Association of the Src homology 2 domain-containing leukocyte phosphoprotein of 76 kD (SLP-76) with the p85 subunit of phosphoinositide 3-kinase

Eun Kyung Shim[a], Chang Suk Moon[a], Gi Yeon Lee[a], Yun Jung Ha[a], Suhn-Kee Chae[b], Jong Ran Lee[a,*]

[a]*Division of Molecular Life Sciences and Center for Cell Signaling Research, Ewha Womans University, 11-1 Daehyun-dong, Seodaemun-gu, Seoul 120-750, South Korea*
[b]*Department of Life Science and Biotechnology, Paichai University, 439-6 Doma 2-dong, Seo-gu, Daejeon 302-735, South Korea*

Received 1 April 2004; revised 13 July 2004; accepted 22 July 2004

Available online 26 August 2004

Edited by Gianni Cesareni

Abstract To investigate additional functions of the T cell adaptor, Src homology 2 (SH2) domain-containing leukocyte protein of 76 kD (SLP-76), we performed a yeast two-hybrid assay using the N-terminal region of SLP-76 fused with the kinase domain of Syk. By screening a human leukemia cDNA library, we identified the p85 subunit of phosphoinositide 3-kinase (PI3K) as one of the interacting molecules. Unlike the SH2 domain of Vav or Nck, tyrosine phosphorylation of SLP-76 at position 113 or 128 was sufficient for it to associate with the N-terminal SH2 of p85. Collectively, these data suggest that SLP-76 may play a role in PI3K signaling pathways.

Keywords: Src homology 2 domain-containing leukocyte protein of 76 kD; Adaptor protein; Yeast two-hybrid screening; Phosphoinositide 3-kinase; Tyrosine phosphorylation; Src homology 2 domain

1. Introduction

Src homology 2 (SH2) domain-containing leukocyte phosphoprotein (SLP-76) is a hematopoietic cell-specific adaptor protein that is phosphorylated by ZAP-70, a member of the Syk family of protein tyrosine kinases (PTK) after ligation of the T cell receptor (TCR) [1,2]. SLP-76 contains motifs that allow for protein–protein interactions and plays an important role in regulating signaling from the TCR [3–5].

An amino-terminal acidic region of SLP-76 (N-SLP) contains tyrosine phosphorylation sites at positions 113, 128, and 145 [4,6–9], and phosphorylation at positions 113 and 128 is known to be critical for transducing the TCR signal and activating the T cells [4]. A proline-rich motif in the central region of SLP-76 binds to the SH3 domain of Grb2 family members [3,10–12], and a carboxyl-terminal SH2 domain (C-SLP) forms signaling complexes by interacting with proteins newly phosphorylated on tyrosine residues [3,13–15]. The schematic diagram of full length SLP-76 is shown in Fig. 1A.

The importance of SLP-76 in T cell signaling has been demonstrated in experiments with the Jurkat human T cell leukemia line and in mice made deficient for SLP-76 expression by homologous recombination [16–18]. When SLP-76 is overexpressed in Jurkat cells, there is a dramatic augmentation of TCR-mediated activation of the full-length IL-2 promoter or of a reporter construct driven by nuclear factor of activated T cells (NFAT) from the IL-2 promoter [3–5]. Overexpression of SLP-76 mutants lacking each of the three structural domains has been shown to be unable to promote TCR-mediated activation events, suggesting that molecular associations involving each of the three domains of SLP-76 are required for optimal function [5]. It is crucial therefore to identify the proteins with which SLP-76 interacts in order to understand how it promotes TCR signaling.

To identify molecules that associate with tyrosine-phosphorylated SLP-76, we performed a yeast two-hybrid assay by screening a human T cell leukemia cDNA library with a fusion of SLP-76 with the kinase domain of Syk PTK (N-SLP-Syk) as bait. We identified the p85 subunit of phosphoinositide 3-kinase (PI3K) as one of the molecules that associates with N-SLP in a Syk-dependent manner. Experiments with mutants lacking various functional domains of these two molecules showed that tyrosine-phosphorylated SLP-76 associates with the SH2 domain near the N-terminus of p85 (N-SH2). Furthermore, unlike the interaction of SLP-76 with the SH2 domain of Vav or Nck, which requires phosphorylation of SLP-76 tyrosines at positions 113 and 128, phosphorylation at either 113 or 128 is sufficient for binding of p85 N-SH2. Collectively, these results indicate that SLP-76 is involved in the PI3K signaling pathway.

2. Materials and methods

2.1. Antibodies and other reagents

Anti-human CD3 monoclonal antibody (mAb) (UCHT1) from BD PharMingen (San Diego, CA) was used for cell stimulation. For immunoprecipitation (IP) and immunoblotting (IB), we used

* Corresponding author. Fax: +82-2-3277-3760.
E-mail address: jrlee@ewha.ac.kr (J.R. Lee).

Abbreviations: SH2, Src homology 2; SLP-76, Src homology 2 domain-containing leukocyte phosphoprotein of 76 kD; PI3K, phosphoinositide 3-kinase; PTK, protein tyrosine kinase; TCR, T cell receptor; Ab, antibody; IP, immunoprecipitation; IB, immunoblotting; WT, wild-type; GST, glutathione *S*-transferase; HEK, human embryonic kidney; PV, pervanadate

0014-5793/$22.00
doi:10.1016/j.febslet.2004.07.090

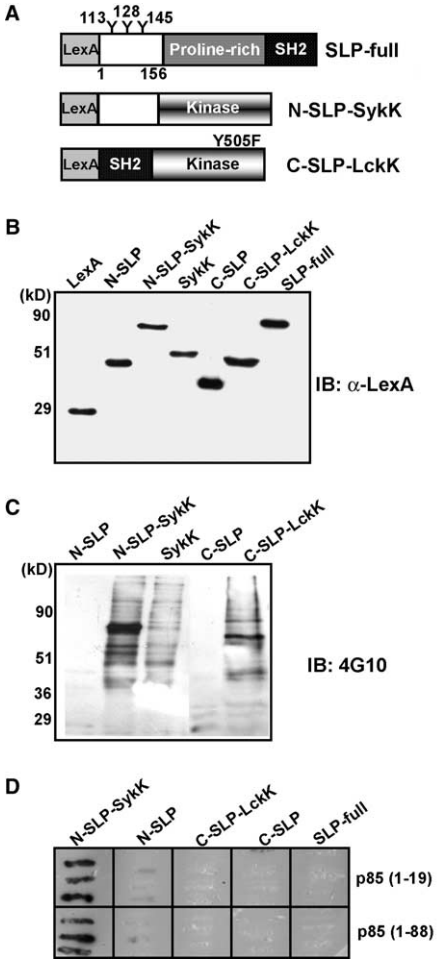

Fig. 1. Expression of SLP-76 bait proteins and analyses of two-hybrid interactions in yeasts. (A) Structure of various LexA fusion SLP-76 derivatives. (B) Yeast cells (EGY48) were transformed with plasmids carrying LexA fusions of various SLP-76 derivatives. The LexA fusion proteins present in cell lysates were fractionated by SDS–8% PAGE and detected by immunoblotting (IB) with anti-LexA Ab. (C) After transformation separately with either SLP-76 or a Syk kinase domain, or with SLP-76 fused to a PTK kinase domain, lysates were subjected to SDS–8% PAGE, and phosphorylation was detected by IB with anti-phosphotyrosine (pY) Ab, 4G10. (D) The two p85 isolates obtained by screening the human Jurkat T leukemia cDNA library with N-SLP-SykK as bait were assayed for interaction with various SLP-76 baits. Yeast cells were transformed with each target and the indicated SLP-76 bait plasmid along with a β-gal reporter gene. The tester strains were streaked onto either induction/selection medium containing X-gal or a non-inducing, non-selective medium, as a loading control (data not shown). Three independent experiments involving independently derived yeast tester strains yielded virtually identical results.

anti-PI3K p85 polyclonal Ab, and anti-myc (9E10) and anti-phosphotyrosine (4G10) mAbs from Upstate Biotechnology, Inc. (Lake Placid, NY), anti-SLP-76 polyclonal Ab and anti-LexA and anti-GST mAbs from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and anti-flag mAb (M2) from Sigma Chemical Co. (St Louis, MO). Secondary Abs, anti-mouse IgG and anti-rabbit IgG conjugated with horseradish peroxidase (HRP) were purchased from Bio-Rad (Hercules, CA). Glutathione-agarose and Coomassie brilliant blue (CBB) R-250 from Sigma Chemical Co. were used for purifying and identifying GST-fusion proteins, respectively. Enhanced chemiluminescence (ECL) reagents and GammaBind G Sepharose were purchased from Amersham Pharmacia Biotech Co. (Arlington Heights, IL).

2.2. Plasmids

Mammalian expression vectors encoding myc-tagged human PI3K p85α and Syk PTK were kindly provided by Drs. A. Toker (Harvard Medical School, Boston, MA) and A. Veillette (McGill University, Montréal, Canada), respectively. The plasmids carrying wild type (Wt) SLP-76 and its mutants cloned into a modified pEF-Bos vector with an amino-terminal sequence encoding the flag epitope [3–5] were generous gifts from Dr. G. Koretzky (University of Pennsylvania, Philadelphia, PA).

Plasmids expressing glutathione *S*-transferase (GST) fusions of truncated p85 and the SH2 domain of Vav, Nck, or Itk were generated by ligating PCR fragments into pGEX-2TK (Amersham Pharmacia Biotech Co., Arlington Heights, IL) between the *Bam*H1 and *Eco*R1 sites. The truncated p85 constructs encoding the sequences indicated in Fig. 3A (SH3-P-B-P including N-terminal SH3-proline rich-Bcr homology-proline rich domains, N-SH2, C-SH2, and N-I-C-SH2 including N-SH2-inter-SH2-C-SH2) were amplified from human p85α cDNA. The SH2 domain constructs encoding amino acids, 671–765, 282–376, and 671–765 of human Vav, Nck, and Itk, respectively, were prepared from Jurkat cDNA by PCR amplification.

A plasmid expressing a LexA DNA-binding domain (BD) fusion of the complete SLP-76 gene was generated by PCR amplification followed by ligation into yeast expression vector pLexA (Clontech laboratories Inc., Palo Alto, CA) at its *Bam*H1 site (pLexA/SLP-76 Wt). The N-SLP fragment amplified by PCR was first ligated into pBluescript SK(+) vector (Stratagene, La Jolla, CA) at the *Bam*H1 site, and further cloned into pLexA between the *Eco*R1 and *Not*1 sites, followed by filling at the *Eco*R1 site to put the sequence back in frame (pLexA/N-SLP). A plasmid expressing a LexA fusion of C-SLP was generated by PCR amplification followed by ligation into pLexA between *Eco*R1 and *Bam*H1 sites (pLexA/C-SLP). To generate the plasmid expressing LexA/N-SLP-SykK, the kinase domain of Syk (amino acids 421–533 of mouse Syk) amplified by PCR was first ligated into pBluscript SK(+) between its *Bam*H1 and *Eco*R1 sites, and further cloned into pLexA/N-SLP between *Not*1 and *Sal*1 sites. To generate the fusion plasmid expressing LexA/C-SLP-LckK(Y505F), the kinase domain of Lck (constitutively active form, F505; amino acids 244–497) was amplified by PCR followed by ligation into pLexA/C-SH2 between *Bam*H1 and *Sal*1 sites. All the constructs were confirmed free of undesired mutations by sequencing.

2.3. Yeast two-hybrid screen and assay

The MATCHMAKER LexA two-hybrid system (Clontech Laboratories Inc.) was used to screen a human leukemia MATCHMAKER LexA cDNA library (Clontech Laboratories Inc.), following the manufacturer's protocol. Briefly, yeast strain EGY48 containing the p80p-lacZ reporter (EGY48 [p80p-lacZ]) was transformed with the LexA/N-SLP-SykK bait and the plasmid human leukemia cDNA library bearing the activation domain (AD) fusion constructed in pB42AD. Transformants were selected on galactose induction medium lacking histidine, leucine, tryptophan, and uracil, and the resulting clones were analyzed for *lacZ* and *LEU2* reporter genes. Plasmid DNA was recovered from positive clones by transforming *E. coli* KC8 and sequenced. To assay interaction of the positive clones with various SLP-76 baits, EGY48 [p80p-lacZ] was transformed with each bait plasmid constructed in pLexA(DNA-BD) and the pB42AD/positive library plasmid. Colony selection and interaction analysis were performed as above.

2.4. Cell culture and T cell activation

Human Jurkat T leukemia cells and human embryonic kidney (HEK) 293T cells were cultured in RPMI 1640 medium and Dulbecco's modified Eagle's medium, respectively, with 10% heat-inactivated fetal calf serum (HI-FCS), 100 units/ml penicillin, 100 μg/ml streptomycin, and 20 mM glutamine. The cell lines were maintained at 37 °C with 5% CO_2/95% O_2. For activation, Jurkat T cells were washed and resuspended in RPMI 1640 medium at 1×10^7/ml and incubated with either control Ab or anti-CD3 Ab for 5 min at 37 °C.

2.5. Transient expression of plasmid DNA

HEK 293T cells were seeded 24 h before transfection at approximately 6×10^5 cells/60 mm culture dish. They were then transfected with 5 μg each of the various expression plasmids by the calcium phosphate precipitation method and the transfected cells were cultured in growth medium for 24 h before performing experiments.

2.6. Immunoprecipitation and immunoblotting

T cell stimulation was terminated by adding an equal volume of ice-cold medium, and cell lysates were prepared in 1% NP-40 lysis buffer that contained protease and phosphatase inhibitors, as described previously [19]. For IB, lysates from 1×10^6 cells were mixed with 2X Laemmli's sample buffer, boiled, and subjected to SDS–10% PAGE. For IP, lysates from 50×10^6 cells were tumbled with GammaBind G sepharose beads conjugated with the individual Abs as indicated. The immune complexes were then subjected to SDS–PAGE, followed by IB.

2.7. Precipitation of GST-fusion proteins

GST fusion proteins containing the SH2 domains of p85, Vav, Nck, or Itk, as well as other domains of p85, were prepared as described elsewhere [20], and bound to glutathione-coated agarose beads. Lysates of HEK 293T cells transfected with various expression plasmids were precleared with 5 μg GST immobilized on 10 μl of glutathione-coated beads, followed by incubation for 2 h at 4 °C with 10 μg of SH2 domain fusion proteins immobilized on 10 μl beads. Proteins bound to the fusion proteins were separated by SDS–PAGE and transferred to nitrocellulose membranes for IB.

3. Results

3.1. The amino-terminal region of SLP-76 (N-SLP) interacts with p85 in a Syk-dependent yeast two-hybrid screen

We prepared LexA fusion bait plasmids that expressed full-length SLP-76 (SLP-full), N-SLP, a fusion of N-SLP with the kinase domain of Syk (N-SLP-SykK), the kinase domain of Syk (SykK), C-SLP, and a fusion of C-SLP with the kinase domain of constitutively active Lck (Y505F) (C-SLP-LckK). Some of these bait plasmids are described schematically in Fig. 1A and protein expression is shown by immunoblotting (IB) yeast lysates with anti-LexA Ab in Fig. 1B. Since three tyrosine residues within N-SLP represent phosphorylation sites for the ZAP-70 and Syk PTKs, but not for the Lck, and since unlike the activation of Syk, ZAP-70 activation requires the activity of LckK [1,2], the kinase domain of Syk was fused to N-SLP to induce phosphorylation of SLP-76. In contrast, the kinase domain of constitutively active Lck was fused to C-SLP to induce phosphorylation of library proteins that may associate with SLP-76 through the SH2 domain, since Lck is a primary PTK that responds to T cell activation [24–27]. IB of yeast lysates with anti-phosphotyrosine Ab revealed that expression of N-SLP-SykK, SykK, or C-SLP-LckK, but not N-SLP or C-SLP, led to tyrosine phosphorylation of yeast proteins (Fig. 1C). To search for molecules that interact with the phosphotyrosine motifs of SLP-76, we expressed the LexA fusion of N-SLP-SykK as bait to screen a human leukemia cDNA library. We isolated 76 positive clones from 1.6×10^8 colony-forming units of the amplified library, representing $\sim 1.9 \times 10^6$ independent clones. Most of the isolates contained cDNA fragments encoding the SH2 domains of Vav-1, Nck-1, or Src-like adaptor protein (SLAP), but two contained different but overlapping fragments encoding the SH2 domain of the p85 subunit of PI3K. These p85 isolates were tested for interaction with the various SLP-76 baits (Fig. 1D). SLP-76 baits not fused with the kinase domain of a PTK interacted weakly if at all with the p85 clones. There was a strong interaction with the N-SLP-SykK, but not with C-SLP-LckK. These results indicate that N-SLP is phosphorylated by Syk kinase and then interacts with p85 through the p85 SH2 domain.

3.2. Tyrosine-phosphorylated SLP-76 associates in vivo with the amino-terminal SH2 domain of p85

To see whether SLP-76 associates with p85 in vivo and whether tyrosine-phosphorylation of SLP-76, not of p85, is required for their interaction, we transiently overexpressed myc-tagged p85 in combination with flag-tagged SLP-76 Wt or a mutant lacking the SH2 domain (SLPΔSH2) in HEK 293T cells (Fig. 2A). When we induced tyrosine-phosphorylation of SLP-76 Wt or SLPΔSH2 by pervanadate (PV) stimulation of the transfected cells, both the Wt and the mutant SLP-76 protein were immunoprecipitated with p85 (Fig. 2A). We also tested for association of the endogenous proteins by performing IP in Jurkat T cells. As shown in Fig. 2B, TCR stimulation with anti-CD3 Ab induced phosphorylation of SLP-76. p85 was detected in precipitates with anti-SLP-76 Ab and SLP-76 was present in immunoprecipitates with anti-p85 Ab (Fig. 2B). These results show that SLP-76 associates with

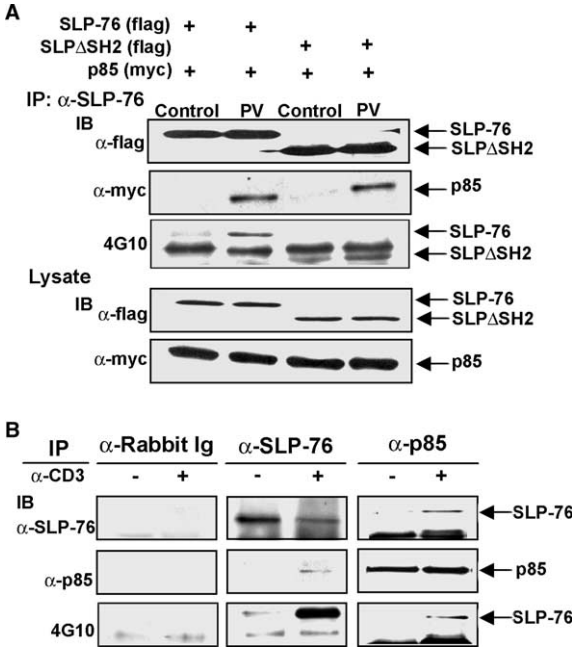

Fig. 2. Tyrosine phosphorylation of SLP-76, but not its SH2 domain, mediates the association with p85 in vivo. (A) HEK 293T cells were transfected with plasmids carrying either full-length SLP-76 (flag) or an SLP-76 mutant (SLPΔSH2, flag) along with p85 (myc). The cells were either left untreated (control), or treated with pervanadate (PV) for 5 min, and lysates were prepared and subjected to SDS–8% PAGE. Protein expression was detected by IB with anti-flag or anti-myc Ab. Phosphorylation induced by the PV treatment was determined by IB with 4G10 Ab (data not shown). Immunoprecipitates with anti-SLP-76 Ab, as well as control anti-mouse Ig (data not shown), were subjected to SDS–8% PAGE. The amounts of IP from untreated or PV treated lysates were determined by IB with anti-flag Ab and tyrosine phosphorylation of SLP-76 following PV treatment was confirmed by IB with 4G10. Association between p85 and SLP-76 was detected by IB using anti-myc Ab. Data shown are representatives of three separate experiments. (B) Jurkat T cells were either left unstimulated (control) or TCR stimulated for 5 min at 37 °C. Lysates were immunoprecipitated with Ab against either SLP-76 or p85. Immunoprecipitates were subjected to SDS–8% PAGE and phosphorylation of SLP-76 following TCR ligation was detected by IB with 4G10. To measure phosphorylation-dependent association of SLP-76 with p85, the same blot was reprobed with either anti-SLP-76, or with anti-p85 Ab to demonstrate equal levels of IP as well as association of SLP-76 with p85. Data are representatives of three independent experiments.

p85 in Jurkat cells and that the association depends on ligation of the TCR.

We next asked which domain(s) of p85 associate(s) with tyrosine-phosphorylated SLP-76. For these experiments, we produced GST-fusions of a set of truncated p85 constructs as described in Fig. 3A and confirmed expression of these proteins by immunoblotting with anti-GST Ab (Fig. 3B). We then performed GST pull-down assays on lysates of HEK 293T cells expressing flag-tagged SLP-76 Wt together with either empty vector or myc-tagged Syk PTK (Fig. 3C). As shown in Fig. 3C and D, SLP-76 was phosphorylated and precipitated with p85 GST-fusion proteins containing the N-SH2 domain when Syk was expressed at the same time. No interaction was detected between SLP-76 and GST on their own (data not shown), further indicating that SLP-76 binds specifically to the N-SH2 of p85.

3.3. Tyrosine phosphorylation of SLP-76 at position 113 or 128 is sufficient for binding to the N-SH2 domain of p85

To confirm the association of the p85 N-SH2 with tyrosine-phosphorylated SLP-76, we overexpressed various flag-tagged SLP-76 mutants in HEK 293T cells together with either empty vector or myc-tagged Syk (Fig. 4A). The SLP-76 mutants included a deletion of the N-terminal acidic region (Δ156), as well as point mutants of the tyrosine motifs (Y113, 128F and Y3F). Lysates of the transfected HEK 293T cells were then

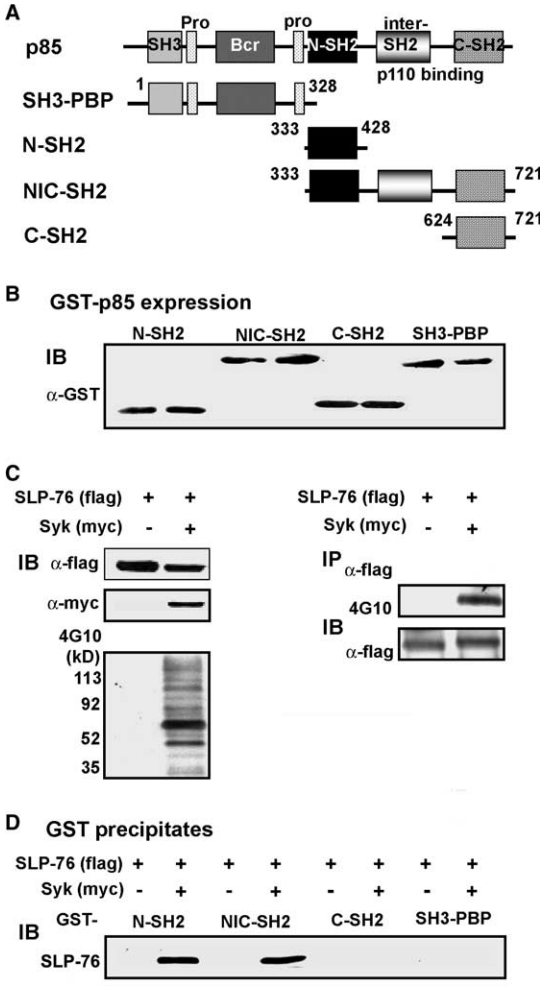

Fig. 3. Association of p85 with phosphorylated SLP-76 via the p85 N-SH2 domain. (A) Structure of p85 and its truncated mutants. (B) GST fusion proteins of p85 domain mutants were expressed and confirmed by IB using anti-GST Ab. (C) SLP-76 was expressed in HEK 293T cells in the presence or absence of Syk. Expression of proteins was detected by IB using anti-flag or anti-myc Ab. Protein phosphorylation induced by Syk kinase was determined by IB lysates of HEK293T cells with 4G10. Phosphorylation of SLP-76 in the presence of Syk was also confirmed by IP of cell lysates with anti-flag Ab followed by IB with 4G10. (D) GST fusions of the p85 domain mutants were incubated with lysates of HEK 293T cells expressing SLP-76 with or without Syk. Association of SLP-76 with p85 was detected by IB using anti-flag Ab. Data shown are representatives of three separate experiments.

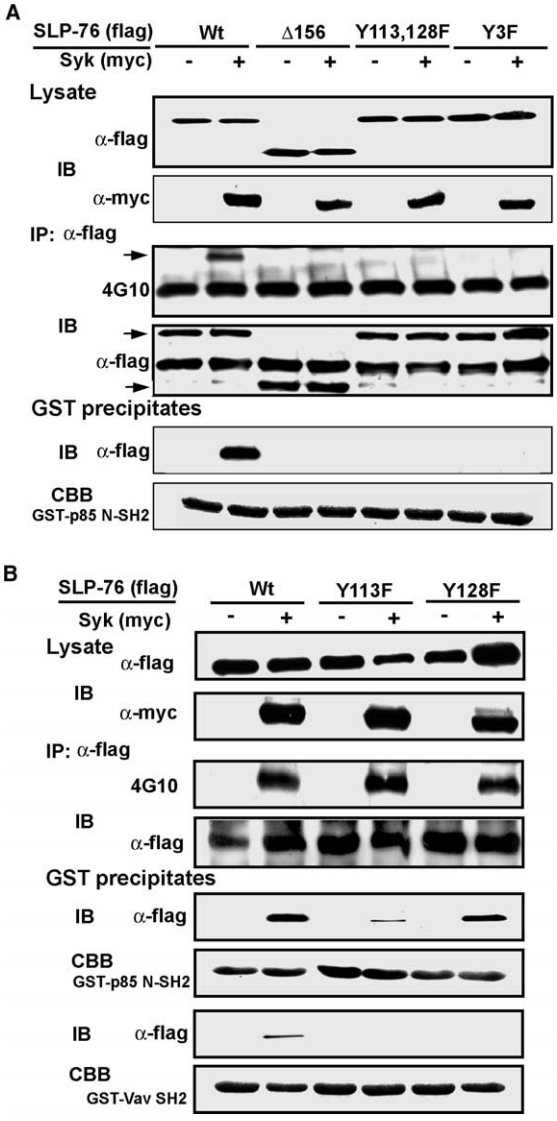

Fig. 4. Phosphorylation of SLP-76 at tyrosines 113 and/or 128 is required for association with p85 N-SH2. SLP-76 Wt, an N-terminal deletion mutant, and various tyrosine substitution mutants were expressed in HEK 293T cells in the presence or absence of Syk. Protein expression was determined by IB with anti-flag or anti-myc Ab and phosphorylation of various SLP-76 induced by Syk kinase was determined by IP with anti-flag Ab followed by IB with 4G10. Association of SLP-76 with p85 N-SH2 (A and B) or Vav SH2 (B) was measured as described in Fig. 3D. GST fusion proteins of p85 N-SH2 and Vav SH2 were expressed and confirmed by staining with CBB. Representative data from three independent experiments are shown.

tumbled with GST-p85 N-SH2 fusion proteins. As expected, SLP-76 lacking its N-terminal region did not interact with p85 N-SH2 (Fig. 4A). Similarly, there was no Syk-dependent association of p85 N-SH2 with a mutant Y113, 128F, in which the tyrosines (Y) at positions 113 and 128 in the two "YESP" motifs were changed to phenylalanine (F), or with a mutant Y3F, with all three Ys, at positions 113, 128, and 145, changed to Fs (Fig. 4A). These results further indicate that phosphorylation of tyrosines at positions 113 and 128 is required for the SLP-76 association with p85.

Since the two "YESP" motifs that are strong substrates for PTKs [2,3] are also required for binding of SLP-76 to the SH2 domain of Vav [6–9], we next compared binding of SLP-76 with the SH2 domains of Vav and p85. For these experiments, the single tyrosine mutants of SLP-76, Y113F and Y128F were expressed in HEK 293T cells together with empty vector or Syk PTK (Fig. 4B). Lysates of the transfected cells were then incubated with the fusion proteins GST-p85 N-SH2 or GST-Vav SH2. As shown previously [7,9] and in Fig. 4B, binding of Vav SH2 to SLP-76 required tyrosine phosphorylation at 113 and 128. In contrast, phosphorylation of tyrosine at either 113 or 128 was sufficient for binding to p85 N-SH2, although phosphorylation at 113 seems to be more influential (Fig. 4B).

We next examined the preference of the SLP-76 tyrosine motifs for binding to other SH2 domains that associate with SLP-76. Since the SH2 domains of Nck and Itk associate with the phosphotyrosines of SLP-76 [21–23], we performed a pull-down assay using GST-fusion proteins of the SH2 domains of Nck and Itk with lysates of HEK 293T cells overexpressing SLP-76 mutants Y113F, Y128F, or Y113, 128F, together with either vector or Syk PTK (Fig. 5). Nck SH2 associated strongly with SLP-76 Wt in a Syk-dependent manner. Binding was, however, dramatically reduced with the single tyrosine mutant, Y113F or Y128F, and completely absent with the double tyrosine mutant, Y113, 128F (Fig. 5). This pattern of binding is similar to that observed with Vav SH2 (Fig. 4B). Although the association was weak, Itk SH2 also bound to SLP-76 in a Syk-dependent binding manner (Fig. 5). As in binding to p85 SH2 (Fig. 4B), the pYESP motif at 113 dominated. These results indicate that the pYESP motifs of SLP-76 differ in their affinities for the SH2 domains of various molecules forming functional molecular complexes during T cell activation.

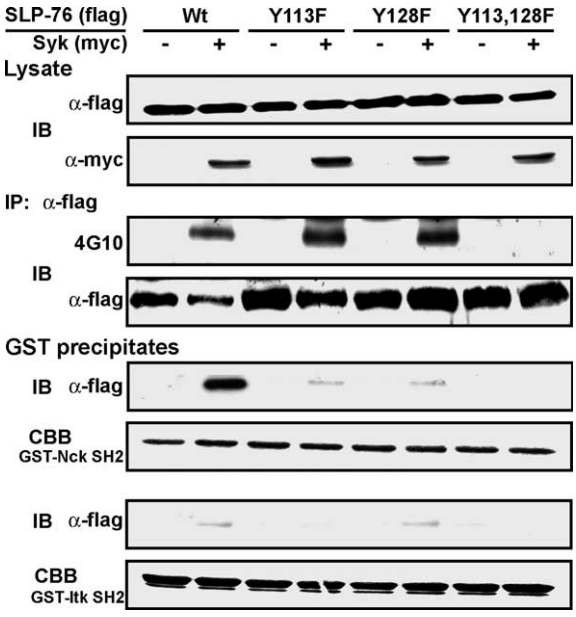

Fig. 5. Differential phosphorylation of SLP-76 tyrosine motifs required for association with the SH2 domain of Nck or Itk. Expression of SLP-76 (Wt or various tyrosine mutants) with or without Syk kinase was determined in HEK 293T cells by IB of lysates with anti-flag or anti-myc Ab. Phosphorylation of various SLP-76 induced by Syk was determined by IP of lysates with anti-flag Ab followed by IB with 4G10. Association of SLP-76 (Wt or mutants) with the SH2 domains of Nck or Itk was detected by IB with anti-flag Ab as described in Fig. 3D. GST fusion proteins of Nck SH2 and Itk SH2 were expressed and confirmed by staining with CBB. The experiments were repeated three times and representative data are shown.

4. Discussion

Ligation of the TCR results in the sequential activation of members of two PTK families such as the Src ($p56^{lck}$/$p59^{fyn}$) and Syk (Syk/ZAP-70) families [24–27]. Tyrosine phosphorylation of receptor-associated ZAP-70 by $p56^{lck}$ is required for upregulation of ZAP-70 catalytic activity required for TCR function [28,29]. Since activation of these kinases is the most membrane-proximal biochemical event known following TCR engagement, and since phosphorylation of cellular proteins by these kinases is essential for all subsequent TCR-initiated signaling pathways, a number of researchers have attempted to identify the substrates of these PTKs. One of the substrates identified is the hematopoietic cell-specific molecule, SLP-76, which is phosphorylated by ZAP-70 [2] and plays key roles in the regulation of TCR signals [3–5]. To identify molecules that associate with the three tandemly repeated DYE(S/P)P sequences of SLP-76 in activated T cells, we screened a human T leukemia cDNA library using the yeast two-hybrid method.

The screen revealed the regulatory subunit of PI3K, p85, as a novel molecule interacting with SLP-76 (Fig. 1). We performed a series of experiments to confirm the association of SLP-76 with p85 in HEK 293T cells, in which we overexpressed various proteins, as well as in Jurkat T cells (Fig. 2). We further demonstrated that one of the phosphotyrosines, at positions 113 and 128, is required for association of SLP-76 with the N-SH2 of p85 (Figs. 3 and 4). We also showed that whereas the association of the SH2 domains of Vav and Nck with SLP-76 requires tyrosine phosphorylation at both 113 and 128, tyrosine phosphorylation at either 113 or 128 is sufficient for association with the SH2 domains of p85 and Itk (Figs. 4 and 5). These results suggest that differences in the extent of tyrosine phosphorylation following T cell activation may lead to the formation of distinct signaling complexes around the SLP-76 adapter molecule.

In summary, this report is the first to show that the T cell adapter, SLP-76, associates with p85, a subunit of PI3K, in an activation-dependent manner and that phosphorylation of tyrosine at 113 preferentially mediates the interaction with the N-SH2 of p85. Further understanding the functional role of this intracellular complex formed upon activation of T cells may help to elucidate a new signaling pathway regulated by SLP-76.

Acknowledgements: We thank Drs. Alex Toker, André Veillette, and Gary Koretzky for providing valuable reagents. This study was supported by a grant from the Korea Health 21 R&D project, Ministry of Health and Welfare, Republic of Korea (00-PJ1-PG3-21200-0045), and another from the Korea Science and Engineering Foundation through the Center for Cell Signaling Research at Ewha Womans University. E.K.S., J.Y.L., and Y.J.H. were supported in part by the Brain Korea 21 Program of the Korea Ministry of Education.

References

[1] Jackman, J., Motto, D., Sun, Q., Tanemoto, M., Turck, C., Peltz, G., Koretzky, G. and Findell, P. (1995) J. Biol. Chem. 270, 7029–7032.
[2] Wardenburg, J.B., Fu, C., Jackman, J.K., Flotow, H., Wilkinson, S.E., Williams, D.H., Johnson, R., Kong, G., Chan, A.C. and Findell, P.R. (1996) J. Biol. Chem. 271, 19641–19644.
[3] Motto, D.G., Ross, S.E., Wu, J., Hendricks-Taylor, L.R. and Koretzky, G.A. (1996) J. Exp. Med. 183, 1937–1943.
[4] Fang, N., Motto, D.G., Ross, S.E. and Koretzky, G.A. (1996) J. Immunol. 157, 3769–3773.
[5] Musci, M.A., Motto, D.G., Ross, S.E., Fang, N. and Koretzky, G.A. (1997) J. Immunol. 159, 1639–1647.
[6] Wu, J., Motto, D.G., Koretzky, G.A. and Weiss, A. (1996) Immunity 4, 593–602.
[7] Tuosto, L., Michel, F. and Acuto, O. (1996) J. Exp. Med. 184, 1161–1166.
[8] Onodera, H., Motto, D.G., Koretzky, G.A. and Rothstein, D.M. (1996) J. Biol. Chem. 271, 22225–22230.
[9] Raab, M., da Silva, A.J., Findell, P.R. and Rudd, C.E. (1997) Immunity 6, 155–164.
[10] Law, C.L., Ewings, M.K., Chaudhary, P.M., Solow, S.A., Yun, T.J., Marshall, A.J., Hood, L. and Clark, E.A. (1997) J. Exp. Med. 189, 1243–1253.
[11] Liu, S.K., Fang, N., Koretzky, G.A. and McGlade, C.J. (1999) Curr. Biol. 9, 67–75.
[12] Asada, H., Ishii, N., Sasaki, Y., Endo, K., Kasai, H., Tanaka, N., Takeshita, T., Tsuchiya, S., Konno, T. and Sugamura, K. (1999) J. Exp. Med. 189, 1383–1390.
[13] Musci, M.A., Hendricks-Taylor, L.R., Motto, D.G., Paskind, M., Kamens, J., Turck, C.W. and Koretzky, G.A. (1997) J. Biol. Chem. 272, 11674–11677.
[14] da Silva, A.J., Li, Z., de Vera, C., Canto, E., Findell, P. and Rudd, C.E. (1997) Proc. Natl. Acad. Sci. USA 94, 7493–7498.
[15] Sauer, K., Liou, J., Singh, S.B., Yablonski, D., Weiss, A. and Perlmutter, R.M. (2001) J. Biol. Chem. 276, 45207–45216.
[16] Yablonski, D., Kuhne, M.R., Kadlecek, T. and Weiss, A. (1998) Science 281, 413–416.
[17] Clements, J.L., Yang, B., Ross-Barta, S.E., Eliason, S.L., Hrstka, R.F., Williamson, R.A. and Koretzky, G.A. (1998) Science 281, 416–419.
[18] Pivniouk, V., Tsitsikov, E., Swinton, P., Rathbun, G., Alt, F.W. and Geha, R.S. (1998) Cell 94, 229–238.
[19] Lee, J.R. and Koretzky, G.A. (1998) J. Immunol. 161, 1637–1644.
[20] Smith, D. and Johnson, K. (1988) Gene (Amst.) 67, 31–40.
[21] Wunderlich, L., Farago, A., Downward, J. and Buday, L. (1999) Eur. J. Immunol. 29, 1068–1075.
[22] Su, Y.W., Zhang, Y., Schweikert, J., Koretzky, G.A., Reth, M. and Wienands, J. (1999) Eur. J. Immunol. 29, 3702–3711.
[23] Bunnell, S.C., Diehn, M., Yaffe, M.B., Findell, P.R., Cantley, L.C. and Berg, L.J. (2000) J. Biol. Chem. 275, 2219–2230.
[24] van Leeuwen, J.E. and Samelson, L.E. (1999) Curr. Opin. Immunol. 11, 242–248.
[25] Clements, J.L., Boerth, N.J., Lee, J.R. and Koretzky, G.A. (1999) Annu. Rev. Immunol. 17, 89–108.
[26] Singer, A.L. and Koretzky, G.A. (2002) Science 296, 1639–1640.
[27] Leo, A., Wienands, J., Baier, G., Horejsi, V. and Schraven, B. (2002) J. Clin. Invest. 109, 301–309.
[28] Chan, A.C., Dalton, M., Johnson, R., Kong, G.-H., Wang, T., Thoma, R. and Kurosaki, T. (1995) EMBO J. 14, 2499–2508.
[29] Wange, R.L., Guitian, R., Isakov, N., Watts, J.D., Aebersold, R. and Samelson, L.E. (1995) J. Biol. Chem. 270, 18730–18733.