Mechanism of Influenza A Virus NS1 Protein Interaction with the p85β, but Not the p85α, Subunit of Phosphatidylinositol 3-Kinase (PI3K) and Up-regulation of PI3K Activity*

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Influenza A virus infection activates the phosphatidylinositol 3-kinase (PI3K)/Akt pathway by binding influenza A virus NS1 protein to the p85β regulatory subunit of PI3K. In this study, we report that NS1 binds to the inter-SH2 (iSH2) domain of p85β. Mutational analyses on p85β iSH2 domain defined that Val-573 is the critical amino acid (AA) that mediates NS1 and p85β interaction. In reciprocal gain of function experiments with p85α, we demonstrated that mutation to Val at Met-582 leads to NS1 binding and increased PI3K activity. Molecular modeling based on our experimental results suggested that, in addition to the interaction interface between the NS1 SH3 binding motif 1 (AA 164–167) and p85β Val-573, AA 137–142 in NS1 might interact with p85β. Indeed, mutations of AA 141 and 142 in NS1 disrupted the interaction between NS1 and p85β. Mutant virus PR8-NS1–141/142 was not able to activate Akt phosphorylation. Furthermore, PI3K assays demonstrated that, in wild-type virus-infected cells, p85β-associated PI3K activity was increased significantly. In contrast, in the mutant virus-infected cells containing mutant NS1 unable to interact with p85β, the p85β-associated PI3K activity up-regulation was not seen, suggesting that PI3K up-regulation is dependent upon the interaction between NS1 and p85β. Competition experiments and the immunoprecipitation studies demonstrated that NS1, p85β, and p110 form a complex in cells. Finally, the mechanism by which binding of NS1 to p85β regulates PI3K activity was discussed based on a predicted structural model of NS1-p85-p110 complex.

Phosphatidylinositol 3-kinases (PI3Ks) have been divided into three classes (1); to date, only those belonging to class I have been subjected to gene targeting. Class I PI3Ks are further divided into class IA and class IB PI3Ks. Class IA enzymes consist of a regulatory subunit (p85) and a catalytic subunit (p110). Three distinct genes encoding class IA PI3K regulatory subunits exist in mammals: Pik3r1 (p85α), Pik3r2 (p85β), and Pik3r3 (p55PIK) (2, 3). p85α is ubiquitously expressed and is thought to be the major responsive pathway for most stimuli, whereas p85β also is widely expressed but at a lower level than p85α (4). There are three class IA catalytic subunits: p110α, p110β, and p110δ. Any of the three isoforms can bind any of the p85-related regulatory subunits (5).

The p85 subunit has five domains: a Src homology 3 (SH3) domain, a domain with homology to breakpoint cluster region (BCR) protein, and two SH2 domains (nSH2 and cSH2) separated by an inter-SH2 (iSH2) (6). The p110α subunit also has five domains: an N-terminal adaptor binding domain (ABD), the Ras binding domain, the putative membrane binding domain C2, a helical domain of unknown function, and a kinase catalytic domain (7, 8). The iSH2 domain of p85 subunit is responsible for tight binding to the ABD domain of the p110 catalytic subunit (9, 10). In quiescent cells, p85 serves two functions on p110: conformational inhibition of its lipid kinase activity and stabilization against the thermal denaturation of p110 (11). In activated cells, p85 uses its SH2 domains to bind phosphotyrosine residues within activated tyrosine kinases, including BCR/ABL and NPM/ALK or adaptor proteins such as Shc, c-Cbl, Crk-L, and Gab2 (12). This results in the translocation of the p85–p110 heterodimer to the plasma membrane and causes steric changes within p85 to release the inhibitory effect on p110 (13). After activation, PI3K phosphorylates the lipid substrate phosphatidylinositol 4,5-bisphosphate to produce phosphatidylinositol 3,4,5-trisphosphate (14–16). Phosphatidylinositol 3,4,5-trisphosphate then serves as a lipid second messenger and is able to regulate phosphorylation of a number of kinases, including the serine/threonine kinase Akt. Akt is activated via phosphorylation at Thr-308 and Ser-473 (17). Phosphorylated Akt plays a central role in modulating diverse downstream signaling pathways associated with cell proliferation, migration, differentiation, and the prevention of apoptosis (18, 19).

Recently, we and others have demonstrated that influenza A virus infection leads to PI3K/Akt pathway activation by interaction of the viral NS1 protein with the p85β subunit of PI3K (20, 21). Furthermore, we identified a polyproline motif PXXP, which is located at amino acids (AA) 164–167 in NS1 (desig-
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...donated SH3 binding motif 1, is essential for mediating NS1-p85β interaction and the activation of the PI3K/Akt pathway (22). Mutant viruses containing SH3 motif 1 mutation in NS1 cannot bind to p85β, and thus are unable to activate the PI3K/Akt pathway. These mutant viruses formed much smaller plaques and grew to 1–1.5 log lower titers than the wild-type (WT) virus (20, 22). In addition, specific inhibitors for PI3K can strongly suppress influenza A virus replication (23, 24). These data demonstrated that the influenza A virus-induced PI3K/Akt pathway is pivotal in supporting virus replication. Studies using mutant viruses and WT virus plus PI3K inhibitors also showed that PI3K/Akt functions to suppress virus-induced apoptosis (22, 25). Heikkinen et al. (26) recently reported the NS1 protein of the 1918 influenza virus and a representative avian H7N3 strain contained a functional SH3 binding motif that mediates binding to the N-terminal SH3 domain of the adapter proteins Crk and CrkL. This binding is associated with enhanced PI3K signaling (26), further supporting the importance of the PI3K/Akt pathway in regulating influenza A virus replication and pathogenesis.

In this study, we further characterized the domains and amino acids on p85β that are required for mediating NS1-p85β interaction. Our data showed that the minimal essential region for NS1 binding on p85β is the iSH2 region (AA 420–615); within this region, Val at AA 573 is critical for NS1-p85β interaction. Mutation of the corresponding AA in p85α from Met to Val led to binding of NS1 to mutated p85α. In addition, AA 141–142 in NS1 was found to be another domain involved in the NS1-p85β interaction. Binding of NS1 to the iSH2 domain of p85β did not compete with p110 for binding with p85β and resulted in the up-regulation of PI3K activity. A molecular model of NS1-p85-p110 suggested binding of NS1 to p85 may sterically interfere with the kinase domain and/or helical domain of p110. Alternatively, binding of NS1 to p85 may disrupt the interactions of C2 region of p110 with p85, thus altering the regulatory effect of p85 on p110. Our data suggested a molecular mechanism by which influenza A virus infection up-regulates PI3K activity.

EXPERIMENTAL PROCEDURES

Cells and Virus—A549 cells (human lung carcinoma cells) and 293T (human embryonic kidney) cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% fetal bovine serum. Influenza A/Puerto Rico/8/34 (H1N1) Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% fetal bovine serum. Influenza A/Puerto Rico/8/34 (H1N1) and 293T (human embryonic kidney) cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% fetal bovine serum...
sequencing of the reverse transcription-PCR product derived from the NS segment.

Antibodies—Rabbit polyclonal NS1 was generated in our laboratory (24). Monoclonal mouse anti-His antibody was purchased from GE Healthcare. Anti-P13K p85 (anti-p85pan rabbit antiserum) and anti-P13K p85, N-SH3, clone AB6 (anti-p85α, does not recognize p85β, mouse monoclonal IgG₁) were purchased from Upstate. Monoclonal P13K p85β (T15, non-cross reactive with p85α), sc56934 antibody and monoclonal HA-probe (F-7), and sc-7392 antibody were purchased from Santa Cruz Biotechnology. Monoclonal anti-FLAG M2 antibody was purchased from Sigma. Phospho-Akt (Ser-473) (193H12) rabbit monoclonal antibody, rabbit polyclonal Akt antibody were purchased from Cell Signaling Technology. Alkaline phosphatase-conjugated anti-rabbit IgG was purchased from Jackson ImmunoResearch. IRDye 680-conjugated goat polyclonal anti-rabbit IgG and IRDye 800-conjugated donkey polyclonal antimouse IgG were purchased from LI-COR Biosciences.

Western Blot Analysis—Western blotting was performed as described previously (24) with minor modifications. Briefly, 30 μg of total protein or a portion of precipitated protein was resolved on SDS-10% PAGE gels and transferred onto nitrocellulose membranes (Bio-Rad). Membranes were blocked for nonspecific binding with Tris-buffered saline (0.1 M Tris, pH 7.6, 0.9% NaCl) containing 0.1% Tween 20 and 10% skim milk (for NS1) or Odyssey blocking buffer (LI-COR Biosciences) for 1 h at room temperature. For examination of NS1, membranes were probed with NS1 (1:2,000) antibody followed by an incubation with alkaline phosphatase-conjugated anti rabbit IgG (1:10,000). The immunoblots were then visualized by incubating with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium premix solution (Sigma). For examination of His₆-tagged p85α, His₆-tagged p85β, FLAG-tagged p85β, HA-tagged p110α, endogenous p85pan, p85α, p85β, pAkt, and Akt, a primary antibody was diluted in Odyssey blocking buffer according to the manufacturer’s suggestion and applied for 2 h at room temperature. Infrared dye-linked secondary antibody (1:15,000) was then added at room temperature for 1 h. The immunoblots were visualized using the Odyssey infrared imaging system. The levels of band density were quantified by using the Odyssey application software, version 2.0. Data are representative of one of three independent experiments unless otherwise indicated.

Transfection and Coimmunoprecipitation Analysis—293T cells were seeded in a 6-well plate at a density of 1 × 10⁶/well. DNA constructs (1 μg per construct unless specified) were transfected using CaCl₂ according to a protocol described previously (30). Twenty-four hours later, cell lysates were prepared as described above and subjected to specific assays.

The generation of whole cell lysates and lysates suitable for immunoprecipitations were carried out as described previously (22). 600 μg of cell lysates was precleared by either 3 μg of rabbit IgG or mouse IgG and protein G-Sepharose beads (Santa Cruz Biotechnology). Three micrograms of specific antibody or IgG control was immobilized to protein G-Sepharose beads and then incubated with precleared cell lysates for 2 h at 4 °C. After extensive washes, the precipitated proteins were subjected to either SDS-PAGE followed by Western blotting with specific antibodies or P13K assay.

GST and Ni-NTA-Sepharose Bead Pulldown Assay—The GST fusion protein was expressed and prepared as described previously (22). 250 μg of each GST fusion protein lysates was bound to 25 μl of a 50% pre-equilibrated glutathione-agarose bead slurry (GE Healthcare) for 1 h at 4 °C, and the beads were then washed three times with radioimmuneprecipitation assay buffer (0.5 M Tris, pH 8.0, 0.15 M NaCl, 0.1% SDS, 1% Nonidet P-40, 1% deoxycholic acid) containing 1 × protease inhibitors mixture, Complete (Roche Applied Science). 100 μl of lysates (1 μg/μl) from infected A549 cells or transfected 293T cells was incubated with glutathione-agarose beads-GST fusion protein complexes in phosphate-buffered saline. After 2-h incubation at 4 °C, beads were washed five times in radioimmuneprecipitation assay. Bound proteins were resolved on SDS-PAGE followed by Western blotting using a specific antibody.

200 μl of cell lysates (0.5 μg/μl) was incubated with 50 μl of 50% nickel-Sepharose 6 Fast Flow (GE Healthcare) slurry. The reaction volume was made up to 500 μl with phosphate-buffered saline and incubated for 2 h at 4 °C. After five washes, bound proteins were resolved by SDS-PAGE followed by Western blotting with specific antibody or Coomassie Blue staining.

P13K Assay—P13K assay was performed as described previously (27). Samples were immunoprecipitated using p85pan, p85α, or p85β antibodies (3 μg), respectively. Immunoprecipitates were washed twice with each of the following: wash 1 (phosphate-buffered saline), wash 2 (100 mM Tris-HCl, pH 7.4, 50 mM LiCl), and wash 3 (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 1 mM EDTA). Excess liquid was removed from the immunoprecipitates. Lipid micelles were generated by sonication of phosphatidylserine and phosphatidylinositol in P13K assay buffer (25 mM Heps, pH 7.4, 10 mM MgCl₂) in a sonication water bath for 20 min. Each sample was incubated with lipid micelles (5 μg of phosphatidylserine plus 2.5 μg of phosphatidylinositol) in P13K assay buffer and 10 μCi of [γ-32P]ATP in a total volume of 50 μl for 15 min at 20 °C while gently rocking. The reaction was stopped by the addition of HCl (to 1.7 M). Lipids were extracted into chloroform:methanol (1:1) and further washed with methanol, 1 N HCl (1:1). Reaction products were dried down, resuspended in chloroform:methanol (1:1), and spotted onto a TLC plate (Silica Gel 60, VWR Canlab). Samples were developed in 1-propanol, water, acetic acid (17:4:7.9:1) in a chromatography chamber for 3 h, dried, and exposed to a phosphorimaging screen. Results were visualized and quantified using Quantity One software (Bio-Rad).

Molecular Modeling—Discovery Studio 2.0 (Accelrys) was used for the modeling study and structure analysis. The crystal coordinates used are p110 ABD/p85 iSH2 (Protein Data Bank code 2V1Y), p110α/p85 iSH2 (Protein Data Bank code 2RD0), and NS1 dimer (Protein Data Bank code 2GX9). To calculate the potential interaction domains between p85β ISH2 and NS1, crystal coordinates of p85β ISH2 (Protein Data Bank code 2V1Y) was used as the templates, after manual replacement of Met-582 in p85α by Val as suggested by our experiments described in the text, the local structure of the mutated residue and its neighbors was optimized using the MODELER program. NS1 was manually docked around this site. Interaction of p85 ISH2 and NS1 was minimized and determined using the ZDOCK program.
 RESULTS

NS1 Binds to the iSH2 Domain of p85β—We and others have previously shown that the influenza A virus NS1 protein interacts with p85β resulting in activation of the PI3K/Akt pathway. To identify the region of p85β that interacts with NS1, different domains of p85β were fused to GST (Fig. 1A). A549 cells were

mock infected or infected with WT virus at a multiplicity of infection (m.o.i.) of 1. Cell lysates were prepared at 6 h post infection (h.p.i.) and were incubated with GST fusion proteins immobilized on glutathione-Sepharose. Pulled down proteins were analyzed by Western blotting using polyclonal NS1 antibody. Fig. 1B shows the iSH2 domain of p85β interacted with NS1 as efficiently as did the full-length p85β (lane 8 versus 10). GST alone and other p85β domains did not interact with NS1. Similar amounts of the GST and GST-p85β fusion proteins were used, as demonstrated by Coomassie Blue staining of the samples resolved by SDS-PAGE.

To examine whether NS1 unaccompanied by other viral components would interact with p85β iSH2 domain, 293T cells were transfected with empty vector or pcDNA3.1-NS1 (22). Cell lysates were prepared at 24 h post transfection and were subjected to a GST pulldown assay. Fig. 1C shows that the iSH2 domain of p85β bound to NS1 protein, which was derived from an expression plasmid (lane 8).

To verify whether the observed interactions would take place in mammalian cells, we cloned each individual domain of p85β into pcDNA4-HisMax vector, resulting in His$_{6}$-tagged p85β domains (Fig. I A) that are driven by a CMV promoter. Thus, 293T cells were co-transfected with pcDNA3.1-NS1 and pcDNA4HisMax-p85β-H3, pcDNA4HisMax-p85β-BCR, pcDNA4HisMax-p85β-NSH2, pcDNA4HisMax-p85β-iSH2, or pcDNA4HisMax-p85β-cSH2 constructs or the pcDNA4-HisMax vector. At 24 h post transfection, cell lysates were prepared and incubated with Ni-NTA-Sepharose beads. Precipitated proteins were analyzed by Western blotting with NS1 or His antibodies. As seen in Fig. 1D, in agreement with the in vitro GST pulldown assay results using infected and transfected cells, the iSH2 domain of p85β interacted with NS1 (lane 14) in mammalian cells. No interactions were detected between the pcDNA4HisMax vector encoded His$_{6}$ protein and NS1 (lane 4). The amount and integrity of the His$_{6}$-tagged p85β and its domains present in the same precipitated samples were also monitored.

Valine 573 in p85β Is the Determinant Residue That Is Essential for NS1-p85β Interaction—It is well known that p110α binds to the iSH2 domain of p85 (10). The observation that NS1 binds to iSH2 domain of p85β prompted us to examine the binding site of NS1 on iSH2 in more detail. We first made three truncation constructs of the p85β iSH2 domain (remaining AA 420–485, 420–550, and 485–615, respectively) that were fused to GST. GST pulldown assay with virus-infected cell lysates indicated that none of these truncated p85β iSH2 domains could interact with NS1 (data not shown), indicating that the entire iSH2 domain is required for the interaction. Given that (i) binding of NS1 requires that iSH2 retain its folded structure and (ii) NS1 does not bind to iSH2 of p85α (20, 22), we made four chimeric iSH2 constructs. As shown in Fig. 2A, each chimeric p85β-iSH2 chimera was comprised of certain regions of p85β iSH2 and remaining AA residues from the p85α iSH2. 293T cells were co-transfected with one of the His$_{6}$-tagged iSH2 chimeras together with pcDNA3.1-NS1. After 24 h, cell lysates were prepared and subjected to Ni-NTA-Sepharose bead pulldown assay followed by Western blotting with NS1 antibody. Fig. 2B shows that, although the WT p85β iSH2 domain could interact with NS1 protein (lane 2), chimera 1 and 2 could not interact with NS1 (lanes 4 and 6). In contrast, chimera 3, which contains the N terminus of p85α iSH2 and AA 454–561 of p85β iSH2, and chimera 4, which contains a large portion of the p85α iSH2 N terminus and AA 555–615 from p85β iSH2, could pull down NS1 (lanes 8 and 10), suggesting that interaction of NS1 with p85β depends on the region between AA 555 and 615 in p85β. As a further step to characterize the molecular mechanism of how NS1 binds to p85β, we then focused our attention on defining the one or more amino acids in p85β that are essential for binding of NS1. The experiments described above have narrowed down the essential region in p85β for NS1 binding to AA 555–615. We compared AA 555–615 of p85β to its corresponding AA sequence in p85α. As shown in Fig. 3A, there are eleven regions that are different between the two isoforms. Thus, eleven mutation constructs were made by replacing each individual different AA sequence in p85β with its counterpart.

**Mechanism of NS1 Up-regulation of PI3K Activity**

| A | Mouse p85β | 420 | 454 | 555 | 615 |
|   | Bovine p85α | 429 | 463 | 564 | 623 |
| Chimaera-1 | 454/463 | 555/564 |
| Chimaera-2 | 555/564 |
| Chimaera-3 | 1000/1045 |
| Chimaera-4 | 1000/1045 |

**B**

Co-transfection:

- NS1+ p85β-iSH2
- NS1+ Chimera-1
- NS1+ Chimera-2
- NS1+ Chimera-3
- NS1+ Chimera-4

Ni-NTA Beads:

- WB: NS1
- WB: His

Fig. 2. NS1 binds to the AA 555–615 region in iSH2 domain of p85β. A, a schematic representation of mammalian cell expressed His$_{6}$-tagged p85β/p85α proteins used in the pulldown assay. Numbers refer to the residues from full-length mouse p85β or bovine p85α. B, 293T cells were co-transfected with pcDNA3.1-NS1 and either the pcDNAHisMax-p85β-iSH2 or pcDNAHisMax-p85β-iSH2 chimera. Ni-NTA bead pulldown assay and Western blotting were performed as described in Fig. 1. A 10% input was loaded as control.
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AA sequence in p85α in the background of pcDNA4HisMax-p85β-iSH2. 293T cells were co-transfected with each mutant plasmid together with pcDNA3.1-NS1. The ability of the mutant p85β-iSH2 to interact with NS1 was assessed by Ni-NTA-Sepharose bead pulldown assay and Western blotting analysis with NS1 antibody. Fig. 3B shows that, of the eleven mutant constructs, only one, p85β-iSH2-P (V573M), was unable to interact with NS1 (lane 10). The rest of the mutations did not affect the interaction of p85β and NS1. These results suggested that residue Val-573 within the p85β iSH2 domain is critical for binding of p85β to NS1 protein.

**Full-length p85β with V573M Mutation Abrogates Its Interaction with NS1, whereas Full-length p85α with Mutation M582V Binds to NS1**—To test whether or not the mutation of the iSH2 domain in full-length p85 can interfere with its interaction with the NS1 protein, we generated two His6-tagged full-length p85α and human p85α (hp85α) C-terminal iSH2 domains. Numbers refer to residues from full-length mouse p85β. Single mutations were introduced into the boxed residue(s) by replacing the AA(s) in p85β with its corresponding AA(s) in p85α. B, 293T cells were co-transfected with pcDNA3.1-NS1 and one of the pcDNAHisMax-mp85β-iSH2 WT or mutants. The Ni-NTA bead pulldown assay and Western blotting were performed as described in Fig. 1. A 10% input was loaded as control.

**FIGURE 3. Residue Val-573 in p85β is critical for NS1 binding.** A, a sequence alignment of mouse p85β (mp85β), human p85β (hp85β), bovine p85α (bp85α), and human p85α (hp85α) C-terminal iSH2 domains. Numbers refer to residues from full-length mouse p85β. Single mutations were introduced into the boxed residue(s) by replacing the AA(s) in p85β with its corresponding AA(s) in p85α. B, 293T cells were co-transfected with pcDNA3.1-NS1 and one of the pcDNAHisMax-mp85β-iSH2 WT or mutants. The Ni-NTA bead pulldown assay and Western blotting were performed as described in Fig. 1. A 10% input was loaded as control.

| A | 550 | 560 | 570 | 550 | 590 | 600 | 610 |
|---|-----|-----|-----|-----|-----|-----|-----|
| mp85β | REIKDNRNKPDLPQRLETRQYLMULCKGKRQPKNEUNGLKEDQYSLIKEDASTHPEDERTU |
| hp85β | REIKDNRNKPDLPQRLETRQYLMULCKGKRQPKNEUNGLKEDQYSLIKEDASTHPEDERTU |
| bp85α | REIKDNRNKPDLPQRLETRQYLMULCKGKRQPKNEUNGLKEDQYSLIKEDASTHPEDERTU |
| hp85α | REIKDNRNKPDLPQRLETRQYLMULCKGKRQPKNEUNGLKEDQYSLIKEDASTHPEDERTU |

**B**

| Co-transfection: | NS1+ iSH2-wt | NS1+ iSH2-M | NS1+ iSH2-N | NS1+ iSH2-O | NS1+ iSH2-P | NS1+ iSH2-Q | NS1+ iSH2-R | NS1+ iSH2-S |
|-----------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|
| Ni-NTA Beads:   | +           | +           | +           | +           | +           | +           | +           | +           |
| WB: NS1         |             |             |             |             |             |             |             |             |
| WB: His         |             |             |             |             |             |             |             |             |

| Co-transfection: | NS1+ iSH2-T | NS1+ iSH2-U | NS1+ iSH2-V | NS1+ iSH2-G |
|-----------------|-------------|-------------|-------------|-------------|
| Ni-NTA Beads:   | +           | +           | +           | +           |
| WB: NS1         |             |             |             |             |
| WB: His         |             |             |             |             |

AA sequence in p85α in the background of pcDNA4HisMax-p85β-iSH2. 293T cells were co-transfected with each mutant plasmid together with pcDNA3.1-NS1. The ability of the mutant p85β-iSH2 to interact with NS1 was assessed by Ni-NTA-Sepharose bead pulldown assay and Western blotting analysis with NS1 antibody. Fig. 3B shows that, of the eleven mutant constructs, only one, p85β-iSH2-P (V573M), was unable to interact with NS1 (lane 10). The rest of the mutations did not affect the interaction of p85β and NS1. These results suggested that residue Val-573 within the p85β iSH2 domain is critical for binding of p85β to NS1 protein.

**Full-length p85β with V573M Mutation Abrogates Its Interaction with NS1, whereas Full-length p85α with Mutation M582V Binds to NS1**—To test whether or not the mutation of the iSH2 domain in full-length p85 can interfere with its interaction with the NS1 protein, we generated two His6-tagged full-length p85α and human p85α (hp85α) C-terminal iSH2 domains. Numbers refer to residues from full-length mouse p85β. Single mutations were introduced into the boxed residue(s) by replacing the AA(s) in p85β with its corresponding AA(s) in p85α. B, 293T cells were co-transfected with pcDNA3.1-NS1 and one of the pcDNAHisMax-mp85β-iSH2 WT or mutants. The Ni-NTA bead pulldown assay and Western blotting were performed as described in Fig. 1. A 10% input was loaded as control.

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and incubated with Ni-NTA beads. Pulled down proteins were analyzed by Western blotting using pAkt, NS1, and total Akt antibodies. Fig. 6B shows that, although mutations of AA 137 and 138 in NS1 did not alter the interaction of NS1 with p85β (lane 4), mutations of AA 139 and 140 in NS1 diminished the NS1-p85β interaction (lane 6). Furthermore, mutations of AA 141 and 142 in NS1 significantly disrupted the interaction of NS1 with p85β (lane 8). In agreement with previous report, NS1 with P164A and P167A mutations did not interact with p85β (lane 12) (22).

To further study the contribution of Leu-141 and Glu-142 in NS1 to p85β binding and the PI3K activation, we generated a mutant virus PR8-NS1–141/142 by reverse genetics (29). This mutant virus encodes NS1 where the original Leu-141 and Glu-142 were replaced by alanines. The ability of the PR8-NS1–141/142 to activate the PI3K/Akt pathway was compared with that of PR8-SH3-mf-1 mutant virus (22). A549 cells were serum-starved overnight and infected by WT or mutant viruses at an m.o.i. of 1. Cell lysates were prepared at 6 h.p.i. and subjected to Western blotting using pAkt, NS1, and total Akt antibodies. As shown in Fig. 6C, a striking amount of phosphorylation of Akt (Ser-473) was detected in A549 cells that were infected with WT virus (lane 4). In contrast, only the basal level of pAkt could be detected in mock-, PR8-NS1–141/142-, and PR8-SH3-mf-1-infected cells (lanes 1–3). Western blotting with NS1 and total Akt antibodies showed a similar amount of NS1 and total Akt in all samples, indicating that the changes in Akt phosphorylation by

NS1 where Asn-137 and Phe-138 were mutated to alanines. Plasmid pcDNA3.1-NS1–137/140 encodes PR8 NS1 protein where Asp-139 and Arg-140 were mutated to alanines. Plasmid pcDNA3.1-NS1–141/142 encodes NS1 where Leu-141 and Glu-142 were mutated to alanines. 293T cells were transfected by pcDNA4HisMax-mp85β and pcDNA3.1-NS1–137/138, pcDNA3.1-NS1–139/140, pcDNA3.1-NS1–141/142, or pcDNA3.1-NS1-SH3-mf-1 (this plasmid encodes mutant NS1 where prolines at AA 164 and AA 167 were replaced by alanines) (22). At 24 h post transfection, cell lysates were prepared infection with different viruses were not due to an altered level of NS1 or Akt.

**NS1-p85-p110 Forms a Complex in the Cells**—The series of experiments described above suggested that NS1-p85β-p110 may form a complex in the influenza A virus-infected cells. This is based on the following observations: (i) the interaction of p85-p110 is very strong and it is unlikely to dissociate the two subunits under physiological conditions (11); (ii) our results show that NS1 binds around Val-573 in p85β (equivalent to AA 582 in p85α), which is located at the C terminus of Helix-2 of...
the iSH2 domain of p85, and (iii) p110 ABD domain basically binds to AA 478–513 in p85α and AA 445–485 in p85β.

To test our hypothesis, we performed the following experiments. First, to examine whether NS1 will compete p110 in binding to p85β, we co-transfected three plasmids: pcDNA4HisMax-p85β, pcDNA3.1-HA-p110α (expressing HA-tagged p110α), and pcDNA3.1-NS1/or vector, into 293T cells. After 24 h, cell lysates were prepared and subjected to Ni-NTA bead pulldown assay followed by Western blotting using NS1, His, or HA antibodies. As shown in Fig. 7A, increasing amounts of NS1 did not prevent binding of p110 to p85β, indicating that binding of NS1 to p85β did not compete for p110 binding to p85β.

To test whether p110, p85, and NS1 would form a complex in mammalian cells, 293T cells were co-transfected with three plasmids: pCMV-3xFLAG-mp85β, pcDNA3.1-HA-p110α, and pcDNA-NS1/or vector. (Because our NS1 antibody was raised by using Hisα-tagged NS1 fusion protein, to avoid the potential cross reaction, FLAG-tagged p85β was used here.) At 24 h post transfection, cell lysates were prepared and immunoprecipitation was carried out using NS1 antibody or rabbit IgG. Immunoprecipitated proteins were subjected to SDS-PAGE followed by Western blotting analysis with HA, FLAG, and NS1 antibodies. As shown in Fig. 7B, NS1 antibody could immunoprecipitate p85β as well as p110α (lane 3), whereas no such proteins were immunoprecipitated by rabbit IgG (lane 4). In cells where no NS1 was expressed, neither NS1 antibody nor rabbit IgG could precipitate p85β and p110α (lanes 6 and 7). We also tested whether this would happen during the virus infection. To this end, 293T cells were cotransfected with pcMV-3xFLAG-mp85β and pcDNA3.1-HA-p110α. At 24 h post transfection, cells were infected with PR8 WT, PR8-SH3-mf-1, or PR8-NS1–141/142 virus at an m.o.i. of 1.6 h later, cell lysates were subjected to immunoprecipitation with NS1 antibody or rabbit IgG. Immunoprecipitated proteins were analyzed by SDS-PAGE followed by Western blotting with FLAG, HA, and NS1 antibodies. As shown in Fig. 7C, in WT virus-infected cells, NS1 antibody could efficiently immunoprecipitate p85β and p110α (lane 2), no p85β and p110α were precipitated by rabbit IgG (lane 3). In cells that were transfected with pCMV-3xFLAG-mp85β and HA-vector or transfected with pcDNA3.1-HA-p110α and pCMV-3xFLAG vector, no such NS1-p85-p110 complex was observed (lanes 5 and 8). In PR8-SH3-mf-1 and PR8-NS1–141/142 virus-infected cells, where mutant NS1 did not interact with p85β, no such ternary complex was detected (lanes 11 and 14). These data suggested that p85-p110 form a complex with WT NS1, regardless if NS1 is derived from plasmid transfection or virus infection.

To exclude the possibility that NS1 interacts with p110, we co-transfected pcDNA3.1-HA-p110α with pcDNA-NS1/or vector. Immunoprecipitation was carried out with cell lysates prepared at 24 h post transfection with NS1 antibody or rabbit IgG. Western blotting of precipitated proteins was done using HA and NS1 antibodies. As seen in Fig. 7D, no NS1 and p110 interactions could be detected (lane 3). It is interesting to note that the amount of p110 in the presence of NS1 was higher than that in the absence of NS1 (lane 2 versus lane 5). This might be due to the direct or indirect stabilization effect of NS1 on p110, or maybe NS1 favors p110 expression. Taken together, these data indicate that NS1 does not bind to p110 directly, and both NS1 and p110 bind to p85β, thus forming a complex of NS1–p85β–p110 in the cells.

FIGURE 5. Hypothetical model of NS1–p85β complex. A, ribbon representation of NS1-p85α–M582V. The crystal structure of p85α iSH2 used here is as reported by Miled et al. (5) (Protein Data Bank File: 2V1Y) with manual replacement of Met-582 to Val. The crystal structure of NS1 is as reported by Bornholdt et al. (31) (Protein Data Bank code: 2GX9). Potential interaction domains in NS1 and p85 are shown in yellow. B, alternate view of the NS1-p85α–M582V complex. The domain around AA 164–167 in NS1 potentially interacts with V582 (equivalent to Val-573 in p85β) as indicated with one star. Domain around AA 137–142 in NS1 potentially interacts with N terminus of iSH2 (area is indicated by two stars). Energy calculations for the two proteins were performed using Discovery Studio 2.0. Blue, sheet; red, helix.
Mechanism of NS1 Up-regulation of PI3K Activity

**A**

| NS1          | 137-142       |
|--------------|---------------|
| NS1-137/138  | NFDRLE        |
| NS1-139/140  | AADRLE        |
| NS1-141/142  | NFAALE        |
| NS1-141/142  | NFDRAA        |

**B**

| Co-transfection: | Ni-NTA Beads: | WB: NS1 | WB: His |
|------------------|---------------|---------|---------|
| +     | +     | +       | +       |
| +   | +   | +       | +       |
| + | + | +       | +       |
| + | + | +       | +       |

**C**

| pAkt          | mock          |
|---------------|---------------|
| 1 2 3 4       | 141/142       |
| 1 2 3 4       | SH3-1         |
| 1 2 3 4       | WT            |

**D**

Binding of NS1 to p85α-M582V or to p85β Leads to Up-regulation of PI3K Activity—Our previous studies showed that influenza A virus infection led to Akt phosphorylation, which was PI3K-dependent (24), and mutant viruses PR8-SH3-mf-1 and PR8-NS1–141/142, whose NS1 proteins did not interact with p85β, could not activate the PI3K/Akt pathway (Fig. 6C) (22). These results suggested that binding of NS1 to p85 results in up-regulation of PI3K activity. To ascertain the biological relevance of NS1-p85 interaction and PI3K enzyme activity, we performed the following experiments. First, we wanted to determine whether binding of NS1 to p85α-M582V would lead to increased PI3K activity, and concurrently, whether abrogation of NS1-p85β-V573M interaction would lead to loss of function for PI3K activity. To this end, 293T cells were transfected with pFLAG3-p85α, pFLAG3-p85α-M582V, pCMV-3xFLAG-p85β, or pCMV-3xFLAG-p85β-V573M. Cells were maintained in serum-free Dulbecco’s modified Eagle’s medium for 24 h and then were either mock or WT virus-infected at an m.o.i. of 2. At 6 h.p.i., cell lysates were subjected to immunoprecipitation with NS1 antibody. PI3K activity in the precipitates was assessed. Fig. 8A shows that, in cells transfected with WT p85α, no PI3K activity could be detected regardless if NS1 is present or not (lanes 1 and 2). In contrast, significant increased PI3K was seen in cells transfected with p85α-M582V and infected with WT virus (lane 3). No PI3K activity was observed in cells transfected with p85α-M582V in the absence of NS1 (lane 4). Conversely, transfection of WT p85β led to an increased PI3K activity by NS1 (lane 5 versus 6); whereas transfection of p85β-V573M did not result in significant changes of PI3K activity in response to NS1 (lanes 7 and 8). The PI3K activity was quantified and plotted in Fig. 8B.

In the second set of experiments, we examined the p85α-, p85α-, and p85β-associated PI3K activities in virus-infected A549 cells. A549 cells were mock infected or infected with WT PR8, PR8-SH3-mf-1, or PR8-NS1–141/142 at an m.o.i. of 2. At 6 h.p.i., cells were harvested, and cell lysates were subjected to immunoprecipitation with p85α, p85α, p85β, and antibodies, respectively. The precipitates were divided into half. For one half of the precipitates, proteins were resolved by SDS-PAGE and Western blotting using p85α, p85β, and p85α antibodies (Fig. 8C). For the other half of the precipitates, PI3K activities were assessed (Fig. 8, D and E). In Fig. 8C, the top panel shows that p85α antibody recognized p85α as well as p85β. In virus-infected cells the ratio of p85α/p85α and p85β/p85α did not change dramatically compared with that in mock infected cells. The middle and bottom panels show that in both mock infected or virus-infected cells, p85α antibody did not cross-react with p85β, and p85β antibody did not recognize p85α either. As shown in Fig. 8D, dramatic PI3K activity changes were only seen in p85β precipitates. In WT virus-infected cells, the p85β-associated PI3K activity was higher than the PI3K activity in mock infected cells (lane 10 versus 9). To obtain more quantitative information about the PI3K activity associated with different p85 isoforms in different virus-infected cells, we normalized the PI3K activity with the p85 level present in the precipitates. Relative PI3K activity in virus-infected cells was expressed as fold increase of that in mock infected cells. As seen in Fig. 8E, in WT virus-infected cells, the p85β-associated PI3K was increased ~5.2-fold compared with that in mock infected cells, no up-regulation of p85β-associated PI3K was observed in PR8-SH3-mf-1 and PR8-NS1–141/142 virus-infected cells (Fig. 8D, lanes 11 and 12). PI3K activities associated either with p85α or p85α in WT, PR8-SH3-mf-1 and PR8-NS1–141/142 virus-infected cells did not change significantly in comparison with mock infected cells.
**Mechanism of NS1 Up-regulation of PI3K Activity**

**A**

| Co-transfection: NS1 plasmid (μg) | Ni-NTA beads: | WB: HA | WB: His | WB: NS1 | Band density of bound p110 |
|----------------------------------|---------------|--------|---------|---------|---------------------------|
| 0.2                              | input + NS1   | IgG    | 1       | 2       | 1.00E+06                  |
| 0.5                              | input + NS1   | IgG    | 1       | 2       | 1.00E+05                  |
| 1.0                             | input + NS1   | IgG    | 1       | 2       | 1.00E+04                  |

**B**

| Co-transfection: HA-p110 + His-p85β + NS1 | HA-p110 + Flag-p85β + NS1 | HA-p110 + Flag-p85β + vector |
|------------------------------------------|----------------------------|-------------------------------|
| Mock                                     | input NS1 IgG             | input NS1 IgG                 |
| WB: Flag                                 |                             |                               |
| WB: HA                                   |                             |                               |
| WB: NS1                                  |                             |                               |

**C**

| Infection (MOI=1): | PR8 WT | SH3-1 | 141/142 |
|--------------------|--------|-------|---------|
| Co-transfection:   | HA-p110 + Flag-p85β | HA-Vector + Flag-p85β | HA-p110 + Flag-p85β |
| IP with:           | input NS1 IgG | input NS1 IgG | input NS1 IgG |
| WB: Flag           |               |               |               |
| WB: HA             |               |               |               |
| WB: NS1            |               |               |               |

**D**

| Co-transfection: | HA-p110 + NS1 | HA-p110 + vector |
|------------------|---------------|------------------|
| IP with:         | input NS1 IgG | input NS1 IgG    |
| WB: HA           |               |                  |
| WB: NS1          |               |                  |

**FIGURE 7. NS1-p85β-p110α forms a complex in cells.** A, 293T cells were co-transfected with pcDNA-HA-p110α, pcDNA4HisMax-mmp85β, and the indicated amount of pcDNA3.1-NS1. At 24 h post transfection, cell lysates were prepared and subjected to Ni-NTA bead pulldown assay. Bound proteins were resolved in SDS-PAGE followed by Western blotting with NS1, HA, and His antibodies, respectively. Band densities of bound p110 were quantified and plotted. B, 293T cells were co-transfected with pcDNA-HA-p110α, pcCMV-3xFLAG-mmp85β, and pcDNA3.1-NS1/141/142 vector. Cell lysates were prepared at 24 h post transfection and subjected to immunoprecipitation with NS1 antibody or rabbit IgG. The precipitated proteins were resolved in SDS-PAGE followed by Western blotting using FLAG, HA, and NS1 antibodies, respectively. C, 293T cells were co-transfected with pcDNA-HA-p110α and pcCMV-3xFLAG-mmp85β. At 24 h post transfection, transfected cells were infected with WT PR8, PR8-SH3-mf-1, or PR8-NS1–141/142 virus at an m.o.i. of 1 for 6 h. Cell lysates were prepared at 24 h post transfection and subjected to immunoprecipitation with either NS1 antibody or rabbit IgG. Immunoprecipitated proteins were analyzed by Western blotting with HA and NS1 antibodies. A 10% input was loaded in all blots and used as control.

**DISCUSSION**

Recent studies have demonstrated that influenza A virus infection activates the PI3K/Akt pathway in the late phase of infection. The PI3K/Akt pathway activation is attributed to the viral NS1 protein, which can specifically interact with PI3K regulatory subunit p85β (20–22, 24). However, the molecular mechanism by which NS1 selectively binds to p85β and up-regulates the kinase activity remains to be elucidated. In our previous study using rat p85α we showed that NS1 binds to the SH3 and cSH2 domains, however, we could not detect binding of NS1 to full-length p85α. In contrast, we observed striking and specific binding of NS1 to p85β (22). As the first step toward understanding how NS1 activates PI3K activity by binding to the p85β subunit, we are more interested in defining the specific domain in p85β that is responsible for binding to NS1. We expressed domains of p85β as GST fusion proteins. In vitro GST pull-down assay with NS1 derived from viral infection (Fig. 1B) or from the plasmid expression (Fig. 1C) clearly showed the iSH2 region of p85β is required for the association with NS1 protein. The same domains of p85β were cloned into a mammalian expression vector, and the data indicated that the p85β iSH2-NS1 interactions also take place *in vivo* (Fig. 1D). To further define regions in the p85β iSH2 domain that are essential for NS1 binding, we initially made three truncation constructs of p85β. However, we could not detect the interactions between NS1 and the truncated p85β iSH2 domains (data not shown). These results suggested that the entire rigid coiled-coil structure of iSH2 is required for NS1 binding. A more detailed analysis of the p85β iSH2 region by exchanging domains between p85α and -β isoforms together with point mutations revealed that residue Val-573 in p85β is critical for the association between p85β and NS1 (Figs. 2 and 3). When full-length p85β was mutated at residue 573 from Val to Met, the corresponding AA in p85α, it lost the ability to interact with NS1. Interestingly, full-length p85α, carrying mutations at AA 582, which original Met was mutated to Val, could bind to NS1. Crystal structure revealed that AA 582 is located close to a flexible linker domain (Fig. 4C), and in WT p85α there is a short coil region consisting of 4 AA (Fig. 4E) (5). A structural analysis of p85α-M582V (Fig. 4D) using Discovery Studio software revealed that changing AA 582 from Met to Val led to a coil region composed of 6 AA (Fig. 4E). This change may alter the orientation or stability of the adjacent domains (Fig. 4, C and D), which favors NS1 binding. This may explain why NS1 binds to p85β and p85α-M582V and does not bind to p85α and p85β-V573M. An alternative hypothesis may be that the longer side chain on Met may prevent NS1 association.
Mechanism of NS1 Up-regulation of PI3K Activity

Influenza A virus infection activates Akt phosphorylation, which is PI3K-dependent (23, 24). Elimination of NS1-p85β interaction leads to abrogation of PI3K/Akt pathway activation (Fig. 6) (22). These data suggested a correlation between the NS1-p85 interaction and the up-regulation of PI3K activity. A series of experiments were carried out to address this question. First, we transfected exogenous p85α, p85α-mutant, p85β, p85β-mutant into cells, transfected cells were left uninfected or WT virus infected. As we expected, p85β-p110 and p85α-mutant-p110 showed increased PI3K activity by interacting with NS1 (Fig. 8A and 8B). We also tried to assess PI3K activity by using exogenous p110 in the similar setting, however, no changes in PI3K activity were detected in response to NS1 (data not shown). This may suggest that other cellular proteins are also involved in regulating p110 activity associated with NS1-p85. Next, to understand the contribution of p85α and p85β to PI3K/Akt activation in virus-infected cells, we assessed the PI3K activities associated with p85pan, p85α, and p85β after virus infection (Fig. 6, C–E). In WT virus-infected cells, p85β-associated PI3K activity increased ~5-fold compared with that in mock infected cells. No changes were observed in p85pan- and p85α-associated PI3K activities.

Based on the experimental results showing that Val-573 (number refers to p85α) is the critical binding site for NS1, we carried out a modeling study to investigate the interaction of NS1 to p85 (Fig. 5). It is intriguing that the model suggested the SH3 binding motif 1 (AA 164–167) in NS1 is the domain that interacts with Val-582 (number refers to p85α). This is consistent with our previous results, that mutant virus PR8-SH3-mf-1, carrying mutations at AA 164 and 167, failed to activate the PI3K/Akt pathway and as a result of its NS1 could not interact with p85β (22). In addition to the SH3 binding motif 1, AA 137–142 in NS1 was also suggested as a potential binding domain to p85. Our mutational analyses confirmed that mutation of AA 141 and 142 in NS1 disrupted the interaction of NS1 with p85β (Fig. 6), and mutant virus PR8-NS1–141/142 was unable to activate Akt phosphorylation. Thus, a novel motif in NS1 that contributes to p85 binding and PI3K activation has been identified. Our data supported the structural model of NS1/p85β iSH2 complex (Fig. 5) where the NS1 AA 164–167 domain interacts with p85β V573 and the NS1 AA 141/142 domain interacts with the N-terminal third of the p85β iSH2 domain. Both interaction interfaces are required for achieving the full interactions between NS1 and p85β, whereas disturbance of either site leads to disruption of NS1 and p85β interaction. This model explains why we could not detect the interactions between NS1 and the truncated p85β iSH2 domains comprising AA 420–485, 420–550, and AA 485–615, respectively (data not shown). These truncated p85β iSH2 domains lack either the N-terminal part of p85β iSH2 or Val-573 residue and thus could not interact with NS1 properly. However, we cannot exclude the possibility that the intact rigid coiled-coil structure of iSH2 is required for binding of NS1.
than the small contribution from p85β, this might also provide an example of how the p85 isoforms have cross-talk and keep a balanced function of p85 in cells.

While this manuscript was being prepared, Hale et al. (33) reported that binding of NS1 to p85β requires residues at the C-terminal end of iSH2 and is independent of p110 binding. These data are consistent with the results presented here. A truncation mutant p85 with elimination of all amino acids C-terminal to residue 571 was reported to have a constitutively increased PI3K activity (34). It was suggested that residues 581–593 in p85 constrain the location of the inhibitory nSH2 domain. Deletion of residues from 571 will remove such orienting constraints (35). Therefore, Hale et al. (33) proposed that binding of NS1 to p85 may have the same effect on p110 activity by masking the contribution of p85 to p110 inhibition. Considering our experimental results showing that NS1 binds to p85β without competing with p110 (Fig. 7), and NS1 binds around Val-573 in p85β (Fig. 3), we performed a modeling study to investigate the molecular basis of how NS1 binds to the p85–p110 complex and alters the p110 kinase activity. The recently resolved crystal structure of a complex between p110α and the p85α iSH2 domain (36) provides a detailed steric position of the p110 domains with respect to the p85 iSH2 domain and, thus, is more suitable for our modeling. Unfortunately, the detailed structure of AA 583–593 in p85α is not available. Therefore, the NS1-p85-p110 model was created by placing the crystal structure of the influenza NS1 dimer (31) around AA 582 of p85α. Three residues that are frequently mutated in cancer are shown in Corey-Pauling-Koltun mode.

FIGURE 9. Hypothetical model of binding of NS1 to p85β-p110 complex leads to up-regulation of PI3K activity. The crystal structure of NS1 (31) (Protein Data Bank code: 2GX9) was manually placed on the crystal structure of the p110α/p85 iSH2 complex (36) (Protein Data Bank code: 2RD0) around AA 582 of p85α. Three residues that are frequently mutated in cancer are shown in Corey-Pauling-Koltun mode.

mutated in cancer. Therefore, we propose that, although no direct interaction of NS1 with p110 was detected (Fig. 7D), NS1 may sterically interact with the kinase domain and/or the helical domain of p110. This interaction may modify the orientation of the kinase domain and/or the helical domain with respect to nSH2 of p85, relieving the inhibitory effect of p85 on p110 activity. It is also noticeable that NS1 is sterically close to the C2 domain of p110. A recent study by Huang et al. (36) found an unexpected feature of the C2 domain of p110 in the p110α/p85 iSH2 crystal structure: the C2 domain interacts with the iSH2 domain of p85 at AA 560 and 564. Because NS1 binds to iSH2 of p85 at AA 582, which is adjacent to the sites where the p110 C2 domain binds, it is possible that binding of NS1 will influence the interaction of C2 with the iSH2 of p85. In this regard, the effect of NS1 binding may be equivalent to that of mutation of Asn-345 of the C2 domain, where disruption of the p110 C2 domain with the iSH2 of p85 would presumably alter the regulatory effect of p85 on p110 (36).

Taken together, this study has localized the amino acids of p85β and NS1 involved in interacting with each other. Moreover, these results provide insights into the molecular basis by which influenza A virus NS1 protein specifically interacts with p85β, but not p85α isoform, leading to up-regulation of PI3K activity. This will allow the use of mutants on both p85β and NS1 to define a more precise role of the PI3K/Akt pathway in influenza A virus pathogenesis.

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