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Adapter Molecule Grb2-Associated Binder 1 Is Specifically Expressed in Marginal Zone B Cells and Negatively Regulates Thymus-Independent Antigen-2 Responses

Shousaku Itoh,* Motoyuki Itoh,2* Keigo Nishida,**† Satoru Yamasaki,* Yuichi Yoshida,* Masahiro Namimatsu,* Sung Joo Park,* Masahiko Hibi,3* Katsuhiko Ishihara,*† and Toshio Hirano4*‡‡

Grb2-associated binder 1 (Gab1) is a member of the Gab/daughter of sevenless family of adapter molecules involved in the signal transduction pathways of a variety of growth factors, cytokines, and Ag receptors. To know the role of Gab1 in hematopoiesis and immune responses in vivo, we analyzed radiation chimeras reconstituted with fetal liver (FL) cells of Gab1−/− mice, because Gab1−/− mice are lethal to embryos. Transfer of Gab1−/− FL cells of 14.5 days post-coitum rescued lethally irradiated mice, indicating that Gab1 is not essential for hematopoiesis. Although mature T and B cell subsets developed normally in the peripheral lymphoid organs, reduction of pre-B cells and increase of myeloid cells in the Gab1−/− FL chimeras suggested the regulatory roles for Gab1 in hematopoiesis. The chimera showed augmented IgM and IgG1 production to thymus-independent (TI)-2 Ag, although they showed normal responses for thymus-dependent and TI-1 Ags, indicating its negative role specific to TI-2 response. Gab1−/− splenic B cells stimulated with anti-β-dextran plus IL-4 plus IL-5 showed augmented IgM and IgG1 production in vitro that was corrected by the retrovirus-mediated transfection of the wild-type Gab1 gene, clearly demonstrating the cell-autonomous, negative role of Gab1. Furthermore, we showed that the negative role of Gab1 required its Src homology 2-containing tyrosine phosphatase-2 binding sites. Cell fractionation analysis revealed that nonfollicular B cells were responsible for the augmented Ab production in vitro. Consistent with these results, the Gab1 gene was expressed in marginal zone B cells but not follicular B cells. These results indicated that Gab1 is a unique negative regulator specific for TI-2 responses. The Journal of Immunology, 2002, 168: 5110–5116.

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5 Abbreviations used in this paper: TD, thymus dependent; TI, thymus independent; BCR, B cell Ag receptor; MZ, marginal zone; FO, follicular; Gab1, Grb2-associated binder-1; SHP-2, Src homology 2-containing tyrosine phosphatase-2; PI-3, phosphatidylinositol 3; FL, fetal liver; MacCAM-1, mucosal addressin cell adhesion molecule-1; X-gal, 5-bromo-4-chloro-3-indolylβ-D-galactopyranoside; TNP, 2,4,6-trinitrophenyl; KLH, keyhole limpet hemocyanin; HA, hemagglutinin; IRES, internal ribosomal entry site; EGFP, enhanced green fluorescent protein; SHIP, Src homology 2 domain-containing inositol-5-phosphatase.

A
tigens that induce humoral immune responses are divided into two groups, thymus-dependent (TD) and thymus-independent (TI) Ags. TI Ags are classified as Ags that stimulate Ab production in the absence of MHC class II-restricted T cell help (1). Most TI Ags are large, multivalent molecules and are found on the surface of pathogens (2). There are two types of TI Ags. A typical TI-1 Ag is an LPS that is recognized by Toll-like receptors on B cells and has mitogenic activity (3). TI-2 Ags, such as a carbohydrate dextran, do not have mitogenic activity, but their repetitive epitopes multivalently cross-link B cell receptors (BCRs) and induce B cell activation without T cell help. TI-2 Ag responses are especially important in the defense mechanisms against capsulated organisms, such as Streptococcus pneumonia, Neisseria meningitides, and Hemophilus influenza (4, 5). The splenic B cell subsets are divided into marginal zone (MZ) B cells, follicular (FO) B cells, and newly formed B cells by the differences in their expression of CD23 and CD21 (6, 7). MZ B cells are CD21hiCD23−, whereas FO B cells are CD21intCD23hi and newly formed B cells are CD21−CD23+. Functional differences of these subsets have been reported (8–10). Among them, MZ B cells play an important role in TI Ag responses (11), particularly to TI-2 Ag (8, 9). Many reports demonstrated the necessity of MZ B cells, but molecular mechanisms regulating TI-2 Ag responses are not well understood.

Grb2-associated binder 1 (Gab1) is a member of the Gab/daughter of sevenless family of adapter molecules (12, 13). Gab1 is phosphorylated on tyrosine upon the stimulation of various receptors for growth factors and cytokines (12–20). Gab1 is also activated upon ligation of B and T cell Ag receptors in cultured cell lines (19, 21). Upon stimulation, Gab1 has been shown to interact with Grb2, Src homology 2-containing tyrosine phosphatase-2 (SHP-2), the p85 phosphatidylinositol 3 (PI-3) kinase, and Shc. Recently it has been reported that Gab1 links the BCR signals to the PI-3 kinase/Akt pathway and to the SHP-2 phosphatase in a B cell line, WEHI231 (22). Targeted disruption of the mouse Gab1...
gene revealed that Gab1 is essential for development of the heart, placenta, and skin and is required for activation of the extracellular signal-regulated kinase/mitogen-activated protein kinase pathway mediated by hepatocyte growth factor, epidermal growth factor, platelet-derived growth factor, and gp130 (23). Furthermore, Gab1 is essential for the migration of myogenic precursor cells through e-Met signaling (24). Although Gab1 has been suggested to play roles in hematopoiesis and in the immune response, the embryonic lethality of Gab1<sup>−/−</sup> mice has made this issue unresolved. To reveal functions of Gab1 proteins in hematopoiesis and in the immune response in vivo, we generated a Gab1-deficient immune system in mice (radiation chimeras) by transplanting fetal liver (FL) cells from Gab1<sup>−/−</sup> mice to x-ray-irradiated mice. Analyses of these chimeras revealed that Gab1 was dispensable in hematopoiesis. Most importantly, immunization of the chimera with TI-2 Ag induced an enhanced production of Abs of the IgM and IgG1 classes, whereas TI-1 and TD antigenic responses were normal. Among the splenic B cell subsets, the expression of the Gab1 gene was restricted to MZ B cells. These results indicated that Gab1 is a specific negative regulator of the TI-2 response.

### Materials and Methods

Reconstitution of chimeras

The establishment of Gab1 knockout mice was described previously, and animals with a mixed background of 129 and C57BL/6 were used in this study. B6C3F<sub>1</sub> mice (H-2<sup>b</sup>) were irradiated with 1300 rad of x-rays and injected i.v. 1 day later with FL from Gab1<sup>−/−</sup> or Gab1<sup>+/−</sup> mice (H-2<sup>b</sup>). Eight to 9 wk after transfer, the reconstituted chimeras were subjected to a variety of analyses. Donor-derived hematopoietic cells in the lymphoid organs were detected as H-2K<sup>−</sup>-negative cells by multicolor flow cytometry analysis. Cells originating from the donors usually repopulated >90% of the hematopoietic cells in the chimeras.

Flow cytometry

Flow cytometry was performed and analyzed as described previously (25). Anti-mouse mAbs, FITC-anti-BP-1 (6C3), anti-CD3 (B3T), PE-anti-CD4 (RM4-4), anti-CD45R (RA3-6B2), and FITC-anti-CD23 (B3B4) were kindly provided by P. W. Kincade (Oklahoma Medical Research Foundation, Oklahoma City, OK) and T. Kinoshita (Osaka University, Osaka, Japan), respectively, and were labeled with FITC or Cy5 (Amersham, Arlington Heights, IL) using fluorescein isothiocyanate- or biotin-streptavidin conjugates. Bridomas using protein G-Sepharose 4B (Pharmacia, Peapack, NJ) were labeled with FITC or Cy5 (Amersham, Arlington Heights, IL) using fluorescein isothiocyanate- or biotin-streptavidin conjugates. Heat-stable Ag (M1/69), anti-CD11b (M1/70), anti-Gr-1 (RB6-8C5), and anti-CD23 (RA3-6B2) were used as the markers for macrophages and lymphocytes. Anti-Thy1, anti-CD43, anti-CD11b, and anti-IgD, anti-IgM, and anti-IgG1 mAbs were used as the markers for B cells or the recipients. For the analysis of the localization of Gab1<sup>+</sup> cells, 35- and 6-μm-thick serial frozen sections were prepared from the spleens of Gab1<sup>−/−</sup> mice and fixed with ethanol. To detect b-galactosidase activity, the 35-μm sections were stained with 0.1% 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (X-gal) in PBS containing 2 mM MgCl<sub>2</sub>, 5 mM K<sub>2</sub>Fe(CN)<sub>6</sub>, and 5 mM K<sub>4</sub>Fe(CN)<sub>6</sub> at room temperature. The marginal lining cells were detected by staining the 6-μm sections with anti-MAdCAM-1, biotinylated F(ab'<sub>2</sub>) donkey anti-rat IgG, and a Vectastain Elite ABC kit.

Analysis of Gab1 gene expression in the sorted B cell subsets by reverse transcription PCR

Spleen cells of wild-type mice were stained with FITC-anti-CD21, PE-anti-CD23, and Quantum Red-anti-CD45R Abs. And MZ B cells (CD45R<sup>−</sup> CD21<sup>−</sup> CD23<sup>−</sup>) and FO B cells (CD45R<sup>−</sup> CD21<sup>−</sup> CD23<sup>+</sup>) were sorted using a FACSVantage cell sorter (BD Biosciences). The purity of both the sorted MZ B cells and the FO B cells was 95%. RNA was isolated from sorted cells lysed with Sepasol-RNA I (Nacalai Tesque, Kyoto, Japan) and reverse transcribed. RT-PCR was conducted as described previously (19). Primer sequences for Gab1 were 5'-GCCGTCGAGAAGAAGGTTGGTTGTGTTG-3' and 5'-GCCGACTGAAAGACGCTTCCATGCA-3'.

### Immunization

To examine the TI responses, 8–9 wk after reconstitution, the chimeric mice were injected i.p. with 50 μg of 2,4,6-trinitrophenyl (TNP)-LPS (prepared in this laboratory) or 20 μg TNP-Ficoll (Biosearch Technologies, Novato, CA). Blood was taken through the retro-orbital plexus every 7 days. For the TD Ag responses, the mice were i.p. injected with 100 μg of DNP-keyhole limpet hemocyanin (KLH) (LSL, Tokyo, Japan) precipitated with 4 mg of alum (LSL) on day 0 and boosted with 20 μg of DNP-KLH in saline on day 21.

B cell proliferation assay

Spleen cells were obtained from mice 8–9 wk after injection and were treated with 0.165 M NH<sub>4</sub>Cl to lyse the erythrocytes. B cells were purified by negative sorting using anti-Thy1, anti-CD43, anti-CD11b, and anti-CD11c Ab-conjugated magnetic beads (Miltenyi Biotech, Auburn, CA) and a MiniMACS column (Miltenyi Biotech). The purity of the splenic B cell preparation was verified by flow cytometry analysis and was >95%. From the purified B cells, FO B and non-FO B cells were further purified by positive and negative sorting using biotin-anti-CD23 Ab (BD Pharmingen) and streptavidin-conjugated magnetic beads (Miltenyi Biotech) and MiniMACS column (Miltenyi Biotech). Non-FO B cell preparation consists of MZ B and newly formed B cells with a ratio of ~1:1.

B cell proliferation assay

Purified splenic B cells (2 × 10<sup>7</sup>/200 μl) were cultured in triplicate in RPMI 1640 supplemented with 10% FBS, 1 mM glutamine, 50 μM 2-ME, and antibiotics. The F(ab')<sub>2</sub> goat anti-mouse IgM (Jackson ImmunoResearch Laboratories), LPS (Escherichia coli 0111:B4; Sigma-Aldrich), anti-δ-dextran (kindly provided by C. M. Snapper, Uniformed Services University of the Health Sciences, Bethesda, MD), or anti-CD40 Ab (HM40-3; BD Pharmingen) was added to the culture medium at various concentrations. Triplicate samples of cells were pulsed with 0.5 μCi/well [<sup>3</sup>H]thymidine (NEN Life Science Products, Boston, MA) for the final 6 h of a 72-h-long culture. The amount of [<sup>3</sup>H]thymidine incorporation was determined using a micro beta scintillation counter (Wallac, Gaithersburg, MD).

Ig production of B cells in vitro

Purified splenic B, FO B, and non-FO B cells (5 × 10<sup>7</sup>/200 μl) were cultured in the presence or absence of anti-δ-dextran (3 ng/ml), anti-δ-dextran plus IL-5 (150 U/ml); a generous gift from K. Takatsu, University of Tokyo, Tokyo, Japan), or anti-δ-dextran plus IL-5 plus IL-4 (1000 U/ml; Life Technologies, Rockville, MD). Supernatants from the cultures were harvested at day 6 and Ab production was measured by ELISA.

### Generation of viral supernatants and transduction of primary B lymphocytes

To construct the Gab1 mutant Gab1<sup>F447/472/589</sup> (phyl), tyrosines 447, 472, and 589 of human Gab1, corresponding to the YXXM motifs, were mutated to phenylalanines by PCR-based mutagenesis using pcDNA3-hemagglutinin (HA)-Gab1 (12, 17). The Gab1 mutant Gab1<sup>F447/472/589</sup> was constructed by replacing tyrosines 627 and 659, corresponding to the YLVD and YVYV motifs, with phenylalanines. The cDNA fragments of HA-tagged wild-type and mutant Gab1 were excised from pcDNA3 and subcloned into the HindIII site of pBluescript SK+ (Strategene, La Jolla, CA). Primers used
for the mutagenesis are available on request. To generate the internal ribosomal entry site (IRES)-enhanced green fluorescent protein (EGFP) fragment, the EGFP cDNA from pC1EGFP (Clontech Laboratories, Palo Alto, CA) was inserted into the Neo and BamHI sites of pBKS-CITE (a gift from H. Niwa, Osaka University). To construct the retroviral vectors, the BglII-Sall fragment of IRES-EGFP was subcloned into the BamHI and Sall sites of pMX (a gift from T. Kitamura, University of Tokyo), then the HA-Gab1 fragments from pBluescript SK− were inserted into the ClaI and EcoRI sites in IRES-EGFP (located at the 5′ end of the IRES sequence). To generate the retroviruses, the 293T-based packaging cell line SBR3432 (a gift from G. Nolan, Stanford University, Stanford, CA) was transfected with the retroviral vectors by a standard calcium phosphate precipitation method. At 24 h after the transfection, the medium was changed to RPMI 1640 containing 10% FBS and antibiotics. The supernatants were harvested after additional 24- and 48-h incubations, with fresh RPMI 1640 medium being added after the first harvest of viral supernatant (26). Purified splenic B cells from Gab1−/− FL chimeras were activated with anti-δ-dextran (3 mg/ml). At 24 h after stimulation, the cells were infected by incubation with the viral supernatants in the presence of 8 μg/ml polybrene (Sigma-Aldrich) and anti-δ-dextran (3 mg/ml). The infected B cells were stimulated with anti-δ-dextran (3 mg/ml), IL-4 (1000 U/ml), and IL-5 (150 U/ml) in 96-well plates at a concentration of 5 × 106 cells/well. After a 6-day incubation, the supernatants were harvested and used to determine the production of Ig. The efficiency of infection was verifed by flow cytometry analysis of the green fluorescent protein-positive cells and was ~50% for each infection.

Results

The Role of Gab1 in hematopoiesis

Gab1 is tyrosine-phosphorylated by the stimulation of stem cell factor, IL-3, erythropoietin, and GM-CSF or by ligation of Ag receptors, suggesting that Gab1 plays a role in hematopoiesis or immune responses in vivo. Because Gab1−/− mice were lethal to embryos, we made radiation chimeras with embryonic day 14.5 FL cells (FL chimeras) to explore the roles of the Gab1-mediated signals in hematopoietic cell differentiation. Transplantation of Gab1−/− FL cells could rescue the lethally irradiated recipient mice, indicating that Gab1 is dispensable, at least cell autonomously, for hematopoiesis. There were no differences in the total cell numbers of the bone marrow, spleen, lymph nodes, and peritoneum between Gab1−/− and Gab1+/− FL chimeras. Flow cytometry analyses of Gab1−/− FL chimeras revealed that, in the bone marrow, the frequencies of myeloid (Gr-1+CD11b+, Gab1+/+; 26 ± 3%; Gab1−/−; 42 ± 7%) and erythroid (TER-119+, Gab1+/+; 27 ± 3%; Gab1−/−; 34 ± 2%) cells increased and those of pre-B cells (CD45R−IgM−), but not pro-B and B cells, decreased (Gab1+/+; 16.3 ± 2%; Gab1−/−; 6.8 ± 2.9%) (Table I and Fig. 1). Total cell numbers of thymus decreased (Gab1+/+; 6.5 ± 1.9 × 106; Gab1−/−; 3.6 ± 1.6 × 106; p < 0.005) due to the reduction of CD4−CD8+ thymocytes (Gab1+/+; 78.5 ± 3.7%; Gab1−/−; 47.6 ± 32.3%). In the spleen, cell numbers of CD4+ or CD8+ T cells, B cell subsets defined by IgM/IgD or CD21/CD23 expressions, and macrophages were normal. In the peritoneum, mature macrophages (F4/80+CD11b+) increased (Gab1+/+; 28 ± 9%; Gab1−/−; 44 ± 11%). These results indicate that the Gab1 signaling pathway was not essential for the generation of mature immunocompetent cells in the peripheral lymphoid organs, although it played some regulatory roles in the early development of erythroid, myeloid, and B lymphoid cells.

Gab1−/− FL chimeras showed higher IgM and IgG1 responses to TI-2 Ag

Because the Gab1−/− FL chimeras had an almost normal population of immunocompetent cells, we examined the Ab production of the chimeras to further clarify the roles of Gab1 in the immune regulation in vivo. The serum Ig levels were normal in the Gab1−/− FL chimeras 9 wk after reconstitution (data not shown). When the Gab1−/− FL chimeras were immunized with TD Ag (DNP-KLH) and TI-1 Ag (TNP-LPS), Ab responses were comparable to those of the controls (Figs. 2 and 3A). In contrast, their Ab productions in response to TI-2 Ag, TNP-Ficoll, showed a higher production of Ag-specific IgM (136 ± 30 μg/ml at day 7) and IgG1 (2.8 ± 0.7 μg/ml at day 14) than did their Gab1+/+ counterparts (IgM: 88 ± 7 μg/ml at day 7; IgG1: 1.3 ± 0.3 μg/ml at day 14) (Fig. 3B), indicating that Gab1 negatively regulates the TI-2 response in vivo.

Gab1−/− B cells showed enhanced Ig secretion in vitro responding to the TI-2-like stimulus anti-δ-dextran

To elucidate the role for Gab1 in B cells, we examined the proliferation of Gab1−/− B cells stimulated with anti-IgM, LPS, and anti-CD40 (Fig. 4A). In each case, the proliferation was similar for purified B cells from the spleens of Gab1−/− and Gab1+/+ FL chimeras. Anti-δ-dextran, which is dextran conjugated with anti-IgD mAb, provides stimuli similar to TI-2 Ags and is used as a model system for studying immune response to TI-2 Ag in vitro (27). First we examined the growth responses to anti-δ-dextran alone or in combination with IL-5 or IL-5 + IL-4 (Fig. 4B). There were no differences between Gab1−/− and Gab1+/+ B cells. To evaluate the role of Gab1 in B cell differentiation in vitro, we stimulated B cells with anti-δ-dextran, anti-δ-dextran plus IL-5, and anti-δ-dextran plus IL-5 plus IL-4 and measured the level of Abs in the supernatant. Only the stimulation with anti-δ-dextran plus IL-5 plus IL-4, which induces a class switch to IgG1, caused the higher production of IgM (28 ± 4 μg/ml) and IgG1 (7.6 ± 0.3 μg/ml) by Gab1−/− B cells compared with Gab1+/+ B cells (IgM: 14 ± 1 μg/ml; IgG1: 4.2 ± 0.1 μg/ml) (Fig. 5A). Although MZ B cells are known to be responsible for TI-2 response, there is a possibility that augmented Ab production is due to FO B, a major population in splenic B cells. To eliminate this possibility, we examined the Ab production of FO B and non-FO B fractions. The

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<td>Bone marrow</td>
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<td>B220+IgM−</td>
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<td>B220+CD3+</td>
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<td>HSA− (fraction A)</td>
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<td>BP-1 HSA− (fraction C′)</td>
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<td>Gr-1 CD11b−</td>
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<td>CD11b− F4/80+</td>
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†, p < 0.05.

*Single-cell suspensions prepared from various tissues were labeled with the indicated Abs as described in Materials and Methods, and were analyzed with FACScan. The percentages of the subset in the H-2Kb-negative donor-derived cells are shown. Values represent the mean ± SD of three to four separate determinations. Statistical analysis was carried out using the Student t test.

p, p < 0.01.

p, p < 0.05.

p, p < 0.005.
amounts of Abs produced by Gab1−/− FO B and those produced by Gab1+/+ FO B were similar, indicating that Gab1−/− FO B is not responsible for the enhanced Ab production in vitro. In contrast, Gab1−/− non-FO B showed higher Ab production (IgM: 27 ± 2 μg/ml; IgG1: 17.6 ± 1.5 μg/ml) than did Gab1+/+ non-FO B (IgM: 22 ± 1 μg/ml; IgG1: 9.7 ± 0.3 μg/ml) (Fig. 5B). Because non-FO B fraction consists of mature MZ B cells and immature, newly formed B cells that die by BCR ligation, Gab1−/− MZ B cells are most likely to be responsible for the enhanced Ab production in vitro. These results consistent with the in vivo data show that Gab1 is a negative regulator specific to the TI-2 response and further suggest its cell-autonomous negative role in B cells.

Gab1 exerted its negative regulation through its SHP-2 binding sites

To confirm the negative regulatory roles for Gab1 in B cell differentiation and to examine which molecular complex with Gab1 is responsible for its negative effect, we made a series of retroviral vectors expressing either wild-type or mutant Gab1, as illustrated in Fig. 6A. Splenic B cells from Gab1−/− FL chimeras were infected with the retrovirus (the frequencies of infected cells were usually ~50% and there was no difference in the infection using each construct) and cultured for 6 days in the presence of anti-ξ-dextran plus IL-5 plus IL-4. The elevated production of IgM and IgG1 was inhibited in the cultured B cells that had been transfected with either wild-type Gab1 or Gab1F447/472/589, a mutant with a
defect in PI-3 kinase binding motifs. However, the expression of Gab1\(^{F627/659}\), which has mutations at the SHP-2 binding motifs, did not rescue the inhibitory effect of Gab1 (Fig. 6B). These data indicate that Gab1 plays negative regulatory roles in B cell differentiation through its SHP-2 binding motifs.

Gab1 was expressed in MZ B cells but not in FO B cells

Although the analyses in vitro suggested that Gab1 negatively regulates the differentiation of MZ B cells, it is not clear why targeting Gab1 resulted in abnormality selective to TI-2 Ag responses. Because the frequency of CD21\(^{+}\)CD23\(^{+}\)MZ B cells in the spleen of Gab1\(^{−/−}\) FL chimeras was normal (Fig. 1 and Table I), we analyzed the structure of the spleen of the Gab1\(^{−/−}\) FL chimera by immunohistochemistry. Dual staining with anti-IgM and anti-

**FIGURE 4.** Normal growth responses of Gab1\(^{−/−}\) B cells. [\(^{3}\)H]Thymidine uptake (cpm) by purified splenic B cells from Gab1\(^{+/+}\) (open bars; \(n = 3\)) or Gab1\(^{−/−}\) (filled bars; \(n = 3\)) FL chimeras is indicated. The mean values of three independent experiments are shown. Error bars indicate SD. B cells were cultured for 72 h in the presence of F(ab\(^{'})\) of goat anti-mouse IgM Ab (A), LPS, anti-CD40 Ab, or various combinations of anti-\(\delta\)-dextran (3 ng/ml), IL-4 (1000 U/ml), and IL-5 (150 U/ml) (B).

**FIGURE 5.** Higher Ig secretion of the IgM and IgG1 classes by Gab1\(^{−/−}\) non-FO B cells stimulated with anti-\(\delta\)-dextran plus IL-4 plus IL-5. A, Purified splenic B cells from Gab1\(^{+/+}\) (open bars) or Gab1\(^{−/−}\) (filled bars) FL chimeras were cultured with combinations of anti-\(\delta\)-dextran (3 ng/ml), IL-4 (1000 U/ml), and IL-5 (150 U/ml). B, Purified non-FO B and FO B cells were cultured with anti-\(\delta\)-dextran plus IL-4 plus IL-5. The culture supernatants were harvested 6 days later, and the concentrations of IgM or IgG1 were measured by ELISA. The ELISAs were performed in triplicate and the data are presented as mean ± SD. Similar results were obtained in three independent experiments. Statistical analysis was conducted using the Student t test. * p < 0.05; ** p < 0.005.

**FIGURE 6.** The SHP-2 binding sites of Gab1 are required for its inhibitory effects on B cell Ig production in response to TI-2-like stimuli in vitro. A, Schematic representation of the retroviral constructs. The gene for green fluorescent protein is indicated downstream as a shaded box. An IRES, which allows for translation of both genes, is indicated. B, Gab1\(^{−/−}\) B cells were infected with retroviral vectors harboring the cDNA for wild-type and mutant Gab1 and were cultured for 6 days. The IgM and IgG1 concentrations in the culture supernatants were measured by ELISA. The ELISAs were performed in triplicate and the data are presented as mean ± SD. Similar results were obtained in three independent experiments.
Gab1 is a scaffolding adapter molecule that is activated downstream of the receptors for various growth factors and cytokines, including stem cell factor, IL-3, IL-6, erythropoietin, GM-CSF, and thrombopoietin, all of which are involved in hematopoiesis (12–22). Although this relationship has led some to suggest a crucial role for Gab1 in hematopoiesis, we found that lethally irradiated mice transplanted with Gab1−/− FL cells survived, which indicated that the role of Gab1 in hematopoiesis was redundant. Another family member, Gab2 (19, 28, 29) or Gab3 (30), may compensate for the missing Gab1 in this process. However, our flow cytometry analysis suggested that Gab1 played some regulatory roles in the early development of erythroid, myeloid, and B lymphoid cells. The frequencies of TER-119+ and CD11b+ cells were higher, whereas those of CD45R+ sIgM− pre-B cells were lower in the bone marrow of Gab1−/− compared with Gab1+/+ FL chimeras. Because Gab1 gene expression was detected in pre-B cells of the bone marrow B lineage cells (data not shown), the positive regulatory roles for Gab1 in the signal transduction pathway of IL-7 were suggested. In contrast, the increase of TER-119+ and CD11b+ cells in Gab1−/− FL chimeras suggested negative regulatory roles for Gab1 in the signal transduction of erythropoietin and GM-CSF, respectively. To address these issues more precisely, the roles of Gab1 family proteins in lymphohematopoiesis are currently under investigation using knockout mice lacking Gab1 or Gab2 or both.

Because the immunocompetent cells in the peripheral lymphoid organs of the Gab1−/− FL chimera developed normally, we were able to analyze the roles for Gab1 in the immune response in vivo. Analysis of the humoral immune responses in the Gab1−/− FL chimera revealed an enhanced production of IgM and IgG1 in response to TI-2 Ag and normal Ig production in response to TI-1 and TD Ag. We concluded that the enhanced TI-2 response was primarily due to a loss of cell-autonomous negative regulation by Gab1 in MB cells because 1) the Gab1 gene was specifically expressed in the MZ B cells of normal mice, 2) MZ B cell development and organization of the MZ were normal in Gab1−/− FL chimeras, 3) a higher production of IgM and IgG1 was also induced in vitro using purified Gab1−/− non-FO B cells stimulated with a combination of cytokines and anti-6-dextran that provides a stimulus similar to that of TI-2 Ag, and 4) Ab production of Gab1−/− B cells induced by cytokines plus anti-6-dextran was inhibited by the retrovirus-mediated expression of wild-type Gab1. Furthermore, we demonstrated that the negative regulatory effect of Gab1 was mediated through its binding sites for SHP-2 but not for PI-3 kinase, suggesting the involvement of SHP-2 in the Gab1-mediated negative regulation. In contrast, Gab2 negatively regulates TCR-evoked activation of the IL-2 promoter in T cells, which is mediated through PI-3 kinase (31). These findings raise the possibility that Gab family proteins regulate B cell and T cell functions in different ways.

MZ B cells comprise a minor subset of B cells that play an important role in the first line of defense against bacterial infection by producing Abs against TI Ags, such as components of the bacterial cell wall (1, 2, 4, 5). MZ B cells localize to the MZ extending outside of the marginal sinus, where microorganisms are carried by the circulation (32). Defective development of MZ B cells has been found in the gene targeting of CD19 (33), Pyk-2 (11), NF-κBp50 (34), and Aiolos (35, 36). Because the targeting of Gab1 did not affect the development of MZ B cells, Gab1 seems to be dispensable for MZ B cell development. Several reports indicate that MZ B cells have unique functional characteristics compared with FO B cells. MZ B cells show higher growth responses and Ab production after LPS stimulation or BCR ligation than do FO B cells (8, 9, 37). Such hyperresponsiveness may be advantageous for the role of MZ B cells as a front-line defender against bacterial infection (38). In contrast, MZ B cells show poorer responses in anti-Ig-induced survival and growth (10). Several negative regulators of MZ B cells have been reported. Analysis of knockout mice of another negative regulatory molecule, Src homology 2 domain-containing inositol-5-phosphatase (SHIP), showed a phenotype partly similar to that of the Gab1−/− FL chimera. SHIP-deficient mice have lower numbers of pre-B cells in

Discussion

Gab1 is a scaffolding adapter molecule that is activated downstream of the receptors for various growth factors and cytokines, including stem cell factor, IL-3, IL-6, erythropoietin, GM-CSF, and thrombopoietin, all of which are involved in hematopoiesis (12–22). Although this relationship has led some to suggest a crucial role for Gab1 in hematopoiesis, we found that lethally irradiated mice transplanted with Gab1−/− FL cells survived, which
the bone marrow, higher numbers of myeloid cells, and enhanced IgG1, IgG2a, and IgG3 Ab responses to Tl-2 Ags, but this enhancement is not cell autonomous (39). These phenotypes are partly explained by the increased expression of SHIP in MZ B cells (37). It is interesting to speculate that there may be a link between the signaling pathways of SHIP and Gab1 in B cells. Targeting of programmed death-1, a member of the Ig superfamily that contains an immunoreceptor tyrosine-based inhibitory motif in its cytoplasmic region, demonstrated that a lack of negative regulation by programmed death-1 resulted in higher TI-2 responses (40) and autoantibody-mediated cardiomyopathy (41). The report indicated that impairment of negative regulation of TI-2 response could result in the development of an autoimmune disease (42). Thus, it is possible that the negative regulation of a very narrow range of humoral immune responses by Gab1 could also be important for preventing the development of autoimmune disease. Collectively, the present results indicate that Gab1 is a unique adapter molecule that is specifically expressed in MZ B cells and negatively regulates their differentiation in response to Tl-2 Ags in vivo.

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