Janus kinase-signal transducer and activator of transcription (JAK-STAT) signals play important roles in cell proliferation, apoptosis, and inflammation, and they recently have been considered as therapeutic targets for suppressing oncogenesis and inflammatory process. Phosphatases including Src homology 2 domain-containing protein-tyrosine phosphatases (SHPs), are well known as negative regulators of the JAK-STAT pathway, but their precise mechanisms are largely unknown. Based on our previous finding that in cultured rat brain microglia, gangliosides induce rapid and transient activation of the JAK-STAT pathway, we hypothesized that raft-mediated SHP-2 activation is involved in transient activation of JAK-STAT signaling by gangliosides. We first used Western blot analysis to confirm that gangliosides rapidly induce the phosphorylation of SHP-2. This was inhibited by pretreatment with the lipid raft disrupter filipin and was restored following filipin removal. Immunostaining using antibodies directed against p-SHP-2 and flotillin-1 revealed ganglioside-induced clustering and polarization of p-SHP-2 in membrane rafts. Raft-associated regulation of SHP-2 was further demonstrated in fractionation experiments using detergent and detergent-free sucrose gradient ultracentrifugation. Rapid SHP-2 recruitment to detergent-insoluble raft fractions by gangliosides was inhibited by filipin, further indicating the involvement of rafts. We also confirmed by immunoprecipitation that SHP-2 rapidly binds in a raft-dependent manner to JAK2 in response to gangliosides. Our study therefore showed that transient activation of the JAK-STAT pathway by gangliosides is accomplished by SHP-2 in a raft-dependent manner in brain microglia.

Src homology 2-containing protein-tyrosine phosphatase 2 (SHP-2) is a member of a subfamily of protein-tyrosine phosphatases that are comprised of two SH2 domains at their N termini and a phosphatase domain at their C termini (1, 2). SHP-2 has been reported to positively regulate the MAP kinase signaling pathway initiated by either interleukin-2 or various growth factors (3, 4) and to negatively regulate JAK-STAT signaling pathways (5, 6). The positive regulation of MAP kinase is achieved through dephosphorylation of the RasGap-binding site on the platelet-derived growth factor receptor, association with other adaptor molecules, or inactivation of inhibitory molecules, all of which eventually lead to Ras-Raf-MAP kinase activation. SHP-2 exerts its negative regulatory effects via direct dephosphorylation of tyrosine-phosphorylated signaling molecules such as JAKs and STATs, which must occur through direct targeting of SHP-2 to tyrosine-phosphorylated or otherwise modified signaling molecules. SHP-2 has been reported to directly interact with growth hormone receptor as well as the gp130 receptor (6, 7) and to regulate Rho activity through its recruitment to a lipid raft (8). Therefore, lipid raft-mediated regulation of SHP-2 is an essential component of many cellular signaling pathways. Lipid rafts, which are detergent-resistant, liquid-ordered membrane domains, are enriched for cholesterol, glycosphingolipids, and phospholipids with relatively long and saturated acyl chains and are reported to serve as platforms for several cellular functions, including vesicular trafficking, signal transduction, and viral entry/infection (9–14). Raft domains contain several types of signaling molecules, including receptor tyrosine kinases, the Src family of non-receptor tyrosine kinases, and G proteins, as well as structural proteins such as members of the caveolin and flotillin families.

Microglia activation, which induces initial neuroinflammation in several neurodegenerative diseases (15–18), is regulated by many signaling pathways, including those involving NF-κB, MAP kinases, or AP-1 (19–22). The regulation of microglial activation is therefore a potential therapeutic target for combating neurodegenerative disease. The mechanisms involved in microglia activation and strategies for suppressing it have been vigorously studied (23–26). We previously reported that a JAK-STAT-mediated inflammatory signal is activated by gangliosides, lipopolysaccharide, and interferon-γ in microglia, with the signal activated by gangliosides being particularly rapid and transient (21, 26). We also reported that an activated JAK-STAT signal is inhibited by curcumin or pexoxime proliferator-activated receptor-γ agonists through interaction of JAK1/2 with SHP-2 (23, 26). However, the precise mechanisms through which SHP-2 is regulated in microglial inflammatory responses and is associated with JAKs remain largely unknown.

Based on our previous results, we propose that raft-associated SHP-2 recruitment and activation may be involved in the rapid and transient activation of the JAK-STAT pathway by gangliosides. In this study, we take advantage of the biochemical characteristics of membrane rafts to demonstrate that SHP-2 phosphorylation and targeting are induced by gangliosides, suppressed by filipin, a disrupter of membrane rafts, and restored upon filipin removal. Using double immunostaining with antibodies against p-SHP-2 and the raft marker flotillin-1, we further demonstrate that p-SHP-2 is recruited to the membrane raft by gangliosides. In addition, we use immunoprecipitation to show that the interaction between SHP-2 and JAK2 is also inhibited by filipin and restored when filipin is removed. Together, these results indicate that SHP-2 phosphorylation and targeting as well as its interaction with JAK2 occur in a raft-dependent manner.
Raft-mediated SHP-2 Regulation in Ganglioside-activated Microglia

**MATERIALS AND METHODS**

*Reagents—Gangliosides mixture (Gmix) was purchased from Matraya (Pleasant Gap, PA). Antibodies against p-SHP-2 (Y580) were purchased from Cell Signaling Technology (Beverly, MA). Antibodies against JAK2 were purchased from UBI (Lake Placid, NY), and antibodies against actin were purchased from Santa Cruz Technology (Santa Cruz, CA). SHP-2 and flotilin-1 antibodies were purchased from Transduction Laboratories (Lexington, KY). Cholera toxin B (GM1), horseradish peroxidase (conjugated), and Filipin complex were obtained from Sigma.*

*Cell Culture—Primary microglia were cultured from the cerebral cortices of 1–3-day old Sprague-Dawley rats as previously described (21, 26). Briefly, the cortices were triturated into single cells in minimal essential medium containing 10% fetal bovine serum (HyClone, Logan, UT) and plated in 75-cm² T-flasks (0.5 hemisphere/flask) for 2–3 weeks. 26). Briefly, the cortices were triturated into single cells in minimal

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*Western Blot Analysis—Cells were washed twice with cold phosphate-buffered saline (PBS) and then lysed in ice-cold RIPA buffer (150 mM NaCl, 10 mM Na₂HPO₄, pH 7.2, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.5 mM Na₃VO₄) containing protease inhibitors (2 mM phenylmethylsulfonyl fluoride, 100 µg/ml leupeptin, 10 µg/ml pepstatin, 2 mM EDTA). The lysate was centrifuged for 20 min at 12,000 × g at 4 °C, and the supernatant was then collected. Proteins were separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane (Schleicher & Schuell BioScience). The membrane was incubated with primary antibodies and horseradish peroxidase-conjugated secondary antibodies (Zymed Laboratories Inc., South San Francisco, CA), and then visualized using an enhanced chemiluminescence (ECL) system.*

*Isolation of Detergent-insoluble Fraction—Cells were washed twice with ice-cold PBS and lysed with HEPES buffer (10 mM sodium HEPES, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride) containing 0.5% Triton X-100. Cells were then incubated for 30 min and centrifuged at 12,000 × g for 30 min at 4 °C. These supernatants were used as the soluble fraction in experiments. The pellets were washed with 1 ml of cold HEPES buffer without detergent, solubilized with lysis buffer (50 mM Tris-HCl, pH 7.4, 0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40, 1 mM EDTA, 1 mM EGTA, 2 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, 10 µg/ml pepstatin, 1 mM Na₃VO₄), and centrifuged at 12,000 × g for 30 min at 4 °C. These supernatants were used as the insoluble fraction in experiments. Each fraction was analyzed by Western blotting.*

*Detergent-free, Discontinuous Sucrose Gradient Ultracentrifugation—To isolate the low-density membrane rafts, discontinuous sucrose gradient ultracentrifugation was performed (27). The cells were washed twice with ice-cold PBS and scraped into 0.5 mM sodium carbonate (pH 11.0). Homogenization was then performed using a loose-fitting Dounce homogenizer (about 40 strokes). The homogenate was then adjusted to 40% sucrose by addition of 2 ml of 80% sucrose prepared in MBS (25 mM Mes, pH 6.5, 0.15 mM NaCl) and placed at the bottom of an ultracentrifuge tube. A 5–35% discontinuous sucrose gradient (4 ml of 5% sucrose; 4 ml of 35% sucrose; both in MBS containing 250 mM sodium carbonate) was formed above the sample and centrifuged at 190,000 × g for 20 h in an SW41 rotor (Beckman Instruments).*

*From the top of each gradient, 1-ml fractions were collected for a total of 12 fractions. Gradient fractions were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes, which were blotted and incubated with anti-SHP-2 antibody.*

*Immunoprecipitation—Cell extracts were prepared using modified RIPA buffer (50 mM Tris-HCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 µg/ml leupeptin, 1 µg/ml pepstatin, 1 mM Na₃VO₄). Lysates weighing 300–500 µg were incubated with 1–2 µg of SHP-2 antibody at 4 °C overnight and precipitated with protein-G-agarose beads (Upstate, Charlottesville, VA) for 2 h at 4 °C. The immunoprecipitated proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane. Western blot analysis was then performed with several antibodies as indicated in the text and figure legends.*

*Immunostaining and Confocal Microscopy—To determine p-SHP-2 and flotilin-1 localization, cells cultured on poly-D-lysine-coated coverslips were washed twice with ice-cold PBS and fixed with 100% methanol at −20 °C. The fixed cells were then washed with PBST (PBS containing 0.1% Triton X-100) and blocked with 10% serum for 30 min at room temperature. Cells were then incubated with primary antibodies overnight at 4 °C and fluorescein- or rhodamine-conjugated secondary antibodies for 2 h, and then mounted and observed under a confocal microscope (Zeiss, Germany).*

*The Measurement of Band Density and Statistical Analysis—The band density of Western blotting was measured by Image Gauge version 4.0 program (FUJI Photo Film Co., Ltd). The graphs were expressed as mean ± S.E. They were analyzed by one-way analysis of variance followed by post-hoc comparisons (Student-Newman-Keuls) using the Statistical Package for Social Sciences 10.0 (SPSS Inc., Chicago, IL).*

**RESULTS**

*SHP-2 Phosphorylation Is Rapidly Induced by Gmix in Rat Primary Microglia and BV2 Murine Microglial Cell Lines—Based on our previous observations that JAK-STAT activation occurs within 5–15 min of Gmix addition and then dissipates after 30 min in cultured rat brain microglia (21, 26), we wanted to determine whether this transient activation is due to the suppression of JAKs by SHP-2. We first assessed SHP-2 phosphorylation using Western blot analysis, because SHP-2 phosphatase activity is regulated by the phosphorylation of the C-terminal tyrosine residues Tyr-542 and Tyr-580 (28). Primary microglia or BV2 murine microglial cells were stimulated with 50 µg/ml Gmix for the times indicated in Fig. 1. The cells were then harvested and cell lysates were subjected to Western blotting using an antibody directed against SHP-2 phosphorylated at Tyr-580. SHP-2 was rapidly phosphorylated by 5 min, but this dissipated after 30 min in both Gmix-treated primary microglia (Fig. 1A) and BV2 microglial cells (Fig. 1B).*

*SHP-2 Phosphorylation by Gmix Is Mediated via Lipid Rafts—Because SHP-2 activation is known to be linked to its raft translocation, we measured raft involvement by pretreating cells with filipin, a cholesterol-binding polyene macrolide that disrupts lipid rafts by dispersing cholesterol, a major raft component. Cells were pretreated with 5 µg/ml filipin for 15 min, washed with serum-free media, and then treated with 50 µg/ml Gmix. Filipin treatment inhibited the phosphorylation of SHP-2 induced by Gmix in both rat primary microglia (Fig. 2A) and BV2 microglial cells (Fig. 2B). Lipid rafts are known to reform after filipin removal through reincorporation of cholesterol (29–31). Therefore, to confirm the regulation of SHP-2 through lipid rafts, we measured SHP-2 phosphorylation after allowing sufficient time for lipid raft reconstruc-
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FIGURE 1. Phosphorylation of SHP-2 is induced by Gmix. Rat primary microglia (A) and murine BV2 microglial cells (B) were treated with 50 μg/ml Gmix for the indicated times. Cells were lysed with RIPA buffer and Western blot analysis was performed following 10% SDS-PAGE. SHP-2 phosphorylation was detected using an antibody directed against phospho-SHP-2 (Tyr-580). The membranes were then stripped and probed with antibodies to SHP-2, as loading controls. The graphs indicate the normalized intensities of p-SHP-2 bands against those of SHP-2 bands measured by Image Gauge technology. Data shown are representative of three independent experiments. **, p < 0.01 for Gmix treatment versus control.

FIGURE 2. Gmix-induced SHP-2 phosphorylation is inhibited by filipin. Rat primary microglia (A) and murine BV2 microglial cells (B) were pretreated with 5 μg/ml filipin for 15 min for disassembly of lipid rafts, and the media was then removed and replaced with fresh serum-free media. Immediately following washing, cells were treated with 50 μg/ml Gmix for 5 min. In BV2 microglial cells, reassembly of lipid rafts occurs 2 h after filipin removal, which is marked “+ (R)” (B). Cell lysates were subjected to Western blot analysis with an antibody directed against p-SHP-2 or actin as loading control. The right graphs indicate the normalized intensities of p-SHP-2 bands against those of actin bands measured by Image Gauge technology. Data are representative of four independent experiments. ***, p < 0.001.

Clustering and Polarization of p-SHP-2 Are Induced by Gmix—Lipid rafts are reorganized and polarized by recruitment of signaling molecules and are coalesced into larger domains. To investigate whether reconstitution of lipid rafts is induced by Gmix and whether p-SHP-2 is present in these rafts, we examined the localization of flotillin-1 and p-SHP-2 using immunocytochemistry visualized with confocal microscopy. Cells treated with or without 50 μg/ml Gmix were incubated with antibodies against the raft marker flotillin-1 and p-SHP-2. The double-stained cells were then observed under a confocal microscope. Immunofluorescence patterns of flotillin-1 and p-SHP-2 were diffusely distributed in the control primary microglia (Fig. 3A, a and b) and BV2 cells (Fig. 3B, a and b). Treatment with 50 μg/ml Gmix for 5 min induced clustering and polarization of flotillin-1 and p-SHP-2 in rat primary microglia (Fig. 3A, d and e) and BV2 microglial cells (Fig. 3B, d and e). The merged images revealed that flotillin-1 and p-SHP-2 are colocalized in Gmix-treated primary microglia (Fig. 3A, f) and BV2 cells (Fig. 3B, f). These results indicate that in Gmix-treated microglia, lipid rafts are reformed and p-SHP-2 is localized at lipid raft.

SHP-2 Is Recruited to Detergent-insoluble Fractions by Gmix—Because lipid rafts are easily isolated by their insolubility in non-ionic detergents at 4 °C, they are also called detergent-resistant microdomains. Whereas many signaling proteins are permanently associated with lipid rafts, some are only temporally associated with rafts via biochemical or biological modifications. SHP-2 is primarily present in the cytosol and is recruited to its target molecules in lipid rafts. Because we observed p-SHP-2 localization at the lipid raft in Fig. 3, we investigated whether SHP-2 is recruited to lipid rafts by Gmix using detergent-insoluble fractionation by the non-ionic detergent Triton X-100. Primary microglia or BV2 microglial cells were treated with 50 μg/ml Gmix for the times indicated in Fig. 4, and cell lysates were then fractionated into insoluble and soluble fractions using 0.5% Triton X-100. These separated fractions were then subjected to Western blot analysis. SHP-2 appeared to be translocated to the detergent-insoluble fraction at 5 min after Gmix treatment in rat primary microglia (Fig. 4A). Similarly, in BV2 microglial cells, Gmix increased the recruitment of SHP-2 to the detergent-insoluble fraction quickly (Fig. 4B). Immunoblotting with flotillin-1 confirmed the presence of lipid rafts in the detergent-insoluble...
fraction. Flotillin-1 was also used as loading control for the detergent-insoluble fractions. Phospho-SHP-2 in the detergent-insoluble fraction was hardly detected under our experimental conditions. This is probably either because of a lack of a sufficient amount of cell lysates or a methodological problem.

SHP-2 Is Recruited by Gmix to the Low-density Fraction during Sucrose Gradient Centrifugation—In addition to detergent-insoluble fractions, lipid rafts are known to be present in the low-buoyant density of sucrose gradients; therefore, we determined whether SHP-2 is also targeted into the low-density fraction by Gmix. Detergent-free sucrose gradient ultracentrifugation was performed as described under “Materials and Methods.” BV2 microglial cells were homogenized in detergent-free buffer, subjected to sucrose gradient ultracentrifugation, and divided into 12 fractions each containing 1 ml. Lipid rafts were detected by Western blot analysis with horseradish peroxidase-linked cholera toxin B, which binds to GM1, a major component of lipid rafts. GM1 was primarily detected in fractions 4 and 5 in BV2 microglial cells, indicating that these fractions contained lipid rafts (Fig. 5A). SHP-2 was detected in the GM1-positive fractions, and Gmix induced rapid association of SHP-2 to the raft region of GM1-positive fractions at 5 min (Fig. 5B). Fig. 5C shows the ratio of the band density of lipid raft-associated SHP-2 (fractions 4 and 5) over total SHP-2. Results of Figs. 4 and 5 indicate that SHP-2 was practically recruited to lipid raft by Gmix.

SHP-2 Translocation to Detergent-insoluble Fractions Is Suppressed by Filipin and Restored upon Filipin Removal—To confirm that the recruitment of SHP-2 is suppressed by disassembly of lipid rafts, we examined the effect of filipin on the translocation of SHP-2 to the detergent-insoluble fraction. As described in the legend to Fig. 2, cells were incubated with filipin for 15 min to allow for disruption of the lipid raft and subsequently incubated in fresh media for 2 h to allow raft reassembly. Cells were separated into insoluble and soluble fractions using Triton X-100 and analyzed by Western blotting. Pretreatment with 5 μg/ml filipin for 15 min suppressed recruitment of SHP-2 by Gmix in rat primary microglia (Fig. 6A) and BV2 microglial cells (Fig. 6B). Two hours after filipin removal, Gmix-induced SHP-2 recruitment was restored, further confirming that SHP-2 targeting is regulated by lipid rafts.

JAK2 Is Recruited to Detergent-insoluble Fractions by Gmix—The above results indicate that SHP-2 is targeted to and phosphorylated in lipid rafts, suggesting that this is where regulation of SHP-2 occurs. We therefore decided to search for an SHP-2 target molecule, JAK2, in the membrane raft using detergent-resistant fractionation. To examine
whether the translocation of JAK2 to lipid rafts is induced by Gmix, primary microglia (Fig. 7A, a) or BV2 microglial cells (Fig. 7B, a) were treated with 50 μg/ml Gmix for the times indicated. The cells were then lysed with 0.5% Triton X-100-containing HEPES buffer and centrifugation was performed to isolate the soluble and insoluble fractions. Each sample was then subjected to Western blot analysis. Gmix-induced recruitment of p-JAK2 and JAK2 to the insoluble fraction occurred by 5 min, but, p-JAK2 was not detected in soluble fractions (Fig. 7, A and B, a). The recruitment of JAK2 into the rafts was also inhibited by filipin in primary microglia and BV2 cells (Fig. 7, A, b and B, b, respectively) and restored following filipin removal in primary microglia. These results indicate that, like SHP-2, p-JAK2 and JAK2 are regulated through lipid rafts.

SHP-2 Targeting to JAK2 Is Regulated by Lipid Rafts—SHP-2, which is known to dephosphorylate phosphotyrosine residues, binds JAKs and negatively regulates the JAK-STAT pathway. To demonstrate that SHP-2 interacts with and inhibits JAK2, we performed immunoprecipitation using an antibody directed against SHP-2. Cell extracts were immunoprecipitated with anti-SHP-2 and subjected to Western blotting with anti-JAK2. In BV2 microglial cells, Gmix increased the association of SHP-2 with JAK2 within 5 min, whereas control levels were restored after 30 min (Fig. 8A). Pretreatment of filipin suppressed the increased binding of SHP-2 with JAK2 by Gmix, and binding was restored following filipin removal in both rat primary microglia (Fig. 8B, a) and BV2 microglial cells (Fig. 8B, b), confirming that SHP-2 association with JAK2 is regulated by lipid rafts. Finally, we tested whether SHP-2 and JAK2 are associated in the detergent-insoluble fractions. BV2 microglial cells were treated with 50 μg/ml Gmix. At the indicated times, cells were lysed and separated into detergent-insoluble and soluble fractions. SHP-2 was isolated from each fraction by immunoprecipitation, and the immunoprecipitates were analyzed by Western blotting using antibody against JAK2 or SHP-2. Although treatment with Gmix for 5 min increased the association of SHP-2 and JAK2 in the insoluble fraction, there was no change in the soluble fraction (Fig. 8C, and data not shown). Taken together, these results suggest that Gmix promotes SHP-2 recruitment to the insoluble fraction and its association with JAK2 in lipid rafts in microglia.

DISCUSSION

In the present study, we have demonstrated that the transient activation of the JAK-STAT signal transduction pathway in ganglioside-stimulated microglia is because of the phosphorylation and lipid raft-mediated association of SHP-2 with JAK-2, suggesting a potential mechanism for the regulation of SHP-2 in activated microglia. JAKs and STATs are traditionally considered interferon signaling molecules, but recent reports suggest that they are also involved in signaling triggered by other cytokines and growth factors and may thus mediate a variety of cellular functions including cell proliferation, apoptosis, and inflammation (32–34). JAK-STAT signaling is tightly regulated by induction of inhibitory molecules such as SOCS family proteins, activation of phosphatases, and degradation through ubiquitin-proteasome pathways (35–37). The rapid and transient activation of the JAK-STAT signaling pathway by gangliosides prompted us to consider regulation by protein tyrosine phosphatases such as SHPs, particularly because we have pre-
microglial cells were treated with 50 μg/ml Gmix and then were separated into detergent-insoluble and -soluble fractions as described under "Materials and Methods." Insoluble fraction was immunoprecipitated using an antibody against SHP-2 and then immunoblotted with antibodies against JAK2 or SHP-2 (C). *, p < 0.05.

FIGURE 8. SHP-2 binding to JAK2 is increased by Gmix in a lipid raft-dependent manner. BV2 microglial cells were treated with 50 μg/ml Gmix for the indicated times (A). Cell lysates were immunoprecipitated with SHP-2 antibody and then subject to Western blot analysis with anti-JAK2 and anti-SHP-2. Filipin pretreatment and removal was performed as described in the legend to Fig. 2 and immunoprecipitated (IP) in rat primary microglia (B, a) and BV2 microglial cells (B, b). The graphs of A and B indicate the normalized intensities of JAK2 bands against those of SHP-2 bands measured by Image Gauge technology. Data are representative of three independent experiments. BV2 microglial cells were treated with 50 μg/ml Gmix and then were separated into detergent-insoluble and -soluble fractions as described under "Materials and Methods." Insoluble fraction was immunoprecipitated using an antibody against SHP-2 and then immunoblotted with antibodies against JAK2 or SHP-2 (C). *, p < 0.05.

RAFT-MEDIATED SHP-2 REGULATION IN GANGLIOSIDE-ACTIVATED MICROGLIA

Viously shown that JAK-STAT signals are negatively regulated by SHP-2 (23, 26). We have shown that SHP-2 phosphorylation is rapidly induced from 5 to 15 min in ganglioside-stimulated microglia. SHPs are autoinhibited in their resting state by an intramolecular interaction between their SH2 domains and their protein-tyrosine phosphatase domain. The protein-tyrosine phosphatase domain is activated following its association with tyrosine-phosphorylated proteins, including membrane receptors and JAKs (38). Recently, Liu et al. (28) demonstrated that the two C-terminal tyrosine residues (Tyr-542 and Tyr-580) are also involved in SHP-2 activation by intramolecularly interacting with SH2 domains. However, the precise mechanisms through which SHP-2 comes in contact with phosphorysor residues and dephosphorylates its target molecules remain to be clarified. Although SHP-2 is a cytosolic protein, it is involved in the dephosphorylation of many membrane proteins. Therefore, SHP-2 must be somehow recruited to the membrane via an adapter protein or conformational changes. The necessity of membrane recruitment suggests the possible participation of lipid rafts. SHP-2 targeting to lipid rafts following cell attachment has been shown to influence cell adhesion and migration by regulating Rho activity (8). Recently, SHP-2 has been reported to be recruited to the low-density membrane fractions in confluent endothelial cells via its inter-

action with annexin II in a cholesterol-dependent manner (39). In this study, we showed that disruption of lipid rafts by filipin inhibits phosphorylation of SHP-2. In addition, SHP-2 is rapidly recruited to the detergent-insoluble, low-density fraction obtained during detergent-free sucrose gradient ultracentrifugation following incubation with Gmix, and this recruitment is inhibited by disrupting the lipid rafts with filipin. Immunocytochemical experiments using phospho-SHP-2 and flotillin-1 antibodies further revealed the induction of raft clustering by Gmix and that phospho-SHP-2 and flotillin-1 colocalized following incubation of cells with Gmix. The present results reveal that although little SHP-2 is translocated into lipid rafts compared with that in lipid rafts, it is sufficient to account for the Gmix-induced, lipid-raft-mediated changes in phosphorylation. Our results are in agreement with a previous report that a small but significant amount of SHP-2 is present in lipid rafts following T cell stimulation and that purified SHP-2 blocks protein tyrosine phosphorylation in T cell membranes (40).

SHP-2 is known to be involved in many cellular signaling pathways in both catalytic-dependent and -independent manners. This protein modulates phosphatidylinositol 3-kinase, NF-kB, JAK-STAT, and MAP kinase in catalytic-dependent ways. Specifically, SHP-2 positively regulates extracellular signal-regulated kinase activation (41, 42) and the phosphatidylinositol 3-kinase/Akt pathway during cell survival (43) in fibroblasts, whereas it negatively regulates hematopoietic cell survival through dephosphorylation of STAT5 (44). Although SHP-2 is generally known to act in a catalytically dependent manner, it has also been reported to have catalytically independent activity. Yu et al. (45) reported that catalytically inactive SHP-2 does not affect phosphatidylinositol 3-kinase activation but rather reduces JAK and ERK pathway signaling induced by interleukin-3, although all three signaling molecules are altered in SHP-2–/– hematopoietic cells, indicating that SHP-2 regulates phosphatidylinositol 3-kinase in a catalytically independent manner. Also, SHP-2 acts as an adapter molecule downstream of the prolactin receptor and is required for recruitment of its substrate (46). Components of the JAK-STAT signaling cascade have previously been reported to be target molecules of SHP-2. SHP-2–/– mutant mice display increased interferon-induced STAT1 activation, suggesting that SHP-2 negatively regulates the JAK-STAT pathway. SHP-2 is also known to be required as an adapter molecule for JAK2 association with its receptor (47). In addition, SHP-2 acts as a dual-specificity phosphatase during the dephosphorylation of STAT1 on tyrosine and serine residues (48), and direct associations between SHP-2 and JAKs have been reported (38, 49). Moreover, our previous studies showed that SHP-2 interacts with JAK and is an important inhibitory molecule of the JAK-STAT pathway. In this study, we demonstrated that SHP-2 interacts specifically with JAK2 and that this interaction is inhibited by filipin. These results suggest that rapid phosphorylation of SHP-2 by Gmix causes the JAK-STAT signal to subside in a raft-dependent manner. Although the present results did not directly show that SHP-2 regulates transient JAK-STAT activation, a recent report that transient activation of STAT1 by oncostatin M was sustained by a mutant gp130 receptor that does not bind SHP-2 (50) is consistent with our findings.

We did not detect p-SHP-2 in the detergent-insoluble fraction by Western blot analysis. Fawcett and Lorenz (51) recently reported that SHP-1 in the lipid rafts is not detected by a monoclonal antibody against the C terminus of SHP-1 but that it is detected by polyclonal antibodies against SHP-1. This suggests that the C terminus of SHP-1 possesses a novel modification that targets it to lipid rafts (51). Because SHP-2 and SHP-1 have similar structures and mechanisms of regulation, we suspect that SHP-2 also has a modification that targets it to lipid rafts.
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The study of biochemically isolated lipid rafts helps clarify pathways for rapid and accurate signaling. However, their significance and existence in vivo are controversial due to a lack of direct evidence (52, 53). Several studies have been carried out in living cells using refined techniques such as fluorescence energy transfer in an attempt to determine their relevance. Friedrichson and Kurzchalia (54) used chemical cross-linking to demonstrate that lipid raft exist at the surface of living cells (54). Recently, however, Glebov and Nichols (55) used fluorescence energy transfer to show that there is no detectable clustering or overall enrichment of glycosylphosphatidylinositol-linked proteins or cholera toxin B binding sites in activated T cells. Despite numerous attempts to confirm the presence of lipid rafts, their nature and existence are still controversial. Nonetheless, the characteristics and specific changes of proteins within biochemically isolated lipid rafts suggest they are sites where signaling molecules are concentrated and redistributed according to various stimuli.

Taken together, our results demonstrate that transient activation of the JAK-STAT pathway by Gmix is regulated by SHP-2 activation and that its association with JAK2 occurs in a raft-dependent manner, thus describing a novel role for SHP-2 targeting to lipid rafts during microglial activation. Because microglial activation plays important roles in several neurodegenerative diseases including Parkinson disease, stroke, and Alzheimer disease, raft-mediated control mechanisms of JAK-STAT inflammatory signaling involved in microglial activation could be a useful therapeutic target to combat neurodegenerative diseases.

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