

Published online ahead of print on ?? October 2006 as DOI 10.1099/vir.0.82419-0

**Influenza A virus NS1 protein activates the phosphatidylinositol 3-kinase (PI3K)/Akt pathway by direct interaction with the p85 subunit of PI3K**

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**Influenza A virus infection activates the phosphatidylinositol 3-kinase (PI3K)/Akt pathway, but the mechanism is not clear. Here, it is reported that influenza A virus NS1 protein is responsible for PI3K/Akt pathway activation. It was demonstrated that the NS1 protein interacts with the p85 regulatory subunit of PI3K via direct binding to the SH3 and C-terminal SH2 domains of p85. Consensus binding motifs for SH3 and SH2 domains were found in influenza A virus NS1, namely an SH2-binding motif (YXXM) at aa 89, SH3-binding motif 1 (PXXP) around aa 164 and SH3-binding motif 2 around aa 212. Mutant virus encoding NS1 protein with mutations in the SH-binding motifs failed to interact with SH domains of p85 and did not activate the PI3K/Akt pathway. The mutant virus is attenuated in Madin–Darby canine kidney cells. Our study has established a novel function of NS1: by interacting with p85 via the SH-binding motifs, NS1 can activate the PI3K/Akt pathway.**

RNA segment 8 of *Influenza A virus* encodes two proteins, NS1 and NS2/NEP (Lamb, 1989). NS1 is a multifunctional protein that is translated from unspliced mRNA (Lamb & Choppin, 1979). Two functional domains have been identified in the NS1 protein: an RNA-binding domain near the N terminus and an effector domain in the C terminus (Qian *et al.*, 1994). The RNA-binding activity of the NS1 protein correlates to its ability to inhibit cellular pre-mRNA splicing (Lu *et al.*, 1994; Qiu *et al.*, 1995). It is also linked to the ability to counteract cellular alpha/beta interferon functions efficiently (Garcia-Sastre *et al.*, 1998) by inhibiting the activation of the protein kinase PKR (Lu *et al.*, 1995; Hatada *et al.*, 1999) and transcription factors NF- $\kappa$ B, IRF-3 and IRF-7 (Talon *et al.*, 2000; Wang *et al.*, 2000). Within the effector domain, two binding sites of cellular proteins were identified. The binding site for CPSF is positioned around aa 186 and the PABII-binding site is located between aa 223 and 237 (Li *et al.*, 2001). These binding sites are required for the inhibition of 3'-end processing of cellular pre-mRNAs; thus, they are important for influenza virus replication (Noah *et al.*, 2003).

Phosphatidylinositol 3-kinases (PI3Ks) are a family of cellular, heterodimeric enzymes that consist of a regulatory subunit (p85) and a catalytic subunit (p110). PI3K is activated by binding of the SH domain in the p85 subunit to autophosphorylated tyrosine kinase receptors, or to non-receptor tyrosine kinases or some viral proteins in the cytoplasm (Carpenter *et al.*, 1993; Street *et al.*, 2004). After activation, the p110 subunit of PI3K phosphorylates the lipid substrate phosphatidylinositol-4,5-bisphosphate to produce phosphatidylinositol-3,4,5-trisphosphate (Toker & Cantley, 1997). This molecule serves as a lipid second messenger and is able to regulate phosphorylation of a number of kinases, including Akt. Akt is activated via phosphorylation at Thr308 and Ser473 (Alessi *et al.*, 1996). Phosphorylated Akt plays a central role in modulating diverse downstream signalling pathways associated with cell survival, proliferation, migration, differentiation and apoptosis (Datta *et al.*, 1999).

The PI3K/Akt pathway is activated by influenza A virus infection and favours virus replication (Ehrhardt *et al.*, 2006). However, the mechanism of how the PI3K/Akt pathway is activated by influenza A virus remains to be elucidated. Study using an influenza mutant virus, deltaNS1, showed that Akt phosphorylation was strongly reduced, suggesting that NS1 might be involved in PI3K/Akt activation (Ehrhardt *et al.*, 2006). We hypothesized that the NS1 protein may have a potential role in activation of the PI3K/Akt pathway by interacting with the p85 subunit of PI3K. This notion is based on the following observations. (i) The p85 subunit of PI3K contains two SH2 domains, one SH3 domain and other protein-interaction domains (Okkenhaug & Vanhaesebroeck, 2001) (Fig. 1a). Sequence analysis revealed that the NS1 protein contains two polyproline motifs with the consensus sequence PXXP positioned at aa 164–167 and 213–216. Such motifs are present in a number of viral and cellular proteins involved in PI3K signalling and form extended helices that bind to SH3 domains found within a diverse group of signalling proteins, including the p85 subunit of PI3K (Pawson, 1995). (ii) The NS1 protein also contains one YXXM motif located at aa 89–93, which is quite similar to the p85 SH2-binding motif YXXM (Songyang *et al.*, 1993). Therefore, NS1 may also bind to p85 SH2 domains and activate the PI3K/Akt pathway.

To examine whether the NS1 protein would interact with the p85 subunit of PI3K *in vivo*, A549 cells were mock-infected or infected with influenza A/PR/8/34 (H1N1) (PR8) at an m.o.i. of 1. Cell lysates were prepared at 6 h post-infection (p.i.), and co-immunoprecipitation using a p85 antibody (Upstate Biotechnology) was performed. Precipitated proteins were subjected to Western blotting with a rabbit polyclonal NS1 antibody generated in our laboratory. Fig. 1(b) shows that, whilst normal rabbit serum failed to precipitate the NS1 protein (lane 3), the p85 antibody did precipitate the NS1 protein (lane 4). No NS1 protein could be immunoprecipitated by the p85 antibody from mock-infected cell lysate (lane 5). Input PR8-infected cell lysate was loaded as control (lane 1). The amount of p85 presented in the immunoprecipitated complex was determined by counterstaining with p85 antibody.

o identify which particular domain of p85 interacts with the NS1 protein, a panel of truncated domains of p85 fused to glutathione S-transferase (GST) (Sossey-Alaoui *et al.*, 2005) were used in a GST pull-down assay with wild-type (wt) PR8-infected A549 cell lysates followed by Western blotting with NS1 antibody. Fig. 1(c) shows that, whilst GST–p85–SH3 did precipitate NS1 (lane 10), GST–p85–SH2C precipitated NS1 to a lesser degree (lane 8). GST–p85–SH2N did not interact with NS1 (lane 6). NS1 was not precipitated either from PR8-infected cell lysates by GST (lane 4) or from mock-infected cell lysates by any purified GST-fusion protein (lanes 3, 5, 7 and 9). Bound proteins were resolved by SDS-PAGE and stained by Coomassie blue to demonstrate an equal amount and integrity of the GST-fusion proteins (lower panel). These data demonstrate that the NS1 protein interacts with the p85 subunit of PI3K via binding to C-terminal SH2 and SH3 domains of p85.

o examine whether NS1 alone would be able to bind to the p85 SH2 and/or SH3 domain, we constructed plasmid pcDNA-NS1, which encodes wt NS1 protein under the control of the

cytomegalovirus promoter. 293T cells were transfected with pcDNA-NS1 or empty vector. Cell lysates were prepared after 48 h transfection and subjected to a GST pull-down assay. Fig. 2(a) shows that NS1 interacted with p85 SH3 and p85 SH2C domains, but not the p85 SH2N domain (lanes 6, 8 and 10). This pattern is in agreement with the observation made when infected cell lysates were used in the pull-down assay (Fig. 1c). Input (10 %) was loaded as control (lane 2). No interactions were detected in vector-transfected samples (lanes 3, 5, 7 and 9). Equal amount and integrity of GST-fusion proteins were verified by Coomassie blue staining.

o ascertain the biological relevance of the observation that the NS1 protein, unaccompanied by other viral components, could interact with p85, we examined whether expression of NS1 alone would be sufficient to activate PI3K/Akt. We attempted to transfect NS1 into A549 cells by cationic lipid transfection reagents, such as Lipofectamine (Invitrogen) and FuGENE 6 (Roche Diagnostics). We observed that, subsequent to transfection, a strong signal of phosphorylated Akt was detected in the vector-control sample as well as in the NS1-expressing sample (data not shown). We then noticed that the cationic lipid reagents increased signal transduction (Giorgione *et al.*, 1998), including marked activation of the kinase activity of the insulin receptor due to the formation of hexagonal phases in the cell membrane (Pramfalk *et al.*, 2004). Thus, we performed electroporation to express NS1 transiently. Thirty micrograms of pcDNA3.1(-) or pcDNA-NS1 was electroporated individually into  $5 \times 10^6$  A549 cells. After electroporation, cells were maintained in medium containing 10 % fetal calf serum for 48 h followed by serum starvation for 24 h. Western blotting was performed by using phospho-Akt(Ser473), Akt (Cell Signaling Technology) or NS1 antibody. Fig. 2(b) shows electroporation of A549 cells with vector-induced visible phosphorylation of Akt; NS1-expressing cells showed a greater degree of Akt phosphorylation. It is notable that electroporated cells expressed an elevated amount of Akt compared with mock- and wt PR8-infected cells; this might result from the stress and cell-death signals resulting from electroporation. Nevertheless, both vector- and NS1-expressing cells had the same level of Akt and, more importantly, NS1-expressing cells had a higher level of phospho-Akt.

sequence analysis revealed that one SH2-binding motif and two SH3-binding motifs are present in the NS1 protein. To address the role of these potential p85-binding motifs in NS1 in the activation of the PI3K/Akt pathway, we generated mutant virus PR8-SH2/SH3mt by a reverse-genetic approach (Hoffmann *et al.*, 2000). Our rationale was that removal of the p85-binding motifs in NS1 would eliminate the binding of NS1 to p85 and thus abrogate PI3K/Akt pathway activation. PR8-SH2/SH3mt encodes NS1 with mutations at aa 89 (from Y to F), 164–167 (from PSLP to ASLA) and 212–216 (from PPLTP to AALTA) (Fig. 3a). Introduction of mutations does not disturb the N-terminal RNA-binding domain or CPSF- and PABII-binding sites, nor does it affect the NS2/NEP mRNA splicing donor site or the NS2/NEP protein sequence. The genotype of PR8-SH2/SH3mt was characterized and confirmed by DNA sequencing of the RT-PCR product derived from the NS gene of PR8-SH2/SH3mt virus. PR8-SH2/SH3mt grown in 9- to 10-day-old embryonated eggs had a titre of  $1.22 \times 10^8$  p.f.u. ml<sup>-1</sup>.

o examine whether PR8-SH2/SH3mt would be able to activate the PI3K/Akt pathway, A549 cells were mock-, wt PR8- or PR8-SH2/SH3mt-infected at an m.o.i. of 1. Cell lysates prepared at 6 h p.i. were subjected to Western blotting. Fig. 3(b) shows that, whilst PR8-SH2/SH3mt produced a slightly lower amount of NS1 than did the wt virus, it failed to induce Akt phosphorylation completely. Neither wt nor mutant virus infection altered the levels of Akt. To ascertain that PR8-SH2/SH3mt does indeed lack the ability to activate PI3K/Akt, phosphorylation of Akt was assessed further in the cells infected with PR8-SH2/SH3mt at higher m.o.i.s. Fig. 3(c) shows that, whilst wt PR8 infection induced a high level of Akt phosphorylation (lane 1), PR8-SH2/SH3mt infection did not lead to Akt phosphorylation at m.o.i.s of 1, 5 or 10 (lanes 2–4). Akt, NS1 and NP expression was monitored from the same samples by Western blotting. An increasing amount of NS1 in cells infected with an increasing amount of PR8-SH2/SH3mt was not achieved; this might be due to a destabilizing effect of the mutations. However, the NP blot shows an increasing amount in these cells, ruling out the possibility of problems with infection.

o investigate the ability of the mutant NS1 encoded by PR8-SH2/SH3mt to interact with SH2 and SH3 domains of p85, a GST pull-down assay was performed by using mock- or PR8-SH2/SH3mt-infected A549 cell lysates prepared at 6 h p.i. Fig. 3(d) shows that none of the SH3, SH2C or SH2N domains of p85 interacted with the mutant NS1 protein. Interaction of wt NS1 and p85 SH3 was included to ascertain the suitability of the conditions used (lanes 11–13). Coomassie blue staining shows that same amount and integrity of GST-fusion proteins were present in each sample.

o examine the possible biological role of NS1–p85 interaction, we examined the replication potential of PR8-SH2/SH3mt in Madin–Darby canine kidney (MDCK) cells by monitoring plaque size and multiple-cycle growth kinetics. Fig. 3(e) shows that PR8-SH2/SH3mt formed small plaques and the virus yield at an m.o.i. of 0.001 was 1.0–1.5 logs lower than that of wt virus, indicating that PR8-SH2/SH3mt is attenuated for growth.

The data presented here demonstrate that influenza A virus NS1 protein can interact directly and specifically with the p85 regulatory subunit of PI3K. This interaction seems to be mediated essentially by the SH3 domain and, to a lesser degree, by the SH2C domain of p85. Furthermore, SH2- and SH3-binding motifs in NS1 are shown to be essential for interaction with p85 and subsequent PI3K/Akt pathway activation. In light of the available three-dimensional (3D) structure of NS1 (Bornholdt & Prasad, 2006), whilst Y89 is located and exposed in the cleft of the two monomers, SH3-binding motif 1 lies exposed in the turn between the sixth  $\beta$ -strand and the large  $\alpha$ -helix. The locations imply that the two motifs are accessible to p85. Residues 206–230 are disordered in the NS1 3D structure, thus we cannot predict whether SH3-binding motif 2 is well positioned for p85 binding. It would be interesting to mutate individual motifs and to investigate the phenotype of the resulting viruses. Ehrhardt *et al.* (2006) reported an early activation of PI3K, when little NS1 is synthesized. This observation may result from double-stranded (ds) RNA, as dsRNA can lead to PI3K/Akt activation via phosphorylation of TLR3 (Sarkar *et al.*, 2004). However, early

activation of PI3K/Akt was not detected in our laboratory. We found that phospho-Akt was elevated at 6 h p.i. and that this level was sustained for the remainder of the infection (data not shown). Nevertheless, the data presented here indicate that NS1–p85 interaction plays a critical role in late-phase activation of PI3K/Akt during influenza A virus infection.

hrhardt *et al.* (2006) proposed a bivalent role of PI3K/Akt in influenza A virus infection. In line with their finding that PI3K is beneficial for virus replication, we showed that PR8-SH2/SH3mt, which lacks the ability to activate PI3K/Akt, is attenuated in tissue culture. Although the exact mechanism by which PI3K/Akt regulates influenza virus replication, and other biological functions of PI3K/Akt in virus infection, remain to be elucidated, our study reveals the mechanism underlying PI3K/Akt activation and adds a novel aspect to the functions of the NS1 protein.

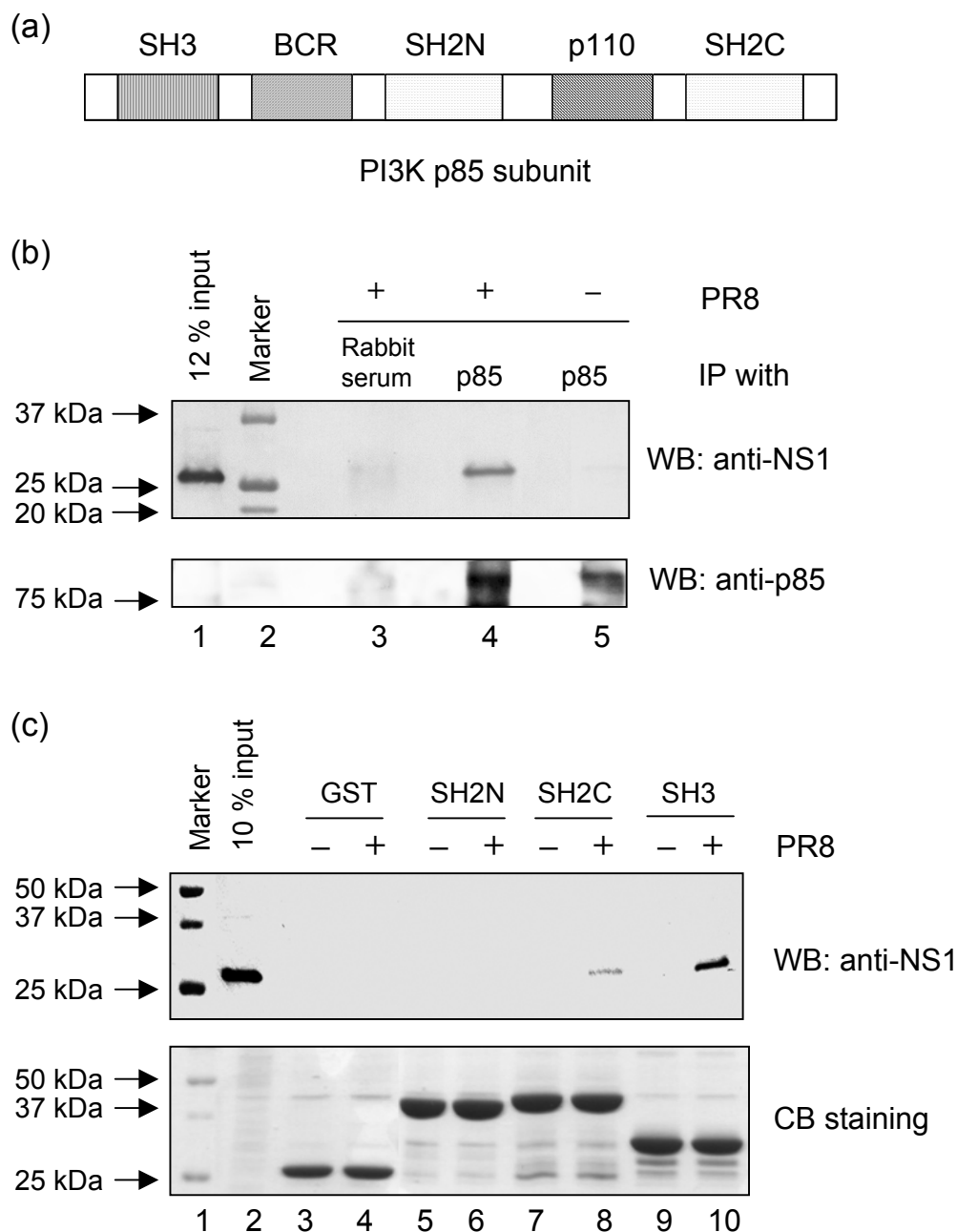
## **ACKNOWLEDGEMENTS**

e thank Dr E. Hoffmann and Dr R. G. Webster (St Jude Children's Research Hospital, Memphis, TN, USA) for providing PR8 plasmids and Dr L. Rameh (Boston Biomedical Research Institute, Watertown, MA, USA) for providing GST-fusion plasmids. We are grateful to Dr B. He (Pennsylvania State University, PA, USA) for critical reading and discussions on the manuscript and to VIDO animal-care staff for rabbit immunization. This study was supported by grants from the Canadian Institutes of Health Research to Y. Z. and the National Veterinary Research and Quarantine Service of South Korea to Y.-K. S. This work is published as VIDO journal series no. 445.

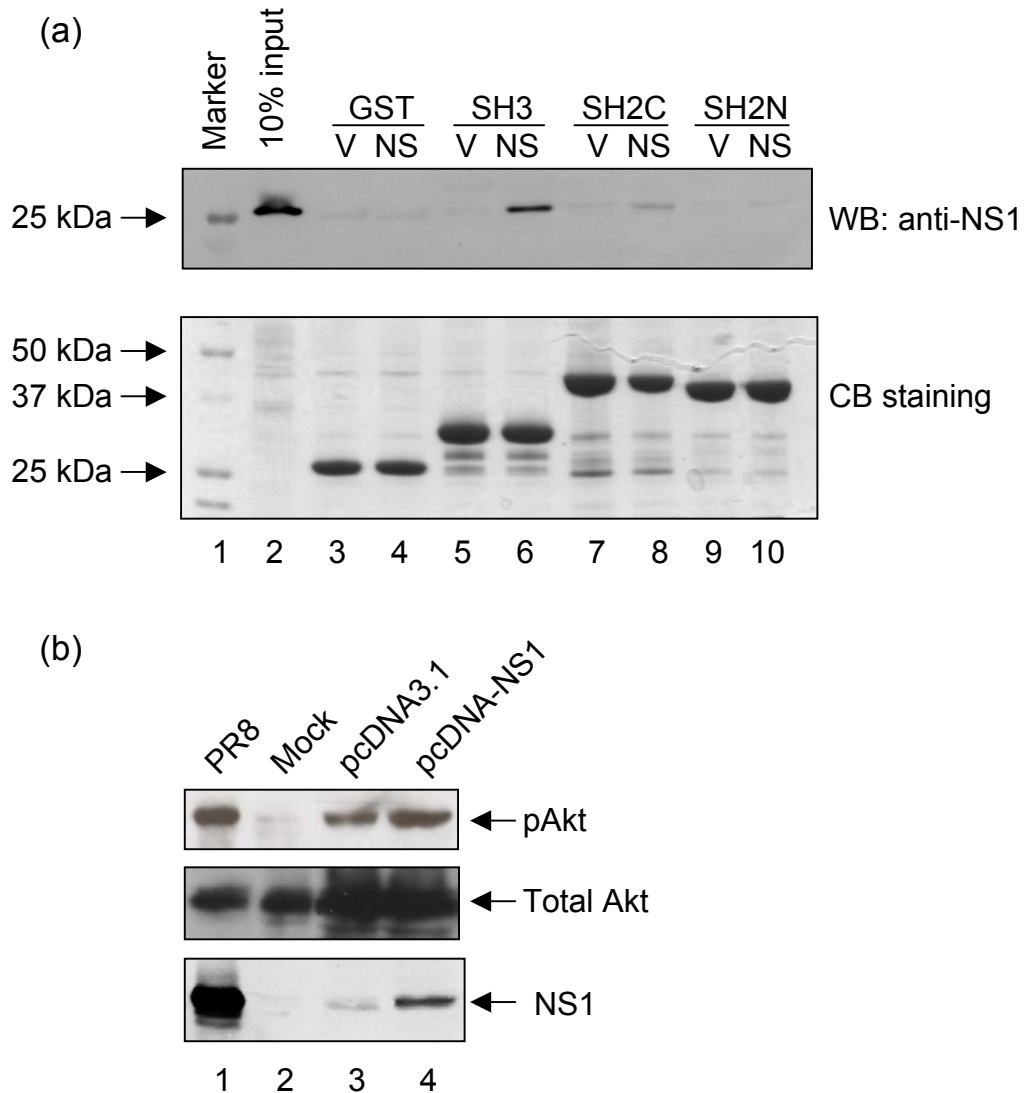
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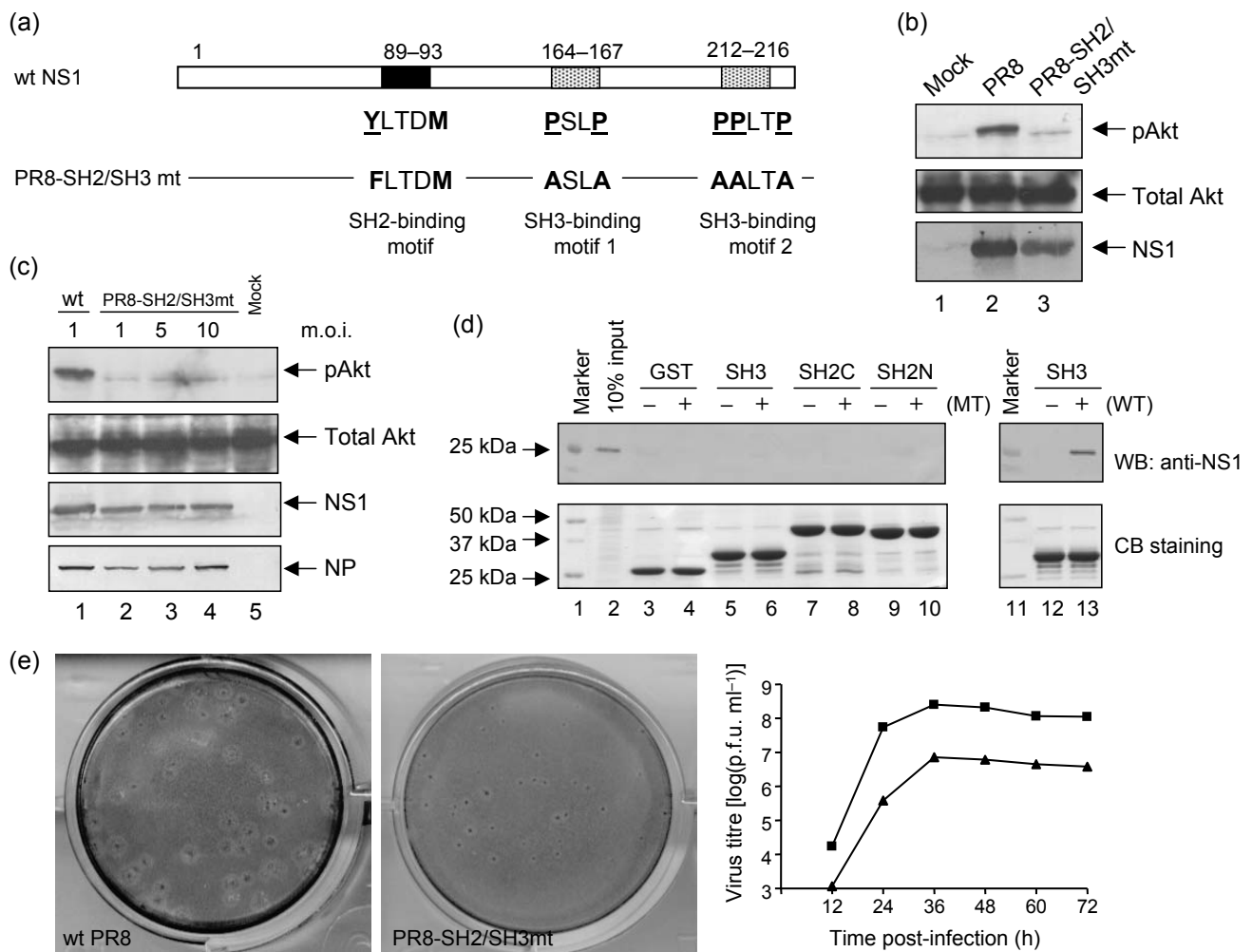
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**Fig. 1.** NS1 protein interacts with the p85 subunit of PI3K. (a) The p85 regulatory subunit of PI3K consists of tandem SH2 domains (SH2N, SH2C), one SH3 domain, a binding domain for PI3K catalytic subunit p110 and other protein-interaction domains, e.g. BCR (breakpoint cluster region homology domain). (b) Co-immunoprecipitation analysis. Cell lysates prepared at 6 h p.i. were incubated with either normal rabbit serum–protein A or p85 antiserum–protein A complexes. Precipitated proteins were subjected to Western blotting with NS1 or p85 antibody. (c) GST pull-down assay with wt virus-infected cell lysates. GST alone or fusions of GST to p85 SH3, p85 SH2C or p85 SH2N domain were immobilized to GA beads and incubated with mock- or wt PR8-infected A549 cell lysates prepared at 6 h p.i. Precipitated proteins were subjected to either Western blotting with NS1 antibody or SDS-PAGE followed by Coomassie blue staining.



**Fig. 2.** NS1 protein alone induces PI3K/Akt activation. (a) 293T cells were transfected with either pcDNA-NS1 or vector and analysed 48 h later by GST pull-down assay as described in the legend to Fig. 1(c). (b) A549 cells were electroporated with pcDNA-NS1 or vector. Seventy-two hours later, cell lysates were subjected to Western blotting with phospho-Akt, Akt or NS1 antibody.



**Fig. 3.** SH-binding motifs in NS1 contribute to PI3K/Akt activation. (a) Schematic diagram showing the locations of one SH2- and two SH3-binding motifs on wt NS1 and the changes in amino acid sequence on NS1 encoded by PR8-SH2/SH3mt. (b) PR8-SH2/SH3mt fails to induce phosphorylation of Akt. A549 cells were mock-, wt PR8- or PR8-SH2/SH3mt-infected at an m.o.i. of 1. Cell lysates prepared at 6 h p.i. were subjected to Western blotting with phospho-Akt, Akt or NS1 antibody. (c) Higher doses of PR8-SH2/SH3mt do not induce Akt phosphorylation. A549 cells were infected with different m.o.i.s of PR8-SH2/SH3mt. Cell lysates prepared at 6 h p.i. were subjected to Western blotting with phospho-Akt, Akt, NS1 or NP antibody. (d) PR8-SH2/SH3mt-infected A549 cell lysates were prepared at 6 h p.i. and subjected to GST pull-down assay as described in the legend to Fig. 1(c). (e) PR8-SH2/SH3mt is attenuated by monitoring plaque size and growth curve of the wt PR8 (■) and PR8-SH2/SH3mt (▲) in MDCK cells.