Hematopoietic lineage cell-specific protein 1 (HS1) is an F-actin- and actin-related proteins 2 and 3 (Arp2/3)- binding protein that undergoes a rapid tyrosine phosphorylation upon B cell antigen receptor (BCR) activation. Density gradient centrifugation of Triton X-100 lysates from B lymphocytes demonstrated that HS1 was translocated in response to BCR cross-linking into lipid raft microdomain along with Arp2/3 complex and Wiskott-Aldrich syndrome protein. HS1-green fluorescent protein was localized in membrane patches enriched with GM1 gangliosides and BCR in the cells treated with anti-IgM antibody. Colocalization of HS1-green fluorescent protein with BCR was also correlated with tyrosine phosphorylation of HS1. Interestingly a murine HS1 mutant at the tyrosine residues Tyr^{388} and Tyr^{405} targeted by Syk failed to respond to BCR cross-linking for either translocation into lipid rafts or colocalization with BCR within cells. Furthermore HS1 was unable to translocate into lipid rafts in a chicken B cell line deficient in Syk. Reintroducing a Syk construct into the Syk knock-out cells recovered effectively both tyrosine phosphorylation and translocation of HS1 into lipid rafts. In contrast, translocation of HS1 into rafts was normal in a Lyn knock-out B cell line, and an HS1 mutant at the tyrosine residue Tyr^{222} targeted by Lyn maintained the ability to partition into rafts upon BCR cross-linking. These data indicate that Syk plays an important role in the translocation of HS1 into lipid rafts and may be responsible for actin assembly recruitment to rafts and subsequent antigen presentations.

Immune response in mammals is initiated by coordinated recognition of foreign antigens through cell surface receptors on lymphocytes. B cell antigen receptor (BCR), the complex of membrane-bound IgM, Igα, and Igβ, is the primary machinery to recognize antigens and provoke signaling cascades for proliferation and maturation of B lymphocytes into specific memory cells. Recent studies indicate that BCR-mediated signaling events often occur in membrane microdomains or lipid rafts that are rich in glycosphingolipid and cholesterol (2–4). Lipid rafts tend to select certain types of proteins and exclude others (4), thereby creating a specific environment wherein a protein can be phosphorylated by local tyrosine kinases and may become prone to interact with other local proteins in the same local environment. In lymphocytes lipid rafts undergo frequent clustering and form membrane patches upon receptor cross-linking. Consequently the lipid raft-associated molecules often display asymmetric distribution. Many processes of membrane clustering appear to be dependent on reorganization of the actin cytoskeleton meshwork, which is associated with lipid rafts on the inner side of the plasma membrane (5). The actin assembly is also necessary for the maintenance of the integrity of lipid raft (6), the antigen transportation into endosomes following BCR cross-linking (7), and the formation of immunological synapses (8). However, the mechanism for the recruitment of actin assembly to lipid rafts is unclear.

Upon receptor cross-linking, actin assembly is rapidly initiated in lipid rafts in a tyrosine phosphorylation-dependent manner (9). Interestingly BCR cross-linking also provokes translocation of BCR itself into lipid rafts in an actin-independent manner (2), suggesting that BCR in the rafts may trigger a signal transduction that ultimately leads to actin assembly. It is known that an early phase of the BCR-mediated signal transduction involves activation of several non-receptor protein-tyrosine kinases including Lyn and Syk, thereby resulting in tyrosine phosphorylation of multiple intracellular proteins (10). One of the prominent phosphorylated proteins upon BCR cross-linking is HS1, which is expressed exclusively in cells of hematopoietic and lymphoid origin (11). HS1 is structurally related to cortactin, a cortical actin-associated protein that is expressed in most adherent cells and implicated in the actin assembly mediated by Arp2/3 complex (12). Like cortactin, HS1 contains a characteristic repeated domain comprised of three and one-half 37-amino acid repeat units, a Src homology 3 (SH3) domain at the C terminus, and an Arp2/3 binding do-

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1 The abbreviations used are: BCR, B cell antigen receptor; Arp2/3, actin-related proteins 2 and 3; HS1, hematopoietic lineage cell-specific protein 1; GFP, green fluorescent protein; RFP, red fluorescent protein; WASP, Wiskott-Aldrich syndrome protein; SH, Src homology; GM1, Galβ1,3GalNAcβ1,4Glcα3,2Galβ1,4Glcα1,1Cer; TRITC, tetra-methylrhodamine isothiocyanate rhodamine; CTB, cholera toxin B; PBS, phosphate-buffered saline.
main at the N terminus (Fig. 1A). Our recent study has demonstrated that HS1 promotes actin assembly and branching by binding to both Arp2/3 complex and F-actin (13). A pathological significance of HS1 binding to F-actin has been indicated in a recent report that aberrant expression of an HS1 mutant lacking a functional domain for F-actin binding is genetically associated with systemic lupus erythematosus, an autoimmune disease characterized by the activation of autoimmune B lymphocytes (14). Studies with HS1 knock-out mice, which displayed a defect in antigen-induced clonal expansion and lymphocyte deletion (15), demonstrated that HS1 is intimately implicated in BCR and T cell receptor signalings. There is also evidence showing that HS1 is required for the apoptotic response to BCR cross-linking because B cells with low levels of HS1 expression are apparently resistant to apoptosis (16). However, the molecular mechanism for the function of HS1 in BCR signaling remains to be established.

Biochemical studies have established HS1 as a prominent substrate of protein-tyrosine kinases Syk and Src family proteins including Lyn, Fgr, Fyn, and Lck (17–20). Direct association of HS1 with Lyn or Lck is evident in T and B lymphocytes and erythroid cells during the differentiation mediated by erythropoietin (21–23). A complex of HS1 and Lck with human immunodeficiency virus type-1 virions was also reported (24). Although HS1 can be targeted by multiple tyrosine kinases, phosphorylation of HS1 at a full level requires a sequential and coordinated process involving Syk and Src. Phosphorylation is first initiated by Syk at Tyr388/Tyr389 in murine HS1 or Tyr378/Tyr387 in human HS1 and subsequently by Src-related kinases at Tyr222, presumably due to an enhanced interaction between phosphorylated HS1 and the SH2 domain of Src kinases (20, 21). However, the physiological role of HS1 phosphorylation has not yet been illustrated. In this study, we show that HS1 was recruited along with Arp2/3 complex and WASP into lipid rafts and associated with BCR upon BCR cross-linking in a tyrosine phosphorylation-dependent manner. We also show that the process of HS1 translocation into lipid rafts requires Syk and its mediated tyrosine phosphorylation. Thus, our data imply that tyrosine phosphorylation of HS1 mediated by Syk may be an important mechanism to induce actin assembly within lipid rafts in response to antigen stimulation.

MATERIALS AND METHODS

Cell Lines and Antibodies—Immature mouse B cell line WEHI-231 was obtained from American Type Culture Collection (ATCC, Manassas, VA) and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 50 μM 2-mercaptoethanol, and penicillin-streptomycin (Invitrogen). Chicken DT40, DT40Syk-/-, and DT40Lyn-/- cells were provided by Dr. Tomohiro Kurosaki (Kansai Medical University, Moriguchi, Japan). Cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 1% chicken serum, 50 μM 2-mercaptoethanol, and penicillin-streptomycin. Rabbit polyclonal antibody specific to mouse HS1 was raised against a peptide corresponding to amino acids 306–320. Mouse anti-Syk monoclonal antibody was from Santa Cruz Biotechnology (Victoria, Canada). Lyn, CD71 (H-300), and histone H1 (AE-4) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-WASP and phosphosyrosine monoclonal antibody (4G10) were from Upstate Biotechnology (Lake Placid, NY). TRITC-conjugated goat F(ab')2 fragment anti-mouse IgM, fluorescein isothiocyanate-conjugated goat F(ab')2 fragment antibody, and peroxidase-conjugated AffiniPure F(ab')2 fragment goat anti-mouse IgM were from Jackson ImmunoResearch Laboratories (West Grove, PA). All anti-IgM antibodies are specifically against the μ chain. Goat anti-mouse IgM and IgG antibodies were from Sigma. Cholera toxin B (CTB) subunit conjugated with peroxidase was from Sigma, and TRITC-CTB conjugate was from List Biological Laboratories (Campbell, CA). Goat anti-chicken IgM antibodies were from Bethyl Laboratories (Montgomery, TX).

Plasmid Constructions—Plasmid pHH117, which encodes HS1-GFP, was prepared by PCR. Briefly a murine HS1 cDNA clone from ATCC was used as the template in PCR using CCAGGAAATCATGTTGGAAGTGTCGAGGTTGGGGGACGATCCAGAGAGGG and CTGAGACTGGG and CGCGGATCCAGGGCGGCGGATGTTTTCGAG (EcoRI and BamHI sites are underlined) as primers. The resulting PCR product was inserted into the EcoRI and BamHI sites of pEFGFP-N1 (Clontech). To prepare virus HS1-GFP, the EcoRI-NotI fragment of HS1-GFP from pHH117 was inserted into the EcoRI and NotI sites of retroviral vector MIGN (a gift of Dr. Robert Hawley, Holland Laboratory), resulting in plasmid pHJ124. To prepare pHJ133 encoding HS1-Flag–GFP, point mutations at Y388F and Y405F were generated using the Transformer site-directed mutagenesis kit (Clontech) based on pHJ117. The resulting DNA fragment was inserted into the EcoRI and NotI sites of MIGN.

Plasmid pHJ172, which encodes human Syk tagged by red fluorescent protein (RFP) at its C terminus, was prepared by PCR using U.G. Summer and D. C. Mueller (University of Victoria, Canada) and B.B. Gluck and J.D. Glenn (Baylor College of Medicine, Houston, TX) as template in PCR using CCGGGAATTCATGTGGAAGTCCATGTTGGAAGTGTCGAGGTTGGGGGACGATCCAGAGAGGG and CGCGGATCCAGGGCGGCGGATGTTTTCGAG (EcoRI and BamHI sites are underlined) as primers. The resulting PCR product was inserted into the EcoRI and BamHI sites of pDsRed-Express-N1 (Clontech). The EcoRI-NotI fragment encoding Syk-RFP was isolated from pHJ172 and further inserted into the EcoRI and NotI sites of retroviral vector MIGN, resulting in plasmid pHJ179.

Virus Preparation and Viral Infection—Retrovirus-packaging cells (293GP) were the gift of Dr. Richard C. Mulligan, Harvard Medical School (Boston, MA) and were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 2 mm l-glutamine, and 1 μg/ml tetracycline. Packaging cells (1 × 10⁵) were transfected with pHJ124 or pHJ133 using Superfect transfection reagent (Qiagen Inc., Valencia, CA). To harvest virus, the transfectants were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 1 mM sodium pyruvate, and 2 mM l-glutamine. The medium of the transfectants was collected at 48, 72, and 96 h after transfection and filtered through a 0.45-μm filter (Gelman Sciences, Ann Harbor, MI). The virus medium was stored at −70 °C.

For viral infection, WEHI-231 cells or DT40 cells were plated at a density of 2 × 10⁶ in 35-mm dishes. On the next day, the medium was replaced with 1 ml of fresh medium containing 8 μg/ml Polybrene and 10 μg/ml spin-label. After 4 h of incubation, the medium was changed to the same fresh medium containing 1 mg/ml G418 for WEHI-231 cells or 2 mg/ml G418 for DT40 cells. Expression of GFP proteins was verified by fluorescence microscopy. To increase the efficiency of infection, the cells were infected with the same viruses for two or three times. After 2 weeks of selection in the medium containing G418, the cells were sorted in a fluorescence-activated cell sorting system (BD Biosciences) according to light scatter and fluorescence intensity. To express human Syk into DT40Syk-/- cells bearing HS1-GFP, the cells were reinjected with the virus pHJ179. Expression of Syk-RFP was verified by fluorescence microscopy and Western blot.

Immunoprecipitation and Immunoblotting—Cells (1 × 10⁶) to be analyzed were washed once with phosphate-buffered saline (PBS), suspended in 1 ml of a serum-free medium, and treated with 20 μg/ml anti-IgM antibodies at 37 °C. After incubation for the times as indicated, the cells were mixed with 6 ml of ice-cold PBS. Following one wash with cold PBS, cells were lysed in 500 μl of TNE buffer (1% Triton X-100, 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM Na₃VO₄, and 5 mM EDTA) containing protease inhibitor mixture (Roche Applied Science) and centrifuged briefly to remove insoluble materials. The supernatants were immunoprecipitated with protein G-Sepharose coupled with HS1 monoclonal antibody or GFP monoclonal antibody. In some experiments, immunoprecipitation was performed with protein A-Sepharose coupled with appropriate polyclonal antibodies. The immunoprecipitates were resolved by SDS-PAGE and transferred to Millipore Immobilon polyvinylidene difluoride membrane, and immunoblotted using appropriate primary antibodies followed by secondary peroxidase-conjugated antibodies (Bio-Rad). Blots were visualized with enhanced chemiluminescence (ECL, Amersham Biosciences) and autoradiography. Gel quantification was performed by densitometric analysis of scanning images using Scion Image software.

Lipid Raft Isolation—Lipid rafts were isolated by lysis of cells in Triton X-100 followed by sucrose density gradient centrifugation as described previously (3). In brief, WEHI-231 or DT40 cells (1 × 10⁶) in 10 ml of growth medium were stimulated with goat anti-IgM antibody (20 μg/ml) at 37 °C for 5 or 15 min. The treated cells were collected and resuspended in 10 ml of ice-cold PBS containing sialic acid inhibitor mixture. The lysates were homogenized with a Dounce homogenizer and centrifuged at 900 × g for 10 min to remove nuclei and cellular debris. All procedures were performed on ice. The clarified supernatants were diluted 1:1 with 85% sucrose in TNE buffer, and 2 ml of the
solution was layered at the bottom of a Beckman 14 × 89-mm centrifuge tube. The lysate was then overlaid with 6 mL of 35% sucrose and 4 mL of 5% sucrose in TNE buffer. The samples were centrifuged at 38,500 rpm in an SW41 rotor for 18 hr at 4 °C. Twelve fractions of 1 mL each were collected from the top of the gradient after centrifugation. Aliquots of each fraction with the same volume were resolved by 10% (v/v) SDS-PAGE and immunoblotted with appropriate antibodies. In some experiments, the blot membrane was stripped and rebotted with antibodies as indicated.

Immunofluorescence Microscopy—To analyze colocalization of HS1-GFP or HS1<sup>Y388F/Y405F</sup>-GFP with GM1, cells grown at log phase were stained at log phase with 25 μg/ml TRITC-CTB for 20 min on ice and centrifuged briefly to remove unbound reagents. The pellets were resuspended in a serum-free medium and then incubated with 20 μg/ml anti-IgM antibodies at 37 °C for 5 min. The stimulated cells was washed once with cold PBS, resuspended in 50 μl of cold PBS, and placed on ice. Before microscopic inspection, 5 μl of the cell samples was transferred to a glass slide, covered with a glass coverslip, and inspected under a confocal microscope. The images captured by the digital camera on the microscope were further processed with Adobe Photoshop software. To analyze colocalization of HS1-GFP or HS1<sup>Y388F/Y405F</sup>-GFP with BCR, cells grown at log phase were stimulated by addition of 25 μg/ml TRITC-conjugated goat anti-mouse IgM followed by incubation at 37 °C for the times as indicated. For the time point at 0 cells, were placed on ice for 5 min and incubated with TRITC-anti-IgM antibody on ice without shifting to 37 °C. The stimulated cells were washed once with cold PBS, resuspended in 50 μl of cold PBS, and examined as above.

RESULTS

HS1 Is Cotranslocated into Lipid Rafts with Arp2/3 Complex and WASP—In an effort to explore the signaling pathway in which HS1 is involved, we examined the potential of HS1 to associate with lipid rafts of WEHI-231 B lymphocytes. The B cells were stimulated with anti-mouse IgM (μ chain) antibody, which cross-links BCR on the cell surface and subsequently triggers a signal cascade to apoptosis (25). The treated cells were lysed in 1% Triton X-100, and the lysates were subjected to sucrose gradient centrifugation (3). Fractions of 1 mL were collected from the top of the tube after centrifugation. Aliquots with equal volumes of the fractions were analyzed by 10% (v/v) SDS-PAGE and transferred to a polyvinylidene difluoride membrane, and immunoblotted with peroxidase-conjugated CTB to detect GM1 ganglioside or blotted with anti-HS1, -Arp3, -WASP, -Lyn, -IgM, and -CD71 antibodies on the same membrane, respectively. Fractions 4–6 were labeled as Raft (fractions); fractions 10–12 are labeled as High (density fractions). As a negative control, the fractions were also probed with anti-histone H1 antibody, showing its exclusive association with high fraction 12. The data shown are representative of three independent experiments. T, total cell lysates.
of each pool were analyzed for HS1 tyrosine phosphorylation by immunoprecipitation of HS1 antibody followed by phosphotyrosine immunoblot. The same membrane was stripped and reblotted with HS1 antibody (bottom panel). Lane 1 in each panel is a negative control wherein no primary antibody was used in the immunoprecipitation. B, B cells were treated as described in Fig. 1B. Fractions 4–6 (R) and 10–12 (H) were pooled together, respectively. Aliquots of each pool were analyzed for HS1 tyrosine phosphorylation by immunoprecipitation of HS1 antibody followed by phosphotyrosine immunoblot.

Results Tyr³⁸⁸ and Tyr⁴⁰⁵ Are Required for HS1 Translocation into Lipid Rafts upon BCR Cross-linking—To study the role of HS1 in BCR signaling in more detail, we examined tyrosine phosphorylation of HS1 in WEHI-231 cells upon BCR cross-linking. As shown in Fig. 2, HS1 underwent a rapid tyrosine phosphorylation that reached a plateau within 5 min followed by a rapid decline and eventual disappearance in 2 h. The induction appeared to be specific for BCR cross-linking. As shown in Fig. 2B, a significant level of phosphorylated HS1 was found in a pooled raft fraction of the stimulated cells.

Next we analyzed whether tyrosine phosphorylation was required for HS1 raft association. As a result, we prepared a GFP-tagged murine HS1 mutant where Tyr³⁸⁸ and Tyr⁴⁰⁵ were replaced by phenylalanine; these residues correspond to Tyr³⁷⁸ and Tyr³⁹⁷ in human HS1 that are targets for Syk protein-tyrosine kinase (19). The resulting mutant, HS1Y³⁸⁸F/Y⁴⁰⁵F-GFP, was introduced into WEHI-231 cells via a retroviral vector. The defect of this mutant in tyrosine phosphorylation was verified by phosphotyrosine immunoblot (Fig. 3A). While HS1-GFP was readily phosphorylated in the cells treated with anti-IgM antibody, HS1Y³⁸⁸F/Y⁴⁰⁵F-GFP failed to show any increase in its phosphorylation level in response to BCR cross-linking. To visualize the distribution of HS1-GFP variants in relation to lipid rafts within cells, cells expressing HS1Y³⁸⁸F/Y⁴⁰⁵F-GFP and HS1-GFP were incubated with TRITC-CTB for GM1 ganglioside staining and anti-IgM antibody for BCR cross-linking. The treated live cells were then examined by confocal microscopy. As a control, cells without IgM antibody treatment were examined in parallel. In most (~75%, n = 300) non-treated cells, HS1-GFP displayed a cell peripheral and punctate staining (Fig. 3B, a and a’), a pattern similar to what has been described for cortactin in adherent cells (29). A punctate staining was also seen with GM1 as detected by TRITC-CTB (Fig. 3B, b and b’). Some GM1 puncta were apparently colocalized with or in proximity to HS1-GFP puncta (Fig. 3B, c and i). After BCR cross-linking, both HS1-GFP and GM1 were evidently translocated into large patches in many cells (~80%, n = 200) (Fig. 3B, d, e, d’, and e’) where their colocalization was more evident (Fig. 3B, f). In contrast, HS1Y³⁸⁸F/Y⁴⁰⁵F-GFP displayed a quite diffuse pattern in the cytoplasm either in most (~95%, n = 200) non-treated cells (Fig. 3B, g and g’) or in the cells treated with anti-IgM antibody (Fig. 3B, j and j’). No colocalization of the mutant with GM1 was detected in patches under either condition (Fig. 3B, i and l).

The lipid raft association with HS1-GFP and HS1Y³⁸⁸F/Y⁴⁰⁵F-GFP mutant was also examined by gradient centrifugation of Triton X-100 lysates of their overexpressors. As shown in Fig. 3C, both HS1-GFP and HS1Y³⁸⁸F/Y⁴⁰⁵F-GFP proteins were mainly found in the high fraction in resting cells. Upon BCR cross-linking, only HS1-GFP but not HS1Y³⁸⁸F/Y⁴⁰⁵F-GFP was...
shifted into the lipid raft fractions. These data indicate that lipid raft association requires functional tyrosine residues Tyr\(^{388}\) and Tyr\(^{405}\).

**Residues Tyr\(^{388}\) and Tyr\(^{405}\) Are Required for HS1 to Colocalize with BCR in Membrane Patches**—Next we examined the colocalization of HS1 with BCR within B cells. As a result, cells expressing HS1-GFP were incubated with TRITC-IgM antibody on ice for 5 min and incubated at 37 °C for an additional 5 min. The fluorescent IgM antibody was used because it can cross-link and label BCR simultaneously. The live cells treated with fluorescent IgM antibody were then inspected by confocal microscopy. As shown in Fig. 4, most BCR staining was found in the periphery of cells when incubated on ice, showing little colocalization with HS1-GFP (Fig. 4c). After cells were shifted to 37 °C for 5 min, BCR accumulated in distinct membrane patches that were distributed asymmetrically in one side of the cell. In these patches BCR was evidently colocalized with HS1-GFP (Fig. 4, d, d’, e, and f). These polarized patches were morphologically reminiscent of the caps that were described for asymmetrical clustering of actin, antigen receptors, and signaling molecules in lymphocytes (30, 31). In contrast, HS1\(^{Y388F/Y405F}\)-GFP failed to colocalize with BCR in the cell under the same conditions (Fig. 4, g–j). The failure of the mutant to translocate into patches was not due to a possible dysfunction with BCR because BCR was translocated normally into the patches within the same cell expressing the HS1 mutant (Fig. 4k). These data indicate that residues Tyr 388 and 405 are indispensable for HS1 to colocalize with BCR and to translocate into patched areas.

**HS1 Translocation into Rafts Requires the Function of Syk Tyrosine Kinase**—The above data implied a role of tyrosine phosphorylation at Tyr\(^{388}\) and Tyr\(^{405}\) residues in HS1 association with lipid rafts. Since these two residues are the targets of Syk (17), we reasoned that Syk might play a role in the translocation of HS1 into lipid rafts as well. As a result, we examined chicken B lymphatic DT40 cells and their derivatives DT40Syk\(^{−/−}\) (32). Because of the lack of appropriate antibodies for chicken HS1, we analyzed HS1-GFP that was introduced into these chicken cells via a retroviral vector. To ensure that HS1-GFP responds to BCR cross-linking properly in chicken cells, we examined tyrosine phosphorylation of HS1-GFP in DT40 cells as significantly as in WEHI-231 cells. In contrast, no apparent phosphorylation of HS1 was found in either stimulated or non-stimulated DT40Syk\(^{−/−}\) cells, although there was apparently a doublet recognized by phosphotyrosine antibody in the cells stimulated by BCR cross-linking. The nature of the doublet is currently not known but is also frequently found in B cells (see also Figs. 1 and 2). Next lipid raft fractions were isolated from these DT40 cells and analyzed for the presence of HS1-GFP in the Triton-insoluble fractions after density gradient centrifugation. As a control, raft and high fractions were probed with histone, which showed an exclusive association with high fraction only (Fig. 5B, right panels). Immunoblotting the fractions with GFP antibodies demonstrated that HS1-GFP was translocated into raft fractions of DT40 cells upon BCR cross-linking in a manner similar to that in WEHI-231 cells (Fig. 5B, left panel). However, the translocation was significantly impaired in either resting or anti-IgM treated DT40Syk\(^{−/−}\) cells. To confirm the result, we reintroduced a Syk construct, which was tagged by RFP at its
C terminus (Syk-RFP), into DT40Syk−/− cells by a retroviral vector. The ectopic expression of Syk-RFP increased tyrosine phosphorylation of HS1 in response to BCR cross-linking (Fig. 5A) and restored effectively BCR cross-linking-mediated translocation of HS1 into lipid rafts as well (Fig. 5B). These data demonstrated that tyrosine phosphorylation mediated by Syk is essential for HS1 recruitment into lipid rafts.

We also analyzed DT40Lyn−/− cells. Interestingly no apparent defect of HS1-GFP translocation into lipid rafts was found in the cells (Fig. 5B). In fact, HS1 even partitioned into lipid rafts in the resting DT40Lyn−/− cells. Tyrosine phosphorylation analysis also revealed that HS1 was markedly phosphorylated in both resting and BCR-stimulated DT40Lyn−/− cells (Fig. 5A), implying that other types of tyrosine kinases may be activated in DT40Lyn−/− cells. Consistent with this view, BCR cross-linking induced only a slight increase in HS1 phosphorylation in DT40Lyn−/− cells (Fig. 5A). To further verify the role of Src family kinases in the translocation of HS1 into rafts, we analyzed a mutant HS1Y222F-GFP in which the residue Tyr222 targeted by Src kinases was mutated to phenylalanine. As shown in Fig. 5C, the mutant was still able to be efficiently translocated into lipid rafts upon BCR cross-linking, indicating that Src family-mediated phosphorylation is not required for HS1 translocation into lipid rafts.

**DISCUSSION**

In this study we provide evidence for the first time showing that HS1, an Arp2/3 complex activator for actin assembly, is recruited to lipid rafts along with WASP and Arp2/3 complex upon BCR cross-linking, indicative of a mechanism for BCR to signal the dynamics of the actin cytoskeleton in lipid rafts. Association of HS1 with lipid rafts is indicated by both biochemical and microscopic studies. Density ultracentrifugation of cell lysates of B lymphocytes demonstrated that significant amounts of HS1 proteins were partitioned to light Triton fractions upon BCR cross-linking by anti-IgM antibody, the property that has been ascribed to many raft-associated proteins (2, 4). Microscopic analysis of live cells further revealed that HS1-GFP proteins are accumulated into asymmetric areas that are enriched with GM1 ganglioside and BCR. The morphology of these patches appears to be similar to previously described raft-associated patches found with other types of B cell lines and splenic B cells (33). Because HS1 binds to and colocalizes with Arp2/3 complex and cortical actin, we suggest that the asymmetric patches enriched with HS1 represent what has been previously described as cap, a membrane structure that is formed upon
receptor stimulation including BCR cross-linking and that accumulates cortical actin along with stimulated receptors in B lymphocytes (9, 31, 34–36). Consistent with the role of HS1 in actin assembly, translocation of HS1 into lipid rafts is accompanied by WASP and Arp2/3 complex, the molecules that are also known to play important roles in the actin reorganization. Previous studies have demonstrated that WASP, a potent activator of Arp2/3 complex, is recruited to lipid rafts in activated T cells and may be implicated in the function of immunosynapses (37). Similarly involvement of Arp2/3 complex in lipid rafts has been implied in raft-enriched vesicle trafficking based on a study with WASP (38). However, our present study shows for the first time a direct association of Arp2/3 complex with lipid rafts. Thus, our data supports the view that lipid rafts provide a physical platform for specific signaling pathways (4) or signaosome wherein HS1 is a component and consolidates with other molecules to modulate the actin cytoskeleton upon receptor stimulation.

Although HS1 is co-stained with lipid rafts and BCR in activated B cells, the mechanism for the recruitment of HS1 to lipid rafts is currently unknown because direct association of HS1 with BCR was not detected by immunoprecipitation (data not shown). Our study with an HS1 variant with mutations at tyrosine residues 388 and 405, which are targeted by Syk, suggests that Syk-mediated tyrosine phosphorylation is important for HS1 translocation into lipid rafts because the mutant fails to partition into either lipid rafts or BCR-enriched patches (Fig. 3). The role of Syk in HS1 association with rafts is further strengthened by the fact that HS1 was neither translocated into rafts nor tyrosine phosphorylated in the cells lacking Syk. Moreover association of Syk itself in the rafts of B cells upon BCR cross-linking has also been recently reported (39). Despite these facts, a strong direct association between Syk and HS1 in response to antigen stimulation remains to be established. It is also possible that Syk-mediated phosphorylation triggers a subsequent event that may promote the interaction between HS1 and other cellular factor(s) that is either directly or indirectly associated with rafts. In this regard, Lyn, which is constitutively associated with rafts (3), appears to be a good candidate. Lyn and its related kinases Lck and Fgr bind to HS1 either through their SH2 domains to phosphorylated residues Tyr775/Tyr777 (Tyr785/Tyr787 in mouse) or their SH3 domains to the proline rich domain of HS1. While binding of Lyn to phosphorylated HS1 is necessary for the phosphorylation at Tyr722 residue, the interaction is transient (20, 21). In contrast, the interaction between the Lyn SH3 domain and HS1 proline rich domain appears to be stable and can be readily detected by co-immunoprecipitation or pull-down assay.2 However, there is

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2 J.-J. Hao and X. Zhan, unpublished data.
Translocation of HS1 into Lipid Rafts

no clear evidence yet whether or not the interaction between Lyn and HS1 through SH3/proline rich domain can be regulated by tyrosine phosphorylation (21). Our data presented here indicate that Lyn itself is not necessary for HS1 to translocate into rafts because HS1 goes to rafts normally in Lyn-deficient cells. While it is possible that other Lyn-related kinases may substitute for the function of Lyn in these cells, the HS1 mutant at Tyr222, which is targeted by Lyn and its related kinases such as Fgr, Fyn, and Lck, is recruited properly to rafts of the cells stimulated by anti-IgM antibody, indicating that phosphorylation at Tyr222 and other Lyn-related kinases may not be indispensable for the recruitment of HS1 to rafts. Interestingly there is a significant number of HS1 proteins that are tyrosine phosphorylated in the cells deficient in Lyn even without stimulation. This may occur due to the function of Syk, which could be activated in the cell line deficient in Lyn, thereby resulting in apparent phosphorylation at Tyr388/Tyr405 by other Lyn-related kinases may substitute for the function of Lyn in these cells, the HS1 mutant at Tyr222, which is targeted by Lyn and its related kinases such as Fgr, Fyn, and Lck, is recruited properly to rafts of the cells stimulated by anti-IgM antibody, indicating that phosphorylation at Tyr222 and other Lyn-related kinases may not be indispensable for the recruitment of HS1 to rafts. Interestingly there is a significant number of HS1 proteins that are tyrosine phosphorylated in the cells deficient in Lyn even without stimulation. This may occur due to the function of Syk, which could be activated in the cell line deficient in Lyn, thereby resulting in apparent phosphorylation at Tyr388/Tyr405 residues. While this is consistent with the role of the Syk-mediated phosphorylation in the translocation of HS1 to rafts, additional studies are required to define the raft-associated molecule(s) that brings HS1 directly to the rafts in a tyrosine phosphorylation-dependent manner.

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