Avian and 1918 Spanish Influenza A Virus NS1 Proteins Bind to Crk/CrkL Src Homology 3 Domains to Activate Host Cell Signaling*

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NS1 (nonstructural protein 1) is an important virulence factor of the influenza A virus. We observed that NS1 proteins of the 1918 pandemic virus (A/Brevig Mission/1/18) and many avian influenza A viruses contain a consensus Src homology 3 (SH3) domain-binding motif. Screening of a comprehensive human SH3 phage library revealed the N-terminal SH3 of Crk and CrkL as the preferred binding partners. Studies with recombinant proteins confirmed avid binding of NS1 proteins of the 1918 virus and a representative avian H7N3 strain to Crk/CrkL SH3 but not to other SH3 domains tested, including p85α and p85β. Endogenous CrkL readily co-precipitated NS1 from cells infected with the H7N3 virus. In transfected cells association with CrkL was observed for NS1 of the 1918 and H7N3 viruses but not A/Udorn/72 or A/WSN/33 NS1 lacking this sequence motif. SH3 binding was dispensable for suppression of interferon-induced gene expression by NS1 but was associated with enhanced phosphatidylinositol 3-kinase signaling, as evidenced by increased Akt phosphorylation. Thus, the Spanish Flu virus resembles avian influenza A viruses in its ability to recruit Crk/CrkL to modulate host cell signaling.

Pandemic as well as seasonal outbreaks of influenza A virus represent major threats to global public health. In the last century three major pandemics have occurred, in 1918, 1957, and 1968, caused by H1N1 (Spanish flu), H2N2 (Asian flu), and H3N2 (Hong Kong flu) viruses, respectively. Of these, the Spanish flu was the most severe and is estimated to have caused over 40 million deaths worldwide (1). Recent human infections by highly pathogenic H5N1 avian influenza A viruses have increased the concern that another global pandemic may occur.

Influenza A virus belongs to the Orthomyxoviridae family of enveloped viruses. Its genome is organized into eight single-stranded, negative-sense RNA segments that code for 11 identified viral proteins (2). NS1 (nonstructural protein 1) is encoded by the shortest RNA segment 8. It is expressed early in viral replication cycle, and it is not a component of the virus particle (2). Instead, NS1 is a multifunctional virulence factor that promotes virus replication in the host cell and helps to evade antiviral immunity (3–5). In particular, NS1 uses several mechanisms to prevent suppression of influenza A virus replication by the type I interferon system of the host.

Recent studies have demonstrated that during influenza A virus infection NS1 protein activates the phosphatidylinositol 3-kinase (PI3K) signaling pathway, apparently via its association with the p85 regulatory subunit of PI3K (6–9). Activation of the PI3K pathway seems to be important for influenza A virus replication, because in cell culture studies recombinant viruses with mutations that prevented binding of NS1 to p85 formed much smaller plaques and grew to 10-fold lower titers than the wild-type virus (9). Moreover, compounds that inhibit PI3K can strongly suppress influenza A virus replication (7, 9, 10).

Hale et al. (9) showed that the tyrosine residue 89 (Tyr–89) of NS1 protein serves a critical role in mediating binding to p85β. This tyrosine lies in the context similar to a YXX[NM] motif, which upon tyrosine phosphorylation can serve as a high affinity binding site for the SH2 domain of p85 (11), but apparently NS1 interacts with p85β in an SH2-independent manner (12). In addition, p85 contains an SH3 domain, and Zhou and co-workers (6, 13) have suggested that a PXKP sequence in NS1, which resembles the consensus of an SH3-binding motif (see below), may also contribute to the p85 interaction.

SH3 domains are small protein modules that mediate inter- and intramolecular protein interactions and are often found in proteins regulating cellular signaling pathways, cytoskeletal organization, and membrane trafficking (14, 15). SH3 domains recognize short proline-rich sequences, which are typically characterized by (+)-XXPHXX (class I) or PXPHX(+) (class II) consensus sequences (where X is any amino acid; (+) indicates a positively charged residue; and Φ indicates a hydrophobic residue) (15–17). Since the discovery that the human immunodeficiency virus type 1 pathogenicity factor Nef regulates the
host cell via binding to SH3 domains of Src family protein kinases (18), SH3 domain binding capacity has been demonstrated for many other proteins encoded by viral as well as bacterial pathogens (19–24).

We noted that, unlike most other NS1 proteins from human strains of influenza, the NS1 sequence of the 1918 pandemic influenza virus (A/Brevig Mission/1/18/H1N1 (25)) contains a perfect class II consensus SH3-binding sequence, and in this regard it resembles many avian strains of influenza (26).

We have recently generated an essentially complete (n = 296) collection of human SH3 domains in the form of a phage display library to allow comprehensive and unbiased identification of preferred SH3 partners for cellular and viral ligand proteins of interest (27). In this study we have made use of this novel research tool to identify the Crk family adapter proteins as high affinity ligands for the NS1 protein of the 1918 virus.

**EXPERIMENTAL PROCEDURES**

**Cells and Viral Infections**—Human embryonic kidney 293FT, human hepatocellular carcinoma Huh-7, and human A549 lung carcinoma cell lines were maintained in Dulbecco’s modified Eagle’s medium high glucose supplemented with 0.6 μg/ml penicillin, 60 μg/ml streptomycin, 10% fetal bovine serum, and 2 mM glutamine. Influenza A virus strains A/mallard/Netherlands/12/2000 (H7N3) and A/Udorn/72 (H3N2) were grown in 11-day-old embryonated eggs, and the virus stock was aliquoted and stored at -70°C. The hemagglutination titers of the viruses were 256, and their infectivity in A549 cells was 1 plaque-forming unit/cell.

Virus infection of A549 cells was carried out in fetal bovine serum and antibiotics for 20 h at a multiplicity of 5 plaque-forming units/cell.

**Plasmid Constructs**—A synthetic gene fragment encoding A/mallard/Netherlands/12/2000 (H7N3) and A/Udorn/72 (H3N2) NS1 genes were generated by overlap PCR mutagenesis, cloned into pEBB-mycN, and verified by sequencing. pEBB-mycN is a derivative of pEBB (from Bruce Mayer, University of Connecticut) (29), in which translation starts upstream of the insert to include a Myc epitope-containing peptide (MEKQLISEEDLGS) at the N terminus. Codon changes to A/Brevig and A/Mallard NS1 gene were generated by overlap PCR mutagenesis, cloned into pEBB-mycN, and verified by sequencing. Bacterial GST and MBP fusion protein expression vectors for NS1 proteins were constructed by inserting the corresponding NS1 cDNAs into pGEX-4T1 (GE Healthcare) and pMAL-c2x (New England Biolabs) vectors, respectively. To generate the plasmid p85α-BP, an oligonucleotide duplex encoding for the peptide CLNCFRPLPPMNPPP (30) was inserted into the polylinker of pMAL-c2x.

A DNA fragment encoding for a 123-amino acid biotin acceptor domain from Propionibacterium shermanii transcarboxylase (start, MKLK; end, IKIG) was PCR-amplified from the PinPoint-Xa1 T-vector (Promega) and inserted between the GST gene and the multiple cloning site of pGEX-4T1 to generate pGEX-PP. Codon optimized cDNAs for the SH3 domains of Crk, CrkL, p85α, p85β, and Eps8L1 derived from the human SH3 library (27) were subsequently cloned in-frame after the biotin acceptor domain in pGEX-PP. To generate C-terminally biotinylated Crk and CrkL expression constructs, human CrkII and CrkL cDNAs with stop codons replaced by a KpnI site were fused with the above-described biotin acceptor domain fragment in pEBB.

**ISRE-Luc reporter plasmid contains a 30-bp interferon-stimulated response element-containing fragment from the ISG15 gene (31) in front of a minimal thymidine kinase promoter driving firefly luciferase expression (obtained from J. Darnell Jr., Rockefeller University, New York). As a control for transfection efficiency and cell viability, we used the plasmid pcDNA-Renilla, which was created by inserting Renilla luciferase cDNA from pRL-null (Promega) into pcDNA3.1/Hygro vector (Invitrogen).

**Antibodies and Other Reagents**—The following antibodies were used in this study: mouse anti-Myc (Sigma), mouse anti-CrkL (Upstate), mouse anti-phospho-Akt(Ser-473) (Cell Signaling Technology), rabbit anti-NP (32), mouse anti-he-magglutinin (HA, Santa Cruz Biotechnology), and guinea pig anti-NS1 (28). Streptavidin IRDye800CW, IRDye 800CW goat anti-mouse IgG, and IRDye680 goat anti-mouse IgG were from LI-COR Biotechnology. Secondary horseradish peroxidase-conjugated anti-guinea pig antibodies were from Jackson ImmunoResearch.

**Recombinant Proteins and Binding Assays**—GST and MBP fusion proteins expressed from pGEX-4T1 and pMAL-c2x protein expression vectors were purified using glutathione-Sepharose 4B (GE Healthcare) or amyllose resin (New England Biolabs), according to the manufacturer’s instructions. Screening of the human SH3 phage library using the GST-NS1 fusion proteins was done as described previously (27). Recombinant protein binding assay was done as in Kärkkäinen et al. (27) with some modifications. MBP-NS1 proteins, MBP-p85α-BP, or plain MBP was coated on 96-well plates (200 ng/well). Wells were blocked with 1.5% bovine serum albumin in Tris-buffered saline (TB5) for 1 h and washed twice with TBS + 0.05% Tween 20 (TBST). MBP proteins were then incubated with 2-fold dilutions of GST-biotinylated SH3 domains in TBS for 1.5 h. Wells were then washed three times with TBST, followed by a 1-h incubation with streptavidin-biotinylated horseradish peroxidase complex (1:2000 dilution in TBS; GE Healthcare). After three washes with TBST, 50 μl of substrate reagent ABTS Single Solution (Invitrogen) was added, and the absorbance at 405 nm was measured 20 min later.
**Protein Pulldowns and Western Blots**—For protein pulldown experiments, 293FT cells were transfected by standard calcium phosphate precipitation method with 10 μg of NS1 expression vectors in 10-cm plates. For avidin pulldown experiments cells were transfected also with 3 μg of Crk or CrkL expression constructs encoding for C-terminally biotinylated proteins. 48 h after transfection, cells were lysed on ice with 1% Nonidet P-40 lysis buffer (150 mM NaCl; 50 mM Tris–HCl, pH 7.9; 1% Nonidet P-40). Cell lysates were used for immunoprecipitation with anti-CrkL antibody and Dynabeads Protein A magnetic beads (Invitrogen) or avidin pulldowns with Tetrailink tetrameric avidin resin (Promega). To examine the phosphorylation status of Akt, Huh-7 cells in 6-well plates were transfected with 4 μg of Myc-tagged NS1 expression plasmids or empty plasmid using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. 48 h after transfection cells were lysed on ice with 1% Nonidet P-40 lysis buffer, and lysates were used for Western blotting to detect phospho-Akt or myc-NS1. For enhanced PI3K activity, 293FT cells were transfected by standard calcium phosphate precipitation method with 10 μg of NS1 expression vectors in 10-cm plates. For avidin pulldown experiments cells were transfected also with 3 μg of Crk or CrkL expression constructs encoding for C-terminally biotinylated proteins. 48 h after transfection, cells were lysed on ice with 1% Nonidet P-40 lysis buffer (150 mM NaCl; 50 mM Tris–HCl, pH 7.9; 1% Nonidet P-40). Cell lysates were used for immunoprecipitation with anti-CrkL antibody and Dynabeads Protein A magnetic beads (Invitrogen) or avidin pulldowns with Tetrailink tetrameric avidin resin (Promega). To examine the phosphorylation status of Akt, Huh-7 cells in 6-well plates were transfected with 4 μg of Myc-tagged NS1 expression plasmids or empty plasmid using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. 48 h after transfection cells were lysed on ice with 1% Nonidet P-40 lysis buffer, and lysates were used for Western blotting to detect phospho-Akt or myc-NS1.

**RESULTS**

Consensus SH3-binding Motif in NS1—To look for potential virally encoded ligands for cellular SH3 proteins, we used the ScanProsite search engine to identify consensus SH3 domain-binding motifs in viral protein sequences in the Swiss-Prot/TrEMBL data base. One interesting protein that was noted to contain a perfect class II SH3-binding motif was NS1 of the 1918 pandemic influenza A virus (A/Brevig Mission/1/18/H1N1; A/Brevig below). Analysis of NS1 protein sequences from other strains of influenza A revealed that this sequence motif is very common among avian influenza A viruses but only rarely found in viruses isolated from humans (Fig. 1). In addition to A/Brevig, only three other human-derived NS1 sequences containing this motif could be found from the NCBI Influenza Virus Resource database. Notably, two of these viruses represented recent zoonotic transmissions from birds with an H5N1 virus (A/Hong Kong/481/97/H5N1 (33)) and an H7N3 virus (A/Canada/rV504/2004/H7N3 (26)). Accordingly, this sequence motif is not present in the NS1 proteins of human influenza A viruses commonly used for laboratory studies, such as A/WSN/33 (H1N1), A/PR/8/34 (H1N1), and A/ Udorn/72 (H3N2) (see Fig. 1).

Identification of SH3 Partners of NS1—To examine their SH3 binding potential and preferences, NS1 proteins encoded by A/Brevig and an avian strain containing the same consensus motif (A/mallard/Netherlands/12/00/H7N3; A/Mallard below) were expressed as GST fusion proteins in *Escherichia coli* and used as ligands for affinity screening of our comprehensive human SH3 phage display library (27). Both proteins bound avidly to SH3 clones in the library as compared with plain GST protein used as a control for nonspecific binding (not shown). Sequencing of the phagemid genomes obtained after a single round of affinity selection with these NS1 proteins revealed that more than 90% of the phages contained the N-terminal SH3 domain of the adapter protein Crk or its close homologue Crkl.

To confirm and study in more detail the NS1/SH3 interactions revealed by phage screening, we generated recombinant GST fusion proteins of the N-terminal SH3 domains of Crk and Crkl. Because the SH3 of p85 has been suggested to bind to PX domain partners in many influenza A NS1 proteins (6) (see Fig. 1) we also generated GST- SH3 fusion proteins of p85α and p85β. In addition, the SH3 of Eps8L1, which is known to prefer atypical PX domain-containing ligands (34), was included as a negative control. Between the GST and SH3 moieties in these

**Enhanced PI3K Activation by NS1 via SH3 Binding**

| A. | B. |
|---|---|
| 1. | 1 MDSNVTSSQEVDIFWFLKRRFQDFQQLDFLIRIRGQRDQ | A/Chicken consensus |
| 2. | MDSNVTSSQEVDIFWFLKRRFQDFQQLDFLIRIRGQRDQ | A/Brevig/Mission/1/18/H1N1 |
| 3. | MDSNVTSSQEVDIFWFLKRRFQDFQQLDFLIRIRGQRDQ | A/Hong Kong/481/97/H5N1 |
| 4. | MDSNVTSSQEVDIFWFLKRRFQDFQQLDFLIRIRGQRDQ | A/Canada/rv504/2004/H7N3 |
| 5. | MDSNVTSSQEVDIFWFLKRRFQDFQQLDFLIRIRGQRDQ | A/Mallard/Netherlands/12/00/H7N3 |
| 6. | MDSNVTSSQEVDIFWFLKRRFQDFQQLDFLIRIRGQRDQ | A/WSN/33/H1N1 |
| 7. | MDSNVTSSQEVDIFWFLKRRFQDFQQLDFLIRIRGQRDQ | A/PR/8/34/H1N1 |
| 8. | MDSNVTSSQEVDIFWFLKRRFQDFQQLDFLIRIRGQRDQ | A/UDORN/72/H3N2 |
| 9. | MDSNVTSSQEVDIFWFLKRRFQDFQQLDFLIRIRGQRDQ | A/H7N3 |

**Figure 1.** Amino acid sequences of selected influenza A virus NS1 proteins. A, alignment of the complete influenza A virus NS1 sequences of A/Brevig Mission/1/18/H1N1 (Brevig) and A/Udorn/72/H3N2 (Udorn) sequences. Residues in Udorn that differ from the corresponding amino acids in Brevig are highlighted in gray. Tyrosine (position 89) and proline (positions 164, 167, 213, and 216) previously implicated in binding to the SH2 and SH3 domains of PI3K-p85 (6, 9) are underlined. The region containing a consensus SH3-binding motif in the Brevig sequence is boxed. B, shown are amino acid sequences within the region boxed in A in NS1 proteins from the indicated influenza A viruses, as well as chicken (based on 1996 isolates) and human (based on 2005 isolates) consensus sequences. The sequence PxPxVx + (where x denotes any amino acid, θ indicates a hydrophobic residue, and + indicates an arginine or a lysine) indicates a class II SH3-binding consensus motif (critical amino acids in boldface).
Enhanced PI3K Activation by NS1 via SH3 Binding

**FIGURE 2. Semi-quantitative analysis of binding of recombinant SH3 domains to NS1 proteins.** A/Mallard (A) and A/Udorn (B) NS1 proteins were expressed as MBP in *E. coli* and used to coat 96-well plates. These wells were incubated with 2-fold dilutions (ranging from 2.5 to 0.16 μM) of the indicated recombinant SH3 domains expressed as biotinylated GST fusion proteins, followed by detection of binding with an enzymatically labeled streptavidin. As a control for nonspecific binding, additional wells were coated with plain MBP and similarly probed with CrkL SH3 domains (CrkL/MBP). In addition, an artificial protein (p85α-BP) consisting of MBP fused to a high affinity p85α-SH3 ligand peptide (CLNCFRPLPPPPPR) (30) was used as an immobilized target protein to test binding of p85α-SH3 and p85β-SH3. As in A and B, wells coated with plain MBP (p85α-SH3/MBP and p85β/MBP) were used as controls (C).

**FIGURE 3. Co-precipitation of A/Brevig, A/Mallard, and A/Udorn NS1 proteins with full-length Crk and CrkL proteins from transfected cells.** Myc-tagged expression vectors for the indicated NS1 proteins were transfected into 293FT cells together with a biotin-acceptor domain-tagged vector for Crk (C) or CrkL (L). Material precipitated with avidin-coated beads from lysates of the co-transfected cells were analyzed by Western blotting using an anti-Myc antibody or a labeled avidin reagent, as indicated. Aliquots of the lysates were collected before avidin pulldown and subjected directly into Western blot analysis (two bottom panels).

fuson proteins, we inserted a biotin acceptor domain from *P. shermanii* transcarboxylase, which during expression in *E. coli* becomes efficiently biotinylated at a single lysine residue, and can be conveniently detected with avidin-based reagents (35). As targets for these SH3 domains, we expressed maltose-binding protein (MBP) fusion constructs A/Udorn, A/Mallard, and A/Brevig NS1 proteins. The latter were coated onto the bottom of 96-well plates and probed with serial dilutions of biotinylated Crk, CrkL, p85α, p85β, or Eps8L1 SH3 domains in an enzyme-linked immunoassorbent assay-like sandwich assay.

As shown in Fig. 2, SH3 domains of Crk and CrkL bound avidly to A/Mallard NS1-coated wells, showing significant binding signals even when tested at sub-micromolar concentrations (see Fig. 2A). By contrast, p85α and p85β SH3 domains were equally negative in binding to A/Mallard NS1 as was Eps8L1 SH3 or CrkL SH3 in binding to the control wells coated with plain MBP. Although the absolute binding to Crk/CrkL SH3 domains was slightly weaker, very similar results were obtained by using A/Brevig NS1 as the immobilized ligand (data not shown). In agreement with the lack of a consensus SH3-binding motif in A/Udorn NS1, none of the tested SH3 domains showed measurable binding to Udorn NS1 protein (Fig. 2B). To confirm the functionality of the p85 SH3 domain proteins used in this study, we fused MBP with a peptide (CLNCFRPLPPPPPR) that has been optimized for binding to p85α using phage display (30). Positive binding to this peptide under identical assay conditions (Fig. 2C) indicated that the failure of p85α and p85β SH3 domains to bind to the NS1 proteins of A/Mallard and A/Udorn (Fig. 2, A and B) as well as A/Brevig and A/WSN (data not shown) was indeed a valid result and not because of a general lack of functionality of our p85 SH3 proteins.

Binding of Crk and CrkL to NS1 in Transfected Cells—To extend these findings to full-length proteins expressed in human cells and to compare NS1-binding by Crk and CrkL, we generated expression vectors for these proteins tagged at their N termini with the transcarboxylase biotin acceptor domain. As in our previous studies on divergent protein interactions (36), this strategy allowed sensitive and equal detection of the tagged Crk and CrkL proteins when co-transfected into 293FT cells together with NS1 proteins from different influenza A viruses (Fig. 3). To ensure equal detection of the different NS1 proteins used in this experiment, these expression constructs were tagged with a Myc peptide epitope. Similar expression of all NS1 proteins in the lysates of transfected cells and equal recovery of the biotinylated Crk and CrkL proteins using avidin-coated beads were observed, whereas the amounts of co-precipitated NS1 proteins differed dramatically (Fig. 3, top panel). Both Crk and CrkL associated strongly with A/Brevig and
A/Mallard NS1 proteins, but they failed to co-precipitate any A/Udorn NS1. Binding to A/WSN NS1 was equally negative (data not shown). Thus, in good agreement with the recombinant protein data involving isolated Crk/CrkL SH3 domains, in transfected cells full-length Crk/CrkL proteins bound well to NS1 proteins containing a class II SH3-binding consensus site but not detectably to NS1 proteins lacking this motif.

CrkL-NS1 Complex in Influenza A Virus-infected Cells—Because of the similar NS1 binding profiles of Crk and CrkL, lack of known differences in their cellular functions, and the availability of a good antibody against Crk, NS1 binding by CrkL was chosen as the subject of our subsequent studies. To examine whether binding of endogenous cellular CrkL with NS1 protein produced during influenza virus infection could be demonstrated, we infected A549 cells with A/Mallard and examined the presence of NS1 in anti-CrkL immunoprecipitates from lysates of these cells. As shown in Fig. 4A, a readily detectable amount of NS1 co-precipitated from the A/Mallard-infected cells using the α-CrkL antibody, corresponding in intensity to the NS1 signal derived from a fraction of the total lysate equaling 10% of the fraction used for the α-CrkL immunoprecipitation. By contrast, no such signal was seen in anti-CrkL immunoprecipitates from uninfected cells or from control (α-HA) immunoprecipitates from the A/Mallard-infected cells, thus confirming the specificity of the observed CrkL/NS1 co-precipitation.

A similar A549 infection experiment was carried out in which A/Udorn was included for comparison. Because of the sequence divergence of the A/Mallard and A/Udorn NS1 proteins, quantitative comparison of the expression levels of these proteins in their native form was not possible using any of the immunological reagents that we tested (data not shown). Therefore, we chose to infect A549 cells with matched titers of A/Mallard and A/Udorn viruses, and subjected the infected cells to metabolic labeling with [35S]methionine. Because A/Mallard and A/Udorn NS1 proteins contain a comparable number of methionine residues, their presence in anti-CrkL immunoprecipitates could be accurately compared by autoradiography (Fig. 4B).

In good agreement with the Western blotting data (Fig. 4A) an NS1-sized protein was co-precipitated by the anti-CrkL antibody from A/Mallard-infected cells but not from uninfected cells (Fig. 4B). A faint signal corresponding to a slightly larger protein (as expected for A/Udorn NS1) could also be detected from A/Udorn-infected cells in overexposures of the autoradiogram shown in Fig. 4B and other similar experiments (data not shown). Thus, despite its lack of direct Crk binding capacity (Figs. 2 and 3), in infected cells A/Udorn NS1 might yet be weakly associated with CrkL. Equal infection of the cells with the A/Mallard and A/Udorn viruses was confirmed by Western blotting using an antiserum against nucleoprotein, which shows high amino acid sequence conservation between different influenza A strains (Fig. 4B, right panel). Together these data confirmed binding of endogenous CrkL to NS1 in virus-infected cells, and underscore the important role of the SH3 binding capacity of NS1 alleles like A/Mallard in promoting this interaction.

Characterization of the Crk/CrkL SH3-binding Site in NS1—To confirm that the class II SH3-binding motif in A/Mallard and A/Brevig NS1 protein was indeed critical for their capacity to bind to the Crk family proteins, we generated a series of point mutated variants of A/Brevig NS1 shown in Fig. 5A. Mutant 1 (M1) contained alanine residues in place of both of the PXXP defining proline residues of the class II consensus motif (P212A/P215A). Mutant 2 (M2) contained a similar double alanine substitution but affected the additional PXXP sequence that is embedded within but is not part of the consensus SH3-binding motif in A/Brevig NS1 (P213A/P216A). These proline residues are conserved in NS1 proteins of most human and avian influenza viruses, including A/Udorn, and were pointed out by Shin et al. (6) as a potential docking site for p85-SH3. Mutants 3 and 4 involved changes in the positively charged residue of the consensus motif. In M3 this charge was reversed by a lysine to glutamic acid substitution (K217E), whereas the lysine to arginine substitution in M4 (K217R) maintained this functionally important positive charge. In fact, arginine is the positively charged residue in most class II SH3-binding sites,
but Crk and CrkL SH3 domains have a characteristic property of preferring a lysine residue in this position (37). Mutant 5 (M5) contained a proline to threonine substitution (P215T) affecting the first proline residue of the consensus motif, thus mimicking the NS1 protein sequence in this region of A/Udorn (and practically all human influenza A virus NS1 proteins; see Fig. 1).

The capacity of Myc epitope-tagged NS1 proteins carrying these mutations to bind to endogenous CrkL protein in transfected 293FT cells was studied by a co-immunoprecipitation assay. As shown in Fig. 5B, NS1 was equally expressed in all transfected cells, but M1 and M3 that carried class II consensus motif-disrupting mutations could not be co-precipitated with CrkL. The single P215T change (M5) resembling NS1 proteins like that of A/Udorn also abolished binding to CrkL. Instead, association of M2 with CrkL was indistinguishable from unmodified A/Brevig NS1 (WT). In agreement with the published binding preferences of Crk/CrkL SH3 (37), binding of M4 to CrkL was indistinguishable of unmodified A/Brevig NS1 (WT). Together with the activity of A/Udorn NS1 of A/Brevig (B), A/Mallard (M), or A/Udorn (U) NS1 or SH3 binding-deficient mutants (M1 in Fig. 5) of A/Brevig (Bm) or A/Mallard (Mm) NS1 proteins, or an empty control plasmid vector (p), in addition, a vector stably expressing Renilla luciferase was included in all cases to monitor transfection efficiency and cell viability. After 22 h, one culture of cells transfected with the control vector was left untreated, and the other plates were stimulated for 7 h with 100 IU/ml of IFN-β, followed by determination of firefly and Renilla luciferase activities of lysates prepared from these cells. ISRE-dependent gene expression is shown as mean values of the ratios of firefly and Renilla luciferase activities. B, Huh-7 cells were transfected with the NS1 or control expression vectors used in A plus a vector for A/WSN/33 NS1 (W). 48 h later the cells were collected and analyzed for their PI3K pathway activity by Western blot analysis of their lysates using an antibody specific for the phosphorylated form of Akt. Uniform expression of the NS1 proteins was confirmed by probing the blots with an anti-Myc tag antibody.

Role of SH3 Binding in Cellular Functions of NS1—To study the functional role and relevance of Crk/CrkL binding by NS1, we tested the activities of A/Brevig and A/Mallard NS1 proteins and their SH3 binding-deficient derivatives in two different cellular functions that have been reported for NS1, namely inhibition of interferon-induced gene expression and activation of the PI3K signaling pathway. The former apparently involves multiple effector functions of NS1, notably the ability of NS1 to interfere with post-transcriptional mRNA processing (38–40). As already discussed, the latter involves association of NS1 with the p85 subunit of PI3K and can be followed by examining the phosphorylation status of the protein kinase Akt/PKB (6, 8, 41).

When Huh-7 cells were transfected with an ISRE-containing reporter plasmid together with an empty control expression vector (p), a robust increase in luciferase activity was observed upon stimulation of these cells with interferon (IFN-β) (compare the two leftmost bars in Fig. 6A). When a vector expressing NS1 of A/Brevig (B), A/Mallard (M), or A/Udorn (U) was included, the IFN-β-induced increase in reporter gene expression was strongly suppressed. Similar inhibition was also seen when SH3 binding-deficient mutants (corresponding to M1 in Fig. 5A) of A/Brevig (Bm) or A/Mallard (Mm) were tested, which together with the activity of A/Udorn in this assay indicated that binding to Crk/CrkL was not required for the capacity of influenza A NS1 proteins to suppress interferon-induced gene expression.

By contrast, SH3 binding by NS1 was found to correlate with a significant increase in Akt phosphorylation in Huh-7 cells transfected with these vectors. As noted by others (6), cationic lipid-mediated transfection of an empty expression plasmid was sufficient to induce a minor activation of PI3K signaling. However, in agreement with earlier reports (6, 8), the use of vectors encoding NS1 of A/Udorn (U) or A/WSN (W) resulted in a significant increase in Akt phosphorylation (Fig. 6B). This effect was abolished by mutating the class II SH3-binding motif (M1 and M3) in addition to the P215T change in M5. A single P215T change (M5) in the class II binding motif of A/Brevig NS1 protein (WT) and its derivatives (M1–M5) carrying the indicated mutations was sufficient to induce a minor activation of PI3K signaling. However, in agreement with earlier reports (6, 8), the use of vectors encoding NS1 of A/Udorn (U) or A/WSN (W) resulted in a significant increase in Akt phosphorylation (Fig. 6B). This effect was abolished by mutating the class II SH3-binding motif (M1 and M3) in addition to the P215T change in M5.
in further accumulation of pAkt as an indication of PI3K activation (see Fig. 6B). By comparison, the levels of pAkt were significantly higher in cells transfected with A/Brevig (B) or A/Mallard (M) NS1 constructs (Fig. 6B and data not shown). This increased capacity of A/Brevig and Mallard NS1 to activate PI3K signaling was lost when the class II consensus SH3-binding motif of these proteins was disrupted by the M1 mutation (lanes B'′ and M'′ in Fig. 6B).

**DISCUSSION**

Influenza A viruses are highly virulent and can infect a broad range of mammalian and avian species. Several influenza A virus gene products such as HA, viral polymerases, nucleoprotein, and NS1 play a role in the virulence of the virus (42, 43). Although the HA protein largely determines the pathogenicity and species specificity of the virus, NS1 protein has a uniform role in regulating host cell responses during the infection independent of the virus type or the animal species the virus is infecting. NS1 protein is a double-strand RNA-binding protein, and it can interfere with the functions of other double-strand RNA-binding proteins such as the RIG-I, protein kinase R, and oligoadenylate synthetases (44–46) that regulate the induction and antiviral effects of interferons, respectively. NS1 protein is also targeted into the host cells nucleus (28), where it can interfere with the processing of host cell pre-mRNAs, including those of antiviral mRNAs rendering them susceptible for degradation (47). Influenza A virus also takes advantage of the host cell signaling pathways activated during the infection in such a way that signal transduction involving, for example, NF-κB, mitogen-activated kinase cascades, and the PI3K pathway is altered to optimize virus replication.

In this study we have shown that the NS1 protein of the 1918 pandemic influenza A virus (A/Brevig) contains a functional SH3 interaction motif that mediates avid binding to the N-terminal SH3 domain of the adapter proteins Crk and CrkL. This sequence motif is common in NS1 proteins of avian influenza A viruses, but besides A/Brevig can be found only in three of the 4505 human-derived NS1 protein sequences available in the NCBI influenza A data base. Interestingly, two of these cases represent zoonotic infections of humans with avian H5N1 and H7N3 viruses.

Amino acid variation in the NS1 gene has been shown to correlate with the pathogenicity of H5N1 strains of avian influenza virus in chickens (48), and the NS1 gene of A/Brevig has been found to be more potent than the reference NS1 proteins in regulating host cell gene expression (49). Thus, although human influenza A infection clearly differs from that of avian hosts in that the capacity of NS1 for Crk/CrkL SH3 binding appears to be generally counter-selected in the formed case, adaptation of the virus to exploit this connection to the cellular signaling machinery also in human cells might provide the virus with increased replicative or pathogenic potential. It was recently shown that the 1918 virus shows a higher replication capacity in tissue culture and in animal models (50, 51).

Several different viral species have been found to stimulate PI3K signaling to increase their replication and/or to prevent apoptotic death of the host cell (for a review, see Ref. 52).

Indeed, NS1-mediated activation of PI3K signaling was recently shown to enhance influenza A virus replication (9, 10) and limit apoptosis in infected cell cultures (8). Our observation that a functional Crk/CrkL-binding motif provided the NS1 proteins of A/Brevig and A/Mallard with an increased ability to induce PI3K signaling could therefore readily explain a potentially higher replicative capacity of viruses expressing Crk/CrkL binding-competent NS1 proteins.

Crk/CrkL proteins were first identified as cellular counterparts of the protein encoded by the v-crk oncogene of the avian sarcoma virus CT10 (53), and have since been found to be ubiquitously involved in numerous signaling pathways regulating diverse functions in different cell types (54). Of note, several studies have reported physical and functional interactions between Crk/CrkL proteins and the PI3K signaling pathway (55–57), thus providing a plausible framework for the mechanism of the observed enhancement of NS1-mediated PI3K activation via the NS1-Crk/CrkL interaction. However, additional roles for the NS1-Crk/CrkL complex in cell biology of influenza A virus should obviously not be ruled out.

Previous studies concerning the activation of the PI3K pathway by NS1 have shown that this regulation involves an association of NS1 with the p85 regulatory subunit of PI3K (6, 8, 9), and subsequent studies even further suggested that there is a direct binding of NS1 to the SH3 domain of p85 (6). However, our studies failed to reveal measurable binding of the SH3 domains of p85α or p85β to NS1 proteins like A/Udorn and A/WSN, which contain the PXXP sequences pointed out by Shin et al. (6) or to A/Brevig or A/Mallard NS1 proteins, which contain the additional class II consensus SH3-binding motif described in this study. Our results do not exclude the possibility that weak binding of p85 SH3 to one of these nonconsensus PXXP motifs in NS1 might play a subtle role in stabilizing or coordinating NS1/p85 binding, but argue against a major role of such contacts in driving NS1-p85 complex formation.

It should be noted, however, that the ability of recombinant p85 SH3 to precipitate NS1 protein from lysates of influenza A-infected cells as reported by Shin et al. (6) does not prove this interaction to be direct rather than mediated by one or more additional factors. In fact, it has been reported that although association of NS1 with p85 can be readily detected in influenza A virus-infected cells, this is less true in the case of NS1 gene-transfected cells, leading Ehrhardt et al. (8) to propose that a cellular bridging factor is required for NS1-p85 complex formation. In this regard, it is interesting to note that although we found A/Udorn NS1 to be completely negative in the CrkL co-precipitation assay in transfected cells (Fig. 3), we did observe a weak association of NS1 and CrkL in cells infected with A/Udorn (data not shown). Thus, in influenza A virus-infected cells expressing high levels of NS1 (as well as other viral proteins that could also be involved) a large protein complex involving NS1, PI3K, and Crk/CrkL might form independently of direct binding of NS1 to Crk/CrkL or the p85 subunit of PI3K. However, NS1 proteins like those of A/Brevig and A/Mallard that can directly recruit Crk/CrkL via SH3 binding would serve to promote and strengthen the assembly of this multiprotein “signalosome” complex.
Enhanced PI3K Activation by NS1 via SH3 Binding

Further characterization of this NS1/PI3K/Crk-containing signalosome is clearly warranted and will shed new light into the cell biology of influenza A replication. Small molecular inhibitors of PI3K are already under evaluation as drugs in cancer (58, 59), and have been shown to reduce influenza A replication in vitro (7, 9). Thus, cellular signaling involving NS1/PI3K/Crk could also provide a useful target for the development of novel anti-influenza drugs. In this regard, possible involvement of the enhanced PI3K activation mediated via Crk/CrkL binding by NS1 proteins of avian strains of influenza in regulating interspecies transmission or pathogenicity in human hosts deserves special attention.

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