Activation of Phosphatidylinositol 3-Kinase Signaling by the Nonstructural NS1 Protein Is Not Conserved among Type A and B Influenza Viruses[▽]

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Recently it has been shown by several laboratories that the influenza A virus nonstructural protein 1 (A/NS1) binds and activates phosphatidylinositol 3-kinase (PI3K). This function of the protein is likely to prevent premature apoptosis induction during viral propagation. Here we show that the B/NS1 protein completely lacks the capacity to induce PI3K signaling. Thus, PI3K activation is another unique function of A/NS1 that is different from the action of its influenza B virus counterpart.

Influenza A virus infections induce a variety of pro- and antiviral-acting signaling processes (12, 13). Very recently the phosphatidylinositol 3-kinase (PI3K) and its downstream effector Akt/protein kinase B have been added to the list of influenza A virus-induced signaling mediators (5, 9, 18). PI3K is involved in a wide variety of cellular events, generating lipid signals downstream of receptors and influencing diverse cellular pathways (20, 21). In the context of an influenza A virus infection, PI3K was identified as a perfect example of a seemingly antiviral signaling component that is misused by the virus to support its replication. While on one hand the kinase regulates virus- and double-stranded RNA (dsRNA)-induced IRF-3 activation (5, 17), on the other hand PI3K activity is hijacked by the virus to support effective virus uptake in early stages as well as preventing apoptosis late in infection (5, 6). It was a striking finding of several laboratories that the nonstructural protein 1 (NS1) of influenza A viruses (A/NS1) induces PI3K activation at advanced stages of the replication cycle (6, 9, 10, 18, 23). Previously, the A/NS1 protein had been known only as a suppressor of signaling events, blocking dsRNA-dependent enzymes (7) and/or interfering with the RNA sensor RIG-I (14–16). These activities lead to suppression of signaling pathways and activation of transcription factors, e.g., of NF- κ B or IRF-3 (11).

Despite the fact that the NS1 protein of influenza B virus (B/NS1) possesses less than 20% amino acid sequence identity to A/NS1, both proteins fulfill similar but not identical functions. B/NS1 acts as an interferon antagonist (2, 7, 19) and inhibits the interferon-inducible protein kinase R (3); however, B/NS1, in contrast to A/NS1, is not able to inhibit polyadenylation, splicing, and nuclear export of cellular mRNA (22). Consequently, the question was raised whether PI3K activation is induced upon influenza B virus infection to the same extent as upon influenza A virus infection and whether B/NS1 acts similarly to A/NS1 to promote PI3K activity and suppress premature apoptosis induction.

Lack of late-stage PI3K activation upon infection with influenza B viruses. To study PI3K activation in influenza B virus-infected cells, we compared the phosphorylation kinetics of the PI3K effector Akt upon infection with influenza A/PR/8/34, B/Lee/40, and B/Maryland/59 viruses. This was investigated using a phosphospecific anti-Akt antibody, detecting Akt when phosphorylated at S473. This occurs in a strictly PI3K-dependent manner (1, 5), thus serving as a marker for activation of the PI3K/Akt pathway. Madin-Darby canine kidney (MDCK) or Vero cells were infected with the different influenza viruses for the times indicated and analyzed for Akt phosphorylation. Detection of the viral NS1 and NP proteins monitored ongoing viral protein synthesis. As shown earlier (5), infection with the influenza A virus strain PR8 resulted in a weak and transient induction of Akt phosphorylation in MDCK and Vero cells 15 min to 1 h postinfection (p.i.), during the phase of viral attachment and entry (Fig. 1A and B, left). Additionally, a much stronger Akt phosphorylation was detected 4 h to 8 h after infection (Fig. 1A and B, left), concomitant with and likely to be caused by A/NS1 expression (6). In influenza B virus-infected cells an early and transient phase of Akt phosphorylation was observed starting from 15 min up to 2 h p.i. (Fig. 1A and B, middle and right). However, in clear contrast to the findings with influenza A viruses, type B virus-infected cells did not show any Akt phosphorylation at 4 h or 8 h p.i., even though B/NS1 is highly expressed. In addition, we directly compared the potentials to activate PI3K during infections with influenza B/Lee/40 virus, the PR8 strain, and the avian influenza A virus strain A/FPV/Bratislava/79 (FPV). MDCK cells were infected for 4 h and 8 h, and lysates were analyzed for Akt phosphorylation as well as A/NS1 and B/NS1 expression. Consistent with the earlier results, we observed again a strong correlation between A/NS1 expression and Akt phosphorylation (Fig. 1C). There was hardly any Akt phosphorylation detectable at 4 h p.i. in FPV-infected cells, which correlated well with a low expression level of the A/NS1 protein at this time point. However, when A/NS1 concentrations had accumulated at 8 h p.i., phosphorylated Akt was easily detected. In PR8-infected cells, A/NS1 expression was already detectable at 4 h p.i., which correlated with an early Akt phosphorylation at this time point. In contrast, influenza B virus-infected cells showed an abundant B/NS1 protein expression at

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FIG. 1. Influenza A and B virus infections induce Akt phosphorylation at different time points. (A and B) MDCK (A) or Vero (B) cells were infected with the influenza virus strains A/Puerto Rico/8/34 (left), B/Lee/40 (middle), or B/Maryland/59 (right) at a multiplicity of infection of 5 for the indicated time points. Subsequently protein lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted onto nitrocellulose membranes. Phosphorylated Akt (Ser473) was detected by Western blotting with a phosphospecific anti-Akt rabbit antiserum (Biosource). Equal protein loading of the kinase was verified using a pan-Akt antiserum (Biosource). Ongoing viral replication was demonstrated by accumulation of the viral nucleoproteins (A/NP and B/NP) or nonstructural proteins (A/NS1 and B/NS1), using specific antibodies detecting NP (Serotec; AA5H and B017), A/NS1 (clone 23-12) (monoclonal antibody generated at the IMV, Münster, Germany), or B/NS1 (rabbit antiserum; T. Wolff, RKI, Berlin, Germany). (C) MDCK cells were left uninfected or infected with the avian influenza virus A/FPV/Bratislava/79 (FPV), the human influenza virus A/Puerto Rico/8/34 (A/PR/8/34), or the influenza B virus strain B/Lee/40 at a multiplicity of infection of 5 for 4 h and 8 h. Phosphorylated Akt (Ser473) was detected by Western blotting with a phosphospecific anti-Akt rabbit antiserum (Biosource). NS1 expression was visualized using antibodies to A/NS1 or B/NS1. Equal protein loading was verified by detecting extracellular signal-regulated kinase 2 (ERK2; Santa Cruz Biotechnology). (D) A549 cells were transfected with empty vector (lane 1) or expression constructs for myc-tagged versions of NS1 of A/PR/8/34 (A/NS1-myc) (lane 2) or NS1 of B/Lee/40 (B/NS1-myc) (lane 3), using Lipofectamine 2000 (Invitrogen) as described earlier (4). Lane 4 represents the untransfected control. Note that cells in panel D were cotransfected with a plasmid expressing wild-type Akt. Equal protein loading was verified by detecting Akt (Biosource) or extracellular signal-regulated kinase 2 (ERK2; Santa Cruz Biotechnology). Equal NS1 production was monitored in Western blot assays detecting myc-tagged A/NS1 (lane 2) and B/NS1 (lane 3) using a mouse-specific antiserum against myc tag (clone 9E10).

4 h and 8 h p.i. that increased with time without any induction of Akt phosphorylation. Taken together, these data indicate that B/NS1 protein expression does not correlate with induction of PI3K/Akt signaling at the selected time points during viral replication.

Influenza B virus protein NS1 does not induce PI3K/Akt signaling. To evaluate the PI3K-activating potential of A/NS1 and B/NS1 when they are expressed as single proteins, A549 cells were transfected with an empty vector (Fig. 1D, lane 1) or plasmids expressing myc-tagged A/NS1 (Fig. 1D, lane 2) or B/NS1 proteins (Fig. 1D, lane 3). PI3K-dependent phosphorylation of Akt was observed only in the presence of A/NS1, while B/NS1 expression failed to activate Akt, although equal levels of the two proteins were expressed (Fig. 1D). Similar results were also obtained when employing constructs expressing A/NS1 and B/NS1 without a tag (data not shown).

Requirement for PI3K activity is restricted to very early stages of the infection cycle of influenza B viruses. A remaining question concerns a potential function of PI3K activation early during influenza B virus infection. Recently we have shown that PI3K plays an important role during influenza A virus entry processes and that the early inhibition of PI3K activity results in decreased virus propagation (5). Since the PI3K/Akt pathway is activated very early upon infection with influenza B viruses and its activity is even more pronounced than that in influenza A virus-infected cells (Fig. 1A and B, middle and right), we analyzed the role of PI3K in propagation of influenza B viruses by the use of PI3K inhibitors. MDCK cells were treated with the specific PI3K inhibitor wortmannin (5 μ M) for different time periods pre- and postinfection. Figure 2 shows that only in the samples where PI3K was inhibited prior to or during very early time points of infection were titers

FIG. 2. Inhibition of the PI3K activity at early but not at later time points of infection results in reduced titers of progeny influenza B virus. MDCK cells were treated with wortmannin (5 μ M; Calbiochem) or dimethyl sulfoxide (Sigma) starting at the indicated time points before and during infection with influenza virus B/Lee/40 (multiplicity of infection of 5) (A) or FPV (multiplicity of infection of 0.05) (B). Supernatants were assayed for progeny virus yields at 9 h p.i. in standard plaque titrations. Virus yields of dimethyl sulfoxide-treated cells were used as a control.

of progeny virus reduced (Fig. 2). While in influenza A virus-infected cells PI3K inhibition led to a pronounced effect on progeny virus titers for up to 2 h p.i. (Fig. 2B) (5), no significant effects were observed in influenza B virus-infected cells if the inhibitor was added later than 30 min p.i. Similar results were obtained using another PI3K inhibitor, LY294002 (50 μ M) (data not shown). This indicates that PI3K activity is required for efficient growth of influenza A viruses at both early and intermediate stages of the replication cycle, while in influenza B virus-infected cells the requirement for PI3K activity is restricted to very early stages.

The B/NS1 protein does not function in the antiapoptotic effect of PI3K activity. The recent finding that A/NS1-induced PI3K activity at later stages of influenza A virus infection

suppresses premature apoptosis induction (6) leads to the assumption that in influenza B virus infections, where the late PI3K activation is missing, this activity is not required. To examine this hypothesis, A549 cells were infected with previously described recombinant influenza A and B viruses expressing either wild-type NS1 proteins (PR8, 230 amino acids, or B/Lee, 281 amino acids) or C-terminally truncated mutant derivatives thereof carrying little more than the dsRNA-binding domain (A/NS1-125 and B/NS1-104) (3, 5) (Fig. 3A). In addition we infected cells with mutant virus strains bearing full deletions of the protein (Δ A/NS1 or Δ B/NS1) (2, 8) (Fig. 3B). At 8 h p.i. phosphorylation of Akt was strongly induced in PR8-infected cells expressing wild-type A/NS1, but not upon infection with the mutants. Conversely, in cells infected with the mutant viruses a strong onset of apoptotic caspase activity was observed as measured by cleavage of poly(ADP-ribose) polymerase (PARP), a prominent substrate of caspases (Fig. 3A and B). This indicates that full-length A/NS1 protein is required to suppress apoptosis. Infection with influenza B/Lee/40 virus led to only a marginal induction of PI3K/Akt activation, while infection with the B/NS1-104 or the Δ B/NS1 virus mutant did not induce Akt phosphorylation. Interestingly, neither wild-type nor mutant influenza B virus infection led to PARP cleavage within the time frame analyzed. From these findings one can speculate that influenza B viruses may have developed a mechanism to avoid or suppress apoptosis induction that is independent of B/NS1. Furthermore, one could also argue that there is a much earlier onset of apoptosis in influenza A virus-infected cells that needs suppression by A/NS1 via induction of PI3K activity. Influenza B viruses might induce apoptosis to a lesser extent or in a delayed fashion and would not require an apoptosis-suppressing function in the stage of replication at which B/NS1 is expressed. With regard to the activation mechanism, it is interesting that the tyrosine at position 89 of A-type viruses that has been shown to be

FIG. 3. Infection with mutant influenza B virus bearing a truncated B/NS1 or without any B/NS1 protein does not result in enhanced apoptosis. A549 cells were left uninfected or infected with the recombinant influenza virus strain A/PR/8/34 or B/Lee/40 (A and B) or corresponding virus mutants expressing truncated NS1 proteins of amino acids 1 to 125 (A/NS1/125, panel A, lane 2) or 1 to 104 (B/NS1/104, panel A, lane 4) or Δ A/NS1 (A/NS1, panel B, lane 3) or Δ B/NS1 (B/NS1, panel B, lane 5) for 8 h (multiplicity of infection of 5). After infection cells were lysed with radioimmunoprecipitation assay buffer and proteins were analyzed in Western blot assays. Phosphorylated Akt (Ser473) and equal loading of the kinase Akt were detected. Ongoing viral replication was demonstrated by accumulation of the viral nucleoproteins (A/NP and B/NP) or nonstructural proteins (A/NS1 and B/NS1) as described above. Cleavage of the caspase substrate PARP as a marker for apoptosis induction was detected with a PARP-specific mouse antibody (BD Transduction Laboratories).

required for interaction with p85 (9, 18) is missing in influenza B viruses. Consistent with that, we do not see any interaction of B/NS1 and p85 α or - β in coimmunoprecipitation experiments (data not shown). Taken together, these data clearly show that the viral A/NS1 protein exerts a function that is lacking in the B/NS1 protein, identifying another distinction in the mechanisms of action of these two viral proteins and, hence, the two influenza virus types.

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