Characterization of the interaction between the influenza A virus polymerase subunit PB1 and the host nuclear import factor Ran-binding protein 5

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The influenza A virus RNA polymerase is a heterotrimer that transcribes and replicates the viral genome in the cell nucleus. Newly synthesized RNA polymerase subunits must therefore be imported into the nucleus during an infection. While various models have been proposed for this process, the consensus is that the polymerase basic protein PB1 and polymerase acidic protein PA subunits form a dimer in the cytoplasm and are transported into the nucleus by the beta-importin Ran-binding protein 5 (RanBP5), with the PB2 subunit imported separately to complete the trimeric complex. In this study, we characterized the interaction of PB1 with RanBP5 further and assessed its importance for viral growth. In particular, we found that the N-terminal region of PB1 mediates its binding to RanBP5 and that basic residues in a nuclear localization signal are required for RanBP5 binding. Mutating these basic residues to alanines does not prevent PB1 forming a dimer with PA, but does reduce RanBP5 binding. RanBP5-binding mutations reduce, though do not entirely prevent, the nuclear accumulation of PB1. Furthermore, mutations affecting RanBP5 binding are incompatible with or severely attenuate viral growth, providing further support for a key role for RanBP5 in the influenza A virus life cycle.

INTRODUCTION

Influenza A virus is the prototypic orthomyxovirus and a serious pathogen of humans and animals. The viral genome consists of eight segments of negative-sense, ssRNA, which are encapsidated as viral ribonucleoprotein complexes (vRNPs) by multiple copies of nucleoprotein (NP) and the trimeric RNA-dependent RNA polymerase (RdRP; consisting of the polymerase basic proteins PB1 and PB2, and the polymerase acidic protein PA) (Palese & Shaw, 2007). Unusually among RNA viruses, orthomyxoviruses replicate their genomes in the nucleus. Consequently, newly synthesized viral proteins must be imported into the nucleus to allow the assembly and export of new vRNPs, as well as to regulate host processes in the infected cell. Of the proteins expressed by influenza A virus, nuclear import is observed for the three polymerase proteins and NP, as well as matrix (M1) protein, non-structural (NS1) protein, nuclear export protein (NEP) and PB1-F2 (Chen et al., 2001; Smith et al., 1987). Nuclear import of structures larger than 20–30 kDa is selective and energy dependent, requiring binding of nuclear import factors (NIFs) through nuclear localization signals (NLSs) on import substrates (Görlich & Kutay, 1999). A number of influenza A virus proteins have been shown to contain NLSs and to bind NIFs (Boulo et al., 2007; Naito et al., 2007; Resa-Infante et al., 2008; Tarendeau et al., 2007). PB1 and PA, despite both having signals that can promote nuclear import when individually expressed (Akkin et al., 1987; Nath & Nayak, 1990; Nieto et al., 1994), do not accumulate efficiently in the nucleus unless expressed together, a requirement that appears to be particularly marked for PB1 (Fodor & Smith, 2004; Huet et al., 2010; Nieto et al., 1992). Although all possible pairwise interactions between polymerase subunits have been detected (Hemerka et al., 2009; Naito et al., 2007), the majority of evidence favours a model of nuclear import and assembly in which PB1 and PA form a dimer in the cytoplasm and then undergo nuclear import, with PB2 being imported separately and completing the trimer in the nucleus (Deng et al., 2005; Huet et al., 2010; Loucaides et al., 2009).

Analyses of cellular proteins co-purified with tagged viral proteins have identified the NIF Ran-binding protein 5 (RanBP5) as a cellular interaction partner of PB1 and of the PB1:PA dimer, but not of the trimeric polymerase or vRNPs (Deng et al., 2006; Mayer et al., 2007). RanBP5 is a member of the importin beta (importin β) superfamily of nuclear import receptors and is also known as importin β3, importin 5 and karyopherin β3 (Deane et al., 1997; Yaseen...
& Blobel, 1997). It functions in non-classical nuclear import, binding cargo directly without an importin alpha (importin \(\alpha\)) adaptor (Jäkel & Görlich, 1998). In common with other importin \(\beta\) family members, RanBP5 facilitates transport of cargo across the nuclear pore complex (NPC), releasing it upon binding RanGTP in the nucleus. Transport across the NPC is directional as RanGTP is present at much higher concentrations in the nucleus than in the cytoplasm (Görlich & Kutay, 1999). In studies of cellular interaction partners additional functional roles for RanBP5 have been posited, including regulating the secretion of apolipoprotein A-1 (Chung et al., 2008) and acting as a chaperone for exposed basic regions of ribosomal proteins (Jäkel et al., 2002).

RanBP5 is involved in the nuclear import of ribosomal proteins (Jäkel & Görlich, 1998) and core histones (Baake et al., 2001; Mühlauser et al., 2001), as well as other cellular cargos (Chung et al., 2008; Deane et al., 1997; Dynes et al., 2004; Fu et al., 2006; Heese et al., 2004; Jäkel & Görlich, 1998; Nakamura et al., 2007; Ross et al., 2003; Waldmann et al., 2007; Yaseen & Blobel, 1997). In addition, RanBP5 has been shown to interact with proteins from a range of viruses (Arnold et al., 2006; Chung et al., 2000; Darshan et al., 2004; Deng et al., 2006; Kluccevsek et al., 2006; Krawczyk et al., 2008; Nelson et al., 2003). In influenza A virus, RanBP5 could be displaced from PB1 or the PB1:PA dimer by the addition of non-hydrolysable RanGTP \textit{in vitro}, and depletion of RanBP5 reduced the nuclear accumulation of PB1 and of PB1:PA. It was therefore suggested that RanBP5 was recruited by the virus as a nuclear import receptor (Deng et al., 2006).

Here, we characterize the interaction between PB1 and RanBP5 in more detail. Using mutational analysis, we mapped the RanBP5-binding site to the N-terminal part of PB1 and identified residues required for binding. Using RanBP5-binding mutants of PB1, we confirmed earlier indirect studies showing that RanBP5 binding promotes nuclear import of PB1, a process that also requires PA. Mutations affecting RanBP5 binding severely attenuated or were incompatible with viral growth, suggesting a key role for RanBP5 in the influenza A virus life cycle. Comparison of different influenza A virus strains suggests that the interaction with RanBP5 is unlikely to be a determinant of host tropism.

### RESULTS

**The RanBP5-binding site is located in the N-terminal 290 aa of PB1**

To characterize the interaction between RanBP5 and PB1, we wished to identify the RanBP5-binding site. Previous studies had shown that PB1 of influenza A/WSN/33 virus (WSN), when fused at its C terminus to a tandem affinity purification (TAP) tag, could co-purify RanBP5 (Deng et al., 2006). We applied the same approach to truncated forms of PB1–TAP (Fig. 1a), using them to co-purify RanBP5 from 293T cells. PB1 was divided into an N-terminal fragment containing the PA-binding and promoter-binding domains (residues 1–290), a middle fragment containing the core RdRP motifs (residues 289–488) and a C-terminal fragment containing the PB2-binding site (residues 488–757) (Jung & Brownlee, 2006; Sugiyama et al., 2009; Ruigrok et al., 2010). Efficient binding of RanBP5 was observed with full-length tagged PB1 and with the N-terminal fragment of PB1, but not with the middle or C-terminal fragments of the protein (Fig. 1b). These relatively short fragments were purified at lower levels than full-length protein. Extension of the N-terminal fragment to include the middle part of the protein (residues 1–488), or fusion of the N-terminal and C-terminal parts of the protein (residues 1–290/488–757) increased tagged protein yield, but did not result in a proportional increase in co-purified RanBP5 (Fig. 1b). Indeed these longer fragments were less able to bind RanBP5 than full-length protein or the N-terminal fragment alone, suggesting that the conformation of the incomplete protein was not optimal for RanBP5 binding. Together, these data suggest that the specific RanBP5-binding activity of PB1 lies predominantly in the first 290 residues.

**The RanBP5-binding site includes residues in a bipartite NLS**

To map the interaction domain more precisely and to identify residues required for RanBP5 binding, we compared the primary amino acid sequence of PB1 to those of known RanBP5-binding sites (Arnold et al., 2006; Chung et al., 2008; Dynes et al., 2004; Fu et al., 2006; Jäkel & Görlich, 1998; Ross et al., 2003; Waldmann et al., 2007). Although there is a general tendency for these regions to be highly basic and, in most cases, unstructured (Choock & Suel, 2010), there was no clear consensus sequence. Accordingly, most known RanBP5-binding sites could not be aligned with the primary sequence of PB1 (by CLUSTALW2, data not shown). However, the \(\beta\)-importin-like-binding (BIB) domain of the ribosomal protein L23a (rPL23a), which has been shown to bind to RanBP5 (Jäkel & Görlich, 1998), aligns to a basic region in the N-terminal fragment of PB1 (Fig. 2a). As this region contains the previously identified bipartite NLS of PB1 (Nath & Nayak, 1990), we hypothesized that the NLS formed a binding site for RanBP5.

To test this hypothesis, we mutated the bipartite NLS of full-length, TAP-tagged PB1, changing pairs of basic residues to alanines. As in a previous study of the NLS (Fodor & Smith, 2004), alanine mutations were introduced at positions 188–189 (NLS1), 208–209 (NLS2) and 188–189 and 208–209 together (NLS12; Fig. 2a). Expression of PB1–TAP, with or without the mutations, did not affect the amounts of RanBP5 detected by Western blotting in unpurified cell lysates (data not shown). After TAP purification, the amount of RanBP5 bound to the NLS1
and NLS2 mutant proteins was reduced compared to wild type (WT), and for the NLS12 double mutant copurification was close to the limits of detection (Fig. 2b). To determine whether these effects were influenced by the TAP tag on PB1, the same mutations were introduced into untagged PB1, which was co-purified with an N-terminally TAP-tagged RanBP5. A similar pattern of binding was observed: mutation of NLS1 and NLS2 had an intermediate effect, and the NLS12 double mutation reduced binding to background levels (Fig. 2c). Thus, these data indicate that residues in the bipartite NLS of PB1 are required for efficient RanBP5 binding.

RanBP5 binding efficiency of PB1 affects nuclear localization

A previous study has shown that depletion of RanBP5 by RNA interference reduces the efficiency of nuclear localization of PB1–GFP in the presence of PA (Deng et al., 2006). However, as RanBP5 has a range of cellular binding partners it is possible that its knockdown could have both direct and indirect effects. Using mutations that reduced RanBP5 binding we could test directly whether RanBP5 binding affected the nuclear localization of PB1. Untagged PB1 and PA were co-expressed in Vero cells and the localization of PB1 determined by indirect immunofluorescence. Whether or not the RanBP5-binding site was mutated, cells with a range of PB1 localizations were observed (Fig. 3a), as previously noted with studies of GFP-tagged PB1 (Fodor & Smith, 2004). Mutations that reduced RanBP5 binding decreased the proportion of cells with exclusively nuclear localization of PB1 and increased the proportion of cells in which PB1 was visible throughout the cell, though with little apparent effect on the small proportion of cells in which PB1 appeared to be excluded from the nucleus (Fig. 3b). Mutations which had a greater effect on RanBP5 binding caused a greater reduction in nuclear localization, consistent with the hypothesis that RanBP5 binding affects the nuclear import of PB1. Despite this correlation, the mutations did not prevent nuclear accumulation: even the NLS12 double mutant, which did not co-purify detectable quantities of RanBP5, accumulated in the nuclei of 40% of PB1-expressing cells. As the antibody used to detect PB1 had a low level of cross-reactivity with PA, we repeated the experiments using PB1–TAP, detected by an epitope in its tag. In this system no cross-reactivity with PA was observed, and as with untagged PB1 the NLS12 mutation reduced, but did not prevent, nuclear localization (data not shown).

The association of PB1 with RanBP5 is not affected by PA

Consistent with previous studies (Deng et al., 2005; Fodor & Smith, 2004; Huet et al., 2010; Loucaides et al., 2009; Nieto et al., 1992), we found that the efficient nuclear accumulation of PB1 required the presence of PA. In the absence of PA, the nuclear accumulation of WT PB1 was
greatly reduced, and exclusively cytoplasmic localization of PB1 was observed in over 70% of cells (Fig. 3). As PA affected the nuclear localization of PB1, it was possible that the change in localization of the mutant proteins was due to reduced binding to PA. However, RanBP5-binding mutations did not reduce the amount of PB1 co-purified with PA–TAP (Fig. 4a), consistent with previous studies showing that the N-terminal 25 aa of PB1 are sufficient to mediate PA binding (He et al., 2008; Obayashi et al., 2008). This suggests that the reduced nuclear accumulation of the PB1 mutants is primarily due to reduced RanBP5 binding. Alternatively, it was possible that PA might contribute to the nuclear localization of PB1 by acting as a cofactor for efficient RanBP5 binding. Increased binding affinity for importins has been shown to increase the efficiency of nuclear import (Hodel et al., 2006; Jans et al., 2000), and we reasoned that if a complex of PB1 and PA had greater affinity for RanBP5 than PB1 alone this would explain the requirement of PA for the efficient nuclear import of PB1. However, the ability of PB1–TAP to co-purify RanBP5 was not increased by PA (Fig. 4b). Moreover, consistent with previous studies (Deng et al., 2006), we were unable to find any evidence that PA bound RanBP5 directly: RanBP5 was only co-purified with PA–TAP in the presence of PB1, and this could be reduced to the limits of detection when the RanBP5-binding site of PB1 was mutated (Fig. 4a). Taken together, these results show that PA does not substantially alter the affinity of PB1 for RanBP5, and that the importin binds to the PB1:PA dimer through PB1.

**Mutations affecting RanBP5 binding prevent efficient viral growth**

In order to determine the effect of mutations that affected RanBP5 binding on viral growth, attempts were made to introduce mutations into WSN virus by reverse genetics. In four separate experiments, in which WT virus was rescued with titres of approximately $10^7$ p.f.u. ml$^{-1}$ as a positive control, no virus was rescued when the NLS1 or NLS12 mutations were encoded by viral RNA (vRNA). A virus containing the NLS2 mutation was rescued on only one of four occasions (Fig. 2). The mean and half-range of two experiments is shown, scaled from 1 (WT) to 0 (background signal with no PB1).
four attempts, with a titre of $7.8 \times 10^2$ p.f.u. ml$^{-1}$ (in the same experiment WT virus was rescued with a titre of $1.1 \times 10^7$ p.f.u. ml$^{-1}$). The presence of the NLS2 mutation was confirmed by sequencing after an additional round of amplification; no other mutations were found at nearby sites, although whole-genome sequencing was not carried out. This NLS2 mutant virus was severely attenuated, producing a smaller plaque size than WT (Fig. 5a) and growing to a maximum titre around three orders of magnitude lower than WT (Fig. 5b). The failure to rescue viruses with the NLS1 or NLS12 mutations, the fact that the NLS2 virus was only rescued on one attempt out of four, and the severe attenuation of the NLS2 virus, indicate that residues required for efficient RanBP5 binding are required for efficient viral growth.

The RanBP5-binding site is conserved and is unlikely to contribute to host tropism

Additional evidence for the functional importance of RanBP5-binding residues was provided by their conservation between strains of influenza A virus from a broad range of host species and initial dates of isolation. The basic residues of the bipartite NLS were invariant in the majority of strains considered (Fig. 6a), although it was noted that position 208 (part of the NLS2 site), though typically lysine, is arginine for WSN and a minority of other strains – a conservative difference. The RNA that encodes these residues has been shown to be capable of considerable synonymous variation (Gog et al., 2007), and is some way from proposed packaging signals or other known cis-acting RNA sequences (Hutchinson et al., 2010). Together, this suggests that it is the conserved amino acid sequence, rather than a particular structural feature of the RNA sequence that encodes it, which is selected for. We therefore concluded that the RanBP5-binding residues provide a fitness advantage and are conserved.

Point mutations in importin-binding sites are associated with the adaptation of influenza A virus from avian to mammalian hosts (Gabriel et al., 2008). However, the conservation we observed for residues involved in RanBP5 binding did not appear to be host specific, and we noted that human and avian forms of RanBP5 are similar: 96% of the Homo sapiens RanBP5 sequence can be aligned to the Gallus gallus sequence, with 85% identity. We therefore
hypothesized that the interaction with RanBP5 does not adapt when influenza moves from avian to mammalian host species. Consistent with this, PB1 proteins from a panel of H5N1 viruses (three avian isolates and one human isolate) were, when fused to a TAP tag, able to co-purify human RanBP5 as efficiently as PB1–TAP from the human-derived, mammalian-adapted laboratory strain WSN (Fig. 6b). This suggested that the RanBP5-binding site does not need to adapt during cross-species transmission of the virus, and that RanBP5 binding is unlikely to be a determinant of host tropism.

**DISCUSSION**

PB1, the core subunit of the influenza A virus polymerase complex, has previously been shown to interact with the β-importin RanBP5 (Deng et al., 2006; Mayer et al., 2007).
Here, we characterized this interaction in detail by identifying residues required for efficient binding to RanBP5. Mutating basic residues in the previously identified bipartite NLS of PB1 (Nath & Nayak, 1990) reduced its ability to bind RanBP5, allowing us to study the significance of this interaction directly. This approach complements and extends earlier studies using RNA interference (Deng et al., 2006), which could not exclude indirect effects of RanBP5 depletion.

Mutating the RanBP5-binding site reduced the ability of PB1 to localize to the nuclei of transfected cells in the presence of PA (Fig. 3). This is consistent with the proposed role for RanBP5 as a NIF for the PB1:PA dimer, and provides direct evidence to corroborate a previous study in which depletion of RanBP5 reduced, but did not prevent, the nuclear localization of PB1-GFP:PA (Deng et al., 2006). Similarly, we found that even the NLS12 mutant protein, which could not co-purify detectable quantities of RanBP5, could accumulate in the nucleus of a substantial fraction of cells. This may reflect the relatively high stringency of the co-purification procedure, suggesting that the double mutant NLS12 can still bind some RanBP5 in a relatively weak and reversible manner, sufficient for nuclear import. Alternatively it may indicate that RanBP5 promotes nuclear import, but that other factors also play a role.

In previous studies PA has been shown to play an important role in the nuclear accumulation of PB1 (Deng et al., 2005; Fodor & Smith, 2004; Huet et al., 2010; Loucaides et al., 2009; Nieto et al., 1992). PB1 alone associates with RanBP5 (Deng et al., 2006), but it is excluded from the nucleus of the majority of cells in the absence of PA. The mechanism by which PA contributes to the nuclear accumulation of the PB1:PA dimer remains unclear, and further work is required to understand the dynamics of nuclear import of these proteins. PA did not increase the amount of RanBP5 co-purifying with PB1, and RanBP5-binding mutations in PB1 reduced its nuclear accumulation but not its binding to PA (Fig. 4). Adaptive mutations in PB2 and NP have been linked to changes in importin specificity (Gabriel et al., 2011), and many RanBP5 cargo proteins bind a range of other β-importins (Chook & Süel, 2010). It is therefore possible that PA binding alters the specificity of PB1 for β-importins. It is also possible that an additional NIF binds to the PB1:PA dimer, possibly using a conformational NLS distributed between the proteins, as seen in the import of signal transducer and activator of transcription (STAT) dimers by importin α5 (Fagerlund et al., 2002). However, there is currently no evidence for other importins interacting with the PB1:PA dimer.

Residues required for the efficient binding of RanBP5 contribute to viral fitness, and are conserved across a broad range of strains (Figs 5 and 6). Comparison of WSN with avian-adapted PB1 proteins suggests that this interaction is unlikely to be a determinant of host tropism, although we note that the KR polymorphism at residue 208 has previously been shown to modulate virulence in mice (Rolling et al., 2009). We were unable to generate recombinant viruses with the NLS1 and NLS12 mutations, and a virus containing the NLS2 mutation had a severe
growth defect. We have not identified a function for these mutated residues other than their interaction with RanBP5.

The mutations did not reduce the binding of PB1 to PA (Fig. 4a). Nor do the mutations overlap known promotor-binding or polymerase motifs (González & Ortíñ, 1999; Jung & Brownlee, 2006; Li et al., 1998; Müller et al., 1994; Poch et al., 1989). A previous study has shown that the NLS2 mutation did not substantially affect the ability of PB1 to transcribe and replicate a template RNA [a negative-sense chloramphenicol acetyltransferase (CAT) gene flanked by the untranslated regions of WSN segment 8] in an RNP reconstitution (Fodor & Smith, 2004); we confirmed this observation using full-length vRNA and cRNA of WSN segment 6 as a template (data not shown). PB1 is a multifunctional protein, and it remains possible that these residues do play additional roles. However, the data currently available suggest that the interaction of PB1 with RanBP5 plays an important role in the influenza A virus life cycle.

The importance of the RanBP5-binding residues for viral growth is hard to reconcile with the relatively small effects they have on the nuclear localization of PB1 in transfected cells. We were particularly surprised that mutations which had only a marginal effect on nuclear localization were incompatible with normal viral growth. As no other function has been identified for the residues, we speculated that RanBP5 binding may have an additional function. At the time of writing no structural data exists for the region of PB1 containing RanBP5-binding residues, but in order to bind RanBP5 these residues must presumably lie on the surface of PB1. We have shown that RanBP5 binds to a highly basic region of PB1, and the RanBP5-binding residues mutated in this study are predicted to be particularly hydrophilic [analysis by EMBOSS PepInfo with a hydrophobicity plot using consensus parameters (Sweet & Eisenberg, 1983); data not shown]. Exposed basic regions of proteins can aggregate with cytoplasmic polyanions such as RNA; consistent with this a fragment of PB1 including the NLS has been shown to bind GTP in vitro (Asano & Ishihama, 1997). RanBP5 has been shown to act as a cytoplasmic chaperone for cellular proteins with exposed basic motifs (Ja¨kel et al., 2002). Although this model has not been directly tested for PB1, the fact that RanBP5 binds to an exposed basic region supports a model in which it shields PB1 from inappropriate interactions prior to the assembly of the complete trimeric polymerase in the nucleus (Deng et al., 2006). In a similar fashion, heat shock protein 90 (Hsp90), which binds to PB1 (Fig. 4b), has been proposed to act as a chaperone during the assembly of the viral polymerase complex (Naito et al., 2007).

In summary, we have identified basic residues in PB1 required for binding to RanBP5. This interaction with RanBP5 contributes to the nuclear accumulation of PB1, though this also requires an interaction of PB1 with PA. The effect of RanBP5-binding mutations on viral growth and the conservation of key residues suggest that this interaction contributes to viral fitness; it does not, however, appear to be a likely determinant of host tropism.

METHODS

Cells, plasmids and antisera. Human embryonic kidney 293T cells, Madin–Darby bovine kidney (MDBK) cells and Vero cells were cultured in minimum essential medium (MEM; PAA), supplemented with 10% FCS and 2 mM L-glutamine, at 37 °C and 5% CO2. Plasmids encoding the genomic segments of influenza A/WSN/33 in pPOLI (Fodor et al., 1999) and its untagged and TAP-tagged proteins in pcDNA (Deng et al., 2005; Engelhardt et al., 2005; Fodor et al., 2002; Fodor & Smith, 2004) have been described previously. Introduction of NLS mutations into pcDNA-PB1 by site-directed mutagenesis has been described previously (Fodor & Smith, 2004); using the same method NLS mutations were made in pcDNA-PB1-TAP and pPOLI-PB1-RT. The PB1 genes of avian influenza viruses (Howard et al., 2007; Kashiwagi et al., 2009; Leung et al., 2010) were subcloned from existing plasmids into a pcDNA3 plasmid (Invitrogen) encoding a C-terminal TAP tag, as described previously (Fodor & Smith, 2004). To produce truncated forms of pcDNA-PB1–TAP, fragments of the WSN PB1 gene were subcloned into pcDNA–GFP–TAP (Engelhardt et al., 2005), replacing the GFP gene. To generate pcDNA–TAP–RanBP5, the human RanBP5 gene (a kind gift of Dr G. Görlich, Max Planck Institute for Biophysical Chemistry, Göttingen) was subcloned into pcDNA–TAP–CTD (Engelhardt et al., 2005), replacing the CTD sequence, and extended by inserting annealed oligonucleotides to include an additional 18 aa found at the N terminus of the longest isoform of RanBP5 (Swiss-Prot accession number O00410-3). The identity of novel constructs was confirmed by sequencing.

The polyclonal antisera rabbit anti-karyopherin β3 (H-300), goat anti-CBP (W-15) and goat anti-PB1 (vK-20) were obtained from Santa Cruz Biotechnology and used for Western blotting. Polyclonal rabbit anti-PB1 (Toyoda et al., 1996), used for immunofluorescence, was a kind gift of Dr Tetsuya Toyoda (Shanghai Pasteur Health Research Foundation). Secondary antibodies were rabbit anti-goat and goat anti-rabbit peroxidase conjugates (Sigma) for Western blotting and donkey anti-rabbit Cy3 IgG (Jackson Immunoresearch) for immunofluorescence.

Purification and detection of TAP-tagged proteins. 293T cells were transfected with pcDNA plasmids encoding the indicated viral proteins using Turbofect (Fermentas). After 2 days, purification of TAP-tagged protein was carried out, as described previously (Deng et al., 2006). Briefly, cells were lysed and incubated with immunoglobulin G-Sepharose (GE Healthcare) at 4 °C; after washing, the TAP tag was cleaved with tobacco etch virus protease (Invitrogen) to release bound proteins. To detect TAP-purified proteins, samples were analysed by SDS-8% PAGE, followed by either silver staining (SilverXpress; Invitrogen) or Western blotting using standard techniques. For Western blotting, proteins were detected by chemiluminescence using specific antibodies, ECL Western blotting Reagents (GE Healthcare) and Super RX film (Fujifilm). Densitometry was carried out on digital images of the developed film using AIDA v.3.27.001 (Raytest). Sample loading of protein gels and exposure times of film were adjusted empirically to ensure the signal from each sample was within a linear range.

Immunofluorescence. Vero cells grown on 13 mm coverslips in 24-well plates were transfected with pcDNA plasmids expressing the indicated proteins using TurboFect (Fermentas). After 2 days, cells were fixed with 4% paraformaldehyde in 0.25 M HEPEs (pH 8) for 15 min at room temperature, permeabilized with 0.2% Triton X-100 for 7 min, and blocked with 3% FCS in PBS. The samples were then labelled with a 1/200 dilution of anti-PB1 (preadsorbed to fixed and permeabilized MDBK cells) for 1 h, washed, then incubated with secondary antibody (1/100 000) and DAPI (20 ng ml−1) for 1 h before rinsing and mounting in Mowiol (Polysciences). Images were
collected using a Zeiss Axioplan microscope; detailed images were taken using a Zeiss ×20 objective with a numerical aperture (NA) of 0.50, and for scoring three images were taken of each coverslip using a Zeiss ×10 objective (NA 0.30). Images were processed with the GNU Image Manipulation Program using consistent settings and anonymized before scoring protein localization.

**Virus rescue and growth curves.** Recombinant influenza A/WSN/33 viruses were rescued in 293T cells using a plasmid-based rescue system, followed by amplification in MDBK cells (Fodor et al., 1999). WT PB1 was expressed from pcDNA for all experiments, and vRNA encoding WT or mutant PB1 was expressed from pPOLI. After amplification of virus, plaque assays were carried out in MDBK cells (Carr et al., 2006). To produce a working stock of the NLS2 mutant virus a second round of amplification was carried out for 48 h in MDBK cells. To determine the growth kinetics of this amplified virus and of WT virus, MDBK cells were infected at an m.o.i. of 0.01 for multi-cycle growth, and viruses harvested at the indicated time points for plaque assay. To confirm the presence of specific mutations in the amplified virus, MDBK cells were infected, and total RNA isolated after 48 h using TRIzol reagent (Invitrogen). The primers 5'-CC-AGCACAAAAATGCTTAAACGCAACTT-3' and 5'-GGTGGTTAGCTTCATTGGCAGGT-3' were used for reverse transcription, PCR, and sequencing of a 1.1 kb section of the PB1 gene; conditions are available on request.

**Sequence analysis.** For comparison of influenza A virus PB1 sequences, the UniProtKB/Swiss-Prot database was searched for full-length influenza A virus PB1 sequences on 10 November 2010; 92 sequences were retrieved (Supplementary Table S1, available in JGV online), aligned using M-COFFEE (Moretti et al., 2005), and logos produced using WebLogo 2.8.2 (Crooks et al., 2004). Fifty-three of the sequences were from human isolates (including human-derived laboratory strains such as WSN), 30 from avian isolates and nine from non-human mammalian isolates. Initial dates of isolation ranged from 1918 to 2007 (median date 1977). For comparison of RanBP5 sequences the longest of the three isoforms of H. sapiens RanBP5 (986 residues; NCBI accession number XP_416978) using LALIGN (Huang & Miller, 1991). The best local alignment produced using default settings was considered (E-value <10^-4).

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