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SHIP2 Is Recruited to the Cell Membrane upon Macrophage Colony-Stimulating Factor (M-CSF) Stimulation and Regulates M-CSF-Induced Signaling

Yijie Wang,* Rosemary J. Keogh,* Melissa G. Hunter,* Christina A. Mitchell,† Randall S. Frey,‡ Kamran Javaid,‡ Asrar B. Malik,‡ Stéphane Schurmans,§ Susheela Tridandapani,* and Clay B. Marsh2*

The Src homology 2-containing inositol phosphatase SHIP1 functions in hemopoietic cells to limit activation events mediated by PI3K products, including Akt activation and cell survival. In contrast to the limited cellular expression of SHIP1, the related isoform SHIP2, is widely expressed in both parenchymal and hemopoietic cells. The goal of this study was to determine how SHIP2 functions to regulate M-CSF signaling. We report that 1) SHIP2 was tyrosine-phosphorylated in M-CSF-stimulated human alveolar macrophages, human THP-1 cells, murine macrophages, and the murine macrophage cell line RAW264; 2) SHIP2 associated with the M-CSF receptor after M-CSF stimulation; and 3) SHIP2 associated with the actin-binding protein filamin and localization to the cell membrane, requiring the proline-rich domain, but not on the Src homology 2 domain of SHIP2. Analyzing the function of SHIP2 in M-CSF-stimulated cells by expressing either wild-type SHIP2 or an Src homology 2 domain mutant of SHIP2 reduced Akt activation in response to M-CSF stimulation. In contrast, the expression of a catalytically deficient mutant of SHIP2 or the proline-rich domain of SHIP2 enhanced Akt activation. Similarly, the expression of wild-type SHIP2 inhibited NF-κB-mediated gene transcription. Finally, fetal liver-derived macrophages from SHIP2 gene knockout mice enhanced activation of Akt in response to M-CSF treatment. These data suggest a novel regulatory role for SHIP2 in M-CSF-stimulated myeloid cells.

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3 Abbreviations used in this paper: PTEN, phosphatase and tensin homolog deleted from chromosome 10 (PTEN), and the inositol 5′-phosphatases SHIP1 and SHIP2. In the case of PTEN and SHIP1, extensive analysis by many investigators has demonstrated that these lipid phosphatases reduce activation of membrane-driven events modulated by the products of PI3K activity (for review, see Refs. 1–3). These products include phosphatidylinositol 3,4,5-trisphosphate (PI-3,4,5-P3), which promotes multiple cellular processes, such as migration, proliferation, and survival (4). Thus, understanding the mechanisms involved in the production and regulation of PI3K products has relevance for human health and disease.

To limit cellular activation by PI3K, inositol phosphatases dephosphorylate key residues on PI3K products. PTEN dephosphorylates PI3,4,5-P3 by removing the 3′-phosphate from the inositol ring, directly repressing the activity of PI3K (5, 6). Similar to PTEN, the inositol 5′-phosphatase SHIP1 reduces signals mediated by PI3K (7). However, in contrast to PTEN, SHIP1 expression is limited to hemopoietic cells and spermatogenetic cells (8) (9, 10). SHIP1 has been reported to regulate the activity of the serine/threonine kinase Akt (11, 12), leading to the inhibition of cell survival and proliferation (13, 14), by converting PI3,4,5-P3 to phosphatidylinositol 3,4-,disphosphate (PI-3,4-P2) (15, 16). The catalytic activity of SHIP1 requires the 3′ position on the inositol ring of PI3,4,5-P3 to be phosphorylated (14, 17), suggesting that PTEN and SHIP1 do not functionally overlap (18). The absence of SHIP1 in transgenic mice results in the accumulation of mononuclear cells in target organs, including the lung, resulting in a shortened life span of these mice compared with littermate animals with normal SHIP1 expression (19–21). In cells from patients with chronic myelogenous leukemia, the expression of SHIP1 is suppressed. Importantly, forced expression of SHIP1 or inhibition of the Abelson tyrosine kinase (Abl) with STI-571 in chronic myelogenous leukemia cell line results in the re-expression of SHIP1 and leads to the death of these cells, suggesting that the reduction in SHIP1 expression plays a causal role in enhanced cellular survival (22, 23).
SHIP2 appears to regulate many of the same processes as SHIP1. For example, in glioblastoma cells, the expression of SHIP2 reduces the activation of Akt and induces cell cycle arrest (29). The regulation of Akt by SHIP2 has important biological effects in animals; transgenic mice lacking SHIP2 have enhanced Akt activation in response to insulin and die from hypoglycemia in utero or within 2 days of birth. The prolonged Akt activation in these animals driven by insulin promotes the hypoglycemia (30).

Although SHIP2 resembles SHIP1 structurally and functionally, SHIP2 is transcribed as an independent gene product (31). Similar to SHIP1, SHIP2 contains a central 5′-inositol phosphatase domain to hydrolyze PI-3,4,5-P3 to generate PI-3,4-P2. The N terminal of SHIP2 contains an Src homology 2 (SH2) domain, which is postulated to bind the phosphorylated tyrosine of ITIM and ITAM. The C-terminal sequence of SHIP2 is significantly different from SHIP1 (for review, see Refs. 1 and 32). In its C-terminal region, SHIP2 contains a proline-rich domain (PRD), capable of interacting with SH3-containing proteins. Also, there is an NPXY site in its C terminus, which, when tyrosine-phosphorylated, can interact with phosphotyrosine binding domain-containing proteins. It has been shown that SHIP2 complexes with the adapter protein p130Cas via its SH2 domain to regulate cell adhesion and spreading (33). Similar to SHIP1, SHIP2 becomes tyrosine-phosphorylated and associates with Src in response to growth factor and cytokine stimulation. However, SHIP1 and SHIP2 also interact with distinct binding partners. For example, SHIP1 selectively binds the SH3 domain of Src and Grb2 (24), whereas SHIP2 selectively binds the SH3 domain of Abl, but not the SH3 domain of Grb2 (23, 34–36).

Previous studies have shown that the PRD of SHIP2 binds filamin to facilitate submembrane localization (37). The PRD also binds c-Cbl and c-Cbl-associated proteins (38).

Because SHIP2 is widely expressed in many cell types, including transformed myeloid cells and primary macrophages, and M-CSF is an important growth factor for these cells, this study focused on the function of SHIP2 in M-CSF-induced signaling. Upon ligand binding to the M-CSF receptors, the receptors dimerize, and auto- and transphosphorylation of tyrosine residues in cytoplasmic domains of the receptor subunits occurs (39, 40). These phosphorylated tyrosine residues initiate a cascade of signaling pathways, including PI3K, Src family kinases, STAT proteins, and Ras/ERK. We have previously demonstrated that M-CSF-induced monocyte survival is promoted in a PI3K-dependent manner, which involved, in part, activation of Akt (41, 42). Because SHIP2 down-regulates Akt activation in response to growth factor stimulation in other cells, we hypothesized that SHIP2 may also negatively regulate M-CSF-induced cellular signaling events.

Previous studies demonstrated that SHIP2 becomes tyrosine-phosphorylated after stimulation with FcγRIIb (43, 44) and FcγRIIIa cross-linking (45, 46). We have shown that the SH2 domain of SHIP2 is necessary for optimal association with FcγRIIa and for optimal SHIP2 tyrosine phosphorylation (45). In this study we focused on the defining the function and molecular mechanism of SHIP2 in regulating signaling events induced by M-CSF. SHIP2 becomes tyrosine-phosphorylated and translocates to the M-CSF receptor after M-CSF stimulation. Furthermore, we demonstrate interaction with filamin in the PRD, not the SH2 domain, of SHIP2, suggesting that filamin binding may account for the membrane translocation of SHIP2. Interestingly, the SH2 domain of SHIP2 does not appear to mediate SHIP2 membrane targeting or function in response to M-CSF, whereas the proline-rich motif is important in regulating membrane targeting. In transient transfection experiments, the expression of SHIP2 reduces Akt activity and inhibits NF-κB-regulated gene transcription in M-CSF-stimulated cells. Finally, fetal liver-derived macrophages from SHIP2-deficient mice have augmented Akt activation in response to M-CSF stimulation. Collectively, these data delineate a novel role for SHIP2 in M-CSF signaling.

Materials and Methods

Reagents and Abs

Recombinant human M-CSF was purchased from R&D Systems (Minneapolis, MN), and endotoxin was removed by END-X B15 endotoxin removal affinity resin (Seikagaku America, Falmouth, MA). RPMI 1640 medium used for mouse bone marrow macrophage culture was obtained from BioWhittaker (Walkersville, MD). RPMI 1640 medium and DMEM were obtained from Invitrogen Life Technologies (Grand Island, NY). For cell culture, FBS was purchased from HyClone (Logan, UT). Polyvinylbtxin B sulfate was obtained from Calbiochem (San Diego, CA). SHIP2 rabbit serum Ab and rabbit polyclonal Ab were gifts from Drs. B. Clarkson and D. Wisniewski (Memorial Sloan-Kettering Cancer Center, New York, NY) (24). The Akt kinase assay kit, anti-phospho-Akt and anti-phosphotyrosine Abs were purchased from Cell Signaling Technology (Beverly, MA). Anti-Xpress Ab was purchased from Invitrogen Life Technologies (Carlsbad, CA). Monoclonal and polyclonal c-Fms/CSF-1R and Akt1 Abs were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-α-actinin 100 was purchased from Bioreagents (Golden, CO). Anti-heparin sulfate (anti-HA) mAb and anti-HA affinity matrix were purchased from Roche (Indianapolis, IN). Anti-fibronectin mAb was obtained from Abcam (Cambridge, MA). All other reagents were obtained from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA) unless otherwise specified. Xpress-tagged cDNAs of murine full-length, wild-type SHIP2 (Xpress-SHIP2 WT), catalytic-inactive SHIP2 (Xpress-SHIP2 D608A), and an SH2 point mutation of SHIP2 (Xpress-SHIP2 R47K) cloned into pCDNA3 vector were provided by Dr. S. Moodie (Metabolon, Hayward, CA). HA-tagged cDNAs of human full-length SHIP2 (HA-SHIP2 WT), truncation mutation of PRD (HA-SHIP2 2PRD) and PRD (HA-SHIP2 PRD) cloned into the pCGN vector were generated as described previously (37). GST-Akt was a gift from Dr. R. B. Pearson (Peter MacCallum Cancer Institute, Melbourne, Australia). Enhanced GFP cDNA was obtained from BD Clontech (Palo Alto, CA).

Cell culture

Cells were cultured in medium supplemented with FBS and antibiotic-antimycotic (1000 U/ml penicillin, 1000 μg/ml streptomycin sulfate, and 250 ng/ml amphotericin B) at 37°C. THP-1 cells and the murine macrophage cell line RAW264 were obtained from American Type Culture Collection (Manassas, VA). THP-1 cells were cultured in RPMI 1640 medium supplemented with 10% FBS, and RAW264 cells were cultured in RPMI 1640 medium supplemented with 3.5% FBS. Murine NIH-3T3 fibroblasts engineered to express the human M-CSF receptor (3T3/fms) were generated as previously described (47). NIH-3T3 and 3T3/fms fibroblasts were maintained in DMEM supplemented with 10% FBS.

Bone marrow macrophage isolation and culture

Femoral and tibial bone marrow-derived macrophages (BMM) were obtained from C57BL/6 mice. Briefly, bone marrow progenitor cells were flushed out with ice-cold RPMI 1640 medium, then plated in RPMI 1640 supplemented with 10% FBS, antibiotic-antimycotic, 10 μg/ml polymyxin B, and 20 ng/ml M-CSF. Cells were cultured at 37°C for 5 days, with the addition of 20 ng/ml M-CSF each day. In culture, mononuclear phagocytes attached to the bottom of the plates and differentiated to macrophages.

BMM were serum-starved for 12–16 h at 37°C before restimulation with 100 ng/ml M-CSF.

Fetal liver macrophage isolation and culture

SHIP2 gene knockout mice were generated as previously reported (30). The mice were bred, and the pregnant mothers were killed on days 17–18 of gestation. Fetal livers were harvested, and single-cell suspensions were obtained by passing the minced liver through an 18-gauge TW syringe, followed by centrifugation. The cells were differentiated into fetal liver-derived macrophages as described for BMM. The embryos were genotyped to identify SHIP2 alleles. Briefly, the DNA was isolated from fetal tissue using the DNeasy Tissue kit (Qiagen, Valencia, CA), and PCR was performed using the following primers: primer 1 5′-cacacaggttccacata-3′; primer 2 5′-gcttgctgatgccgtag-3′; and primer 3 5′-cgaattatattttgttgcg-3′. Primers 1 and 2 were used to identify the wild-type allele of 530 bp, and primers 2 and 3 were used to identify the knockout allele of 270 bp. Fetal liver cells were cultured at 37°C for 6 days, with the addition of 20 ng/ml M-CSF each day. The cells were removed from culture dish using

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Accutase (Chemicon International, Temecula, CA), resuspended in a fresh plate, and serum-starved for 12–16 h at 37°C before restimulation with 100 ng/ml M-CSF.

**Preparation of human alveolar macrophages**

Human alveolar macrophages were obtained from healthy lifetime non-smoking donors by bronchoalveolar lavage. Cells were washed twice with PBS, counted, and analyzed by Diff-Quick staining for purity. Cell preparations were >95% positive for macrophages.

**Immunoprecipitation and Western blotting**

THP-1 cells were stimulated and lysed in ice-cold TN1 buffer (50 mM Tris (pH 8.0), 10 mM EDTA, 10 mM Na3PO4, 10 mM NaF, 1% Triton X-100, 125 mM NaCl, 1 mM Na3VO4, and 10 μg/ml each of aprotinin and leupeptin), whereas NIH-3T3 cells, 3T3/FLS cells, and BMM were lysed in ice-cold Akt A buffer (50 mM Tris-HCl (pH 7.5), 1% Triton X-100, 1 mM EDTA (pH 8.3), 1 mM EGTA (pH 8.0), 50 mM NaF, 10 mM β-glycerophosphate, 5 mM sodium pyrophosphate, 1 mM Na3VO4, 10 μg/ml each of aprotinin and leupeptin, and 1 mM PMSF) on ice for 15 min. Nuclei were removed by centrifugation at 16,000 x g for 10 min, a protein assay (Bio-Rad, Hercules, CA) was performed on the supernatants, and equal amounts of protein were immunoprecipitated with the appropriate Abs overnight at 4°C. Immune complexes were collected with protein G beads (Invitrogen Life Technologies, Grand Island, NY) at 4°C for 1 h. The beads were washed three times in ice-cold lysis buffer, boiled in Laemmli sample buffer (62.5 mM Tris (pH 6.8), 2% SDS, 10% glycerol, 0.01% bromophenol blue, and 2-ME), and separated by SDS-PAGE. The proteins were then transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH), probed with the indicated Ab, and detected by ECL (Amersham Biosciences, Piscataway, NJ). Membranes were either exposed to BioMax x-ray films (Eastman Kodak, Rochester, NY) or analyzed by Fluor S Multiimager (Bio-Rad).

**Subcellular fractionation**

Cells were washed with ice-cold PBS and resuspended in 500 μl of buffer A (20 mM Tris (pH 7.5), 5 mM MgCl2, 1 mM EGTA, 20 mM β-glycerophosphate, 1 mM PMSF, 1 mM Na3VO4, and 20 μg/ml aprotinin and leupeptin). The cells were sonicated four times for 5 s each time and then centrifuged at 700 x g for 5 min. The supernatant was centrifuged at 100,000 x g for 30 min in a TLA-120 rotor (Beckman Coulter, Fullerton, CA) at 4°C. The supernatant (cytosol fraction) was removed and saved. The pellet containing the membrane fraction was washed in buffer A, then resuspended in buffer A with 1% Nonidet P-40 and rotated at 4°C for 1 h. Samples were centrifuged again at 100,000 x g for 30 min, and the supernatant (membrane fraction) was recovered.

**Akt in vitro kinase assays**

Akt in vitro kinase activity was measured using the assay kit from Cell Signaling Technology following the manufacturer’s protocol. In this method, Akt kinase activity was assayed using a peptide derived from glycogen synthase-3-kinase (GSK3) as a substrate. The reaction products were analyzed on a 12% SDS-polyacrylamide gel. After electrophoresis, proteins were transferred onto a nitrocellulose membrane and probed with phospho-GSK3 Ab.

**Transit transfection of 3T3/fms cells**

Xpress-tagged SHIP2 constructs or HA-tagged SHIP2 constructs were transfected into 3T3/fms cells using Effectene Transfection Reagent from Qiagen. The manufacturer’s protocol was followed with minor modification. Briefly, the cells were transfected and incubated in DMEM supplemented with 10% FBS for 48 h at 37°C; cells were then quenched in serum-free medium for another 6 h before stimulation with 100 ng/ml M-CSF. For cotransfection experiments, a ratio of 1:4 (GST-Akt:SHIP2) was used. Cells were harvested after 48 h, lysed, and immunoprecipitated with glutathione-Sepharose beads (Amersham Biosciences).

**Transit transfection of THP-1 cells and luciferase assay**

Xpress-tagged cDNA and HA-tagged cDNA constructs were transfected into THP-1 cells using Nucleofector Kit V (Amaxa, Cologne, Germany). The manufacturer’s protocol was followed with minor modification. Briefly, the cells were transfected with 4 μg of SHIP2 constructs or empty vector (1 μg of EF1α-Luc vector), then incubated in RPMI supplemented with 10% FBS for 24 h at 37°C. Cells were then quenched in serum-free medium for 4 h and activated with 100 ng/ml M-CSF for 5 h at 37°C. Luciferase activity was measured with the Luciferase Assay System using Luminometer (Promega, Madison, WI). Data are presented as the relative fold increase in M-CSF-stimulated sample readout over nonstimulated sample readout and are expressed as the mean ± SD.

**Statistical analysis**

The ECL signal was quantitated with Quantity One densitometry program (Bio-Rad). Phosphorylation data were normalized and expressed as the fold change from nonstimulated samples. All data are expressed as the mean ± SD derived from at least three independent experiments. Statistical analysis was performed with paired two-tailed Student’s t test. Statistical significance was defined as p < 0.05.

**Results**

**SHIP2 is tyrosine-phosphorylated upon M-CSF stimulation**

Previous work from our group demonstrated that SHIP2 is expressed in transformed myeloid cells and primary macrophages, and that LPS treatment induces human PBMC to express this phosphatase. Moreover, SHIP2 tyrosine phosphorylation is induced by FcγRIIa clustering in human monocytic THP-1 cells (45). To determine the effect of M-CSF stimulation on the function of SHIP2, we first evaluated whether M-CSF induced SHIP2 tyrosine phosphorylation in macrophages and monocytic cell lines. As shown in Fig. 1, SHIP2 was constitutively expressed in human alveolar macrophages (Fig. 1A), human myeloid THP-1 cells (Fig. 1B), ex vivo murine BMM (Fig. 1C), and the murine macrophage cell line RAW264 (Fig. 1D). Human M-CSF induced SHIP2 tyrosine phosphorylation in these cells. Tyrosine phosphorylation of SHIP2 was maximal 2–5 min after M-CSF stimulation and decreased after 30 min.

Murine NIH-3T3 fibroblasts engineered to express human M-CSF receptor (3T3/FLS) were next used for transfection studies. Because 3T3/FLS cells do not express SHIP1, we used this cell line as a model to dissect the function of SHIP2 in M-CSF signaling without interference from endogenous SHIP1. After M-CSF stimulation, SHIP2 was tyrosine-phosphorylated in murine NIH-3T3/FLS cells expressing human M-CSF receptors, but not in native 3T3 fibroblasts (Fig. 1E). We confirmed that the human M-CSF receptor is expressed in 3T3/FLS fibroblasts, but not in native NIH-3T3 fibroblasts (Fig. 1F). These data suggest that SHIP2 is tyrosine-phosphorylated in response to M-CSF stimulation in cells bearing M-CSF receptors.

**SHIP2 down-regulates NF-κB-dependent gene transcription in human monocytic THP-1 cells**

Having shown M-CSF induces SHIP2 tyrosine phosphorylation, we next assessed whether SHIP2 affected M-CSF-regulated functional events in THP-1 human monocytic cells. It is known that M-CSF is involved in activating the NF-κB transcription factor in human PBMC (48) and T cells (49). Furthermore, in human monocytic THP-1 cells, SHIP1 down-regulates NF-κB-dependent gene transcription initiated by M-CSF stimulation, and this regulation requires the SH2 domain of SHIP1 (50). Because SHIP1 and SHIP2 have the same enzymatic activity, we analyzed whether SHIP2 also regulates NF-κB-mediated gene transcription.

First, the NF-κB binding element coupled with firefly luciferase gene (NF-κB-Luc) was transiently transfected into THP-1 cells along with plasmids encoding Xpress-tagged empty vector (pcDNA3), wild-type SHIP2 (Xpress-SHIP2 WT), catalytically inactive SHIP2 (Xpress-SHIP2 D608A), or SH2 nonfunctioning mutant SHIP2 (Xpress-SHIP2 R47K) cDNA. Luciferase activity was measured after stimulating the transfected cells with M-CSF for 5 h to determine the amount of NF-κB-mediated gene transcription (Fig. 2A). The data showed a 2-fold increase in NF-κB-mediated gene transcription.
transcription after M-CSF stimulation in cells expressing NF-κB-Luc and empty vector pcDNA3. Wild-type, full-length SHIP2 reduced NF-κB transcriptional activity to the basal level, whereas catalytically inactive Xpress-SHIP2 D608A enhanced NF-κB transcriptional activity by 2-fold over wild-type SHIP2-transfected cells. Interestingly, Xpress-SHIP2 R47K lacking the functional SH2 domain of SHIP2 decreased NF-κB transcriptional activity to the same degree as wild-type SHIP2. These data suggest that SHIP1 and SHIP2 regulate downstream events through different protein interaction domains, and that SHIP2 does not need functional SH2 domains for its catalytic activity.

Secondly, NF-κB-Luc was transiently transfected into THP-1 cells along with plasmids encoding HA-tagged empty vector (pCGN), wild-type SHIP2 (HA-SHIP2 WT), or the PRD of SHIP2 (HA-SHIP2 PRD) cDNA. NF-κB-mediated gene transcription was evaluated by quantifying luciferase activity in cells stimulated with M-CSF for 5 h (Fig. 2B). Consistent with the above observations, the expression of wild-type SHIP2 decreased NF-κB transcriptional activity by 2-fold compared with that in mock-transfected cells (pCGN; p < 0.05). Importantly, expression of the PRD domain of SHIP2 significantly enhanced NF-κB transcriptional activity in M-CSF-stimulated THP-1 cells compared with transfection of wild-type SHIP2 in these cells (p < 0.05). These data imply that the PRD of SHIP2 plays an important role in regulating NF-κB-mediated gene transcription in response to M-CSF.

SHIP2 localizes to the M-CSF receptor after M-CSF stimulation

Because the phosphoinositide substrates of SHIP2 are located on the inner wall of the plasma membrane and membrane targeting of this phosphatase is important for its function, we next determined whether SHIP2 interacts with the M-CSF receptor upon M-CSF stimulation. The results in Fig. 3A (upper panel) indicate that native SHIP2 associated with the M-CSF receptor after stimulation in 3T3/fms cells. The recruitment of SHIP2 to the M-CSF receptor correlated with tyrosine phosphorylation of the receptor (middle panel). As shown in Fig. 3B, transfected Xpress-tagged SHIP2 also associated with the M-CSF receptor after M-CSF stimulation of 3T3/fms cells, suggesting that SHIP2 may target to the M-CSF receptor.

Of note, 3T3/fms fibroblasts were used as a model for M-CSF signaling studies for SHIP2, because these cells do not express native SHIP1 and have high transfection efficiency (80% as assayed by transfection of the enhanced GFP (data not shown). In addition, these experiments did not examine the association between SHIP2 and the M-CSF receptor in primary murine macrophages, human THP-1 cells, or human monocytes. Although these
cells are important models, the expression of the M-CSF receptor on the surface of these cells is rapidly down-regulated by the addition of M-CSF (51, 52), and these cells are very difficult to transfect, making them difficult models for these studies.

**SHIP2 membrane localization is dependent on the PRD, but not on the SH2 domain**

We next explored the molecular mechanism of the regulation of SHIP2. Deletion constructs of SHIP2 were used to determine which interaction domains regulate membrane localization and function during M-CSF receptor-mediated activation.

The following SHIP2 constructs were transiently transfected into 3T3/fms cells: Xpress-SHIP2 WT, Xpress-SHIP2 D608A, or Xpress-SHIP2 R47K. It was found that each of the transfected SHIP2 gene products localized to the membrane upon M-CSF stimulation (Fig. 4A). Interestingly, after transfection, the HA-SHIP2 ΔPRD gene product did not localize to the cell membrane upon M-CSF stimulation, whereas the HA-SHIP2 PRD product moved to the cell membrane upon M-CSF stimulation (Fig. 4B). To ensure that membrane and cytosolic preparations were adequately separated, these fractions were stained with Abs directed against the membrane protein α-adaptin and the cytosolic protein ERK. As shown in Fig. 4C, α-adaptin was largely found in the membrane fraction, whereas ERK was found in the cytosolic fraction. As a control, these transfection studies were repeated in 3T3 fibroblasts lacking human M-CSF receptors. Transfected SHIP2 did not translocate to the membrane in these cells regardless of the SH2 domain, of SHIP2 is necessary to facilitate membrane translocation in response to M-CSF stimulation.

**SHIP2 reduces the phosphorylation and activation of Akt in M-CSF-treated cells**

To further test the importance of the PRD of SHIP2 in membrane targeting and the subsequent functional effects of SHIP2, the following experiments were performed. Cells were cotransfected with various mutant constructs of SHIP2 along with GST-Akt, then stimulated with M-CSF, and Akt phosphorylation was measured.

In the first set of experiments, the function of the catalytic domain and SH2 domain of SHIP2 was evaluated by transiently transfecting GST-Akt along with wild-type SHIP2, catalytic mutant SHIP2, or SH2 mutant SHIP2 constructs. The transfected cells were harvested 48 h after transfection and stimulated with M-CSF. The expressed GST-Akt protein was isolated using glutathione-Sepharose beads and subjected to SDS-PAGE. As shown in Fig. 5A, GST-Akt was isolated with glutathione-Sepharose beads and subjected to SDS-PAGE.
the expression of catalytic deficient Xpress-SHIP2 D608A enhanced the phosphorylation of Akt in response to M-CSF stimulation. These results suggest that the catalytically inactive mutant SHIP2 successfully competes with endogenous SHIP2 to liberate Akt activation by M-CSF.

Notably, SH2 mutant of SHIP2 (Xpress-SHIP2 R47K) reduced Akt activity in M-CSF-stimulated cells almost to the level of the wild-type construct, indicating that the SH2 domain of SHIP2 was not critical for regulating Akt phosphorylation in response to M-CSF stimulation. The membrane was probed with total Akt to show equal loading (Fig. 5A, middle panel). The graph in Fig. 5A (lower panel) represents pooled densitometric analysis from three experiments identical with that in the upper panel of Fig. 5A. To ensure equal expression of the Xpress-tagged SHIP2 proteins, cell lysates were subsequently immunoprecipitated with anti-Xpress Ab and resolved by SDS-PAGE, and the membrane was probed with the anti-SHIP2 Ab (Fig. 5B).

In addition to measuring Akt phosphorylation, Akt kinase activity using GSK3 as a substrate is also shown. Expression of the catalytic-deficient SHIP2 construct (Xpress-SHIP2 D608A) enhanced Akt kinase activity in M-CSF-stimulated cells, whereas expression of either wild-type SHIP2 or the SH2 mutant SHIP2 (Xpress-SHIP2 R47K) reduced Akt kinase activity in response to M-CSF in 3T3/fms cells (Fig. 5C). In control experiments performed in 3T3 cells that do not express M-CSF receptors, M-CSF had no effect on Akt activation (Fig. 5D). These data are further evidence that the SH2 domain of SHIP2 is not required for its phosphatase activity.

In a second set of experiments, HA-SHIP2 WT, HA-SHIP2 PRD, or empty vector (HA-pCGN) was transiently transfected into 3T3/fms cells. As expected, the expression of HA-SHIP2 WT decreased phosphorylation of Akt in M-CSF-stimulated cells. In contrast, transfection of HA-SHIP2 PRD into 3T3/fms cells enhanced Akt activation in response to M-CSF stimulation (Fig. 6A). To ensure equal transfection of the HA-tagged SHIP2 proteins, cell lysates were subsequently immunoprecipitated with anti-HA Ab and resolved by SDS-PAGE, and the membrane was probed with the anti-SHIP2 Ab (Fig. 6B). These observations suggest that the PRD of SHIP2 is critical for its biological function.

The PRD of SHIP2 mediates the binding of SHIP2 and filamin in 3T3/fms cells

Because SHIP2 can translocate to cell membrane and bind to the M-CSF receptor, and this process may be facilitated by the PRD of SHIP2, we next investigated the membrane targets of SHIP2. The binding of SHIP2 and filamin is proposed to regulate SHIP2 membrane targeting and PI3K signaling in the cytoskeleton (37). We therefore investigated whether SHIP2 associates with filamin through PRDs and whether this association is induced by M-CSF. 3T3/fms cells were transiently transfected with Xpress-SHIP2, Xpress-SHIP2 R47K or the vector control pcDNA3 in one set of experiments and with HA-SHIP2 WT, HA-SHIP2 PRD, or the empty vector HA-pCGN in another set of experiments. The cells were harvested 48 h after transfection and stimulated with M-CSF. Cell lysates were isolated and immunoprecipitated with anti-Xpress or anti-HA Ab and resolved by SDS-PAGE, and the membrane was probed with the anti-filamin Ab. As shown in Fig. 7, wild-type SHIP2 (both Xpress-tagged and HA-tagged) and SH2 mutant SHIP2 (Xpress-SHIP2 R47K) associated with filamin, whereas PRD mutant SHIP2 (HA-SHIP2 ΔPRD) failed to interact with filamin regardless of whether the receptor was activated.
FIGURE 5. SHIP2 suppresses Akt phosphorylation and kinase activity. A, 3T3/fms fibroblasts (1 × 10⁷/condition) were transiently cotransfected with GST-Akt and Xpress-tagged pcDNA3 vector, Xpress-SHIP2 WT, Xpress-SHIP2 D608A, or Xpress-SHIP2 R47K. Cells were transfected for 48 h and quiesced for 6 h on ice before stimulation with M-CSF (100 ng/ml) for 2 min. Cell lysates were incubated with glutathione-Sepharose beads to isolate GST-Akt. Phosphorylation of GST-Akt was assessed by Western blotting with combined anti-phospho-Ser473 Akt Ab and anti-phospho-Thr 308 Akt Ab (upper panel) or anti-Akt Ab (middle panel). The lower panel represents pooled densitometric analysis of Akt phosphorylation from three independent experiments identical with those shown in the upper panel. Akt phosphorylation was higher in mock-transfected cells vs cells transfected with Xpress-SHIP2 WT or Xpress-SHIP2 R47K when stimulating with M-CSF (p < 0.05 vs wild-type SHIP2 and SH2 mutant R47K SHIP2). B, Xpress-tagged proteins were isolated from cell lysates with anti-Xpress Ab, the proteins were separated by SDS-PAGE, followed by Western transfer, and the membrane was probed with anti-SHIP2 Ab. C, GSK3 phosphorylation after M-CSF stimulation of cells transfected with Xpress-SHIP2 WT, Xpress-SHIP2 D608A, or Xpress-SHIP2 R47K. Phosphorylation was detected using anti-phospho-GSK3 (pGSK3) Ab. Membranes were reprobed with anti-Akt Ab to ensure equal loading. The lower panel represents pooled densitometric analysis of GSK3 phosphorylation from three experiments identical with those shown in the upper panel. GSK3 phosphorylation was greater in mock-transfected cells vs Xpress-SHIP2 WT or Xpress-SHIP2 R47K cells when stimulated with M-CSF (p < 0.05 vs GST-Akt plus SHIP2 WT or GSK-Akt plus SHIP2 R47K). D, NIH-3T3 fibroblasts (1 × 10⁷/condition) lacking M-CSF receptor (3T3) or with M-CSF receptor (3T3/mcs) were stimulated with M-CSF (100 ng/ml). Cell lysates were immunoprecipitated with anti-Akt Ab. After SDS-PAGE and membrane transfer, the membrane was probed with anti-Akt Ab. The cell lysates were subsequently immunoprecipitated with anti-M-CSF receptor (anti-M-CSFR) Ab, and the membrane was probed with anti-M-CSFR Ab. Shown are representative data from three independent experiments.
of M-CSF stimulation. Thus, the association of SHIP2 and filamin does not appear to depend on the SH2 domain of SHIP2 or M-CSF.

SHIP2 knockout mice macrophages enhance sensitivity in respond to M-CSF

We next obtained SHIP2 gene deletion mice to test the function of SHIP2 in M-CSF signaling in cells still expressing native SHIP1. Because homozygous deficiency in SHIP2 gene leads to prenatal death or death 1–2 days after birth (30), macrophages were derived from fetal liver on days 17–18 of gestation. The fetal liver cells were differentiated into macrophage with addition of 20 ng/ml M-CSF each day for 6 days. In contrast, cells isolated similarly, but not exposed to M-CSF, did not have macrophage-like cells (Fig. 8A). We confirmed that the fetal liver-derived macrophages expressed M-CSF receptors (Fig. 8B, upper panel) and responded to M-CSF by phosphorylating Akt (Fig. 8B, middle and lower panels). Furthermore, M-CSF-stimulated fetal liver cells were subjected to flow cytometric analysis as defined by surface expression of CD11b, CD14, CD16, and CD32 Ags (data not shown). We confirmed the genotype of each embryo before additional analysis of the macrophages from fetal liver cells (Fig. 8C). We analyzed the presence of SHIP2 and SHIP1 protein in the cell lysate from wild-type and SHIP2-null mice with Western blot analysis (Fig. 8D). The cells from SHIP2 knockout mice and their wild-type littermates were stimulated with 100 ng/ml M-CSF for 0–60 min. In these studies, Akt phosphorylation and GSK3 phosphorylation through the kinase activity of Akt from these cells were used to define Akt activity (Fig. 8E). The results show that macrophages from SHIP2 knockout mice had greater activation of Akt and prolonged activation of Akt compared with wild-type cells.

Discussion

The PI3K product PI-3,4,5-P3 plays an important role in regulating a variety of cellular responses to extracellular signals. The concentration of this signaling intermediate is low in resting cells, but rapidly

FIGURE 6. The PRD of SHIP2 is crucial in suppressing Akt phosphorylation. A, 3T3/fms fibroblasts (1 × 10⁵/conidition) were transiently cotransfected with GST-Akt and HA-tagged pCGN vector, HA-SHIP2 WT, or HA-SHIP2 PRD. Cells were transfected for 48 h and quiesced for 6 h on ice before stimulation with M-CSF (100 ng/ml) for 2 min. Cell lysates were incubated with glutathione-Sepharose beads, and phosphorylation of GST-Akt was assessed by Western blotting with anti-phospho-Akt Ab (upper panel) and anti-Akt Ab (middle panel). The lower panel represents pooled densitometric analysis of GST-Akt phosphorylation from three experiments identical with those shown in the upper panel. *, p < 0.05 vs GST-Akt plus SHIP2 WT. B, HA-tagged proteins were isolated from the lysates using anti-HA Ab, the proteins were separated by SDS-PAGE, followed by Western transfer, and the membrane was probed with anti-SHIP2 Ab. Shown are representative data from two independent experiments.

FIGURE 7. The PRD is critical for the binding of SHIP2 with endogenous filamin. A, 3T3/fms fibroblasts (1 × 10⁵/conidition) were transiently transfected with Xpress-tagged pcDNA3 vector, Xpress-SHIP2 WT, or Xpress-SHIP2 R47K. Forty-eight hours post-transfection, the cells were quiesced for 6 h on ice before stimulation with M-CSF (100 ng/ml) for 5 min. Cell lysates were immunoprecipitated with anti-Xpress Ab, and the proteins were separated by 8% SDS-PAGE. The membrane was probed with mouse anti-filamin Ab (upper panel), stripped, and reprobed with mouse anti-Xpress Ab (lower panel). B, 3T3/fms fibroblasts (1 × 10⁵/conidition) were transiently transfected with HA-tagged pCGN vector, HA-SHIP2 WT, or HA-SHIP2 ΔPRD. Cells were transfected for 48 h and quiesced for 6 h on ice before stimulation with M-CSF (100 ng/ml) for 5 min. Cell lysates were immunoprecipitated with anti-HA Ab, and the proteins were separated by 8% SDS-PAGE. The membrane was probed with mouse anti-filamin Ab (upper panel), stripped, and reprobed with mouse anti-HA Ab (lower panel). Shown are representative data from two independent experiments.
The activity was measured using GSK3 as substrate, and the reaction mixture was restimulated with M-CSF (100 ng/ml) for different time periods. Equal amounts of protein from the different conditions were resolved by Western blotting. The membrane was probed with SHIP2 Ab (lower panel) and then for SHIP2 (middle panel). Shown are duplicate data from two independent experiments.

Several studies suggested that in response to growth factor stimulation, SHIP1 and SHIP2 have overlapping functions, including negatively regulating Akt activity and NF-κB gene transcription in response to M-CSF (1, 53). However, in contrast to SHIP1, which relies on SH2 interactions with proteins such as Lyn for membrane targeting and function in response to M-CSF stimulation (22, 54, 55), SHIP2 requires different binding partners to facilitate membrane targeting. We have demonstrated that wild-type SHIP2 constitutively associates with filamin. The deletion of the PRD in SHIP2 abolished this association and reduced the function of SHIP2 in M-CSF signaling. This observation is consistent with previous reports that SHIP2 interacts with filamin in the yeast two-hybrid system. Filamin appears to play an important role in membrane location of SHIP2, because SHIP2 is only found in the cytosolic cell fraction in filamin-deficient cells (37). All mammalian cells have three major filamin isoforms (A, B, and C), which are primarily localized in the cortical cytoplasm subjacent to the plasma membrane and are differentially expressed in various tissues (56, 57). Filamin functions as a cross-linking protein between actin filaments to regulate cell migration and membrane stability (58, 59). Thus, the association of the PRD of SHIP2 with filamin may also play an important role in the negative regulation of Akt by SHIP2 in M-CSF-stimulated cellular signaling. In our cell system, filamin is not highly expressed (data not shown); therefore, we cannot exclude the fact that some other cytoskeletal proteins may also interact with SHIP2 and assist its translocation to the cell membrane. Although the association with filamin is constitutive, the association of SHIP2 with the M-CSF receptor occurs only after M-CSF stimulation. We are currently investigating the roles of both events in the regulation of M-CSF signaling. Recently, Kobayashi et al. (60) reported that SHIP2 predominantly regulates Akt2, not Akt1, phosphorylation at the plasma membrane in response to insulin in 3T3-L1 adipocytes, suggesting that there may also be alternative targets that SHIP1 and SHIP2 primarily regulate.

In contrast to SHIP2, SHIP1 relies on a functional SH2 domain for the catalytic function. In addition, SHIP1 is tightly regulated by domains in the C-terminal portion of the protein (9). Studies have shown that the C terminus of SHIP1 plays a role in hydrolysis of phosphatidylinositol 4,5-trisphosphate and inhibits the degranulation of mast cells (61). It is clear that noncatalytic portions of the protein phosphatase are important for its activity (22, 54, 62). Given that SHIP1 and SHIP2 vary most in the C-terminal region (32), it is
Moreover, in cells transformed by the restricting activation of PI3K and Akt in insulin signaling (30). The hypoglycemia is related to an important role of SHIP2 in cence in SHIP2 die of hypoglycemia prenatally or soon after birth. The hypoglycemia is related to an important role of SHIP2 in many organs. These animals die prematurely of lung injury and respiratory failure (19, 21). In contrast, animals deficien in SHIP2 die of hypoglycemia prenatally or soon after birth.

The regulation of cell survival and that of downstream signaling events by SHP1 and SHP2 may be modulated differently; this needs to be further investigated. In SHP1-deficient mice, the Akt phosphorylation level is prolonged for up to 60 min upon M-CSF stimulation and decreases by 120 min, possibly due to the presence of other phosphatases, such as SHIP2 and PTEN.

We found that SHIP2 is present in SHP1-deficient BMM (data not shown), but does not replace SHIP1 in reducing Akt phosphorylation in response to M-CSF stimulation for the first 60 min (50). Similarly, in SHP2-deficient murine macrophages, SHIP1 is present, and in response to M-CSF stimulation, Akt activation was enhanced and prolonged. Interestingly, a difference between these two macrophage types is the basal activity of Akt. In SHP1-deficient cells, Akt appeared to have constitutive activity in these macrophages, whereas basal Akt activity was reduced in SHP2-deficient cells to that seen in wild-type cells.

In summary, this study demonstrates that SHP2 participates in the negative regulation of M-CSF-induced cellular activation both in vitro and ex vivo. Moreover, the catalytic domain of SHP-2 is necessary for the negative regulation of M-CSF-stimulated Akt activation and NF-κB gene transcription. However, although the SH2 domain of SHP1 is important in the negative regulation of M-CSF signaling, the PRD of SHP2 seems to primarily regulate membrane translocation and M-CSF receptor binding. These data begin to elucidate a novel functional role for SHP2 in M-CSF-stimulated cellular activation and suggest a distinct mechanism of regulation from that of SHP1. Future studies will clarify the different functional roles for SHP1 and SHP2 in response to M-CSF in myeloid cells.

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SHIP2 REGULATES M-CSF-INDUCED SIGNALING


