THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 278, No. 25, Issue of June 20, pp. 22657–22663, 2003

Printed in U.S.A.

SHIP-2 Inositol Phosphatase Is Inducibly Expressed in Human Monocytes and Serves to Regulate Fcγ Receptor-mediated Signaling*

Received for publication, March 21, 2003, and in revised form, April 8, 2003
Published, JBC Papers in Press, April 10, 2003, DOI 10.1074/jbc.M302907200

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SHIP-2, a recently identified inositol 5′-phosphatase, shares high level homology with SHIP-1. Although the role of SHIP-1 has been extensively studied, the role of SHIP-2 in myeloid cell functions is not known. Here, we have analyzed the expression patterns, molecular mechanism of activation, and function of SHIP-2 in human myeloid cell Fcγ receptor (FcγR) signaling. We report that SHIP-2 is expressed in transformed myeloid cells and in primary macrophages, but not in peripheral blood monocytes. Treatment of peripheral blood monocytes with bacterial lipopolysaccharide induced expression of SHIP-2 in a dose-dependent manner. FcγRIIa clustering in THP-1 cells induced SHIP-2 tyrosine phosphorylation, suggesting a role for SHIP-2 in modulating FcγR-mediated function. Consistent with this notion, overexpression of wild-type SHIP-2 (but not catalytically deficient SHIP-2) in THP-1 cells almost completely abrogated NFκB-mediated gene transcription in response to FcγRIIa clustering. Furthermore, FcγRIIa-induced Akt activation was blocked by wild-type SHIP-2, but not by a catalytically deficient mutant of SHIP-2. Additional experiments analyzing the molecular mechanism of SHIP-2 induction by FcγRIIa revealed that SHIP-2 associated with the phosphorylated FcγRIIa immunoreceptor tyrosine-based activation motif via the SHIP-2 SH2 domain. Thus, an SH2 domain mutant of SHIP-2 failed to associate with FcγRIIa or to become tyrosine-phosphorylated upon FcγRIIa clustering. Finally, we also demonstrate that SHIP-2 phosphorylation was induced by FcγRI clustering in THP-1 cells. These findings unravel a novel level of regulation of FcγR-mediated activation of human myeloid cells by the expression and function of the inositol phosphatase SHIP-2.

Clustering of FcγR[1] on monocytes and macrophages by immune complexes initiates a series of signaling events that result in phagocytosis of the immune complex (1). The process of phagocytosis is accompanied by the generation of reactive oxygen and nitrogen radicals, as well as the production of inflammatory cytokines, all of which cause tissue damage. Thus, the phagocytic process must be subject to tight regulation.

Human monocytes and macrophages express three classes of FcγR (2). FcγRI, FcγRIIa, and FcγRIII are activating receptors that are associated with an immunoreceptor tyrosine-based activation motif (ITAM). In contrast, FcγRIIb is an inhibitory receptor that bears in its cytoplasmic tail a tyrosine-based inhibitory motif that predominantly recruits negative regulatory phosphatases and causes down-regulation of activation events. In addition to the regulatory role of FcγRIIb, recent studies have revealed that the FcγR ITAMs are often capable of simultaneously activating positive and negative regulatory proteins such that the final biologic response is tempered. We (3) and others (4) have demonstrated that FcγR ITAMs are capable of recruiting and activating the inositol phosphatase SHIP-1. Other studies have shown that the protein-tyrosine phosphatase SHP-1 (5) and the dual phosphatase PTEN (6) also serve to regulate FcγR-mediated macrophage functions. Thus, it is clear that FcγR-mediated activation of macrophages is subject to multiple levels of regulation, which are not fully understood. In this report, we present molecular details of a novel regulatory influence exerted by the recently identified inositol phosphatase SHIP-2.

FcγR clustering in monocytes and macrophages initiates a biochemical cascade that begins with the activation of the Src kinases that phosphorylate the ITAMs of FcγR (7). Once phosphorylated, the ITAMs serve as docking sites for several signaling enzymes and enzyme-adapter complexes, including the Syk tyrosine kinase (8); the p85 subunit of phosphatidylinositol 3-kinase (9); and the Ras adapter molecule Shc (3, 10), which serves to recruit the Grb2-Sos complex, leading to activation of the Ras pathway. Association of phosphatidylinositol 3-kinase with the ITAM places the enzyme in proximity with its lipid substrates, resulting in the generation of the important lipid second messenger PtdIns-3,4,5-P_3. PtdIns-3,4,5-P_3 is critical for the activation of PH domain-containing enzymes such as Btk (11); the Tec family tyrosine kinase involved in intracellu-

* This work was supported by National Institutes of Health Grants P30 CA16058, HL63800, and P01 CA095426. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: FcγR, Fcγ receptor; ITAM, immunoreceptor tyrosine-based activation motif; SH, Src homology; SHIP, SH2 domain-containing inositol phosphatase; PtdIns-3,4,5-P_3, phosphatidylinositol 3,4,5-trisphosphate; PH, pleckstrin homology; PBMs, peripheral blood monocytes; LPS, lipopolysaccharide; GST, glutathione *S*-transferase; WCLs, whole cell lysates.The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

lar calcium mobilization, Vav (12); the guanine nucleotide exchange factor for Rac; and Akt, the serine/threonine kinase that is involved in cell survival (13, 14) and in the activation of NFκB (15). The hydrolysis of PtdIns-3,4,5-P_3 by SHIP-1 has been demonstrated to down-regulate the activity of the above PH domain-containing enzymes and the downstream functional outcomes (11, 13, 14).

SHIP-2 is an inositol 5′-phosphatase that has high level homology to SHIP-1 in its catalytic region (16–18). The molecules are largely divergent in the C-terminal region, consisting of a proline-rich domain that associates with unique SH3 domain-containing proteins (19). In addition, whereas SHIP-1 has two tyrosine residues in the C terminus that conform to an NP*X*Y motif shown to bind phosphotyrosine-binding domains upon phosphorylation, SHIP-2 has only one NP*X*Y motif (20). Thus, while enzymatically similar, these two enzymes likely differ in functions that are related to their protein interactions via the C-terminal region. These two molecules also differ in their expression patterns: SHIP-1 is expressed predominantly in hematopoietic cells, whereas SHIP-2 is much more ubiquitously expressed (20). Recent studies have revealed a role for SHIP-2 in regulating insulin receptor signaling (21–23). Other studies have demonstrated a role for SHIP-2 in mediating the inhibitory effect of FcγRIIb in B cells (21, 24, 25). However, the expression and function of SHIP-2 in monocytes and macrophages are not known.

In this study, we first demonstrate that SHIP-2 was expressed in human alveolar macrophages, but was almost undetectable in peripheral blood monocytes derived from the same donors. Interestingly, expression of SHIP-2 in PBMs was induced by bacterial LPS in a dose-dependent manner. Second, FcγRIIa clustering in the human myeloid cell line THP-1 induced tyrosine phosphorylation of SHIP-2, suggesting that SHIP-2 may play a role in FcγR-mediated function. Analyzing the functional consequence of SHIP-2 in FcγRIIa signaling, we report that overexpression of wild-type SHIP-2 (but not catalytically deficient SHIP-2) completely abrogated NFκB-dependent gene transcription in response to FcγRIIa clustering in THP-1 cells. Transient cotransfection experiments in COS-7 cells demonstrated that wild-type SHIP-2 down-regulated FcγRIIa-induced Akt phosphorylation. Additional experiments analyzing the molecular mechanism of SHIP-2 activation by FcγRIIa demonstrated that the SH2 domain of SHIP-2 was necessary for optimal association of SHIP-2 with FcγRIIa and for optimal tyrosine phosphorylation of SHIP-2. Finally, we also demonstrate that FcγRI clustering resulted in phosphorylation of SHIP-2, suggesting that SHIP-2 may regulate both FcγRIIa- and FcγRI-mediated myeloid cell function.

EXPERIMENTAL PROCEDURES

Cells, Antibodies, and Reagents—THP-1, U937, and COS-7 cells were obtained from American Type Culture Collection. COS-7 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. All other cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum. Anti-FcγRI antibody 197 and anti-FcγRIIa antibody IV.3 were obtained from Medarex (Annandale, NJ). Rabbit polyclonal anti-SHIP-2 antibody was a generous gift from Dr. Bayard Clarkson (Memorial Sloan Kettering Cancer Center, New York, NY) (19). Goat polyclonal anti-SHIP-2 antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phosphotyrosine, anti-phospho-Akt, and anti-Akt antibodies were purchased from Cell Signaling Technology (Beverly, MA). Rabbit polyclonal anti-Syk antibody was purchased from Upstate Biotechnology, Inc. (Charlottesville, VA). Anti-Xpress antibody was purchased from Invitrogen.

Isolation of PBMs—CD14-positive PBMs were isolated as described previously (26). Briefly, peripheral blood mononuclear cells were first isolated by density gradient centrifugation over Histopaque (Sigma). Monocytes were then purified from the peripheral blood mononuclear cells by negative selection using the MACs monocyte isolation kit (Miltenyi Biotech). For this, peripheral blood mononuclear cells were first treated with FcγR blocking reagent (human IgG), followed by a hapten/antibody mixture (mixture of hapten-conjugated monoclonal anti-CD3, anti-CD7, anti-CD19, anti-CD45RA, anti-CD56, and anti-IgE antibodies). The labeled cells were further treated with MACS anti-hapten magnetic microbeads conjugated to monoclonal anti-hapten antibody. The cells were then passed over a MACS column, and the effluent was collected as the negative fraction representing enriched monocytes.

Preparation of Human Alveolar Macrophages—Macrophages were obtained from healthy donors by bronchoalveolar lavage. Cells were washed twice with phosphate-buffered saline, counted, and analyzed by Diff-Quick staining for purity. Cell preparations were >95% positive for macrophages.

Immunoprecipitation and Western Blotting—THP-1 cells and transfected COS-7 cells were activated by clustering FcγRI and or FcγRIIa with monoclonal antibody 197 or Fab fragments of antibody IV.3 and goat $F(ab')_2$ anti-mouse Ig secondary antibody. Resting and activated cells were lysed in buffer A (50 mM Tris (pH 8.0), 10 mM EDTA, 10 mM $Na_4P_2O_7$, 10 mM NaF, 1% Triton X-100, 125 mM NaCl, 10 mM Na_3VO_4, and 10 μg/ml each aprotinin and leupeptin), and post-nuclear lysates were incubated overnight with the antibody of interest and protein G-agarose beads (Invitrogen) or goat $F(ab')_2$ anti-mouse Ig covalently linked to Sepharose, depending on the antibody. Immune complexes bound to beads were washed with buffer A and boiled in SDS sample buffer (60 mM Tris (pH 6.8), 2.3% SDS, 10% glycerol, 0.01% bromphenol blue, and 2% 2-mercaptoethanol) for 5 min. Proteins were separated by SDS-PAGE, transferred to nitrocellulose filters, probed with the antibody of interest, and developed by enhanced chemiluminescence.

Immunoblot Data Quantitation—The ECL signal was quantitated using a scanner and a densitometry program (Scion Image). To quantitate the phosphorylation signals in the activated samples, we first subtracted background, normalized the phosphorylation signal to the amount of total precipitated protein, and plotted the values obtained by expressing them as -fold increase over the values in unstimulated samples.

Transfection—COS-7 cells were transfected as described previously (3). Briefly, cells were grown on culture dishes to 60–70% confluency. cDNA for FcγRIIa in pCEXV3 (kindly provided by Dr. J. Ravetch, Rockefeller University, New York) and cDNAs for wild-type SHIP-2, the inactive SH2 domain mutant of SHIP-2, and the inactive catalytic domain mutant of SHIP-2 in pcDNA3 (generously provided by Dr. Shonna Moodie, Metabolex Inc., Hayward, CA) (27) were mixed in various combinations with LipofectAMINE 2000 reagent (Invitrogen). The DNA mixture was added to cells in serum-free Dulbecco's modified Eagle's medium and incubated for 3 h at 37 °C in a CO_2 incubator. The medium was then replaced with Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. The cells were harvested 24 h later and analyzed for expression of the transfected cDNAs by Western blotting. Having ensured that the various transfectants expressed comparable levels of protein, we then examined the ability of the wild-type and mutant SHIP-2 molecules to associate with the FcγRIIa ITAM and to become tyrosine-phosphorylated. In other experiments, GST-Akt (a kind gift from Dr. R. B. Pearson, Peter MacCallum Cancer Institute, Melbourne, Australia) (28) was cotransfected with the above molecules (2 μg of FcγRIIa, 5 μg of SHIP-2, and 1 μg of GST-Akt) to analyze the influence of SHIP-2 on Akt activation by FcγRIIa.

Transfection of THP-1 Cells and Luciferase Assays—For analysis of SHIP-2 influence on NFκB transcriptional activity, THP-1 cells were transfected as described previously (3). Briefly, THP-1 cells were electroporated (310 V, 950 microfarads; Bio-Rad Gene Pulser II) with 5 μg of wild-type SHIP-2 or catalytically deficient SHIP-2 in pcDNA3, 1 μg of NFκB-luciferase plasmid, and 0.5 μg of pEGFP to normalize for transfection efficiency. Transfectants were harvested 24 h later and activated by clustering FcγRIIa by the methods described above for 6 h at 37 °C. The cells were lysed in 100 μl of cell culture lysis reagent (Promega). Luciferase activity was measured using the Promega luciferase assay reagent. Data are represented as graphs indicating the percent induction of NFκB activity in cells activated by clustering FcγRIIa over the activity in cells that were not activated. Data points are expressed as the mean ± S.D. of three independent experiments. Statistical analysis was performed by Student's *t* test.

RESULTS

SHIP-2 Expression Pattern in Myeloid Cells—Expression of SHIP-2 in myeloid cells was assessed by Western blotting protein-matched lysates obtained from the THP-1 and U937 hu-

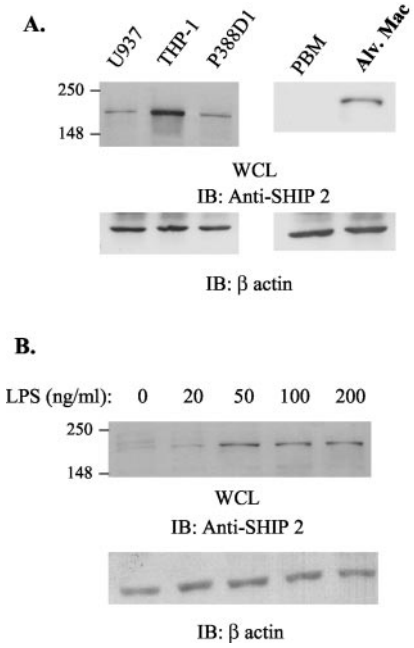

FIG. 1. **Expression of SHIP-2 in myeloid cells.** *A*, protein-matched WCLs from the human monocytic cell lines U937 and THP-1, the mouse myeloid cell line P388D1, and human PBMs and alveolar macrophages (*Alv. Mac*) were separated by SDS-PAGE and analyzed by Western blotting with anti-SHIP-2 antibody (*upper panels*). The same membranes were reprobed with anti-β-actin antibody to ensure equal loading of protein in all lanes (*lower panels*). *B*, human PBMs were cultured overnight in the presence of increasing doses of LPS, and protein-matched lysates were probed using anti-SHIP-2 antibody (*upper panel*) and then reprobed with anti-β-actin antibody (*lower panel*). These results are representative of four independent experiments. *IB*, immunoblot.

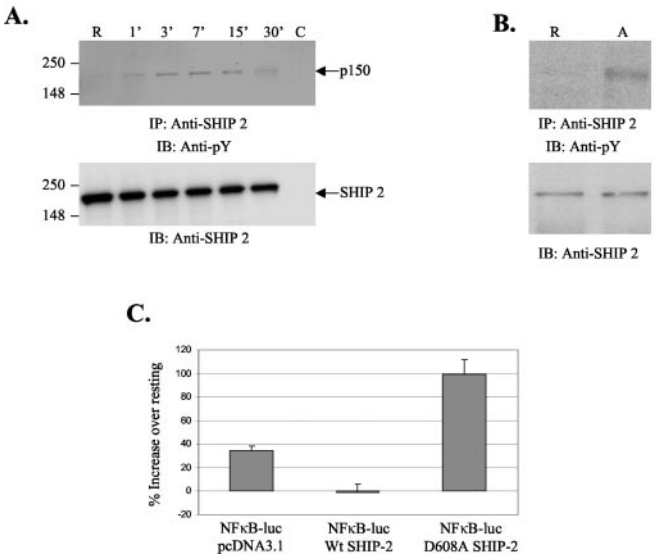

FIG. 2. **SHIP-2 is tyrosine-phosphorylated upon FcγRIIa clustering and inhibits NFκB-mediated gene transcription.** *A*, THP-1 cells were activated for the various time points indicated by clustering FcγRIIa with Fab fragments of monoclonal antibody 1V.3, followed by goat anti-mouse IgG. SHIP-2 was immunoprecipitated (*IP*) from resting (*R*) and activated (*A*) cells and analyzed by Western blotting with anti-phosphotyrosine (*pY*) antibody (*upper panel*). The same membrane was reprobed with anti-SHIP-2 antibody to ensure equivalent loading in all lanes (*lower panel*). The *C lane* is a control immunoprecipitation with normal goat serum. These results are representative of three independent experiments. *B*, PBMs were activated for 5 min by clustering FcγRIIa, and SHIP-2 phosphorylation was assessed by Western blotting with anti-phosphotyrosine antibody (*upper panel*). The results from a reprobe with anti-SHIP-2 antibody are also shown (*lower panel*). *C*, THP-1 cells were transiently transfected with plasmids encoding the NFκB binding element coupled to the luciferase (*luc*) gene along with either empty vector or plasmids encoding wild-type (*Wt*) SHIP-2 or D608A SHIP-2 (catalytically deficient mutant). Cells were activated by clustering FcγRIIa for 5 h, and luciferase gene induction was measured in a luciferase enzyme assay. Results are shown as percent increase in luciferase activity in the activated samples over the activity in resting samples. The graph represents the mean ± S.D. of values from three independent experiments. Statistical analysis was performed with a paired two-tailed Student's *t* test. *IB*, immunoblot.

man monocytic cell lines, the P388D1 mouse myeloid cell line, and primary human PBMs and alveolar macrophages. The results shown in Fig. 1*A* (*upper panels*) demonstrate that SHIP-2 was expressed in the transformed myeloid cell lines and in primary human alveolar macrophages. Interestingly, PBMs appeared to express very low levels of SHIP-2 compared with alveolar macrophages from the same donor. The results were consistently observed in five separate donors. A reprobe of the membranes with anti-β-actin antibody (Fig. 1*A*, *lower panels*) showed equivalent loading of protein in the different lanes. Treatment of PBMs with LPS, a reagent often used to activate PBMs, resulted in increased expression of SHIP-2 in a dose-dependent manner (Fig. 1*B*, *upper panel*). These data suggest that SHIP-2 is inducibly expressed in human monocytes.

SHIP-2 Is Tyrosine-phosphorylated and Serves to Down-regulate FcγRIIa-mediated Function—To test whether SHIP-2 is involved in FcγR-mediated myeloid cell activation, SHIP-2 phosphorylation was assessed in THP-1 cells stimulated by clustering FcγRIIa (Fig. 2*A*). For this, FcγRIIa receptors were clustered using Fab fragments of monoclonal anti-FcγRIIa antibody IV.3, followed by $F(ab')_2$ fragments of goat anti-mouse IgG. SHIP-2 was immunoprecipitated from resting and activated cells with goat polyclonal anti-SHIP-2 antibody and analyzed by Western blotting with anti-phosphotyrosine antibody. The results indicate that FcγRIIa clustering induced SHIP-2 phosphorylation within 1 min and that the phosphorylation signals peaked at ~7 min and subsided by 30 min post-stimulation (Fig. 2*A*, *upper panel*). A reprobe of the same membrane with anti-SHIP-2 antibody revealed equal loading of SHIP-2 in all lanes. The *seventh lane* is a negative control immunoprecipitation with normal goat IgG (Fig. 2*A*, *lower panel*). In a second set of experiments, human PBMs cultured overnight with 100 ng/ml LPS were stimulated by clustering FcγRIIa, and SHIP-2 phosphorylation was assessed by Western blotting (Fig. 2*B*). As shown in Fig. 2*B* (*upper panel*), SHIP-2 phosphorylation was induced by FcγRIIa clustering in PBMs. Fig. 2*B* (*lower panel*) demonstrates equivalent loading of SHIP-2 in the two lanes.

Having ensured that SHIP-2 is phosphorylated during FcγRIIa signaling, we next assessed whether SHIP-2 influences FcγRIIa-mediated functional outcomes. We (3) and others (10) have previously reported that FcγR clustering induces NFκB-mediated gene transcription, which is down-regulated by the inositol phosphatase SHIP-1. Because SHIP-1 and SHIP-2 are both capable of hydrolyzing PtdIns-3,4,5-P_3 and influence downstream events, we analyzed whether SHIP-2 plays a role in regulating NFκB-mediated gene transcription. In these experiments, we analyzed NFκB-dependent transcription from a reporter plasmid encoding the luciferase gene in the presence of excess wild-type SHIP-2 or the catalytically deficient SHIP-2 mutant D608A. Thus, THP-1 cells were transiently cotransfected with the NFκB-luciferase plasmid and with plasmids encoding wild-type SHIP-2 or catalytically deficient SHIP-2. The transfected cells were activated by clustering FcγRIIa for 5 h at 37 °C. Transcription of the luciferase gene was measured by a luciferase enzyme assay as described above. The results are expressed as percent increase in luciferase activity in cells activated by clustering FcγRIIa over the activ-

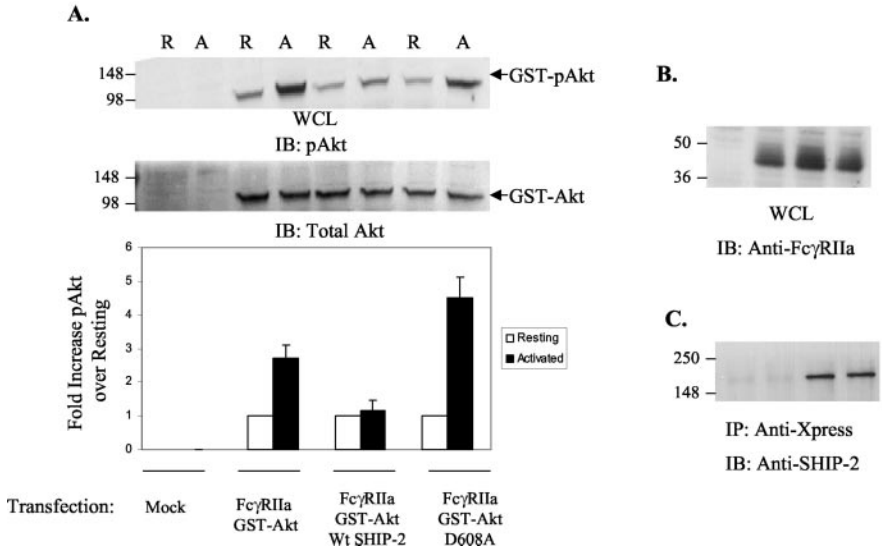

FIG. 3. **SHIP-2 inhibits FcγRIIa-mediated Akt phosphorylation.** COS-7 cells were transiently transfected to express FcγRIIa and GST-Akt along with wild-type (*Wt*) or D608A SHIP-2. *A*, the transfectants were activated by clustering FcγRIIa, and serine phosphorylation of Akt was assessed by Western blotting WCLs with anti-phosphoserine Akt (*pAkt*) antibody (*upper panel*). The same membrane was reprobed with anti-total Akt antibody (*middle panel*). The graph represents a quantitative measure of GST-Akt phosphorylation expressed as -fold increase in activated (*A*) cells over that in resting (*R*) cells (*lower panel*). Data represent the mean ± S.D. of values obtained from three independent experiments. Statistical analysis was performed using a paired two-tailed Student's *t* test. *B*, WCLs from the four transfectants were analyzed for the presence of transfected FcγRIIa by Western blotting with anti-FcγRIIa antibody. *C*, expression of transfected Xpress-tagged SHIP-2 was assessed by probing anti-Xpress immunoprecipitates (*IP*) with anti-SHIP-2 antibody. *IB*, immunoblot.

ity in resting cells (Fig. 2*C*). The data indicate that NFκB-dependent transcription of the luciferase gene occurred upon FcγRIIa clustering. However, overexpression of wild-type SHIP-2 blocked the induction of gene transcription ($p = 0.027$, presence of exogenous wild-type SHIP-2 *versus* pcDNA3 empty vector). Importantly, gene transcription was significantly enhanced in the presence of dominant-negative, catalytically deficient SHIP-2 ($p = 0.006$, presence of exogenous catalytically deficient SHIP-2 *versus* pcDNA3 empty vector), suggesting that SHIP-2 serves to down-regulate NFκB-dependent gene transcription by FcγRIIa.

Recent studies have demonstrated an upstream role for Akt in NFκB activation (15). Because Akt is a PH domain-containing enzyme whose activity is regulated by PtdIns-3,4,5-P_3, a substrate of SHIP-2, we next analyzed Akt activation in the presence of overexpressed wild-type SHIP-2 or catalytically deficient SHIP-2. For these experiments, COS-7 fibroblasts were used to achieve high levels of transfection. Thus, COS-7 cells were transiently transfected to express epitope (GST)-tagged Akt along with FcγRIIa (COS-7 cells do not express any endogenous FcγR) and either wild-type SHIP-2 or the catalytically deficient SHIP-2 mutant D608A. The transfected cells were harvested 24 h later and activated by clustering FcγRIIa by the methods described above. Whole cell lysates from resting and activated cells were separated by SDS-PAGE and analyzed by Western blotting with anti-phosphoserine Akt antibody (Fig. 3*A*, *upper panel*). GST-Akt migrated at ~95 kDa in comparison with endogenous Akt, which migrated at 65 kDa. The slower migration of GST-Akt allowed for analysis of the effect of overexpression of the SHIP-2 proteins on cotransfected GST-Akt. The membrane was reprobed with anti-Akt antibody to detect the total amount of GST-Akt present in each lane (Fig. 3*A*, *middle panel*). Fig. 3*A* demonstrates that GST-Akt was serine-phosphorylated upon FcγRIIa clustering (*fourth lane*) in comparison with the resting sample (*third lane*). Overexpression of wild-type SHIP-2 down-regulated FcγRIIa-induced Akt phosphorylation (compare the *fifth* and *sixth lanes*). In contrast, overexpression of catalytically deficient SHIP-2 resulted in enhanced phosphorylation of Akt in response to FcγRIIa clustering (*eighth lane*), suggesting that catalytically deficient SHIP-2 functions in a dominant-negative manner, overcoming the inhibition of Akt by endogenous SHIP-2 (*fourth lane*). Akt phosphorylation was quantitated by normalizing phospho-Akt (GST) signals to total Akt (GST) signals as described under "Experimental Procedures." The results are expressed as -fold increase in Akt phosphorylation in activated cells in comparison with that in resting cells. Fig. 3*A* (*lower panel*) represents the mean ± S.D. of values obtained from three independent experiments. The inhibition of Akt phosphorylation by wild-type SHIP-2 was statistically significant ($p = 0.02$). To ensure that transfected FcγRIIa was expressed to comparable levels in the different transfectants, WCLs were probed with anti-FcγRIIa antibody (Fig. 3*B*). Likewise, expression of Xpress-tagged wild-type and catalytically deficient SHIP-2 was comparable in the transfected cells as shown in Fig. 3*C*. These data suggest that SHIP-2 down-regulates FcγRIIa-induced Akt phosphorylation.

SHIP-2 Associates with the Phosphorylated ITAM of FcγRIIa—SHIP-2 is a cytoplasmic protein whose activity is regulated by subcellular localization, *i.e.* SHIP-2 must translocate to the membrane to access its lipid substrates (20). We therefore tested whether FcγRIIa provides a docking site for SHIP-2 in the following experiments. First, we used biotinylated peptides derived from the ITAM of FcγRIIa that were either non-phosphorylated or doubly phosphorylated at the tyrosine residues. These peptides were applied to THP-1 lysates, and the peptide-bound material was analyzed by Western blotting with anti-SHIP-2 antibody. As shown in Fig. 4*A* (*upper panel*), SHIP-2 associated with the doubly phosphorylated FcγRIIa ITAM peptide, but not with the non-phosphorylated peptide. A WCL from THP-1 cells is shown in the *third lane*. To ensure that the binding of SHIP-2 to the phosphorylated ITAM peptide was specific, the same membrane was reprobed with anti-Syk antibody (Fig. 4*A*, *lower panel*). As previously reported, the phosphorylated ITAM of FcγRIIa bound Syk (9).

In a second set of experiments, we investigated whether native FcγRIIa associates with SHIP-2 upon receptor cluster-

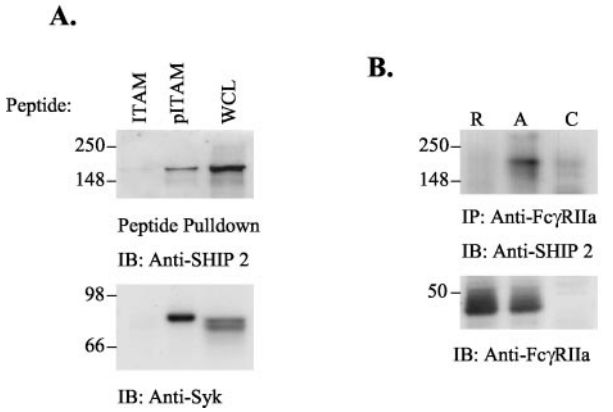

FIG. 4. **SHIP-2 associates with FcγRIIa.** *A*, THP-1 cells lysates were incubated with biotinylated peptides corresponding to the non-phosphorylated or phosphorylated ITAM (*pITAM*) of FcγRIIa. The peptide-bound material was analyzed for the presence of SHIP-2 by Western blotting with anti-SHIP-2 antibody (*upper panel*). The same membrane was reprobed with anti-Syk antibody (*lower panel*). The *third lane* is a WCL from the THP-1 cells. *B*, FcγRIIa was immunoprecipitated from resting (*R*) and activated (*A*) THP-1 cells that had been activated for 5 min by clustering FcγRIIa. The immunoprecipitated (*IP*) proteins were separated by SDS-PAGE and analyzed by Western blotting with anti-SHIP-2 antibody (*upper panel*). The membrane was reprobed with anti-FcγRIIa antibody (*lower panel*). These data are representative of three independent experiments. *IB*, immunoblot.

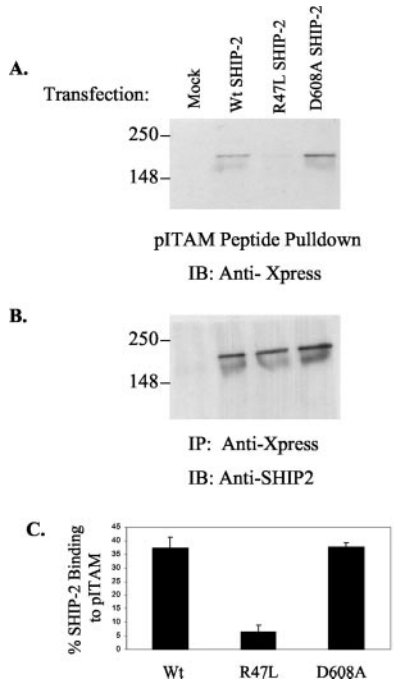

FIG. 5. **The SH2 domain of SHIP-2 is necessary for optimal SHIP-2 association with FcγRIIa ITAM.** *A*, Xpress-tagged SHIP-2 proteins (wild-type (*Wt*), SH2 domain mutant R47L, catalytically deficient mutant D608A) were expressed in COS-7 fibroblasts by transient transfection. The phosphorylated ITAM (*pITAM*) peptide of FcγRIIa was applied to COS-7 lysates, and binding of the SHIP-2 proteins was assessed by Western blotting with anti-Xpress antibody. *B*, parallel samples were immunoprecipitated (*IP*) with anti-Xpress antibody and probed with anti-SHIP-2 antibody to compare the level of expression of the SHIP-2 proteins in the different transfectants. *C*, the percent SHIP-2 protein that bound the phosphorylated ITAM peptide was quantitated by dividing the density of the bands in *A* by the density of the bands in *B*. The graph represents the mean ± S.D. of values from three independent experiments. *IB*, immunoblot.

ing. Here, THP-1 cells were activated by clustering FcγRIIa for 5 min. FcγRIIa was immunoprecipitated from resting and activated cells, separated by SDS-PAGE, and analyzed for the presence of SHIP-2 by Western blotting. The results show that SHIP-2 was capable of associating with FcγRIIa in an activation-dependent manner (Fig. 4*B*, *upper panel*). A reprobe of the same membrane indicated equal loading of FcγRIIa in both resting and activated samples. Taken together, the results suggest that SHIP-2 translocates to the membrane upon FcγRIIa clustering by associating with the phosphorylated ITAM of FcγRIIa.

The SH2 Domain of SHIP-2 Is Required for Association with the FcγRIIa ITAM—The above finding that SHIP-2 associates with the phosphorylated ITAM of FcγRIIa suggests that the interaction may be mediated via the SH2 domain of SHIP-2. To formally test this possibility, COS-7 fibroblasts were transfected to express epitope (Xpress)-tagged wild-type SHIP-2, R47L SHIP-2 (SH2 domain mutant), or D608A SHIP-2 (catalytically deficient mutant). The tagged SHIP-2 proteins were assessed for their ability to bind the FcγRIIa ITAM in a peptide binding assay. Thus, the peptide-bound material was separated by SDS-PAGE and immunoblotted with anti-Xpress antibody. The results indicate that the SH2 domain mutant of SHIP-2 was unable to associate optimally with the phosphopeptide (Fig. 5*A*, *third lane*). In contrast, both wild-type SHIP-2 and catalytically deficient SHIP-2 were able to efficiently bind the phosphorylated ITAM peptide (*second* and *fourth lanes*). No signal was seen in the *first lane*, as these cells were not transfected with the Xpress-tagged SHIP-2 proteins. To ensure that the lack of binding observed with the SH2 domain mutant was not due to lack of expression of the transfected protein, parallel immunoprecipitations were performed with anti-Xpress antibody and analyzed by Western blotting with anti-SHIP-2 antibody (Fig. 5*B*). Fig. 5*C* is a quantitative measure of the percent of Xpress-tagged SHIP-2 that bound to the phosphorylated ITAM of FcγRIIa. The data represent the mean ± S.D. of values obtained from three independent experiments.

The SH2 Domain of SHIP-2 Is Necessary for FcγRIIa-induced SHIP-2 Tyrosine Phosphorylation—As an additional approach to determine the importance of the SH2 domain of SHIP-2 in its association with FcγRIIa and thereby its translocation to the membrane, we next assessed whether SH2 domain mutants of SHIP-2 would become phosphorylated upon FcγRIIa clustering. Here, COS-7 cells were transfected to express FcγRIIa along with wild-type SHIP-2, R47L SHIP-2 (SH2 domain mutant), or D608A SHIP-2 (catalytically deficient mutant). SHIP-2 proteins were immunoprecipitated from resting and activated cells, which had been activated by clustering FcγRIIa, using anti-Xpress antibody and analyzed by Western blotting with anti-phosphotyrosine antibody. The results indicate that, although both wild-type SHIP-2 and catalytically deficient SHIP-2 became tyrosine-phosphorylated upon FcγRIIa clustering, the SH2 domain mutant of SHIP-2 failed to become phosphorylated (Fig. 6*A*, *upper panel*). A reprobe of the membrane with anti-SHIP-2 antibody showed the presence of SHIP-2 in all lanes (Fig. 6*A*, *middle panel*). The phosphorylation level of SHIP-2 was quantitated as described under "Experimental Procedures," normalized for the amount of total SHIP-2 present in each lane, and expressed as -fold increase in SHIP-2 phosphorylation in the activated samples over that in resting samples. The graph in Fig. 6*A* (*lower panel*) represents the mean ± S.D. of values obtained from three independent experiments. To ensure that the lack of phosphorylation of the SH2 domain mutant of SHIP-2 was not due to lack of expression of FcγRIIa in these cells, WCLs from the transfectants were probed with anti-FcγRIIa antibody (Fig. 6*B*). Taken together, these data indicate that the SH2 domain of SHIP-2 is necessary for the association of SHIP-2 with FcγRIIa.

SHIP-2 Is Phosphorylated by FcγRI Clustering in THP-1 Cells—In additional experiments, THP-1 cells were activated

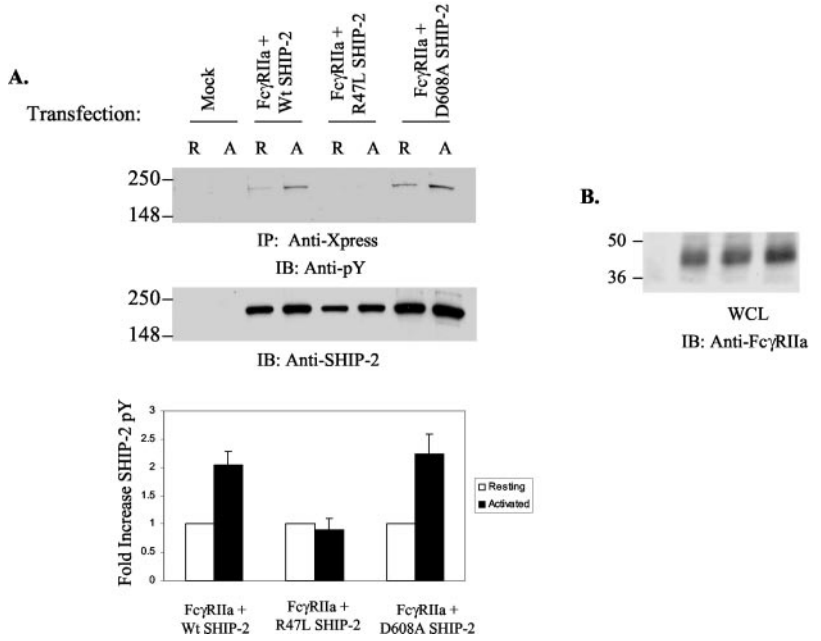

FIG. 6. **The SH2 domain of SHIP-2 is necessary for optimal phosphorylation of SHIP-2 by FcγRIIa clustering.** COS-7 cells were transiently transfected to express FcγRIIa along with wild-type (*Wt*), R47L, or D608A SHIP-2. *A*, the transfected cells were activated for 5 min by clustering FcγRIIa, and phosphorylation of SHIP-2 was assessed by Western blotting anti-Xpress immunoprecipitates (*IP*) with anti-phosphotyrosine (*pY*) antibody (*upper panel*). The same membrane was reprobed with anti-SHIP-2 antibody to ensure equal loading in the resting (*R*) and activated (*A*) samples (*middle panel*). The graph is a quantitative measure of the phosphorylation levels of the different SHIP-2 proteins normalized for the amount of total SHIP-2 present in each lane (*lower panel*). Values represent the mean ± S.D. from three independent experiments. *B*, WCLs from the four transfectants were analyzed for the expression of the transfected FcγRIIa by Western blotting. *IB*, immunoblot.

by clustering FcγRI with monoclonal antibody 197, followed by goat anti-mouse IgG. SHIP-2 phosphorylation was assessed by Western blotting with anti-phosphotyrosine antibody (Fig. 7*A*, *upper panel*). Fig. 7*A* (*lower panel*) is a reprobe with anti-SHIP-2 antibody to ensure equal loading of SHIP-2 in all lanes. The results indicate that SHIP-2 was indeed invoked upon FcγRI clustering in THP-1 cells.

Because our data indicated that SHIP-2 associated with the phosphorylated ITAM of FcγRIIa, we next analyzed whether a similar association might exist between SHIP-2 and the phosphorylated ITAM of the FcγR γ-chain. For this, biotinylated peptides corresponding to the ITAM of the γ-chain that were either non-phosphorylated or doubly phosphorylated were applied to THP-1 lysates. The peptide-bound material was analyzed by Western blotting with anti-SHIP-2 antibody. The results shown in Fig. 7*B* (*upper panel*) indicate that the phosphorylated ITAM of the γ-chain was incapable of association with SHIP-2. To ensure that the phosphopeptide used in these experiments is functional, the same membrane was reprobed with anti-Syk antibody (Fig. 7*B*, *lower panel*). As shown, the phosphopeptide efficiently bound Syk, consistent with earlier reports (2). These data suggest that SHIP-2 translocation to the membrane in response to FcγRI clustering might involve adapter molecules or membrane-associated proteins other than the γ-chain. Further analysis is needed to resolve these issues.

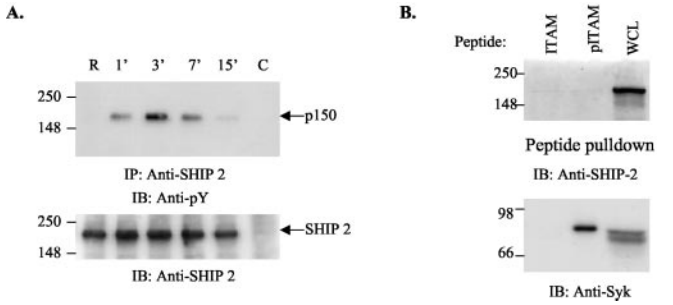

FIG. 7. **SHIP-2 is tyrosine-phosphorylated upon FcγRI clustering in THP-1 cells.** *A*, THP-1 cells were activated for the time points indicated by clustering FcγRI with monoclonal antibody 197, followed by goat anti-mouse IgG. SHIP-2 was immunoprecipitated (*IP*) from resting (*R*) and activated cells and analyzed for phosphorylation by Western blotting with anti-phosphotyrosine (*pY*) antibody (*upper panel*). The same membrane was reprobed with anti-SHIP-2 antibody to ensure equivalent loading of SHIP-2 in all lanes (*lower panel*). The *sixth lane* is a control (*C*) immunoprecipitation with isotype-matched antibody. *B*, non-phosphorylated and doubly phosphorylated (*pITAM*) peptides corresponding to the ITAM of the FcγR γ-subunit were applied to THP-1 lysates. The peptide-bound material was separated by SDS-PAGE and analyzed for binding of SHIP-2 by Western blotting with anti-SHIP-2 antibody (*upper panel*). The same membrane was reprobed with anti-Syk antibody (*lower panel*). These data are representative of three independent experiments. *IB*, immunoblot.

DISCUSSION

FcγR-mediated phagocytosis is regulated by a complex set of phosphatases, each using a distinct molecular mechanism for mediating its inhibitory function. Thus, recent reports have implicated the inositol phosphatase SHIP-1 in modulating FcγR-mediated macrophage function, including phagocytosis of IgG-opsonized particles (4, 29) and induction of gene transcription (3). Likewise, Durden and co-workers have demonstrated a role for the protein-tyrosine phosphatase SHP-1 (5) and for PTEN (6) in down-regulating phagocytosis. Although it is clear that to limit the tissue damage caused by the reactive oxygen and nitrogen radicals and the inflammatory cytokines that accompany phagocytosis, this process must be subject to tight regulation, the mechanisms involved in this regulation are not fully understood.

In this study, we have analyzed the molecular details of a novel level of regulation exerted by the inositol phosphatase SHIP-2. Our results indicate that SHIP-2 associates with the ITAM of FcγRIIa via the SH2 domain of SHIP-2 and becomes tyrosine-phosphorylated. As in the case of SHIP-1, tyrosine phosphorylation of SHIP-2 does not influence SHIP-2 enzyme activity. However, because membrane translocation of SHIP-2 is necessary for SHIP-2 tyrosine phosphorylation by Src kinases (30) and because membrane translocation puts SHIP-2 in the proximity of its lipid substrates, tyrosine phosphorylation of SHIP-2 serves as a correlate of SHIP-2 activation. Our data also demonstrate that SHIP-2 serves to down-regulate FcγRIIa-induced activation of Akt and NFκB-dependent gene transcription, as overexpression of wild-type SHIP-2 (but not catalytically deficient SHIP-2) almost completely abrogates these two events. Both SHIP-1 and SHIP-2 mediate their inhibitory effects via the SH2 domains, which are reported to have 54% homology. Thus, it must be noted that the enhanced activation of NFκB-dependent luciferase gene induction by the

dominant-negative SHIP-2 mutant D608A used in the transfection studies in THP-1 cells (Fig. 2*C*) may be the result of competition of D608A SHIP-2 with both endogenous SHIP-1 and SHIP-2.

In contrast to the association of SHIP-2 with the FcγRIIa ITAM, we were unable to detect any binding of SHIP-2 to the phosphorylated ITAM of the FcγRI-associated γ-chain, despite the fact that SHIP-2 was efficiently phosphorylated upon FcγRI clustering (Fig. 7). Co-immunoprecipitation experiments revealed a very weak association of SHIP-2 with the γ-chain that became apparent only when the Western blot was overexposed (data not shown). Based on the results of Fig. 7, we suggest that SHIP-2 association with the γ-chain is likely mediated via adapter molecules or that the association may be weak and transient and is disrupted by the detergent-based lysis buffer. In a recent report, Mitchell and co-workers (31) demonstrated an association of SHIP-2 with the actin-binding protein filamin. This association is mediated via the C-terminal proline-rich domain of SHIP-2 and the SH3 domain of filamin. Because filamin is known to be associated with FcγRI (reviewed in Ref. 32) in macrophages, it is possible that FcγRI clustering recruits SHIP-2 via filamin. Nonetheless, our data indicate that FcγRI clustering induces tyrosine phosphorylation of SHIP-2, suggesting a role for SHIP-2 in regulating FcγRI-mediated events.

Interestingly, SHIP-2 expression in human PBMs is almost undetectable, but is induced upon activation of these cells with bacterial lipopolysaccharide. Similar findings were recently reported by Cambier and co-workers (24) in murine B cells. It is tempting to speculate that the induction of SHIP-2 by LPS is a mechanism employed by Gram-negative bacteria to suppress the host immune response so that the pathogen may evade the phagocytic machinery. Numerous earlier studies have reported that LPS attenuates FcγR-mediated phagocytosis (33, 34). However, the molecular mechanism by which LPS mediates its suppressive effect has thus far remained elusive. Further studies are underway to investigate the details of LPS induction of SHIP-2 expression and the functional consequence on FcγR-mediated phagocytosis.

Finally, is not clear why myeloid cells express two inositol 5′-phosphatases, SHIP-1 and SHIP-2. Our data suggest that these two molecules may not be coexpressed at all times. Furthermore, although the results of this study demonstrate overlapping functions for SHIP-1 and SHIP-2, *i.e.* inhibition of Akt and NFκB activation, it is likely that these two phosphatases mediate additional and non-overlapping effects on signaling pathways. Indeed, previous studies have demonstrated that the C-terminal proline-rich regions of SHIP-1 and SHIP-2 are quite different, leading to association of a distinct sets of SH3 domain-containing molecules (19), which likely influence distinct signaling pathways. We are currently investigating the functional differences between SHIP-1 and SHIP-2 in FcγR-mediated activation of monocytes and macrophages.

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