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Scaffolding Adapter Grb2-Associated Binder 2 Requires Syk to Transmit Signals from FcεRI

Min Yu,* Cliff A. Lowell,† Benjamin G. Neel,* and Haihua Gu2*

Scaffolding adapter Grb2-associated binder 2 (Gab2) is a key component of FcεRI signaling in mast cells, required for the activation of PI3K. To understand how Gab2 is activated in FcεRI signaling, we asked which protein tyrosine kinase is required for Gab2 phosphorylation. We found that Gab2 tyrosyl phosphorylation requires Lyn and Syk. In agreement with published results, we found that Fyn also contributes to Gab2 tyrosyl phosphorylation. However, Syk activation is defective in Fyn−/− mast cells, suggesting that Syk is the proximal kinase responsible for Gab2 tyrosyl phosphorylation. Then, we asked which domains in Gab2 are required for Gab2 tyrosyl phosphorylation. We found that the Grb2-Src homology 3 (SH3) binding sites are required for Gab2 tyrosyl phosphorylation. Using a protein/lipid overlay assay, we determined that the Gab2 PH domain preferentially binds the PI3K lipid products, PI3, 4,5P3 and PI3, 4P2. Furthermore, the Grb2-SH3 binding sites and PH domain binding to PI3K lipid products are required for Gab2 function in FcεRI-evoked degranulation and Akt activation. Our data strongly suggest a model for Gab2 action in FcεRI signaling. The Grb2 SH3 binding sites play a critical role in bringing Gab2 to FcεRI, whereupon Gab2 becomes tyrosyl-phosphorylated in a Syk-dependent fashion. Phosphorylated Gab2 results in recruitment and activation of PI3K, whose lipid products bind the PH domain of Gab2 and acts in positive feedback loop for sustained PI3K recruitment and phosphatidylinositol-3,4,5-trisphosphate production, required for FcεRI-evoked degranulation of mast cells. The Journal of Immunology, 2006, 176: 2421–2429.

M ast cells are the major effector cells for the allergic response. FcεRI, the high-affinity receptor for IgE present on mast cells and basophils, binds the Fc portion of IgE. FcεRI becomes activated when IgE prebound to FcεRI is cross-linked by the binding of multivalent Ag. Activated FcεRI triggers the release of performed granules (degranulation) and lipid-derivatized mediators that evoke the immediate hypersensitivity reaction. FcεRI cross-linking also results in activation of gene expression for various cytokines and chemokines that can further enhance the initial hypersensitivity response.

FcεRI consists of one ligand-binding α-chain and three associated subunits (one β-chain and two γ-chains). The β- and γ-chains contain ITAMs without any intrinsic enzymatic activity. It is generally accepted that upon FcεRI cross-linking, FcεRI-associated Src family protein tyrosine kinase (SFK) Lyn becomes activated and phosphorylates the β- and γ-chain ITAMs. Phosphorylated ITAMs in the β-chain recruits and activates additional Lyn whereas phosphorylated ITAMs in the γ-chain recruit and activate another key protein tyrosine kinase (PTK) Syk, which ultimately triggers various mast cell responses (1). A recent report showed that another SFK, Fyn, also is important for FcεRI-initiated signaling and biological responses (2). Subsequent to these initial signaling events, critical downstream signaling molecules, including PI3K, become activated, leading to various mast cell responses. Although much is known about initial PTK activation following FcεRI cross-linking, it is still not well-understood how this results in the activation of various mast cell responses.

Recent studies indicate that adapter proteins including linker for activation of T cells (LAT) (3), Src homology 2 domain-containing leukocyte protein of 76 kDa (4), Grb2-associated binder (Gab) 2 (5), and non-T cell activation linker/linker for activation of B cells (6, 7) play key roles in mediating FcεRI-evoked responses in mast cells. Gab2 belongs to the Gab2/daughter of sevenless family of scaffolding adapters that include mammalian Gab1, Gab2, and Gab3, Drosophila daughter of sevenless, and Caenorhabditis elegans Soc-1 (8, 9). Like its relatives, Gab2 contains an N-terminal pleckstrin homology (PH) domain, several proline-rich motifs, and multiple tyrosyl phosphorylation sites. The PH domain is a modular domain known to bind inositol phospholipids (10). The Gab1 PH domain preferentially binds phosphatidylinositol-3,4,5-trisphosphate (PIP3) (11, 12), and is essential for Gab1 function in branching morphogenesis (12). The Gab2 PH domain is critical for recruiting Gab2 to the phagocytic cup in macrophages (13), but dispensable for Gab2 function in IL-3/GM-CSF signaling (14). Although the lipid-binding specificity of Gab2 PH has not been determined, Gab2 PH domain recruitment to the phagocytic cup is sensitive to pretreatment with wortmannin, a specific inhibitor of PI3K, suggesting that Gab2 PH may also bind PI3K lipid products (13). Two of the proline-rich motifs in Gab2 are binding sites for the SH3 of Grb2 (15, 16). Grb2 binding to Gab2 plays a key role in recruiting Gab2 to its upstream receptors. In IL-3/GM-CSF signaling, the Gab2/Grb2 complex via Grb2 Src homology 2 (SH2) domain interacts with tyrosyl-phosphorylated Shc. Shc via its

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* Abbreviations used in this paper: SFK, Src family protein tyrosine kinase; PTK, protein tyrosine kinase; LAT, linker for activation of T cells; Gab, Grb2-associated binder; PH, pleckstrin homology; PIP3, phosphatidylinositol-3,4,5-trisphosphate; SH, Src homology; PTB, phosphotyrosine binding; fc, β common; BMNC, bone marrow-derived mast cell; HA, hemagglutinin; WT, wild type; NEAA, nonessential amino acid; SCF, stem cell factor; PI, phosphatidylinositol.
phosphotyrosine-binding (PTB) domain interacts with the tyrosyl-phosphorylated IL-3 β common (βc). Therefore, Gab2 is recruited to the βc via the Shc/Grb2 complex (14). Gab2/Grb2 complex via the Grb2 SH2 domain is recruited to tyrosyl-phosphorylated BCR-Abelson tyrosine kinase and plays key role in mediating BCR-Abelson tyrosine kinase transformation (17).

Upon receptor activation, Gab2 becomes tyrosyl-phosphorylated and associated with SHP-2, a tyrosine phosphatase, required for optimal activation of Erk (18, 19) and immediate early gene transcription (20). Tyrosyl-phosphorylated Gab2 also binds p85, the regulatory subunit of PI3K, resulting in activation of the PI3K pathway (14, 21).

Previously, we found that Gab2 is required for FcεRI-triggered responses including degranulation and cytokine gene expression in bone marrow-derived mast cells (BMMC) (5). Gab2 is also required for mast cell development in specific tissues mostly likely via signaling through c-Kit (5, 22). One key roles of Gab2 in the FcεRI-initiated response is to activate the PI3K pathway (5), which is required for mast cell degranulation (23). Interestingly, Gab2 has also been found to be more important for FcεRI-evoked chemokine gene expression (24). A recent report also suggests that Gab2 regulates degranulation by activating small GTPase RhoA and microtubule polymerization (25). Consistent with the important role of Gab2 in FcεRI-signaling responses, Gab2 is found to be in the same specialized membrane domain (osmiophilic patches) with FceRIβ upon FcεRI cross-linking (26). However, it is still not clear how Gab2 is recruited to the activated FcεRI and becomes activated to participate in the FcεRI-initiated degranulation response. Furthermore, the PTK required for Gab2 tyrosyl phosphorylation remains controversial. One group reported that Gab2 tyrosyl phosphorylation is dramatically increased in Lyn−/− BMMC (2), another group claimed that Gab2 phosphorylation remains normal in Lyn−/− BMMC (27). Although Lyn is reportedly required for Gab2 phosphorylation (2), it is not clear whether Gab2 is a direct substrate of Lyn. In this study, we investigated how Gab2 becomes tyrosyl-phosphorylated and which PTKs are responsible for Gab2 tyrosyl phosphorylation.

Materials and Methods

Constructs

The Gab2R232C mutant cDNA was generated by PCR using the pBluescript (Stratagene) HA-Gab2 as template and the following primers: 1) 5′-CCA GCA TTT TCT CCA CGC GT A GCG-3′ is the primer encoding the R→C 32 mutation; 2) 5′-ATC GGA TCC CCG AAT AGC AGC GGC-3′ encoding the 5′ end of Gab2 cDNA; and 3) 5′-GCT GTA GAA GTA GAT CAC CAA-3′ encoding the 3′ end of Gab2 and the hemagglutinin (HA) epitope Tag. The PCR product was ligated into pBluescript and verified by DNA sequencing. The HA-Gab2 wild-type (WT) and R32C fragments released by restriction enzyme digestion from pBluescript and the HA-Gab2Grb2 fragment released from a pEBB plasmid (17) were cloned into the retrovector pMxS-puro (a gift from T. Kitomura, Tokyo University, Tokyo, Japan) or MSCV-IRES-GFP (13). To generate the pGex-4T-3 vector, pBAMII was digested with XhoI and XbaI and ligated into BamHI and XhoI-digested pGex-4T-3. The GST-Shc-SH2 and GST-Grb2 constructs were provided by Dr. J. Rivera (National Institutes of Health, Bethesda, MD). Rab-11 anti-Syk Abs were provided by Dr. J. Cambier (National Jewish Medical and Research Center, Denver, CO) or purchased from Cell Signaling Technology. Anti-Shc, Akt1/2, Lyn, and anti-GST rabbit Abs were purchased from BD Transduction Laboratories. Anti-Shc and anti-Akt Abs were provided by Dr. J. Cambier (National Jewish Medical and Research Center, Denver, CO) or purchased from Cell Signaling Technology. Anti-phospho-Akt (Ser473)- and -Syk Abs were purchased from Santa Cruz Biotechnology. Anti-phospho-Akt (Ser473)- and -Syk Abs were purchased from Cell Signaling Technology. Monoclonal anti-phosphotyrosine Ab (4G10) was obtained from Upstate Biotechnology. Anti-She rabbit Abs were purchased from BD Transduction Laboratories. The She inhibitor piceatannol was obtained from Calbiochem. Cells were lysed in 1% Nonidet-P40 lysis buffer as described previously (5). Total cell lysates or immunoprecipitates were resolved by SDS-PAGE, immunoblotted with indicated primary Abs, followed by HRP-conjugated anti-rabbit or anti-mouse IgG (Amersham Biosciences), and developed by ECL Amersham Biosciences). Bands in Western blot were quantified by densitometry analysis using NIH Image 1.63F software. The intensities of phospho-Akt, phospho-Gab2, and phospho-Syk were normalized to the corresponding total Akt, Gab2, and Syk signal, respectively.

Flow cytometry analysis (FACS)

BMMCs were prebound with anti-DNP IgE, washed with PBS containing 3% FBS, and stained with FITC-conjugated anti-mouse IgE rat mAb R35-72 (IgG1) or FITC-concatameric IgG1 as control. Stained cells were analyzed using a FACScan (BD Biosciences). BMMCs were stained with rat mAb FITC-concatameric IgG1 or FITC-isotype control Ab to measure surface expression of c-Kit. All these FITC-conjugated Abs were purchased from BD Pharmingen.

Mice cell degranulation assays

BMMCs (5 × 105) were sensitized with anti-DNP IgE supernatants overnight, washed, resuspended in modified Tyrode’s buffer, stimulated with 0.1 μg/ml DNP-RI and c-Kit. Five- to 8-wk-old BMMC cultures were passaged in RPMI 1640 supplemented with 10% FBS, 5% T-Stat supplement (BD Biosciences), 0.1 mM NEAA, 2 mM t-glutamine, 0.1 mM NEAA, 1 mM sodium pyruvate, 1000 U/ml penicillin, 100 μg/ml streptomycin, and 50 μM 2-ME. The anti-DNP IgE hybridoma cell line (10⁶/ml), a gift from Dr. F.-T. Liu (University of California–Davis, Sacramento, CA), was grown in PHMH-II protein-free hybridoma medium (In-vitrogen Life Technologies) for 4–5 days. The resultant supernatant that typically contains 10–20 μg/ml anti-DNP-IgE was used to sensitize mast cells at a 1/10–20 dilution overnight. For cell stimulation, sensitized BMMC were washed, stimulated with DNP-RI and 1% BSA for 3–4 h at 37°C, and resuspended in modified Tyrode’s buffer (135 mM NaCl, 5 mM KCl, 1 mM MgCl2, 1.8 mM CaCl2, 10 mM HEPES (pH 7.4), 5.6 mM glucose, and 0.1% BSA) before stimulation with indicated concentrations of DNP (Sigma-Aldrich).
Retroviral infection of BMCC and MC9

PMXs-puro and MSCV-ires-GFP retroviral plasmids were transfected into the ecotropic packaging cell line PlateE (31) (provided by T. Kitamura, Tokyo University, Tokyo, Japan) using Fugene reagent (Roche). Virus-containing culture supernatants were collected 2 days later. Bone marrow cells cultured in IL-3-containing IMDM medium for 12 days were spin-infected (2500 rpm, 90 min) with PMXs-puro virus supernatants in the presence of 4 μg/ml polybrene, and then incubated at 37°C for 18–24 h. Infected cells were selected in the presence of 0.8 μg/ml puromycin for 10–14 days, and then cultured in the absence of puromycin for 4–5 wk. For infection of MC9 with MSCV-ires-GFP viruses, MC9 cells were resuspended in fresh medium and incubated with an equal volume of virus supernatant in the presence of 4 μg/ml polybrene at 37°C overnight. One week later, GFP-positive infected cells were purified by FACS.

Protein/lipid overlay assay

Bacterial-expressing GST fusion proteins were purified as described (32). GST-fusion proteins purified by glutathione agarose beads (Sigma-Al
drich) were washed, eluted with an equal volume of 20 mM glutathione (Sigma-Aldrich), resolved by SDS-PAGE, and quantified by staining with Coomassie brilliant blue.

Phosphatidylinositol (PI) lipids were purchased from Echelon Biosciences, reconstituted in chloroform:methanol:water (1:2:0.8), and spotted onto HyBond C extra membranes (Amerham Biosciences) at 100, 50, 25, and 12.5 pmol/spot, respectively. Membranes were air-dried, blocked in TBST (150 mM NaCl, 10 mM Tris-Cl (pH 8.0), 0.05% Tween 20) and with 3% fat-free BSA (Sigma-Aldrich) for 1 h, incubated with 1 μg/ml purified GST-fusion proteins in TBST + 3% fat-free BSA overnight, and then blotted with anti-GST mAb.

Results

Lyn is required for Gab2 phosphorylation

We first asked which PTKs are required for Gab2 tyrosyl phosphorylation. It is generally believed that Lyn is the initial tyrosine kinase activated upon FcεRI cross-linking (1). However, whether Lyn is required for Gab2 tyrosyl phosphorylation remains controversial. Flow cytometry analysis showed that Lyn+/+ and Lyn−/− BMCC have similar surface expression of IgE receptor and c-Kit (Fig. 1A). However, while Gab2 was robustly tyrosyl-phosphorylated in Lyn+/+ BMCC, it was hardly tyrosyl-phosphorylated upon FcεRI cross-linking in Lyn−/− BMCC (Fig. 1B). We also found, in agreement with published reports (33), that Syk activation is severely inhibited in Lyn−/− BMCC (data not shown). These data suggest that Lyn and/or Syk are required for Gab2 tyrosyl phosphorylation. Interestingly, FceRI cross-linking still induced similar mobility shift of Gab2 in Lyn+/+ and Lyn−/− BMCC (Fig. 1B). In response to various stimuli, Gab2 also shows a decreased in mobility shift in SDS-PAGE, which is mainly due to serine and threonine phosphorylation (14, 34, 35). This result suggests that loss of Lyn does not affect serine and threonine phosphorylation (Gab2).

Syk is required for Gab2 phosphorylation

Because Lyn is required for Syk activation in response to FcεRI activation, we asked whether Gab2 tyrosyl phosphorylation also depends on Lyn. We first examined Gab2 tyrosyl phosphorylation in WT BMCC pretreated with piceatannol, a pharmacological inhibitor of Syk. Piceatannol pretreatment completely eliminated FcεRI-evoked Syk and Gab2 tyrosyl phosphorylation, and Gab2 mobility shift, respectively (Fig. 2A). To further support a role of Syk in Gab2 tyrosyl phosphorylation, we assessed Gab2 tyrosyl phosphorylation in Syk+/+ and Syk−/− BMCC. FACS analysis indicated that Syk+/+ and Syk−/− BMCC express similar levels of cell surface IgE receptor and c-Kit (Fig. 2B), suggesting that loss of Syk has no effect on mast cell differentiation (data not shown). However, in agreement with the effect of piceatannol, FcεRI-evoked Gab2 tyrosyl phosphorylation and mobility shift was completely blocked in Syk−/− BMCC (Fig. 2C). We also found that Syk becomes coimmunoprecipitated with Gab2 in WT BMCC upon FcεRI cross-linking (Fig. 2D). Gab2 association with Syk has also been reported when BaF3 cells were stimulated through integrin cross-linking (36). In contrast, SCF-stimulated Gab2 tyrosyl phosphorylation occurred normally in Syk−/− BMCC compared with Syk+/+ BMCC (data not shown). Collectively, these data suggest that Syk, rather a SFK, is the proximal PTK required specifically for FcεRI-evoked Gab2 tyrosyl and serine/threonine phosphorylation.

Decreased FcεRI-evoked Gab2 tyrosyl phosphorylation correlates with decreased Syk activation in Fyn−/− BMCC

A recent report suggested that Fyn is the kinase that responsible for FcεRI-evoked Gab2 tyrosyl phosphorylation and activation of PI3K (2). Therefore, we examined FcεRI evoked Gab2 tyrosyl phosphorylation and Akt activation in Fyn+/+ and Fyn−/− BMCC.
FIGURE 2. Syk is required for FceRI-evoked tyrosyl phosphorylation of Gab2. A, Syk inhibitor blocks FceRI-evoked Gab2 tyrosyl phosphorylation. DNP-IgE-sensitized WT BMMC were pretreated with DMSO (−) or 100 μg/ml Syk inhibitor piceatannol (+) for 1 h, and stimulated with 10 ng/ml DNP for 5 min. Lysates were immunoprecipitated with anti-Gab2- or Syk-specific Abs, reprobed with anti-Gab2 and Syk Abs, respectively. B, Syk+/+ and Syk−/− BMMC express similar IgE receptor and c-Kit. Syk+/+ and Syk−/− BMMC were analyzed for cell surface IgE binding (top) and c-Kit (bottom) by FACS as described in Materials and Methods. C, FceRI-evoked Gab2 tyrosyl phosphorylation is completely inhibited in Syk−/− BMMC. BMMC were sensitized by anti-DNP-IgE, starved, and stimulated with 10 ng/ml DNP for the indicated times. Gab2 tyrosyl phosphorylation was analyzed as in Fig. 1B. D, Syk becomes associated with Gab2 upon FceRI cross-linking. WT BMMC were sensitized by anti-DNP-IgE, starved, and stimulated with 10 ng/ml DNP for 1 min. Lysates were immunoprecipitated with anti-Gab2 serum, immunoblotted with anti-pTyr, and reprobed with anti-Gab2 or Syk Abs. Data shown here represent one of the three independent experiments with similar results.

BMMC. In agreement with the previous report, loss of Fyn did not affect surface expression of IgE receptor and c-Kit in BMMC (Fig. 3A). In addition, FceRI-evoked degranulation (data not shown) and Akt phosphorylation (Fig. 3B) were decreased in Fyn−/− BMMC compared with Fyn+/+ BMMC as reported (2). Gab2 tyrosyl phosphorylation was also reduced in Fyn−/− BMMC (Fig. 3C), but not completely inhibited as we found in Syk−/− BMMC. Importantly, FceRI-evoked total Syk tyrosyl phosphorylation (Fig. 3D) was decreased in Fyn−/− BMMC compared with Fyn+/+ BMMC. These data suggest that the FceRI-evoked Syk activation is impaired in Fyn−/− BMMC. Collectively, these data indicate that Fyn is required for the optimal activation of Syk in response to FceRI engagement. Since we found that Syk is absolutely required for Gab2 tyrosyl phosphorylation (Fig. 2, A and B), our data strongly support a model that Fyn, via Syk, indirectly contributes to the FceRI-evoked Gab2 tyrosyl phosphorylation.

Gab2 PH domain binding sites are required for Gab2 tyrosyl phosphorylation

Next, we asked which domain(s) in Gab2 is required for FceRI-evoked Gab2 tyrosyl phosphorylation. To address the role of Grb2 SH3 binding sites, we expressed HA-tagged Gab2 WT or a mutant of Gab2 (Gab2ΔGrb2), in which the Grb2-SH3 domain binding sites are mutated, in the MC/9 mast cell line. Upon FceRI cross-linking, HA-tagged Gab2 WT or Gab2ΔGrb2 were immunoprecipitated by anti-HA Ab followed by anti-phosphorylation tyrosine immunoblotting (Fig. 4A). HA-Gab2 WT became robustly tyrosyl-phosphorylated and associated with Shc and Grb2. By contrast, HA-Gab2ΔGrb2 showed only weak tyrosyl phosphorylation (~75% decrease). HA-Gab2ΔGrb2 also failed to associate with Shc and Grb2. Thus, the Grb2-SH3 domain binding sites are required for Gab2 association with Shc and the majority of the Gab2 tyrosyl phosphorylation in response to FceRI cross-linking.

She was reported to bind a phosphorylated ITAM peptide derived from the FceRI β-chain (37). However, it is not clear whether the Shc SH2 domain or the PTB domain interacts with the tyrosyl-phosphorylated FceRI β-chain. To address this question, we incubated purified GST-fusion proteins containing either the Shc SH2 domain or PTB with lysates from unstimulated or FceRI-activated mast cells. Only the GST-Shc SH2 domain was able to immunoprecipitate the FceRI β-chain, as validated by antiphosphotyrosine and anti-FceRI β immunoblotting (Fig. 4B). These data suggest that Shc, via its SH2 domain, is recruited to FceRI β-chain. Shc then becomes tyrosyl-phosphorylated and recruits the Grb/Gab2 complex.

Gab2 PH domain also contributes to FceRI-evoked Gab2 tyrosyl phosphorylation

Our previous published data suggest that the Gab2 PH domain may bind PI3K lipid products, and is critical for recruiting Gab2 to the phagocytic cup in macrophages (13). However, the phospholipid-binding specificity of Gab2 PH has not been determined. To address this question, we performed protein/lipid overlay assays by incubating the GST-Gab2 PH domain fusion protein with membranes spotted with different amounts of PI lipids (Fig. 5A). The GST-Gab2 PH domain preferentially interacted with PIP3 and PIP4,2, and to a lesser extent PIP5 and PIP4,5P2. As a control for the lipids spotted on the membrane, the GST-Grp1PH domain interacted only with PIP3, consistent with published reports about this PH domain’s specificity (38). To verify the PI lipid-binding specificity of Gab2 PH, we generated a mutant (R32C) Gab2 PH domain in which R32 is mutant to C. R32, a residue found conserved in other PH domains, is required for PI lipid binding (39). Importantly, the GST-Gab2 PH R32C fusion protein failed to bind PIP3 and PIP4,2 in the lipid overlay assay (Fig. 5A). These data indicate that the Gab2 PH domain binds preferentially to PIP3,4,5P3 and PIP3,4,2, the lipid products of class IA PI3K, similar to the Gab1 PH domain as reported (11, 12).

Next, we assessed the role of the Gab2 PH domain in FceRI-triggered tyrosyl phosphorylation. We expressed HA-tagged...
FIGURE 4. Grb2-SH3 binding sites are required for FcεRI-evoked Gab2 tyrosyl phosphorylation and association with Shc. A, MC9 cells were infected MSCV-HA Gab2WT and MSCV-HA-Gab2ΔGrb2. Infected cells were sensitized with DNP, starved, and stimulated with DNP for 5 and 10 min. Lysates were immunoprecipitated with anti-HA Ab, immunoblotted with anti-pTyr, and reprobed with anti-HA Abs. B, Shc SH2 domain can bind to FcεRI β upon FcεRI cross-linking. BMMC were sensitized, starved, and stimulated with DNP. Lysates were incubated with GST alone, and GST-ShcPTB or GST-ShcSH2 fusion protein, immunoblotted with anti-pTyr, and reprobed with anti-FcεRI β Ab. Data shown here represent one of the three independent experiments with similar results.
ever, compared with Gab2WT expressing cells, phosphorylation of Akt was decreased by ~60% in Gab2ΔGrb2 cells and Gab2R32C cells (at 10–15 min). As Akt is a downstream effector of PI3K, the results suggest that both Gab2 mutants of Gab2 cannot fully activate PI3K. Thus, both Grb2 SH3 binding sites and the Gab2 PH domain binding to PI3K lipid products are critical for FcεRI-evoked responses such as activation of PI3K and degranulation.

**Discussion**

Gab2 is one key tyrosyl-phosphorylated scaffolding adapter protein that plays a critical role in FcεRI-evoked mast cell responses including degranulation, cytokine, and chemokine gene transcription (5, 24, 25). In our present study, we have shown that FcεRI-evoked Gab2 tyrosyl phosphorylation requires Syk by using the pharmacologic inhibitor of Syk and Syk-deficient mast cells. Although Lyn and Fyn are also critical for Gab2 tyrosyl phosphorylation, they act likely through activation of Syk. By expressing the mutant of the Gab2 protein in mast cells, we found that the Grb2-SH3 domain-binding sites and the PH domain are required for FcεRI-evoked Gab2 tyrosyl phosphorylation, Akt activation, and degranulation. We showed that the Grb2-SH3 binding sites are required for Gab2 interaction with Shc while the Gab2 PH domain can preferentially bind the PI3P and PI3,4P2.

**Gab2 recruitment to FcεRI**

A previous study using immunogold labeling suggested that Gab2 becomes associated with a pool of the membrane domain containing FcεRI β and p85, the regulatory subunit of PI3K, upon FcεRI cross-linking (26). Our study provides a biochemical explanation for how Gab2 is recruited to the FcεRI β complex. Upon FcεRI cross-linking, the ITAM in the cytoplasmic tail of the β-chain becomes tyrosyl-phosphorylated. Shc is recruited to the FcεRI β via its SH2 domain and becomes tyrosyl-phosphorylated. Phosphorylated Shc provides a docking site for the Grb2 SH2 domain and recruits Gab2 that is in a constitutive complex with Grb2 via the Grb2 SH3 domain (16, 20). Subsequently, Gab2 becomes tyrosyl-phosphorylated in an Syk-dependent manner and recruits SH2 domain-containing signaling molecules including PI3K. The Gab2-activated PI3K lipid products bind to the Gab2 PH domain around the FcεRI β, further potentiating recruitment. In this positive feedback loop, Gab2 contributes to the sustained activation of PI3K, required for FcεRI-evoked mast cell responses (Fig. 7). This model is similar to the role of Shc in recruiting Gab2 to IL-3/CSF β although Shc binds βc via its PTB domain (14). Likewise, the function of Gab2 PH binding to PI3P is analogous to the proposed model for Gab1 action in epithelial growth factor receptor signaling (11).

Previously, phosphorylated FcεRI β was reported to interact with adapter protein Shc (37). Shc is also reported to become tyrosyl-phosphorylated and associated with Grb2 upon FcεRI cross-linking (40). However, it is not clear how Shc interacts with FcεRI β and what role Shc plays in FcεRI signaling. We showed here that Shc can interact with the phosphorylated FcεRI β specifically via its SH2 domain, not the PTB domain (Fig. 4B). This result is consistent with the predication by Scanote (41) that the cytoplasmic tail of FcεRI β only contains tyrosine-containing motifs (Tyr217, Tyr223, and Tyr227) that may bind the Shc SH2 domain. Consistent with Shc recruiting Gab2 to FcεRI β (Fig. 7), we found that Gab2 increases its association with Shc upon FcεRI cross-linking. A mutant of Gab2 (ΔGrb2), that cannot bind Grb2, lost its association with Shc and tyrosyl phosphorylation (Fig. 4A). Therefore, the FcεRI-evoked Gab2 tyrosyl phosphorylation requires Gab2 association with the Shc/Grb2 complex.
A previous study strongly suggested that the Gab2 PH domain binds PI3K lipid products and is critical for Gab2 function in FcγR1-mediated phagocytosis (13). Here, we showed that the Gab2 PH domain can preferentially bind PIP3, and PI3,4P2, the lipid products of PI3K, and the mutant Gab2 PH R32C domain lost its binding to these lipids (Fig. 5A). Gab1 and Gab2 PH domains share ∼70% of the sequence identity. Especially, the regions in PH domain involved in direct PI lipid binding are conserved (20).

Using the same protein/lipid overlay assay, we found that the Gab1 PH domain also binds preferentially PIP3 and PI3,4P2 (data not shown), in agreement with previously published work using a lipid vesicle-binding assay (11), validating the method of protein/lipid overlay assay in determining PI lipid-binding specificity for the Gab2 PH domain. We also found consistently, although to a lesser extent, that the GST-Gab2 PH domain binds to PI5P (Fig. 5A), a PI lipid with essentially unknown function (42). Likewise, the Gab1 PH domain binds to PI5P in the similar preference in this in vitro-binding assay (data not shown). Future study is needed to determine the function of PI5P binding to Gab2 PH domain.

FIGURE 6. Grb2-SH3 domain binding sites and the Gab2 PH domain are required for FcεRI-evoked degranulation and Akt activation in BMMC. A, Expression of Gab2 Δ Grb2 or Gab2 R32C cannot rescue the defective FcεRI-evoked degranulation in Gab2−/− BMMC. Gab2−/− BMMC were infected with vector, Gab2WT, Gab2 Δ Grb2, and Gab2 R32C pMXs-puro-viruses as described in Materials and Methods. BMMC were sensitized with anti-DNP-IgE, and stimulated with DNP for 10 min. Degranulation was measured as percentage of β-hexosaminidase release (top). Cell lysates were immunoblotted with anti-Gab2 Abs (bottom), indicating similar expression of Gab2WT, Gab2 Δ Grb2, and Gab2 R32C. B, Expression of Gab2 Δ Grb2 cannot rescue the defective Akt activation compared with Gab2WT in Gab2−/− BMMC. BMMC were sensitized with anti-DNP-IgE, starved, and stimulated with DNP for the indicated times. Lysates were immunoblotted with Abs against phospho-Akt and Gab2, and reprobed with Abs against Akt. Akt phosphorylation was quantified by densitometry was shown (bottom). C, Expression of Gab2 R32C cannot rescue the defective Akt activation compared with Gab2 WT in Gab2−/− BMMC. BMMC were sensitized with anti-DNP-IgE, starved, and stimulated with DNP for the indicated times. Lysates were immunoblotted with Abs against phospho-Akt and Gab2, and reprobed with Abs against Akt. Akt phosphorylation quantified by densitometry was shown (bottom). Data shown here represent one of the three independent experiments with similar results.

FIGURE 7. Model of Gab2 action in FcεRI signaling. Upon cross-linking of IgE prebound FcεRI by Ag, FcεRI-associated SFK (Lyn and Fyn) are activated. Then, the ITAM in β-chain becomes phosphorylated and recruits Shc via the Shc SH2 domain. Shc becomes phosphorylated and interacts with Grb2 via the Grb2 SH2 domain, and subsequently recruits Gab2, which is in a constitutive complex with Grb2 via the Grb2 SH3 domain. Gab2 becomes tyrosyl-phosphorylated in a Syk-dependent manner and becomes associated with PI3K that produces PIP3. The Gab2 PH domain binds to PIP3, resulting in a sustained Gab2-signaling complex around FcεRI and FcεRI-evoked mast cell responses.
look into how PISP binds to the Gab PH domain and whether this regulates Gab protein function.

**Kinases phosphorylate Gab2**

Our data strongly suggest that Syk is the major tyrosine kinase responsible for Gab2 tyrosyl phosphorylation upon FcεRI engagement whereas Lyn and Fyn contribute to Gab2 tyrosyl phosphorylation by acting upstream of Syk. A previous study suggested that FcεRI-triggered Gab2 tyrosyl phosphorylation requires Fyn while Syk is dispensable. Furthermore, Gab2 became associated with Fyn upon FcεRI engagement (2). However, under the condition that we immunoprecipitated almost all of Gab2 or Fyn from mast cell lysates using Abs against Gab2 or Fyn, we were unable to detect Gab2 association with Fyn (data not shown). Importantly, we found that FcεRI-triggered Syk tyrosyl phosphorylation was reduced accordingly in Fyn−/− BMMC compared with Fyn+/+ BMMC (Fig. 3D), suggesting that Fyn is required for optimal activation of Syk.

Because our result shows that Syk is absolutely required for FcεRI-evoked Gab2 tyrosyl phosphorylation (Fig. 2C), there are several possibilities regarding how Fyn and Syk control Gab2 tyrosyl phosphorylation. A simple model is that Fyn regulates the activation of Syk, which phosphorylates Gab2 directly (Fig. 7). Alternatively, Syk phosphorylates Gab2 at several sites including the site that recruits Fyn. Upon interacting with Gab2, Fyn may phosphorylate other tyrosine residues in Gab2. Lastly, Syk may contribute to Gab2 tyrosyl phosphorylation by controlling the phosphorylation of the tyrosine residue in FcεRI β that recruits the Shc/Grb2/Gab2 complex as discussed above (Fig. 7). Consistent with this idea, a previous study revealed that the FcεRI-evoked Shc/Grb2 complex depends on Syk (40). At present, we could not determine whether Syk also indirectly contributes to tyrosyl phosphorylation of Gab2 through activation of other protein kinases, such as Tec family kinase member Btk and Itk (1), present in mast cells. Syk is required for activation of Btk in mast cells (43). The use of Btk and/or Itk-deficient mast cells are required to address the potential role of Tec family kinase FcεRI-evoked Gab2 tyrosyl phosphorylation in the future.

Our results also indicate that Lyn is required for Gab2 phosphorylation. Because Lyn acts upstream of Syk in FcεRI signaling (1), it is logical to conclude that Lyn controls Gab2 phosphorylation mainly through activation of Syk. We cannot exclude the possibility that Lyn may phosphorylate Gab2 directly. Consistent with this idea, Gab2 has been suggested to mediate G-CSF-invoked differentiation in an Lyn-dependent manner (44). However, our result does not agree with published reports from Rivera’s group (2) which showed that Lyn negatively regulates FcεRI-evoked Gab2 tyrosyl phosphorylation through the activation of C-terminal Src kinase, an inhibitory PTK for SFK, which inhibits FcεRI-evoked Lyn activation in mast cells (45). There are several possibilities that may account for this discrepancy. The conditions for generating BMMC are different. We typically use only IL-3-containing medium to generate BMMC from bone marrow. However, FcεRI-evoked Gab2 tyrosyl phosphorylation was still severely impaired in Lyn−/− BMMC derived from the same medium condition (IL-3 + SCF) as described (2). The Lyn−− mice used in our study were generated differently from the Lyn−− mice used in the Parravicini et al. (2) study. In the former case, the promoter of region of the Lyn gene was disrupted (46). In the latter case, exon 3-7 of the Lyn gene was eliminated (47). However, both Lyn−− mice are null for Lyn protein. The possibility that we favor is the difference of genetic background of these two Lyn−− mice. The Lyn−− mice used in our study was in the mixed background of sv129/J and C57BL6 while the mice used in the Parravicini et al. (2) study was in the pure C57BL6 background, which could contribute to the different responses of the mast cells derived. Notably, the FcεRI-evoked degranulation is impaired in Lyn−− BMMC in our study (data not shown) while the degranulation was enhanced in Lyn−− BMMC used in the Parravicini et al. (2) study. One possible implication from this discrepancy is the genetic background that may contribute to different expression and/or activation of different SFKs that can activate parallel pathways critical for FcεRI-evoked responses in mast cells (48).

Besides tyrosyl phosphorylation, our data also indicates that Syk is required, while Lyn or Fyn is dispensable, for FcεRI-evoked serine/threonine phosphorylation. Published reports showed that activation of the PI3K (14, 34) and/or Erk (35) pathways contributes to serine phosphorylation in Gab2. Serine phosphorylation on Gab2 has been suggested as a negative feedback mechanism to turn off the Gab2-initiated positive signaling by tyrosyl phosphorylation in response to growth factor and cytokine stimulation (34, 35). It will be important to study in the future how serine/threonine phosphorylation affects Gab2 functions in FcεRI-evoked responses in mast cells.

The Lyn/Syk/LAT pathway and the alternative pathway involving Fyn/Gab2/PI3K have been suggested to play critical roles in FcεRI-evoked degranulation of mast cells (2). Our result indicates that Syk, at least a pool of Syk, is critical for the activation of the Gab2/PI3K in the FcεRI-evoked degranulation response (Fig. 7). Furthermore, our data provide a further biochemical basis for developing potential drugs that specifically inhibit Syk activation (49) to treat allergy.

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**Disclosures**

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**References**


