High Expression of Inhibitory Receptor SHPS-1 and Its Association with Protein-tyrosine Phosphatase SHP-1 in Macrophages*

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SHPS-1 (or SIRP) is a member of the immunoglobulin (Ig) superfamily abundantly expressed in neurons and other cell types. Within its cytoplasmic domain, it possesses at least two immunoreceptor tyrosine-based inhibitory motifs, which are targets for tyrosine phosphorylation and mediate the recruitment of SHP-2, an Src homology 2 (SH2) domain-containing protein-tyrosine phosphatase. Since other immunoreceptor tyrosine-based inhibitory motifs-containing receptors have critical roles in the negative regulation of hemopoietic cell functions, we wanted to examine the expression of SHPS-1 in cells of hematological lineages. By analyzing a panel of hemopoietic cell lines, evidence was provided that SHPS-1 is abundantly expressed in macrophages and, to a lesser extent, in myeloid cells. No expression was detected in T-cell or B-cell lines. Expression of SHPS-1 could also be documented in normal ex vivo peritoneal macrophages. Further studies showed that SHPS-1 was an efficient tyrosine phosphorylation substrate in macrophages. However, unlike in non-hemopoietic cells, tyrosine-phosphorylated SHPS-1 in macrophages associated primarily with SHP-1 and not SHP-2. Finally, our analyses allowed us to identify several isoforms of SHPS-1 in mouse cells. In part, this heterogeneity was due to differential glycosylation of SHPS-1. Additionally, it was caused by the production of at least two distinct shps-1 transcripts, coding for SHPS-1 polypeptides having different numbers of Ig-like domains in the extracellular region. Taken together, these findings indicate that SHPS-1 is likely to play a significant role in macrophages, at least partially as a consequence of its capacity to recruit SHP-1.

Protein tyrosine phosphorylation is involved in a wide variety of cellular responses, including growth factor-induced proliferation and differentiation, G-protein-coupled receptor signal transduction, and antigen/Fe receptor signaling (reviewed in Ref. 1). As the mechanisms inducing protein tyrosine phosphorylation are becoming well understood, significant interest has been directed toward identifying the processes involved in their negative regulation. As a result, numerous protein-tyrosine phosphatases (PTPs) have been identified (reviewed in Ref. 2). In some cases such as the hemopoietic cell phosphatase SHP-1, PTPs have been shown to be potent inhibitors of cell signaling.

Generally, there is little known about the modes of recruitment and activation of PTPs in physiological situations. Nonetheless, a significant advance in our comprehension of these processes was provided by the discovery that certain inhibitory receptors on hemopoietic cells, such as FcyRIIB and killer inhibitory receptors, contain within their cytoplasmic domain a motif capable of inhibiting receptor-mediated signal transduction (Refs. 3 and 4; reviewed in Ref. 5). This sequence, termed immunoreceptor tyrosine-based inhibitory motif (ITIM), is (V/I)TXXYY(L/V) (where V is valine, I is isoleucine, T is threonine, L is leucine, Y is tyrosine, and X is any residue). Phosphorylation of the tyrosine in the ITIM triggers binding and activation of Src homology 2 (SH2) domain-containing phosphatases like the protein-tyrosine phosphatases SHP-1 and SHP-2 and the inositol phosphatase SHIP (reviewed in Ref. 6). There is now strong genetic and biochemical evidence indicating that the inhibitory function of ITIM-containing receptors is mediated via these phosphatases.

Through molecular cloning, several additional receptors with potential ITIMs have been identified. Whereas most are restricted to hemopoietic cells, one receptor, named SHPS-1 or SIRP (hereafter termed SHPS-1), was first identified in non-hemopoietic cell types, especially neuronal cells (7–9). The sequence of SHPS-1 is highly conserved across species. It possesses three extracellular Ig-like domains, a single hydrophobic transmembrane sequence, and an intracellular domain of ~115 amino acids containing at least two ITIMs. Tyrosine phosphorylation of SHPS-1 was reported to occur in response to various stimuli, including activation of TPK receptors such as insulin receptor and epidermal growth factor receptor (8), exposure to mitogens (7) and cell adhesion on fibronectin-coated plastic (7). This phosphorylation was shown to trigger the binding of SHP-2, a PTP expressed in all cell types (7, 8). Whereas the exact physiological role of SHPS-1 remains to be clarified, there are convincing indications that it can inhibit receptor-mediated growth signals (8).

As other ITIM-containing receptors are involved in modulating hemopoietic cell functions, we wished to examine the distribution of SHPS-1 in hemopoietic cells. Although SHPS-1 was absent in most hemopoietic cell types, we observed that it

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF072543 and AF072544.

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1 The abbreviations used are: PTP, protein-tyrosine phosphatase; ITIM, immunoreceptor tyrosine-based inhibitory motif; SH2, Src homology 2; PAGE, polyacrylamide gel electrophoresis; PNGase, peptide-N-glycosidase F.
characterization of SHPS-1 in macrophages

accumulated in large amounts in macrophages. However, contrary to non-hemopoietic cells where SHPS-1 associates primarily with SHP-2 (Refs. 7 and 8), tyrosine-phosphorylated SHPS-1 from macrophages bound mostly to SHP-1. Our studies also uncovered multiple SHPS-1 isoforms in mouse cells. This heterogeneity was the result of differential glycosylation of the SHPS-1 protein, as well as of the existence of alternative shps-1 transcripts coding for distinct SHPS-1 polypeptides.

materials and methods

Cells and Tissues—The panel of hemopoietic cell lines used herein has been described elsewhere (10). Peritoneal macrophages were isolated from 8-week-old C57BL/6 mice after priming with an intraperitoneal injection of thioglycollate. Cells were harvested after 5 days of incubation. Cytotoxic fluorescent analyses demonstrated that >90% of cells expressed high affinity receptors for IgG and were positive for Mac-1 (data not shown). Tissues were obtained from 4-week-old C57BL/6 mice.

cDNA Cloning—A mouse C57BL/6 fetal thymocyte cDNA library (kindly provided by Dr. Louis Matis, Alexion Pharmaceuticals, New Haven, CT) was screened with a polymerase chain reaction-generated radiolabeled probe corresponding to the first Ig-like domain of mouse SHPS-1 (9). Positive clones were analyzed by sequencing (data not shown).

Ribonuclease Protection Assays—RNAs were isolated from tissues or cells of C57BL/6 mice using either the guanidinium isothiocyanate method (11) or the RNeasy Kit (Qiagen, Germany). Equivalent amounts of RNA (30 μg) were tested in ribonuclease (RNase) protection assays, as described previously (10). Radiolabeled antisense RNA probes corresponding to nucleotides 421–635 (first Ig-like region) or 1351–1626 (third Ig-like and transmembrane domains) in the mouse shps-1 sequence reported by Yamao et al. (9) were produced using the Riboprobe System (Promega, Madison, WI).

Antibodies—Polyclonal antisera directed against the first Ig-like domain (α1g) or the cytoplasmic domain (αcyt) of SHPS-1 were obtained by immunizing rabbits with TrypE fusion proteins encompassing the corresponding amino acid sequences. Rabbit antibodies reacting against SHP-1, SHP-2, phospholipase C-γ1, Cbl, Vav, Syk, Csk, Lyn, Scl, SLP-76, and phosphotyrosine have been described elsewhere (12–16, 24). An antiserum directed against the SH3 domain of the p85 subunit of phosphatidylinositol 3′-kinase was kindly provided by Dr. Tony Pawson, Samuel Lunenfeld Research Institute, Toronto, Ontario, Canada. The anti-Grb-2 serum was purchased from Santa Cruz Biotechnology, Santa Cruz, CA. Anti-Myc monoclonal antibody 9E10 was reported elsewhere (17).

Immunoprecipitations and Immunoblots—Cells or tissues were lysed in 1× TNE buffer (50 mM Tris, pH 8.0, 1% Nonidet P-40; 2 mM EDTA, pH 8.0) containing 10 μg per ml each of the protease inhibitors leupeptin, aprotinin, N-tosyl-l-phenylalanyl chloromethyl ketone, N-p-tosyl-l-lysyl chloromethyl ketone, and phenylmethylsulfonyl fluoride as well as the phosphatase inhibitors sodium fluoride (50 mM) and sodium orthovanadate (1 mM). Proteins were recovered by immunoprecipitation using the indicated antibodies. Immune complexes were then collected with formalin-fixed Staphylococcus aureus protein A (Pansorbin; Calbiochem) and washed three times in TNE buffer containing 1 mM sodium orthovanadate. In the case of mouse monoclonal antibody 9E10, S. aureus protein A was coupled to rabbit anti-mouse IgG prior to incubation with immunoprecipitates. Proteins were subsequently eluted in sample buffer, boiled, electrophoresed in 8% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) gels, and transferred onto Immobilon membranes (Millipore, Mississauga, Ontario, Canada) for immunoblotting. For immunoblots of total cell lysates, cells were lysed in boiling SDS-containing sample buffer, and lysates corresponding to equivalent cell numbers were separated by SDS-PAGE. Immunoblots were performed according to a previously described protocol (18), using 125I-protein A (Amersham Canada, Oakville, Ontario, Canada). Immunoreactive products were detected by autoradiography. All quantitations were done with a PhosphorImager (BAS 2000; Fujifilm).

Deglycosylation—After immunoprecipitation, immune complexes were collected with S. aureus protein A as described above. Following several washes, samples were treated for 2 h at 37 °C with 1000 units of peptide-N-glycosidase F (PNGase F; New England Biolabs, Beverly, MA), according to the protocol provided by the manufacturer. After elution in sample buffer, immunoprecipitates were boiled and resolved in 10% SDS-PAGE gels.

Transfections—The wild-type shps-1 cDNA and the shps-1 cDNA lacking nucleotides 849–1502 from the sequence of Yamao et al. (Ref. 9; shps-1) were cloned from a C57BL/6 mouse thymocyte cDNA library. A myc-tagged version of shps-1 cDNA was created by polymerase chain reaction. These cDNAs were inserted in the multiple cloning site of pNT-Neo, a mammalian expression vector that contains an SRE promoter and the neomycin resistance gene (neo). 70Z/3 pre-B cells or BI-141 T-cells were transfected by electroporation (260 V, 960 microfarads), using linearized DNA (50 μg). Transfected cells were selected by growth in medium containing G418 (0.5 mg/ml for 70Z/3 and 0.75 mg/ml for BI-141).

Treatment with Protein-Tyrosine Phosphatase Inhibitor PIC—J774A cells were incubated for 10 min at 37 °C with 20 μg/l bpv (PIC), a synthetic analog of the protein-tyrosine phosphatase inhibitor pervanadate (kindly given by Dr. Barry Posner, McGill University, Montréal, Québec, Canada). They were then processed for immunoprecipitation according to the protocol outlined above.

results

Cloning of SHPS-1 cDNAs from C57BL/6 Mouse—To study the expression of SHPS-1 in hemopoietic cells, mouse shps-1 cDNAs were first cloned using a C57BL/6 mouse-derived cDNA library. Approximately 100 recombinants were screened by hybridization with a radiolabeled DNA probe corresponding to the first Ig-like domain of SHPS-1. A total of 38 positive clones were identified and characterized by sequencing (data not shown). The deduced amino acid sequence of a representative clone is shown in Fig. 1A. When compared with two previously reported mouse shps-1 cDNAs, our cDNA showed differences in the predicted sequence for the SHPS-1 protein. This was especially noticeable in the first Ig-like domain, where ~10% of residues were different. A similar degree of divergence could also be observed between the two shps-1 cDNAs reported earlier. Although the exact basis for this variation is not known, the fact that all our cDNA clones had identical sequences (data not shown) and that the three known types of cDNAs had been characterized from distinct strains suggested that it may reflect some degree of polymorphism of the shps-1 gene in the mouse.

The accumulation of shps-1 transcripts in mouse tissues was next examined by RNase protection assay (Fig. 1B). Total cellular RNA (30 μg) from a variety of C57BL/6 tissues was incubated overnight with a radiolabeled antisense riboprobe corresponding to the first Ig-like region of SHPS-1. After digestion with RNase A, the protected probe fragments were resolved by electrophoresis and detected by autoradiography. The expected fragment of 214 nucleotides was present in all mouse tissues tested (lanes 1–5), including thymus (lane 1) and spleen (lane 2). Notably, the highest levels of shps-1 RNA were found in spleen (lane 2) and brain (lane 3). Similar results were obtained when a probe corresponding to the cytoplasmic domain of SHPS-1 was used (data not shown).

Expression of SHPS-1 in Hemopoietic Tissues—To assess further the distribution of SHPS-1, the levels of protein expression were examined. Polyclonal antibodies directed against the first Ig-like domain (Iγ1) or the cytoplasmic portion (cyt) of SHPS-1 were produced in rabbits, as outlined under “Material and Methods.” When used in immunoblots (Fig. 2A), these two types of antisera recognized an ~120-kDa immunoreactive polypeptide in lysates of a T-cell line transfected with a shps-1 cDNA (lanes 2 and 4) but not in untransfected cells (lanes 1 and 3). The antisera directed against the cytoplasmic domain of SHPS-1 (Fig. 2B, lanes 3 and 4) could also efficiently immunoprecipitate SHPS-1 from Nonidet P-40-containing cell lysates. However, those against the first Ig-like domain (lanes 1 and 2) were unable to recognize SHPS-1 under these conditions. This phenomenon likely indicated that the antibodies against the first Ig-like domain reacted with epitopes that required denaturation of SHPS-1 to be accessible.

Next, the expression of SHPS-1 in mouse tissues was ascer-
Tissues from C57BL/6 mice were lysed in Nonidet P-40-containing buffer, and SHPS-1 polypeptides were recovered by immunoprecipitation with a polyclonal antiserum against the cytoplasmic domain. After electrophoresis in 8% SDS-PAGE gels, immunoreactive products were detected by anti-SHPS-1 immunoblotting using another antiserum directed against the same portion of the molecule (Fig. 3A). We found that thymus (lane 1) and spleen (lane 2) contained appreciable amounts of a 120-kDa polypeptide consistent with SHPS-1 (7, 8). A similar product was observed in liver (lane 4). The electrophoretic migration of these polypeptides was fairly diffuse, in keeping with the previous documentation that SHPS-1 is extensively glycosylated (7, 8). Lesser amounts of an 50-kDa product were also detected in these tissues. Contrary to spleen, thymus,
and liver, brain tissue (lane 3) contained immunoreactive products with different molecular masses. It possessed two major species of 95 and 42 kDa, in addition to minor products of 120, 75, 60, and 35 kDa. Comparable results were obtained when an antiserum produced against the first Ig-like domain of SHPS-1 was used for immunoblotting (Fig. 3B), although this antiserum was clearly less sensitive.

As SHPS-1 is a glycoprotein (7, 8), the possibility that the heterogeneity in the apparent molecular mass of SHPS-1 was due to differences in glycosylation was tested. After immunoprecipitation, SHPS-1 polypeptides were treated for 2 h at 37 °C in the presence of peptide:N-glycosidase F (PNGase F), which removes asparagine-linked sugars. They were then resolved in 10% SDS-PAGE gels and detected by anti-SHPS-1 immunoblotting (data not shown). The positions of pre-stained molecular mass markers are shown on the right, whereas those of the various forms of SHPS-1 are indicated on the left. Exposure: 17 h.

**SHPS-1 Is Abundantly Expressed in Macrophages**—The abil-
ity to detect SHPS-1 in thymus and spleen suggested that it might be expressed in hemopoietic cells. However, as these tissues contain multiple cell lineages including non-hemopoietic cells, it was still conceivable that SHPS-1 is not expressed in hemopoietic cells. To address this issue further, the expression of SHPS-1 was evaluated in a series of mouse hemopoietic cell lines. Cells were lysed directly in boiling sample buffer, and lysates from equivalent cell numbers were separated by SDS-PAGE. The presence of SHPS-1 was detected by immunoblotting with anti-SHPS-1 antibodies (Fig. 4A).

Among the various cell lines examined, T-cells (lanes 1 and 2), B-cells (lanes 3–8), and mast cells (lanes 9 and 10) generally lacked SHPS-1. There was one exception with the B-cell line WEHI-231 (lane 6), which possessed a modest amount of SHPS-1. By contrast, SHPS-1 was abundantly expressed in the macrophage cell lines J774A and RAW264.7 (lane 11 and 12). Accumulation of SHPS-1 was also documented in another macrophage cell line, 1C-21 (data not shown). Moderate levels of expression were also detected in the myelomonocytic cell line WEHI-3B (lane 13) and the factor-dependent myeloid cell line B6SutA1 (lane 14), whereas no SHPS-1 was found in 32D, another factor-dependent myeloid cell line (lane 15). Finally, low levels of SHPS-1 were observed in the undifferentiated leukemia cell line L1210 (lane 16) but not in the erythroleukemic cell line MEL (lane 17). In general, the SHPS-1 protein detected in these cells migrated at ~120 kDa. However, in B6SutA1 (lane 14), it had an apparent mass of ~130 kDa. The exact basis for this difference is not known. In addition to a 120-kDa product, the macrophage cell lines (lanes 11 and 12) also contained large amounts of an immunoreactive species of ~50 kDa.

We wished to evaluate whether the polypeptides of 120 and 50 kDa found in macrophages were also glycosylated versions of p65 and p35. To this end, immunoprecipitated SHPS-1 molecules from J774A cells were subjected to deglycosylation as outlined above. A subsequent anti-SHPS-1 immunoblot (Fig. 4B) demonstrated that the molecular masses of these two glycoproteins were indeed diminished to 65 and 35 kDa after enzymatic removal of asparagine-linked sugars (lane 2). Contrary to normal mouse tissues, however (Fig. 3C), J774A contained approximately equal quantities of p65 and p35.

To examine whether SHPS-1 was also expressed in normal macrophages, its presence was evaluated in peritoneal macrophages. Cells were isolated from C57BL/6 mouse after intraperitoneal injection of thioglycollate, and the accumulation of SHPS-1 was ascertained by immunoblotting of total cell lysates with anti-SHPS-1 antibodies (Fig. 5). This analysis showed that ex vivo peritoneal macrophages (lane 2) expressed SHPS-1 in amounts analogous to those of J774A (lane 2). Once again, two distinct SHPS-1 molecules, gp120 and gp50, could be observed in these cells.

*p65 and p35 Are Likely Encoded by Different shps-1 Transcripts*—Whereas part of the heterogeneity in the molecular weight of SHPS-1 was explained by differences in glycosylation, two distinct SHPS-1 polypeptides, p65 and p35, remained after deglycosylation. To gain insight into the nature of these products, the C57BL/6 mouse library tested above was assessed for the existence of alternative *shps-1* cDNAs. Notably, one cDNA exhibited an internal deletion of 654 nucleotides...
The characterization of SHPS-1 in macrophages involved the study of transcripts for this molecule. The SHPS-1 polypeptide encoded by this cDNA is expected to lack the second and third Ig-like domains, thus having a predicted mass of ~32 kDa before glycosylation. A similar cDNA was recently cloned from mouse brain by Comu et al. (19).

We first wanted to ensure that transcripts corresponding to this cDNA variant existed in tissues and cells. Thus, RNase protection assays were conducted on RNA samples isolated from spleen, brain, or peritoneal macrophages, using riboprobes corresponding either to the conserved first Ig-like domain (Fig. 7A) or to the third Ig-like domain and transmembrane sequence (Fig. 7B). This last riboprobe is expected to produce a 270-nucleotide fragment when protected by wild-type shps-1 transcripts, while yielding a smaller 123-nucleotide fragment in the presence of the deleted version of shps-1 RNAs.

In this experiment, we found that a single fragment was protected in all cells and tissues when the probe corresponding to the first Ig-like domain was used (Fig. 7A). By contrast, two different fragments of 270 and 123 nucleotides were observed with the probe covering the third Ig-like region and the transmembrane domain (Fig. 7B). This was especially obvious in peritoneal macrophages (Fig. 7B, top panel, lane 3), where shps-1 RNAs were 5 to 6 times more abundant than in spleen (lane 1) or brain (lane 2). Nonetheless, a longer autographic exposure of this gel (bottom panel) showed that the 123-nucleotide species was also present in spleen (lane 1) and brain (lane 2).

To test whether the deleted shps-1 cDNA could encode p35, wild-type and deleted (Δ) cDNAs were individually transfected by electroporation in the mouse B-cell line 70Z/3, which normally lacks SHPS-1 (Fig. 4A, lane 5). Stably transfected cells were selected by growth in medium supplemented with G418 (0.5 mg/ml). An anti-SHPS-1 immunoblot of total cell lysates (Fig. 8A) showed that, contrary to parental 70Z/3 cells (lane 1), cells bearing the full-length shps-1 cDNA (lane 3) expressed an SHPS-1 polypeptide that comigrated with the larger molecule found in J774A (lane 4). In contrast, cells bearing the deleted cDNA (lane 2) displayed a species of SHPS-1 coinciding with the smaller product from J774A (lane 4).

To support further the notion that p35 was encoded by the shorter shps-1 cDNA, we tested the effect of deglycosylation (Fig. 8B). This assay demonstrated that, when deglycosylated, the molecule produced by this cDNA (lane 4) resolved at the same position as the 35-kDa polypeptide from J774A (lane 6). In comparison, the polypeptide encoded by the full-length cDNA (lane 5) comigrated with the 65-kDa protein found in the macrophage cell line (lane 6). Hence, these results indicated that p35 is likely an alternative form of SHPS-1 lacking the second and third Ig-like domains.

Tyrosine-phosphorylated SHPS-1 from Macrophages Primarily Associates with the Protein-tyrosine Phosphatase SHP-1—Previous studies have shown that tyrosine phosphorylation of SHPS-1 in fibroblasts led to its association with the SH2 domain-containing protein-tyrosine phosphatase SHP-2 (7, 8). On the basis of this observation, we wanted to ascertain whether SHPS-1 could interact with signal transduction molecules in macrophages, especially the SH2 domain-containing phosphatases SHP-1 and SHP-2 (Fig. 9). As the stimuli leading to SHPS-1 tyrosine phosphorylation in macrophages have not been determined, such a modification was provoked using the potent protein-tyrosine phosphatase inhibitor PIC, an analog of pervanadate. J774A cells were incubated for 10 min at 37 °C in the presence or absence of 20 µM PIC. After several washes, they were lysed in Nonidet P-40-containing buffer, and lysates were immunoprecipitated with antibodies directed against SHP-1, SHP-2, or SHPS-1. The interaction of SHPS-1 with SHP-1 or SHP-2 was revealed by immunoblotting with anti-phosphotyrosine (Fig. 9A) or anti-SHPS-1 (Fig. 9B) antibodies. This assay demonstrated that, when deglycosylated, the polypeptide that comigrated with the larger molecule found in J774A (lane 4) expressed an SHPS-1 polypeptide that comigrated with the larger molecule found in J774A (lane 6).

The expression of shps-1 RNAs in tissues and cells from C57BL/6 mice was determined by RNase protection assay, using probes corresponding either to the first Ig-like domain of SHPS-1 (A) or to the third Ig-like domain and transmembrane regions (B). See "Materials and Methods" for further details. The migrations of DNA molecular weight standards are shown on the right. The locations of the undigested probes are indicated by arrows on the left, and those of the predicted protected fragments are depicted by arrowheads. B, the smaller protected fragment corresponding to the deleted version of shps-1 transcripts is indicated by a double arrowhead. A longer exposure of the corresponding portion of the gel is also shown at the bottom. Exposures: A, 8 h; B, top panel: 8 h; bottom panel: 3 days.

Analyses of total cell lysates showed that J774A cells treated with PIC (Fig. 9A, lane 10) underwent a marked increase in protein tyrosine phosphorylation, in comparison with cells that had not been treated (lane 9). Importantly, the phosphotyrosine content of SHPS-1 was also strongly enhanced by incubation with the phosphatase inhibitor (lane 7). Immunoprecipitation with anti-SHP-1 antibodies (lane 5) showed that, in addition to a 65-kDa tyrosine-phosphorylated polypeptide consistent with SHP-1, several phosphotyrosine-containing proteins migrating between 110 and 130 kDa co-purified with SHP-1 in PIC-treated cells. The most prominent of these products co-migrated with tyrosine-phosphorylated SHPS-1 (lane 7). Likewise, in addition to SHPS-1, anti-SHPS-1 immunoprecipitates (lane 7) possessed a tyrosine-phosphorylated protein resolving at the same position as SHP-1 (lane 5). Contrary to SHP-1, SHP-2 immunoprecipitates (lane 6) exhibited only a few minor tyrosine-phosphorylated polypeptides, whereas immunoprecipitates obtained with preimmune serum (lane 8) did not contain any phosphotyrosine-containing protein. No tyrosine-phosphorylated products were immunoprecipitated from untreated cells with any of these antisera (lanes 1–4).
Characterization of SHPS-1 in Macrophages

When identical immunoprecipitates were subjected to immunoblotting with anti-SHPS-1 serum (Fig. 9B), it was confirmed that anti-SHP-1 immunoprecipitates from PIC-treated cells (lane 5) contained large amounts of SHPS-1. Little or no SHPS-1 was bound to SHP-1 in untreated cells (lane 1). It is worth noting that both the large (gp120) and the small (gp50) isoforms of SHPS-1 became associated with SHP-1 in PIC-treated cells (lane 5). The shorter variant of SHPS-1 could also be seen to co-precipitate with SHP-1 in the anti-phosphotyrosine immunoblot of Fig. 9A (lane 5), although it was somewhat obscured by the heavy chain of Ig. The anti-SHPS-1 immunoblot (Fig. 9B) also demonstrated that a small quantity of SHPS-1 was present in anti-SHP-2 precipitates from PIC-treated cells (lane 6). This amount was ~20 times lower than that complexed with SHP-1 (lane 5). Finally, this experiment showed that there was a noticeable retardation in the electrophoretic mobility of the two isoforms of SHPS-1 in cells incubated with the phosphatase inhibitor (compare lanes 9 and 10). In all likelihood, this alteration reflects the change in tyrosine phosphorylation of SHPS-1 induced by PIC treatment.

Next, the specificity of the interaction of SHPS-1 with SHP-1 and, to a lesser extent, SHP-2 was evaluated. J774A cells were treated with PIC as described above, and the association of SHPS-1 with various signaling molecules was determined by immunoblotting of the indicated immunoprecipitates with anti-SHPS-1 antibodies (Fig. 10). Contrary to SHP-1 (lane 1) and SHP-2 (lane 2), most other signal transduction molecules tested failed to bind detectably to SHPS-1. These included phospholipase C-γ1 (lane 3), the adaptor molecules Hip (lane 4), She (lane 10), and SLP-76 (lane 12), the guanine nucleotide exchange factor Vav (lane 5), phosphatidylinositol 3’-kinase (lane 6), and the protein-tyrosine kinases Syk (lane 7) and Lyn (lane 9). It is noteworthy, however, that SHPS-1 also co-immunoprecipitated with the inhibitory protein-tyrosine kinase Csk (lane 8) and, in smaller quantities, with the adaptor molecule Grb-2 (lane 11).

Finally, we wanted to obtain evidence that SHPS-1 could associate with SHP-1 under more physiological conditions. To this end, SHP-1 was immunoprecipitated from spleen tissue, and its binding to SHPS-1 was examined by immunoblotting with anti-SHPS-1 antibodies (Fig. 11A). We found that ~10% of SHPS-1 was constitutively bound to SHP-1 in spleen (lane 2), whereas little or none was associated with SHP-2 (lane 3) and p50csk (lane 4). To examine whether this association was caused by constitutive tyrosine phosphorylation of SHPS-1, an identical set of immunoprecipitates was probed by anti-phosphotyrosine immunoblotting (Fig. 11B). This experiment revealed that SHPS-1 (lane 1) was indeed tyrosine-phosphorylated in splenic cells. The abundance of tyrosine-phosphorylated SHPS-1 (lane 1) was equivalent to that of the ~120-kDa phosphotyrosine-containing protein bound to SHP-1 (lane 2), suggesting that most of the tyrosine-phosphorylated SHPS-1 in spleen was associated with SHP-1 (lane 2). The existence of SHPS-1-SHP-1 complexes was also documented in peritoneal macrophages (data not shown).

DISCUSSION

Previous studies have shown that the transcripts coding for SHPS-1 (or SIRP) were abundantly expressed in brain, as well as in several tissues such as lung, liver, and kidney (7–9). Although these findings allowed the identification of SHPS-1 as the first ITIM-containing receptor in non-hemopoietic cells, the observation that shps-1 RNA (7–9; this report) and protein (this report) were present in spleen and thymus suggested that SHPS-1 might be contained in hemopoietic cells. In order to identify the hematological cell types expressing SHPS-1, we have screened a series of cell lines by immunoblotting with anti-SHPS-1 antibodies. The results of this analysis indicated that most hemopoietic cell lines tested, including T-cells, B-cells, and mast cells, lacked SHPS-1 expression. However, high amounts of SHPS-1 were found in macrophage-derived cell lines. Lower quantities were uncovered in immature myeloid cell lines. Importantly, high levels of SHPS-1 were also documented in ex vivo peritoneal macrophages, indicating that expression of this molecule was clearly a feature of normal macrophages.

Collectively, these data implied that the major hemopoietic cell type containing SHPS-1 is the macrophage. However, they did not formally exclude the possibility that SHPS-1 is also present in other hemopoietic cells and that its expression is lost.
during cell line establishment. This notion was especially suggested by the observation that, although thymic tissue contains relatively few cells belonging to the macrophage lineage, it expressed appreciable levels of SHPS-1. It should be pointed out, however, that T-cells from the thymus were unlikely to be responsible for this expression, since partially purified mouse thymocytes expressed significantly lower amounts of SHPS-1, when compared with total thymus. Hence, it is possible that other thymic cell types such as stromal cells or epithelial cells might contain SHPS-1.

Whereas other studies are needed to elucidate the distribution of SHPS-1 in hemopoietic cells other than macrophages, recent data on the expression of SHPS-1 in bovine tissues provide further clarification (20). Brooke et al. (20) found that a bovine hemopoietic cell antigen previously termed MyD-1 was actually SHPS-1. By immunostaining of normal tissues, they determined that the MyD-1 epitope was expressed in bovine monocytes and epidermal dendritic cells but not in T-cells or B-cells. Thus, combined with our results, these findings do imply that within hemopoietic cells, SHPS-1 is mostly expressed in the monocyte-macrophage lineage.

Throughout our studies, we observed a marked heterogeneity in the molecular mass of SHPS-1. This variation was explained in part by differences in glycosylation of SHPS-1 between spleen, thymus, hemopoietic cells, and liver on the one hand and brain tissue on the other. In addition, we obtained evidence that there were two types of SHPS-1 protein produced by two distinct shps-1 transcripts. Whereas one protein (p65) was expected to have three Ig-like domains in its extracellular region, the other (p35) should contain only one (Fig. 6B). It should also be mentioned that Kharitonenkov et al. (8) provided data suggesting that multiple shps-1/sirp genes exist in humans. Although we failed to obtain similar evidence for a gene family in an inbred mouse strain (Fig. 1A), such a phenomenon would certainly add further complexity to the SHPS-1 protein family. Although the significance of the diversity in the SHPS-1 family is not known, it is compelling to postulate that the various isoforms of SHPS-1 interact with different extracellular ligands and, therefore, potentially mediate different functions.

In cells of neuronal, fibroblastic, or epithelial lineage, tyrosine phosphorylation of SHPS-1 was reported to trigger the
recruitment of the SH2 domain-containing phosphatase SHP-2 (7, 8), most probably via the ITIMs of SHPS-1. In contrast, we found that tyrosine-phosphorylated SHPS-1 from macrophages and spleen was primarily bound to SHP-1, a related PTP that accumulates at high levels in hemopoietic cells (reviewed in Ref. 2). There was significantly less binding to SHP-2. This difference might reflect the distinct patterns of expression of SHP-1 and SHP-2. Indeed, whereas there is little or no expression of SHP-1 in fibroblasts and neuronal cells, it abounds in hemopoietic cells. It is also possible that distinct sites of tyrosine phosphorylation on SHPS-1 mediate the recruitment of SHP-1 and SHP-2 and that these sites are differentially phosphorylated in macrophages and non-hemopoietic cells. Although additional studies are needed to test these possibilities, the fact that SHPS-1 has the capacity to bind both SHP-1 and SHP-2 is rather appealing, since there is much evidence that these two PTPs mediate distinct biological effects (reviewed in Ref. 2). Whereas SHP-1 is generally viewed as a negative regulator of cell signaling, SHP-2 has been mostly implicated in the positive regulation of intracellular signal transduction. It is also worth mentioning that a small quantity of Csk, a PTK capable of inhibiting Src family kinases (Ref. 21; reviewed in Ref. 22), was also found to associate with SHPS-1 in cells treated with the PTP inhibitor PIC. The capacity to recruit Csk may provide yet another mechanism by which SHPS-1 could suppress intracellular protein tyrosine phosphorylation.

Obviously, the physiological stimuli leading to tyrosine phosphorylation of SHPS-1 in macrophages remain to be identified. As is the case for fibroblasts (7, 8), it is imaginable that activation of receptor tyrosine kinases induces SHPS-1 tyrosine phosphorylation in these cells. However, we found that stimulation of the colony-stimulating factor-1 receptor, a PTK receptor abundantly expressed on such cells, caused only a small increase in the phosphotyrosine content of SHPS-1.2 Alternatively, SHPS-1 tyrosine phosphorylation may be regulated by interactions with components of the extracellular matrix, as reported for non-hemopoietic cells (7). Once again though, we observed that plating of macrophages on fibronectin-coated dishes resulted only in a minimal increment in SHPS-1 tyrosine phosphorylation.2 Presumably, other types of stimuli are primarily responsible for inducing SHPS-1 tyrosine phosphorylation in macrophages.

The determination of the physiological processes leading to tyrosine phosphorylation of SHPS-1 in macrophages may also shed light on its function in these cells. Based on studies performed in NIH 3T3 fibroblasts (8), SHPS-1 may have an inhibitory role in macrophages, as a consequence of its ability to recruit SHP-1. It is also likely that SHPS-1 is a receptor regulated by extracellular ligands. These ligands could control its conformation, cellular localization, and/or phosphorylation. Conversely, SHPS-1 may itself be a “ligand” for receptors expressed on other cells. As a result, it may have the capacity to affect surrounding cells. Support for this notion was provided by an earlier report in which purified p94, a brain antigen later found to be SHPS-1 (19), promoted neurite outgrowth when added to neuronal cell cultures (23). Moreover, it was recently observed that SHPS-1 on bovine monocytes interacted with a yet undetermined ligand on CD4+ T-cells, thus improving antigen presentation (20).

In summary, the results presented in this report show that SHPS-1 is abundantly expressed in macrophages, where it can undergo tyrosine phosphorylation and associate with the protein-tyrosine phosphatase SHP-1. They also demonstrate that multiple isoforms of SHPS-1 are present in mouse cells. This diversity is caused by the existence of at least two distinct

Fig. 10. Association of SHPS-1 with various signaling molecules in PIC-treated J774A cells. Cells were stimulated with PIC as for Fig. 9. The ability of SHPS-1 to associate with a variety of signaling molecules was determined by immunoblotting of the indicated immunoprecipitates with anti-SHPS-1 (A) or anti-phosphotyrosine (P.tyr) (B) antibodies. The positions of prestained molecular weight markers are indicated on the right and those of the various forms of glycosylated SHPS-1 and heavy chain of Ig are shown on the left. Exposures: 16 h.

Fig. 11. Binding of SHPS-1 to SHP-1 in normal mouse spleen tissue. After lysis of mouse spleen tissue in detergent-containing buffer, the association of SHPS-1 with signaling molecules was ascertained by immunoblotting of the indicated immunoprecipitates with either anti-SHPS-1 (A) or anti-phosphotyrosine (P.tyr) (B) antibodies. The positions of prestained molecular mass markers are shown on the right, and those of the major isoforms of SHPS-1 and heavy chain of Ig are indicated on the left. Exposures: 16 h.
shps-1 transcripts, as well as by differential glycosylation of SHPS-1. In light of these findings, it seems likely that SHPS-1 plays a significant, albeit complex, role in a variety of cell types, particularly in macrophages.

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