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Molecular Basis for a Direct Interaction between the Syk Protein-tyrosine Kinase and Phosphoinositide 3-Kinase*

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After engagement of the B cell receptor for antigen, the Syk protein-tyrosine kinase becomes phosphorylated on multiple tyrosines, some of which serve as docking sites for downstream effectors with SH2 or other phosphotyrosine binding domains. The most frequently identified binding partner for catalytically active Syk identified in a yeast two-hybrid screen was the p85 regulatory subunit of phosphoinositide 3-kinase. The C-terminal SH2 domain of p85 was sufficient for mediating an interaction with tyrosine-phosphorylated Syk. Interestingly, this domain interacted with Syk at phosphotyrosine 317, a site phosphorylated *in trans* by the Src family kinase, Lyn, and identified previously as a binding site for c-Cbl. This site interacted preferentially with the p85 C-terminal SH2 domain compared with the c-Cbl tyrosine kinase binding domain. Molecular modeling studies showed a good fit between the p85 SH2 domain and a peptide containing phosphotyrosine 317. Tyr-317 was found to be essential for Syk to support phagocytosis mediated by FcγRIIA receptors expressed in a heterologous system. These studies establish a new type of p85 binding site that can exist on proteins that serve as substrates for Src family kinases and provide a molecular explanation for observations on direct interactions between Syk and phosphoinositide 3-kinase.

Syk is a 72-kDa protein-tyrosine kinase that plays a central role in coupling immune recognition receptors to multiple downstream signaling pathways (1, 2). This function is a property of both its catalytic activity and its ability to participate in interactions with effector proteins containing SH2[1] domains. For example, after the engagement of antigen receptors on B cells, Syk is phosphorylated on three tyrosines that lie within the linker B region, which separates the N-terminal tandem pair of SH2 domains from the catalytic domain (3). Phosphorylation of the first, at Tyr-317 (numbering based on the murine Syk sequence), is catalyzed primarily by Lyn, a Src family kinase. This creates a docking site for c-Cbl, a potential negative regulator of Syk-dependent signaling (4, 5). Indeed, mutant forms of Syk containing substitutions of Phe for Tyr at position 317 exhibit enhanced activity in B cells and mast cells (3, 5, 6). To date, c-Cbl is the only protein identified that is capable of binding to Syk at pTyr-317. Phosphorylation of Tyr-342 and 346 forms a docking site for multiple SH2 domain-containing proteins including phospholipase C-γ, Vav, and Fgr (7–10). Mutant forms of Syk containing substitutions of Phe for Tyr-342 or both Tyr-342 and 346 exhibit a reduced ability to couple immune recognition receptors to the activation of downstream effectors such as phospholipase C-γ2 in B cells and mast cells (10, 11).

Syk also is required for the activation of phosphoinositide 3-kinase (PI3K) in response to a variety of signals (12–18) including engagement of the B cell antigen receptor (BCR) (12) and macrophage or neutrophil Fcγ receptors (13, 14). Furthermore, the expression of a constitutively active TEL-Syk fusion protein in atypical myelodysplastic syndrome leads to the constitutive activation of PI3K (19). The mechanisms by which Syk is coupled to the activation of PI3K appear to be either direct or indirect. In B cells, the BCR-stimulated activation of PI3K can be accomplished through the phosphorylation of adaptor proteins such as BCAP, CD19, or Gab1, which creates binding sites for the p85 regulatory subunit (20–22). Signals transmitted by many IgG receptors require the activities of both Syk and PI3K and their recruitment to the site of the clustered receptor (23). In neutrophils and monocytes, a direct association of p85 with phosphorylated immunoreceptor tyrosine-based activation motif sequences on FcγRIIA was proposed as a mechanism for the recruitment of PI3K to the receptor (24), whereas in platelets, an association of PI3K with receptor-bound Syk has been suggested (25). A thrombin-stimulated association of Syk with PI3K in platelets also has been reported (26). In cells expressing TEL-Syk, PI3K binds directly to the fusion protein, which is constitutively activated and phosphorylated because of dimerization mediated by the TEL SAM or helix-loop-helix domain (19).

The mechanism by which a direct interaction of Syk with

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The atomic coordinates and structure factors (code 1H90) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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[1] The abbreviations used are: SH2, Src homology 2; BCR, B cell receptor for antigen; CblN, N-terminal domain of c-Cbl; E3, ubiquitin-protein isopeptide ligase; EGFP, enhanced green fluorescence protein; GST, glutathione *S*-transferase; His-SykL, linker B region of Syk expressed with a hexahistidine tag; p85CSH2, C-terminal SH2 domain of p85; PDGF, platelet-derived growth factor; PI3K, phosphoinositide 3-kinase; pTyr, phosphotyrosine; SykL, Syk linker; TKB, tyrosine kinase binding.

PI3K could occur is not immediately obvious. The SH2 domains of p85 preferentially recognize the sequence p*YXX*M as a binding motif (27). Although Syk has three such motifs, none of these is apparently phosphorylated *in vivo* or *in vitro* (3, 28). In this study, we report that p85 can, in fact, interact directly with Syk in an interaction mediated by the SH2 domains of p85 binding to specific phosphotyrosines on Syk. In fact, we found that p85 was the major Syk-binding protein identified in yeast two-hybrid screens using libraries from two different sources. Interestingly, the binding of the C-terminal SH2 domain of p85 to pTyr-317 was a major contributor to this interaction. *In vitro*, the apparent affinity of the p85 C-terminal SH2 domain for pTyr-317 was greater than that of the c-Cbl tyrosine kinase binding (TKB) domain. Molecular modeling was used to investigate the potential structural determinants for recognition between the p85 C-terminal SH2 domain and pTyr-317 of Syk.

MATERIALS AND METHODS

Yeast Two-hybrid Analyses—The cDNAs for wild-type Syk (Syk(WT)), Syk(Y317F), and the Syk catalytic domain (SykK, amino acids 370–629), were amplified by PCR from the corresponding pGEM vectors (3) and inserted into the pGBKT7 yeast expression vector (Clontech) to generate the various Syk-GAL4 DNA binding domain fusions. A DNA fragment digested from pGEM-Syk(K396R) with EcoRI and BglII was inserted into predigested pGBKT7-Syk(WT) to generate pGBKT7-Syk(K396R). The cDNA for the human Lck SH2 domain (amino acids 126–234) was PCR amplified and inserted into the yeast expression plasmid pACT2 to generate the LckSH2-GAL4-transcriptional activation domain fusion. Y187 yeast cells transformed with a bone marrow cDNA library were purchased from Clontech and mated with AH109 yeast cells transformed with pGBKT7-Syk(WT). Plasmids were eluted from colonies that grew on stringent selection media (His^-, Leu^-, Trp^-, and Ade^-), amplified in competent bacterial cells, isolated, and sequenced. For analysis of interactions between specific proteins, Y187 yeast cells were transformed with the pACT2 plasmid containing the cDNA of interest and mated with AH109 cells transformed with the pGBKT7 plasmid containing the appropriate DNA insert. Mated transformants were grown on stringent selection media.

Protein Interactions with GST-Syk—Insect viruses for the expression of GST-Syk fusion proteins were described previously (29). A baculovirus for the expression of p85α was generously provided by Lewis Cantley (Harvard University). Sf9 cells expressing GST fusion proteins were lysed by sonication in lysis buffer containing 20 mM Hepes, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, and 10 μg/ml each aprotinin and leupeptin. GST fusion proteins were isolated by adsorption to glutathione-agarose. Immobilized GST-Syk, GST-p42.5, and GST-p35 were autophosphorylated *in vitro* by incubation at 37 °C for 15 min in kinase buffer (50 mM Hepes, pH 7.5, 10 mM $MnCl_2$, 1 mM Na_3VO_4, 0.05 mM [γ-^{32}P]ATP, and 10 μg/ml each aprotinin and leupeptin). Immobilized proteins were incubated with lysates of Sf9 cells expressing p85α and then washed extensively with wash buffer containing 20 mM Hepes, pH 7.5, 250 mM NaCl, 5 mM EDTA, 0.5 M LiCl, and 1% Nonidet P-40. Bound proteins were eluted in SDS-sample buffer, separated by SDS-PAGE, and detected by Western blotting with anti-p85 (Upstate Biotechnology, Inc.) or anti-GST (Santa Cruz Biotechnology, Inc.) antibodies. In some assays, resin-bound, phosphorylated GST-Syk fusion proteins were eluted with 20 mM glutathione, and the soluble fusion proteins were incubated with either immune complexes containing p85α bound to anti-p85 immobilized on protein A-Sepharose or His-tagged LckSH2 bound to His-select HC nickel affinity gel (Sigma). The p85-anti-p85 immune complexes were prepared by incubating protein A-Sepharose beads containing anti-p85 antibody with lysates of Sf9 cells expressing p85. The vector for expression of the His-tagged Lck SH2 domain (LckSH2) in bacteria was constructed by insertion of the appropriate PCR-amplified fragment into NdeI and XhoI double digested pRGT7 (provided by Dr. Hyunho Chung, LG Chem). Beads were washed as above. Bound fusion proteins were separated by SDS-PAGE and detected by autoradiography.

Interaction of Proteins with Cellular Syk Family Kinases in Cell Lysates—Jurkat T cells, DG75 B cells, and THP-1 monocytes were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 50 μM 2-mercaptoethanol, 2 mM glutamine, 1 mM sodium pyruvate, 100 IU/ml penicillin G, and 100 μg/ml streptomycin. The chicken DT40 B cell line stably expressing a Myc epitope-tagged murine Syk was described previously (3). Cells were treated for 15 min at 37 °C in the presence or absence of pervanadate (0.1 mM sodium orthovanadate and 0.5 mM H_2O_2). THP-1 cells also were preincubated for 30 min at room temperature with 2 mM dithiobis(succinimidyl propionate) (Pierce Biotechnology). Cells (1×10^7) were lysed with 0.5 ml of lysis buffer as described above. For coimmunoprecipitation experiments, lysate supernatants were incubated at 4 °C for 4 h with protein A-Sepharose preadsorbed to the anti-p85α antibody. The protein/bead mixture was washed three times with wash buffer and then incubated in kinase buffer prior to separation by SDS-PAGE. Bound Syk was detected by Western blotting and by autoradiography. Lysates of Jurkat, DG75, or DT40 B cells that had been treated with or without pervanadate also were incubated with fusion proteins of GST linked to the C-terminal SH2 domain of p85α (p85CSH2), the N terminus of c-Cbl (GST-CblN) or an inactive mutant of c-Cbl (CblN(G306E)) immobilized to glutathione-agarose. The pGEX4T-2-p85CSH2 plasmid coding p85CSH2 (amino acids 565–724) for expression in bacteria was constructed by in-frame insertion of PCR-amplified p85CSH2 cDNA into pGEX4T-2 (Amersham Biosciences). Plasmids for the expression of GST-CblN and GST-CblN(G306E) were generously supplied by Hamid Band (Northwestern University).

Interactions with the Syk Linker Region—The cDNA for the linker region of Syk (SykL, amino acids 257–363) was amplified by PCR and inserted into the pET15b expression plasmid (Novagen) to generate pET15b-His-SykL. For the purification of His-SykL, transformed bacterial cells (50-ml culture) were lysed on ice by sonication in 10 ml of denaturing cell lysis buffer (0.1 M sodium phosphate, pH 8.0, 8 M urea). 10 ml of cell lysate was loaded on the nickel-resin column. After washing with the same buffer, target proteins bound to the resin were renatured with buffer containing 50 mM sodium phosphate, pH 7.5, and 150 mM sodium chloride. The resin-bound His-SykL was phosphorylated *in vitro* by incubation in kinase buffer containing a mixture of GST-Syk and Lck. Lck was isolated by immunoprecipitation from lysates of Sf9 cells infected with a baculovirus directing the expression of Lck (30). Phosphorylated His-SykL was finally eluted from the resin with elution buffer (50 mM sodium phosphate, pH 8.0, 0.3 M sodium chloride, 250 mM imidazole) and incubated with GST-p85CSH2, GST-FgrSH2, or GST-CblN immobilized onto glutathione-agarose. Bound SykL was detected by autoradiography after SDS-PAGE or by Western blotting with a phosphospecific antibody recognizing Syk phosphorylated on Y317 (Cell Signaling Technologies). A bacterial expression plasmid for the GST-Fgr SH2 domain was generously provided by Dr. Cheryl L. Willman (University of New Mexico).

Interactions with Synthetic Peptides—Synthetic polypeptides corresponding to sequences surrounding Y317, TVSFNPYEPTGGP, TVSFNPpYEPTGGP, and TVSFNPpYEPELAP, were purchased from Advanced ChemTech. Peptides were covalently coupled to Affi-Gel 10 (Bio-Rad) by incubating 15 mg of each peptide with 500 μl of resin in 10 mM Hepes, pH 7.5, at 4 °C for 12 h. The coupling reaction was stopped by the addition of 0.1 ml of 1 M ethanolamine HCl, pH 8.0. For binding studies, 100 μl of the peptide-resin suspension was mixed with purified GST-p85CSH2, GST-FgrSH2, or GST-CblN at 4 °C for 2 h and washed three times with buffer containing 10 mM Hepes, pH 7.5, 150 mM NaCl, and 5 mM EDTA. Bound proteins were separated by SDS-PAGE and detected by Western blotting with an anti-GST antibody.

Phosphopeptide Mapping—For the identification of Syk-derived phosphopeptides, Syk or SykL, phosphorylated *in vitro*, were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The radiolabeled bands were excised and digested with trypsin. Phosphopeptides were either separated by electrophoresis on an alkaline 40% polyacrylamide gel or were first adsorbed to GST-p85CSH2 immobilized on glutathione-agarose as described previously prior to separation. The phosphopeptides were detected by autoradiography and their identities determined by their migration positions as described previously (3).

Molecular Modeling—The peptide pYEPTG, derived from residues 317–321 of Syk linker B, was modeled in the phosphopeptide binding site of p85CSH2 based on the crystal structure (PDB entry 1H9O) for the complex of this SH2 domain with the peptide pYVPML from the platelet-derived growth factor (PDGF) receptor. Initial heavy atom coordinates were obtained for the Syk-derived peptide by retaining coordinates from 1H90 for all atoms common to both peptides. Initial positions for heavy atoms unique to pYEPTG were built without steric overlap using the molecular graphics program QUANTA. The peptide N terminus was acetylated, and the C terminus was amidated. Hydrogen atom positions were built with the HBUILD facility of CHARMM. The p85CSH2·pYEPTG complex was conformationally relaxed using molecular dynamics and energy minimization calculated with the CHARMM program and the all hydrogen CHARMM27 force field. To preserve the

effects of solvation during the relatively short time period of the conformational relaxation, the crystallographic water molecules were included in the molecular mechanics calculations using the TIP3P water model. The molecular mechanics protocol included 1,000 steps of Powell energy minimization, followed by 50 ps of molecular dynamics with a temperature ramp from 400 to 100 K and 1000 steps of Powell energy minimization. The root mean square coordinate difference between the initial structure modeled directly from 1H90 and the final structure from conformational relaxation was 1.2 Å for main chain atoms and 2.0 Å for all heavy atoms.

Phagocytosis Assay—COS-7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Phagocytosis assays in COS-7 cells transfected with expression vectors coding for FcγRIIA and the various Syk mutants were as described previously (31). In brief, cells were transiently transfected using Lipofectamine with plasmids coding for FcγRIIA and either enhanced green fluorescence protein (EGFP) or wild-type or mutant Syk fused to EGFP (Syk-EGFP). The expression levels of EGFP, Syk-EGFP, and FcγRIIA were compared by flow cytometry with FcγRIIA levels measured using an allophycocyanin-conjugated anti-CD32 monoclonal antibody (FLI8.26) (Pharmingen). Transfected cells were incubated with sheep red blood cells coated with IgG at a subagglutinating concentration at a target to effector ratio of 200:1 for 2 h. After uningested red blood cells were lysed in water, COS-7 cells were spun onto glass slides, fixed, stained with Wright Giemsa stain, and counted for internalized red blood cells. Alternatively, cell lysates were prepared and immunoblotted for activated Akt using an anti-phospho-Akt antibody (Cell Signaling Technologies).

RESULTS

The p85 Regulatory Subunit of PI3K Interacts with Syk in a Yeast Two-hybrid Screen—To identify proteins capable of interacting directly with Syk, we screened a human bone marrow cDNA library using the yeast two-hybrid method. We reasoned that proteins capable of binding to sites of tyrosine-phosphorylation on Syk would interact with the catalytically active form of the kinase because Syk can catalyze the autophosphorylation of all of the relevant tyrosines that also are phosphorylated *in vivo* (3, 28). This approach requires that a fusion protein of Syk with the GAL4 DNA binding domain be active when expressed in yeast and capable of autophosphorylation. To explore this, we first examined the ability of Syk to interact in the two-hybrid screen with a protein consisting of the GAL4 transactivation domain fused to the SH2 domain derived from Lck, a Src family kinase. The Lck SH2 domain has been shown previously to bind to phosphorylated forms of Syk (32) and the Syk family kinase, Zap-70 (33). We expressed in yeast fusion proteins containing either catalytically active, wild-type Syk, or a catalytically inactive mutant (Syk(K396R)). In a two-hybrid screen, wild-type Syk, but not Syk(K396R), interacted with the Lck SH2 domain fusion protein (Fig. 1). This is consistent with a model in which Syk can undergo autophosphorylation in yeast to create a site for interaction with proteins containing SH2 domains. The two-hybrid screen was then carried out using the bone marrow cDNA library, which produced a number of positive clones of which nearly 50% could be accounted for by one of eight different cDNAs coding for various regions of either the α or β isoforms of p85 (Fig. 1*C*). Similar results were observed when a human mammary gland library was substituted for the bone marrow library (data not shown). These results indicate that p85 is capable of a direct physical interaction with Syk.

The Binding of p85 Requires the Tyrosine Phosphorylation of Syk—Because the ability of Syk to bind p85 physically had not been described in detail previously, we explored the mechanism of this interaction. To test for a role for protein phosphorylation, we used the yeast two-hybrid screen to compare the binding of p85 SH2 domains with wild-type or catalytically inactive Syk (Syk(K396R)). Both the Lck SH2 domain and Fgr were included as prey for positive controls and p53 as bait to confirm specificity. Fgr is an Src family kinase that also was positively identified in our bone marrow two-hybrid screen and was recently identified as a protein that interacts selectively with Syk to negatively regulate the integrin-mediated spreading of macrophages on ICAM-1 (9). The small ribosomal subunit protein, S10, which was also identified in the screen as a protein that reacts with multiple baits in an apparently nonspecific fashion, was also included. Neither the C-terminal SH2 domain nor constructs containing both the N + C-terminal SH2 domains of either the α or β isoforms of p85 were capable of interacting with either Syk(K396R) or p53 (Fig. 1*D*). Similarly, neither the Lck SH2 domain nor Fgr interacted with Syk(K396R) or p53 as expected. This result suggested that the interaction of Syk with p85 was mediated by SH2 domains

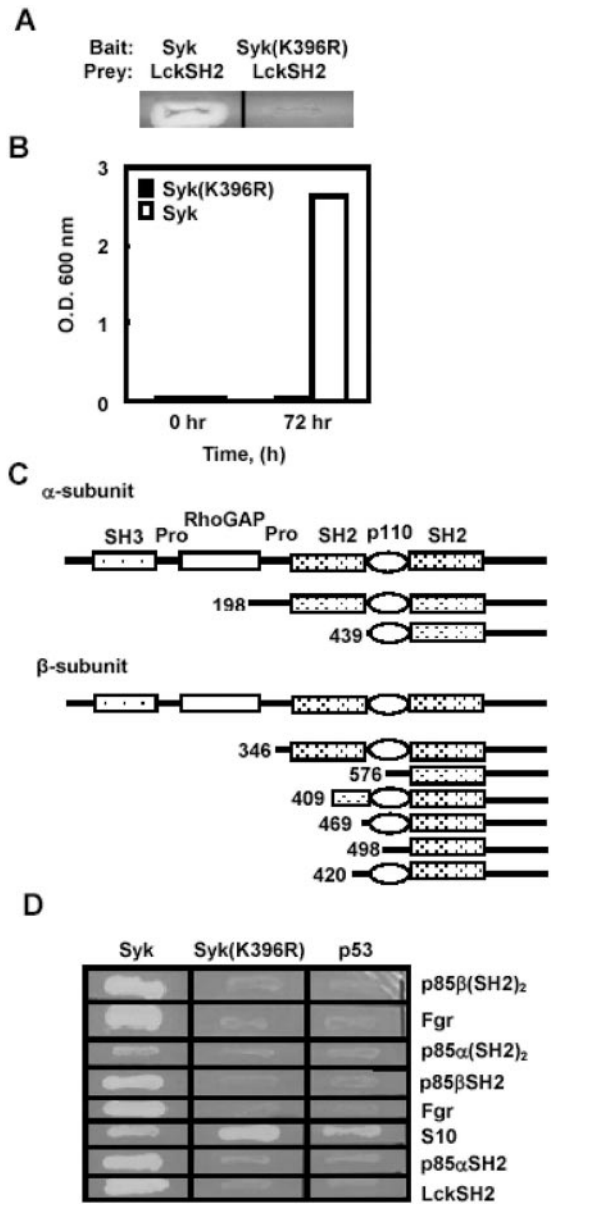

FIG. 1. **The p85 subunit of PI3K interacts with catalytically active Syk in yeast.** *A*, yeast transformants expressing the Lck SH2 domain fused to the GAL4 transcriptional activation domain (*LckSH2*) and either wild-type Syk or catalytically inactive Syk(K396R) fused to the GAL4 DNA binding domain were grown on plates in media deficient in adenine and histidine. *B*, growth of transformants described above in liquid selection media. *C*, schematic diagram of eight independent clones of p85 identified in a yeast two-hybrid screen using a bone marrow cDNA library. *D*, yeast transformants expressing Syk, Syk(K396R), or p53 fused to the GAL4 DNA binding domain and the indicated proteins fused to the GAL4 transcriptional activation domain were grown on plates containing media deficient in adenine and histidine.

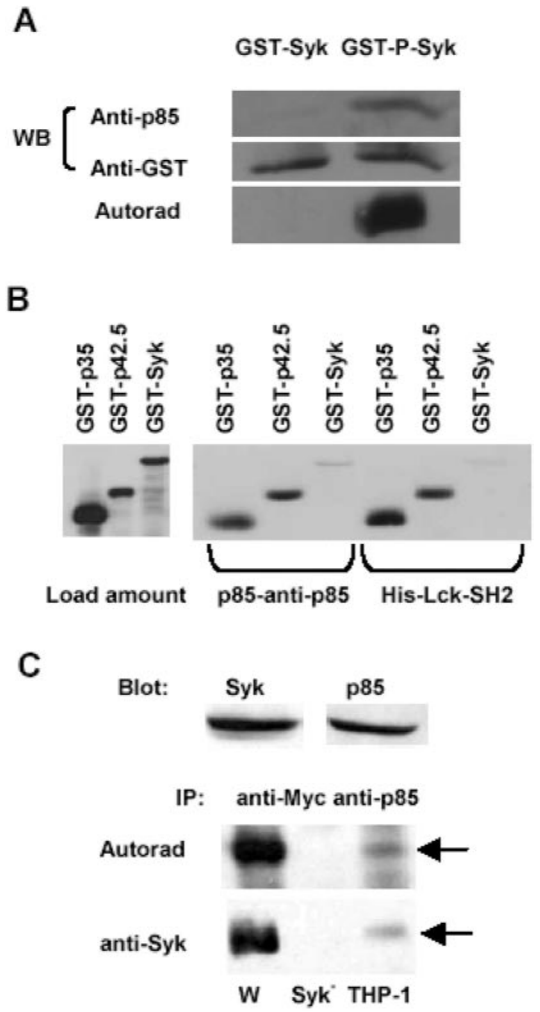

FIG. 2. **The p85 subunit of PI3K binds to phosphorylated Syk *in vitro*.** *A*, lysates from insect cells infected with a baculovirus expressing p85 were incubated with GST-Syk bound to glutathione-agarose that had been preincubated with or without [γ-^{32}P]ATP. Bound proteins were separated by SDS-PAGE. Adsorbed p85 was detected by Western blotting (*WB*) with an anti-p85 antibody. Bound GST-Syk or phosphorylated GST-Syk (*GST-P-Syk*) was detected by Western blotting with an anti-GST antibody. Phosphorylated Syk was detected by autoradiography (*Autorad*). *B*, GST-Syk and two truncated forms of GST-Syk, GST-p35 and GST-p42.5, were preincubated with buffer containing [γ-^{32}P]ATP to allow autophosphorylation. ^{32}P-Labeled proteins were incubated with a p85-anti-p85 immune complex bound to protein A-Sepharose or a hexahistidine-tagged Lck SH2 domain bound to nickel-resin. Bound phosphoproteins were detected by autoradiography. *C*, endogenous Syk and p85 in THP-1 cells were detected by Western blotting using anti-Syk or anti-p85 antibodies (*top panel*). Syk-deficient DT40 B cells (*Syk*$^-$), Syk-deficient DT40 cells expressing Syk with a Myc-epitope tag (*W*), or THP-1 cells were preincubated with pervanadate, lysed, and then incubated with anti-Myc epitope or anti-p85 antibodies immobilized on protein A-agarose. Immune complexes were incubated with [γ-^{32}P]ATP to allow Syk autophosphorylation to occur. Proteins were separated by SDS-PAGE. Syk was detected by autoradiography or by Western blotting with an anti-Syk antibody. The migration positions of Syk are indicated by the *arrows*.

interacting with sites of tyrosine phosphorylation on Syk.

To confirm an interaction biochemically, we examined the ability of a GST-Syk fusion protein to bind to p85. GST-Syk was adsorbed to glutathione-agarose and then was left either untreated or was incubated with [γ-^{32}P]ATP to allow autophosphorylation. Resin-bound Syk proteins were then incubated with lysates of insect cells expressing full-length, untagged p85α. Proteins bound to the resin were separated by SDS-PAGE and detected by Western blotting with anti-p85 or anti-GST antibodies. As shown in Fig. 2*A*, p85 was adsorbed to resin containing bound, phosphorylated Syk, but was not adsorbed to resin containing the unphosphorylated kinase despite the presence of equivalent amounts of bound Syk. These results are consistent with a requirement for phosphorylation for the interaction of Syk with p85.

To verify this interaction further, we examined the ability of phosphorylated Syk to bind to immobilized p85. Three forms of Syk, GST-Syk (full-length), GST-p42.5 (GST fused to Syk residues 256–629), and GST-p35 (GST fused to Syk residues 327–629) were isolated, phosphorylated using [γ-^{32}P]ATP (Fig. 2*B*), and then incubated with p85 bound to an anti-p85 antibody immobilized on protein A-Sepharose. GST-Syk, GST-p42.5, and GST-p35 all bound to immobilized p85 (Fig. 2*B*). For a comparison of relative affinities, a His_6-tagged Lck SH2 domain was expressed in bacteria, immobilized on a nickel affinity-resin, and used in the same pull-down experiment. The Lck SH2 domain also interacted with all three versions of phosphorylated Syk. These results are consistent with a direct physical interaction between Syk and p85.

To test for a possible interaction in intact cells, THP-1 monocytes were treated in the presence or absence of pervanadate and a cleavable cross-linking reagent. PI3K was immunoprecipitated using an anti-p85 antibody. The presence of Syk in the resulting immune complex was detected both by Western blotting with an anti-Syk antibody and by autophosphorylation resulting from the incubation of the complex with buffer containing [γ-^{32}P]ATP. As shown in Fig. 2*C*, Syk protein and activity could be detected in anti-p85 immune complexes indicating either a direct or indirect interaction between the two proteins.

The C-terminal SH2 Domain of p85 Binds Directly to Phosphorylated Syk—An examination of the clones identified in the yeast two-hybrid screen indicated that the C-terminal SH2 domain of p85 (p85CSH2) alone was sufficient for mediating an interaction with catalytically active Syk. To confirm this, we incubated immobilized GST-p85CSH2 with lysates from pervanadate-treated Syk-deficient DT40 B cells, Syk-deficient DT40 B cells stably expressing murine Syk, human DG75 B cells, or human Jurkat T cells. As illustrated in Fig. 3, *A* and *B*, both murine and human Syk were capable of binding to immobilized GST-p85CSH2. No protein reacting with anti-Syk antibodies was detected in lysates from Syk-deficient cells. Human Syk also was able to bind to the Fgr SH2 domain. GST-p85CSH2 also bound to phosphorylated Zap-70 as illustrated by the fact that Zap-70 could be adsorbed to resin containing bound GST-p85CSH2 from lysates of pervanadate-treated, but not untreated, Jurkat T cells (Fig. 3*C*). These results indicated that the C-terminal SH2 domain of p85 was sufficient for mediating an interaction with the phosphorylated Syk and Zap-70 tyrosine kinases.

Binding of the C-terminal SH2 Domain of p85 to Syk in Yeast Requires Tyr-317—The consensus sequence recognized by the SH2 domains of p85 is pY*XX*M (27). Syk possesses three Y*XX*M motifs, all of which are present within the catalytic domain at residues 383–386, 567–570, and 596–599. To determine whether p85 could interact with one of these motifs, we monitored the ability of the C-terminal SH2 domain of p85α or p85β to interact with the Syk catalytic domain alone (residues 370–629) using the yeast two-hybrid assay. None of the constructs containing the C-terminal SH2 domain demonstrated any ability to bind to the Syk catalytic domain (Fig. 4A). Neither Fgr nor the Lck SH2 domain interacted with the Syk catalytic domain in this assay (data not shown).

Extensive studies on the characterization of Syk have provided evidence for a critical role for the linker B region as an adaptor for binding proteins with SH2 domains. The first of

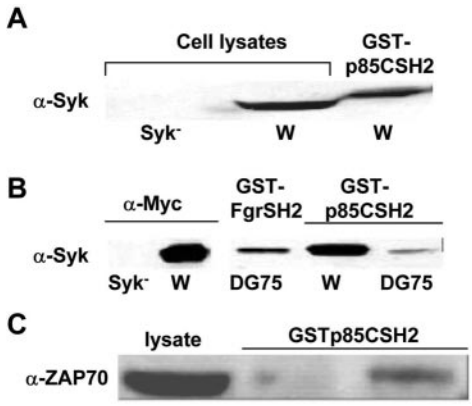

FIG. 3. **The C-terminal SH2 domain of p85 interacts with Syk and Zap-70.** *A*, lysates from pervanadate-treated DT40 cells expressing Myc-tagged murine Syk (*W*) were incubated with a GST-C-terminal p85 SH2 domain fusion protein (*GSTp85CSH2*) bound to glutathione-agarose. Syk bound to GST-p85CSH2 was detected by Western blotting using an anti-Syk antibody. The migration position of Syk was verified by Western blotting of detergent lysates from Syk-deficient DT40 cells (*Syk*$^-$) or DT40 cells expressing Myc-tagged Syk (*W*). *B*, lysates from pervanadate-treated, human DG75 B cells, Syk-deficient DT40 B cells, or DT40 cells expressing Myc-tagged murine Syk were incubated with anti-Myc epitope antibody bound to protein A-Sepharose or with the GST-Fgr SH2 domain (*GSTFgrSH2*) or GST-p85CSH2 bound to glutathione-agarose. Bound proteins were separated by SDS-PAGE and detected by Western blotting with anti-Syk antibody. *C*, lysates from human Jurkat T cells preincubated with (*right*) or without (*middle*) pervanadate were incubated with GST-p85CSH2 bound to glutathione-agarose. Zap-70 bound to GST-p85CSH2 was detected by Western blotting using an anti-Zap-70 antibody. The migration position of Zap-70 was verified by Western blotting of detergent lysates from Jurkat cells with the anti-Zap-70 antibody.

three major sites of autophosphorylation within the Syk linker B region that constitutes a recognized binding site is Tyr-317. We tested, using the yeast two-hybrid assay, the ability of a mutant form of Syk in which Tyr-317 was replaced by Phe (Syk(Y317F)) to bind to forms of p85 containing either the N + C-terminal SH2 domains or only the C-terminal SH2 domain. As a control, Fgr, which was shown previously to interact with Syk in the region surrounding Tyr-342 and Tyr-346 (9), was also examined. Fgr interacted with both Syk and Syk(Y317F) as expected (Fig. 4*B*). The region of p85 containing both N- and C-terminal SH2 domains also interacted with both bait constructs. Interestingly, the p85 C-terminal SH2 domain failed to interact with Syk(Y317F). This result suggested that pTyr-317 in the Syk linker B region was required for the interaction with the p85 C-terminal SH2 domain, whereas other tyrosine residues in the linker region contributed to the interaction with the N-terminal SH2 domain.

Binding of the C-terminal SH2 Domain of p85 to the SykL B Region Requires the Phosphorylation of Tyr-317—An interaction of p85 with Syk pTyr-317 was unexpected because the sequence surrounding this residue does not resemble a consensus binding site for the p85 SH2 domain. To confirm further an interaction between the p85 C-terminal SH2 domain and the SykL B region, we examined the ability of GST-p85CSH2 to bind to a His_6-tagged SykL B polypeptide (His-SykL) consisting of a hexahistidine tag at the N terminus of the SykL B region comprising amino acids 257–363. His-SykL was expressed in bacteria, immobilized by adsorption to nickel-resin, and phosphorylated with [γ-^{32}P]ATP using a combination of GST-Syk and Lck, both expressed in insect cells. The sites phosphorylated on His-SykL were identified by phosphopeptide mapping as Tyr-317, Tyr-342, and Tyr-346 (Fig. 4*C*). The *in vitro* phosphorylated His-SykL was then incubated with GST-p85CSH2 and the GST-Fgr SH2 domain immobilized on glutathione-agarose. Phosphorylated His-SykL was bound by both SH2

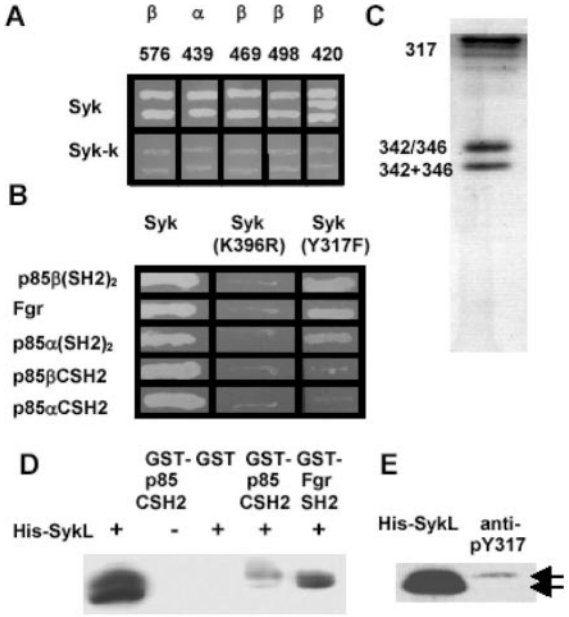

FIG. 4. **The C-terminal SH2 domain of p85 does not interact with the kinase domain of Syk, but interacts with a linker region site of phosphorylation.** *A*, full-length Syk (*Syk*) or only the kinase domain of Syk (*Syk-k*) was used as bait for testing an interaction with the C-terminal SH2 domain of p85. The transformants containing Syk or Syk-k and the various forms of p85 which contain only the C-terminal SH2 domain (the starting amino acid for each construct is listed at the *top*; see Fig. 2) were grown on plates with media lacking histidine and adenine. *B*, full-length Syk, Syk(K396R), or Syk(Y317F) was used as bait for testing an interaction with Fgr or with the α and β isoforms of p85 containing only the C-terminal SH2 domain (*p85CSH2*) or both N- and C-terminal SH2 domains (*p85(SH2)$_2$*). Transformants were grown on selection media lacking both histidine and adenine. *C*, the hexahistidine-tagged Syk linker B region (*His-SykL*) was phosphorylated *in vitro* using a combination of Lck and GST-Syk as catalysts. ^{32}P-His-SykL was separated by SDS-PAGE, transferred to nitrocellulose, and digested with trypsin. The pool of digested peptides was separated on an alkaline 40% polyacrylamide gel and subjected to autoradiography. The migration positions of peptides containing pTyr-317, pTyr-342, and pTyr-346 are indicated. Note that Tyr-342 and Tyr-346 are present on the same tryptic peptide. *D*, ^{32}P-His-SykL was subjected to SDS-PAGE and detected by autoradiography (*first lane*). GST, GST-p85CSH2, or GST-FgrSH2 bound to glutathione-agarose was incubated with (+) or without (−) ^{32}P-His-SykL. Bound proteins were separated by SDS-PAGE and detected by autoradiography. *E*, ^{32}P-His-SykL bound to GST-FgrSH2 was separated by SDS-PAGE, transferred to Immobilon P, and detected by autoradiography or by Western blotting with an anti-pTyr-317-Syk specific antibody. The positions of the two differentially migrating forms of ^{32}P-His-SykL are indicated by *arrows*.

domains, indicating that one or more of the linker B phosphorylation sites could mediate these interactions.

Interestingly, His-SykL migrated on SDS-polyacrylamide gels as a doublet after phosphorylation (Fig. 4*D*). The SH2 domain of Fgr, which binds to Syk at pTyr residues 342 and 346 (9), bound both of the two differentially migrating forms of His-SykL. This indicated that both migrating forms contained these phosphoamino acids. In contrast to the GST-Fgr SH2 domain, GST-p85CSH2 associated preferentially with the slower mobility form of His-SykL (Fig. 4*D*). Syk, when phosphorylated on Tyr-317, but not at other sites, also exhibits a reduced mobility on SDS-PAGE (3). To determine whether the slower migrating form of His-SykL also resulted from the phosphorylation of Tyr-317, the phosphorylated linker was separated by SDS-PAGE and immunoblotted with an anti-phosphopeptide antibody that recognizes selectively the region of Syk phosphorylated at Tyr-317. This antibody detected only the upper band (Fig. 4*E*), confirming that only this slower migrating form of His-SykL contained phosphorylated Tyr-317.

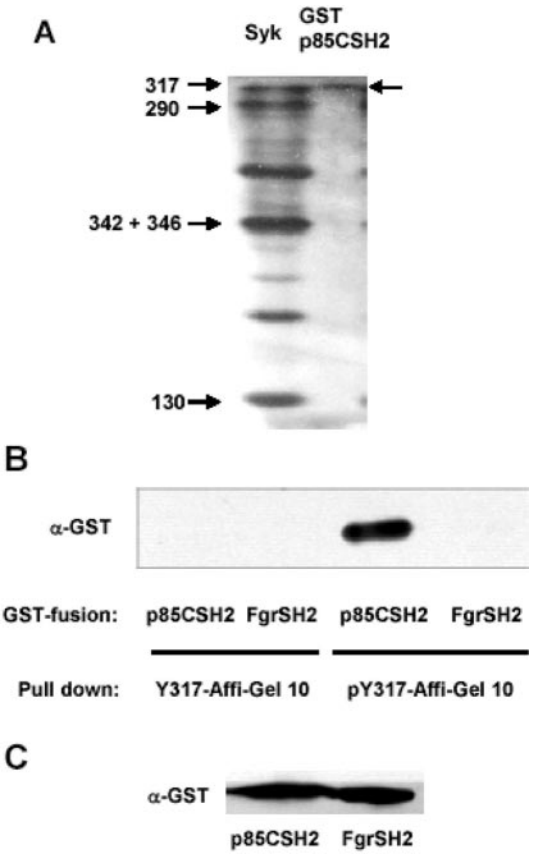

FIG. 5. **Phosphopeptides containing Tyr-317 bind to the C-terminal SH2 domain of p85.** *A*, Syk, autophosphorylated *in vitro* with buffer containing [γ-^{32}P]ATP, was digested with trypsin. The resulting phosphopeptides were either separated directly by electrophoresis on an alkaline 40% polyacrylamide gel or adsorbed to glutathione-agarose resin containing immobilized GST-p85CSH2 and eluted prior to separation. The migration positions of phosphopeptides containing the indicated phosphotyrosine residues are indicated. *B* and *C*, GST-p85CSH2 or GST-FgrSH2 was incubated with immobilized peptides containing either Tyr-317 or pTyr-317. GST fusion proteins bound to peptides were detected by Western blotting using an anti-GST antibody. The relative amounts of GST-p85CSH2 and GST-FgrSH2 applied to the resins were compared by Western blotting with an anti-GST antibody.

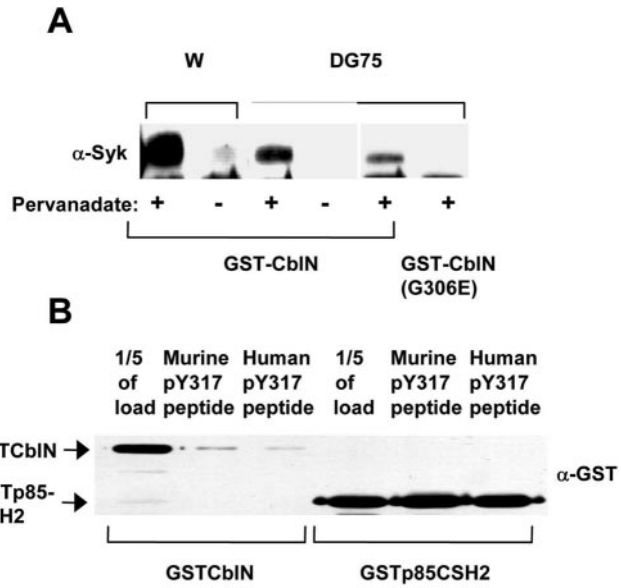

FIG. 6. **The c-Cbl TKB domain binds to Syk and Syk-derived phosphopeptides.** *A*, lysates from DT40 cells expressing Myc epitope-tagged Syk or DG75 cells that had been pretreated with (+) or without (-) pervanadate were incubated with GST-fusion proteins containing a functional (*GSTCblN*) or nonfunctional (*GSTCblN(G306E*)) c-Cbl N-terminal TKB domain bound to glutathione-agarose. Bound Syk was detected by Western blotting with an anti-Syk antibody. *B*, GSTCblN or GSTp85CSH2 were incubated with immobilized phosphopeptides corresponding to the sequences surrounding pTyr-317 from either the murine or human Syk. Bound proteins were detected by Western blotting using an anti-GST antibody. The migration positions of GSTCblN and GSTp85CSH2 are indicated by the *arrows*.

Consequently, the C-terminal SH2 domain of p85 interacted preferentially with forms of the SykL containing pTyr-317.

The C-terminal SH2 Domain of p85 Binds to Phosphopeptides Containing pTyr-317—The specificity of the p85CSH2 domain for sites of tyrosine phosphorylation on Syk was verified using two phosphopeptide binding assays. In the first assay, tryptic phosphopeptides derived from autophosphorylated Syk were incubated with GST-p85CSH2 bound to glutathione-agarose. Phosphopeptides that bound to GST-p85CSH2 were eluted and separated by 40% alkaline SDS-PAGE and detected by autoradiography. The migration positions of the bound phosphopeptides were compared with those of phosphopeptides derived from a total tryptic digest of phosphorylated Syk. Only the peptide containing pTyr-317 was detected (Fig. 5*A*). As described previously, the Fgr SH2 domain selects only the phosphopeptide containing both pTyr-342 and pTyr-346 in this same assay (9). We have found that the Lck SH2 domain exhibits the same binding specificity as the Fgr SH2 domain (data not shown).

For the second phosphopeptide binding assay, two synthetic 13-residue peptides were prepared. One contained Tyr-317 and the 6 amino acids N- and C-terminal to this residue. The second was an identical peptide, but it contained phosphotyrosine in place of Tyr-317. Both were immobilized to Affi-Gel 10 and then incubated with either purified GST-p85CSH2 or GST-FgrSH2. Western blotting with anti-GST antibodies identified the bound SH2 domains. Neither SH2 domain fusion protein bound the immobilized, unphosphorylated peptide (Fig. 5*B*). GST-p85CSH2, but not GST-FgrSH2, bound to the immobilized pTyr-317-containing peptide, despite the application of similar amounts of fusion protein to the beads (Fig. 5*C*). The result of these combined peptide binding assays is consistent with the conclusions drawn from the yeast two-hybrid screen and protein binding studies indicating that p85 interacts with Syk through the linker B region, with the C-terminal SH2 domain binding preferentially through pTyr-317.

The C-terminal SH2 Domain of p85 Binds More Tightly Than the c-Cbl TKB Domain to Phosphopeptides Containing pTyr-317—The only reported protein capable of binding to pTyr-317 is c-Cbl. This led us to compare the interaction of Syk and c-Cbl with that of Syk and p85CSH2. The N-terminal regional of c-Cbl, which contains the SH2-like TKB domain, was expressed in bacteria as a GST fusion protein and immobilized on glutathione-agarose. The *in vitro* phosphorylated His-SykL was adsorbed to resin containing immobilized GST-CblN as described above for the SH2 domains of p85 and Fgr. However, we were unable to detect any bound phospho-His-SykL (data not shown). To determine whether the GST-CblN construct retained the ability to bind to tyrosine-phosphorylated proteins, we incubated the immobilized domain with lysates of either untreated or pervanadate-treated DT40 B cells that expressed murine Syk or human DG75 B cells. Bound Syk was detected by Western blotting using an anti-Syk antibody. The association of Syk with immobilized GST-CblN was detected when lysates were obtained from pervanadate-treated cells, but not from untreated cells (Fig. 6*A*). Furthermore, Syk was able to associate with GST-CblN, but not with GST-CblN(G306E), a form of CblN that contains an inactive TKB domain. This result suggests that GST-CblN has a functional

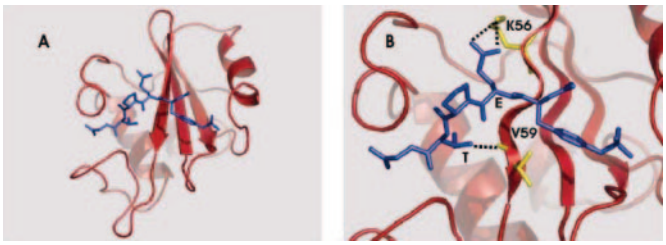

FIG. 7. **Molecular modeling of the interaction between p85CSH2 and a Syk-derived phosphopeptide.** *A*, proposed structure of a complex between the Syk-derived phosphopeptide, pYEPTG (*blue*) and p85CSH2 (*red*). *B*, detailed view of interactions of Glu and Thr side chains with Lys-56 and Val-59 main chain of p85CSH2.

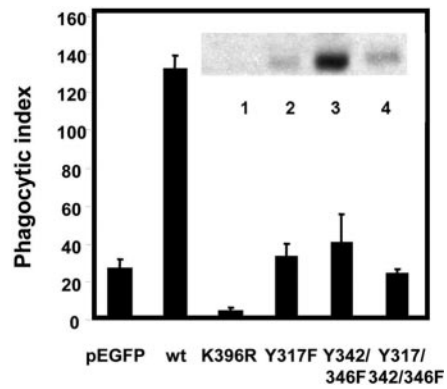

FIG. 8. **Phagocytosis of IgG-sensitized sheep red blood cells by COS-7 cells expressing FcγRIIA and Syk.** COS-7 cells were transiently transfected with plasmids coding for FcγRIIA and EGFP or Syk-EGFP containing the indicated point mutations. The phagocytic index (number of red blood cells internalized/100 COS-7 cells) was determined. *Inset*, lysates of COS-7 cells expressing wild-type Syk-EGFP (*lanes 1 and 3*), EGFP (*lane 2*) or Syk-EGFP(Y317/342/346F) (*lane 4*) that were unstimulated (*lane 1*) or activated for 5 min (*lanes 2–4*) were immunoblotted with antibodies against anti-phospho-Akt.

phosphotyrosine interaction domain and can bind either directly or indirectly to phosphorylated Syk.

The binding of GST-CblN to immobilized synthetic phosphopeptides corresponding to the 13 amino acids surrounding pTyr-317 from both murine and human Syk was then compared with the binding of GST-p85CSH2 (Fig. 6*B*). Both phosphopeptides were immobilized on Affi-Gel 10 and then incubated with purified GST-CblN or GST-p85CSH2. In this assay, GST-CblN exhibited a relatively weak interaction with both phosphopeptides, whereas the binding of GST-p85CSH2 was much more extensive. There was no significant difference in the interaction of either GST-p85CSH2 or GST-CblN with murine *versus* human Syk-derived phosphopeptides. This analysis suggests that GST-CblN does have the capacity to interact with the pTyr-317-containing peptide, although the association is considerably weaker than that of GST-p85CSH2.

Structure Model of the p85CSH2·Syk Peptide Complex—The Syk sequence pYEPTG is not a consensus sequence for phosphopeptides that bind p85CSH2. Molecular modeling was therefore used to consider possible atomic interactions that could stabilize this complex. A model for pYEPTG bound to p85CSH2 was generated using molecular graphics starting from the crystallographic coordinates of p85CSH2 in complex with the phosphopeptide pYVPML from PDGF receptor (PDB 1H9O). The manually built model was conformationally relaxed using molecular dynamics and energy minimization. Modeling pYEPTG produced no large forces so the final structure, shown in Fig. 7, is similar overall to the complex with the PDGF receptor peptide. The intermolecular nonbonded energies are highly favorable, and excellent steric and chemical complementarity exists between the Syk peptide and p85CSH2. A number of intermolecular interactions are present (Fig. 7*B*). A salt bridge is formed between Glu of the peptide and p85 Lys-56. Thr of the peptide forms a hydrogen bond with the main chain NH of p85 Val-59. Interactions with pTyr present in the 1H9O are maintained, as well as a hydrogen bond between His-94 Nδ1 and the main chain carbonyl of the peptide Tyr+4 residue Gly. The goodness of the fit between p85CSH2 and Syk peptide suggests that p85CSH2 recognition of pYEPTG is structurally reasonable.

Tyr-317 Is Required for Coupling the FcγRIIA Receptor to Phagocytosis in COS-7 Cells—Syk Tyr-317 plays a negative role in coupling the BCR and FcεRI receptors to downstream signaling pathways in B cells and mast cells, respectively. In these cells, PI3K is reported to bind to hematopoietic cell-specific adaptor proteins phosphorylated in response to receptor engagement. We explored a role for Tyr-317 in a Syk-dependent signaling process reconstituted in a nonhematopoietic cell. COS-7 epithelial cells expressing FcγRIIA can phagocytose IgG-opsinized red blood cells by a mechanism that is dependent on the expression of exogenous Syk and on the activity of endogenous PI3K (31). COS-7 cells were cotransfected with plasmids coding for human FcγRIIA and Syk or a mutant form of Syk lacking one or more linker B region sites of tyrosine phosphorylation. All forms of Syk were expressed fused to EGFP to mark transfected cells. The percentage of cells expressing Syk-EGFP and the intensity of the fluorescence were analyzed using flow cytometry as described previously (31) and were determined to be similar among the different experimental groups. As shown in Fig. 8, phagocytosis of IgG-coated red blood cells was dependent on the expression of wild-type Syk-EGFP. Cells transfected with either EGFP alone or a catalytically inactive kinase (Syk-EGFP(K396R)) failed to phagocytose red cells. Interestingly, the elimination of Tyr-317 also substantially abrogated phagocytosis. Thus, in this model system, Tyr-317 plays a positive role in signaling. Forms of Syk lacking the pair of tyrosines at positions 342 and 346 or lacking all three linker B sites at Tyr-317, Tyr-342, and Tyr-346 also were unable to couple the receptor to phagocytosis. Elimination of the linker B sites of tyrosine phosphorylation also uncoupled receptor engagement from the activation of Akt, which lies downstream from PI3K. These data indicate that both of the protein docking sites within the Syk linker B region play critical roles in FcγRIIA-mediated phagocytosis in COS-7 cells.

DISCUSSION

In hematopoietic cells, Syk couples multiple membrane-associated receptors to intracellular signaling pathways. Syk mediates signaling by interacting with these receptors via its tandem SH2 domains and functioning both as a catalyst for protein phosphorylation and as an adaptor for the formation of protein complexes. Sites of tyrosine phosphorylation within the linker B region at residues 317, 342, and 346 have been identified as docking sites for the SH2 domain-containing proteins c-Cbl, phospholipase C-γ, Fgr, and Vav (4, 5, 7–10). Other proteins with SH2 domains that have been reported to interact with Syk include PI3K, Lck, Lyn, CrkL, Grb2, Gab2, SHP1, and SHP2 (26, 32, 34–38).

The yeast two-hybrid system can be used as a tool for evaluating protein-protein interactions mediated by phosphorylation sites on Syk. Syk expressed in yeast as a GAL4 fusion protein can participate in phosphotyrosine-dependent binding events as evidenced by the interaction of catalytically active, but not inactive, Syk with SH2 domains of Lck, Fgr, and p85. This differential binding was not the result of a lack of expression of the catalytically inactive Syk because we were able to identify in our screen cDNAs coding for two proteins that could

interact with Syk in a specific manner, but did so independent of its catalytic activity.[2] Of those proteins identified in the screen which bound to Syk, the p85 regulatory subunit of PI3K was by far the most frequently encountered. Nearly 50% of all positive clones identified from two different libraries contained cDNAs for either the p85α or the p85β isoform. This was not a function of the overabundance of one particular clone in the library because multiple, independent clones coding for various portions of p85 were identified. This suggests a robust interaction between Syk and p85.

Based on the domains encoded by the identified genes in the two-hybrid analysis, the C-terminal SH2 domain of p85 alone was sufficient for mediating an interaction with Syk. Consistent with this observation, a GST-p85CSH2 fusion protein could bind phosphorylated Syk or Zap-70 present in cell lysates. The failure of the p85CSH2 to bind to either Syk(K396R) or Syk(Y317F) first suggested that a specific interaction occurred between the C-terminal SH2 domain of p85 and pTyr-317 of Syk. This specificity was confirmed by a number of biochemical interaction studies. An immobilized GST-p85CSH2 domain fusion protein was capable of binding to the isolated, phosphorylated linker B region *in vitro*, but only if Tyr-317 was phosphorylated. Similarly, GST-p85CSH2 bound to a single phosphopeptide derived from autophosphorylated Syk, and this peptide contained pTyr-317. Finally, an immobilized phosphopeptide corresponding to the region surrounding Syk pTyr-317 bound to soluble GST-p85CSH2.

The sequence that includes Y317, pYEPT, does not match the consensus sequence for binding to either SH2 domain of p85. The N- and C-terminal p85 SH2 domains are structurally related and fall within a single class of SH2 domain (27, 39), and both exhibit a preference for the sequence pY*XX*M (27). However, a Syk construct that contains the three Y*XX*M sequences found within the catalytic domain, but lacks the phosphorylation sites within linker B, does not bind p85 in yeast. Other docking sites for p85 have been identified which also do not match this strict consensus sequence. For example, p85 interacts with the nonconsensus sequences, pYVHV and pYVNV, on the HGF/SF receptor (40), pYLVL of the EPO receptor (41), and pYVNT of Tie2 (42).

Modeling of the pYEPTG sequence derived from the SykL B region onto p85CSH2 resulted in a low energy complex that showed a number of sequence-specific intermolecular interactions. In particular the peptide Glu and Thr side chains interact with Lys-56 and Val-59 main chain, respectively, of p85CSH2 (Fig. 7*B*). Although the consensus recognition sequence for p85CSH2, a group III SH2 domain, includes Met at position pTyr+3, the Syk linker B sequence contains Thr at the +3 position following pTyr-317. Nonetheless, Thr appears reasonable given that the model shown here positions the methyl group of Thr in the region otherwise occupied by Met and the hydroxyl group within hydrogen bonding distance of the main chain amide group of Val-59.

The C-terminal p85 SH2 domain bound more readily to a pTyr-317-containing phosphopeptide than did CblN. Prior to this analysis, c-Cbl was the only protein identified which could interact with Syk at Tyr-317. c-Cbl and its family member, Cbl-b, act to regulate negatively the activity of Syk in mast cells and/or B cells and stimulate its ubiquitination by acting as an E3 ligase (43–45). An interaction of the Cbl TKB domain with Syk Tyr-317 is supported by several observations. In assays conducted both *in vitro* and in yeast, the Cbl TKB domain interacted with Syk in a manner largely dependent on the phosphorylation of Tyr-317 (equivalent to Tyr-323 in human Syk or Tyr-316 in the porcine enzyme) (4, 46). Furthermore, the down-regulation of human Syk by c-Cbl when coexpressed in COS-7 cells and the inhibition of BCR signaling by overexpressed c-Cbl both required the presence of Tyr-317 (4, 5). The conserved Asn-315 found at position pTyr−2 within the SykL is consistent with the consensus sequence for a Cbl docking site as determined by a phosphopeptide library screen (47).

CblN was capable of binding to an immobilized peptide containing pTyr-317, but not as effectively as p85CSH2. An x-ray crystal structure of the c-Cbl TKB domain bound to a related phosphopeptide derived from the linker region of Zap-70 suggested that a small hydrophobic residue at pTyr+4, in addition to an Asp or Asn at pTyr−2, would also be important for high affinity binding (48). Leu is present at this site in human Syk, but it is replaced by Gly in both the murine and rat enzymes. This difference in sequence did not account, however, for the comparatively lower affinity of CblN compared with P85CSH2 for the murine Syk-derived peptide because CblN bound to a comparable extent to phosphopeptides based on either the human or murine Syk sequences.

It is likely that the N-terminal SH2 domain of p85 also can participate in an interaction with phosphorylated Syk because the C-terminal SH2 domain alone was unable to bind in yeast to a Syk(Y317F) mutant, whereas a protein containing both domains was competent to bind. Also, full-length p85 was able to bind a phosphorylated form of Syk (GST-p35) that lacked Tyr-317. This indicates a difference in the binding specificities of the two SH2 domains. Indeed, differences in the binding specificities of the N- and C-terminal domains have been observed previously in other systems. For example, p85 binds to the cytoplasmic tail of the PDGF receptor at tyrosines 740 and 751. The N-terminal SH2 domain exhibits a strong preference for Tyr-751, whereas the C-terminal domain does not discriminate between the two sites (49). Furthermore, binding of p85 to the insulin-like growth factor I receptor is largely mediated only by the C-terminal SH2 domain, whereas both domains interact with sites on the insulin receptor (50).

The binding of the p85 SH2 domains to Syk provides a mechanism by which signals could be transmitted directly from activated, phosphorylated Syk to PI3K. However, studies in B cells and mast cells suggest that an indirect coupling of Syk to PI3K mediated by adaptor proteins is the primary mechanism for the regulation of PI3K by activated Syk in these systems. Indeed, the expression of a Syk(Y317F) mutant in either cell type enhances receptor-mediated signaling (3, 5, 6). Forms of Syk or Zap-70 that lack either all sites of tyrosine phosphorylation in the linker B region or the entire linker region are active and demonstrate an enhanced ability to couple antigen receptors to the phosphorylation of the adaptor proteins BLNK and SLP-76. Interestingly, we found that in nonhematopoietic COS-7 cells expressing exogenous FcγRIIA, Syk-dependent phagocytosis requires the linker B sites of tyrosine phosphorylation including Tyr-317. This is the first system identified in which Tyr-317 plays a positive role in signaling and is consistent with a role for Tyr-317 in the activation of PI3K, which is also required for phagocytosis in these cells. In a recent study on the capabilities of Syk linker B mutants to reconstitute signaling in mast cells from Syk-deficient mice, we found that the elimination of Tyr-317 was able to correct multiple signaling defects arising from the loss of Tyr-342 and Tyr-346 with the notable exception of Akt activation.[3] Consistent with this observation, elimination of linker B tyrosines also attenuated the FcγRIIA-induced activation of Akt in COS-7 cells.

[2] Q. Zhou, K. D. Moon, and R.L. Geahlen, unpublished observation.

[3] Simon, M., Vanes, L., Geahlen, R. L., and Tybulewicz, V. L. J. (2005) *J. Biol. Chem.*, in press.

Our observation of a direct interaction between Syk and p85 provides a reasonable explanation for the constitutive association of TEL-Syk with p85 in cells that express the fusion protein (19). The *TEL* gene is frequently involved in rearrangements that yield TEL-protein-tyrosine kinase fusion proteins that become constitutively active because of oligomerization of the TEL HLH/SAM domain (51). In atypical myelodisplastic syndrome with a t(9,12)(q22;p12) translocation, the *TEL* gene has become fused in-frame with the gene coding for Syk, resulting in the formation of a TEL-Syk fusion protein (52). The resulting protein is constitutively active, phosphorylated on tyrosine, and bound to p85, leading to the activation of PI3K (19). Because Syk can catalyze the phosphorylation of all three linker B tyrosines that play a role in the binding of p85 (28), it is likely that the oligomerization of the TEL-Syk protein results in its autophosphorylation at these sites resulting in the recruitment of p85. This is consistent with the observation that mutated forms of TEL-Syk lacking the dimerization domain are not constitutively phosphorylated and do not bind p85 (19).

The preferred specificity determinant of p*YXX*M for the p85 SH2 domains matches the consensus sequence for substrate recognition by the insulin receptor tyrosine kinase, suggesting that the insulin receptor is particularly well suited for the phosphorylation of proteins that provide docking sites for p85 (53). In contrast, cytoplasmic Src family tyrosine kinases often catalyze the phosphorylation of sites recognized by proteins with group I SH2 domains (54). It is interesting to note that Syk Tyr-317, which is within the sequence pYEPT, is in B cells a prominent phosphorylation site for Lyn, a Src family kinase (10). Sequences related to pYEPT thus may serve in general as a distinct class of sites on p85-binding proteins that connect the activities of Src family kinases to the activation of PI3K.

REFERENCES

1. Turner, M., Schweighoffer, E., Colucci, F., Di Santo, J. P., and Tybulewicz, V. L. (2000) *Immunol. Today* **21,** 148–154
2. Latour, S., and Veillette, A. (2001) *Curr. Opin. Immunol.* **13,** 299–306
3. Keshvara, L. M., Isaacson, C., Yankee, T. M., Sarac, R., Harrison, M. L., and Geahlen, R. L. (1998) *J. Immunol.* **161,** 5276–5283
4. Lupher, M. L., Rao, N., Lill, N. L., Andoniou, C. E., Miyake, S., Clark, E. A., Druker, B., and Band, H. (1998) *J. Biol. Chem.* **273,** 35273–35281
5. Yankee, T. M., Keshvara, L. M., Sawasdikosol, S., Harrison, M. L., and Geahlen, R. L. (1999) *J. Immunol.* **163,** 5827–5835
6. Sada, K., Zhang, J., and Siraganian, R. P. (2000) *J. Immunol.* **164,** 338–344
7. Law, C.-L., Chandran, K. A., Sidorenko, S. P., and Clark, E. A. (1996) *Mol. Cell. Biol.* **16,** 1305–1315
8. Deckert, M., Tartare-Deckert, S., Couture, C., Mustelin, T., and Altman, A. (1996) *Immunity* **5,** 591–604
9. Vines, C. M., Potter, J. W., Xu, Y., Geahlen, R. L., Costello, P. S., Tybulewicz, V. L., Lowell, C. A., Chang, P. W., Gresham, H. D., and Willman, C. L. (2001) *Immunity* **15,** 507–519
10. Hong, J., Yankee, T. M., Harrison, M. L., and Geahlen, R. L. (2002) *J. Biol. Chem.* **277,** 31703–31714
11. Zhang, J., Berenstein, E., and Siraganian, R. P. (2002) *Mol. Cell. Biol.* **22,** 8144–8154
12. Beitz, L. O., Fruman, D. A., Kurosaki, T., Cantley, L. C., and Scharenberg, A. M. (1999) *J. Biol. Chem.* **274,** 32662–32666
13. Crowley, M. T., Costello, P. S., Fitzer-Attas, C. J., Turner, M., Meng, F., Lowell, C., Tybulewicz, V. L., and DeFranco, A. L. (1997) *J. Exp. Med.* **186,** 1027–1039
14. Raeder, E. M., Mansfield, P. J., Hinkovska-Galcheva, V., Shayman, J. A., and Boxer, L. A. (1999) *J. Immunol.* **163,** 6785–6793
15. Jiang, K., Zhong, B., Ritchey, C., Gilvary, D. L., Hong-Geller, E., Wei, S., and Djeu, J. Y. (2003) *Blood* **101,** 236–244
16. Jiang, K., Zhong, B., Gilvary, D. L., Corliss, B. C., Vivier, E., Hong-Geller, E., Wei, S., and Djeu, J. Y. (2002) *J. Immunol.* **168,** 3155–3164
17. Ding, J., Takano, T., Gao, S., Han, W., Noda, C., Yanagi, S., and Yamamura, H. (2000) *J. Biol. Chem.* **275,** 30873–30877
18. Pogue, S. L., Kurosaki, T., Bolen, J., and Herbst, R. (2000) *J. Immunol.* **165,** 1300–1306
19. Kanie, T., Abe, A., Matsuda, T., Kuno, Y., Towatari, M., Yamamoto, T., Saito, H., Emi, N., and Naoe, T. (2004) *Leukemia* **18,** 548–555
20. Okada, T., Maeda, A., Iwamatsu, A., Gotoh, K., and Kurosaki, T. (2000) *Immunity* **13,** 817–827
21. Buhl, A. M., and Cambier, J. C. (1999) *J. Immunol.* **162,** 4438–4446
22. Ingham, R. J., Holgado-Madruga, M., Siu, C., Wong, A. J., and Gold, M. R. (1998) *J. Biol. Chem.* **273,** 30630–30637
23. Cooney, D. S., Phee, H., Jacob, A., and Coggeshall, K. M. (2001) *J. Immunol.* **167,** 844–854
24. Chacko, G. W., Brandt, J. T., Coggeshall, K. M., and Anderson, C. L. (1996) *J. Biol. Chem.* **271,** 10775–10781
25. Chacko, G. W., Duchemin, A. M., Coggeshall, K. M., Osborne, J. M., Brandt, J. T., and Anderson, C. L. (1994) *J. Biol. Chem.* **269,** 32435–32440
26. Yanagi, S., Sada, K., Tohyama, Y., Tsubokawa, M., Nagai, K., Yonezawa, K., and Yamamura, H. (1994) *Eur. J. Biochem.* **224,** 329–333
27. Songyang, Z., Shoelson, S. E., Chaudhuri, M., Gish, G., Pawson, T., Haser, W. G., King, F., Roberts, T., Ratnofsky, S., Lechleider, R. J., Neel, B. G., Birge, R. B., Fajardo, J. E., Chou, M. M., Hanafusa, H., Schaffhausen, B., and Cantley, L. C. (1993) *Cell* **72,** 767–778
28. Furlong, M. T., Mahrenholz, A. M., Kim, K. H., Ashendel, C. L., Harrison, M. L., and Geahlen, R. L. (1997) *Biochim. Biophys. Acta* **1355,** 177–190
29. Peters, J. D., Furlong, M. T., Asai, D. J., Harrison, M. L., and Geahlen, R. L. (1996) *J. Biol. Chem.* **271,** 4755–4762
30. Nadler, M. J. S., Harrison, M. L., Ashendel, C. L., Cassady, J. M., and Geahlen, R. L. (1993) *Biochemistry* **32,** 9250–9255
31. Kim, J. S., Peng, X., De, P. K., Geahlen, R. L., and Durden, D. L. (2002) *Blood* **99,** 694–697
32. Couture, C., Deckert, M., Williams, S., Russo, F. O., Altman, A., and Mustelin, T. (1996) *J. Biol. Chem.* **271,** 24294–24299
33. Williams, B. L., Irvin, B. J., Sutor, S. L., Chini, C. C. S., Yacyshyn, E., Wardenburg, J. B., Dalton, M., Chan, A. C., and Abraham, R. T. (1999) *EMBO J.* **18,** 1832–1844
34. Sidorenko, S. P., Law, C. L., Chandran, K. A., and Clark, E. A. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 359–363
35. Oda, A., Ochs, H. D., Lasky, L. A., Spencer, S., Ozaki, K., Fujihara, M., Handa, M., Ikebuchi, K., and Ikeda, H. (2001) *Blood* **97,** 2633–2639
36. Saci, A., Liu, W. Q., Vidal, M., Garbay, C., Rendu, F., and Bachelot-Loza, C. (2002) *Biochem. J.* **363,** 717–725
37. Yu, W. M., Hawley, T. S., Hawley, R. G., and Qu, C. K. (2002) *Blood* **99,** 2351–2359
38. Ganju, R. K., Brubaker, S. A., Chernock, R. D., Avraham, S., and Groopman, J. E. (2000) *J. Biol. Chem.* **275,** 17263–17268
39. Campbell, S. J., and Jackson, R. M. (2003) *Protein Eng.* **16,** 217–227
40. Ponzetto, C., Bardelli, A., Maina, F., Longati, P., Panayotou, G., Dhand, R., Waterfield, M. D., and Comoglio, P. M. (1993) *Mol. Cell. Biol.* **13,** 4600–4608
41. He, T. C., Zhuang, H., Jiang, N., Waterfield, M. D., and Wojchowski, D. M. (1993) *Blood* **82,** 3530–3538
42. Kontos, C. D., Stauffer, T. P., Yang, W.-P., York, J. D., Huang, L., Blanar, M. A., Meyer, T., and Peters, K. G. (1998) *Mol. Cell. Biol.* **18,** 4131–4140
43. Rao, N., Ghosh, A. K., Ota, S., Zhou, P., Reddi, A. L., Hakezi, K., Druker, B. K., Wu, J., and Band, H. (2001) *EMBO J.* **20,** 7085–7095
44. Paolini, R., Molfetta, R., Beitz, L. O., Zhang, J., Scharenberg, A. M., Piccoli, M., Frati, L., Siraganian, R., and Santoni, A. (2002) *J. Biol. Chem.* **277,** 36940–36947
45. Sohn, H. W., Gu, H., and Pierce, S. K. (2003) *J. Exp. Med.* **197,** 1511–1524
46. Deckert, M., Elly, C., Altman, A., and Liu, Y.-C. (1998) *J. Biol. Chem.* **273,** 8867–8874
47. Lupher, M. L., Songyang, Z., Shoelson, S. E., Cantley, L. C., and Band, H. (1997) *J. Biol. Chem.* **272,** 33140–33144
48. Meng, W., Sawaskikosol, S., Burakoff, S. J., and Eck, M. J. (1999) *Nature* **398,** 84–90
49. Panayotou, G., Gish, G., End, P., Truong, O., Gout, I., Dhand, R., Fry, M. J., Hiles, I., Pawson, T., and Waterfield, M. D. (1993) *Mol. Cell. Biol.* **13,** 3567–3576
50. Tartare-Deckert, S., Murdaca, J., Sawka-Verhelle, D., Holt, K. H., Pessin, J. E., and Van Obberghen, E. (1996) *Endocrinology* **137,** 1019–1024
51. Kim, C. A., Phillips, M. L., Kim, W., Gingery, M., Tran, H. H., Robinson, M. A., Faham, S., and Bowie, J. U. (2001) *EMBO J.* **20,** 4173–4182
52. Kuno, Y., Abe, A., Emi, N., Iida, M., Yokozawa, T., Towatari, M., Tanimoto, M., and Saito, H. (2001) *Blood* **97,** 1050–1055
53. Shoelson, S. E., Chatterjee, S., Chaudhuri, M., and White, M. F. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 2027–2031
54. Songyang, Z., Carraway, K. L., Eck, M. J., Harrison, S. C., Feldman, R. A., Mohammadi, M., Schlessinger, J., Hubbard, S. R., Smith, D. P., Eng, C., Lorenzo, M. J., Ponder, B. A. J., Mayer, B. J., and Cantley, L. C. (1995) *Nature* **373,** 536–539