Gab1 is a member of the docking/scaffolding protein family which includes IRS-1, IRS-2, c-Cbl, p130Cas, and p62dok. These proteins contain a variety of protein-protein interaction motifs including multiple tyrosine residues that when phosphorylated can act as binding sites for Src homology 2 (SH2) domain-containing signaling proteins. We show in the RAMOS human B cell line that Gab1 is tyrosine-phosphorylated in response to B cell antigen receptor (BCR) engagement. Moreover, tyrosine phosphorylation of Gab1 correlated with the binding of several SH2-containing signaling proteins to Gab1 including Shc, Grb2, phosphatidylinositol 3-kinase, and the SHP-2 tyrosine phosphatase. Far Western analysis showed that the SH2 domains of Shc, SHP-2, and the p85 subunit of phosphatidylinositol 3-kinase could bind directly to tyrosine-phosphorylated Gab1 isolated from activated RAMOS cells. In contrast, the Grb2 SH2 domain did not bind directly to Gab1 but instead to the Shc and SHP-2 associated with Gab1. We also show that Gab1 is present in the membrane-enriched particulate fraction of RAMOS cells and that Gab1/signaling protein complexes are found in this fraction after BCR engagement. Thus, tyrosine-phosphorylated Gab1 may recruit cytosolic signaling proteins to cellular membranes where they can act on membrane-bound targets. This may be a critical step in the activation of multiple BCR signaling pathways.

Signaling by the B cell antigen receptor (BCR) regulates several key steps in B cell development and activation (1). In particular, the binding of antigens to the BCR is important for initiating the differentiation of resting B cells into antibody (Ab)-secreting plasma cells. The BCR exerts its effects by activating multiple signal transduction pathways. These include the well characterized Ras, phospholipase C-γ (PLC-γ), and phosphatidylinositol (PtdIns) 3-kinase pathways, as well as other signaling pathways involving Vav and the HS-1 protein (1, 2).

An important question is how these signaling pathways are turned on when antigens bind to the BCR. The BCR consists of a membrane-bound immunoglobulin (Ig) that is associated with the invariant Ig-α and Ig-β poly peptides (3). The membrane Ig contains the antigen binding site while the disulfide-linked Ig-α/β heterodimer can initiate all signal transduction events characteristic of the BCR (4). Clustering of the BCR by multivalent antigens or by anti-Ig Abs results in the activation of protein-tyrosine kinases, which then phosphorylate a large number of substrates. Two of these substrates are the Ig-α and Ig-β chains of the BCR, which are phosphorylated on tyrosine residues within the immunoreceptor tyrosine-based activation motifs (ITAMs) in their cytoplasmic tails (5). These phosphotyrosine (Tyr(P))-containing sequences provide binding sites for the Src homology 2 (SH2) domains of both the Src family tyrosine kinases and the Syk tyrosine kinase. Recruitment of these tyrosine kinases to Ig-α/β is essential for tyrosine phosphorylation of other substrates and for activation of BCR signaling pathways (1, 6, 7).

Many of the signaling pathways initiated by the BCR also rely on the translocation of cytosolic signaling proteins to cellular membranes where they can act on membrane-associated targets. For example, the Ras activator SOS is a cytosolic enzyme that must translocate to the membrane in order to activate Ras, which is tethered to the inner face of the plasma membrane. Similarly, both PLC-γ and PtdIns 3-kinase are cytosolic enzymes whose lipid substrates are located in the inner leaflet of the plasma membrane. Thus, bringing together the components of signaling pathways is likely to be the key event in initiating signal transduction.

A major mechanism by which cytosolic signaling proteins are recruited to cellular membranes involves the binding of SH2 domains in the signaling enzyme or associated adapter protein to Tyr(P)-containing sequences in membrane-associated proteins. The Tyr(P)-containing ITAM sequences in Ig-α/β are all pYXX(I/L) (single-letter code, where X is any letter), which is optimal for recruiting the Src and Syk kinases, but would not be able to recruit the diverse array of SH2 domain-containing proteins important for BCR signaling (8). One way in which
this problem is overcome is by phosphorylation of other membrane-associated proteins that can provide docking sites for signaling proteins. For example, BCR ligation induces tyrosine phosphorylation of the transmembrane proteins CD19 and CD22, allowing them to bind PtdIns 3-kinase, Vav, and the SHP-1 tyrosine phosphatase (9–11). Another class of proteins termed docking or scaffolding proteins may also play a role in recruiting signaling proteins to cellular membranes.

The docking protein family includes IRS-1, IRS-2, p130cas, c-Cbl, and p62Dok. These proteins have multiple protein-protein interaction motifs including proline-rich sequences, SH3 domains, and pleckstrin homology (PH) domains. Some docking proteins can associate with membranes, and this may be due in part to their PH domains binding membrane lipids (12). The most striking feature of docking proteins is that they are phosphorylated on multiple tyrosine residues in response to receptor signaling and can then bind SH2 domain-containing signaling proteins. For example, in response to insulin stimulation, IRS-1 is phosphorylated on several tyrosine residues, which allows it to bind Fyn, PtdIns 3-kinase, SHP-2, Grb2, and Nck (13). Similarly, the BCR uses c-Cbl and p130cas as docking proteins for PtdIns 3-kinase and for the Crk and Grb2 adapter proteins (14–17).

A recently identified member of the docking protein family is the Grb2-associated binder 1 (Gab1) protein (18). Gab1 is most closely related to the IRS-1 docking protein. Like IRS-1, Gab1 has a PH domain as well as a C-terminal region that is rich in proline and serine residues. Gab1 also has two PXXPXR motifs, which may allow it to bind SH3 domain-containing proteins. Gab1 is tyrosine-phosphorylated in response to both insulin and epidermal growth factor (EGF) stimulation, and this allows it to bind the SH2 domains of Grb2, PtdIns 3-kinase, PLC-γ, and the SHP-2 tyrosine phosphatase (18). In this report we investigated whether the BCR uses Gab1 as a docking protein in the RAMOS human B cell line, a cell line that has been used extensively to study BCR signaling (14–17, 19, 20).

We show that BCR ligation induces strong tyrosine phosphorylation of Gab1 and causes the SH2 domain-containing proteins She, Grb2, PtdIns 3-kinase, and SHP-2 to bind to Gab1. These Gab1/signaling protein complexes are present in the membrane-enriched particulate fraction of activated RAMOS B lymphoma cells. Thus, the BCR may use Gab1 to recruit multiple signaling proteins to cellular membranes where they can act on membrane-associated targets.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—Abs to Gab1 were produced by immunizing rabbits with a glutathione S-transferase (GST)-Gab1 fusion protein (18) or were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). The 4G10 anti-Tyr(P) monoclonal antibody (mAb) was from Upstate Biotechnology, Inc. The anti-SHP-2, anti-Cbl, and anti-IRS Abs, as well as the anti-Grb2 mAb, were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The anti-Cas mAb was from Transduction Laboratories (Lexington, KY). The anti-p85 Ab was from Lewis Williams (Chiron Corp., Emeryville, CA). Rabbit IgG (control) was purified from normal rabbit serum using protein A-Sepharose (Sigma). Rabbit IgG (control) was purified from normal rabbit serum using protein A-Sepharose (Sigma). Rabbit IgG (control) was purified from normal rabbit serum using protein A-Sepharose (Sigma). Rabbit IgG (control) was purified from normal rabbit serum using protein A-Sepharose (Sigma).

**GST Fusion Proteins**—Bacteria harboring plasmids encoding GST fused to the Grb2 SH2 domain were from G. Kretzky (University of Iowa, Iowa City, IA), while bacteria carrying a plasmid encoding GST fused to the tandem SH2 domains of SHP-2 were obtained from F. Jirik (University of British Columbia, Vancouver, Canada). Fusion protein production was induced with isopropyl-1-thio-β-D-galactopyranoside, and the fusion proteins were purified from bacterial lysates using glutathione-Sepharose 4B (Pharmacia, Bnie d’Urfe, Quebec, Canada).

**Preparation of Particulate and Soluble Fractions**—Cells were stimulated and washed as above, resuspended in sonication buffer (20 mM Tris-HCl, pH 8, 150 mM NaCl, 1 mM EDTA, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 1 μg/ml aprotonin, 0.1% Triton X-100), and sonicated. The sonication-resistant material was removed by centrifugation. Protein concentrations were determined using the bicinchoninic acid assay (Pierce).

**Far Western Blotting Assays**—Anti-Gab1 immunoprecipitates were separated by SDS-PAGE and transferred to nitrocellulose filters. The filters were blocked and probed as above. The filter with several changes of TBS, pH 2, for 1 h. The stripped filters were then blocked and probed as above.

**Cell Culture and Stimulation**—RAMOS cells were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 2 mM t-glutamine, 1 mM sodium pyruvate, and 50 μg 2-mercaptoethanol. The cells were resuspended to 2.5 × 107/ml in modified Hepes-buffered saline (21) and stimulated with goat anti-human IgM Abs (BioCan, Mississauga, Ontario, Canada) at a final concentration of 1 μg/ml.

The stimulation was stopped by adding cold phosphate-buffered saline containing 1 mM Na3VO4. After washing, the cells were solubilized at 5 × 107/ml 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 Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 1 μg/ml aprotinin, 10 μg/ml soybean trypsin inhibitor, 1 μg/ml aprotinin), and broken with five 5-s bursts using a needle probe and a Misonix XL sonicator (Farmingdale, NY). The efficiency of cell disruption was monitored by trypan blue staining. Unbroken cells and nuclei were removed by centrifuging at 14,000 rpm for 2 min at 4 °C. The post-nuclear supernatant was centrifuged at 60,000 rpm for 20 min in a Beckman TL-100 ultracentrifuge. The supernatant was removed, and Triton X-100 was added to a final concentration of 1%. The pellet containing the particulate fraction was rinsed with sonication buffer and then resuspended in sonication buffer containing 1% Triton X-100. The pellet was dispersed by brief sonication, and detergent-insoluble material was removed by centrifuging at 14,000 rpm for 3 min in the cold.

**Immunoprecipitations and Immunoblotting**—Extracts from 1–2 × 107 cells (5 × 107 cells for cell fractionation experiments) were pre-cleared for 30 min at 4 °C with 10 μl of protein A-Sepharose and then mixed with Abs (1–2 μg) for 2 h at 4 °C. Immune complexes were collected by adding 10 μl of protein A-Sepharose and mixing for an additional hour. The beads were washed three times with Triton X-100 lysis buffer before eluting bound proteins with SDS-PAGE sample buffer containing 100 mM dithiothreitol. Proteins were separated on 1.5-mm-thick SDS-PAGE mini-gels and transferred to nitrocellulose filters for 75 min at 70 V. Molecular mass standards were visualized by staining with Ponceau S (Sigma). For anti-Tyr(P) blots, filters were blocked with 5% bovine serum albumin (BSA) in TBS (10 mM Tris-HCl, pH 2, for 1 h. The stripped filters were then blocked and probed as above.

**Far Western Blotting Assays**—Anti-Gab1 immunoprecipitates were separated by SDS-PAGE and transferred to nitrocellulose filters. The filters were blocked with 10% nonfat milk powder in TBS and incubated with 10% nonfat milk powder in TBS and incubated with 10% nonfat milk powder in TBS. The filters were then blocked and probed as above.

**Far Western Blotting Assays**—Anti-Gab1 immunoprecipitates were separated by SDS-PAGE and transferred to nitrocellulose filters. The filters were blocked with 10% nonfat milk powder in TBS and incubated with 10% nonfat milk powder in TBS and incubated with 10% nonfat milk powder in TBS. The filters were then blocked and probed as above.

**Far Western Blotting Assays**—Anti-Gab1 immunoprecipitates were separated by SDS-PAGE and transferred to nitrocellulose filters. The filters were blocked with 10% nonfat milk powder in TBS and incubated with 10% nonfat milk powder in TBS and incubated with 10% nonfat milk powder in TBS. The filters were then blocked and probed as above.
Gab1 Is Tyrosine-phosphorylated and Associates with Tyrosine-phosphorylated Proteins in Response to BCR Ligation—If the BCR uses Gab1 as a docking site for SH2 domain-containing proteins, then Gab1 should be phosphorylated on tyrosine residues in response to BCR engagement. We initiated BCR signaling by adding anti-IgM Abs to RAMOS human B lymphoma cells and then examined the phosphorylation state of Gab1 by anti-Tyr(P) immunoblotting. We found that a 110–120-kDa protein that had the same electrophoretic mobility as Gab1 was strongly tyrosine-phosphorylated within 2 min of adding anti-IgM antibodies to the cells. This response persisted for at least 30 min (Fig. 1). Re-probing these blots with anti-Gab1 Abs showed that BCR ligation caused a decrease in the electrophoretic mobility of Gab1 (Fig. 1), consistent with the idea that Gab1 is phosphorylated after BCR engagement. However, since BCR ligation also induces tyrosine phosphorylation of p130 Cas and p120 Cbl, we wished to rule out the possibility that the tyrosine-phosphorylated 110–120-kDa band seen in the anti-Gab1 immunoprecipitates was either Cas or Cbl that had co-precipitated with Gab1. We found that neither Cas nor Cbl could be detected in anti-Gab1 immunoprecipitates (Fig. 2), even after very long exposures. Although we cannot definitively rule out the possibility that an unidentified tyrosine-phosphorylated 110–120-kDa protein co-precipitated with Gab1, the simplest interpretation of our results is that BCR ligation induces tyrosine phosphorylation of Gab1.

Our finding that Gab1 is tyrosine-phosphorylated in response to BCR ligation suggests that it could act as a docking site for SH2 domain-containing signaling proteins. Since many of these proteins are themselves tyrosine-phosphorylated in response to receptor signaling, we asked whether tyrosine-phosphorylated proteins associated with Gab1 in anti-IgM-stimulated RAMOS cells. We found that tyrosine-phosphorylated proteins of approximately 150, 90–97, 70, 55, and 50 kDa bound to Gab1 after BCR ligation (Fig. 3). We had previously shown that BCR engagement causes Shc (21) and PtdIns 3-kinase2 to associate with tyrosine-phosphorylated proteins of 110–120 kDa. Therefore, we asked whether these signaling proteins associated with Gab1 in activated RAMOS B cells.

The Shc and Grb2 Adapter Proteins Associate with Gab1 after BCR Engagement—The p52 and p46 forms of the Shc adapter protein are strongly tyrosine-phosphorylated in response to BCR engagement, and phosphorylated Shc is a major target for the SH2 domain of the Grb2 adapter protein in activated B cells (21). Since Grb2 associates with the Ras activator SOS, Shc may play a key role in activating Ras by directing Grb2/SOS complexes to the plasma membrane where Ras is located. Given the potentially important role of Shc in BCR signaling, we asked whether the Gab1-associated phosphoproteins of approximately 55 and 50 kDa were the two isoforms of Shc.

Immunoblotting anti-Gab1 immunoprecipitates with anti-Shc Abs showed that both the p52 and p46 forms of Shc bound to Gab1 in anti-IgM-stimulated cells but not in unstimulated RAMOS cells (Fig. 4A). Re-probing these filters with an anti-Tyr(P) mAb showed that the Gab1-associated phosphoproteins of approximately 55 and 50 kDa had the same electrophoretic mobility as tyrosine-phosphorylated Shc. Thus, both isoforms of Shc bind to Gab1 after BCR ligation.

The binding of Shc to Gab1 after BCR engagement suggests that Gab1 could be a docking site for Grb2/SOS complexes in activated B cells. Therefore, we determined whether Grb2 bound to Gab1 in B cells. Fig. 4B shows that there was a small amount of Grb2 associated with Gab1 in unstimulated RAMOS cells but that BCR stimulation significantly increased the amount of Grb2 bound to Gab1. Thus, Gab1 may participate in BCR-mediated Ras activation by providing a docking site for Grb2/SOS complexes.

PtdIns 3-Kinase Associates with Gab1 after BCR Engagement—PtdIns 3-kinase is another cytosolic signaling enzyme that must be recruited to the plasma membrane in order to gain access to its substrates. PtdIns 3-kinase phosphorylates inositol phospholipids, and we have shown that BCR ligation

2 R. J. Ingham and M. R. Gold, unpublished results.
indicated to the phosphorylated 110–120-kDa protein in this figure had the same electrophoretic mobility as Gab1. Molecular mass standards (in kDa) are indicated to the left.

Fig. 3. Gab1 is tyrosine-phosphorylated and associates with tyrosine-phosphorylated proteins in response to BCR ligation. RAMOS cells were incubated for 2 min with (+) or without (−) anti-IgM Abs. Cell lysates were immunoprecipitated with either the anti-Gab1 Ab or with an irrelevant affinity-purified rabbit Ab (control) and then analyzed by immunoblotting with the 4G10 anti-Tyr(P) mAb. The filter was stripped and reprobed with the anti-Gab1 Ab (not shown). The phosphorylated 110–120-kDa protein in this figure had the same electrophoretic mobility as Gab1. Molecular mass standards (in kDa) are indicated to the left.

increases the levels of PtdIns 3-kinase products in B cells (22). To determine whether Gab1 might also be a docking site for PtdIns 3-kinase, we immunoprecipitated Gab1 from RAMOS cells and assayed for the presence of the p85 subunit of PtdIns 3-kinase. Immunoblotting with anti-p85 Abs showed that BCR ligation caused PtdIns 3-kinase to associate with Gab1 in anti-IgM-stimulated RAMOS cells. The precipitated proteins were analyzed by immunoblotting with the anti-p85 Ab (A) or in vitro PtdIns 3-kinase enzyme assays using PtdIns as a substrate (B). Reaction products from the lipid kinase assays were separated by TLC and radiolabeled PtdIns-phosphate (PtdIns-PO4) was visualized by autoradiography. Note that the anti-p85 immunoprecipitations in these experiments were not quantitative and are intended only to indicate the migration of p85 and PtdIns-PO4. In other experiments, we estimated that approximately 5% of the total cellular p85 associated with Gab1 in anti-IgM-stimulated RAMOS cells.

We found that SHP-2 bound to Gab1 in anti-IgM-stimulated cells but not in unstimulated cells (Fig. 6A). Reprobing these blots with the anti-Tyr(P) mAb indicated that SHP-2 had the same electrophoretic mobility as the 70-kDa Gab1-associated phosphoprotein.2 This suggested that SHP-2 might be tyrosine-phosphorylated in response to BCR ligation. Fig. 6B shows that SHP-2 was strongly tyrosine-phosphorylated in response to BCR cross-linking. Moreover, tyrosine-phosphorylated proteins of approximately 150 and 110–120 kDa co-precipitated with SHP-2 from anti-IgM-stimulated cells. The 110–120-kDa protein is likely Gab1. The identity of the 150-kDa protein is not known, but a tyrosine-phosphorylated protein of similar molecular mass associates with Gab1 in anti-IgM-stimulated cells (Fig. 3). Thus, SHP-2 is a target of BCR-activated tyrosine kinases and uses Gab1 as a docking site.

The SH2 Domains of Shc, PtdIns 3-Kinase, and SHP-2 Bind Directly to Gab1, whereas the Grb2 SH2 Domain Binds to Gab1-associated SHP-2 and Shc—Gab1 contains tyrosine residues, which if phosphorylated, would be consensus binding sites for the SH2 domains of Shc, Grb2, PtdIns 3-kinase, and SHP-2. Tyrosine 162 of Gab1 is part of an optimal sequence (YQIL) for binding the Shc SH2 domain (23), tyrosine 48 is part of a YKND motif that is optimal for binding the Grb2 SH2 domain (8), tyrosines 447, 472, and 589 are all part of YYPM sequences that are optimal for binding the SH2 domains of PtdIns 3-kinase (8), and tyrosine 627 is part of a YLDL sequence that is optimal for binding the SH2 domains of SHP2 (24). This suggests that BCR-induced tyrosine phosphorylation of Gab1 could create binding sites for the SH2 domains of Shc, Grb2, PtdIns 3-kinase, and SHP-2 and that this could be the basis for the association of these proteins with Gab1 in activated RAMOS cells. To directly test this model, we performed Far Western assays in which we probed blots of anti-Gab1 immunoprecipitates with GST fusion proteins containing the SH2 domains of Shc, Grb2, PtdIns 3-kinase, and SHP-2.
Fig. 7 shows that a GST fusion protein containing the SH2 domain of Shc could bind directly to a 110–120-kDa protein that was immunoprecipitated with anti-Gab1 Abs. A, cell lysates were immunoprecipitated with the anti-Gab1 Ab. “Cell lysate” represents 40 μg of total cellular protein (∼10^6 cell equivalents). The filter was probed with an anti-SHP-2 Ab. B, cell lysates were immunoprecipitated with an anti-SHP-2 Ab or with an irrelevant affinity-purified rabbit Ab (control). Immunoprecipitates were probed with the 4G10 anti-Tyr(P) mAb (upper panel). The filter was then stripped and reprobed with the anti-SHP-2 Ab (lower panel). Molecular mass standards (in kDa) are indicated to the left.

Fig. 6. SHP-2 is tyrosine-phosphorylated and associates with Gab1 after BCR ligation. RAMOS cells were incubated for 2 min with (+) or without (−) anti-IgM Abs. A, cell lysates were immunoprecipitated with the anti-Gab1 Ab. “Cell lysate” represents 40 μg of total cellular protein (6 × 10^6 cell equivalents). The filter was probed with an anti-SHP-2 Ab or with an irrelevant affinity-purified rabbit Ab (control). Immunoprecipitates were probed with the 4G10 anti-Tyr(P) mAb (upper panel). The filter was then stripped and reprobed with the anti-SHP-2 Ab (lower panel). Molecular mass standards (in kDa) are indicated to the left.

myeloid cell line Grb2 has been reported to bind to tyrosine-phosphorylated SHP-2 (25). Reprobing our blots with Abs to SHP-2 and to SHP-2 domain proteins containing the Shc SH2 domain (A), the tandem SH2 domains of SHP-2 (B), the N-terminal SH2 domain of the PtdIns 3-kinase p85 subunit (C), the C-terminal SH2 domain of the PtdIns 3-kinase p85 subunit (D), or the Shc phosphotyrosine-binding domain (E). Re-probing these blots with the anti-Gab1 Ab showed that the 110–120-kDa band recognized by the fusion proteins in panels A–D had the same electrophoretic mobility as Gab1. Molecular mass standards (in kDa) are indicated to the left.

Gab1 Is a Membrane-associated Docking Protein in B Cells—Our data suggest that Gab1 is a multifunctional docking protein that binds Shc, Grb2, PtdIns 3-kinase, and SHP-2 in response to BCR ligation. Since all of these proteins must translocate from the cytosol to cellular membranes in order to activate signaling pathways, we asked whether Gab1 is associated with cellular membranes and whether it acts as a membrane docking site for these signaling proteins in B cells.
To test this, we separated RAMOS cells into a soluble cytosol fraction and a membrane-enriched particulate fraction. Fig. 9A shows that Gab1 was present in both the soluble and particulate fractions of RAMOS cells. In resting cells, the majority of Gab1 was in the soluble fraction, but there was a significant portion in the particulate fraction that may be able to act as a docking site for signaling proteins. Indeed, reprobing the blot in Fig. 9A with the anti-Tyr(P) mAb showed that substantial amounts of the Gab1/tyrosine phosphoprotein complexes were present in both the particulate and soluble fractions of anti-IgM-stimulated cells (Fig. 9B).

In Figs. 3–6, we showed that BCR ligation caused Shc, Grb2, PtdIns 3-kinase, and SHP-2 to bind to Gab1. The anti-Tyr(P) blot in Fig. 9B suggested that complexes of Gab1 with these signaling proteins were present in the membrane fraction of activated RAMOS cells. To assess this directly, we immunoprecipitated Gab1 from the particulate and soluble fractions of RAMOS cells and looked for its association with these signaling proteins (Fig. 10). Complexes of Shc, Grb2, PtdIns 3-kinase, and SHP-2 with Gab1 were clearly present in both the particulate and soluble fraction of anti-IgM-stimulated RAMOS cells. Thus, tyrosine-phosphorylated Gab1 is associated with cellular membranes and provides binding sites that can recruit Shc, Grb2, PtdIns 3-kinase, and SHP-2 to cellular membranes in response to BCR engagement.

### DISCUSSION

We and others have previously shown that the BCR uses the Cas and Cbl docking proteins to co-localize components of signaling pathways (14–17, 26). In this report, we show that the recently described Gab1 protein is a major docking site for a number of proteins involved in BCR signaling in the RAMOS human B cell line. In response to BCR ligation, Gab1 is tyrosine-phosphorylated and binds Shc, Grb2, PtdIns 3-kinase, and the SHP-2 tyrosine phosphatase. We estimated that between 1% and 5% of the total Grb2, PtdIns 3-kinase, and SHP-2 in RAMOS cells bound to Gab1 after BCR ligation, while up to 10% of the Shc was bound to Gab1 in activated RAMOS cells. This is similar to the level of Grb2 and PtdIns 3-kinase reported to associate with the Cbl docking protein in activated RAMOS cells (14, 15). Further analysis showed that a significant portion of these Gab1/signaling protein complexes were in the membrane-enriched particulate fraction of anti-IgM-stimulated RAMOS cells. Thus, the BCR may use Gab1 to recruit cytosolic signaling enzymes to cellular membranes where their substrates are located. This could be an important step in the activation of signaling pathways by the BCR.

Docking proteins such as IRS-1, Cas, and Cbl are phosphorylated on multiple tyrosine residues after receptor signaling, allowing them to bind the SH2 domains of a variety of signaling proteins. In this way, receptors use docking proteins to form signaling complexes and to recruit signaling proteins to specific cellular locations. We have shown that the BCR uses Gab1 in this manner in RAMOS cells. BCR-induced tyrosine phosphorylation of Gab1 creates binding sites for the SH2 domains of Shc, PtdIns 3-kinase, and SHP-2. Thus, Gab1 could concentrate these cytosolic signaling proteins at specific sites in the cell. A significant portion of Gab1 was present in the membrane-enriched particulate fraction of both unstimulated and anti-IgM-stimulated RAMOS cells. While this particulate fraction contains cellular membranes and is often interpreted as
being a membrane-enriched fraction, it will be important to confirm by microscopy that Gab1 associates with membranes. Nevertheless, given this proviso, our results suggest that Gab1 is a membrane-associated docking protein that the BCR uses to recruit cytosolic signaling proteins to cellular membranes. Although it is not clear how docking proteins such as Gab1 bind to cellular membranes, their PH domains may be involved. PH domains can bind the inositol phospholipids PtdIns(4,5)P₂, PtdIns(3,4)P₂, and PtdIns(3,4,5)P₃ (12). PtdIns(4,5)P₂ is present in the membranes of both unstimulated and anti-IgM-stimulated B cells (22) and may be responsible for the constitutive membrane association of Gab1 in B cells. BCR signaling also increases the levels of the PtdIns 3-kinase products PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃ (22), and this may provide additional membrane binding sites for Gab1. 

Our results suggest that Gab1 could play a role in several key BCR signaling pathways including the Ras pathway. Activation of Ras involves the recruitment of Grb2-SOS complexes to the plasma membrane where Ras is located. We have previously shown that a major target for the Grb2 SH2 domain in anti-IgM-stimulated RAMOS B cells is tyrosine-phosphorylated She and that BCR ligation induces the formation of She-Grb2-SOS complexes (21). However, it was not clear how She was recruited to the plasma membrane after BCR engagement. In vitro, the She SH2 domain can bind very strongly to the phosphorylated ITAM motifs of the BCR Ig-α/β subunit (27, 28). While D’Ambrosio et al. (28) have shown that She and Ig-α/β can be co-precipitated from B cells, we have been unable to detect this interaction.³ In contrast, we could readily detect Gab1-She complexes in both detergent extracts and particulate fractions from activated RAMOS cells. Moreover, we could show that the She SH2 domain could bind directly to Gab1 precipitated from anti-IgM-stimulated RAMOS cells. Gab1 has an optimal binding site (YQLI) for the SH2 domain in anti-IgM-stimulated RAMOS cells. Gab1 in B cells binds to the plasma membrane after BCR ligation and contributes to the association of She with Gab1. This may account for the large amount of She that binds to Gab1 in anti-IgM-stimulated RAMOS B cells. In addition to showing that She binds to Gab1 in the particulate fraction of anti-IgM-stimulated RAMOS cells, we showed that the Grb2 SH2 domain can bind directly to the Gab1-associated She. This would leave the SH3 domains of Grb2 free to bind SOS. Thus, Gab1 could recruit She-Grb2-SOS complexes to the plasma membrane in B cells. This is in contrast to other Grb2/docking protein interactions. For example, Grb2 binds to Cbl via its SH3 domains (14, 15), suggesting that Cbl, unlike Gab1, may sequester Grb2 and prevent it from binding SOS.

Our data also show that Gab1 may recruit Grb2-SOS complexes to the plasma membrane in B cells by using SHP-2 as an adapter protein in place of She. We found that BCR ligation caused SHP-2 to bind to Gab1 and that this interaction was mediated by the SHP-2 SH2 domains. SHP-2 was strongly phosphorylated on tyrosine residues after BCR ligation, and the Grb2 SH2 domain could bind directly to the Gab1-associated SHP-2. Thus, Gab1 may use both She and SHP-2 to recruit Gab1 to cellular membranes. 

³ S. Barbazuk and M. R. Gold, unpublished results.
Phosphorylated protein. However, we were unable to detect PLC- 
this was a good candidate for the 150-kDa Gab1-associated
IgM-stimulated RAMOS cells may also be signaling proteins.
sine-phosphorylated proteins that associate with Gab1 in anti-
kinase (36). The unidentified 150-kDa and 90–97-kDa tyro-

SHP-2 is tyrosine-phosphorylated in B cells and the first evi-
dation of the PtdIns 3-kinase pathway by the BCR. BCR ligation
as a tyrosine phosphatase.
protein has been implicated as a substrate of SHP-2 in NIH
SHP-2 has the potential to dephosphorylate Gab1 and nega-
tively regulate the formation of Gab1/signaling protein com-
plexes. Consistent with this idea, a 120-kDa SHP-2-associated
protein has been implicated as a substrate of SHP-2 in NIH
3T3 cells (29). It remains to be determined whether the pri-
mary role of SHP-2 in BCR signaling is as an adapter protein or
as a tyrosine phosphatase.
Finally, we have shown that Gab1 may contribute to activation
of the PtdIns 3-kinase pathway by the BCR. BCR ligation
leads to increased levels of PtdIns 3-kinase products (22) in B
cells. These lipid second messengers have been implicated in
a number of important cellular processes including the preven-
tion of apoptosis (30), membrane trafficking (31), and the
activation of Ras (32). Since the substrate for PtdIns 3-kinase,
PtdIns(4,5)P2, is in the plasma membrane, PtdIns 3-kinase
must be recruited from the cytoplasm to the plasma membrane
after BCR engagement. Previous work has identified the trans-
membrane protein CD19 as well as the Cbl docking protein as
major binding sites for PtdIns 3-kinase in activated B cells (9,
14, 33). In this report, we have shown that Gab1 is another
major binding site for PtdIns 3-kinase in activated B cells (9,
13, 37–39), this is the first report that Gab1 is involved in
immunoreceptor signaling. Given its role in BCR signaling, it is
likely that Gab1 also has an important role in signaling by
other immunoreceptors such as the T cell receptor and the mast
cell receptor for IgE.

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