Critical role for scaffolding adapter Gab2 in FcγR-mediated phagocytosis

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Grb2-associated binder 2 (Gab2), a member of the Dos/Gab subfamily scaffolding molecules, plays important roles in regulating the growth, differentiation, and function of many hematopoietic cell types. In this paper, we reveal a novel function of Gab2 in Fcγ receptor (FcγR)-initiated phagocytosis in macrophages. Upon FcγR activation, Gab2 becomes tyrosyl phosphorylated and associated with p85, the regulatory subunit of phosphatidylinositol 3-kinase (PI3K), and the protein–tyrosine phosphatase; RAW, RAW 264.7; SFK, Src family tyrosine kinase; WT, wild type; Wort, wortmannin.

Key words: macrophages; PI3K; phagocytosis; FcγR; PH domain

Article

Introduction

Macrophages are professional phagocytes that internalize large particulate antigens through various cell surface receptors during innate and adaptive immune responses. Fcγ receptors (FcγR)1 on macrophages bind the Fc portion of IgG molecules and evoke the phagocytosis of IgG-coated (opsonized) particles. FcγR engagement triggers increased actin polymerization near the plasma membrane. This newly polymerized actin fills the pseudopod that extends around and, finally, engulfs the particle. Cross-linking of FcγRs also triggers the secretion of reactive oxygen intermediates and inflammatory cytokines involved in the killing of microorganisms or infected cells.

FcγRs belong to the family of multi-chain immune recognition receptors that includes the T and B cell antigen receptors, and FcεRI, the high affinity receptor for IgE (Nadler et al., 2000). Murine macrophages express two activating FcγRs, a high affinity receptor, FcγRI, and a low affinity receptor, FcγRII. Both of these receptors are multimers comprised of a ligand binding α chain and associated signaling subunits that contains immune receptor–based tyrosine motifs (ITAMs). Engagement of FcγRs during phagocytosis or by antibody cross-linking activates membrane-associated Src family tyrosine kinases (SFKs), which phosphorylate ITAMs. Although other SFKs are present in macrophages, Lyn appears to catalyze the majority of the initial tyrosine phosphorylation on FcγR activation (Fitzer-Attas et al., 2000). Another key tyrosine kinase in FcγR signaling, Syk, is recruited to the phosphorylated ITAMs and becomes activated (Fitzer-Attas et al., 2000). Consistent with their critical roles in FcγR-initiated tyrosine phosphorylation, macrophages from mice with a deletion of SFKs show dramatically decreased rates of FcγR-mediated phagocytosis (Fitzer-Attas et al., 2000). Syk is required for completion of FcγR-mediated phagocytosis because Syk−/− macrophages fail to close the phagocytic cup (Crowley et al., 1997; Kiefer et al., 1998).

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*Abbreviations used in this paper: BMM, bone marrow–derived macrophages; FcγR, Fcγ receptor; Gab2, Grb2-associated binder 2; ITAM, immune receptor–based tyrosine motif; PH, pleckstrin homology; PI3K, phosphoinositide 3-kinase; PIP3, phosphatidylinositol-3,4,5-trisphosphate; RAW, RAW 264.7; SFK, Src family tyrosine kinase; WT, wild type; Wort, wortmannin.

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Although these initial activation events in FcγR signaling are well understood, many details of how SFK and Syk activation translate into various responses, including phagocytosis, remain unclear. Specifically, the important kinase substrates in FcγR-evoked phagocytosis and how these targets couple to downstream pathways important for FcγR signaling, remain to be elucidated.

Phosphatidylinositol kinases are critical for the progression and completion of FcγR-mediated phagocytosis. Phosphatidylinositol-4,5-bisphosphate synthesis is required for efficient phagocytosis (Botelho et al., 2000; Coppolino et al., 2002). Phosphatidylinositol 3-kinase (PI3K) also plays a key role in FcγR-mediated phagocytosis, where it is thought to promote pseudopod extension (Araki et al., 1996; Cox et al., 1999). Furthermore, FcγR-mediated phagocytosis is essentially blocked in “engineered phagocytic fibroblasts” lacking both the p85α and p85β subunits of PI3K, which lack 90% of class IA PI3K activity (Vieira et al., 2001). Studies using GFP fusion proteins that bind phosphatidylinositol-3,4-bisphosphate and/or phosphatidylinositol-3,4,5-trisphosphate (PIP3; i.e., Akt PH-GFP or Gab1 PH-GFP) have shown that PIP3 lipid generation is highly restricted to the phagocytic cup during FcγR-mediated phagocytosis (Marshall et al., 2001). Despite the essential role of PI3K in FcγR-evoked phagocytosis, it is not clear how PI3K is activated at the phagocytic cup.

Recently, we found that Grb2-associated binder 2 (Gab2), a member of Gab/Dos subfamily of scaffolding adapters, is a key regulator of PI3K activation in FcεRI-mediated mast cell signaling (Gu et al., 2001). Gab2 also plays important roles downstream of cytokine (IL-3/GM-CSF; Gu et al., 2000), growth factor (KitL and CSF-1) receptors (Liu et al., 2001; Nishida et al., 2002), and multi-chain immune recognition receptors (TCR and FcεRI; Pratt et al., 2000; Gu et al., 2001; Yamasaki et al., 2001). Like other Gab2/Dos family members, Gab2 contains an amino-terminal pleckstrin homology (PH) domain, several proline-rich motifs (PXXP), and multiple tyrosine phosphorylation sites (Gu et al., 1998). Although the phosphoinositide binding specificity of the Gab2 PH domain has not been determined, the highly similar Gab1 PH domain preferentially binds PIP3. Two of the proline-rich motifs in Gab2 are Grb2-SH3 domain binding sites (Lock et al., 2000; Schaeper et al., 2000), and are important for coupling Gab2 to upstream receptors (Gu et al., 2000). Upon receptor activation, Gab2 becomes tyrosyl phosphorylated and recruits SH2 domain–containing signal relay molecules, including Shp-2 and p85. Gab2 association with Shp-2 is important for cytokine-induced immediate early gene expression (Gu et al., 1998) and growth factor–induced MAPK activation (Liu et al., 2001). Gab2 association with p85, the regulatory subunit of class IA PI3K, is critical for cytokine- and FcεRI-evoked PI3K activation (Gu et al., 2000, 2001).

Because FcεR and FcεRI signaling are similar (Nadler et al., 2000) and Gab2 is expressed in macrophages (Gu et al., 1998), we hypothesized that Gab2 might play a role in FcεR-evoked macrophage responses. Here, we show that Gab2 becomes tyrosyl phosphorylated and associates with Shp-2 and p85 upon FcεR cross-linking in bone marrow–derived macrophages (BMM). Lyn is the major kinase responsible for Gab2 tyrosyl phosphorylation upon FcεR activation. Importantly, BMM from Gab2−/− mice show impaired FcεR-evoked phagocytosis of IgG-opsonized sheep RBC. Furthermore, Gab2−/− BMM display reduced FcεR-evoked activation of Akt, a downstream target of PI3K, suggesting that Gab2 participates in FcεR initiated phagocytosis by regulating PI3K activity in the phagocytic cup. Moreover, Gab2, primarily via its PH domain, is recruited to the nascent phagosomal cup, where it remains until phagosome closure. Finally, expression of a Gab2 mutant that is unable to bind PI3K decreases Akt PH domain recruitment to the phagocytic cup. Collectively, our data establish Gab2 as a key component of the FcεRI-mediated phagocytic machinery in macrophages, and indicate that it functions to control local generation of PIP3 lipids at the phagocytic cup.

Results

Gab2 is critical for FcγR-initiated phagocytosis and signal transduction

To begin to study the involvement of Gab2 in FcγR-initiated phagocytosis, we examined Gab2 phosphorylation and interaction with downstream signaling molecules. BMM were stimulated through FcγRII/III using anti-FcγR antibodies, followed by antibody cross-linking at 37°C for different periods of time. Stimulated BMM were lysed, immunoprecipitated with anti-Gab2 antiserum, and Gab2 immune complexes were resolved by SDS-PAGE and subjected to immunoblotting with antiphosphotyrosine (pTyr) antibodies. Upon FcγR engagement, Gab2 became rapidly tyrosyl phosphorylated and associated with p85 and Shp-2 (Fig. 1 A).

Next, we asked which tyrosine kinase is required for FcγR-induced Gab2 tyrosyl phosphorylation. Because Lyn is the major initial kinase in FcγR-signaling (Fitzer-Attas et al., 2000), we examined Gab2 tyrosyl phosphorylation in BMM from Lyn−/− mice. The majority of FcγR-evoked Gab2 tyrosyl phosphorylation was inhibited in Lyn−/− BMM, compared with Lyn+/+ BMM (Fig. 1 B), suggesting that Lyn is the major kinase required for Gab2 tyrosyl phosphorylation. However, we cannot distinguish whether Lyn directly phosphorylates Gab2 or a Lyn-activated PTK mediates Gab2 phosphorylation. Notably, FcεRI-evoked Gab2 tyrosyl phosphorylation is not decreased in bone marrow–derived mast cells from Lyn−/− mice (Parravicini et al., 2002). Although FcεRI and FcγRs activate the similar SFKs, these disparate results indicate that signaling from the two classes of FcRs to downstream effectors are distinct.

Gab2 association with p85 is critical for FcεRI-induced PI3K activation and mast cell function (Gu et al., 2001). Therefore, we asked whether FcγR-initiated phagocytosis is impaired in BMM from Gab2−/− mice. BMM were incubated with IgG-opsonized RBCs for 5, 10, and 20 min at 37°C (Fig. 1 C). In Gab2+/+ BMM, significant phagocytosis can be seen by 5 min, and phagocytosis continues to increase with time. However, phagocytosis was impaired significantly in Gab2−/− BMM, as indicated by the lower number of RBCs inside Gab2−/− BMM at each time point (Fig. 1 C). Quantification revealed a 50–65% decrease in
Gab2 is critical for FcγR-mediated phagocytosis in BMM. (A) FcγR cross-linking evokes Gab2 tyrosyl phosphorylation and complex formation. BMM from a wild-type (+/+) mouse were starved and stimulated by FcγR cross-linking for the indicated times. Lysates were immunoprecipitated with anti-Gab2 antibodies, and immune complexes were immunoblotted with anti-pTyr (4G10) antibodies. The same blot was reprobed with anti-Gab2, p85, and SHP-2 antibodies, respectively. (B) Lyn is required for Gab2 tyrosyl phosphorylation upon FcγR stimulation. BMM from Lyn+/+ and Lyn−/− mice were stimulated and Gab2 tyrosyl phosphorylation analyzed as in A. (C and D) FcγR-mediated phagocytosis is impaired in Gab2−/− BMM. Gab2+/+ and Gab2−/− BMM were incubated with opsonized RBCs for the indicated times (C). Bar, 10 μm. The average phagocytic index from three fields per time point is shown (D). Similar results were obtained from three different experiments. (E) Gab2−/− BMM have normal cell surface expression of CD16/CD32 and Mac-1. Gab2+/+ and Gab2−/− BMM were incubated with FITC-labeled anti-Mac-1 or PE-labeled anti-CD16/CD32 and analyzed by flow cytometry. (F) Complement-mediated phagocytosis is unimpaired in Gab2−/− BMM. Gab2+/+ and Gab2−/− BMM were exposed to complement-opsonized RBCs for 10, 20, and 45 min. The phagocytosis index was calculated according to Materials and methods. (G) PIRB expression is normal in Gab2−/− BMM. Lysates from Gab2+/+ and Gab2−/− BMM were immunoblotted with anti-PIRB antibodies. The same blot was reprobed with anti-p85 antibodies as a loading control. The error bars represent the SD.

However, phagocytosis is still present in Gab2−/− BMM, indicating that Gab2 is not absolutely required for FcγR-mediated phagocytosis. In contrast, complement-mediated phagocytosis is not impaired in Gab2−/− BMM compared with Gab2+/+ BMM (Fig. 1 F), suggesting that Gab2 functions specifically in FcγR-mediated phagocytosis in macrophages.

Cell surface expression of FcγR and another macrophage marker, Mac-1, were normal in Gab2−/− BMM (Fig. 1 E). A previous work suggested a role for Gab2 in CSF-1 induced macrophage differentiation, based on diminished expression of the inhibitory receptor PirB in cells overexpressing a putative dominant negative mutant of Gab2 (Liu et al., 2001). However, PirB levels were normal in Gab2−/− BMM (Fig. 1 G), indicating that Gab2 is not required for CSF-1-induced BMM. Therefore, impaired FcγR-mediated phagocytosis in Gab2−/− BMM most likely reflects a key role for Gab2 in FcγR-evoked functional responses.

To define where Gab2 acts downstream of FcγR, we examined the biochemical responses of Gab2−/− BMM upon FcγR cross-linking. Overall tyrosyl phosphorylation of cel-
Figure 2. Gab2 is important for FcyR-induced activation of PI3K and Erk pathways. Gab2+/+ and Gab2−/− BMM were starved and stimulated by FcyR cross-linking for the indicated times. (A) Gab2−/− BMM show normal levels of total tyrosyl phosphorylation upon FcyR stimulation. Lysates from Gab2+/+ and Gab2−/− BMM were immunoblotted with anti-pTyr antibodies. As a control for loading, the blot was reprobed with anti-Erk2 antibodies. (B) Syk activation is normal in Gab2−/− BMM. Syk immunoprecipitates were immunoblotted with anti-pTyr antibodies. The same blot was reprobed with anti-Syk antibodies. (C) FcyR-induced activation of Akt and Erk is impaired in Gab2−/− BMM. Lysates from FcyR-activated BMM indicated above were immunoblotted with anti–phospho (Ser 473)-Akt, and anti–phospho-Erk antibodies, followed by reprobing the same blot with anti-Akt and Erk antibodies, respectively. (D) Gab1 expression is up-regulated in Gab2−/− BMM. (top) FcyRII and FcyRII surface expression is similar in Gab2+/+ and Gab2−/− BMM. BMM from Gab2+/+ and Gab2−/− mice (C57/B6 background) were pretreated with isotype control or anti-Ly17.2 (FcyRII) antibodies for 30 min at 4°C, incubated with PE-anti-CD16/32 antibodies, followed by flow cytometric analysis. (bottom) BMM pretreated with isotype control (iso) or anti-Ly17.2 antibodies were activated by cross-linking with anti-CD16/32 antibodies for 10 min. Lysates from activated BMM were immunoblotted.
lular proteins was essentially similar in Gab2−/−, compared with Gab2+/+, BMM (Fig. 2 A), suggesting that Gab2 deficiency does not affect the activation of proximal tyrosine kinases. Consistent with this notion, FcγR-evoked Syk tyrosyl phosphorylation was not decreased in Gab2−/− BMM (Fig. 2 B).

PI3K activity is required for completion of the phagocytic cup during FcγR-evoked phagocytosis (Araki et al., 1996). As an indirect assessment of the PI3K pathway, we monitored Akt activation, which requires the lipid products of PI3K, using phospho (Ser-473)-Akt antibodies. Upon FcγR triggering, Akt phosphorylation increases dramatically in Gab2+/+ BMM. However, Akt phosphorylation was inhibited significantly (~50%) in Gab2−/− BMM (Fig. 2 C). These data suggest that Gab2 association with p85 probably is important for the FcγR-evoked PI3K activation required for phagocytosis.

To further support this notion, we examined the effects of PI3K inhibition on phagocytosis by exposing wild-type (WT) BMM to increasing doses of LY294002 (Fig. 2 F). At 5 μM LY 294002, phagocytosis was inhibited by 70%, whereas Akt phosphorylation was decreased by ~66%. These results are quite similar to our observation of an ~60% decrease in phagocytosis (Fig. 1, C and D) and an ~50% decrease in Akt phosphorylation (Fig. 2 C) in Gab2−/− BMM, and argue that decreased PI3K (as monitored by Akt activation) can account for the phagocytic defect in Gab2−/− BMM.

Erk activation also was reduced dramatically in Gab2−/− BMM (Fig. 2 C). This result is consistent with the key role of Gab proteins for Erk activation by many growth factor and cytokine receptors (Itoh et al., 2000; Sachs et al., 2000), most likely through Gab–Shp–2 interaction (Cunnick et al., 2000; Maroun et al., 2000; Schaeper et al., 2000; Liu et al., 2001). Interestingly, Erk activation by FcεRI is normal in Gab2−/− mast cells (Gu et al., 2001), indicating that Gab2 requirement for MAPK activation depends on the cell type and the receptor signaling involved.

In most of our experiments, we used anti-CD16/CD32 cross-linking to activate FcγR. Anti-C16/CD32 cross-linking activates FcγRIII and FcγRII. These receptors elicit distinct biological responses, with FcγRIII playing a positive role in phagocytosis and FcγRII having a negative regulatory function (Schiller et al., 2000). To test the role of Gab2 specifically in FcγRIII signaling, we blocked FcγRII by pretreating BMM with anti-Ly17.2 antibodies, which recognize FcγRII, or isotype controls, followed by anti-CD16/CD32 cross-linking (Fig. 2 E). Flow cytometric analysis revealed that surface CD16/CD32 expression was similar in Gab2+/+ and Gab2−/− BMM pretreated with isotype control antibodies. Anti-Ly17.2 pretreatment caused a similar reduction in anti-CD16/CD32 surface staining in Gab2+/+ and Gab2−/− BMM (Fig. 2 E, top). This result indicates that, as expected, anti-Ly17.2 pretreatment blocked surface FcγRII in these BMM. In addition, the surface expression of FcγRIII, and FcγRII by inference, is similar in Gab2+/+ and Gab2−/− BMM.

Next, we examined Akt activation by immunoblotting using phospho-Akt antibodies (Fig. 2 E, bottom). As expected from our earlier results, we found that anti-CD16/CD32–induced Akt phosphorylation was lower in Gab2−/− compared with Gab2+/+ BMM pretreated with isotype control antibodies. Moreover, pretreatment with anti-Ly17.2 antibodies did not affect anti-CD16/CD32–evoked Akt phosphorylation in Gab2+/+ or Gab2−/− BMM, strongly suggesting that FcγRIII signaling is impaired in Gab2−/− BMM.

**Mechanism of Gab2 action in FcγR-mediated phagocytosis**

To gain further insight into Gab2 participation in FcγR signaling, we investigated its subcellular localization during phagocytosis. We subjected the macrophage cell line RAW 264.7 (RAW) to transient transfection with the GFP-tagged Gab2 construct. GFP-Gab2 was visualized by confocal microscopy in real time after addition of IgG-opsonized RBCs (Fig. 3 A; Video 1 available at http://www.jcb.org/cgi/content/full/jcb.200212158/DC1). Before initiation of phagocytosis, GFP-Gab2 was distributed evenly throughout the cell. However, when opsonized RBCs made contact with the transfected RAW cells, there was a marked increase of the GFP signal at the site of contact. Subsequently, the GFP signal began to extend into the nascent phagosome (Fig. 3 A, arrowheads) until it completely surrounded the RBCs upon phagosome closure. The GFP-Gab2 signal remained in the sealed phagosome (Fig. 3 A, arrows) for additional 1.5–2 min. The control GFP protein remained cytosolic during phagocytosis (unpublished data). An HA-tagged Gab2 construct showed the same pattern of recruitment to the phagosome (Fig. 4 A).

We asked which domain in Gab2 is required for its localization to the phagocytic cup. To assess the role of the PH domain and Grb2-SH3 domain binding sites, we transfected RAW cells with HA-tagged Gab2 WT, ΔPH, and ΔGrb2 mutants, respectively, and examined their localization by immunofluorescence using anti-HA antibodies (Fig. 3 B). Quantification (see Materials and methods) revealed that Gab2 WT-HA was enriched 6.7-fold in phagocytic cups (Fig. 3 C). In contrast, Gab2 ΔPH-HA was virtually unable to be recruited to phagocytic cups, exhibiting only a 1.6-fold enrichment. Gab2 ΔGrb2-HA enrichment (5.2-fold) in phagocytic cups was decreased slightly (but significantly, P < 0.01) compared with Gab2 WT-HA (Fig. 3 C). These data indicate that the Gab2 PH domain is critical, whereas the Grb2-SH3 binding sites also contribute to the efficient recruitment of Gab2 to the phagocytic cup. Consistent with

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with anti–phospho (Ser 473)-Akt antibodies, followed by reprobing with anti-Akt antibodies. (F) Inhibition of FcγR-evoked phagocytosis and Akt activation by PI3K inhibition. BMM from wild-type mice were pretreated with the indicated concentrations of LY294002 (LY) for 30 min, and subjected to FcγR-mediated phagocytosis for 20 min at 37°C (top) and FcγR cross-linking for 7 min at 37°C (bottom). Bar, 10 μm. The percent inhibition of phagocytosis by different LY294002 concentrations is indicated under each condition. Lysates from activated BMM were immunoblotted with anti–phospho (Ser 473)-Akt antibodies, and then reprobed with anti-Akt antibodies (bottom).
The requirement of the PH domain for targeting Gab2 to the phagosome, a GFP-Gab2 PH domain fusion protein also localized to the phagocytic cup in a similar fashion to GFP-Gab2 WT (Fig. 3 D; Video 2 available at http://www.jcb.org/cgi/content/full/jcb.200212158/DC1). Thus, our data indicate that the Gab2 PH domain is sufficient for phagocytic cup localization.

Because the Gab1 PH domain binds preferentially to PIP3 (Marou et al., 1999; Rodrigues et al., 2000), we want to test whether Gab2 recruitment depends on PIP3, we analyzed the localization of Gab2 WT-HA and GFP-Gab2 PH during phagocytosis in RAW cells pretreated with the specific PI3K inhibitor wortmannin (Fig. 4 A and B, Wort). For comparison, we also quantified recruitment of the PIP3 probe, Akt-PH-GFP, in control and Wort-treated cells (Fig. 4 C). Recruitment of GFP-Gab2 PH to phagocytic cups was inhibited by ~90%, suggesting that recruitment of Gab2 to the phagocytic cup is mainly via Gab2 PH domain binding to PI3K lipid products. Consistent with this notion, a Gab2 mutant, DMPH, with point mutations in the presumptive PI lipid binding (based on Btk PH domain structure) surface of its PH domain, fails to be recruited to the phagocytic cup (Fig. 4 F). However, Wort pretreatment inhibited Gab2 WT-HA recruitment by only ~50% (Fig. 4 C). Together, our data indicate that a PIP3-independent mechanism, most likely involving the Grb2 binding site on Gab2, also contributes to Gab2 recruitment to the phagocytic cups.

Previously, we used a GFP-Akt PH construct to demonstrate increased PI3 lipid production in the nascent phagosome (Marshall et al., 2001). Interestingly, when Gab2-HA and GFP-Akt-PH proteins were coexpressed transiently in RAW cells, we found that they colocalized completely in phagocytic cups during FcyR-mediated phagocytosis (Fig. 4 D). Again, Wort abolished the coenrichment of these components in phagocytic cups (unpublished data). These data further support the conclusion that Gab2 is localized to the site where the PIP3 lipids are produced during phagocytosis.
To test the functional role of Gab2 recruitment to the phagocytic cup, we examined the effect of expression of a Gab2 mutant (Δp85), which cannot bind p85, on Akt PH domain recruitment to the phagocytic cup (Fig. 4 G). Expression of Gab2 Δp85, compared with Gab2 WT, inhibited Akt PH domain recruitment. This result suggests that Gab2, on recruitment to the phagocytic cup and complex formation with p85, contribute to PI3 lipid production in the nascent phagosome.

Discussion

Here, we have demonstrated a new function for Gab2 in FcγR-mediated phagocytosis. Macrophages from Gab2−/− mice show impaired Fcγ-mediated phagocytosis. Upon FcγR engagement, Gab2 is recruited to the nascent phagosome mainly via its PH domain, interacting most likely with PIP3 (Fig. 5). Subsequently, Gab2 becomes tyrosyl phosphorylated in a Lyn-dependent manner and recruits SHP-2 and p85. Gab2 association with p85 is important for producing additional, wild-type levels of PIP3 in the phagocytic cup. These data support a model in which Gab2 functions as a key component of a positive feedback loop for sustained PIP3 production, which, in turn, is required for pseudopod extension and closure of the phagocytic cup (Araki et al., 1996; Cox et al., 1999).

Our results establish a critical role for the Gab2 PH domain in recruitment of Gab2 to the phagocytic cup. Consistent with this notion, recruitment of Gab2 PH domain to the phagocytic cup is blocked by treatment with PI3K inhibitors (Fig. 4 B). Although the binding specificity of Gab2 PH for PI lipids remains to be determined directly, the Gab1 PH domain is known to bind PIP3 (Maroun et al., 1999; Rodrigues et al., 2000). Because the PH domains of Gab1 and Gab2 are highly related (~70% amino acid identity), especially in the regions involved in PIP3 binding (Gu et al., 1998), it is likely that Gab2 PH also binds PIP3. In addition, a Gab2 mutant, Gab2DMPH, with point mutations in the PI lipid binding surface (29 Trp → Ser and 32 Arg → Cys) fails to be recruited to the phagocytic cup (Fig. 4 F). The same mutations severely impair Gab1 PH domain binding to PIP3 (Maroun et al., 1999).

However, our data also indicate that Gab2 can be recruited to the phagocytic cup in a PIP3-independent manner. Most likely, the Grb2 SH3 binding sites in Gab2 contribute to PIP3-independent recruitment because phagosomal recruitment of the Gab2ΔGrb2 mutant is slightly decreased (Fig. 3 C). We have shown previously that recruitment of Gab2 to the βc chain of the IL-3R/IL-5R/GM-CSFR (Gu et al., 2000) or to BCR/ABL (Sattler et al., 2002) requires Grb2 binding to Gab2. In contrast, in FcγR signaling, the PH domain is crucial for recruitment to the phagosome, whereas Grb2 binding to Gab2 plays a supportive role. Therefore, our results demonstrate for the first time a physiologically important function for the Gab2 PH domain.

There are at least two ways by which the Grb2 binding sites might mediate Gab2 recruitment. Grb2 could bring the Grb2–Gab2 complex to activated FcγRs in the nascent phagosome in a tyrosyl phosphorylation–dependent manner similar to the mechanism of Gab2 recruitment to the IL3R βc chain (Gu et al., 2000). However, to sustain recruitment, Gab2 must bind PI3 lipids in the phagocytic cup via its PH domain; otherwise Gab2 falls off the phagocytic cup quickly. The localization of the Gab2ΔPH (Fig. 3 C) and Gab2DMPH (Fig. 4 F) are consistent with this model. Al-
Figure 4. **PIP3 is required for efficient recruitment of Gab2 in nascent phagosomes.** (A and B) Gab2 recruitment to the phagocytic cups correlates with PIP3 binding to Gab2 PH domain. RAW cells expressing Gab2-HA (A, green) or Gab2-PH-GFP (B, green) in the process of engulfing RBCs (A, blue; B, red). In the presence of the PI 3-kinase inhibitor, Wort, Gab2-PH-GFP (green) does not accumulate in phagocytic cups (B), whereas Gab2-HA recruitment to phagocytic cups is inhibited by ~50% (A). Phagocytic cups are actin- and RBC-rich. (C) The accumulation of Gab2-HA, Gab2-PH-GFP, and Akt-PH-GFP proteins in the phagocytic cups in the absence (C) or presence of Wort (W) was quantified as described in Materials and methods. The error bars represent the SD. (D) Gab2-HA (red) colocalizes with GFP-Akt PH (green) in phagocytic cups (blue, RBC) in RAW cells. Bars: (A, B, D, and F) 10 μm. (E) Inhibition of PI3K activation by LY294002 does not affect FcγR-induced Gab2 tyrosyl phosphorylation. Wild-type BMM were starved or pretreated with 25 μM LY294002 (LY) for 20 min and activated for 5 min by FcγR cross-linking. Lysates were immunoprecipitated with anti-Gab2 antibodies followed by immunoblotting with anti-pTyr and Gab2 antibodies, respectively. Lysates were also immunoblotted with anti–phospho (Ser 473)-Akt and anti–phospho-Erk antibodies. (F) Gab2 with mutations in its phospholipid binding surface cannot be recruited to the phagocytic cup. RAW cells expressing GFP-Gab2 and GFP-Gab2 DMPH in the process of internalizing IgG-opsonized RBCs are shown. Note that although GFP-Gab2 is recruited to the nascent phagosome, GFP-Gab2 DMPH fails to be recruited. (G) Expression of Gab2Δp85 inhibits Akt PH domain recruitment to the phagocytic cup. RAW cells cotransfected with GFP-Akt PH and Gab2-HA or Gab2Δp85-HA plasmids were allowed to internalize IgG-opsonized RBCs. The accumulation of GFP-Akt PH in the phagocytic cup was quantified as described in Materials and methods. The data shown are the average from analyzing the indicated number of phagocytic cups in Gab2- and Gab2Δp85-transfected cells. The error bars represent the SD.
alternatively, Grb2 might dimerize Gab2 or cause a conformational change in Gab2 via the Grb2 SH3 binding site, which might allow the Gab2 PH domain to interact with the phagocytic cup in a PIP3-independent manner. For example, the Gab2 PH domain might bind to non-PI3 lipids at a lower affinity than to PI3 lipids. In that case, a dimerized Gab2 might allow the Gab2 PH domain to interact with the nascent phagosome, where it becomes phosphorylated and activates PI3K to generate additional PIP3 in the nascent phagosome. Alternatively, small amounts of Gab2 (or other Gab family members) might be transiently recruited to the activated FcγR via the PI3K-independent mechanism, where it becomes tyrosyl phosphorylated and associated with p85, thereby generating PI3K locally in the nascent phagosome. Notably, an analogous model has been proposed for EGFR signaling involving Gab1 (Rodrigues et al., 2000). Consistent with this model, pretreatment of BMM with LY294002 has no effect on FcγR-evoked Gab2 tyrosyl phosphorylation (Fig. 4 E). This model also is consistent with the partial recruitment of wild-type Gab2 in the presence of Wort (Fig. 4, A and C).

Because phagocytosis and the PI3K–Akt pathway activation are only partially inhibited Gab2−/− BMM (Figs. 1 and 2), other Gab/Dos family members may also function in BMM. Indeed, Gab1 was not only present in BMM but its expression was increased at approximately twofold in Gab2−/− BMM (Fig. 2 D). We also found that GFP-Gab1 was recruited to the nascent phagosome (unpublished data), although possibly at lower efficiency than Gab2. Notably, Gab1 association with p85 is important for EGF and NGF activation of PI3K (Holgado-Madruga et al., 1997; Rodrigues et al., 2000). Thus, our data suggest that Gab1 may partially substitute for Gab2, explaining the residual FcγR signaling and function in Gab2−/− BMM, and conceivably, the normal macrophage differentiation in Gab2−/− mice. A recent report indicates that the third mammalian Gab/Dos family member, Gab3, also is expressed in BMM (Wolf et al., 2002), although its function in FcγR signaling remains unclear.

Finally, in view of the scaffolding function of Gab2, it is possible that other Gab2 binding proteins are recruited to the nascent phagosome, where they are responsible for other aspects of the phagocytic process, such as cytoskeletal remodeling, fission, or phagosomal maturation and/or cytolysosome gene expression. Additional work will be required to address these possibilities.

Materials and methods
Reagents and antibodies
Anti-Gab2 (Gu et al., 1998) and PriB (Timms et al., 1998) antibodies were generated as described previously. Anti–phospho-Akt (Ser-473) and anti–phospho-Erk1/2 rabbit antibodies were purchased from Cell Signaling Technology. Rabbit polyclonal anti-Akt1/2, Erk2, and Shp2 rabbit antibodies were purchased from Santa Cruz Biotechnology, Inc. Polyclonal anti-p85 rabbit antiserum was generated as described previously. Anti–phospho-Akt (Ser-473) and anti–phospho-Erk1/2 rabbit antibodies were purchased from Santa Cruz Biotechnology, Inc. Polyclonal anti-Gab1 antibodies and monoclonal anti-pTyr (4G10) antibodies were obtained from Cell Signaling Technology. Rabbit polyclonal anti-Akt1/2, Erk2, and Shp-2 rabbit antibodies were purchased from Upstate Biotechnology. Anti-p85 rabbit antiserum was a gift from Dr. L. Cantley (Beth Israel-Deaconess Medical Center, Boston, MA) and anti-Syk rabbit IgG was provided by Dr. J. Cambier (National Jewish Center, Denver, CO). Purified rat anti–murine CD16/CD32 (mAb 2.4G2), PE-labeled rat anti–murine CD16/CD32, and FITC-labeled anti–murine Mac-1 were obtained from BD Biosciences. Murine monoclonal anti–rat IgG2b and C5-deficient serum were obtained from Sigma-Aldrich. Rabbit anti–sheep RBC antiserum, anti–sheep RBC IgM, and sheep RBCs were obtained from ICN Biomedicals. LY294002 and Wort were purchased from BIOMOL Research Laboratories, Inc. Anti–FcyRII mAbs, directed against mouse Ly-17.2 in C57/B6 mice, were provided by Dr. J.P. Kinet (BDMC; Schiller et al., 2000).

Plasmids
pEBB-derived plasmids expressing HA-tagged Gab2 WT, ΔPH, ΔGrb2, and Δp85 mutants were described previously (Gu et al., 1998; Pratt et al., 2000; Sattler et al., 2002). pEGFP AktPH was described previously (Marshall et al., 2001). The GFP-Gab2, GFP-Gab2 PH domain (1–116

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**Figure 5. Model of Gab2 action in FcγR-mediated responses.** FcγR crosslinking results in the activation of the receptor-associated protein tyrosine kinase including Lyn and the generation of low level PI3K lipid products in the nascent phagosome. Gab2 is recruited to the nascent phagosome mainly via its PH domain, which likely binds PI3K lipid products initially present in the phagocytic cup. Next, Gab2 becomes tyrosyl phosphorylated likely by Lyn and/or Lyn-dependent protein tyrosine kinase(s). Phosphorylated Gab2 recruits SHP-2 and PI3K to the phagocytic cup. Gab2–PI3K complex contributes to the generation of additional PI3K lipid products, which are required for the progressive formation and the closure of the phagocytic cup. It remains to be determined whether SHP-2 or other Gab2-associated signal molecule plays a role in FcγR-evoked responses.
amino acids), and GFP-Gab2DMPH (29 Trp → Ser and 32 Arg → Cys) fusion constructs were generated using the plasmid pEGFP-C2 (CLONTECH Laboratories, Inc.). Six oligo-primers were used to generate the three GFP-Gab2 constructs. Primer 1, 5'-ATCCTCAGATATGACCCCGG-GACATGAGC-3' primer 2, 5'-TCACTGATCAGATACGGGCAG-CCT-3'; primer 3, 5'-TCACTGATAGAAGCCAGATCGGCA-3'; primer 4, 5'-AGGCGCTACGCGTCCAAGAAATGCTGGTTTA-3'; primer 5, 5'-ATCGATCAGATATGACCCCGG-GACATGAGC-3'; primer 6, 5'-GCTTAC-GAAGCTTGGGATCC-3'. The full-length Gab2 cDNA in pBluescript plasmid (Gu et al., 1998) was used as the template for PCR to generate various Gab2 DNA fragments. For generating the GFP-Gab2 construct, the full-length Gab2 cDNA was amplified by PCR using primers 1 and 2, digested with XhoI, and cloned into XhoI-digested pEGFP-C2 plasmid. For generating the GFP-Gab2 PH construct, the Gab2 PH domain cDNA was amplified by PCR using primers 1 and 3, digested with XhoI, and cloned into XhoI-digested pEGFP-C2 plasmid. For generating the GFP-Gab2 PH construct, the Gab2 PH domain cDNA was amplified by PCR using primers 1 and 2, digested with XhoI, and cloned into XhoI-digested pEGFP-C2 plasmid. The Gab2 PH construct was isolated from the pBluescript KS (+) plasmid, purified using the QiaEX II Tips Kit (Qiagen), and cloned into pBluescript (Marshall et al., 2001). Transfected RAW cells were used for experiments.

**BMM culture**

Gab2−/− mice (sv129/J × C57Bl/6J background) were generated as in Gu et al. (2001). Gab2−/− mice, in C57B6 background, were generated from the original 129 × B6 hybrids by backcrossing for five generations to wild-type C57B6 mice. Lys−/− mice were provided by Dr. T. Yamamoto (University of Tokyo, Tokyo, Japan). Littermate wild-type and Gab2−/− mice or Lys−/− mice with 2–4- mo old, were killed. BMM were prepared for phagocytosis assays as described previously (Fitzer-Attas et al., 2000), 6–7 d after the initial isolation of the bone marrow, macrophages were detached from the dishes and replated for the assays to be performed.

**FcγR and complement-mediated phagocytosis by BMM**

To opsonize RBCs with IgG, RBCs were washed and resuspended in veronal-buffered saline (Sigma-Aldrich) containing 1:500 rabbit anti-RBC serum, and incubated at 37°C for 1.5–2 h. To opsonize RBCs with complement, BMMs were incubated with anti sheep RBC IgM for 1 h (1:5,000) and followed by incubation with 10% C5-deficient serum for 20 min. Opsonized RBCs were washed with veronal-buffered saline and resuspended in DME at ~5 × 107 RBCs/ml. BMM (7–8-d old) were seeded in 24-well plates (3 × 104/well) for 24 h, starved for 2 h, and incubated with 0.4 ml of opsonized RBCs at 37°C for the indicated times. Phagocytosis was terminated by lysing nonphagocytosed RBCs with H2O for 40 s. BMM were fixed and photographed under a phase-contrast microscope (Olympus). Phagocytosis was quantified by randomly counting the total number of RBCs in four fields (≈100 macrophages/field) under a 20× objective lens. The phagocytic index was calculated by dividing the total number of RBCs by the total number of macrophages in the same field.

**Stimulation of BMM for biochemical analysis**

BMM (7–9-d old) were starved in DME + 1% BSA for 2 h. Anti-CD16/CD32 antibodies (5 μg/ml) were added to BMM and incubated on ice for 40 min. The media were discarded and DME containing 1% BSA and 6 μg/ml anti-rat IgG2b was added to the cells. Cross-linking of FcγR was initiated by shifting the plates to 37°C for the indicated times.

**Immunoprecipitation and immunoblotting**

BMM lysates, prepared in NP-40 lysis buffer, were clarified and immunoprecipitated with the indicated antibodies for 2.5–3 h as described previously (Gu et al., 2000). Immunocomplexes were recovered on protein A- or protein G-agarose beads (Amersham Biosciences) and immunoblotted with the indicated antibodies. Immunoblots were developed using ECL (NEL Life Science Products).

**Transfection of RAW cells**

RAW cells were transiently transfected with the indicated DNAs using FuGENE 6 reagent (Roche Molecular Biochemicals) as described previously (Marshall et al., 2001). Transfected RAW cells were used for experiments 24 h later.

**Immunofluorescence and confocal microscopy**

Immunofluorescent staining of RAW cells was described previously (Botelho et al., 2000). HA-tagged proteins were detected with monoclonal anti-HA antibody ascites (1:10,000 dilution of HA.11; Covance Research Company), followed by fluorochrome-conjugated donkey anti-mouse antibodies (Jackson Immunoresearch Laboratories). Filamentous actin was detected with fluorochrome-conjugated phalloidin (Molecular Probes), and IgG-coated RBCs were detected with Cy3-conjugated donkey anti-rabbit antibodies (Jackson Immunoresearch Laboratories).

Immunofluorescence analysis and real-time observation of GFP-transfected RAW cells was accomplished by using a confocal microscope (model LSM510; Carl Zeiss Microimaging, Inc.). Phagocytosis was initiated by the addition of RBCs at a concentration of 2–5 RBCs/macrohage. Cells were scanned every 30 s with the 488-nm laser line at low power (<12%) to minimize photobleaching, and recording was accomplished with the time series function of LSM510 software.

**Quantification of Gab2 recruitment to the phagocytic cup**

Single confocal cross sections of cells expressing the indicated tagged proteins were acquired at nonsaturating settings and used to quantify the recruitment of these proteins to sites of particle internalization. The signal intensity (grayscale value) of each protein at the phagocytic cup, defined as a region of the cell where polymerized actin and RBCs localized, and “resting plasma membrane,” defined as nonphagocytic, nonruffling plasma membrane (or cell periphery), was quantified using Image J v. 1.28 (National Institutes of Health, Bethesda, MD). The ratio of the signals recorded from the phagocytic cup and the “resting plasma membrane” was then calculated as a measure of enrichment of each construct at the phagocytic cup, normalizing for variability in protein expression between cells.

**Online supplemental material**

Video 1 shows the recruitment of GFP-Gab2 to the phagocytic cup. RAW cells transfected with the GFP-Gab2 construct are in process of engulfing IgG-opsonized RBC. Images are acquired every 30 s.

Video 2 shows the recruitment of GFP-Gab2PH to the phagocytic cup. RAW cell transfected with the GFP-Gab2PH construct is in process of engulfing IgG-opsonized RBC. Images are acquired every 30 s. Videos are available at http://www.jcb.org/cgi/content/full/jcb.200212158/DC1.

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