Binding of Influenza A Virus NS1 Protein to the Inter-SH2 Domain of p85β Suggests a Novel Mechanism for Phosphoinositide 3-Kinase Activation*

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Influenza A virus NS1 protein stimulates host-cell phosphoinositide 3-kinase (PI3K) signaling by binding to the p85β regulatory subunit of PI3K. Here, in an attempt to establish a mechanism for this activation, we report further on the functional interactions between NS1 and p85β. Complex formation was found to be independent of NS1 RNA binding activity and is mediated by the C-terminal effector domain of NS1. Interestingly, the primary direct binding site for NS1 on p85β is the inter-SH2 domain, a coiled-coil structure that acts as a scaffold for the p110 catalytic subunit of PI3K. In vitro kinase assays, together with protein binding competition studies, reveal that NS1 does not displace p110 from the inter-SH2 domain, and indicate that NS1 can form an active heterotrimetric complex with PI3K. In addition, it was established that residues at the C terminus of the inter-SH2 domain are essential for mediating the interaction between p85β and NS1. Equivalent residues in p85α have previously been implicated in the basal inhibition of p110. However, such p85α residues were unable to substitute for those in p85β with regards NS1 binding. Overall, these data suggest a model by which NS1 activates PI3K catalytic activity by masking a normal regulatory element specific to the p85β inter-SH2 domain.

Lipid second messengers generated by phosphoinositide 3-kinases (PI3Ks) regulate an array of protein kinase signaling cascades that, in turn, control diverse cellular processes such as cell survival, metabolism, proliferation, and inflammation/immunity (1, 2). Class IA PI3Ks are dimeric enzymes consisting of a p110 catalytic subunit tethered to a smaller, non-catalytic, regulatory subunit (typically p85α or p85β). The interaction of p85 with p110 functions to both stabilize heat-labile p110, and suppress its enzymatic activity (3). Thus, within cells, p85 and p110 proteins exist as obligate heterodimers (3, 4), and subsequent activation of PI3K must occur via inter- or intramolecular allosteric changes.

The p85 regulatory subunits contain an N-terminal SH3 (Src homology 3) domain, a BH (B-cell receptor homology) domain flanked by proline-rich sequences, and two SH2 (Src homology 2) domains, which are on either side of the p110-binding inter-SH2 (iSH2) domain (Fig. 1A) (2, 5). All the domains of p85 contribute to the regulation of p110, and stimulatory signals (such as growth factors and hormones) act through multiple mechanisms in order to modulate the basal inhibition of PI3K. For example, tyrosine phosphorylation of consensus YXXM motifs in activated growth factor receptors (or their specific adapter substrates) provides docking sites for the two p85 SH2 domains, and relieves the effect of p85 on p110 (6, 7). Additionally, binding of GTPases (such as Cdc42 and Rac) to the p85 BH domain, or binding of Src family kinases to the p85 proline-rich motifs, have also been shown to increase the activity of the p85:p110 heterodimer (8–10). In contrast, a novel adapter protein, Ruk, interacts with the N-terminal SH3 domain of p85 and negatively regulates PI3K function (11). Furthermore, direct phosphorylations of p85 also determine p110 activity: Src kinase-mediated phosphorylation of tyrosine 688 alleviates p110 inhibition (12), while autophosphorylation of serine 608 restores inhibition (13). Thus, docking of adapter proteins to particular domains, or phosphorylation of key residues, probably induces conformational changes in p85 that are somehow transmitted to p110 and direct its catalytic activity.

It is clear that many important chronic- and acute- disease causing viruses hijack the PI3K signaling pathway to facilitate their efficient replication (reviewed in Ref. 14). As a parallel to the host-cell regulatory control of PI3K, viruses also activate this pathway by a variety of mechanisms. For example, tyrosine-phosphorylated motifs in the middle-T antigen of polyoma virus act to bind the SH2 domains of p85 and consequently stimulate PI3K activity (15). The NS5A protein of hepatitis C virus interacts with the SH3 domain of p85 to release p85-mediated inhibition of p110 (16), and the HIV-1 Nef protein appears to target a region in the C-terminal-half of p85 (17).

We (and others) (18–21) have recently shown that cellular PI3K signaling is also activated during influenza A virus infections. This effect is mediated solely by the viral non-structural (NS1) protein (18, 19), which binds directly and specifically to the p85β regulatory subunit of PI3K (18, 22). NS1 functionally interacts with a plethora of viral and host-cell factors (summa-
rized in Fig. 1B), although in virus-infected cells a major role for NS1 is to limit host innate immunity (reviewed in Ref. 23, 24). Under tissue culture conditions, NS1-mediated PI3K activation is clearly important for the efficient propagation of some (but not all) strains of influenza A virus (18, 19, 25). Indeed, recent data from other groups suggest that active PI3K signaling may contribute to the reduced induction of apoptosis in influenza A virus-infected cells (21, 22, 26). However, given the diverse array of PI3K-regulated physiological processes (2), together with the unique isoform selectivity displayed by NS1 (18, 22), it is likely that other cell type-specific consequences of NS1-activated PI3K may also exist.

Previously, we have demonstrated that two amino acid residues in the C-terminal effector domain of NS1 (tyrosine 89 and methionine 93) are necessary for binding p85α (18). Here, in an attempt to derive the mechanism by which NS1 activates PI3K, we report further on the domains of NS1 and p85β that mediate the interaction.

**EXPERIMENTAL PROCEDURES**

**Cells and Antibodies**—293T, 1321N1, and 1321N1 cells stably expressing the V5-tagged influenza A virus NS1 protein (strain A/Puerto Rico/8/34; PR8) or the V5-tagged NS1-Y89F mutant, have been detailed previously (18). Mouse anti-V5 and anti-p85β antibodies were purchased from Serotec, mouse anti-HA was from BabCo, and rabbit anti-Myc antibody was from Santa-Cruz Biotechnology. Rabbit anti-NS1 antibody was generated against recombinant full-length PR8/p85α protein expressed and purified from *Escherichia coli* (Scottish National Blood Transfusion Service).

**Plasmids**—Mammalian expression vectors for C-terminally V5-tagged wild-type PR8/p85 and PR8/p85α/Y89F have been described previously (18). Arginine 38 and lysine 41 were both changed to alanine by PCR mutagenesis of the relevant wild-type PR8/p85 α cDNA. Tyrosine 89 was changed to glutamic acid by a similar procedure. For mammalian expression vectors encoding N-terminally Myc-tagged domains of bovine p85β, cDNA fragments corresponding to each domain (SH3 (amino acids 1–100), nSH2 (amino acids 313–433), iSH2 (amino acids 433–564), and cSH2 (amino acids 611–724)) were amplified by PCR from pMT2.bov.p85β (provided by B. Vanhaesebroeck (Queen Mary University of London, UK)) and ligated between the NcoI and NotI sites of pEHISTEV. The pGADT7.p110α/iSH2 (amino acids 440–616), His6-tagged βα-iSH2 (amino acids 433–610), nSH2 (amino acids 313–433), iSH2 (amino acids 440–616), and cSH2 (amino acids 611–724)) were amplified by PCR from pMT2.bov.p85β (provided by B. Vanhaesebroeck, Queen Mary University of London, UK) and ligated between the NcoI and NotI sites of pGEX4T3 (Amersham Biosciences). For Hisα-tagged pGEX4T3 (Amersham Biosciences). For Hisα-tagged α-iSH2 (amino acids 440–616), Hisiβ-tagged β-iSH2 (amino acids 433–610), and Hisiβ-tagged β-564 (amino acids 433–564), the relevant cDNAs were PCR amplified from pCDNA3.bov.p85α (provided by B. Vanhaesebroeck, Queen Mary University of London, UK) or pMT2.bov.p85β (as appropriate), and ligated between the Ncol and NotI sites of pEHISTEV (provided by H. Lui, University of St. Andrews, UK).

**RESULTS**

The RNA-binding Domain of NS1 Is Not Essential for the Interaction with p85β—The ability of influenza A virus NS1 protein to bind RNA has been mapped to a number of basic residues in the first 73 amino acids of the protein (29, 30). In particular, arginine 38 probably interacts directly with the RNA...
**NS1 Binds the Inter-SH2 Domain of p85**

**A**

| RBD       | Effector |
|-----------|----------|
| SH3       | p85β     |
| BH        | 1        |
| nSH2      | 73       |
| iSH2      | 123-127  |
| cSH2      | 222-230  |
| p110      | 724      |

**B**

1-81 PABIL-binding

Y89 p85β-binding

144-188 CPSF-binding

223-237 PABII-binding

FIGURE 1. Schematic representations of p85β and NS1. A, p85β regulatory subunit of PI3K (724 amino acids long) consists of an N-terminal SH3 domain, a BH domain, and two SH2 domains (N-terminal: nSH2, and C-terminal: cSH2), which flank the inter-SH2 (iSH2) domain. The iSH2 domain binds the p110 catalytic subunit of PI3K. Asterisks denote two proline-rich regions within the p85β protein (2). B, influenza A virus NS1 protein is 230–237 amino acids long depending upon the strain. The N-terminal 73 amino acids form a functional RNA-binding domain (RBD), while the C-terminal effector domain mediates interactions with host-cell proteins. NS1 contains two nuclear localization sequences (black circles, Ref. 46), and a nuclear export sequence (white circle, Ref. 47). Residues involved in RNA-binding (arginine 38 and lysine 41, Refs. 30) are also implicated in the inhibition of cellular 2′-5′-oligo (A) synthetase/RNase L (32), the inhibition of Jun N-terminal kinase (48), and the interaction with/inhibition of RIG-I (33). Additionally, NS1 contains binding sites for: poly(A)-binding protein I (PABI, Ref. 49), p85β (18), protein kinase R (PKR, Ref. 50), 30-kDa subunit of cleavage and polyadenylation specificity factor (CPSF, Ref. 51), poly(A)-binding protein II (PABII, Ref. 52), and PDZ domain-containing proteins (53).

**NS1 Binds the Inter-SH2 Domain of p85β**—To determine the domain of p85β that NS1 targets, we generated N-terminal

To confirm that the entire RNA-binding domain of NS1 is not essential for binding p85β, we tested the in vitro interaction of p85β with recombinant *E. coli* expressed GST-PR8/NS1 protein lacking the first 72 amino acids of NS1 (termed GST-PR8/NS1Δ72). GST-PR8/NS1 fusion proteins (WT, Y89F, or Δ72) were immobilized onto glutathione-agarose beads and used to affinity isolate an excess of baculovirus-expressed p85β. SDS-PAGE and Coomassie Blue staining revealed that a single 90-kDa protein (p85β) was specifically isolated by both WT GST-PR8/NS1 and GST-PR8/NS1Δ72, but not by the GST-PR8/NS1-Y89F mutant (Fig. 2B). These data indicate that the C-terminal effector domain of NS1 alone is sufficient to form a stable complex with p85β.

**FIGURE 2. The RNA-binding domain of NS1 is not required for the interaction with p85β.** A, 293T cells were transfected for 48 h with empty vector (−), a plasmid expressing WT V5-tagged PR8/NS1, or plasmids expressing V5-tagged PR8/NS1 proteins with the amino acid substitutions Y89F or R38AK41A. Soluble antigen extracts were immunoprecipitated with anti-V5 antibody and precipitates separated by SDS-PAGE followed by transfer to polyvinylidene difluoride membrane. Endogenous p85β and V5-tagged NS1 proteins were detected using specific mAbs. B, equal amounts of soluble Sf9 cell lysates (expressing p85β) were mixed with recombinant GST-NS1/Y89F, GST-NS1/W, or GST-NS1Δ72 protein immobilized onto glutathione-agarose beads. After washing, protein complexes were dissociated from the beads and separated by SDS-PAGE through 4–12% polyacrylamide gradient gels. Polypeptides were stained with Coomassie Blue, and protein identification was confirmed by mass spectrometry. Note that polypeptides marked with asterisks are truncated forms of the full-length fusion proteins. Molecular mass markers (kDa) are indicated to the right.
(ii) unlabeled PR8/NS1 was used because of the similar molecular weights of PR8/NS1 and $\beta$-iSH2; and (ii) $\beta$-iSH2 synthesized using this system is expressed in two forms: full-length His$_{6}$-tagged $\beta$-iSH2 and untagged $\beta$-iSH2.) SDS-PAGE followed by phosphorimager analysis showed that both forms of $\beta$-iSH2 were efficiently precipitated from lysate mixtures containing both $\beta$-iSH2 and PR8/NS1, but not from lysates containing $\beta$-iSH2 only (Fig. 3B). In addition, we also expressed and purified a recombinant His$_{6}$-tagged form of $\beta$-iSH2 from E. coli (see construct in Fig. 4A), and assessed its ability to compete with full-length p85$\beta$ for the binding of WT GST-PR8/NS1. As shown in Fig. 3C, the binding of increasing amounts of $\beta$-iSH2 to E. coli expressed GST-PR8/NS1 prevented the subsequent interaction of GST-PR8/NS1 with baculovirus-expressed p85$\beta$. Overall, these data reveal that $\beta$-iSH2 is the primary site of interaction between NS1 and p85$\beta$.

**FIGURE 3.** NS1 binds the inter-SH2 domain of p85$\beta$. A, 293T cells were transfected for 48 h with plasmids expressing Myc-tagged domain constructs of p85$\beta$: SH3, nSH2, iSH2, or cSH2. Soluble antigen extracts were mixed with GST-NS1 glutathione-agarose beads and the resulting pull-downs were analyzed by SDS-PAGE followed by immunoblotting using an anti-Myc mAb. Input GST-NS1 amount was confirmed by detecting NS1 with a specific pAb. The original soluble antigen extracts were also probed with an anti-Myc mAb in order to assess relative expression of the Myc-tagged constructs. B, in vitro synthesized [35S]methionine-labeled $\beta$-iSH2 was mixed with unlabeled PR8/NS1 and immunoprecipitated using anti-NS1 pAb. Immunoprecipitation of $\beta$-iSH2 alone acted as a negative control. Protein complexes were separated by SDS-PAGE through 4–12% polyacrylamide gradient gels and subjected to phosphorimager analysis. Note that these in vitro translations produce both full-length His$_{6}$-tagged $\beta$-iSH2 (upper band) and untagged $\beta$-iSH2 (lower band). C, inter-SH2 domain of p85$\beta$ is the primary site of interaction for NS1. GST-NS1 immobilized onto glutathione-agarose beads was mixed with 2-fold increasing amounts of purified His$_{6}$-tagged $\beta$-iSH2 (E. coli expressed). After washing, a fixed amount of soluble p85$\beta$-expressing SF9 cell lysate was added. Subsequent protein complexes were separated by SDS-PAGE, and GST-NS1 and $\beta$-iSH2 were visualized by Coomassie Blue staining. Levels of precipitated full-length p85$\beta$ were determined by immunoblot analysis. Molecular mass markers (kDa) are indicated to the right.

**FIGURE 4.** Purification of an untagged NS1Δ72-$\beta$-iSH2 complex. A, schematic representation of the GST-NS1Δ72 and His$_{6}$-tagged $\beta$-iSH2 constructs. Numbers refer to residues from full-length PR8/NS1 and p85$\beta$. Sites of TEV protease cleavage are indicated. B, SDS-PAGE analysis of the purification process. Co-expressed GST-NS1Δ72 and His$_{6}$-$\beta$-iSH2 from E. coli were purified on glutathione-agarose beads and eluted using 10 mM glutathione (i). The eluate was further purified on Ni-NTA resin and eluted in 100 mM imidazole (ii). After TEV protease cleavage (iii), a second glutathione-agarose column was used to remove free GST (~25 kDa) and uncleaved GST-NS1Δ72 (iv). A second Ni-NTA column was used to remove His$_{6}$-TEV (~25 kDa), uncleaved His$_{6}$-$\beta$-iSH2 (~25 kDa), and the small ~2 kDa cleavage product from His$_{6}$-$\beta$-iSH2 (band z). The flow-through (v) contained only two polypeptide species as determined by Coomassie Blue staining, untagged NS1Δ72 (band y) and untagged $\beta$-iSH2 (band x). Molecular mass markers (kDa) are indicated to the right. C, interaction is independent of NS1 tyrosine 89 phosphorylation. 293T cells were transfected for 48 h with empty vector (−), a plasmid expressing WT V5-tagged PR8/NS1, or plasmids expressing V5-tagged PR8/NS1 proteins with the amino acid substitutions Y89F or Y89E. Soluble antigen extracts were immunoprecipitated with anti-V5 antibody and precipitates separated by SDS-PAGE followed by transfer to polyvinylidene difluoride membrane. Endogenous p85$\beta$ and V5-tagged NS1 proteins were detected using specific mAbs.
**NS1 Binds the Inter-SH2 Domain of p85β**

In Vitro Purification of a Complex Containing only the C-terminal Effector Domain of NS1 and β-iSH2—To further confirm direct binding between the C-terminal effector domain of NS1 and β-iSH2, we purified an untagged complex of these proteins. Constructs encoding GST-PR8/NS1Δ72 and His$_6$-tagged β-iSH2 (see Fig. 4A) were co-expressed in *E. coli*, and glutathione-agarose beads were used to affinity isolate GST-PR8/NS1Δ72 together with any associated proteins. It should be noted that both constructs have a cleavage sequence for the tobacco etch virus (TEV) protease between the protein of interest and the affinity tag. Glutathione eluted the bound proteins (Fig. 4B, lane i), and this eluate was further purified on Ni-NTA resin. After imidazole elution (Fig. 4B, lane ii), the GST and His$_6$ tags were cleaved from the recombinant proteins by TEV protease (Fig. 4B, lane iii). A second glutathione-agarose column removed free GST and uncleaved GST-PR8/NS1Δ72 (Fig. 4B, lane iv), while a second Ni-NTA column removed His$_6$-tagged TEV, uncleaved His$_6$-β-iSH2, and the small ~2-kDa cleavage product from His$_6$-β-iSH2. The flow-through from this column contained only two polypeptide species (as revealed by SDS-PAGE and Coomassie Blue staining, Fig. 4B), which were confirmed as untagged PR8/NS1Δ72 and untagged β-iSH2 by mass spectrometry.

The observation that an NS1:β-iSH2 complex can be formed in vitro from *E. coli* expressed recombinant proteins argues against a phosphorylation-dependent interaction. However, our previous studies had identified that a Y89F mutation in NS1 completely abrogates its interaction with p85β, both in vitro and in vivo (18). As this was highly suggestive of a role for tyrosine 89 phosphorylation in the binding of NS1 to p85β, we investigated this requirement further. Constructs encoding V5-tagged PR8/NS1 with a Y98E amino acid substitution (a mimic for the negative charge of phosphotyrosine), V5-tagged PR8/NS1-Y98F, or V5-tagged WT PR8/NS1 were transiently expressed in human 293T cells and subsequently immunoprecipitated with anti-V5 antibody. Western blot analysis of the immunoprecipitates revealed that only WT PR8/NS1 (and not the Y98F or Y98E mutants) was able to precipitate endogenous p85β (Fig. 4C). Together with the direct in vitro binding results from Fig. 4A, the data imply that phosphorylation of tyrosine 89 is not a determinant of the NS1:p85β interaction, and might even suggest that phosphorylation of tyrosine 89 could potentially prevent binding.

Binding of NS1 to β-iSH2 does not displace the p110 catalytic subunit of PI3K. In our original identification of p85β as a direct binding partner for NS1, we did not detect a polypeptide band corresponding to any p110 isoform in NS1 immunoprecipitates from HEp2 cells (18). This was surprising given that class IA PI3Ks are obligate p85:p110 heterodimers (3, 4). One explanation for this result may be that HEp2 cells express relatively low levels of p110α protein as compared with other commonly used laboratory cell lines (36). However, given that NS1 was found to interact directly with the p110-binding (iSH2) domain of p85β, the possibility that NS1 might displace p110 subunits from p85β was investigated. To determine initially if NS1 could associate with a functional p110-containing complex, a sensitive kinase activity assay was used. V5-tagged WT PR8/NS1 or PR8/NS1-Y98F were immunoprecipitated from 1321N1 cells stably expressing the appropriate construct (18), and these immunoprecipitates were subjected to in vitro PI3K activity assays (immunoprecipitates from naïve 1321N1 cells acted as a negative control). As shown in Fig. 5A, significant PI3K activity was readily detectable in anti-V5 immunoprecipitates from lysates.
expressing WT PR8/NS1, but not from naïve lysates, or from lysates expressing PR8/NS1-Y89F (which is unable to bind p85β, Ref. 18). The ability of WT PR8/NS1 (but not PR8/NS1-Y89F) to precipitate PI3K activity is most likely caused by the indirect co-precipitation of a p110 catalytic subunit via p85β. To directly establish this, we next investigated the in vitro formation of a heterotrimeric complex comprising NS1, β-iSH2, and the p85-binding domain of p110α (also known as the p110α adaptor binding domain, p110α-ABD). Initially, [35S]methionine-labeled β-iSH2 and HA-tagged p110α-ABD were individually or co-synthesized in reticulocyte lysates and immunoprecipitated using anti-HA antibody. SDS-PAGE and phosphorimager analysis showed that β-iSH2 could be specifically co-precipitated together with p110α-ABD (Fig. 5B), indicating that the β-iSH2 construct was able to interact with p110α-ABD. We then individually synthesized [35S]methionine-labeled β-iSH2 or p110α-ABD, as well as unlabeled PR8/NS1. Reticulocyte lysates expressing these proteins were mixed in various combinations and immunoprecipitated with anti-NS1 antibody. Although some minor nonspecific absorption of both β-iSH2 and p110α-ABD was observed (Fig. 5C, lanes 4 and 5), as expected only β-iSH2 (and not p110α-ABD) was efficiently co-precipitated directly with PR8/NS1 (Fig. 5C, lanes 6 and 7). However, a significant amount of p110α-ABD was clearly evident in immunoprecipitates of PR8/NS1 that contained co-precipitating β-iSH2 (Fig. 5C, lane 8). This confirms that the binding of PR8/NS1 to β-iSH2 does not prevent interactions between β-iSH2 and p110α-ABD, and suggests that a heterotrimeric complex consisting of NS1, p85β, and p110α could potentially form.

Binding of NS1 to β-iSH2 requires residues at the C-terminal end of β-iSH2. As the interaction between NS1 and β-iSH2 is independent of the p110-binding site (Fig. 5C), we hypothesized that NS1 must bind elsewhere on the molecule. It has previously been shown that truncation of p85α at residue 571 can cause constitutive p110 catalytic activity (37), which also occurs in the context of an isolated p85α nSH2-iSH2 fragment (38). Thus, as residues downstream of 571 in the iSH2 domain of p85α contribute to p85-mediated inhibition of PI3K activation, we investigated if the same region in p85β was required for binding to NS1. Plasmids were generated to express α-iSH2, β-iSH2, a β-iSH2 construct lacking residues downstream of amino acid 564 (equivalent to truncation at residue 571 in p85α), termed β-564, and a β-iSH2 construct with residues downstream of amino acid 564 substituted for the equivalent residues in p85α-iSH2 (termed β/α-iSH2) (see constructs in Fig. 6A). [35S]Methionine-labeled polypeptides were synthesized in vitro and individually mixed with unlabeled PR8/NS1 before immunoprecipitation with anti-NS1 antibody. As expected from our previous observations (18), β-iSH2, but not α-iSH2, was specifically co-precipitated with PR8/NS1 (Fig. 6B, lanes 1–6). Interestingly, the truncated β-iSH2 construct (β-564) was not precipitated by PR8/NS1 (Fig. 6B, lanes 7–9), and binding of this construct to PR8/NS1 could not be rescued by addition of the corresponding C-terminal α-iSH2 residues (Fig. 6B, lanes 10–12). The inability of PR8/NS1 to bind α-iSH2, β-564, or β/α-iSH2 is unlikely to be caused by gross nonspecific disruption of their structures, as these constructs could be co-precipitated with in vitro synthesized HA-tagged p110α-ABD (Fig. 6C). These data strongly indicate that the C-terminal end of β-iSH2 is specifically required for NS1 binding. However, this C-terminal fragment of β-iSH2 alone could not be co-precipitated with PR8/NS1 (data not shown). Thus, the C terminus of β-iSH2 is absolutely essential, but probably not sufficient, to mediate the interaction with NS1.

**DISCUSSION**

We have demonstrated that the direct binding of NS1 to p85β is mediated via the C-terminal effector domain of NS1.
NS1 Binds the Inter-SH2 Domain of p85β

and the iSH2 domain of p85β. This concurs with our previous results which identified tyrosine 89 and methionine 93 (both in the C-terminal domain of NS1) as essential for binding p85β (18). We were initially interested in these two residues as they formed part of a putative motif with similarity to the well-documented PI3K SH2-binding motif, phospho-YYXM (39). However, results from this and other studies indicate that the activation of PI3K by NS1 probably does not involve tyrosine-phosphorylated NS1 occupying a p85β SH2 domain. First, an interaction could not be detected between NS1 and either of the two p85β SH2 domains. Secondly, NS1 with glutamic acid substituted as a mimic of phosphotyrosine at residue 89 was unable to bind full-length p85β, and the interaction domains of NS1 and p85β could be expressed and purified as a complex entirely from E. coli. Thirdly, previous YXXM phosphopeptide binding studies have shown relatively conserved pockets in both p85 SH2 domains, which are strongly selective for methionine only at the Tyr+3 position (39). In NS1, the relevant methionine is at the Tyr+4 position and is unlikely to be presented in the appropriate orientation. Finally, structural analysis of the PR8/NS1 effector domain indicates that while tyrosine 89 appears exposed, methionine-93 is mostly buried within the NS1 homodimer (40). It is therefore not possible for methionine 93 to be directly involved in the interaction with p85β without a major conformational change in NS1. The observation that mutation of methionine 93 abrogates p85β binding may therefore be due to destabilization of the NS1 homodimer, as this residue could be important for maintaining functional integrity of the NS1 structure. We are currently investigating this possibility.

Competition assays from this work clearly establish that the principal direct binding site for NS1 in p85β is the iSH2 domain. Previous biochemical studies (5, 41), together with a recently determined x-ray crystallographic structure (42), show that iSH2 is a rigid 100–110 Å coiled-coil consisting primarily of two anti-parallel α-helices. The helices are ~70 amino acids long (helix-1; residues 434–505, and helix-2; residues 511–581), and are connected by a loop of 5 residues (506–510) (5). At the C-terminal end of helix-2 is a ~30 residue “tail” (helix-3) that links the coiled-coil to the cSH2 domain (42). The acidic nature of this stretch of amino acids may be responsible for it folding back and packing against exposed basic residues at the C terminus of helix-2 (5). Studies to determine the p110-binding site on p85β have indicated essential residues in helix-1 as 445–485 (and in particular 475–477) (5, 42, 43). Important residues for p110 binding in helix-2 (adjacent to the binding site on helix-1) are 525–534 (42, 43) (Fig. 7A).

Truncation of p85α at residue 571 removes part of helix-2/helix-3 from the iSH2 domain and the whole of cSH2, but leaves the p110-binding site intact. Consequently, this mutant leads to constitutive activation of the resulting p85α:p110α heterodimer (37). It is not absolutely clear why this mutant (unlike full-length p85α) is unable to repress PI3K function. However, one possibility is that deletion of the autoinhibitory phosphorylation site at serine 608 (in the “tail” of iSH2) leads to the deregulated phenotype (13, 44). Alternatively, specific inter-or intrasubunit interactions that repress p110 activity might be lost (38, 45). For example, biophysical results achieved using isolated recombinant p85α nSH2-iSH2 constructs suggest that

FIGURE 7. A, sequence alignment of the p85α and p85β inter-iSH2 domains. Amino acid sequence alignment of the bovine p85α (SwissProt data bank accession number: P23727) and p85β (SwissProt data bank accession number: P23726) iSH2 domains used in this study. Numbers correspond to residues in the full-length proteins. Assignment of structural helices and residues involved in p110 binding is shown above the sequences. Amino acids that differ between human and bovine p85 homologues are highlighted in gray. Sequence identity and homology is shown beneath the sequences. Assignment of structural helices and residues involved in p110 binding is shown above the sequences. Amino acids that differ between human and bovine p85 homologues are highlighted in gray. Sequence identity and homology is shown beneath the sequences. Only 10 amino acids differ between p85α and p85β in the region of iSH2 that is required for NS1 binding. B, putative NS1-binding site of iSH2. Crystal structure of p85α-iSH2 in complex with p110α-ABD, as recently solved by Miled et al. (42). The p85α-iSH2 helices are colored dark gray, while p110α-ABD is a lighter shade of gray. Residues of α-iSH2 equivalent to those required for NS1 to bind β-iSH2 are highlighted. The image was generated using MacPyMol (Protein Data Bank file: 2V1Y).
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the nSH2 domain of p85α is normally in close contact with residues 581–593 of iSH2, a conformation which could present nSH2 in such a way as to inhibit p110 activity (38). Indeed, a model has recently been proposed wherein kinase activity is negatively regulated by a charge-charge interaction between the p85 nSH2 domain and the p110 helical domain (42) (Fig. 8A). Thus, normal PI3K activation may result from disruption of the nSH2:p110 interaction by competitive receptor-mediated phosphopeptide binding to nSH2 (42) (Fig. 8B). In the context of truncated p85α lacking residues downstream of 571, nSH2 is unlikely to be positioned in such a way as to suppress p110 lipid kinase function (38).

It is intriguing to find that the binding of influenza A virus NS1 protein to the p85β iSH2 domain requires residues equivalent to those lost in the constitutively active p85α mutant (i.e. part of helix-2 and the acidic “tail” of helix-3) (Fig. 7, A and B). Thus, our data are suggestive of a novel mode-of-action whereby NS1 may potentially bind to this region and mask its contribution to p110 inhibition. Currently the mechanistic details can only be speculated upon: NS1 may modify inter- and intramolecular contacts within the PI3K heterodimer, displace an unknown repressive element, or recruit additional cellular co-stimulatory factors to the complex. However, given the model recently proposed by Miled et al. (42), it is tempting to think that NS1 simply displaces nSH2 from the p110 helical domain (Fig. 8C). Such direct targeting of a mechanical aspect of PI3K regulation would be an efficient way to activate the kinase, as the multiple inputs that normally regulate p85:p110 function would be short-circuited (6–9, 11–13). For example, stimulation of PI3K by NS1 during virus infection would be independent of phosphopeptide binding, thus ensuring that signaling was not linked to host activity, and could occur even if the infected cells were quiescent (when normal receptor signaling might be low). Additionally, NS1 might mask the inhibitory serine 608 autophosphorylation site, and in this way circumvent any automatic negative feedback by p110 (13).

From our data we cannot rule out the possibility that other regions of either full-length NS1 or full-length p85β may play some minor role in the functional interaction in vivo. For example, the RNA-binding domain of NS1 is not absolutely essential for complex formation, but residues within this domain could contribute to NS1:p85β stability and/or the activation of PI3K signaling. Indeed, Ehrhardt et al. (26) noted that transient expression of an NS1 construct containing amino acid substitutions at arginine 38 and lysine 41 induced less PI3K activity (as determined by phospho-Akt levels) than the wild-type construct (26). Additionally, Shin et al. (20) have previously reported the independent co-precipitation of NS1 with both the SH3 and cSH2 domains of p85 (isoform not specified), and have also identified a putative SH3-binding poly-proline motif in the C-terminal effector domain of NS1 (22). Although we did not identify such interactions under our experimental conditions, it is possible that the binding of NS1 to p85β is relatively complex, and dynamic interplay between NS1 and multiple domains of p85β occurs in vivo. Indeed, the hypothetical model proposed here (Fig. 8C), would position NS1 at the C terminus of β-iSH2, potentially bringing it into close proximity with both the SH3 and cSH2 domains of p85. Thus, the observations made by Shin et al. (20, 22) may yet be compatible with this model.

To our knowledge, analogous host-cell or viral proteins that regulate PI3K by interacting with the p85β (or even p85α) iSH2
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domain have yet to be identified. In this regard, it is intriguing to speculate that NS1 may mimic the normal function of an unknown cellular protein. Our ongoing biochemical and structural work aims to establish the mechanism by which NS1-mediated PI3K activation occurs. We are also actively seeking to determine the molecular and biological basis for p85β isoform specificity displayed by NS1, which itself is remarkable given the high protein sequence identity between the p85α and p85β ISH2 domains (Fig. 7A). It is likely that such studies will provide yet another example of how viruses can help us understand the normal regulation of cellular signaling pathways.

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