Influenza A virus NS1 protein binds p85β and activates phosphatidylinositol-3-kinase signaling

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Influenza A virus NS1 is a multifunctional protein, and in virus-infected cells NS1 modulates a number of host-cell processes by interacting with cellular factors. Here, we report that NS1 binds directly to p85β, a regulatory subunit of phosphatidylinositol-3-kinase (PI3K), but not to the related p85α subunit. Activation of PI3K in influenza virus-infected cells depended on genome replication, and showed kinetics that correlated with NS1 expression. Additionally, it was found that expression of NS1 alone was sufficient to constitutively activate PI3K, causing the phosphorylation of a downstream mediator of PI3K signal transduction, Akt. Mutational analysis of a potential SH2-binding motif within NS1 indicated that the highly conserved tyrosine at residue 89 is important for both the interaction with p85β, and the activation of PI3K. A mutant influenza virus (A/Udorn/72) expressing NS1 with the Y89F amino acid substitution exhibited a small-plaque phenotype, and grew more slowly in tissue culture than WT virus. These data suggest that activation of PI3K signaling in influenza A virus-infected cells is important for efficient virus replication.

Akt phosphorylation | multifunctional NS1 protein | reverse genetics

Influenza A viruses are globally important human and animal respiratory pathogens that are responsible for both seasonal "flu" outbreaks, and periodic world-wide pandemics (1). The multifunctional NS1 protein of influenza A is widely regarded as a virulence factor (2), and contributes significantly to disease pathogenesis by modulating many virus and host-cell processes (3–5). A major role of NS1 is as a type I IFN antagonist: NS1 down-regulates host innate IFN-mediated antiviral responses during infection (3–5). To perform such activities, NS1 functionally interacts with virus- and/or cell-derived factors: e.g., NS1 blocks the activation of two IFN-inducible antiviral proteins: 2′-5′-oligoadenylate synthetase (2′-5′-OAS), and the dsRNA-dependent protein kinase R (PKR). In infected cells, the binding of NS1 to dsRNA (a putative by-product of viral RNA genome replication) has been implicated in the inhibition of cellular 2′-5′-OAS (6). dsRNA-binding by NS1 may also be a requirement for blocking the activation of PKR *in vitro* (7), but studies now suggest that this inhibition, both *in vitro* and *in vivo*, may occur via direct interaction of NS1 with PKR (8, 9).

The biological activities of NS1 are likely to be strain- and/or cell-type specific (10). For influenza virus A/Udorn/72 (Ud), it has been shown that NS1 interacts with two cellular proteins involved in mRNA processing and transport: the 30-kDa subunit of the cleavage and polyadenylation specificity factor (CPSF30), and poly(A)-binding protein II (PABII) (11, 12). The direct binding of Ud/NS1 to these two proteins blocks the posttranscriptional processing of the 3′ ends of cellular pre-mRNAs, inhibiting nucleo-cytoplasmic mRNA export, and limiting both IFN-β production and the host response to IFN (11–14). However, studies with influenza virus A/Puerto Rico/8/34 (PR8) suggest that during infection NS1 blocks the pretranscriptional nuclear translocation of both IRF-3 and NF-κB (thus limiting IFN-β promoter activation), possibly by binding and sequestering dsRNA away from intracellular sensors (15, 16). Interestingly, PR8/NS1 was unable to block the activation of an IFN-stimulated response element (ISRE) reporter construct in response to exogenous IFN-α (10). In contrast, the NS1 proteins of other influenza virus strains, including A/Victoria/3/75 (Vic), efficiently limit ISRE activation probably by blocking a posttranscriptional process (10). These data suggest that, unlike other NS1 proteins, PR8/NS1 is incapable of inhibiting the posttranscriptional processing of cellular pre-mRNAs, and thus may limit the synthesis of IFN-β by a pretranscriptional mechanism that is distinct from the posttranscriptional process adopted by many other strains.

We generated a HEp2 cell line that constitutively expresses PR8/NS1 as part of our studies on viral evasion of host innate immunity. It was found that PR8/NS1 binds specifically to the p85β regulatory subunit of phosphatidylinositol-3-kinase (PI3K), and subsequently activates PI3K signaling. These observations were extended by finding that Ud/NS1 and Vic/NS1 also specifically bind p85β. PI3K is a heterodimeric protein/lipid kinase that consists of a regulatory subunit (usually p85α, p85β, or p55γ), and a p110 catalytic subunit (α, β, γ, or δ) (17). Activation of PI3K causes the generation of the second messenger phosphatidylinositol (3,4,5)-triphosphate, which acts to recruit pleckstrin homology domain-containing proteins (such as the key downstream mediator of PI3K, Akt), to membranes, where they are further activated by phosphorylation (18–20). The modulation of host-cell PI3K signaling is a target for many acute- and chronic- disease-causing viruses, and induces a number of physiological changes within cells that aid virus replication (i.e., regulation of virus/host gene transcription, protein synthesis, or cell survival) (21). We observed that activation of PI3K also aids the replication of influenza A virus. By using reverse genetics, a mutant influenza virus (Ud strain) was generated that contains an amino acid substitution in NS1 at the highly conserved tyrosine residue 89. This mutant is unable to bind p85β or activate PI3K signaling, and forms smaller plaques and grows less efficiently than WT Ud influenza virus.

Results

The NS1 Protein of Influenza A Virus Interacts with p85β. A HEp2 cell line was isolated that constitutively expresses a V5 epitope-tagged form of PR8/NS1, an influenza virus protein that has been reported to limit both the production and downstream effects of IFN (3, 4). Characterization of the PR8/NS1-expressing cell line confirmed previously observed NS1 functions: NS1 limits PKR activation in response to virus infection or synthetic dsRNA (7, 9, 22) (Fig. 1*a*), and NS1 localizes predominantly to the nucleus (10, 16, 23) (Fig. 1*b*). Antibody to the V5-tag was used to immunoprecipitate NS1 from cell lysates [or as a control, the V5-tagged V protein of Sendai virus (SeV); also

Conflict of interest statement: No conflicts declared.

Abbreviations: PKR, protein kinase R; PR8, influenza virus A/Puerto Rico/8/34; PI3K, phosphatidylinositol-3-kinase; SeV, Sendai virus; p.i., postinfection; MDCK, Madin–Darby canine kidney.

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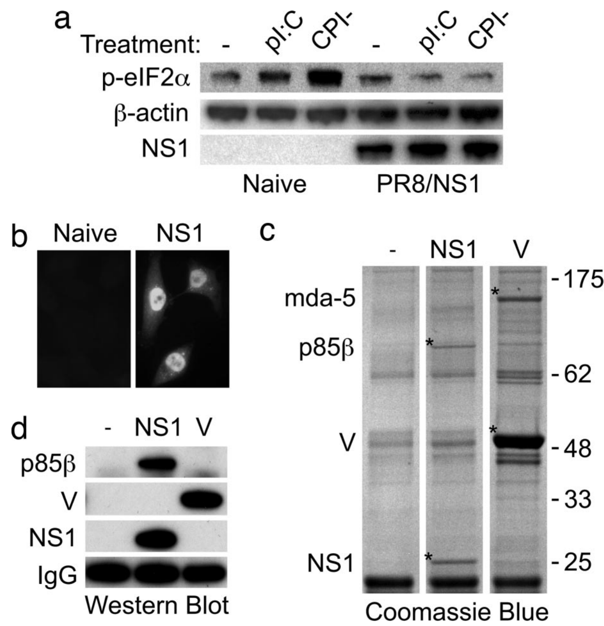

Fig. 1. Characterization of an NS1-expressing HEp2 cell line. (*a*) PKR activation in naïve or PR8/NS1-expressing HEp2 cells was assessed by the induction of phospho-eIF2α (Ser-51) in response to mock-transfection (−), transfection of poly I:C (pI:C), or RNA virus infection (multiplicity of infection of 5 PFU per cell, CPI−). Twenty hours after treatment, monolayers were harvested, and protein lysates were separated by SDS/PAGE followed by transfer to PVDF membrane. Phospho-eIF2α (Ser-51) and β-actin were detected by using specific antibodies. NS1 expression was detected by using an anti-V5 mAb. (*b*) Predominant nuclear localization of V5-tagged NS1 in NS1-expressing HEp2 cells was confirmed by indirect-immunofluorescence using an anti-V5 mAb. (*c*) p85β interacts with NS1. Soluble antigen extracts from $\approx 2 \times 10^7$ naïve, NS1-, or SeV/V-expressing HEp2 cells were immunoprecipitated with anti-V5 mAb cross-linked to protein G Sepharose. Precipitated proteins were separated by electrophoresis through 4–12% polyacrylamide gradient gels and visualized by Coomassie blue staining. Polypeptide bands denoted by an asterisk were excised and identified by mass spectrometry. Molecular mass markers (in kilodaltons) are indicated on the right. (*d*) Immunoprecipitates were prepared as in *c* and separated by electrophoresis followed by transfer to PVDF membrane. p85β was detected by using a specific mAb. Anti-V5 mAb was used to detect V5-tagged NS1 and SeV/V.

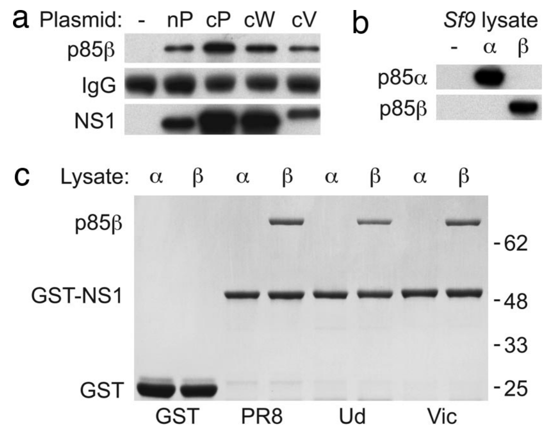

Fig. 2. The NS1 proteins of several influenza virus strains bind efficiently to p85β but not p85α. (*a*) 293T cells were transfected for 48 h with empty vector (−), a plasmid expressing the N-terminal V5-tagged NS1 protein of PR8 (nP), or plasmids expressing the C-terminal V5-tagged NS1 proteins of PR8 (cP), WSN (cW), or Vic (cV). Soluble antigen extracts were immunoprecipitated with anti-V5 antibody and precipitates separated by SDS/PAGE followed by transfer to PVDF membrane. Endogenous p85β was detected by using a specific mAb. Anti-V5 mAb was used to detect V5-tagged NS1 proteins. (*b*) *Sf9* cells were infected (or mock, −) with recombinant baculoviruses expressing either p85α or p85β. Equal amounts of soluble lysate were separated by electrophoresis, transferred to PVDF membrane, and probed for p85α or p85β expression using specific mAbs. (*c*) Equal amounts of soluble *Sf9* cell lysate from *b* were mixed with GST or GST-NS1 (PR8, Ud, or Vic) immobilized onto glutathione-agarose beads. After washing, protein complexes were dissociated from the beads and separated by SDS/PAGE through 4–12% polyacrylamide gradient gels. Polypeptides were stained with Coomassie blue, and protein identification was confirmed by mass spectrometry. Molecular mass markers (in kilodaltons) are indicated on the right.

constitutively expressed in a HEp2 cell line]. Analysis of the coimmunoprecipitated proteins by mass spectrometry showed that, as expected, mda-5 associated with SeV/V (24) (Fig. 1*c*). A parallel analysis of cellular proteins coprecipitated with the PR8/NS1 protein identified a polypeptide species of ≈90 kDa (Fig. 1*c*). This band could not be observed in pull-downs from either naïve or SeV/V-expressing extracts. Mass spectrometry identified the ≈90-kDa protein as p85β, a regulatory subunit of PI3K (probability-based Mowse score of 520, the next nearest score being 111; only scores over 45 are considered significant). p85α, a related PI3K subunit that is ≈55% identical to p85β, was not identified in the band. The NS1:p85β interaction was confirmed by immunoblot analysis of similar immunoprecipitates (Fig. 1*d*).

The NS1 Proteins of Different Influenza A Viruses Bind p85β but Not p85α. To determine whether the interaction of p85β with NS1 is a feature common to other influenza virus strains, we investigated the coprecipitation of p85β with the NS1 proteins of the related H1N1 strain A/WSN/33 (WSN), or the H3N2 strain A/Victoria/3/75 (Vic). We were unable to isolate a cell line stably expressing Vic/NS1, possibly due to toxicity caused by this protein inhibiting cellular pre-mRNA processing (12, 25); a function not shared by PR8/NS1 (10). Therefore, 293T cells were transfected with plasmids expressing either the C-terminal V5-tagged NS1 protein of each strain, N-terminal V5-tagged PR8/NS1, or empty vector (negative control). Analysis of proteins immunoprecipitated with the V5 antibody showed that endogenous p85β interacted with the NS1 proteins of all three influenza A virus strains (Fig. 2*a*). The position of the V5-tag (N- or C-terminal) had no effect on the interaction.

To confirm the specific and direct binding of NS1 to p85β *in vitro*, a GST-NS1 fusion protein pull-down assay was used. Recombinant GST or GST-NS1 (NS1 of PR8, Vic, or Ud) was expressed and purified from *Escherichia coli* and immobilized onto glutathione-agarose beads. Equal amounts of these beads were then used as bait to affinity isolate an excess of baculovirus-expressed p85α or p85β (the expression of these proteins in infected *Sf9* cells was confirmed before the assay by immunoblot; Fig. 2*b*). Strikingly, a single protein band of ≈90 kDa was specifically isolated from the p85β expressing lysate by each GST-NS1 (PR8, Vic, and Ud) but not by GST alone (Fig. 2*c*). The identity of the ≈90-kDa polypeptide was confirmed as p85β by mass spectrometry. Because GST-NS1 and p85β were the only protein bands visible in this pull-down experiment, the interaction between NS1 and p85β is likely to be direct. Interestingly, no proteins were precipitated by GST-NS1 from the p85α-expressing lysate (Fig. 2*c*).

NS1 Expression Induces the Phosphorylation of Akt (Ser-473) in a PI3K-Dependent Manner. Akt is a serine/threonine protein kinase that serves as a key downstream mediator of PI3K signaling (18, 20). In response to active PI3K, Akt is recruited to membranes and phosphorylated at residues Thr-308 and Ser-473 (by PDK1 and the putative PDK2, respectively) (18, 19). As NS1 was found to bind the p85β regulatory subunit of PI3K, we investigated whether PI3K/Akt signaling was activated after influenza A

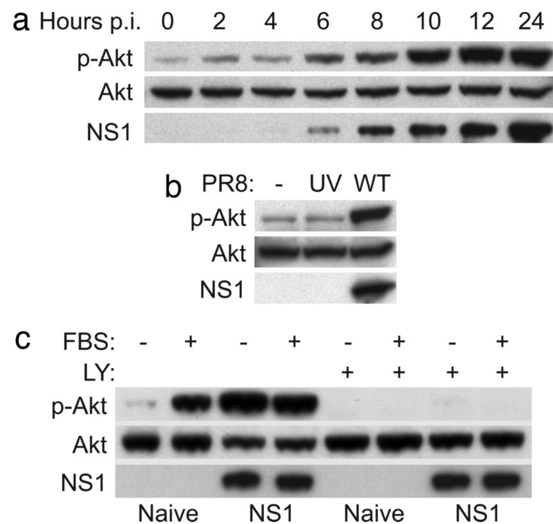

Fig. 3. NS1 induces the phosphorylation of Akt (Ser-473) in a PI3K-dependent manner. (*a*) Time course of Akt phosphorylation at Ser-473 after influenza A virus (PR8) infection. Confluent serum-starved monolayers of 1321N1 cells were infected with influenza virus at a multiplicity of infection of 5 PFU per cell, and total cell lysates were harvested at various times p.i. Lysates were separated by SDS/PAGE followed by transfer to PVDF membrane. Phospho-Akt (Ser-473) was detected by using a specific mAb. Total Akt was detected by using a pAb and acted as a loading control. NS1 expression was detected by using a specific pAb. (*b*) UV-inactivated influenza virus (PR8) does not induce Akt phosphorylation. Serum-starved 1321N1 monolayers were infected (or mock, −) as in *a* with either untreated (WT) or UV-inactivated (UV) virus. Lysates were prepared 20 h p.i. and analyzed as in *a*. (*c*) Serum-starved naïve or PR8/NS1-expressing 1321N1 monolayers were treated (or mock) with 5% FBS for 1 h. In a duplicate experiment, cells were also treated with 25 μM LY294002 (a specific PI3K inhibitor; LY). Cell lysates were analyzed as in *a*. NS1 was detected by using an anti-V5 mAb.

virus infection. Serum-starved monolayers of 1321N1 cells (used widely in PI3K activation studies) were infected with influenza virus (PR8), and the phosphorylation state of Akt (Ser-473) was analyzed in total cell lysates at various times postinfection (p.i.) (Fig. 3*a*). Increasing levels of phosphorylated Akt were observed during the time-course of infection, and phospho-Akt levels appeared concomitant with the expression of NS1 (an increase in phospho-Akt was first detectable ≈6 h p.i.). The induction of phospho-Akt depended on influenza virus genome replication, as prior UV-inactivation of the virus prevented phosphorylation of Akt (Fig. 3*b*).

To determine whether NS1 is responsible for the influenza virus activation of PI3K, a 1321N1 cell line was isolated that constitutively expressed PR8/NS1, and the phosphorylation state of Akt within these cells was investigated. In naïve 1321N1 cells, Akt phosphorylation (Ser-473) decreased rapidly in response to serum-withdrawal (Fig. 3*c*, lanes 1 and 2). In contrast, there was no apparent reduction in phospho-Akt when NS1-expressing cells were similarly serum-starved (Fig. 3*c*, lanes 3 and 4). This finding indicates that NS1 expression alone can induce the phosphorylation of Akt without the absolute requirement for additional virus-derived factors. Furthermore, we found that the phosphorylation of Akt by NS1 depends on PI3K activation, as treatment of cells with the specific inhibitor of PI3K, LY294002 (26), was able to negate the effect of NS1 expression (Fig. 3*c*, lanes 5–8).

We next investigated whether commercially available inhibitors of PI3K, such as LY294002 and wortmannin (27), specifically affected the replication of influenza A virus. In low multiplicity multistep growth curves, neither drug had a detrimental affect on the paramyxovirus, PIV-5, whereas a dramatic

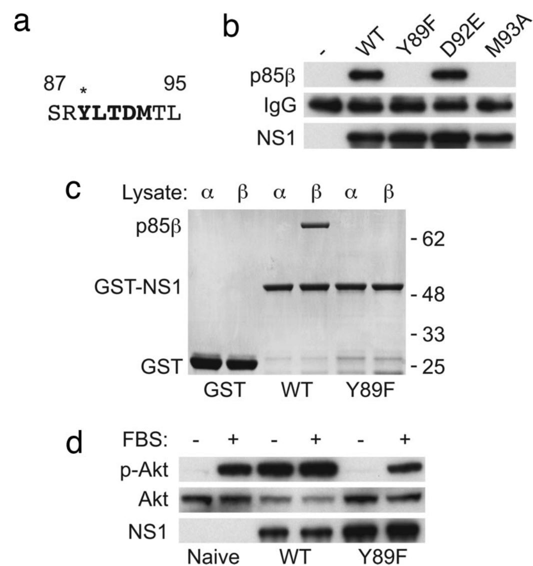

Fig. 4. Binding of p85β and activation of PI3K requires residues from a Y*XX*M-like motif in NS1. (*a*) Amino acid sequence of PR8/NS1 residues 87–95. The putative Y*XX*M-like motif is shown in bold. Y89 (denoted by an asterisk) is totally conserved among all NS1 proteins of influenza A strains sequenced to date. (*b*) Binding of NS1 to p85β requires Y89 and M93. 293T cells were transfected for 48 h with either empty vector (−), a plasmid expressing WT V5-tagged PR8/NS1, or plasmids expressing V5-tagged PR8/NS1 proteins with single amino acid substitutions (Y89F, D92E, or M93A). Soluble antigen extracts were immunoprecipitated with anti-V5 antibody and precipitates separated by SDS/PAGE followed by transfer to PVDF membrane. p85β was detected by using a specific mAb. Anti-V5 mAb was used to detect the V5-tagged NS1 mutants. (*c*) WT GST-PR8/NS1 and GST-PR8/NS1 with the Y89F mutation were used as bait to precipitate p85α or p85β from baculovirus-infected *Sf9* cell lysates. Protein complexes were analyzed as for Fig. 2*c*. (*d*) Expression of NS1 with the single amino acid substitution Y89F does not induce the phosphorylation of Akt at Ser-473. Serum-starved naïve, WT PR8/NS1-expressing (WT), or mutant PR8/NS1-expressing (Y89F) 1321N1 monolayers were treated (or mock-treated) with 5% FBS for 1 h. Cell lysates were separated by SDS/PAGE followed by transfer to PVDF membrane. Phospho-Akt (Ser-473) was detected by using a specific mAb. Total Akt was detected by using a pAb and acted as a loading control. NS1 (WT and Y89F) was detected by using an anti-V5 mAb.

decrease in the yield of PR8 was noted (Fig. 6, which is published as supporting information on the PNAS web site). However, because it has very recently been shown that inhibitors of PI3K activation may block influenza virus entry (28), the interpretation of studies using these compounds remains unclear. Interestingly, the authors of this paper (28) also noted that, unlike WT PR8, a recombinant PR8 virus lacking the NS1 ORF (termed delNS1) was unable to induce the phosphorylation of Akt at Ser-473 (28). This finding correlates with the data presented here, which indicates NS1 alone can activate PI3K/Akt signaling.

Binding of p85β and Activation of PI3K Requires Tyrosine-89 in the Effector Domain of NS1. Tyrosine-phosphorylated Y*XX*M motifs are considered consensus docking sites for the SH2 domains of PI3K p85 subunits (29). The amino acid sequence of PR8/NS1 contains a single tyrosine residue (Y89), which is four residues upstream of a methionine (M93) (Fig. 4*a*). Alignment of 1,546 NS1 protein sequences available in the public domain indicated that Y89 is absolutely conserved among all influenza A strains. Given the similarity in sequence between NS1 residues 89–93 and classical Y*XX*M motifs, the importance of this sequence for binding p85β was investigated. As the substitution of aspartic acid for glutamic acid at position 92 has been previously identified as a potential pathogenicity factor in some H5N1 isolates

(30), it was also included in a panel of NS1 mutants that were assessed for their ability to interact with endogenous p85β. V5-tagged NS1 constructs were expressed transiently in 293T cells and immunoprecipitated with anti-V5 antibody. Coprecipitating p85β was detected by immunoblot analysis. As shown in Fig. 4*b*, both WT PR8/NS1 and PR8/NS1-D92E precipitated p85β efficiently. In contrast, PR8/NS1 constructs containing Y89F or M93A substitutions were unable to precipitate p85β. The requirement for Y89 in binding p85β was further investigated by expressing and purifying a GST-PR8/NS1-Y89F fusion protein, and testing its *in vitro* interaction with baculovirus-expressed p85β. As expected, the NS1-Y89F mutant did not bind to either p85α or p85β (Fig. 4*c*). Similarly, a Ud/NS1-Y89F construct was also unable to bind either p85α or p85β (data not shown).

To investigate whether NS1-Y89F was able to activate PI3K/Akt signaling, a 1321N1 cell line that constitutively expresses the PR8/NS1-Y89F mutant protein was isolated. The intracellular localization of PR8/NS1-Y89F in these cells was, like that of WT NS1, predominantly nuclear (data not shown). Serum starvation of both naïve and NS1-Y89F expressing cells for 1 h caused a marked reduction in phospho-Akt (Ser-473) levels (Fig. 4*d*, lanes 1, 2, 5, and 6). In contrast, there was no apparent reduction in Akt phosphorylation when cells expressing WT NS1 were similarly serum-starved (Fig. 4*d*, lanes 3 and 4). These data indicate that the binding of p85β (and activation of PI3K) absolutely requires the highly conserved tyrosine residue at position 89 of NS1.

Characterization of a Recombinant Influenza A Virus Expressing NS1 with a Y89F Amino Acid Substitution. To study the importance of NS1-mediated PI3K activation during influenza A virus replication, reverse genetics was used to engineer recombinant viruses (rWSN and rUd) that expressed their strain-specific NS1 proteins with Y89F amino acid substitutions. The rUd NS1-Y89F mutant virus was clearly attenuated: it formed small plaques in Madin–Darby canine kidney (MDCK) cells (Fig. 5*a*), and grew to infectious titers ≈10-fold lower than WT rUd virus during single-step growth analysis (Fig. 5*b*). Hemagglutination (HA) assays showed a similar reduction in rUd NS1-Y89F titers, indicating that the attenuation was not due to an increase in the number of defective virus particles (data not shown). Western blot analysis confirmed that PI3K activation (i.e., phosphorylation of Akt at Ser-473) did not occur in cells infected with the rUd NS1-Y89F virus (Fig. 5*c*). Interestingly, the rWSN NS1-Y89F virus did not appear to exhibit an attenuated phenotype under tissue culture conditions (data not shown).

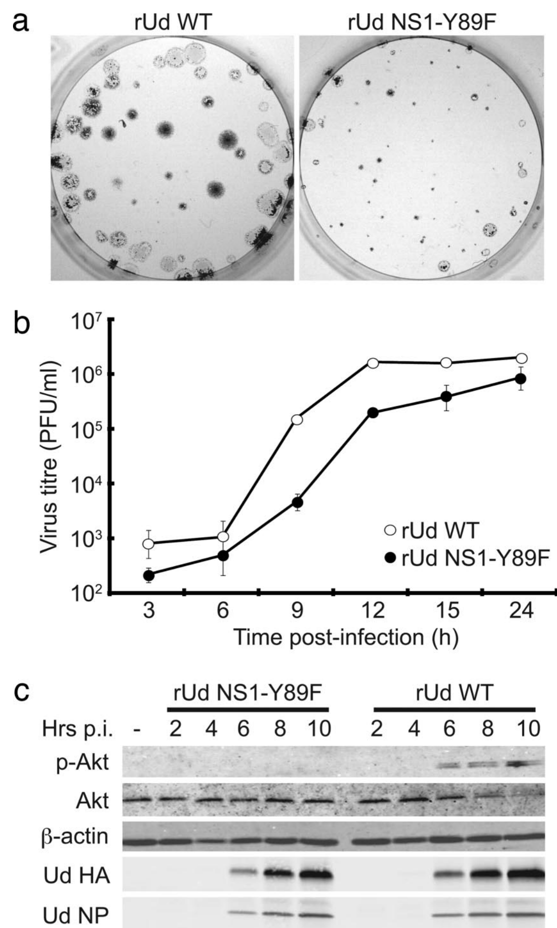

Fig. 5. Characterization of a recombinant Ud virus (rUd) expressing NS1 with the Y89F amino acid substitution. (*a*) rUd NS1-Y89F forms smaller plaques than WT rUd virus. Confluent MDCK monolayers were infected at equal multiplicities of infection with either WT rUd or rUd NS1-Y89F. Plaques were fixed 4 days p.i. and immunostained with goat anti-A/Udorn/72 virus serum. (*b*) Single-step growth analysis of WT rUd (open circles) and rUd NS1-Y89F (filled circles). Recombinant Ud viruses were used to infect MDCK cell monolayers (in the absence of TPCK-trypsin) at a multiplicity of infection of 3 PFU per cell. Virus-containing supernatants were harvested at various times p.i. and titrated by plaque assay. The mean titers from three independent experiments are shown. Error bars represent standard deviation. (*c*) Phosphorylation of Akt in response to infection with either WT rUd or rUd NS1-Y89F viruses. Confluent serum-starved monolayers of 1321N1 cells were infected at a multiplicity of infection of 5 PFU per cell, and total cell lysates were harvested at various times p.i. Lysates were separated by SDS/PAGE followed by transfer to PVDF membrane. Phospho-Akt (Ser-473) was detected by using a specific mAb. Total Akt was detected by using a pAb, and β-actin acted as a loading control. Ud hemagglutination and NP were detected by using goat anti-Ud serum.

Discussion

In this study, we report the direct interaction of influenza A virus NS1 protein with the p85β regulatory subunit of PI3K. Recently, the x-ray crystallographic structure of the NS1 effector domain has been solved (31). Y89, the absolutely conserved residue that is essential for PI3K binding and activation, lies exposed within a cleft formed at the interface between the two NS1 monomers (see Fig. 7, which is published as supporting information on the PNAS web site). Its location on the border of an extended chain region/helix means that it is well positioned for a role in binding p85β. However, residue M93 is mostly buried within the NS1 molecule, and therefore may not be directly involved in mediating the NS1:p85β interaction; rather, it may be important for maintaining the functional integrity of the NS1 structure. Thus, a linear Y*XX*M-like motif in NS1 may not be an absolute requirement for binding p85β. The structural constraints relating to the NS1:p85β interaction require further investigation.

The biological consequences of NS1 binding and activating PI3K remain to be elucidated, but given that Y89 is totally conserved, it seems probable that the activation of PI3K is a necessary function for all natural non-laboratory-adapted strains of influenza A virus. However, whereas rUd NS1-Y89F exhibited a small plaque phenotype and grew to titers lower than WT rUd virus, rWSN NS1-Y89F did not exhibit an altered phenotype in tissue culture cells. The reasons for this remain unclear. One possibility may be that viruses such as Ud require the early activation of PI3K to help counter the effects of blocking the posttranscriptional processing of cellular pre-mRNAs. In contrast, viruses such as A/PR8 (and A/WSN by relatedness), which may have lost their ability to inhibit mRNA processing (10) during species or cell adaptation, could have retained their capacity to activate PI3K despite this property not being essential for their growth in tissue culture. Alternatively, the need to activate PI3K may be a requirement primarily for efficient virus replication and spread *in vivo*.

Class IA PI3Ks are involved in many host-cell signal transduction processes, and ultimately induce the phosphorylation of a number of substrates, including BAD, caspase-9, GSK-3β, NF-κB, FKHR, MDM2, mTOR, and p70 S6-kinase (18, 20, 32). The finding that NS1 binds p85β, but not p85α, is an intriguing one. These two p85 isoforms are encoded by separate genes, yet exhibit $\approx$55% protein sequence identity and high structural homology. Both are present in a wide range of human tissues, but p85β expression is $\approx$40% that of p85α (33). p85α is thought to be involved in the major response pathway for most PI3K-mediated stimuli: the physiological role of p85β *in vivo* is largely unknown. A number of studies have attempted to establish distinct roles for p85α and p85β in the regulation of different PI3K-dependent or -independent pathways (33–37), but to our knowledge (other than a potential function in limiting T cell expansion; ref. 36), p85β has yet to be specifically associated with a role directly relevant to virus infection.

Recently, NS1 has been shown to limit the induction of numerous specific transcriptional events associated with the maturation of human dendritic cells (DCs) in response to virus infection (38). In particular, expression of IL-12 p35 (a subunit of the proinflammatory and immunoregulatory cytokine IL-12) was found to be inhibited in DCs infected with recombinant viruses expressing NS1 (38). IL-12 is a key regulator of host innate and adaptive immune responses during infection: it enhances the cytolytic activity of natural killer (NK) cells and $CD8^+$ T cells, and contributes to the optimal production of IFN-γ (39). Therefore, it is worth noting that IL-12 synthesis by DCs has previously been shown to be negatively regulated by PI3K-mediated signaling (40).

It will be of great interest to determine the precise downstream events (both generic and cell-type specific) that are mediated by the NS1:p85β induced activation of PI3K. The activation mechanism, involvement of p110 catalytic subunits, and possible kinase-independent functions of the NS1:p85β complex remain to be elucidated. Not only should these studies give us general insights into the biology of influenza A virus infection, but they may potentially aid our understanding of cellular PI3K signaling.

Materials and Methods

Cells, Viruses, and PI3K Inhibitors. HEp2, 293T, 1321N1, and MDCK cells were grown as monolayers in DMEM supplemented with 10% FBS. The WT parainfluenza virus-5 strain (PIV-5) and the IFN-sensitive strain (CPI−) were propagated and titrated in Vero cells. Influenza A virus strains PR8, A/Udorn/72, and A/WSN/33 were propagated in 10-day-old chicken eggs and titrated by plaque assay in MDCK cells as described (41). WT and mutant influenza A viruses (Ud and WSN genetic backbones) were generated by reverse genetics from cDNAs as described (41, 42). Tyrosine 89 in the NS1 proteins of both WSN and Ud was changed to phenylalanine by PCR mutagenesis of the relevant NS segment cDNA (pHH21 vector). UV-inactivation of viruses was carried out as described (10). LY294002 (Calbiochem, San Diego, CA), LY303511 (Alexis Biochemicals, Lausanne, Switzerland), and wortmannin (Sigma, St. Louis, MO) were used as directed by the manufacturer. Transfection of cells by plasmids (and synthetic dsRNA; poly I:C) were carried out as described (24).

Generation of Cell Lines Expressing Recombinant Proteins. cDNAs encoding for the NS1 proteins of influenza strains PR8, A/Victoria/3/75 (Vic), A/WSN/33 (WSN), and A/Udorn/72 (Ud) were amplified by PCR from existing NS gene clones (41, 42) [PR8 and Vic clones were provided by S. Goodbourn, St. George's, University of London (10)]. Silent splice acceptor mutations were made by overlap PCR as described by others (43). PCR products were ligated between the SpeI and NdeI sites of modified bicistronic expression vectors, derived from the self-inactivating lentivirus vector pHR-SIN-CSGW (44). The vectors express the recombinant proteins with a V5-tag fused to either the N or C terminus (45, 46). The same strategy allowed the generation of a vector expressing the V protein of Sendai virus (Fushimi strain). As required, overlap PCR was used to introduce specific site-directed point mutations into the NS1 encoding cDNAs. The integrity of each construct was confirmed by sequencing. Generation of lentiviruses and production of stable cell lines constitutively expressing the protein of interest were carried out as described (44).

GST-NS1 Affinity Isolation of p85β. Recombinant baculoviruses expressing bovine p85α and p85β were kindly provided by B. Vanhaesebroeck (Ludwig Institute for Cancer Research, London, U.K.) (47). *Spodoptera frugiperda* (*Sf9*) cells were maintained, infected, and harvested as described (48). cDNAs encoding for the relevant NS1 protein were amplified from lentivirus vectors by PCR using gene-specific primers. Each forward primer contained a coding region (GAAAACCTG-TATTTTCAGGGCGCC) for the cleavage sequence of tobacco etch virus (TEV) protease. Digested PCR products were ligated between the EcoRI and NotI sites of pGEX-4T3 (Amersham Pharmacia, Piscataway, NJ) and transformed into *E. coli* strain BL-21 for optimum expression. GST-fusion proteins were expressed and purified onto glutathione–agarose beads (Sigma) and used to capture baculovirus-expressed proteins as described (48, 49).

Immunoanalyses and SDS/PAGE. Immunofluorescence, immunoprecipitation, SDS/PAGE, and immunoblot analysis were carried out as described (48, 50). Plaque staining for immunological specificity (A/Udorn/72) was also performed as described (41). Covalently cross-linked immunoaffinity matrices were prepared as directed (51). V5, p85α, and p85β antibodies were purchased from Serotec (London, U.K.). Akt, phospho-Akt, and phospho-eIF2α antibodies were from Cell Signaling Technology (Beverly, MA). The β-actin antibody was from Sigma. Rabbit anti-NS1 antibody was provided by P. Digard (University of Cambridge, Cambridge, U.K.) (52). Goat serum raised to purified Ud virus (goat anti-Ud) was used to detect hemagglutination and NP.

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