, 110 nucleotides of the *Alu* domain of *Pyrococcus horikoshii* SRP RNA, *Candida albicans* $tRNA^{Asn}$, U-rich RNA (5′-GGCCAUCCUGU$_7$ CCCU$_{11}$CU$_{19}$-3′)[29], ph-RNA of 81 nucleotides[30], and short ph-RNA of 36 nucleotides comprising just the conserved 3′ and 5′ ends with a short linker and circular single stranded DNA (M13mp18) (Fermentas). Crystals diffracting to a maximum of 2 Å resolution were obtained at 20 °C by the hanging-drop method using a protein solution of 5–10 mg ml^{-1} in 20 mM Tris-HCl, pH 8.0, 100 mM NaCl and 2.5 mM $MnCl_2$ and a reservoir composition of 1.2 M Li_2SO_4, 100 mM MES, pH 6.0, 10 mM Mg acetate and 3% ethylene glycol. Diffraction data were collected on beamlines ID14-4 and ID23-1 at the European Synchrotron Radiation Facility (ESRF). The structure was solved by the single-wavelength anomalous diffraction (SAD) method using a gadolinium-chloride-soaked crystal, and initial phases were improved by three-fold non-crystallographic symmetry averaging. Data were also measured on a native crystal at the manganese K edge to reveal the location and identity of bound manganese ions through anomalous difference Fourier synthesis. The structure described is that of a native crystal soaked with additional $MnCl_2$ at 10 mM. The final R factor (R_{free}) is 0.217 (0.268). Of the three molecules in the asymmetric unit, A has the best defined metal structure and D is the most disordered. Further details are available in the Supplementary Methods including a table of crystallographic statistics (Supplementary Table 1).

Received 10 November; accepted 22 December 2008.
Published online 1 February 2009.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements We thank the ESRF, EMBL and MRC-France for access to synchrotron facilities and the Partnership for Structural Biology for an integrated structural biology environment. The work was partially funded by the EU FLUPOL contract (SP5B-CT-2007-044263) and the ANR FLU INTERPOL contract (ANR-06-MIME-014-02). A.D. has a PhD fellowship from the MENRT. We thank J.-L. Decout for advice about the inhibitor, E. Kowalinski and C. Swale for RNA transcripts, and C. Petosa and J. Perona for a critical reading of the manuscript.

Author Contributions A.D., D.B., T.C. and A.A.M. performed the experiments, D.J.H. and F.B. supervised the search for a soluble fragment of PA, R.W.H.R. and S.C. supervised the biochemical characterization of PA-Nter, and S.C. supervised the crystallography. S.C. wrote the paper with the help of A.D., D.B., T.C. and R.W.H.R.

Author Information Atomic coordinates and structure factors have been deposited with the Protein Data Bank (PDB) under accession codes 2W69 and R2W69SF. Reprints and permissions information is available at www.nature.com/reprints. The authors declare competing financial interests: details accompany the full-text HTML version of the paper at www.nature.com/nature. Correspondence and requests for materials should be addressed to S.C. (cusack@embl.fr).