SHPS-1 Induces Aggregation of Ba/F3 Pro-B Cells Via an Interaction with CD47

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SHPS-1 Induces Aggregation of Ba/F3 Pro-B Cells Via an Interaction with CD47

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SHPS-1 (SH2-domain bearing protein tyrosine phosphatase (SHP) substrate-1), a member of the inhibitory-receptor superfamily that is abundantly expressed in macrophages and neural tissue, appears to regulate intracellular signaling events downstream of receptor protein-tyrosine kinases and integrin-extracellular matrix molecule interactions. To investigate the function of SHPS-1 in a hematopoietic cell line, SHPS-1 was expressed in Ba/F3 cells, an IL-3-dependent pro-B-cell line that lacks endogenous SHPS-1 protein. Interestingly, expression of either SHPS-1, or a mutant lacking the intracellular domain of SHPS-1 (ΔCT SHPS-1), resulted in the rapid formation of macroscopic Ba/F3 cell aggregates. As the integrin-associated protein/CD47 was shown to be a SHPS-1 ligand in neural cells, we investigated whether CD47 played a role in the aggregation of SHPS-1-expressing Ba/F3 cells. In support of this idea, aggregate formation was inhibited by an anti-CD47 Ab. Furthermore, erythrocytes from control, but not from CD47-deficient mice, were able to form rosettes on SHPS-1-expressing Ba/F3 cells. Because erythrocytes do not express integrins, this result suggested that SHPS-1-CD47 interactions can take place in the absence of a CD47-integrin association. We also present evidence that the amino-terminal Ig domain of SHPS-1 mediates the interaction with CD47. Although SHPS-1-CD47 binding likely triggers bidirectional intracellular signaling processes, these results demonstrate that this interaction can also mediate cell-cell adhesion. *The Journal of Immunology, 2000, 164: 3652–3658.

The signal-regulatory proteins (SIRPs)³ are a family of immunoreceptor tyrosine-based inhibition motif (ITIM)-containing transmembrane glycoproteins displaying sequence diversity in their three extracellular Ig-like domains (1). Belonging to the inhibitory-receptor superfamily (2), SIRPs, which include SIRPα1/SHPS-1 (SH2-domain bearing protein tyrosine phosphatase (SHP) substrate-1), were initially identified based on their ability to become tyrosine phosphorylated and to interact with the SH2-domain-containing protein-tyrosine phosphatase SHP-2 following activation of growth factor-receptor protein-tyrosine kinases (1, 3). SHPS-1, for example, was cloned from insulin-stimulated rat fibroblasts (1, 3). Other groups have also identified SIRPs based on the ability of these molecules to function in neural cell adhesion (P84, BIT), as macrophage fusion receptors, and as Ag presentation-stimulating molecules (4–7).

SHPS-1 encodes a 65-kDa protein that is heavily glycosylated, giving rise to a variety of isoforms that differ in m.w. according to the cell line or tissue type examined (1, 3). The extracellular region of SHPS-1 consists of an amino-terminal Ig variable (V) type domain and two Ig constant (C) type domains (3, 6, 8). The 112-aa intracellular domain of SHPS-1 contains four tyrosine residues that include phosphorylation sites that are evident following stimulation of cells with growth factors and other stimuli, including platelet-derived growth factor, epidermal growth factor, insulin, CSF-1, or lysophosphatidic acid (1, 3, 9–14). Two of the potential phosphorylation sites lie within ITIMs, the latter being characterized by the consensus sequence I/LxYxxI/V. ITIMs, an essential component of the inhibitory-receptor superfamily, are found within the cytosolic domains of a variety of molecules, including killer cell Ig-like receptors, C-type lectin inhibitory receptors, paired Ig-like receptors, FcγRIIIB, and CD22 (15). Although SHPS-1 may be directly phosphorylated by activated receptor protein-tyrosine kinases, the presence of Src family kinases has been shown to be necessary for SHPS-1 phosphorylation following integrin clustering, and Src kinases were able to phosphorylate SHPS-1 in vitro (13, 16). Phosphorylation of ITIM-associated tyrosine residues enables the recruitment of phosphatases, such as SHP-1, SHP-2, or SHP (SH2 domain-containing inositol phosphatase), which in turn dephosphorylate specific protein substrates involved in mediating a variety of physiological effects.

An important step in understanding SHPS-1 function was the identification of a ligand for SHPS-1 (P84) in neural cells. Using a mouse brain cDNA library for expression cloning, CD47 (also known as the integrin-associated protein, IAP) was found to interact with the extracellular region of P84 (17). P84 is localized to sites of synaptogenesis, suggesting it might be involved in regulation of synapse formation or maintenance (6, 18). CD47 is a 50-kDa protein expressed on most mammalian cells (19–21), featuring one Ig-like domain in the extracellular region and five transmembrane domains with a short cytoplasmic tail.

Because SHPS-1 is highly expressed in cells of the macrophage lineage and at lower levels in other hematopoietic cells (5, 8, 22), we investigated the consequences of SHPS-1 expression in a pro-B-cell line, Ba/F3, that does not express detectable levels of endogenous SHPS-1 protein. Expression of SHPS-1 in Ba/F3 cells

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3 Abbreviations used in this paper: SIRP, signal-regulatory protein; ITIM, immunoreceptor tyrosine-based inhibition motif; SHP, SH2 domain-bearing protein tyrosine phosphatase; SHPS-1, SHP substrate-1; ΔCT SHPS-1, deleted cytoplasmic tail mutant of SHPS-1; IAP, integrin-associated protein; GFP, green fluorescent protein; WT, wild type.
resulted in the rapid formation of large cell aggregates. Using several different experimental approaches, we present evidence that CD47 is a ligand for SHPS-1 on Ba/F3 cells, and that the aminoterminal Ig domain of SHPS-1 mediates this interaction.

Materials and Methods

Cells and Abs

The murine pro-B cell line Ba/F3 was kindly provided by Dr. J. W. Schrader (Biomedical Research Centre, University of British Columbia) and cultured in Ba/F3 medium (RPMI 1640 (Life Technologies, Gaithersburg, MD) supplemented with 10% FBS (Intergen, Purchase, NY) and 20% WEHI-3 conditioned medium containing IL-3, 50 μM 2-ME, 50 μM penicillin, and 50 μg/ml streptomycin (Life Technologies)) at 37°C in 5% CO2. The rat IgG2a anti-mouse CD47 Ab (mIAP301) has been described (23, 24). The rat IgG2a anti-mouse CD8a Ab (Ly-2, clone 53-67; Pharmingen, San Diego, CA) served as the isotype control. The mouse monomclonal anti-Flag (M2) Ab was obtained from Sigma (St. Louis, MO).

Plasmids and plasmid constructions

The rat full-length SHPS-1 cDNA, having an introduced carboxy-terminal Myc epitope tag, was generously provided by Takashi Matozaki (Kobe University School of Medicine, Kobe, Japan) (3). To generate an extracellular epitope tag that would enable FACs analysis of transfected populations, sequences encoding the Flag tag were introduced into the SHPS-1 cDNA 3’ of the signal peptide. A sense primer “Bam” spanning a region 5’ of the ATG (5’-TCTCCTTGATGCCGCGTCACCCGCGCACTG-3’) and the antisense primer “FL2” at the end of the signal peptide (5’-AGAGAAGGGTTGCTATGC TGCTTGTAGTCCTAGTC TTCCG C-3’) were used in the PCR (it canalicus in the sequences denote the restriction enzyme sites). The PCR product was digested with BamHI/HindIII. The remaining sequence of SHPS-1 including the Myc tag was generated using the sense primer “H2” (5’-CTGTCACAATGTCAGCTCAG GCTGACAAATCAGTGTC-3’) and the antisense primer “FFS” (5’-GTGGTGAATTCTGCAAGGTTCCTC-3’). This PCR product was then digested with HindIII/EcoRI and ligated with the BamHI/HindIII fragment into BamHI/EcoRI-digested pBluescript (Stratagene, La Jolla, CA). The BamHI/EcoRI SHPS-1 fragment was excised and ligated into BamHI/EcoRI-digested pCDEF vector (Clontech, Palo Alto, CA). The Flag staining and FACS. Wells containing clones showing the highest fluorescence were then expanded and isolated by cell sorting. The Flag staining and FACS fractions were stained with a secondary Ab and analyzed by FACs.

Transfections and cell selection

A total of 1 × 106 Ba/F3 cells were washed once with PBS and resuspended in 800 μl of ZAP buffer (25 mM HEPES, 0.75 mM Na2HPO4, 140 mM KCl, 5 mM NaCl, 2 mM MgCl2, and 0.5% Ficoll 400) with 12 μg of Powl-linearized plasmid, and electroporated at 300 mV and 960 μF using a Bio-Rad Gene Pulser (Bio-Rad, Richmond, CA). Cells were resuspended in 30 ml of Ba/F3 medium. Cells were allowed to recover for 20 min at room temperature before returning to the 37°C incubator. After 48 h cells were centrifuged, washed with PBS, and resuspended in Ba/F3 medium with 1 mg/ml genetin (G418) (Life Technologies) selection and plated in 96-well plates. After 3–4 wk of selection, plates were screened using anti-Flag staining and FACS. Wells containing clones showing the highest fluorescence signals were then expanded and isolated by cell sorting (see below). To generate green fluorescent protein (GFP)-expressing Ba/F3 cells, 1 × 106 Ba/F3 cells were transfected with Powl-linearized pCDEF vector containing the GFP cDNA. After G418 selection, Ba/F3 clones expressing high levels of GFP were isolated by cell sorting.

Flow cytometry and cell sorting

A total of 1 × 106 cells were washed twice with FACS buffer (2% PBS in PBS) and resuspended in 100 μl of FACS buffer. Cells were stained with 1 μg Ab (anti-Flag or anti-CD47) for 1 h on ice then washed twice with FACS buffer and incubated with goat anti-mouse IgG FITC for anti-Flag stained cells, or goat anti-rat FITC for anti-CD47 stained cells, for 30 min on ice. Cells were then washed twice with FACS buffer and resuspended in 300 μl of FACS buffer before analysis on a FACSort (Becton Dickinson, Mountain View, CA) flow cytometer equipped with CellQuest software program (Becton Dickinson). For cell sorting, ~1 × 106 cells were collected and resuspended in Ba/F3 medium with 1 mg/ml G418.

Immunoblotting

Ba/F3 cells transfected with the empty pCDEF vector or with this vector containing wild-type (WT) SHPS-1 or the ΔCT SHPS-1 deletion mutant construct were washed once in PBS and lysed in Nonidet P-40 (NP-40) lysis buffer (1% NP-40, 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, and 10% glycerol), 100 μg/ml PMSF, 1 μg/ml aprotinin, 1 μg/ml pepstatin, 1 μg/ml leupeptin, 40 μg/ml bestatin, 10 μg/ml soybean trypsin inhibitor (Boehringer Mannheim, Indianapolis, IN), 10 mM sodium fluoride, 1 mM sodium vanadate, and 1 mM sodium molybdate. After 50 min on ice, cell lysates were centrifuged at 10,000 × g for 15 min. Lysates were then separated by SDS-PAGE, then transferred to nitrocellulose membranes by electrobobbling. Membranes were blocked for 1 h in 5% BSA in Tris-buffered saline (TBS), washed in TBS with 1% Tween 20 (TBST), and incubated with anti-Flag M2 (10 μg/ml) in 1 h in TBS. The membrane was washed with TBST and incubated with a HRP conjugated secondary Ab (anti-mouse Ab (Dako, Carpinteria, CA) for 1 h and washed with TBST. Enhanced chemiluminescence (Amersham, Arlington Heights, IL) detection was performed according to manufacturers instructions.

Ba/F3–RBC adhesion assay

Poly-l-lysine at 100 μg/ml (500 μl) was added to wells of 24-well plates and incubated at room temperature for 1 h, then washed twice with diH2O. Wells were then washed with 1% BSA to block non-specific adhesion. RBCs were resuspended in 1% BSA in PBS at 3 × 106 cells/ml; 500 μl of cells was added to the poly-l-lysine-coated wells. After a 15 min incubation plates were centrifuged at 100 × g for 4 min, and nonadherent cells were aspirated. The RBCs were washed twice with PBS and resuspended in PBS at 2 × 105 cells/ml; 500 μl of cells was then added to the Ba/F3–RBC adhesion assay. Plates were incubated at room temperature for 15 min, then centrifuged at 100 × g for 5 min. RBCs not bound were aspirated and the surface washed three times with PBS. Immobilized cells were imaged using a CCD camera and Northern Eclipse 5.0 software (Empix Imaging, Mississauga, Ontario, Canada).

Generation of recombinant GST-V1SHPS-1 and cell adhesion assay

The amino-terminal V domain of rat SHPS-1 was tagged with Flag-epitope at the carboxy terminus and fused to GST by PCR using the sense primer “G1” (5’-GGTGGGATCCAGATACTTCAATGAGATTGTTTCTGTTGCGGAGGAGAAGAAGAGGAAATCGGAGTATTGCGCTTGTAGTCCTAGTC TTCCG C-3’) and the antisense primer “G2” (5’-GTGGTGAATTCTGCAAGGTTCCTC-3’) with an amino oligonucleotide (5’-GCGCGCTCAGTCAATTGCTTGTAGTATGCGCAGAAGAAGGAAATCGGAGTATTGCGCTTGTAGTCCTAGTC TTCCG C-3’). The amplified product was digested with BamHI/EcoRI and cloned in-frame into BamHI/EcoRI-digested pGEX-2T (Pharmacia, Piscataway, NJ) to generate GST-tagged V1SHPS-1. The construct thus had GST fused in-frame with amino acids 32–147 of SHPS-1. The GST fusion construct was sequenced before protein isolation. Bacterial expression and fusion protein purification was performed as previously described (25). For the cell adhesion assay, 100 μg GST or GST-V1SHPS-1 protein was spotted on a 1 × 1 cm nitrocellulose filter under vacuum aspiration. The filter was washed with PBS and placed in wells of a 24-well plate with 4% BSA in PBS solution for 30 min. Blood from control and CD47-deficient mice was collected in heparinized tubes and washed three times with 1% BSA in PBS. RBCs were counted and resuspended in 1% BSA in PBS at 3 × 106 cells/ml; 500 μl of 3 × 106 cells/ml RBCs were added to the immobilized Ba/F3 cells. Plates were incubated at room temperature for 15 min, then centrifuged at 100 × g for 5 min. RBCs not bound were aspirated and the surface washed three times with PBS. Immobilized cells were imaged using a CCD camera and Northern Eclipse 5.0 software (Empix Imaging, Mississauga, Ontario, Canada).
The lysis solution was transferred to a microtiter plate, and OD$_{405}$ was measured on a Bio-Kinetics microplate reader (Bio-Tek, Burlington, VT).

Results

Expression of SHPS-1 in Ba/F3 cells results in cellular aggregation

To investigate the effects of SHPS-1 expression within a hematopoietic cell line, we expressed SHPS-1 in the pro-B cell line Ba/F3. This cell line lacks detectable endogenous SHPS-1 protein (Ref. 22 and our observations) and therefore provided a model system where different SHPS-1 mutants could be evaluated. Both the full-length SHPS-1 (WT SHPS-1) and a cytoplasmic deletion mutant with amino acids 400–509 deleted (ΔCT SHPS-1) were introduced into Ba/F3 cells and oligoclonal populations with high level expression identified. Out of these populations, cells showing a two-log shift by FACS analysis were sorted to obtain cell subpopulations having similar levels of both proteins (Fig. 1A). Cell surface expression was confirmed by anti-Flag Ab staining and FACS analysis of the transfected cells (Fig. 1B). During their growth, Ba/F3 cells expressing the full-length SHPS-1 displayed a spontaneous aggregation phenotype that resulted in the formation of large cell clusters (Fig. 1B). Cells transfected with a SHPS-1 cDNA that lacked the Flag epitope tag at the amino terminus were also able to form aggregates, demonstrating that this phenotype was not Flag-dependent (data not shown). Aggregate formation occurred rapidly. Thus, when aggregates were disrupted to yield a single cell suspension, and then replated, SHPS-1-expressing cells, but not control Ba/F3 cells, were able to begin reaggregating within 1–2 h. Cells transfected with empty pCDEF vector failed to aggregate, growing as a single cell suspension like the parental Ba/F3 cells (Fig. 1B). The ability to form aggregates varied with SHPS-1 expression level, with cells expressing relatively low levels of SHPS-1 showing only a weak tendency to cluster (data not shown). The expression of ΔCT SHPS-1, the mutant lacking the intracellular domain of this molecule, at levels similar to those for SHPS-1, also produced Ba/F3 cell aggregation (Fig. 1B), indicating that this process was likely independent of the SHPS-1 intracellular domain.

Cell aggregation of SHPS-1 expressing Ba/F3 cells involves a heterophilic association

Cell cluster formation could have been due either to a SHPS-1-SHPS-1 (homophilic) interaction, or to a heterophilic interaction between SHPS-1 and another molecule. To evaluate these alternative possibilities, ΔCT SHPS-1-expressing Ba/F3 cells were co-cultured at a 1:1 ratio with Ba/F3 cells expressing the fluorescent marker GFP for 24 h. As seen in Fig. 2A, the SHPS-1-transfected cells produced clusters. When these were viewed under fluorescence, GFP-expressing Ba/F3 cells were also evident within the clusters (Fig. 2B). The ability of SHPS-1-expressing Ba/F3 cell to include GFP expressing cells within the aggregates, while not excluding the possibility of a homophilic interaction, suggested that SHPS-1 might be able to bind to another cell surface molecule on Ba/F3 cells.

Anti-CD47 Ab blocks the aggregation of SHPS-1-expressing Ba/F3 cells

Jiang et al. (17) reported that SHPS-1 (P84) was one of the ligands for CD47 in neural cells; therefore we examined Ba/F3 cells for CD47 expression, verifying that these cells express CD47 and that expression levels of this molecule were not altered by SHPS-1 expression (data not shown). To investigate whether SHPS-1-expressing Ba/F3 cell aggregation was due to an interaction between SHPS-1 and CD47, the Ab mIAP301, known to block CD47-mediated cell migration (23, 24), was added to the cells. At 50 μg/ml, mIAP301, but not an isotype control, was able to block aggregation of SHPS-1-expressing cells (Fig. 3). The anti-CD47 Ab was effective at preventing aggregation at the lowest concentration.
tested (1 μg/ml), whereas the isotype control was ineffective, even at concentrations of 100 μg/ml (data not shown). As a further specificity control, the anti-Flag mAb was unable to inhibit cell-cell aggregation of cells expressing Flag epitope-tagged SHPS-1 (data not shown). This result suggested that a heterophilic interaction was likely responsible for aggregate formation of SHPS-1-transfected cells, and the result of a specific SHPS-1-CD47 interaction.

FIGURE 2. Heterophilic interactions of Ba/F3 cells expressing ΔCT SHPS-1 with GFP-Ba/F3 cells. ΔCT SHPS-1 Ba/F3 cells were co-cultivated with GFP-expressing Ba/F3 cells at a 1:1 ratio. Photographs of representative experiments were taken after 24 h. A. Cells photographed without fluorescence. B. Cells photographed with fluorescence to reveal the GFP-expressing Ba/F3 cells contained within the SHPS-1-expressing Ba/F3 cell clusters.

RBCs from C57BL/6 mice but not from CD47-deficient mice form rosettes with ΔCT SHPS-1 Ba/F3 cells. Ba/F3 cells transfected with vector-alone, or ΔCT SHPS-1, were co-cultivated with RBCs from control or CD47-deficient mice. RBCs expressing CD47 were able to form rosettes only with Ba/F3 cells expressing the extracellular domain of SHPS-1. The photographs show one representative set of experiments of five.

FIGURE 3. Anti-CD47 Ab (mIAP301) inhibits the aggregation of Ba/F3 cells expressing WT SHPS-1 and ΔCT SHPS-1. Anti-CD47 or an isotype control Ab was added at 50 μg/ml to a volume of 200 μl that contained 1 × 10⁶ Ba/F3 cells transfected with either vector alone, SHPS-1, or ΔCT SHPS-1. Photographs were taken after incubation for 16 h.

FIGURE 4. SHPS-1 mediates the formation of rosettes of control erythrocytes with ΔCT SHPS-1 Ba/F3 cells. Ba/F3 cells transfected with vector-alone, or ΔCT SHPS-1, were co-cultivated with RBCs from control or CD47-deficient mice. RBCs expressing CD47 were able to form rosettes only with Ba/F3 cells expressing the extracellular domain of SHPS-1. We evaluated whether SHPS-1 expression on the surface of Ba/F3 cells could mediate cell-cell adhesion with another hematopoietic cell type, RBCs. The latter are known to express CD47 (20, 21). RBCs from control mice were incubated with vector-alone transfected Ba/F3 cells, or with cells expressing the ΔCT SHPS-1 protein. In this experiment, RBCs were able to form rosettes only with ΔCT SHPS-1 expressing Ba/F3 cells (Fig. 4). RBCs from control mice were also able to form rosettes with Ba/F3 cells that expressed full-length SHPS-1 (data not shown). To determine...
whether rosetting was due primarily to a SHPS-1-CD47 interaction, the assay was also performed employing RBCs obtained from CD47-deficient mice. These RBCs were unable to associate with ΔCT SHPS-1-expressing Ba/F3 cells (Fig. 4), indicating that RBC-Ba/F3 cell adhesion required the presence of both SHPS-1 and CD47. Furthermore, as RBCs do not express integrins (20), the results suggested that SHPS-1-CD47-dependent cell-cell binding occurs independently of this class of cell adhesion molecule.

The amino-terminal Ig domain of SHPS-1 is sufficient to mediate association with CD47

When RBCs from control mice were incubated with Ba/F3 cells expressing a SHPS-1 mutant lacking the amino-terminal IgV domain, no rosettes were obtained (data not shown). This suggested that the IgV domain was responsible for mediating the association between SHPS-1 and CD47. To investigate this further, we generated a Flag-epitope-tagged SHPS-1 mutant that lacked the two membrane proximal IgC domains. Interestingly, despite repeated attempts, we were unable to isolate cells expressing this protein at levels (by FACS or by immunoblotting of cell lysates) comparable to those of the other SHPS-1 proteins. Thus, the ability of a GST-fusion protein (GST-V1SHPS-1) containing the IgV domain of SHPS-1 (amino acids 32–147) to bind CD47-expressing RBCs was evaluated. The GST or GST-V1SHPS-1 proteins were immobilized on nitrocellulose filters and incubated with the RBCs obtained either from control or CD47-deficient mice. Filters with immobilized GST-V1SHPS-1 incubated with control RBCs were visibly covered with these cells, whereas GST control filters, and GST-V1SHPS-1 filters incubated with cells from CD47−/− mice failed to retain as many RBCs and appeared blank on visual inspection. Cells remaining associated with the filters were lysed to enable the quantitation of RBC-derived hemoglobin by spectrophotometry (Fig. 5A). As further evidence of the role of CD47 in the RBC adhesion process, GST-V1SHPS-1 binding of RBCs was inhibited by preincubation of these cells with the anti-CD47 Ab, but not with the isotype control (Fig. 5B). It is also notable that Ba/F3 cells transfected with a SHPS-1 mutant lacking the amino-terminal IgV domain failed to aggregate (data not shown), further suggesting that this domain is both necessary and sufficient for the interaction between SHPS-1 and CD47.

Discussion

Our findings and those of others (5–7, 17, 18) point to a role for the extracellular domain of SHPS-1 in the regulation of cell adhesion phenomena. Similarly, through association with molecules such as the protein-tyrosine phosphatase SHP-1 or SHP-2, the cytosolic domain of SHPS-1 appears capable of regulating cell adhesion-induced intracellular signaling events (3, 13, 16, 26). Thus, in fibroblastic cells, SHP-2 recruitment to tyrosine-phosphorylated SHPS-1 appeared to be required for normal phosphorylation and activation of the focal adhesion kinase (FAK) in response to integrin-extracellular matrix ligand interactions (13, 16). This effect likely involved SHP-2-catalyzed Src pY527 dephosphorylation with the ensuing activation of this kinase (16). In addition, it was recently shown that an adapter protein complex, composed of SKAP55hom/R (Src-kinase-associated protein of 55-kDa homologue) and FYB/SLAP-130 (Fyn-binding protein/SLP-76-associated protein of 130 kDa), as well as the PYK2 protein-tyrosine kinase were able to associate with SHPS-1 (26). These findings prompted the speculation that the increased adhesiveness of SHP-1-deficient motheaten macrophages (26, 27) might be directly correlated with the increased phosphorylation of the adapter complex, and/or increased activity of PYK2, seen in these cells. These results indicate that a primary function of SHPS-1 may be in the regulation of subcellular signaling pathways subsequent to cell adhesion. In addition to regulating cell adhesion and receptor protein-tyrosine kinase-induced intracellular signaling pathways, the extracellular domain of SHPS-1 also appears to be involved in cell adhesion events. For example, Brooke et al. (5) demonstrated that COS-7 cells transfected with SHPS-1 were capable of binding CD4+ T cells, and that this binding was inhibited by an anti-SHPS-1 Ab. Also, macrophage fusion receptor, the cell surface target of Abs selected for their ability to block macrophage fusion, was identified to be an isoform of SHPS-1 (7). Thus, it appears that SIRP family molecules, such as SHPS-1, in addition to regulating cell adhesion-induced intracellular processes, are also capable of mediating cell adhesion events via their extracellular domains.

Recently, CD47 (IAP) was identified as a ligand for neural cell-expressed P64 (SHPS-1) (17). In this paper we have shown that CD47 is also capable of being a ligand for SHPS-1 in the hematopoietic cell line, Ba/F3. Interestingly, Ba/F3 cell aggregation was also observed in cells expressing a mutant of SHPS-1 that lacked the intracellular region of this molecule. This result suggested that SHPS-1 was unlikely to be acting via the induction of intracellular events, such as stimulation of an "inside-out" effect on integrins, to bring about the aggregate formation. However, given that CD47 has been shown to be closely associated with specific integrins (19, 28), it remained possible that the Ba/F3 cell aggregation might in part be due to an integrin-cell adhesion molecule interaction. Although this was not formally excluded in our study, the ability of SHPS-1-expressing Ba/F3 cells to bind CD47-expressing RBCs argues against this possibility. As RBCs do not express detectable...
integrins (20), we propose that a CD47-integrin complex is not required for CD47 recognition by SHPS-1, or even for the observed cell-cell adhesion that resulted in SHPS-1-induced Ba/F3 cell aggregation. The ability of the GST-V1SHPS-1 fusion protein to bind CD47-positive (but not CD47-negative) RBCs further supports the notion that SHPS-1-CD47-mediated aggregation can occur in the absence of integrins. Interestingly, our finding that the bacterially expressed amino-terminal SHPS-1 Ig-like domain was able to bind CD47-expressing RBCs also suggests that the recognition of CD47 by SHPS-1 may not be dependent on the glycosylation of this domain. Given the large number of SIRP isoforms that appear to be encoded by the human genome (1), it will be of considerable interest to determine whether CD47 is a universal ligand for this family, or alternatively, whether the sequence variations in the extracellular domains of the various isoforms specify interactions with ligands other than CD47.

Arguing that the SHPS-1-CD47 interaction leading to Ba/F3 cell aggregation was mediated by the amino-terminal IgV domain of SHPS-1, we found that a bacterially expressed GST-fusion protein containing this domain was capable of binding RBCs obtained from Cd47<sup>+/+</sup> mice, but not from Cd47<sup>−/−</sup> mice. Interestingly, Veillette et al. (22) identified an isoform of SHPS-1 in macrophages that lacked the two membrane-proximal Ig domains, raising the possibility that expression of this single Ig-like domain in this cell type might have a specific physiological role. Although the function of this particular isoform is as yet unknown, our results suggest it may indeed be capable of binding CD47. Another protein structurally related to SHPS-1, PECAM (platelet-endothelial cell adhesion molecule), also mediates both homophilic and heterophilic interactions via the amino-terminal Ig loop, a region that is required for the transendothelial migration of hemopoietic cells (29). Likewise, the finding that the amino-terminal Ig domains of both killer cell Ig-like receptor p58 and CD22 inhibitory receptors are responsible for binding to HLA-C allotypes and sialic acid ligands, respectively (30, 31), raises questions about the precise function of the membrane proximal IgC domains in these various molecules.

CD47 has been implicated in the regulation of both cell adhesion and migration; for example, it is required for postadhesive events in the transepithelial or transendothelial migration of neutrophils (32–34). In keeping with these results, mice rendered deficient in CD47 by gene targeting demonstrated an impaired ability of leukocytes to migrate into sites of infection (23). Because macrophages have also been shown to express SHPS-1, and SHPS-1-CD47 associations can lead to cell-cell association, it is plausible that SHPS-1 will be found to play an important role in adhesion-mediated migration of macrophages, or other hemopoietic cell types, across epithelial or endothelial cell layers. Furthermore, as CD47 was identified as a receptor for the extracellular matrix molecule thrombospondin (35–37), it would be of interest to determine whether SHPS-1-CD47 binding prevents CD47 binding to this angiogenesis inhibitor.

CD47 is able to function as a costimulatory molecule in the responses of T-lymphocytes to TCR stimulation (38–40). This appears to occur by at least two mechanisms. First, CD47 promotes the adhesion of T cells to APC. In support of this, COS-7 cells expressing SHPS-1 were able to bind CD47<sup>+</sup> T cells (5). Second, CD47 also appears capable of regulating T cell function via intracellular signaling pathways initiated by CD47 interaction with ligands. Interestingly, like CD47, SHPS-1 has been shown to play a costimulatory role in Ag presentation. Thus, not only were dendritic cells expressing SHPS-1 more effective in stimulating T cell responses than were their SHPS-1-deficient counterparts, but SHPS-1-blocking Abs were capable of blocking APC-induced T cell activation (5). Thus, it is conceivable that direct interactions between SHPS-1 and CD47 may be critical to the normal T cell-dependent immune responses. As many CD47-dependent processes have been defined via the use of monoclonal anti-CD47 Abs capable of blocking or stimulating various cell responses (23, 24, 32–34, 38), it is plausible that binding of CD47 by SHPS-1 will be able to alter signaling pathways lying downstream of CD47. The converse is also possible; namely, that CD47 binding to SHPS-1 at regions of cell-cell contact will be found to regulate both the subcellular localization and tyrosine-phosphorylation of SHPS-1.

Note. While this manuscript was under review, Seiffert et al. (41) demonstrated that hematopoietic cells were able to bind recombinant SIRPα protein, and that this was likely mediated by a SIRPα-CD47 interaction.

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References


