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Mutational Analysis of the Mechanism of Negative Regulation by Src Homology 2 Domain-Containing Protein Tyrosine Phosphatase Substrate-1 of Phagocytosis in Macrophages

Hiroshi Ikeda,* Hideki Okazawa,* Hiroshi Ohnishi,* Yoji Murata,* Per-Arne Oldenborg,† and Takashi Matozaki2*‡

Src homology 2 domain-containing protein tyrosine phosphatase substrate-1 (SHPS-1) is a transmembrane protein predominantly expressed in macrophages. The binding of CD47 on RBCs to SHPS-1 on macrophages is implicated in inhibition of phagocytosis of the former cells by the latter. We have now shown that forced expression in mouse RAW264.7 macrophages of a mutant version (SHPS-1-4F) of mouse SHPS-1, in which four tyrosine phosphorylation sites are replaced by phenylalanine, markedly promoted FcR-mediated phagocytosis of mouse RBCs or SRBCs. Forced expression of another mutant form (SHPS-1-ΔCyto) of mouse SHPS-1, which lacks most of the cytoplasmic region, did not promote such phagocytosis. Similarly, forced expression of a rat version of SHPS-1-4F, but not that of rat wild-type SHPS-1 or SHPS-1-ΔCyto, in RAW264.7 cells enhanced FcR-mediated phagocytosis of RBCs. Tyrosine phosphorylation of endogenous SHPS-1 as well as its association with Src homology 2 domain-containing protein tyrosine phosphatase-1 were not markedly inhibited by expression of SHPS-1-4F. Furthermore, the attachment of IgG-opsonized RBCs to RAW264.7 cells was markedly increased by expression of SHPS-1-4F, and this effect did not appear to be mediated by the interaction between CD47 and SHPS-1. These data suggest that inhibition by SHPS-1 of phagocytosis in macrophages is mediated, at least in part, in a manner independent of the transinteraction between CD47 and SHPS-1. In addition, the cytoplasmic region as well as tyrosine phosphorylation sites in this region of SHPS-1 appear indispensable for this inhibitory action of SHPS-1. Moreover, SHPS-1 may regulate the attachment of RBCs to macrophages by an as yet unidentified mechanism. The Journal of Immunology, 2006, 177: 3123–3132.

Macrophages are professional phagocytes that play an important role in innate and acquired immunity as a result of their ability to internalize and degrade pathogens (1, 2). They also contribute to preservation of tissue integrity and function by engulfing apoptotic bodies and toxic particles (1, 3). Phagocytosis occurs by extension of the plasma membrane of the macrophage around an extracellular target and subsequent internalization of the object within a membrane-bounded intracellular phagosome (4). The mechanisms by which phagocytosis is triggered by the interaction of ligands with specific receptors on the macrophage surface, including FcγRs (for IgG-coated particles), complement receptors (for C3bi-opsonized particles), scavenger receptors, and phosphatidylinerse receptors (for apoptotic cells), have been relatively well characterized (1, 3–5). In contrast, the molecular mechanisms for negative regulation of phagocytosis in macrophages remain largely unknown. However, we and others have recently shown that the interaction of Src homology 2 domain-containing protein tyrosine phosphatase (SHP)3 substrate-1 (SHPS-1) on macrophages with CD47 on a phagocytic target triggers such negative regulation of phagocytosis in macrophages (6–9).

SHPS-1 (10, 11), also known as signal regulatory protein α (12), brain Ig-like molecule with tyrosine-based activation motif (13), and p84 neural adhesion molecule (14), is a member of the Ig superfamily of proteins. It was initially discovered as a tyrosine-phosphorylated transmembrane protein that binds and serves as a substrate for SHP-1 or SHP-2 (10, 15), both of which are SHPs. The putative extracellular region of SHPS-1 comprises three Ig-like domains, whereas its cytoplasmic region contains four YXX(L/V/I) motifs, which are putative tyrosine phosphorylation sites and binding sites for the Src homology 2 domains of SHP-1 and SHP-2 (10–12, 15). SHPS-1 is especially abundant in macrophages and neurons (13, 14, 16, 17).

CD47, which has been implicated as a ligand for SHPS-1 (18, 19), was originally identified in association with αβ integrin and is also a member of the Ig superfamily, possessing an Ig-V-like extracellular domain, five putative membrane-spanning segments, and a short cytoplasmic tail (20). CD47 is highly expressed on the surface of RBCs, where it associates with the Rh protein complex instead of with integrins (21). The rate of clearance of transfused
CD47-deficient RBCs from the bloodstream was found to be markedly increased compared with that apparent for wild-type (WT) RBCs (6, 7). Furthermore, the phagocytosis of CD47-deficient RBCs by either splenic or bone marrow-derived macrophages was markedly enhanced in an in vitro assay (6, 7). Conversely, we recently showed that the rate of clearance of transfused RBCs from the bloodstream was markedly increased in mice that express a mutant SHPS-1 protein lacking most of the cytoplasmic region, compared with that apparent for WT mice (9). In addition, phagocytosis of opsonized RBCs by peritoneal macrophages (PEMs) derived from the SHPS-1 mutant mice was enhanced compared with that apparent with WT PEMS (8). Furthermore, the phagocytic response is enhanced in viable moth-eaten mice, in which the activity of SHP-1 is reduced (7). Together, these observations suggest that the binding of CD47 on RBCs to SHPS-1 on macrophages inhibits the phagocytosis of the former cells by the latter, and that SHP-1, which forms a complex with SHPS-1, participates in negative regulation of phagocytosis in macrophages.

It has remained unclear, however, whether negative regulation of phagocytosis by SHPS-1 is dependent on its transinteraction with CD47 on phagocytic targets. Indeed, fibroblasts derived from the SHPS-1 mutant mice described above were found to be defective with regard to their abilities to migrate, spread, and reorganize their actin cytoskeleton (22), suggesting that SHPS-1 may have a regulatory role unrelated to its transinteraction with CD47. In addition, it has remained unknown whether the formation of a complex between SHPS-1 and SHP-1 indeed contributes to negative regulation of phagocytosis in macrophages, given that the substrate for SHP-1 in such regulation has not been identified (8).

To address unresolved issues, we have now investigated the effects of forced expression of mutant versions of mouse SHPS-1 that lack either the tyrosine phosphorylation sites (mSHPS-1-1ΔCyto) or almost the entire cytoplasmic region (mSHPS-1-1ΔCyto) on FcγR-mediated phagocytosis in RAW264.7 mouse macrophage cells. In addition, we tested the effects of expression of the corresponding mutants of rat SHPS-1, which do not bind mouse CD47, on FcγR-mediated phagocytosis in RAW264.7 cells.

Materials and Methods

Abs and reagents

Hybridoma cells producing a rat mAb to mouse SHPS-1 (p84) were provided by C. Lagenaur (University of Pittsburgh, Pittsburgh, PA). A mouse mAb to rat SHPS-1 (9F10) was generated with a rat SHPS-1 fusion protein (the extracellular region of rat SHPS-1 fused to the Fc region of human IgG) as Ag, as described previously (23). Hamster mAbs to mouse SHPS-1 (1SD9 and FG2) (24) were provided by S. Nagata (Osaka University, Osaka, Japan). Rabbit polyclonal Abs to SHPS-1 (anti-SHPS-1 Cyto) were obtained from ProSci. Rabbit polyclonal Abs to SHP-1 or SHP-2 were from Santa Cruz Biotechnology. A mouse mAb to phosphotyrosine (p84) to SHPS-1 (2×106) was from Cell Signaling Technology. Normal rat, mouse, and hamster IgG as well as HRP-conjugated secondary Abs were from Jackson ImmunoResearch Laboratories. The mAb p84 was conjugated to NHS-LC biotin from Pierce. Streptavidin-FITC was from BD Pharmingen, and rabbit polyclonal Abs to mouse RBCs were from Cedarlane Laboratories. A rat mAb to FcγRII/III (2×106) was isolated from the culture supernatant of hybridoma cells (provided by Y. Kaneko, Gunma University, Gunma, Japan). Glutaraldehyde-stabilized, IgG-opsinized SRBCs were obtained from InterCell Technologies. Wortmannin and piceatannol were from Sigma-Aldrich. Fluorescein-conjugated zymosan particles were from Invitrogen Life Technologies.

Cells, cell culture, and generation of RAW264.7 cells expressing WT or mutant SHPS-1 by retrovirus infection

All cells were maintained at 37°C under a humidified atmosphere of 5% CO2 in air. The mouse macrophage cell line RAW264.7 (provided by Y. Kaneko, Gunma University, Gunma, Japan) was cultured in RPMI 1640 (Sigma-Aldrich) supplemented with 10% FBS.

To generate an expression plasmid for the mSHPS-1-1ΔCyto mutant of mouse SHPS-1, in which all four tyrosine residues (Tyr149, Tyr269, Tyr439, and Tyr605) in the cytoplasmic region had been changed to phenylalanine by site-directed mutagenesis, we subcloned the corresponding full-length cDNA (provided by T. Noguchi, Kobe University, Hyogo, Japan) into pTracer-CMV (Invitrogen Life Technologies), yielding pTracer-CMV-mSHPS-1-1ΔCyto. The expression of this plasmid was induced by puromycin (provided by Y. Kaneko, Gunma University, Gunma, Japan). The mAb 2.4G2 to FcγR (1×106) were transiently transfected with 2×106 g/ml of each pMX-puro vector (provided by T. Kitamura, University of Tokyo, Tokyo, Japan). The production of retroviruses encoding the various SHPS-1 proteins and infection of cells with these viruses were performed, as described (27). Plat-E packaging cells (28) (provided by T. Kitamura, University of Tokyo, Tokyo, Japan) were maintained under a humidified atmosphere of 5% CO2 and 95% air at 37°C in DMEM supplemented with 10% FBS, puromycin (1 μg/ml) (Invitrogen Life Technologies), penicillin (100 U/ml), and streptomycin (100 μg/ml). The cells were trypsinized and transfected with 5 μg of each pMX-puro vector containing the cDNA for the cytoplasmic region of mSHPS-1-1ΔCyto, which was then digested with BamHI and EcoRI, and the resulting fragments were ligated.

The production of retroviruses encoding the various SHPS-1 proteins and infection of cells with these viruses were performed, as described (27). Plat-E packaging cells (28) (provided by T. Kitamura, University of Tokyo, Tokyo, Japan) were maintained under a humidified atmosphere of 5% CO2 and 95% air at 37°C in DMEM supplemented with 10% FBS, puromycin (1 μg/ml) (Invitrogen Life Technologies), penicillin (100 U/ml), and streptomycin (100 μg/ml). The cells were transiently transfected with 3 μg of each pMX-puro vector containing the cDNA with the use of FuGene 6 (Roche). Fresh medium (RPMI 1640 supplemented with 10% FBS) was added to the cells 16 h after transfection, and the culture supernatants (3 ml) were harvested after incubation for an additional 24 h. RAW264.7 cells were infected with the retrovirus-containing culture supernatants supplemented with polybrene (hexadimethrine bromide; Sigma-Aldrich) at 10 μg/ml. The culture medium was refreshed 24 h after infection and was replaced with serum-supplemented RPMI 1640 containing puromycin (2 μg/ml) after incubation of the cells for an additional 24 h. Colonies were isolated after 14 days. Several cell lines expressing WT or mutant SHPS-1 proteins were identified by flow cytometry and immunoblot analysis with Abs to SHPS-1.

Flow cytometry

The surface expression of SHPS-1 on RAW264.7 cells was examined by flow cytometry, as described (8, 9). In brief, cells were detached from culture dishes by treatment with 0.01% EDTA and then washed with ice-cold PBS. They (0.5 × 10^6) were incubated with the rat mAb 2.4G2 to FcγR (1 μg/ml) for 30 min on ice, washed twice with ice-cold PBS, and incubated for 30 min on ice with a biotinylated mAb (p84) to SHPS-1 (2 μg/ml). After two washes with ice-cold PBS, the cells were incubated with streptavidin-FITC (1/2000 dilution), washed twice with PBS, suspended in 1 ml of PBS, and analyzed with a FACSCalibur flow cytometer (BD Biosciences). Data were processed with CellQuest software (BD Biosciences).

Opsonization of mouse RBCs

IgG-opsinized mouse RBCs were prepared by incubation of washed RBCs with rabbit polyclonal IgG to mouse RBCs (3 μg/ml) for 20 min at 37°C. Opsonized cells were finally washed with and resuspended in RPMI 1640 before assays.
In vitro phagocytosis assay

Phagocytosis was assayed in vitro, as previously described (8, 9). In brief, opsonized or nonopsonized normal mouse RBCs or RBCs from CD47-deficient mice (6, 7), or IgG-opsonized SRBCs, suspended in 200 μl of RPMI 1640, were overlaid on RAW264.7 cells that had been plated at a density of 1.25 × 10^5 cells/well in 24-well plates. After incubation for the indicated times at 37°C, the RAW264.7 cells were washed with PBS and then incubated for 5 min at room temperature with hemolysis buffer (154 mM NH₄Cl (pH 7.3), 10 mM KHCO₃, and 0.1 mM EDTA) to remove attached, but not phagocytosed, RBCs. After fixation of the cells with 4% paraformaldehyde in PBS, phagocytosis was detected by phase-contrast light microscopy and quantified as the phagocytic index (the percentage of RAW264.7 cells that had engulfed particles). For attachment of zymosan, the cells were incubated for 1 h at 37°C in fresh FBS. Zymosan particles were added to adherent RAW cells at a final concentration of 25 μg/ml and allowed to be ingested for 1 h at 37°C in assay medium. After the incubations, adherent cells were washed three times with PBS and then incubated for 1 h at 37°C with 0.25% trypsin/1 mM EDTA to remove attached, but not phagocytosed, particles. The intracellular particles were detected by fluorescent microscopy and quantified as the phagocytic index (the percentage of RAW264.7 cells that had engulfed particles).

**Assay for attachment of RBCs or zymosan to RAW264.7 cells**

Opsonized RBCs from WT or CD47-deficient mice in 200 μl of RPMI 1640 were overlaid on RAW264.7 cells that had been plated at a density of 1.25 × 10^5 cells/well in 24-well plates. After incubation for 20 min at 4°C, the RAW264.7 cells were washed twice with PBS to remove nonattached RBCs and fixed with 4% paraformaldehyde in PBS. The attachment of RBCs was then detected by phase-contrast light microscopy and quantified as the attachment index (the number of attached RBCs per 100 RAW264.7 cells). For attachment of zymosan, the cells were incubated with zymosan (25 μg/ml) for 1 h at 4°C, and then washed twice with PBS to remove nonattached particles. The attachment of zymosan particles was then detected by fluorescent microscopy and quantified as the attachment index (the number of attached particles per 100 RAW264.7 cells).

**Immunoprecipitation and immunoblot analysis**

RAW264.7 cells were washed with ice-cold PBS and then lysed on ice in 400 μl of lysis buffer (20 mM Tris-HCl (pH 7.6), 140 mM NaCl, 1 mM EDTA, and 1% Nonidet P-40) containing 1 mM PMSF, aprotinin (10 μg/ml), and 1 mM sodium vanadate. The lysates were centrifuged at 10,000 × g for 15 min at 4°C, and the resulting supernatants were subjected to immunoprecipitation and immunoblot analysis.

For immunoprecipitation, the supernatants were incubated for 3 h at 4°C with Ab-coupled protein G-Sepharose beads (20 μl of beads) (Amersham Biosciences). The beads were then washed three times with 1 ml of wash buffer (50 mM HEPES-NaOH (pH 7.6), 150 mM NaCl, and 0.1% Triton X-100), suspended in Laemmli sample buffer, boiled for 5 min, and subjected to SDS-PAGE, followed by immunoblot analysis with various Abs and an ECL detection system (Amersham Biosciences). The intensity of an immunoblot band was determined by densitometric analysis that was performed by using NIH Image version 1.62.

**Statistical analysis**

Data are presented as means ± SE and were analyzed by Student’s t test. A p value of <0.05 was considered statistically significant.

**Results**

**Effects of CD47 ablation in RBCs or of mAbs to SHPS-1 on FcγR-mediated phagocytosis of RBCs by RAW264.7 cells**

We first examined the expression of SHPS-1 in the mouse macrophage cell line RAW264.7. Flow cytometric analysis with a mAb (p84) that recognizes the extracellular region of mouse SHPS-1 revealed the presence of SHPS-1 immunoreactivity on the cell surface (Fig. 1A). In addition, immunoblot analysis with the p84 mAb or with polyclonal Abs (anti-SHPS-1 Cyto) that recognize the cytoplasmic region of SHPS-1 also revealed the presence of SHPS-1 (~120 kDa) in lysates of RAW264.7 cells (Fig. 1A). These results thus indicated that SHPS-1 is abundant in RAW264.7 cells.

We next performed in vitro phagocytosis assays with RBCs from WT donor mice or from CD47-deficient mice. The phagocytosis of CD47-deficient RBCs by bone marrow-derived macrophages or PEMs in vitro was previously shown to be markedly enhanced compared with that apparent with WT RBCs (7, 8). Little phagocytosis of nonopsonized RBCs from either WT or CD47-deficient donor mice was apparent with RAW264.7 cells even after...
FIGURE 2. Effects of forced expression of mSHPS-1-4F or mSHPS-1-ΔCyto on the phagocytic response of RAW264.7 cells. A. Schematic representation of WT (mSHPS-1-WT) and mutant (mSHPS-1-4F and mSHPS-1-ΔCyto) forms of mouse SHPS-1 (upper panel). Numbers indicate amino acid residues. ECR, extracellular region; TM, transmembrane region; CPR, cytoplasmic region. Four tyrosine residues (Y) in the cytoplasmic region, which are putative phosphorylation sites and are mutated to phenylalanine (F) in mSHPS-1-4F, are indicated. Whole cell lysates of parental RAW264.7 cells (RAW), clones 13 (m4F13) and 28 (m4F28) of RAW264.7 cells expressing mSHPS-1-4F, or clones 8 (mΔCyto8) and 11 (mΔCyto11) of RAW264.7 cells expressing mSHPS-1-ΔCyto were subjected to immunoblot analysis with the mAb p84 to SHPS-1, polyclonal Abs to the cytoplasmic region of SHPS-1 (anti-SHPS-1 Cyto), or polyclonal Abs to SHP-1 (anti-SHP-1) or to SHP-2 (anti-SHP-2), as indicated (lower panel). Data are representative of three independent experiments. B-D, Parental RAW264.7 cells or cells expressing mutant forms of mouse SHPS-1 were assayed for their phagocytic responses in vitro with nonopsonized (B) or IgG-opsonized (C) RBCs from WT donor mice or with IgG-opsonized SRBCs (D). The phagocytic index was determined after incubation of cells for 60 min (B and C) or 10 min (D) at 37°C. Data are means ± SE of triplicates from an experiment that was repeated a total of three times with similar results. *, p < 0.05 for the indicated comparisons vs the corresponding value for parental cells (Student’s t test).

Effects of forced expression of mSHPS-1-4F or mSHPS-1-ΔCyto on phagocytosis by RAW264.7 cells

The cytoplasmic region of SHPS-1 contains four putative tyrosine phosphorylation sites that bind the protein tyrosine phosphatase SHP-1 or SHP-2 (10–12) (Fig. 2A). The formation of a complex between SHPS-1 and SHP-1 is implicated in negative regulation of phagocytosis in macrophages (6–9). To further investigate the role of the cytoplasmic region of SHPS-1 in the negative regulation of phagocytosis by this protein, we generated RAW264.7 cells that express either mSHPS-1-4F (m4F cells) or mSHPS-1-ΔCyto (mΔCyto cells); all four tyrosine residues (Y436, Y460, Y477, Y501) in the cytoplasmic region are replaced by phenylalanine in mSHPS-1-4F, whereas mSHPS-1-ΔCyto (aa 1–404) lacks almost the entire cytoplasmic region (Fig. 2A). Several cell lines expressing mSHPS-1-4F or mSHPS-1-ΔCyto were obtained, from which we chose two m4F lines (clones 13 and 28) as well as two mΔCyto lines (clones 8 and 11) for further analysis.

Immunoblot analysis of cell lysates with the p84 mAb to mouse SHPS-1 revealed that the total amount of mouse SHPS-1 protein (both endogenous and exogenous) in m4F cells or mΔCyto cells was markedly greater than that in parental RAW264.7 cells (Fig. 2A). In addition, the amount of mSHPS-1-ΔCyto in each mΔCyto cell line was much greater than that of mSHPS-1-4F in either m4F cell line. Immunoblot analysis with rabbit polyclonal Abs to the cytoplasmic region of SHPS-1 (anti-SHPS-1 Cyto) showed that the amount of endogenous SHPS-1 in mΔCyto cells was similar to that in parental RAW264.7 cells (Fig. 2A). In addition, the abundance of SHP-1 or SHP-2 was not altered by forced expression of either mSHPS-1-4F or mSHPS-1-ΔCyto were obtained, from which we chose two m4F lines (clones 13 and 28) as well as two mΔCyto lines (clones 8 and 11) for further analysis.

To investigate the effect of forced expression of mSHPS-1-4F or mSHPS-1-ΔCyto on phagocytosis by RAW264.7 cells, we performed the in vitro phagocytosis assay with nonopsonized or IgG-opsonized mouse RBCs as the target. Minimal phagocytosis of nonopsonized RBCs was apparent with parental RAW264.7 cells or mΔCyto cells after incubation for 60 min (Fig. 2B). In contrast, the phagocytic response of m4F28 cells was slightly, but significantly greater than that of parental cells (Fig. 2B). IgG-opsonized mouse RBCs underwent phagocytosis to a marked extent by the parental and transfected RAW264.7 cell lines (Fig. 2C), with the...
phagocytic response of m4F13 or m4F28 cells being significantly greater than that of the parental cells (Fig. 2C).

We also examined the phagocytosis of IgG-opsonized SRBCs by the various cell lines. We have shown previously that mouse SHPS-1 fails to bind to CD47 expressed on the surface of SRBCs (8). The phagocytic response of m4F13 or m4F28 cells with IgG-opsonized SRBCs, however, was still greater than that of parental RAW264.7 cells (Fig. 2D). As with IgG-opsonized mouse RBCs, the phagocytic response of mαCyto8 or mΔCyto1 cells with IgG-opsonized SRBCs was similar to that of the parental cells (Fig. 2D). These data suggested that forced expression of mSHPS-1-4F, but not that of mSHPS-1-ΔCyto, promoted the phagocytic response of RAW264.7 cells. In addition, this effect appeared to be largely independent of the interaction between CD47 on mouse RBCs and SHPS-1 on RAW264.7 cells.

Effects of forced expression of rat SHPS-1-4F or rat SHPS-1-ΔCyto on phagocytosis by RAW264.7 cells

To further examine the effects of SHPS-1-4F or SHPS-1-ΔCyto, we generated RAW264.7 cells that express either WT rat SHPS-1 (rat WT cells) or the mutants rat SHPS-1-4F (rat 4F cells) or rat SHPS-1-ΔCyto (rat ΔCyto cells) (Fig. 3A). Several cell lines expressing WT or mutant rat SHPS-1 proteins were obtained, and a representative of each type was chosen for further analysis. The binding of a mouse CD47-Fc fusion protein to rat SHPS-1 was markedly reduced compared with that to mouse SHPS-1 (data not shown).

Immunoblot analysis with the p84 mAb to mouse SHPS-1, which does not recognize rat SHPS-1 (data not shown), revealed that the expression levels of endogenous SHPS-1 in rat WT, rat 4F, and rat ΔCyto cells were similar to that in parental RAW264.7 cells (Fig. 3A). In contrast, immunoblot analysis of cell lysates with the 9F10 mAb, which recognizes rat SHPS-1, but not mouse SHPS-1 (data not shown), revealed that the expression levels of rat SHPS-1-4F and rat SHPS-1-ΔCyto were markedly greater than that of rat WT SHPS-1 (rat SHPS-1-WT) in the respective transfected cells (Fig. 3A). Immunoblot analysis with polyclonal Abs (anti-SHPS-1 Cyto) that recognize the cytoplasmic regions of both mouse and rat SHPS-1 revealed that the total amount of SHPS-1 protein in rat WT or rat 4F cells was much greater than that in parental RAW264.7 cells (Fig. 3A). The abundance of SHP-1 or SHP-2 was not altered by forced expression of either rat SHPS-1-WT, rat SHPS-1-4F, or rat SHPS-1-ΔCyto (Fig. 3A). Flow cytometric analysis confirmed that the WT and mutant forms of rat SHPS-1 were expressed on the surface of the corresponding transfected cell lines and that the surface expression of endogenous mouse SHPS-1 was largely unaffected by that of the rat proteins (data not shown).

We then examined the effect of forced expression of rat SHPS-1-WT or the corresponding mutants on phagocytosis by RAW264.7 cells. In contrast to the minimal level of phagocytosis of nonopsonized mouse RBCs apparent after incubation for 60 min with parental RAW264.7, rat WT, or rat ΔCyto cells, a small, but significant phagocytic response was observed with rat 4F cells (Fig. 3B). In addition, the phagocytic response with IgG-opsonized mouse RBCs was markedly greater for rat 4F cells than for parental RAW264.7 cells, whereas the responses of rat WT and rat ΔCyto cells were similar to that of the parental cells (Fig. 3C), even though the amount of rat SHPS-1-ΔCyto in rat ΔCyto cells was similar to or even slightly greater than that of rat SHPS-1-4F in rat 4F cells (Fig. 3A).
The phagocytic response with IgG-opsonized SRBCs was also markedly greater for rat 4F cells than for parental RAW264.7 cells, whereas the responses of rat WT and rat ΔCyto cells were similar to that of the parental cells (Fig. 3D). These data indicated that forced expression of rat SHPS-1-4F, like that of mSHPS-1-4F, promoted FcγR-mediated phagocytosis in RAW264.7 cells. In addition, they suggested that the cytoplasmic region, rather than the extracellular region, of SHPS-1-4F mediates this effect.

**Effect of forced expression of CD8-mSHPS-1-4FCyto on phagocytosis by RAW264.7 cells**

To clarify the mechanism by which SHPS-1-4F promotes FcγR-mediated phagocytosis in RAW264.7 cells, we next generated cells (CD8-m4FCyto cells) that express a chimeric protein comprising the extracellular and transmembrane regions of human CD8 fused to the cytoplasmic region of mSHPS-1-4F (CD8-mSHPS-1-4FCyto) (Fig. 4A). Several cell lines expressing this chimeric protein were obtained, two of which (clones 13 and 14) were chosen for further analysis. Immunoblot analysis with polyclonal Abs to the cytoplasmic region of SHPS-1 revealed that CD8-m4FCyto cells indeed expressed the ~50-kDa chimeric protein, which did not affect the expression of endogenous SHPS-1 (Fig. 4A). The phagocytosis of IgG-opsonized mouse RBCs by each CD8-m4FCyto cell line was markedly enhanced compared with that apparent with parental RAW264.7 cells (Fig. 4B). These results suggested that expression of the cytoplasmic region of mSHPS-1-4F is sufficient for the promotion of FcγR-mediated phagocytosis in RAW264.7 cells.

**Effects of forced expression of SHPS-1-4F on tyrosine phosphorylation of endogenous SHPS-1 in RAW264.7 cells**

To investigate further the molecular mechanism by which SHPS-1-4F promotes FcγR-mediated phagocytosis in RAW264.7 cells, we examined the effect of forced expression of mSHPS-1-4F on pervanadate-induced tyrosine phosphorylation of SHPS-1 and the consequent formation of a complex between SHPS-1 and SHP-1. Lysates of pervanadate-treated or untreated parental RAW264.7 or m4F13 cells were subjected to immunoprecipitation with polyclonal Abs to SHP-1. Immunoblot analysis of the resulting precipitates with a mAb to phosphotyrosine revealed only a minimal level of protein tyrosine phosphorylation in those prepared from either parental or m4F13 cells that had not been exposed to pervanadate (Fig. 5A). In contrast, tyrosine phosphorylation of a ~120-kDa protein was apparent in the immunoprecipitates prepared from both pervanadate-treated cell lines. Reprobing of the same blot with the p84 mAb to SHPS-1 confirmed that the ~120-kDa protein was indeed SHPS-1 (Fig. 5A). The densitometric analysis revealed that amount of tyrosine-phosphorylated SHPS-1 that formed a complex with SHP-1 in m4F13 cells was slightly reduced compared with that apparent in the parental cells; however, such reduction was not statistically significant.

We also subjected lysates of pervanadate-treated or untreated parental or rat 4F cells to immunoprecipitation with the p84 mAb to mouse SHPS-1. Immunoblot analysis and densitometric analysis of the resulting precipitates also revealed that the amount of tyrosine-phosphorylated SHPS-1 that formed a complex with SHP-1 in response to pervanadate treatment was slightly reduced compared with that apparent in the parental cells; however, such reduction was not statistically significant (Fig. 5B). These data suggested that forced expression of SHPS-1-4F in RAW264.7 cells does not markedly inhibit the tyrosine phosphorylation of endogenous SHPS-1 and its formation of a complex with SHP-1.

**Effects of inhibitors of Syk or PI3K on the enhancement of phagocytosis in RAW264.7 cells by SHPS-1-4F**

Downstream signaling mediated by Syk or PI3K triggers FcγR-dependent phagocytosis (4, 29–31). Treatment with the Syk inhibitor piceatannol (23, 32) inhibited FcγR-mediated phagocytosis in parental RAW264.7 cells as well as the enhancement of this process apparent in m4F13 cells (Fig. 6A). In addition, treatment with wortmannin, a PI3K inhibitor (23, 33), also inhibited FcγR-mediated phagocytosis in parental RAW264.7 cells and abolished the enhancement of this process apparent in m4F13 cells (Fig. 6B).

**Effects of forced expression of mSHPS-1-4F or mSHPS-1-ΔCyto on the attachment of RBCs to RAW264.7 cells**

We next examined RBC attachment to RAW264.7 cells by incubating the latter with IgG-opsonized or nonopsonized mouse RBCs for 20 min at 4°C. A minimal level of attachment of nonopsonized RBCs to either parental, m4F, or mΔCyto cells was detected (data not shown). In contrast, IgG-opsonized RBCs attached to a marked extent to each of these cell lines, with the attachment to m4F cells
FIGURE 5. Effects of forced expression of SHPS-1-4F on pervanadate-induced tyrosine phosphorylation of endogenous SHPS-1 and its association with SHP-1. A. Serum-deprived parental RAW264.7 cells or m4F13 cells were incubated for 5 min at 37°C in the absence or presence of 2 mM pervanadate, after which cell lysates were subjected to immunoprecipitation with polyclonal Abs to SHP-1. The resulting precipitates were subjected to immunoblot analysis with the mAb PY100 to phosphotyrosine (anti-pY), the p84 Ab to SHPS-1, and polyclonal Abs to SHP-1 (anti-SHP-1). B. Serum-deprived parental RAW264.7 cells or rat 4F cells were incubated for 5 min at 37°C in the absence or presence of 2 mM pervanadate, after which cell lysates were subjected to immunoprecipitation with the mAb p84 to SHPS-1. The resulting precipitates were subjected to immunoblot analysis as in A. Results in A and B are representative of three separate experiments. Densitometric analysis was also performed. The ratio of the band intensity of phospho-SHPS-1 to that of SHP-1 (pY/SHP-1), of phospho-SHPS-1 to mSHPS-1 (SHPS-1/p84) for each lane was calculated, and results were expressed as a percentage of the value with pervanadate-treated parental RAW264.7 cells (right panels in A and B). Data are means ± SE of three separate experiments. NS, for the indicated comparisons vs the corresponding value for parental RAW264.7 cells (Student’s t test).

being significantly greater than that to parental RAW264.7 cells (Fig. 7A). The extent of such attachment to either parental or m4F13 cells was greatly reduced for IgG-opsonized RBCs from CD47−/− mice compared with that apparent for IgG-opsonized WT RBCs (Fig. 7B), suggesting that the interaction of CD47 on RBCs with SHPS-1 on RAW264.7 cells contributes to the attachment of the former cells to the latter. However, the attachment of opsonized CD47-deficient RBCs to m4F13 cells was still significantly increased compared with that apparent with the parental cells. Furthermore, the increased association of IgG-opsonized RBCs to m4F13 cells was not affected by either piceatannol or wortmannin (Fig. 7C). The attachment of IgG-opsonized RBCs to rat 4F cells, but not that to rat ΔCyto cells, was also significantly increased compared with that observed with parental RAW264.7 cells (Fig. 7D). The promotion of phagocytosis by SHPS-1-4F may thus result in part from increased attachment of RBCs to RAW264.7 cells. In addition, this increased RBC attachment appears to be largely independent of the CD47-SHPS-1 interaction.

We finally examined the phagocytic response with nonopsonized or serum-opsonized zymosan. In contrast to the minimal level of phagocytosis of nonopsonized zymosan apparent after incubation for 60 min with parental RAW264.7 or mΔCyto cells, a small, but significant phagocytic response was observed with m4F cells or CD8-m4FCyto cells (Fig. 8A). In addition, the phagocytic response with serum-opsonized zymosan was markedly greater for m4F cells or CD8-m4FCyto cells than for parental RAW264.7 cells or mΔCyto cells, whereas the responses of rat ΔCyto cells were similar to that of the parental cells (Fig. 8B). We also examined the attachment of zymosan to RAW264.7 cells by incubating the latter with serum-opsonized or nonopsonized zymosan for 1 h at 4°C. A minimal level of attachment of nonopsonized zymosan to either parental, m4F, mΔCyto, or CD8-m4FCyto cells was detected (data not shown). In addition, the level of attachment of serum-opsonized zymosan to these cell lines was low, with the attachment to m4F or CD8-m4FCyto cells being slightly greater than that to parental RAW264.7 cells or mΔCyto cells (Fig. 8C).

Discussion

We have shown that the extent of phagocytosis by RAW264.7 cells of IgG-opsonized CD47−/− mouse RBCs was markedly increased compared with that of IgG-opsonized CD47+/+ RBCs. In addition, mAbs that block the interaction between CD47 and SHPS-1 enhanced the phagocytosis of IgG-opsonized mouse
RBCs by RAW264.7 cells. These results are essentially consistent with previous observations with primary cultured macrophages (6–8), and they suggest that the binding of CD47 on RBCs to SHPS-1 on RAW264.7 cells negatively regulates FcγR-mediated phagocytosis by these latter cells.

We have now further shown that forced expression in RAW264.7 cells of mSHPS-1–4F, which does not bind SHP-1 or SHP-2 (25, 26), markedly promoted the phagocytosis of IgG-opsonized mouse RBCs. Given that binding of CD47 to SHPS-1 negatively regulates phagocytosis by RAW264.7 cells, expression of mSHPS-1–4F appears to prevent the inhibition of FcγR-mediated phagocytosis by endogenous SHPS-1 in a dominant-negative manner. In contrast, forced expression of mSHPS-1–ΔCyo did not promote FcγR-mediated phagocytosis by RAW264.7 cells. These results thus indicate that the cytoplasmic region as well as mutation of its tyrosine phosphorylation sites are required for the dominant-negative effect of mSHPS-1–4F. The inhibitory action of mSHPS-1–4F is therefore not attributable simply to its prevention of the interaction between CD47 (on RBCs) and endogenous SHPS-1 (on RAW264.7 cells). Indeed, forced expression of

![Figure 7](http://www.jimmunol.org/) Effects of forced expression of SHPS-1–4F or SHPS-1–ΔCyo on the attachment of IgG-opsonized mouse RBCs to RAW264.7 cells. A, IgG-opsonized mouse RBCs were incubated for 20 min at 4°C with parental RAW264.7 cells or cells expressing mutant versions of mouse SHPS-1, after which the attachment index (the number of attached RBCs per 100 RAW264.7 cells) was determined. B, IgG-opsonized RBCs derived from either WT or CD47-deficient donor mice were incubated for 20 min at 4°C with parental RAW264.7 or mF13 cells, after which the attachment index was determined. C, Parental RAW264.7 or mF13 (m4F) cells were incubated for 30 min at 37°C in the absence or presence of 10 μM piceatannol or 50 nM wortmannin. They were then assayed for the attachment of IgG-opsonized mouse RBCs during incubation for 20 min at 4°C in the continued absence or presence of inhibitor. D, IgG-opsonized mouse RBCs were incubated for 20 min at 4°C with parental RAW264.7 cells or cells expressing WT or mutant versions of rat SHPS-1, after which the attachment index was determined. All data are means ± SE of triplicates from experiments that were repeated a total of three times with similar results. NS: * p < 0.05 for the indicated comparisons vs the corresponding value for parental RAW264.7 cells (Student’s t test).

![Figure 8](http://www.jimmunol.org/) Effects of forced expression of SHPS-1 mutants on phagocytosis and the attachment of zymosan by RAW264.7 cells. Parental RAW264.7 cells or cells expressing mutant forms of mouse SHPS-1 were assayed for their phagocytic responses in vitro with nonopsonized (A) or serum-opsonized (B) zymosan particles. The phagocytic index was determined after incubation of cells for 1 h at 37°C. C, Serum-opsonized zymosan particles were incubated for 1 h at 4°C with parental RAW264.7 cells or cells expressing mutant forms of mouse SHPS-1, after which the attachment index (the number of attached RBCs per 100 RAW264.7 cells) was determined. Data are means ± SE of triplicates from an experiment that was repeated a total of three times with similar results. * p < 0.05 for the indicated comparisons vs the corresponding value for parental cells (Student’s t test).
mSHPS-1-4F also promoted phagocytosis of IgG-opsonized SRBCs by RAW264.7 cells, even though mouse SHPS-1 does not bind to CD47 on the surface of SRBCs (8).

Forced expression of rat SHPS-1-4F also promoted FcγR-mediated phagocytosis of mouse RBCs by RAW264.7 cells, even though the ability of rat SHPS-1 to bind mouse CD47 is markedly reduced compared with that of mouse SHPS-1. In addition, forced expression of either rat SHPS-1-WT or rat SHPS-1-ΔCyto failed to promote FcγR-mediated phagocytosis by RAW264.7 cells. Expression of rat SHPS-1-4F also promoted phagocytosis of IgG-opsonized SRBCs by RAW264.7 cells. Forced expression of mSHPS-1-4F also promoted the phagocytosis of zymosan by RAW264.7 cells. Moreover, forced expression of the CD8-mSHPS-1-4FCyto chimera, comprising the extracellular and transmembrane regions of human CD8 fused to the cytoplasmic region of mSHPS-1-4F, promoted FcγR-mediated phagocytosis by RAW264.7 cells. These data further support the notion that the cytoplasmic region as well as mutation of its tyrosine phosphorylation sites are sufficient for the dominant-negative effect of SHPS-1-4F. In addition, they suggest that SHPS-1 may negatively regulate FcγR-mediated phagocytosis in macrophages, at least in part, in a manner independent of its interaction with CD47 on the phagocytic target.

Forced expression of SHPS-1-4F (either mouse or rat versions) did not markedly alter the extents of both the tyrosine phosphorylation of endogenous SHPS-1 and its association with SHP-1 induced by treatment of cells with pervanadate. The results suggest that expression of SHPS-1-4F may impede the physical interaction of endogenous SHPS-1 with its downstream pathway, but not with tyrosine kinase responsible for its phosphorylation in RAW264.7 cells. Such an effect might require the entire cytoplasmic region of SHPS-1, explaining why expression of SHPS-1-ΔCyto failed to promote phagocytosis in RAW264.7 cells.

It was suggested previously that tyrosine phosphorylation of SHPS-1 and its association with SHP-1 induce activation of SHPS-1 at sites near FcγRs in macrophages, and that SHP-1 negatively regulates FcγR-mediated activation of Syk or of PI3K, thereby inhibiting FcγR-mediated phagocytosis (7, 8). Indeed, we have now shown that inhibitors of Syk or PI3K blocked the enhancement of phagocytosis by SHPS-1-4F in RAW264.7 cells. Expression of SHPS-1-4F may therefore inhibit the tyrosine phosphorylation of SHPS-1 and its formation of a complex with SHP-1, thereby preventing inhibition by SHP-1 of the activation of Syk, PI3K, and their downstream signaling molecules that mediate FcγR-dependent phagocytosis in RAW264.7 cells. The FcγR-mediated tyrosine phosphorylation of Syk or Cbl, the latter of which binds to the p85 subunit of PI3K in response to FcγR ligation (34), or tyrosine phosphorylation by Syk of adapter proteins such as SLP-76 or BLNK (23, 35), might be regulated by the SHPS-1-SHP-1 complex.

We found that the attachment of IgG-opsonized RBCs to RAW264.7 cells was markedly increased by expression of SHPS-1-4F, but not by that of either SHPS-1-WT or SHPS-1-ΔCyto. Promotion of phagocytosis by SHPS-1-4F thus appears to be attributable in part to an increased attachment of opsonized RBCs to RAW264.7 cells. The mechanism by which SHPS-1-4F facilitates the attachment of RBCs to RAW264.7 cells remains unclear, however. The binding of CD47 on apoptotic cells to SHPS-1 on macrophages is implicated in the tethering of the former cells to the latter (24). Indeed, the attachment of IgG-opsonized CD47+/+/ RBCs to either parental RAW264.7 cells or mF13 cells was markedly reduced compared with that of WT RBCs, suggesting that the interaction between CD47 (on RBCs) and SHPS-1 (on RAW264.7 cells) also contributes to the tethering of the former cells to the latter. In contrast, the increase in the extent of RBC attachment to RAW264.7 cells induced by expression of SHPS-1-4F was not abolished by ablation of CD47 in the RBCs. In addition, inhibitors of Syk or PI3K did not prevent this effect of SHPS-1-4F, even though they did abolish its promotion of FcγR-mediated phagocytosis. These data suggest that forced expression of SHPS-1-4F most likely inhibits the down-regulation by endogenous SHPS-1 (and SHP-1) of the expression of an as yet unidentified protein on the surface of RAW264.7 cells that binds and tethers RBCs.

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Disclosures

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References


