The Inositol 5′-Phosphatase SHIP Binds to Immunoreceptor Signaling Motifs and Responds to High Affinity IgE Receptor Aggregation

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Immunoreceptors such as the high affinity IgE receptor, FcεRI, and T-cell receptor-associated proteins share a common motif, the immunoreceptor tyrosine-based activation motif (ITAM). We used the yeast tribrid system to identify downstream effectors of the phosphorylated FcεRI ITAM-containing subunits β and γ. One novel cDNA was isolated that encodes a protein that is phosphorylated on tyrosine, contains a Src-homology 2 (SH2) domain, inositol polyphosphate 5-phosphatase activity, three NXXY motifs, several proline-rich regions, and is called SHIP. Mutation of the conserved tyrosine or leucine residues within the FcεRI β or γ ITAMs eliminates SHIP binding and indicates that the SHIP-ITAM interaction is specific. SHIP also binds to ITAMs from the CD3 complex and T cell receptor ζ chain in vitro. SHIP protein possesses both phosphatidylinositol-3,4,5-trisphosphate 5′-phosphatase and inositol-1,3,4,5-tetrakisphosphate 5′-phosphatase activity. Phosphorylation of SHIP by a protein-tyrosine kinase, Lck, results in a reduction in enzyme activity. FcεRI activation induces the association of several tyrosine phosphoproteins with SHIP. SHIP is constitutively tyrosine-phosphorylated and associated with Shc and Grb2. These data suggest that SHIP may serve as a multifunctional linker protein in receptor activation.

The aggregation of immunoreceptors by antigen initiates a complex response leading to cellular activation (1). Receptors on T-, B-, and mast cells each contain subunits with similar primary amino acid sequence within their cytokine domains, comprising the immunoreceptor-based tyrosine activation motif (ITAM), whose consensus is [(E/D)XX](Y)[(E/D)XX] (2, 3). Both tyrosine residues within the ITAM are rapidly phosphorylated by protein kinases after receptor aggregation. The biphotorylated ITAM then binds directly to cytosolic tyrosine kinases such as Syk in B-cells and mast cells and ZAP70 in T-cells, thereby activating their tyrosine kinase activity (4, 5). In mast cells, the FcεRI subunits β and γ each possess a single ITAM, which, when biphotorylated on tyrosine, binds to Syk (6–8).

We have used a novel genetic approach, the yeast tribrid system (8), to isolate cDNAs that encode proteins that interact with the tyrosine-phosphorylated FcεRI γ ITAM. The yeast two-hybrid system facilitates the study of protein-protein interactions but is limited to the investigation of proteins that are properly expressed and modified in the host, Saccharomyces cerevisiae. S. cerevisiae does not employ tyrosine phosphorylation as a major regulatory modification of proteins (9, 10). This limits the utility of the two-hybrid system, especially in the area of signal transduction, where tyrosine phosphorylation is a critical component of the process. In order to study protein-protein interactions that are dependent on tyrosine phosphorylation or on other post-translational or allosteric modifications, the yeast tribrid system was developed (8, 11).

In the yeast tribrid system, a third plasmid is introduced, which directs the synthesis of a protein-tyrosine kinase, which catalyzes the phosphorylation of a tyrosine-containing protein, facilitating the interaction with the SH2 domain-containing protein. The interaction is detected through the use of a transcriptional activation assay, as is used in the two-hybrid system (12). The tribrid system lends itself to a variety of studies, including the characterization of phosphotyrosine-SH2 domain interactions, identification of ligands for SH2 domains, cloning of novel protein-tyrosine kinases, and the identification of novel SH2-domain-containing proteins, to name a few. In this study, the tribrid system was used to isolate novel cDNAs that encode proteins that interact with the tyrosine-phosphorylated FcεRI γ chain.

One of the cDNAs isolated encodes SHIP, a novel ITAM-binding protein that is part of the FcεRI activation pathway, as revealed by its association with several additional tyrosine phosphoproteins following FcεRI stimulation. SHIP also forms complexes with the signaling molecules Shc and Grb2 in vitro. The cloned gene product is a phosphatidylinositol-3,4,5-trisphosphate and inositol-1,3,4,5-tetrakisphosphate 5′-phosphatase; PIP3, phosphatidylinositol 3,4,5-trisphosphate; Ins, inositol; PtdIns, phosphatidylinositol; PI 3-kinase, phosphoinositide-3-OH-kinase.

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The abbreviations used are: ITAM, immunoreceptor tyrosine-based activation motif; SH2 and SH3, Src homology 2 and 3, respectively; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase; TBS, Tris-buffered saline; 5-phosphatase, inositol polyphosphate 5-phosphatase; PTB domain, phosphotyrosine-binding domain; PIP2, phosphatidylinositol 3,4-

biphosphatase; PIP3, phosphatidylinositol 3,4,5-trisphosphate; Ina, inositol; PtdIns, phosphatidylinositol; PI 3-kinase, phosphoinositide-3-OH-kinase.
phatase. SHIP associates with tyrosine-phosphorylated ITAMs in the yeast tridrib system and with tyrosine-phosphorylated ITAM peptides in vitro.

EXPERIMENTAL PROCEDURES

Commercially Available Reagents—Monoclonal antibodies recognizing Shc and glutathione S-transferase fusion proteins were purchased from Santa Cruz Biotechnology. The anti-Grb2 monoclonal antibody and anti-RasGAP polyclonal antibody were obtained from Upstate Biotechnology, Inc. Antibodies to phospholipase C-Y, Nck, Sox1, and SAM-68 were purchased from Transduction Laboratories. Peroxidase-conjugated anti-rabbit and anti-mouse secondary antibodies were obtained from Jackson Immunoresearch.

Isolation of SHIP—A cDNA library from RBL-2H3 cells (8) was screened using LexA-FceRIγ as bait (p4108; Ref. 8) in host strain S-260 as described (8). Three overlapping clones were identified (p4187, p4193, and p4186), and the DNA sequences were determined. The 3′-end was monitored by screening a ZAP cDNA library with a fragment of p4187. The 3′-end was then amplified from RBL-2H3 RNA by reverse transcription-PCR (Life Technologies, Inc.) using specific primers that hybridized to the coding region only p4187 and the 3′-untranslated region. The fragment amplified was ~1-kilobase pair longer than that recovered from the λ clone. After subcloning into pCR (Invitrogen), a portion of the PCR-amplified fragment was ligated into pKaslXhol digest to generate p4802. These sequence data are available from GenBank™ under accession number U55192. We refer to this cDNA as SHIP.

Plasmid Constructions—Two-step PCR was used to construct mutant LexA-ITAM fusions in a manner identical to that described previously (8). The T-cell receptor γ chain was PCR-amplified from peripheral blood lymphocyte DNA.2 The p4187 (ASH2) plasmid was generated by PCR and contains nucleotides 885-2570 of the complete SHIP sequence (encoding amino acids 219-790). GST-SHIP was generated by PCR and encodes amino acids 4-356 of SHIP joined to the GST sequence (encoding amino acids 21-790). GST-SHIPN was generated in a vector for expression of LexA and Vmw65 fusion proteins p4187 (the original SHIP isolate from the tridrib screen, terminating at amino acid 790) or p4802 (encoding the full-length SHIP terminating at amino acid 1190) with p4140 (encoding Lck; Ref. 8) or pRS415 (vector for p4140). Cells were grown overnight in liquid urea Leu minus medium containing 2% galactose as the sole carbon source. After centrifugation, the cells were washed, resuspended in H2O, and diluted to an A260 of 0.3 in 50 ml of urea Leu minus medium containing 2% galactose as the sole carbon source. After 4 h, the cells were harvested by centrifugation, enzymatically treated to generate sphero- phorins with zymolyase and gluclase, and lysed in lysis buffer (10 mM imidazole, pH 7.2, 1 mM MgCl₂, 1 mM EDTA, 0.3% m/sucrose, 1% Triton X-100). After clarification by centrifugation, an unrelated rabbit antibody was added for 30 min, followed by protein G-Sepharose beads (Pharmacia). After centrifugation, anti-GST-Vmw65 antiserum raised against a GST fusion protein containing amino acids 410-490 of HSV1 Vmw65 protein was added, followed by protein G-Sepharose beads. The beads were incubated for 1 h with the antibody/extract mixture and then washed twice with lysis buffer, twice with lysis buffer lacking detergent, and then twice with 5-phosphate assay buffer (50 mM Tris, pH 7.5, 3 mM MgCl₂). The beads were aspirated to dryness and stored frozen at ~70°C until assayed.

To measure isostilopolysphosphate 5-phosphatease (5-phosphatease) activity, beads were resuspended in 50 l of 5-phosphate assay buffer and split in half for Ins1,3,4,5-P₄,5-phosphate and PtdIns3,4,5-P₃-phosphate assay, respectively. The Ins1,3,4,5-P₄,5-phosphate assay was conducted in a 50-l reaction mixture containing 50 mM Tris, pH 7.5, 8 mM MgCl₂, 800 cpm of TLC-purified PtdIns[3,4,5]P₃, 0.5 μg/mL phosphatidylserine, and 25 μl of bead slurry. The PtdIns3,4,5,6-P₄ was prepared as described (17, 18). The reaction mixture was incubated at 37°C for 30 min with constant rotation. The product of the reaction (Ins1,3,4,5-P₄) was separated and measured as described (14-16). The PtdIns3,4,5,6-P₄ was conducted in a 50-l reaction mixture containing 50 mM Tris, pH 7.5, 10 mM MgCl₂, 800 cpm of TLC-purified PtdIns3,4,5,6-P₄, 0.5 μg/mL phosphatidylserine, and 25 μl of bead slurry. The PtdIns3,4,5,6-P₄ was prepared as described (17, 18). The reaction mixture was incubated at 37°C for 30 min with constant rotation. The reaction was stopped, and the product PtdIns3,4,5-P₄ was separated by TLC (17, 18). The percentage of hydrolysis of PtdIns3,4,5-P₄ was measured by densimetric analysis of autoradiographs of TLC plates.

Peptide Binding Assays—ITAM-containing biotinylated 23-mer carboxyamide peptides (Table I) were prepared as described (13). Each peptide (10 μg/mL in 50 μl of 50 mM Tris pH 7.5, 100 mM NaCl (TBS) was incubated with 60 liters of streptavidin-agarose beads (Sigma) with rotation for 1 h at 4°C. After washing with TBS, the beads were preincubated for 30 min at 4°C with 0.25% bovine serum albumin in TBS for 15 min, washed three times with TBS, and incubated with 0.25 μg of purified GST-SHIPN in 200 μl of TBS containing 1% Nonidet P-40 and 10% glycerol for 1 h with rotation. The beads were washed three times and then boiled in 1 × Laemli sample buffer and analyzed by immunoblot after separation by SDS-PAGE and immunoblotting with anti-GST antibodies (Santa Cruz Biotechnology). All immunoblots were developed using horseradish peroxidase-coupled secondary antibodies and enhanced chemiluminescence (Amersham Corp.).

Immunoprecipitation—RBL-2H3 monolayers were incubated with biotinylated rat IgE (a gift of M. Basu, Roche) at 1 μg/mL for 30 min in Iscove’s modified Dulbecco’s medium, 10% fetal calf serum, the cells were rinsed twice with medium, and the receptors were aggregated with 10 μg/mL egg white avidin for 10 min. The cells were rinsed twice with phosphate-buffered saline and lysed in 1 mL of 50 mM Tris pH 8, 150 mM NaCl, 1 mM Na₂VO₃, 10 mM NaF, and protease inhibitors (sodium orthovanadate, and, to inhibit GST, 0.1 mM ethylenediaminetetraacetic acid). The cells were incubated with unrelated rabbit serum for 30 min at 4°C and precipitated with protein G-Sepharose (Pharmacia), followed by incubation with 2 μg of affinity-purified rabbit anti-SHIP antibodies for 1 h. Control immunoprecipitations utilized 2 μg of affinity-purified anti-GST antibodies. Immunoprecipitates were adsorbed to protein G-Sepharose (Pharmacia) for 1 h, washed with buffer, boiled in Laemmli sample buffer, and electrophoresed on a 4–20% SDS-PAGE.

GST Fusion Protein Chromatography—Extracts of RBL-2H3 cells that were treated with biotinylated IgE and egg white avidin or left untreated were prepared as above and incubated with 1 μg of purified GST fusion proteins (Santa Cruz Biotechnology) for 1 h at 4°C. Glutathione-Sepharose beads (10 μl) were added, and the incubation continued for an additional 30 min to 1 h. The beads were collected by centrifugation, washed in buffer, and boiled in sample buffer. The samples were then subjected to electrophoresis on a 4–20% SDS-polyacrylamide gel, transferred to polyvinylidene difluoride or nitrocellulose, and processed for immunoblotting with affinity-purified anti-SHIP antibodies (1 μg/mL).

Inositolpolysphosphate 5-phosphate Activity—S. cerevisiae strain S-260 was transformed with Vmw65 fusion protein plasmids p4187 (the original SHIP isolate from the tridrib screen, terminating at amino acid 790) or p4802 (encoding the full-length SHIP terminating at amino acid 1190) with p4140 (encoding Lck; Ref. 8) or pRS415 (vector for p4140). Cells were grown overnight in liquid urea Leu minus medium containing 2% galactose. The next morning, the cells were centrifuged, washed once with H₂O, and diluted to an A400 of 0.3 in 50 ml of urea Leu minus medium containing 2% galactose as the sole carbon source. After 4 h, the cells were harvested by centrifugation, enzymatically treated to generate sphero- phorins with zymolyase and gluclase, and lysed in lysis buffer (10 mM imidazole, pH 7.2, 1 mM MgCl₂, 1 mM EDTA, 0.3% m/sucrose, 1% Triton X-100). After clarification by centrifugation, an unrelated rabbit antibody was added for 30 min, followed by protein G-Sepharose beads (Pharmacia). After centrifugation, anti-GST-Vmw65 antiserum raised against a GST fusion protein containing amino acids 410-490 of HSV1 Vmw65 protein was added, followed by protein G-Sepharose beads. The beads were incubated for 1 h with the antibody/extract mixture and then washed twice with lysis buffer, twice with lysis buffer lacking detergent, and then twice with 5-phosphate assay buffer (50 mM Tris, pH 7.5, 3 mM MgCl₂). The beads were aspirated to dryness and stored frozen at ~70°C until assayed.

RESULTS

Isolation and Analysis of SHIP cDNA—We utilized the yeast tridrib system in order to identify proteins that interact with the activated FceRIγ chain. A LexA-FceRIγ fusion protein (containing the 42 C-terminal amino acids of the FceRIγ chain) was used as a “bait,” the prey protein being a biotinylated version of FcεRIγ on mouse IgE, and a Vmw65 activation domain fusion-cDNA expression library from the mast cell line RBL-2H3 was screened in S. cerevisiae strain S-260 for positive

2 M. Labinus and J. P. Kochan, unpublished results.
interactors. Of 500,000 colonies screened, five were identified that required the tyrosine kinase Lck for interaction with the LexA-γ fusion protein and did not interact with other negative control baits such as LexA-lamin C, LexA-SNF1, and LexA-SIR4. Three of these clones contained similar sequences and were characterized further. When other LexA-ITAM fusions were used as baits, the cDNAs identified were found to interact with both the FcɛRI β and γ subunits, as well as the T-cell receptor γ chain (Table I).

The three LexA-γ interactors contained overlapping sequences encoding a novel protein (a schematic diagram is shown in Fig. 1). All three cDNAs had similar 5′-end points but differed in their 3′-end points (p4187 terminating at nucleotide 2570, p4193 at 1778, and p4195 at 1211, respectively). The cDNAs had open reading frames extending throughout the length of the insert. The 3′-end of the transcript was cloned by screening a similar library from RBL-2H3 cells with a 3′-end fragment of the longest cDNA and assembled with reverse transcription-PCR-amplified cDNA from RBL-2H3 cells. The 5′-end was cloned by screening a similar λ cDNA library. The entire clone encompassed 4.9 kilobase pairs and encoded a protein of 1190 amino acids, with a predicted molecular weight of 133,000. The 5′-end contains an in-frame stop codon, suggesting that there is no additional coding sequence in this region. 3′ to the TGA stop codon are several other stop codons in all three reading frames.

A comparison of the predicted peptide sequence of the cDNA to nucleic acid and protein sequence data bases revealed extensive similarities to two classes of proteins. The first region, amino acids 7–110, displayed 38% identity to Cak and other SH2-containing proteins of the Src family (group I; Refs. 20 and 21). The second region (amino acids 270–715) is similar (40% identical) to a class of proteins possessing inositol-5-phosphatase activity. These proteins display a variety of substrate specificities, but all are able to hydrolyze the 5′-phosphate group from a particular species of inositol phosphate or inositol phosphospholipid (14–16, 22–24). The C-terminal half of the protein contains numerous tyrosine residues (14 between amino acids 555 and 867) and three NXXY motifs (at amino acids 555, 914, and 1017). NXXY motifs of other proteins, when phosphorylated on the tyrosine residue, have been shown to bind to phosphotyrosine-binding (PTB) domains in a manner different from that of SH2 domains (25–28). The C terminus is also very proline-rich and has several regions that (by visual inspection) could make good Src-homology domain 3 (SH3) ligands. We refer to this cDNA as SHIP.

The tissue distribution of rat SHIP mRNA was determined by screening a Northern blot of poly(A)+ mRNA with the longest SHIP clone identified in the trbrid screen (Fig. 2). Lung and spleen tissue displayed strongly reactive bands at approximately 4.9 kilobase pairs, with lesser amounts present in all tissues tested (heart, brain, skeletal muscle, kidney, and testis). Extremely low mRNA levels were observed in liver tissue. The **SHIP SH2 Domain Binds to ITAMs**—The fact that all three cDNAs isolated in the screen had similar 5′-end points suggested that the N terminus contained a region of the protein that was important for binding to the LexA-ITAM fusion protein. The shortest of these cDNAs identified (p4195) contained amino acids 1–338, excluding the possibility that any portion of the protein distal to amino acid 338 is required for an interaction to be detected (see Fig. 1). The most obvious region of SHIP that could facilitate the interaction with the phosphorylated LexA-ITAM fusion is the SH2 domain. To test this hypothesis, a deletion mutant of the longest of the three clones was constructed to remove just the SH2 domain. This SHIP(ΔSH2)
Table II
Amino acid sequences of the biotinylated 23-mer carboxamide peptide corresponding to the ITAMs of the invariant CD3 chains and \( \xi \) chain of the TCR

| ITAM | Sequences* |
|------|------------|
| ITAM\( \alpha \) | Biotinyl-C8-QQGQNLKYNEQLNLGREE YVDVL-NH\(_2\) |
| ITAM\( \alpha \)-PP | Biotinyl-C8-QQGQNLKYNEQLNLGREE TDVLD-NH\(_2\) |
| ITAM\( \beta \) | Biotinyl-C8-KNPQEGLYNQLKQMAEASEEG-NH\(_2\) |
| ITAM\( \gamma \) | Biotinyl-C8-GKGDQGLYQGLSTAKD TDAHL-NH\(_2\) |
| ITAM\( \delta \) | Biotinyl-C8-GKGDQGLYQGLSTAKD TDAHL-NH\(_2\) |
| ITAM\( \varepsilon \) | Biotinyl-C8-PPVPNPDEYPIRKGGRL YSGLM-NH\(_2\) |
| ITAM\( \zeta \) | Biotinyl-C8-LLRDQVYQLPRDDDAQ YSHLG-NH\(_2\) |
| ITAM\( \eta \) | Biotinyl-C8-LLRDQVYQLPRDDDAQ YSHLG-NH\(_2\) |

* C8 represents the caprylic acid spacer, and \( \overline{\gamma} \) represents a phosphorylated tyrosine residue.

The SH2 domain of SHIP can bind to ITAMs, since direct peptide selection by the GST-SHIP-(4–356) fusion protein indicated preference for the motif Y(Y/D)X(L/I)V, in general agreement with the ITAM consensus of YXXL (Fig. 4).

SHIP Associates with Signaling Proteins in RBL-2H3 Cells—To determine the association of SHIP with other proteins in RBL-2H3 cells, extracts from untreated or FceRI-stimulated cells were immunoprecipitated with affinity-purified rabbit anti-SHIP antibodies. As shown in Fig. 5, proteins of 145, 45, and 52 kDa are observed co-precipitating with SHIP (but not with control antibody) in untreated cells. Longer exposures of the same gel reveal a protein of 90 kDa that is also specifically precipitated by anti-SHIP antibodies. After FceRI aggregation, several additional phosphorylated proteins of 30–40 kDa and \( \sim 60 \) kDa are present in addition to the 145-, 52-, and 45-kDa proteins. The 145-kDa species co-migrates with SHIP (Fig. 5, anti-SHIP blot), and reprobing of this blot confirms this (data not shown), indicating that SHIP is itself a tyrosine phosphoprotein.

**Fig. 3.** The SHIP SH2 domain binds to phosphorylated ITAM peptides. A, association of GST-SHIP (amino acids 4–356) with the ITAMs of the T-cell receptor-associated \( \gamma \) chain. B, association of GST-SHIP-(4–356) with the ITAMs of the CD3 complex. Lane C, control with streptavidin beads without peptide; lane 1, ITAM\( \gamma \); lane 2, ITAM\( \delta \); lane 3, ITAM\( \varepsilon \); lane 4, ITAM\( \zeta \); lane 5, ITAM\( \eta \); lane 6, ITAM\( \zeta \); lane 7, ITAM\( \gamma \); lane 8, ITAM\( \delta \); lane 9, ITAM\( \zeta \); lane 10, ITAM\( \gamma \). The arrow indicates the position of GST-SHIP-(4–556).
bodies specific for the FcεRI β and γ did not reveal any association with either of these subunits, although tyrosine phosphoproteins were detected in these samples. Similarly, immunoprecipitation of extracts from unstimulated and stimulated RBL-2H3 cells with anti-FcεRI-β or anti-FcεRI-γ antibodies did not reveal any SHIP associated with the FcεRI (data not shown).

In order to further explore the interaction of SHIP and Grb2, GST fusion proteins to a variety of SH2 and SH3 domain-containing proteins were used as affinity reagents to determine the spectrum of potential SHIP interactors. As shown in Fig. 6, the GST-Grb2 (SH3SH2SH3) and GST-phospholipase Cγ (SH2SH2SH3) fusion proteins specifically precipitate SHIP from RBL-2H3 extracts of both unstimulated and stimulated cells. GST fusion proteins containing the N-terminal SH2 domain of phosphoinositide-3-OH-kinase (PI 3-kinase), the SH2 domain of SHIP, and SH2 domains of SH-PTP2 (data not shown) did not display any binding to SHIP. A GST-Grb2 SH2 domain fusion and a GST-phospholipase Cγ-1 SH2 domain fusion do not precipitate SHIP, indicating that the interaction with these proteins is not mediated by an SH2 domain. In addition, a GST-phospholipase Cγ-1 SH3 domain fusion protein does precipitate SHIP, suggesting that SHIP is associating with SH3 domains.

SHIP Is an Inositol-5-phosphatase—To test whether SHIP possessed inositol-5-phosphatase activity, as was suggested by its similarity to other proteins, SHIP was expressed in S. cerevisiae. Immunoprecipitates from S. cerevisiae bearing either the original SHIP plasmid (encoding amino acids derived from the 5'-untranslated region through amino acid 790) or a reconstituted full-length clone (encoding amino acids from the 5'-untranslated region through amino acid 1190) were incubated with inositol 1,3,4,5-tetrakisphosphate or phosphatidylinositol 3,4,5-trisphosphate, and the products were analyzed.

The full-length SHIP protein contains 5'-phosphatase activity (Fig. 7, A and B, column 4), while the truncated form does not (Fig. 7, A and B, column 2). No enzymatic activity was observed when inositol 1,4,5-trisphosphate was used as a substrate (data not shown).
not shown).

To test the effect of tyrosine phosphorylation on SHIP enzymatic activity, a similar experiment was repeated with immunoprecipitates from yeast expressing SHIP in the presence or absence of the tyrosine kinase Lck. Co-expression of the tyrosine kinase Lck with SHIP results in the tyrosine-phosphorylation of SHIP (data not shown) and a 2–3-fold reduction in the phosphoprotein that specifically recognizes individual phosphotyrosine motifs within the FcRI ITAMs (Fig. 7, lane 2). The results clearly demonstrate a loss of 5'-phosphatase activity when SHIP is tyrosine-phosphorylated. Control immunoprecipitations (Fig. 7B, lanes 3c and 4c) display a small amount of associated 5'-phosphatase activity. This could be due to nonspecific binding of SHIP to the antibody beads, due to the large overexpression of SHIP from the pGAL promoter.

DISCUSSION

The molecular details of signaling through the IgE receptor are still being defined. To unravel the underlying mechanisms involved, it seems crucial to identify the molecules participating in the FcεRI signal transduction cascade. To this end, we have employed a genetic strategy, the yeast tribrid system, to identify proteins that interact with the activated FcεRI. We have identified one cDNA, encoding SHIP, a 145-kDa tyrosine phosphoprotein that specifically recognizes individual phosphotyrosine motifs within the FcεRI ITAMs (Table I).

SHIP binds to a variety of ITAMs, both in the yeast tribrid system (Table I) and in vitro (Table II). The interaction is dependent on the tyrosine phosphorylation of the ITAM and is not due to nonspecific phosphotyrosine binding, since mutation of the conserved leucine residue within the YXXL abolishes interaction with SHIP, just as it does for ZAP70 (29). Direct peptide binding studies reveal that the SH2 domain of SHIP binds to the CD3 complex, whereas the T cell receptor (TCR) δ chain phospho-ITAM peptides tested, while nonphosphorylated peptides do not. In addition, the results obtained with the yeast tribrid system clearly demonstrate a specificity for SHIP binding to the ITAM motif YXXL. Within the ITAM, SHIP displays preferential binding to the C-terminal phosphotyrosine of the FcεRI β and γ ITAMs (Table I). The optimal ligand for the SHIP SH2 domain, Y(Y/D)X(L/V), is consistent with ITAM-binding ability. However, there may be other SHIP ligands present in the cell, since ITAMs typically do not contain an aromatic residue C-terminal to the phosphotyrosine (30). These results suggest a potential mechanism for SHIP binding to the hemiphostyrosine ITAMs and participating in the IgE response in a manner distinct from that of Syk, which only binds tightly to doubly phosphorylated ITAMs.

SHIP is tyrosine-phosphorylated and bound to Shc in both resting and FcεRI-stimulated RBL 2H3 cells (Fig. 5). Li and co-workers (31) noted a 145-kDa protein that acquires additional phosphate in response to FcεRI aggregation. Our experiments cannot exclude the possibility that SHIP phosphorylation is increased following receptor stimulation. However, the high basal state of SHIP tyrosine phosphorylation would make this difficult to detect. Co-immunoprecipitating with SHIP are the p46 and p52 forms of the signaling adaptor protein Shc, which is also basally tyrosine-phosphorylated in RBL-2H3 cells (32), unlike other cell types (33, 34). Shc contains two phosphotyrosine-binding motifs, an SH2 domain, and a PTB domain (25, 35, 36). SHIP may thus serve as a bridge between the hemiphostyrosine ITAM and participating in the IgE response in a manner distinct from that of Syk, which only binds tightly to doubly phosphorylated ITAMs.

Also present in anti-SHIP immunoprecipitates is the adaptor protein Grb2 in both resting and FcεRI-activated RBL-2H3 cells. Grb2 is typically bound to Sos, which serves as a GTP exchange factor for Ras. Although GST-Grb2 fusion proteins are able to precipitate Sos (32) from RBL-2H3 cells, anti-SHIP immunoprecipitates containing Grb2 do not contain Sos. This may be a reflection of the sensitivity of the reagents available, since others have had difficulty in observing Sos-Shc co-immunoprecipitation (38). Alternatively, the Grb2-SHIP association may be regulatory in nature, as a means of dissociating the Grb2-Sos signaling complex. GST-Grb2 fusion proteins bind to SHIP, and this interaction requires more than the SH2 domain of Grb2, since the GST-Grb2 SH2 domain fusion protein does not precipitate SHIP (Fig. 6). The proline-rich region of the C terminus of SHIP is an excellent candidate for an SH3 ligand and may serve as a binding site for the Grb2 SH3 domain.
The association between Shc and SHIP is only detected after stimulation with growth factors. These observations may reflect a fundamental difference between RBL-2H3 cells and other cell types. When all data are considered together, a more complete story of SHIP activity emerges. Damen et al. (17) used the Grb2 N-terminal SH3 domain to purify mouse SHIP, and Kavanaugh et al. (18) used a strategy based on expression cloning via Grb2-binding ability to clone cDNAs encoding a class of proteins they term SIP. It thus appears that Grb2 binding is mediated by one (or more) of the polyproline stretches at the C terminus of SHIP, consistent with the observation that Grb2-SHIP binding is not detected with the Grb2 SH2 domain alone (Fig. 6). Furthermore Kavanaugh et al. (18) showed that binding of SIP to Grb2 required both SH3 domains but not the SH2 domain. Lioubin et al. (41), using a strategy similar to ours to identify Shc PTB domain ligands, showed that the region of SHIP containing the NPXY motif (at amino acid 914) was sufficient for Shc binding.

SHIP is basally tyrosine-phosphorylated in RBL-2H3 cells, in agreement with other reports (17, 18, 40). We demonstrate that FceRI stimulation results in SHIP association with several additional tyrosine phosphoproteins, which is not observed in growth factor-stimulated cells (17, 18, 40). These results demonstrate that SHIP can associate with receptor-activated proteins and that SHIP may play a role in the signaling events associated with FceRI activation.

Other investigators have observed a tyrosine phosphoprotein of 145 kDa (termed pp145) in a complex with tyrosine-phosphorylated Shc in cells treated with cytokines or stimulated through Fc receptors (33, 34, 38, 42–46). In B cells and macrophages, both IL-4 and antigen induce the association of Shc with pp145. It remains to be proven whether all of the 145-kDa phosphoproteins described in these experiments are identical, but their behavior suggests a common mechanism for association of Shc with SHIP in response to receptor activation.

SHIP is an inositol-5'-phosphatase that will hydrolyze phosphatidylinositol 3,4,5-trisphosphate in addition to the soluble inositol 1,3,4,5-tetrakisphosphate. The 5-phosphatase activity observed in this protein does not require tyrosine phosphorylation, since immunoprecipitations from S. cerevisiae (which has few tyrosine phosphoproteins) not expressing Lck do not interfere with its activity. Although it is difficult to quantify, the observation that the specific activity of SHIP is reduced when tyrosine-phosphorylated suggests that receptor activation (and concurrent SHIP phosphorylation) would lead to a decrease in SHIP activity.

SHIP is able to dephosphorylate phosphatidylinositol 3,4,5-trisphosphate (PIP3), the product of PI 3-kinase, to phosphatidylinositol 3,4-bisphosphate (PIP2). PIP2 has been reported to stimulate protein kinase C isozymes (47), and SHIP activity would result in an increase in PIP2. The observation that SHIP is negatively regulated by tyrosine phosphorylation would be consistent with PI 3-kinase activation in response to receptor activation. Since SHIP activity is decreased, the 5-phosphatase may play a negative regulatory role in receptor activation, similar to protein-tyrosine phosphatases. Such a mechanism would lead to a synergistic increase in the levels of PIP3, since PI 3-kinase is activated and 5-phosphatase activity is inhibited. Given the importance of PI 3-kinase activation in cell signaling in RBL-2H3 cells (48), SHIP may function as a negative regulator of PI 3-kinase. Since SHIP is able to bind to hemophagocytic ITAMs, Fc receptor activation would provide a docking site for SHIP, facilitating its translocation to the membrane, where the substrate for its enzymatic activity is found. Its association with Grb2, but not with Sos, suggests that it may serve to sequester Grb2 during the signal shut-off period.

The recent report that the PI 3-kinase binds to PIP3 directly via its SH2 domain (21) also raises the possibility that SHIP, by virtue of its PIP3 to PIP2 catalytic activity, may act to alter the pattern of SH2 domain-containing proteins with the activated receptor complex at the membrane. The observation that SHIP will bind to the SH3 domain of phospholipase Cγ-1 (Fig. 6) suggests that SHIP may allow SHP to regulate or be regulated by phospholipase C activation and/or localization. The involvement of several different phospholipid modifying enzymes in signal transduction (PIP3, phospholipase Cγ, and SHIP) may reflect the importance of membrane dynamics in signal transduction from cell surface receptors.

SHIP is also able to hydrolyze inositol 1,3,4,5-tetrakisphosphate, and the product of this enzymatic reaction, Ins(1,3,4,5)P4, may be involved in cell signaling as well. The exact nature of the role of Ins(1,3,4,5)P4 is not apparent at present.

The precise role of SHIP in signal transduction from FcɛRI is not yet clear. However, it is reasonable to conclude that SHIP is an important component of the receptor signal transduction apparatus in several hematopoetic lineages. Three distinct types of SHIP interactions, via SH2, SH3, and PTB domains have been identified, providing support that SHIP has a complex adapter function. Further experiments examining the role of SHIP in receptor activation or deactivation will reveal additional insights into the varied overlapping signal transduction pathways in the cell.

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