The Gab1 Docking Protein Links the B Cell Antigen Receptor to the Phosphatidylinositol 3-Kinase/Akt Signaling Pathway and to the SHP2 Tyrosine Phosphatase*

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Received for publication, November 22, 2000, and in revised form, January 17, 2001

B cell antigen receptor (BCR) signaling causes tyrosine phosphorylation of the Gab1 docking protein. This allows phosphatidylinositol 3-kinase (PI3K) and the SHP2 tyrosine phosphatase to bind to Gab1. In this report, we tested the hypothesis that Gab1 acts as an amplifier of PI3K- and SHP2-dependent signaling in B lymphocytes. By overexpressing Gab1 in the WEHI-231 B cell line, we found that Gab1 can potentiate BCR-induced phosphorylation of Akt, a PI3K-dependent response. Gab1 expression also increased BCR-induced tyrosine phosphorylation of SHP2 as well as the binding of Grb2 to SHP2. We show that the pleckstrin homology (PH) domain of Gab1 is required for BCR-induced phosphorylation of Gab1 and for Gab1 participation in BCR signaling. Moreover, using confocal microscopy, we show that BCR ligation can induce the translocation of Gab1 from the cytosol to the plasma membrane and that this requires the Gab1 PH domain as well as PI3K activity. These findings are consistent with a model in which the binding of the Gab1 PH domain to PI3K-derived lipids brings Gab1 to the plasma membrane, where it can be tyrosine-phosphorylated and then act as an amplifier of BCR signaling.

Engagement of the B cell antigen receptor (BCR) activates multiple signaling pathways that regulate B cell development, survival, activation, and proliferation (1–5). The initiation of many BCR signaling pathways involves the recruitment of cytoplasmic signaling enzymes to the plasma membrane, where they can act on membrane-associated substrates. For example, phosphatidylinositol 3-kinase (PI3K) and phospholipase C \( \gamma \) must be recruited to the plasma membrane to act on their substrate, the membrane lipid phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)). There are multiple ways in which receptors can recruit cytoplasmic signaling enzymes to the plasma membrane. One important mechanism involves the binding of SH2 domains in signaling proteins to phosphotyrosine-containing sequences on membrane-associated docking/scaffolding proteins. Since the SH2 domains of different proteins bind different phosphotyrosine-containing sequences (6, 7), the set of signaling enzymes a receptor recruits depends on the spectrum of phosphotyrosine-containing sequences it generates on membrane-associated proteins. For example, tyrosine phosphorylation of its Ig-\( \alpha \) and Ig-\( \beta \) subunits allows the BCR to recruit the Syk tyrosine kinase (8, 9). However, the absence of other phosphotyrosine-containing sequences in Ig-\( \alpha \) and Ig-\( \beta \) prevents the direct recruitment of other SH2 domain-containing proteins to the BCR. The BCR overcomes this problem by using its associated tyrosine kinases to phosphorylate appropriate tyrosine residues on other membrane-associated docking proteins (e.g. CD19). In addition, adaptor proteins (e.g. BLNK and Shc) that are tyrosine-phosphorylated after BCR signaling can couple SH2 domain-containing signaling proteins with membrane-associated docking proteins (10, 11). These SH2 domain-phosphotyrosine interactions are essential for the BCR to recruit PI3K, phospholipase C\( \gamma \), mSOS (son of sevenless homolog), and other signaling enzymes to the plasma membrane. Signaling enzymes can also be recruited to the plasma membrane via their pleckstrin homology (PH) domains (12). PH domains bind membrane phospholipids, in particular the PI3K products phosphatidylinositol 3,4,5-trisphosphate (PIP\(_{3,4,5}\)) and phosphatidylinositol 3,4-bisphosphate. PH domain-mediated recruitment to the plasma membrane is important for the BCR to activate the Akt and Btk kinases (13–17). In addition to promoting their membrane localization, PIP\(_{3,4,5}\) can also contribute to the activation of PH domain-containing signaling enzymes such as phospholipase C\( \gamma \), mSOS, and Vav (18, 19). Thus, PI3K-derived lipids play a role in the activation of many protein; PBS, phosphate-buffered saline; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase.

* This work was supported in part by grants from the Canadian Institutes of Health Research (to M. R. G. and L. M.) and by grants from the National Institutes of Health and the American Cancer Society (to A. J. W.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡§§ Recipient of a postdoctoral fellowship from the Ministerio de Educacion y Ciencia de Espana.
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different BCR signaling pathways. The recruitment of PI3K to the plasma membrane, where its substrates are located, is therefore a key event in BCR signaling.

The BCR uses multiple docking/scaffolding proteins to recruit PI3K to the plasma membrane. BCR engagement results in tyrosine phosphorylation of the cytoplasmic domain of CD19, a transmembrane protein; and this creates binding sites for the SH2 domains of PI3K (20). Cytosolic docking proteins that are recruited to the plasma membrane also contribute to the ability of the BCR to direct PI3K to the plasma membrane. Cbl, which uses its SH2-like domain to bind to phosphorylated Syk (21), is tyrosine-phosphorylated after BCR engagement and binds PI3K (22, 23). The BCR also uses the Gab1 docking protein to recruit PI3K as well as other signaling enzymes to the plasma membrane (24). We have previously shown that Gab1 is tyrosine-phosphorylated after BCR ligation and that this allows the SH2 domains of PI3K, the SHP2 tyrosine phosphatase, and the Shc adaptor protein to bind directly to Gab1 (24). Grb2 can then bind via its SH2 domain to the tyrosine-phosphorylated SHP2 and Shc that are bound to Gab1.

Gab1 belongs to a family of docking/scaffolding proteins that includes the closely related Gab2 protein as well as insulin receptor substrate-1 and -2 and the Drosophila DOS (daughter of sevenless) protein (25–27). All of these proteins contain a PH domain, a cluster of binding sites for the PI3K SH2 domain, and one or more binding sites for the SH2 domain of SHP2. The presence of a PH domain in these docking proteins suggests that they are recruited to the plasma membrane when receptor signaling stimulates the production of PI3K-derived lipids. Once at the plasma membrane, these docking proteins can be tyrosine-phosphorylated, allowing them to recruit additional PI3K complexes as well as other signaling enzymes. In this report, we tested the hypothesis that Gab1 functions in this way as a PI3K-dependent amplifier of BCR signaling. We found that overexpressing Gab1 increased the ability of the BCR to signal via PI3K and SHP2. We also show that the BCR recruits Gab1 to the plasma membrane in a PI3K-dependent manner and that the PH domain of Gab1 is required for membrane recruitment of Gab1 and for its ability to amplify BCR signaling.

EXPERIMENTAL PROCEDURES

Antibodies and Other Reagents—Goat anti-mouse IgM antibodies were obtained from BioCan (Mississauga, Ontario, Canada). Anti-Gab1 antibodies and the anti-phospho-proline monoclonal antibody 4G10 were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Antibodies to SHP2, Grb2, and the p85 subunit of PI3K were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Antibodies against Akt and antibodies that specifically recognize Akt phosphorylated on serine 473 (anti-phospho-Akt) were purchased from New England Biolabs (Mississauga). LY294002 and wortmannin were from BIOMOL Research Labs Inc. (Plymouth Meeting, PA).

Cell Culture—WEHI-231 cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 50 μg/ml 2-mercaptoethanol, 1 mM pyruvate, and 2 mM glutamine. AtT20/BCR/Syk cells (28) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum.

cDNAs Encoding Mutant Forms of Gab1—Polymerase chain reaction overlap extension (29) was used to generate cDNAs encoding a mutant form of Gab1 that cannot bind PI3K (Gab1ΔPI3K) (30) as well as a mutant form of Gab1 that cannot bind SHP2 (Gab1ΔSHP2) (31). The Gab1ΔPI3K protein has tyrosine-to-phenylalanine substitutions at amino acids 448, 473, and 590 that eliminate the three YXXM sequences that the PI3K SH2 domains can bind to. The Gab1ΔSHP2 protein has a tyrosine-to-phenylalanine substitution at amino acid 628 that eliminates the YXXD sequence that the SHP2 SH2 domains can bind to. A cDNA encoding a truncated form of Gab1 that includes amino acids 116–695 but lacks the N-terminal PH domain (Gab1ΔPH) has been described previously (32).

Expression of Gab1 cDNAs in WEHI-231 Cells—cDNAs encoding wild-type Gab1, Gab1ΔPI3K, Gab1ΔSHP2, and Gab1ΔPH were excised from the pLTR2 vector using BamHI and NotI and subcloned into the pMX retroviral expression vector (33). The resulting plasmids, as well as pMX with no insert, were transfected into the BOSC23 packaging cell line (34). The retroviral particles released into the culture supernatant were then used to infect WEHI-231 cells as described (35). After retroviral infection, the WEHI-231 cells were cultured in the presence of 0.25 μg/ml puromycin for 2 days to select for infected cells. The resulting bulk populations of infected WEHI-231 cells were maintained in culture medium with 0.25 μg/ml puromycin. Expression of the various Gab1 proteins in these bulk populations was confirmed by immunoblotting. Previous analyses using enhanced green fluorescent protein (EGFP) cDNA cloned into pMX showed that >95% of the puromycin-resistant WEHI-231 cells obtained after retroviral infection express the transduced gene (35).

Expression of Gab1-EGFP Fusion Proteins in AtT20/BCR/Syk Cells—cDNAs encoding wild-type Gab1 and Gab1ΔPH were subcloned into the pEGFP-C2 vector (CLONTECH), as described previously (32), to generate fusion proteins with EGFP at the N terminus. AtT20/BCR/Syk cells were transfected as described (36) with 50 μg of the plasmid encoding either Gab1-EGFP or Gab1ΔPH-EGFP in addition to 20 μg of pWZLBlG (a gift from Dr. Steven Robbins, University of Calgary), a plasmid encoding resistance to blasticidin. After culturing the cells for 10–14 days in the presence of 2 μg/ml blasticidin (Invitrogen, Carlsbad, CA), individual colonies of blasticidin-resistant cells were isolated as described (28).

Preparation of Cell Lysates—WEHI-231 cells were resuspended to 2.5 × 10⁷/ml in modified HEPES-buffered saline (25 mM sodium HEPES, pH 7.2, 125 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1 mM Na₂HPO₄, 0.5 mM MgSO₄, 1 mM glucose, 2 mM glutamine, 1 mM sodium pyruvate, and 50 μM 2-mercaptoethanol) and stimulated with goat anti-mouse IgM antibodies at a final concentration of 100 μg/ml. Reactions were stopped by adding cold phosphate-buffered saline (PBS) to the cells. After washing, the cells were solubilized in Triton X-100 lysis buffer (1% Triton X-100, 20 mM Tris-HCl, pH 8, 137 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM Na₂VO₃, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM leupeptin, and 1 μg/ml aprotinin). Detergent-insoluble material was removed by centrifugation, and protein concentrations were determined using the bicinchoninic acid assay (Pierce).

Prior to being stimulated, AtT20/BCR/Syk cells were washed with PBS and cultured overnight in Dulbecco's modified Eagle's medium with 0.2% fetal calf serum. The cells were then washed with PBS and incubated for 30 min at 37 °C in modified HEPES-buffered saline (see above) to further reduce signaling due to serum growth factors. The cells were washed again with PBS, and 10 μl of 37 °C modified HEPES-buffered saline were added to each dish. BCR signaling was initiated by adding goat anti-mouse IgM antibodies to a final concentration of 20 μg/ml. Reactions were terminated by aspirating the medium, washing the cells twice with cold PBS, and then solubilizing the cells with Triton X-100 lysis buffer containing protease and phosphatase inhibitors.

Immunoprecipitation and Immunoblotting—For immunoprecipitations, extracts from 1–2 × 10⁶ cells (0.5–1 mg of protein) were mixed with 1–2 μg of specific antibodies plus 10 μl of protein A-Sepharose for 1 h at 4 °C. The beads were pelleted and washed three times with Triton X-100 lysis buffer before bound proteins were eluted with SDS-polyacrylamide gel electrophoresis sample buffer containing 0.1 M dithiothreitol. Immunoprecipitated proteins were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and analyzed by immunoblotting as described (24). Immunoreactive bands were visualized using enhanced chemiluminescence detection. Akt activation was analyzed by immunoblotting total cell lysates (60 μg of protein) with anti-phospho-Akt antibodies. The filters were then stripped and reprobed with anti-Akt antibodies. The relative levels of Akt phosphorylation were determined by densitometry using an Alpha Innotech gel documentation system (Canberra Packard, Mississauga, CA). Individual colonies of blasticidin-resistant cells were isolated as described (28).

Fluorescence studies on Gab1-EGFP-expressing AtT20/BCR/Syk Cells—The cells were grown to near confluency on 10-mm poly-d-lysine-coated glass coverslips. After culturing overnight in Dulbecco's modified Eagle's medium plus 0.2% fetal calf serum, the cells were then washed with PBS and incubated at 37 °C. The cells were then washed twice with PBS and incubated at 37 °C for 20 min. The cells were then fixed in 4% paraformaldehyde in PBS and permeabilized in 0.1% Triton X-100 for 5 min. The cells were then incubated with goat anti-mouse IgM antibodies at a final concentration of 20 μg/ml. Reactions were stopped by aspirating the medium, washing the cells twice with cold PBS, and then solubilizing the cells with Triton X-100 lysis buffer containing protease and phosphatase inhibitors. Immunoprecipitation and Immunoblotting—For immunoprecipitations, extracts from 1–2 × 10⁶ cells (0.5–1 mg of protein) were mixed with 1–2 μg of specific antibodies plus 10 μl of protein A-Sepharose for 1 h at 4 °C. The beads were pelleted and washed three times with Triton X-100 lysis buffer before bound proteins were eluted with SDS-polyacrylamide gel electrophoresis sample buffer containing 0.1 M dithiothreitol. Immunoprecipitated proteins were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and analyzed by immunoblotting as described (24). Immunoreactive bands were visualized using enhanced chemiluminescence detection. Akt activation was analyzed by immunoblotting total cell lysates (60 μg of protein) with anti-phospho-Akt antibodies. The filters were then stripped and reprobed with anti-Akt antibodies. The relative levels of Akt phosphorylation were determined by densitometry using an Alpha Innotech gel documentation system (Canberra Packard, Mississauga, CA).
Gab1 Links the BCR to the PI3K/Akt Signaling Pathway—We have previously shown that BCR ligation results in tyrosine phosphorylation of Gab1 and that this allows PI3K to bind via its SH2 domains to Gab1 (24). Since Gab1-PI3K complexes are found in the membrane-enriched particulate fraction of the RAMOS human B cell line after BCR ligation (24), this led us to propose that Gab1 acts as a docking protein that recruits PI3K to the plasma membrane after BCR engagement. This would presumably result in increased production of PI3K-derived lipids that can activate downstream targets such as the Akt protein kinase. To test this idea that Gab1 functionally links the BCR to the PI3K/Akt signaling pathway, we expressed Gab1 in the WEHI-231 murine B lymphoma cell line. WEHI-231 cells normally express the closely related Gab2 protein (Ref. 25 and data not shown), but express little or no Gab1 (Fig. 1). Fig. 1 shows that this transfected Gab1 protein was tyrosine-phosphorylated after BCR ligation in the WEHI-231 cells and that it associated with a number of other tyrosine-phosphorylated proteins. As in RAMOS cells (24), the 72-kDa tyrosine-phosphorylated protein that coprecipitated with Gab1 after BCR stimulation in WEHI-231 cells is the SH2 phosphorytase (see Fig. 9B), whereas the 46- and 52-kDa tyrosine-phosphorylated proteins are two isoforms of the Shc adaptor protein (data not shown). Thus, the transfected Gab1 expressed in the WEHI-231 cells appears to function similarly to the endogenous Gab1 present in other B cell lines such as RAMOS.

We then asked whether expressing Gab1 in WEHI-231 cells could potentiate the ability of the BCR to activate the PI3K/Akt signaling pathway. We used phosphorylation of Akt on serine 473 as a readout since we had previously shown that this response is dependent on PI3K activity (37). Fig. 2 shows that overexpressing Gab1 in WEHI-231 cells increased the ability of the BCR to stimulate Akt phosphorylation. Densitometric analysis showed that Gab1 expression increased anti-IgM antibody-stimulated Akt phosphorylation by ~2-fold at all time points between 1 and 30 min. Thus, Gab1 expression increased the magnitude of BCR-induced Akt phosphorylation as opposed to merely changing the kinetics of this response.

The ability of Gab1 to potentiate BCR-induced Akt phosphorylation depended on its ability to bind PI3K (Fig. 3). This was shown by expressing in WEHI-231 cells a mutant form of Gab1 (Gab1ΔPI3K) in which all three potential binding sites for the PI3K (P-Akt) had been ablated by tyrosine-to-phenylalanine mutations. Fig. 3 shows that Gab1ΔPI3K did not bind PI3K after BCR ligation and that its ability to potentiate BCR-induced Akt phosphorylation was much less than that of wild-type Gab1. Compared with WEHI-231 cells infected with retroviruses containing the empty pMX vector, Akt phosphorylation at 2 min after anti-IgM antibody addition was 97 ± 21% (n = 10) higher in cells expressing wild-type Gab1, but only 27 ± 16% (n = 3) higher in cells expressing Gab1ΔPI3K. Thus, the ability of Gab1 to bind PI3K allows it to link the BCR to PI3K-dependent signaling events such as Akt phosphorylation.

Gab1 Requires Its PH Domain to Link the BCR to the PI3K/Akt Signaling Pathway—PI3K must be recruited to the plasma membrane to convert PIP2 to PIP3, and to promote the activation of Akt. The ability of Gab1 to potentiate BCR-induced Akt activation presumably reflects the ability of Gab1 to recruit PI3K to the plasma membrane. This implies that Gab1 is localized at the plasma membrane in anti-IgM antibody-stim-
Gab1 is required for Gab1 to be tyrosine-phosphorylated, bind PI3K, and potentiate Akt phosphorylation after BCR engagement. Vector control cells and WEHI-231 cells expressing either wild-type (wt) Gab1 or a mutant form of Gab1 that lacks the PH domain (Gab1ΔPH) were incubated with or without anti-IgM antibodies for 2 min. A, cell lysates were immunoprecipitated (ippt) with anti-Gab1 antibodies, and the precipitated proteins were analyzed by immunoblotting with the anti-Tyr(P) monoclonal antibody 4G10. The filters were then stripped and reprobed with anti-Gab1 antibodies to show that similar amounts of Gab1 and Gab1ΔPH had been precipitated. Tyrosine-phosphorylated Gab1, SHP2, and Shc are indicated by the arrows. B, the binding of PI3K to Gab1 was assessed by immunoprecipitating cell lysates with anti-Gab1 antibodies, followed by immunoblotting with antibodies against the p85 subunit of PI3K. The filters were then reprobed with anti-Gab1 antibodies to show that similar amounts of wild-type Gab1 and Gab1ΔPH had been precipitated. C, Akt phosphorylation was analyzed by immunoblotting with an anti-phospho-Akt antibody. The filter was then reprobed with anti-Akt antibodies to show that equal amounts of Akt were present in each lane. Molecular mass standards (in kilodaltons) are indicated to the left. All of the experiments were performed on the same set of cell lysates. One of three independent experiments that yielded similar results is shown.

To test this model, we expressed in WEHI-231 cells a truncated form of Gab1 that lacks the PH domain (Gab1ΔPH). We found that the Gab1ΔPH protein was very poorly phosphorylated in response to BCR engagement and that, consequently, it bound other tyrosine-phosphorylated proteins to a much lesser extent than the wild-type Gab1 protein (Fig. 4A). In particular, the Gab1ΔPH protein did not bind significant amounts of PI3K after BCR ligation (Fig. 4B), and this correlated with its inability to significantly increase BCR-induced Akt phosphorylation (Fig. 4C). Anti-IgM antibody-stimulated Akt phosphorylation was only 11 ± 8% (n = 2) higher in the cells expressing Gab1ΔPH than in the vector control cells. Taken together, these data suggest that Gab1 uses its PH domain to come to the plasma membrane after BCR engagement and that this is required for Gab1 to be tyrosine-phosphorylated, to bind PI3K, and to contribute to Akt activation.

**BCR Signaling Can Cause Translocation of Gab1 to the Plasma Membrane**—The requirement that Gab1 have a PH domain to participate in BCR signaling is consistent with the idea that Gab1 is recruited to the plasma membrane in response to the production of PIP₃ by PI3K. Since it is difficult to analyze the translocation of cytoplasmic proteins to the plasma membrane in B cells, we tested this hypothesis by expressing a Gab1-EGFP fusion protein in a derivative of the AtT20 endocrine cell line that had been transfected with DNAs encoding all four chains of the BCR as well as the Syk tyrosine kinase (28). We have previously shown that many aspects of BCR signaling, including PI3K-dependent Akt activation (28, 37), can occur in these AtT20/BCR/Syk cells. EGF fusion proteins containing either full-length Gab1 (Gab1-EGFP) or Gab1 lacking its PH domain (Gab1ΔPH-EGFP) were expressed in the AtT20/BCR/Syk cells (Fig. 5A).
We then assessed whether these proteins behaved similarly to the wild-type Gab1 and truncated Gab1PH proteins that were expressed in the WEHI-231 B cell line. When the BCR on the AtT20/BCR/Syk cells was cross-linked with anti-IgM antibodies, we found that the Gab1-EGFP protein became phosphorylated on tyrosine residues and associated with tyrosine-phosphorylated proteins (Fig. 5B). Reprobing these blots showed that these Gab1-associated proteins were SHP2 (Fig. 5B) and the p46 and p82 forms of Shc (data not shown). In contrast to the wild-type Gab1-EGFP fusion protein, the Gab1ΔPH-EGFP protein showed very little BCR-induced tyrosine phosphorylation, and its ability to bind SHP2 and Shc did not increase after BCR engagement (Fig. 5B). This is consistent with our finding that the PH domain of Gab1 is required for its BCR-induced tyrosine phosphorylation in WEHI-231 cells (Fig. 4). We went on to show that expressing the wild-type Gab1-EGFP protein in AtT20/BCR/Syk cells increased BCR-induced Akt phosphorylation by ∼2-fold at all time points, whereas expressing Gab1ΔPH-EGFP had a much smaller effect (Fig. 6). Thus, the wild-type Gab1-EGFP and Gab1ΔPH-EGFP proteins expressed in AtT20/BCR/Syk cells appear to function identically to the wild-type Gab1 and Gab1PH proteins, respectively, that were expressed in the WEHI-231 B cell line. In response to BCR engagement, wild-type Gab1-EGFP was tyrosine-phosphorylated, bound SHP2 and Shc, and linked the BCR to Akt activation.

As in WEHI-231 cells, all of these responses were dependent upon the PH domain of Gab1. Thus, the expression of Gab1-EGFP fusion proteins in AtT20/BCR/Syk cells can be used as a model system to investigate whether BCR engagement results in translocation of Gab1 to the plasma membrane.

The subcellular localization of the Gab1-EGFP and Gab1ΔPH-EGFP fusion proteins in AtT20/BCR/Syk cells was analyzed by confocal microscopy. In unstimulated AtT20/BCR/Syk cells, the Gab1-EGFP protein was mostly cytoplasmic, with very little of the protein accumulating at the margins of the cells that would correspond to the plasma membrane (Figs. 7, a–c; and 8a). After BCR engagement, however, there was a significant increase in the amount of Gab1-EGFP at the margins of the cells (Figs. 7, d–f; and 8, b and c). This likely represents translocation of Gab1-EGFP to the plasma membrane of the cells since staining nonpermeabilized AtT20/BCR/Syk cells with antibodies to the cell-surface BCR yields a similar pattern of fluorescence (40). The BCR-induced translocation of Gab1-EGFP to the plasma membrane was most evident at 15 min after adding anti-IgM antibodies to the cells (Figs. 7, d–f; and 8, b and c), but could also be observed at 3, 5, and 10 min after initiating BCR signaling (data not shown).

To test whether the BCR-induced recruitment of Gab1 to the plasma membrane is dependent upon the PH domain of Gab1, we analyzed the subcellular distribution of the Gab1ΔPH-EGFP protein. We found that the Gab1ΔPH-EGFP protein was mostly cytoplasmic in unstimulated cells (Fig. 7, g–i) and that its subcellular distribution did not change significantly upon BCR engagement (j–l). The Gab1ΔPH-EGFP protein did not accumulate at the margins of the cells after BCR engagement. Thus, BCR signaling can recruit Gab1 to the plasma membrane, and this membrane translocation requires that Gab1 have a PH domain. Our finding that the PH domain of Gab1 is required both for its recruitment to the plasma membrane and for its ability to potentiate Akt phosphorylation is consistent with the idea that Gab1 must localize to the plasma membrane to amplify BCR signaling.

To further test our model that BCR-induced recruitment of Gab1 to the plasma membrane depends on the binding of the Gab1 PH domain to PI3K-derived lipids, we investigated whether it could be blocked by PI3K inhibitors. We found that pretreating AtT20/BCR/Syk cells with LY294002 caused significant inhibition of BCR-induced translocation of Gab1-EGFP to the plasma membrane (Fig. 8). Similar results were obtained when wortmannin was used to inhibit PI3K activity (data not shown). The partial inhibition of BCR-induced translocation of Gab1 to the plasma membrane may be due to small amounts of PIP3 being produced even in the presence of LY294002 or wortmannin. This might be sufficient to cause some membrane translocation of Gab1 since the Gab1 PH domain binds PIP3 with high affinity (41–43).

**Gab1 Regulates the Function of the SHP2 Tyrosine Phosphatase**—We have previously shown that the SHP2 tyrosine phosphatase also binds to Gab1 after BCR ligation (24). Far West-
The cells were fixed and analyzed by confocal microscopy. Two different representative confocal sections are shown for the anti-IgM antibody-treated samples, whereas a single representative section is shown for the unstimulated samples. The scale bar in B represents 10 μm. The scale is the same for all panels. One of two experiments with similar results is shown.

western analysis indicated that this interaction is mediated by the binding of the SHP2 SH2 domains to tyrosine-phosphorylated Gab1 (24). We also showed that the SHP2 that binds to Gab1 after BCR ligation is strongly phosphorylated on tyrosine residues (24). Since both tyrosine phosphorylation of SHP2 and engagement of its SH2 domains increase its phosphatase activity (44, 45), Gab1 may be an important regulator of both the enzymatic activity and subcellular localization of SHP2. Tyrosine phosphorylation of SHP2 also allows it to act as an adaptor protein that can bind the SH2 domain of Grb2 (46). We have shown that BCR ligation causes Grb2 to bind to the tyrosine-phosphorylated SHP2 associated with Gab1 (24). Together, these findings suggest that Gab1 regulates the ability of SHP2 to function both as a phosphatase and as an adaptor protein. To test this model, we investigated whether expressing Gab1 in WEHI-231 cells would enhance both the tyrosine phosphorylation of SHP2 and its ability to bind Grb2.

We found that expressing Gab1 in WEHI-231 cells greatly potentiated both the tyrosine phosphorylation of SHP2 and its ability to bind Grb2. Although BCR ligation caused modest tyrosine phosphorylation of SHP2 in vector control cells, very strong tyrosine phosphorylation of SHP2 was seen in Gab1-expressing WEHI-231 cells (Fig. 9A). The ability of Gab1 to potentiate BCR-induced tyrosine phosphorylation of SHP2 required the Gab1 PH domain. Expressing Gab1ΔPH did not increase SHP2 tyrosine phosphorylation (Fig. 9A). This presumably reflects the fact that the Gab1ΔPH protein is not tyrosine-phosphorylated to a significant extent after BCR ligation (Fig. 4A) and therefore does not bind SHP2 (Fig. 9B).

Since expressing wild-type Gab1 increased BCR-induced tyrosine phosphorylation of SHP2, we investigated whether this correlated with an increase in the ability of Grb2 to bind to SHP2. Fig. 10 shows that expressing Gab1 in WEHI-231 cells greatly increased the anti-IgM antibody-induced binding of Grb2 to SHP2. This effect was dependent on the binding of SHP2 to Gab1. Expressing a mutant form of Gab1 that lacks the major site of SHP2 binding (Gab1ΔSHP2) and therefore does not bind SHP2 after BCR ligation (Fig. 10, lower panels) caused only a small increase in the binding of Grb2 to SHP2 (upper panels). Thus, the ability of Gab1 to bind SHP2, and to promote its tyrosine phosphorylation, facilitates the binding of Grb2 to SHP2. This may be an important mechanism by which Grb2 and Gab2-associated proteins such as mSOS and SHIP are recruited to the plasma membrane by the BCR.

**DISCUSSION**

The Gab1 and Gab2 docking/scaffolding proteins participate in signaling by a variety of tyrosine kinase-linked receptors, including the T and B cell antigen receptors, a number of cytokine receptors, and the receptors for growth factors such as epidermal growth factor, hepatocyte growth factor, nerve growth factor, and insulin (24, 25, 30, 32, 38, 41, 47–53). In response to signaling by these receptors, the Gab1 and/or Gab2 protein becomes tyrosine-phosphorylated and then binds SH2 domain-containing signaling proteins, including PI3K (24, 25, 30, 32, 48, 49, 51); the SHP2 tyrosine phosphatase (24, 25, 30, 32, 48–54); and the Shc (24, 25, 50), Grb2 (24, 25, 49, 50, 53, 54), and CrkL (55) adaptor proteins. In this report, we have shown that Gab1 can functionally link the BCR to signaling events involving PI3K and SHP2. We also showed that the PH domain of Gab1 is required for BCR-induced recruitment of Gab1 to the plasma membrane, for tyrosine phosphorylation of Gab1, and for the binding of SH2 domain-containing signaling proteins, including PI3K and SHP2.
proteins to Gab1. This suggests that BCR-induced recruitment of Gab1 to the plasma membrane allows Gab1 to act as a docking/scaffolding protein that recruits PI3K and SHP2 to the plasma membrane, where they can contribute to BCR signaling.

Receptor-induced recruitment of Gab1 to the plasma membrane can occur by at least two different mechanisms (32). The first mechanism involves the binding of the Gab1 PH domain to PIP3 and is therefore dependent on prior activation of PI3K. The PH domain of Gab1 selectively binds PIP3 (41), and PI3K activation is sufficient to recruit either full-length Gab1 or the isolated Gab1 PH domain to the plasma membrane (32, 42). Moreover, both epidermal growth factor- and serum-induced membrane translocation of Gab1 can be blocked by PI3K inhibitors (32, 41). In contrast, hepatocyte growth factor-induced recruitment of Gab1 to the plasma membrane does not require the PH domain of Gab1 and is independent of PI3K (32, 43). Instead, Gab1 binds directly to the cytoplasmic domain of the activated hepatocyte growth factor receptor, c-Met. This is mediated by a novel phosphorytserine-binding domain within Gab1 termed the Met-binding domain (54). Gab1 can also be bridged to c-Met by Grb2, with the Grb2 SH3 domains binding Gab1 and the Grb2 SH2 domain binding tyrosine-phosphorylated c-Met (54). It is possible that Grb2 also links Gab1 to other receptors either by directly binding phosphorylated receptors or by binding to tyrosine-phosphorylated Shc that is associated with a receptor. Thus, recruitment of Gab1 to the plasma membrane can be mediated either by the N-terminal PH domain of Gab1 or by the Met-binding domain and/or Grb2-binding sites of Gab1, both of which are located in the C-terminal portion of the protein (49, 54). The PH domain-mediated membrane recruitment is dependent upon production of PIP3 by PI3K, whereas membrane recruitment mediated by the Met-binding domain or Grb2 is tyrosine-phosphorylation-dependent, but independent of PIP3 production.

Our microscopy data (Figs. 7 and 8) suggest that the BCR-induced translocation of Gab1 to the plasma membrane is mediated by the binding of PIP3 to the PH domain of Gab1. First, a truncated form of Gab1-EGFP that lacks the PH domain was not recruited to the plasma membrane of AtT20/BCR/Syk cells after BCR engagement, whereas plasma membrane recruitment was readily observed for the full-length Gab1-EGFP protein. In addition, we found that pretreating AtT20/BCR/Syk cells with either LY294002 (Fig. 8) or wortmannin (data not shown) significantly reduced BCR-induced translocation of Gab1-EGFP to the plasma membrane. The use of two different PI3K inhibitors minimizes the possibility that this inhibition was due to effects of these compounds on cellular processes other than the production of PIP3 by PI3K. Taken together, these data indicate that BCR-induced translocation of Gab1 to the plasma membrane is a PI3K-dependent event.

Since the ability of Gab1 to bind PI3K and to potentiate BCR-induced phosphorylation of Akt also depends on the Gab1 PH domain, it suggests that Gab1 is a PI3K-dependent amplifier of BCR signaling. We propose that Gab1 does not initiate BCR-induced PI3K signaling, but instead increases the amount of PI3K that the BCR recruits to the plasma membrane, an event that is necessary for PI3K to phosphorylate its lipid substrates. The initial recruitment of PI3K to the plasma membrane after BCR engagement in B cells is most likely mediated by CD19, a transmembrane protein. BCR signaling results in tyrosine phosphorylation of CD19, and this allows CD19 to bind the SH2 domains of PI3K (20). PI3K may also be recruited to the plasma membrane by Cbl. Cbl is strongly phosphorylated on tyrosine residues after BCR engagement and binds significant amounts of PI3K (22, 23). Once PI3K is recruited to the plasma membrane by binding via its SH2 domains to CD19 or Cbl, it can phosphorylate PIP3. The resulting PIP3 can then bind the PH domain of Gab1 and recruit Gab1 to the plasma membrane. Our experiments using the truncated form of Gab1 that lacks the PH domain suggest that membrane recruitment of Gab1 is required for tyrosine kinases to phosphorylate Gab1. Tyrosine phosphorylation of Gab1 creates binding sites for the SH2 domains of PI3K, allowing Gab1 to recruit additional PI3K molecules to the plasma membrane. In this way, Gab1 can amplify the ability of the BCR to signal via PI3K. The PI3K that is bound to Gab1 may further amplify PI3K signaling by producing PIP3, which can bind to the Gab1 PH domain and stabilize the association of Gab1 with the plasma membrane.

In this report, we also show that Gab1 can link the BCR to an important PI3K-dependent signaling event, the phosphorylation of Akt on serine 473. Phosphorylation of Akt at this site is required for its full activation and is strongly correlated with an increase in the enzymatic activity of Akt (14, 56, 57). Akt is a multifunctional kinase that regulates many important processes. In particular, Akt is the primary mediator of the anti-apoptotic/pro-survival functions of PI3K (15). This appears to be true in B cells as well. Pogue et al. (58) have recently shown that overexpressing Akt prevents BCR-induced apoptosis in the DT40 chicken B cell line. The anti-apoptotic/pro-survival functions of Akt may reflect its ability to regulate the function of a variety of proteins involved in cell survival and apoptosis, including NF-κB, forkhead family transcription factors, and the Bel-2 family member Bad (15). Akt activation may also increase protein synthesis, an important aspect of cell growth and activation, by either directly or indirectly regulating the activity of glycogen synthase kinase-3, the mammalian TOR/FRAP kinase, and p70 S6 kinase (15, 56). Thus, Gab1 may play an important role in BCR signaling by amplifying the ability of the BCR to activate Akt.

In addition to amplifying BCR signaling via PI3K, we found that Gab1 can also amplify the ability of the BCR to signal via the SHP2 tyrosine phosphatase. We have previously shown that SHP2 binds via its SH2 domains to Gab1 in anti-Ig antibody-stimulated RAMOS B cells (24). The binding of tyrosine-phosphorylated peptides to the SH2 domains of SHP2 has been shown to increase the phosphatase activity of SHP2 (45). The
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phosphatase activity of SHP2 is also increased upon tyrosine phosphorylation of SHP2 (44); and in this report, we have shown that overexpression of Gab1 greatly increases BCR-induced tyrosine phosphorylation of SHP2. In contrast, the Gab1APH protein, which cannot localize to the plasma membrane, did not bind SHP2 or potentiate BCR-induced phosphorylation of SHP2. This suggests that the activated, tyrosine-phosphorylated form of SHP2 that binds to Gab1 is associated with the plasma membrane. Consistent with this idea, we have previously shown that Gab1-SHP2 complexes are present in the membrane-enriched particulate fraction of anti-Ig antibody-stimulated RAMOS B cells (24). Thus, in addition to increasing the enzymatic activity of SHP2, Gab1 appears to recruit SHP2 to the plasma membrane. Cell fractionation studies by Frearson and Alexander (59) have shown that the majority of SHP2 substrates in T cells are in the membrane fraction. Gab1 may therefore play an important role in directing SHP2 to where its major substrates are located.

Although few substrates of SHP2 have been identified, recent reports have shown that Gab1 and Gab2 can be dephosphorylated by SHP2 in vitro (48). This suggests that SHP2 could negatively regulate the ability of Gab1/2 to bind PI3K or other SH2 domain-containing signaling proteins. This form of regulation may be common to all members of the Gab1/2 family of docking/scaffolding proteins. Both insulin receptor substrate-1 and DOCK can be dephosphorylated by SHP2 (27, 60), and SHP2 has been shown to negatively regulate the binding of PI3K to insulin receptor substrate-1 (61).

In addition to being a phosphatase, tyrosine-phosphorylated SHP2 can also act as an adaptor protein that binds the SH2 domain of Grb2 (46). This presumably allows SHP2 to recruit Grb2-mSOS complexes that can activate Ras. Consistent with this idea, in many cell types, SHP2 is a positive regulator of the ERK mitogen-activated protein kinase (25, 59, 62), a downstream target of Ras. Moreover, it has been shown that overexpression of Gab1 can potentiate receptor-induced activation of ERK and that this depends on the ability of Gab1 to bind SHP2 (48, 52, 53, 63). A physiological role for Gab1 in ERK activation has been confirmed by studies showing that ERK activation is depressed in embryos from Gab1 knockout mice (64). To date, we have not observed an effect of Gab1 overexpression on BCR-induced ERK activation. This may reflect the possibility that activation of Ras by the BCR is mediated primarily by RasGRP (65), a diacylglycerol-regulated Ras exchange factor, and not by mSOS. Gab1-mediated recruitment of Grb2-mSOS complexes may therefore contribute to the activation of other signaling pathways. mSOS has been shown to activate the Rac1 GTPase (18), and we are currently testing whether overexpression of Gab1 potentiates BCR-induced activation of JNK, a downstream target of Rac1 signaling (66). Gab1 overexpression has been reported to potentiate activation of JNK by the epidermal growth factor and hepatocyte growth factor receptors (41, 67).

Both Gab1 and Gab2 are expressed in B cells (24, 25, 48), with Gab1 being more highly expressed in some cell lines (e.g., the RAMOS human B cell line) and Gab2 being more highly expressed in other cell lines (e.g., the WEHI-231 murine B cell line). An important question therefore is whether Gab1 and Gab2 are functionally equivalent. Both Gab1 and Gab2 have N-terminal PH domains that are 73% identical (90% similar) at the amino acid level. Moreover, the organization of the potential binding sites for the SH2 domains of PI3K and SHP2 is highly conserved between Gab1 and Gab2. This suggests that the BCR could use either Gab1 or Gab2 to recruit the same signaling proteins. Tyrosine-phosphorylated Gab2 has been shown to bind PI3K and SHP2 in other cell types (25, 48). Although BCR engagement results in tyrosine phosphorylation of Gab1 (25, 48), the ability of Gab2 to bind PI3K and SHP2 in anti-Ig antibody-stimulated B cells has not been evaluated. We are currently analyzing whether Gab2 binds PI3K and SHP2 after BCR signaling and whether overexpression of Gab2 increases BCR-induced phosphorylation of Akt. These experiments will reveal whether Gab1 and Gab2 play equivalent roles in BCR signaling.

In summary, our data suggest the following model for how Gab1 and perhaps the closely related Gab2 protein participate in BCR signaling. In response to BCR engagement, PI3K is recruited to CD19 or other membrane-associate docking proteins and produces PI3P$_2$. This PI3P$_2$ can bind to the PH domain of Gab1 and recruit Gab1 to the plasma membrane. Once at the plasma membrane, Gab1 can be tyrosine-phosphorylated by BCR-regulated tyrosine kinases. Phosphorylation of the appropriate tyrosine residues on Gab1 allows it to bind the SH2 domains of Src, PI3K, and SHP2, thereby recruiting these proteins to the plasma membrane. Recruitment of PI3K to the plasma membrane allows it to convert PI3P$_2$ to PI3P$_3$, resulting in further activation of PI3K-dependent signaling events such as the activation of Akt. Recruitment of SHP2 to the plasma membrane would allow it to be phosphorylated by BCR-regulated tyrosine kinases, thereby increasing its specific activity. SHP2 may then dephosphorylate membrane-associated substrates including Gab1 itself. Although this might limit Gab1-mediated signaling, tyrosine phosphorylation of the Gab1-associated SHP2 and Shc would allow Grb2-mSOS complexes and Grb2-SHIP complexes to be recruited to the plasma membrane. Since the ability of mSOS to act as an exchange factor that activates Rac1 is increased by the binding of PI3P$_2$ to its PH domain, Gab1-mediated colocalization of mSOS and PI3K may facilitate the activation of Rac1 by the BCR. Similarly, colocalization of PI3K and SHIP by Gab1 may allow for efficient production of phosphatidylinositol 3,4-bisphosphate, a lipid that binds to a subset of PH domain-containing proteins. Thus, Gab1 and perhaps Gab2 may act as PI3K-dependent amplifiers of multiple BCR signaling pathways.

Acknowledgment—We thank Dr. Elaine Humphrey (Electro Microscopy Facility at the University of British Columbia) for confocal microscopy training and for assistance with data collection and analysis.

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The Gab1 Docking Protein Links the B Cell Antigen Receptor to the Phosphatidylinositol 3-Kinase/Akt Signaling Pathway and to the SHP2 Tyrosine Phosphatase


doi: 10.1074/jbc.M010590200 originally published online January 22, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M010590200

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