

Science Signaling



Online issue 10 September 2013

The Scaffold Protein Prohibitin Is Required for Antigen-Stimulated Signaling in Mast Cells

Do Kyun Kim,^{1*} Hyuk Soon Kim,^{1*} A-Ram Kim,¹ Geun Hyo Jang,¹ Hyun Woo Kim,¹ Young Hwan Park,¹ Bokyoung Kim,¹ Yeong Min Park,¹ Michael A. Beaven,² Young Mi Kim,³ Wahn Soo Choi^{1†}

The protein prohibitin (PHB) is implicated in diverse cellular processes, including cell signaling, transcriptional control, and mitochondrial function. We found that PHB was abundant in the intracellular granules of mast cells, which are critical for allergic responses to antigens. Thus, we investigated whether PHB played a role in signaling mediated by the high-affinity receptor for antigen-bound immunoglobulin E (IgE), FcεRI. PHB-specific small interfering RNAs (siRNAs) inhibited antigen-mediated signaling, degranulation, and cytokine secretion by mast cells *in vitro*. Knockdown of PHB inhibited the antigen-dependent association of the tyrosine kinase Syk with FcεRI and inhibited the activation of Syk. Fractionation studies revealed that PHB translocated from intracellular granules to plasma membrane lipid rafts in response to antigen, and knockdown of PHB suppressed the movement of FcεRIγ and Syk into lipid rafts. Tyrosine phosphorylation of PHB by Lyn was observed early after exposure to antigen, and point mutations in PHB indicated that Tyr¹¹⁴ and Tyr²⁵⁹ were required for the recruitment of Syk to FcεRIγ and mast cell activation. In mice, PHB-specific siRNAs inhibited antigen-initiated mast cell degranulation, passive cutaneous anaphylaxis, and passive systemic anaphylaxis. Together, these results suggest that PHB is essential for FcεRI-mediated mast cell activation and allergic responses *in vivo*, raising the possibility that PHB might serve as a therapeutic target for the treatment of allergic diseases.

INTRODUCTION

Prohibitin (PHB; also known as PHB1 and BAP32), a member of the Band-7 family of proteins, is highly conserved, is ubiquitously expressed, and localizes to the mitochondria, cytosol, nucleus, and plasma membrane (1–4). PHB has a critical role in mediating apoptosis, cellular senescence, and mitochondrial biogenesis (5, 6). PHB is required as a scaffold protein that ensures the interaction between Ras and Raf at the plasma membrane, thus leading to Ras-mediated activation of Raf, as well as downstream activation of the extracellular signal-regulated kinase (ERK) pathway (7). PHB also interacts with the insulin receptor, phosphatidylinositol 3,4,5-trisphosphate (PIP₃), and the serine and threonine kinase Akt (also known as protein kinase B), and it plays a modulatory role in the phosphatidylinositol 3-kinase (PI3K)–Akt pathway (8, 9). The ERK and PI3K–Akt pathways are also engaged in mast cell activation through the high-affinity receptor for immunoglobulin E (IgE) (FcεRI); however, whether PHB plays any role in FcεRI-mediated signals and allergic responses remains undetermined.

Mast cells are the major effector cells that mediate allergic responses (10–12). After stimulation of mast cells by antigen, mast cells secrete a large number of granules that contain histamine and other preformed inflammatory mediators, as well as newly formed mediators such as eicosanoids and cytokines. These mediators play central roles in allergic responses (13). The multivalent binding of antigen to IgE bound to FcεRI at the plasma membrane results in the aggregation of FcεRI complexes and the rapid phosphorylation of tyrosine residues in the immunoreceptor tyrosine-based

activation motifs (ITAMs) of the β and γ chains of FcεRI by the Src family kinase (SFK) Lyn, or possibly by other SFK members. This leads to the recruitment of additional Lyn molecules to the β chain and of another protein tyrosine kinase, Syk, to the γ chains (14–16). Fully activated Syk is essential for the activation of key proteins downstream of FcεRI, including the transmembrane adaptor protein linker for activation of T cells (LAT) (17–20), phospholipase C (PLC) (21, 22), Src homology 2 (SH2) domain-containing leukocyte protein of 76 kD (SLP-76) (23), and Gab2 (24). Subsequent events include calcium (Ca²⁺) mobilization and activation of the mitogen-activated protein kinases (MAPKs) ERK, c-Jun N-terminal kinase (JNK), and p38, as well as the PI3K–Akt pathway. These signaling events eventually lead to the release of inflammatory mediators to induce allergic responses.

Here, we report the presence of PHB in mast cells and present evidence of a role for PHB in FcεRI-mediated signaling, degranulation, and cytokine release *in vitro*, as well as in anaphylaxis *in vivo*. We found that PHB was critical for the recruitment of Syk to the γ subunit of FcεRI (FcεRIγ), most likely by acting as an adaptor protein to enable the phosphorylation of FcεRIγ by Lyn, and as a consequence, PHB also appeared to be essential for the activation of Syk and downstream signaling molecules, including PLC-γ, Gab2, Akt, ERK1/2, JNK, and p38. These results suggest that PHB, which translocated from granules to the plasma membrane during stimulation, acts as an essential adaptor molecule for FcεRI-mediated signaling pathways.

RESULTS

PHB enhances mast cell degranulation and cytokine release in response to antigen, but not to lipopolysaccharide or stem cell factor

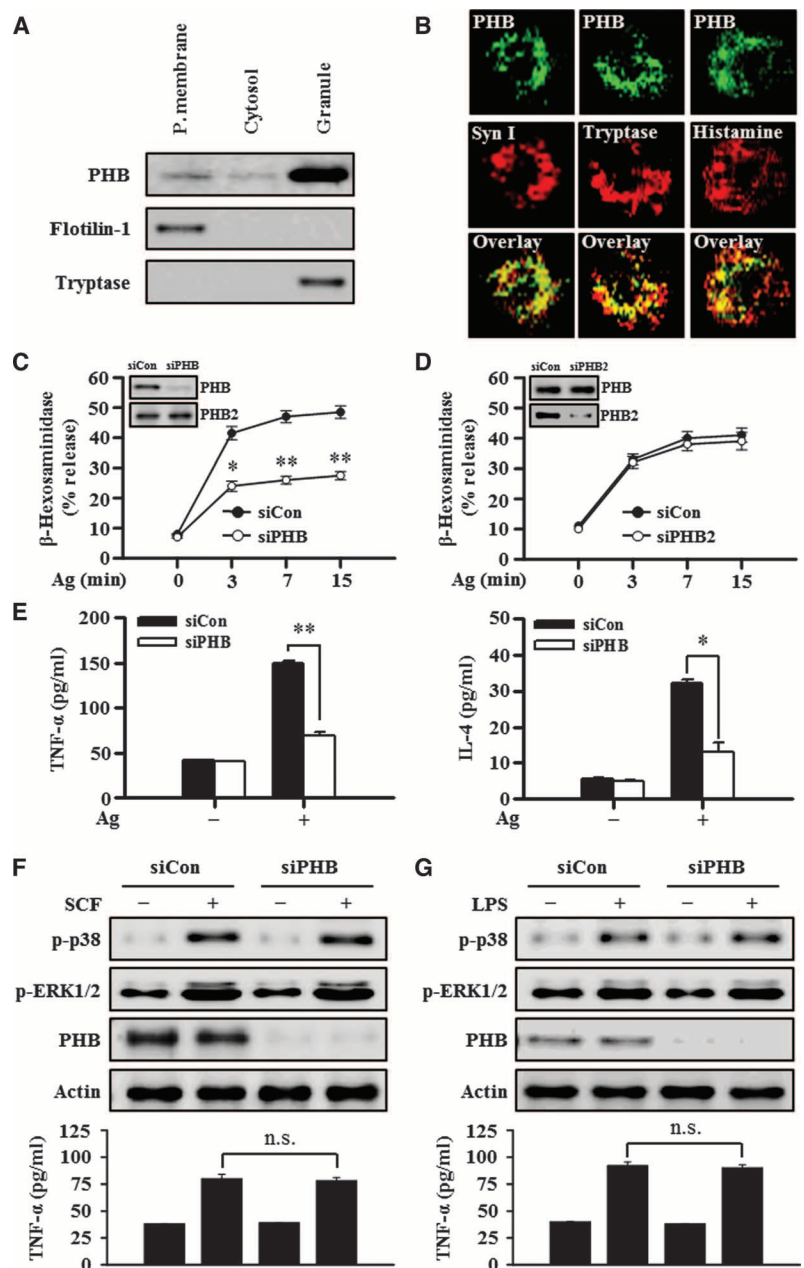
Our analysis of the location of PHB in mast cells by sucrose density gradient centrifugation of mouse bone marrow–derived mast cells (BMMCs)

¹Department of Immunology and Physiology and Functional Genomics Institute, College of Medicine, Konkuk University, Chungju 380-701, Korea. ²Laboratory of Molecular Immunology, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD 20892, USA. ³College of Pharmacy, Duksung Women's University, Seoul 132-714, Korea.

*These authors contributed equally to this work.

†Corresponding author. E-mail: wahnchoi@kku.ac.kr

Fig. 1. Analysis of the location of PHB in mast cells and the effects of PHB knockdown on degranulation and cytokine release in response to antigen, SCF, or LPS. (A) BMMC fractions were separated by sucrose density gradient centrifugation and subjected to Western blotting analysis with antibodies specific for flotillin-1 (a plasma membrane marker), tryptase (a granule marker), and PHB. (B) Confocal microscopy images of the localization of PHB (in green) together with typical granule markers, tryptase, synaptotagmin-1, and histamine (in red). (C to G) BMMCs were transiently transfected with PHB-specific (siPHB), PHB2-specific (siPHB2), or control siRNAs (siCon). Inset: Western blotting analysis of the abundances of PHB and PHB2. (C and D) IgE-sensitized cells were left untreated or were stimulated with antigen (Ag) for the indicated times, and then samples were analyzed to determine the percentage of the granule constituent β -hexosaminidase that was released into the culture medium. (E) IgE-sensitized cells were left untreated or were stimulated with antigen for 6 hours before the amounts of TNF- α and IL-4 secreted into the culture medium were determined by enzyme-linked immunosorbent assay (ELISA). (F and G) BMMCs were left untreated or were stimulated with SCF (100 ng/ml) or LPS (500 ng/ml) for (Top) 3 min before being analyzed by Western blotting to assess the extent of phosphorylation of p38 and ERK1/2, or (Bottom) 6 hours before being analyzed by ELISA to measure the amount of TNF- α secreted by the cells. All of the Western blots and confocal images are representative of three independent experiments. Data in (C) and (D) to (G) are means $\pm$ SEM from three independent experiments. * P < 0.05, ** P < 0.01; n.s., not significant.



indicated that PHB was located largely in granules, with little, if any, PHB present in the plasma membrane or cytosolic fractions (Fig. 1A). This finding was verified by confocal microscopic analysis, which showed that PHB mostly colocalized with typical granule marker proteins, synaptotagmin-1 and tryptase, as well as histamine (Fig. 1B), a classic granule constituent in mast cells.

We next investigated whether PHB regulated the Fc ϵ RI-dependent release of bioactive mediators from BMMCs in experiments in which we transfected cells with small interfering RNAs (siRNAs) specific for PHB or PHB2, which is an isoform of PHB. We achieved selective knockdown of PHB (Fig. 1C, inset) and PHB2 (Fig. 1D, inset) with their respective siRNAs; however, we observed a substantial reduction in the extent of degranulation only in BMMCs transfected with PHB-specific siRNAs (Fig. 1C), and not PHB2-specific siRNAs (Fig. 1D). The antigen-dependent release of interleukin-4 (IL-4) and tumor necrosis factor α (TNF- α) by BMMCs was substantially reduced in cells transfected with PHB-specific siRNAs compared to that in BMMCs transfected with control siRNAs (Fig. 1E). In contrast, knockdown of PHB did not inhibit the release of TNF- α when BMMCs were stimulated by stem cell factor (SCF) or lipopolysaccharide (LPS) (Fig. 1, F and G), which typically stimulate the release of cytokines without inducing degranulation in mast cells (25, 26). Two critical signals necessary for the production of TNF- α , the phosphorylation and activation of (i) p38 MAPK and (ii) ERK1/2, were similarly unaffected by PHB depletion in SCF- or LPS-stimulated cells (Fig. 1, F and G). These results imply that granule-localized PHB was critical for mast cell activation in response to Fc ϵ RI-

mediated signaling, but not SCF or LPS signaling, which are presumed to be mediated by the receptors c-Kit and Toll-like receptor 4, respectively. Therefore, we examined in more detail the effects of PHB on Fc ϵ RI-generated signals.

PHB enhances Fc ϵ RI-mediated activation of Syk and Syk-dependent signaling

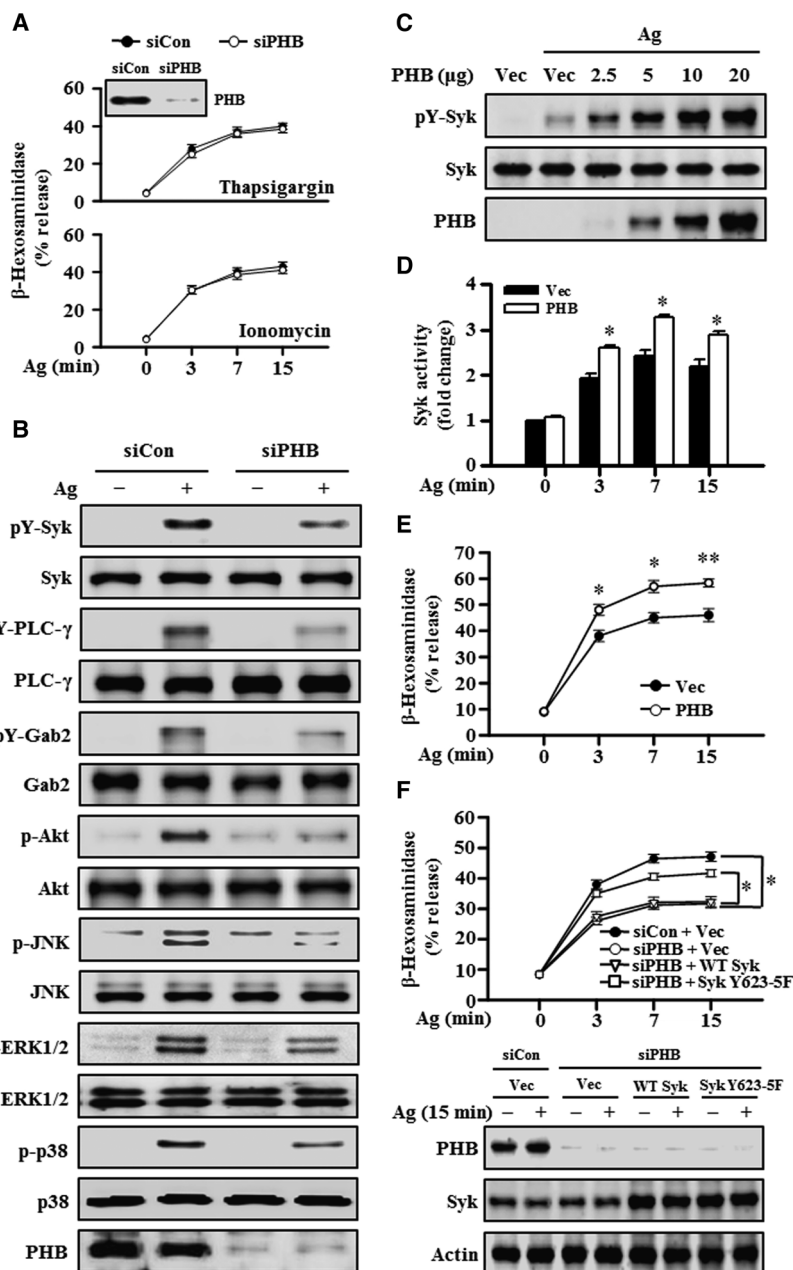
An increase in the concentration of cytosolic Ca^{2+} through the activation of PLC- γ is an essential signal for the degranulation of antigen-stimulated mast cells (27). We found that degranulation was not affected in BMMCs transfected with PHB-specific siRNA that were stimulated with thapsigargin or ionomycin (Fig. 2A), which mobilize Ca^{2+} independently of PLC- γ , which

Fig. 2. FcεRI-mediated activation of Syk and downstream signals depends on PHB. (A and B) BMMCs were transfected with PHB-specific siRNAs (siPHB) or control siRNAs (siCon) 48 hours before each experiment. (A) BMMCs were stimulated for 0, 3, 7, and 15 min with 300 nM thapsigargin or 1 μM ionomycin before the cells were analyzed to determine the amount of β-hexosaminidase that the cells released. Inset: Western blotting analysis of the abundance of PHB. (B) IgE-primed BMMCs were stimulated with antigen (25 ng/ml) for 7 min before being analyzed by Western blotting with antibodies specific for the indicated proteins. (C to E) BMMCs were transfected with empty vector (Vec; 20 μg) or with the indicated amounts of plasmid encoding wild-type PHB (PHB) 24 hours before the following experiments were performed. (C) IgE-sensitized BMMCs that were left untreated or were stimulated with antigen for 7 min were subjected to Western blotting analysis with antibodies specific for the indicated proteins. (D) BMMCs were stimulated with antigen for the indicated times before the cells were lysed and subjected to immunoprecipitation with anti-Syk antibody. The activities of immunoprecipitated Syk were measured by an ELISA-based tyrosine kinase assay kit. (E) The extent of release of β-hexosaminidase was determined after BMMCs were stimulated with antigen for the indicated times. (F) BMMCs were transfected with PHB-specific siRNAs or control siRNAs. Forty-eight hours later, the cells were transfected with 10 μg of empty vector or with 10 μg of plasmid encoding wild-type (WT) Syk or the Syk Y623-625F mutant. Cells were then analyzed to determine the extent of release of β-hexosaminidase. Bottom: Western blotting analysis of the abundances of Syk, PHB, and actin. Data in (A) and (D) to (F) are means ± SEM from three independent experiments. **P* < 0.05, ***P* < 0.01. Western blots in (A), (B), (C), and (F) are representative of three independent experiments.

suggested that PHB acted upstream of the Ca²⁺ signal. Indeed, antigen-stimulated phosphorylation of Syk, PLC-γ, and Gab2 and of downstream signaling molecules, including Akt, ERK1/2, p38, and JNK, was substantially suppressed by knockdown of PHB (Fig. 2B). In contrast, transfection of BMMCs with plasmid encoding PHB resulted in enhanced antigen-dependent phosphorylation (Fig. 2C) and activity (Fig. 2D) of Syk compared to that in vector-transfected BMMCs. An increase in PHB abundance also substantially augmented the extent of antigen-stimulated degranulation of BMMCs transfected with plasmid encoding PHB compared to that of vector-transfected BMMCs (Fig. 2E). Furthermore, transfection of BMMCs with plasmid encoding Syk Y623-625F, a constitutively active mutant of Syk (28), but not with wild-type Syk, restored degranulation lost as a result of PHB-specific siRNAs (Fig. 2F). Collectively, these results suggest that PHB enhances FcεRI-mediated signals before or at the level of Syk in the signaling pathway.

PHB is essential for the activation of Syk, but not Lyn, Fyn, or Fgr

Because the initial events after aggregation of FcεRI by antigen include the activation of SFKs, such as Lyn, Fyn, or Fgr in addition to Syk (29),



we investigated the effects of PHB knockdown on the activation of these kinases in antigen-stimulated cells. We found that siRNA-mediated knockdown of PHB did not impair the activation of Lyn, Fyn, or Fgr in response to antigen stimulation, but did result in the substantial suppression of Syk activation (Fig. 3A), which suggests that PHB specifically acts at the level of Syk.

PHB is critical for the recruitment of Syk to FcεRIγ in antigen-stimulated mast cells

Aggregation of FcεRI molecules stimulates the phosphorylation of ITAMs within FcεRIγ by Lyn, or possibly by another SFK member (30), and thus provides docking sites for Syk. The recruitment of Syk to the phosphorylated ITAMs results in a conformational change in Syk that unlocks

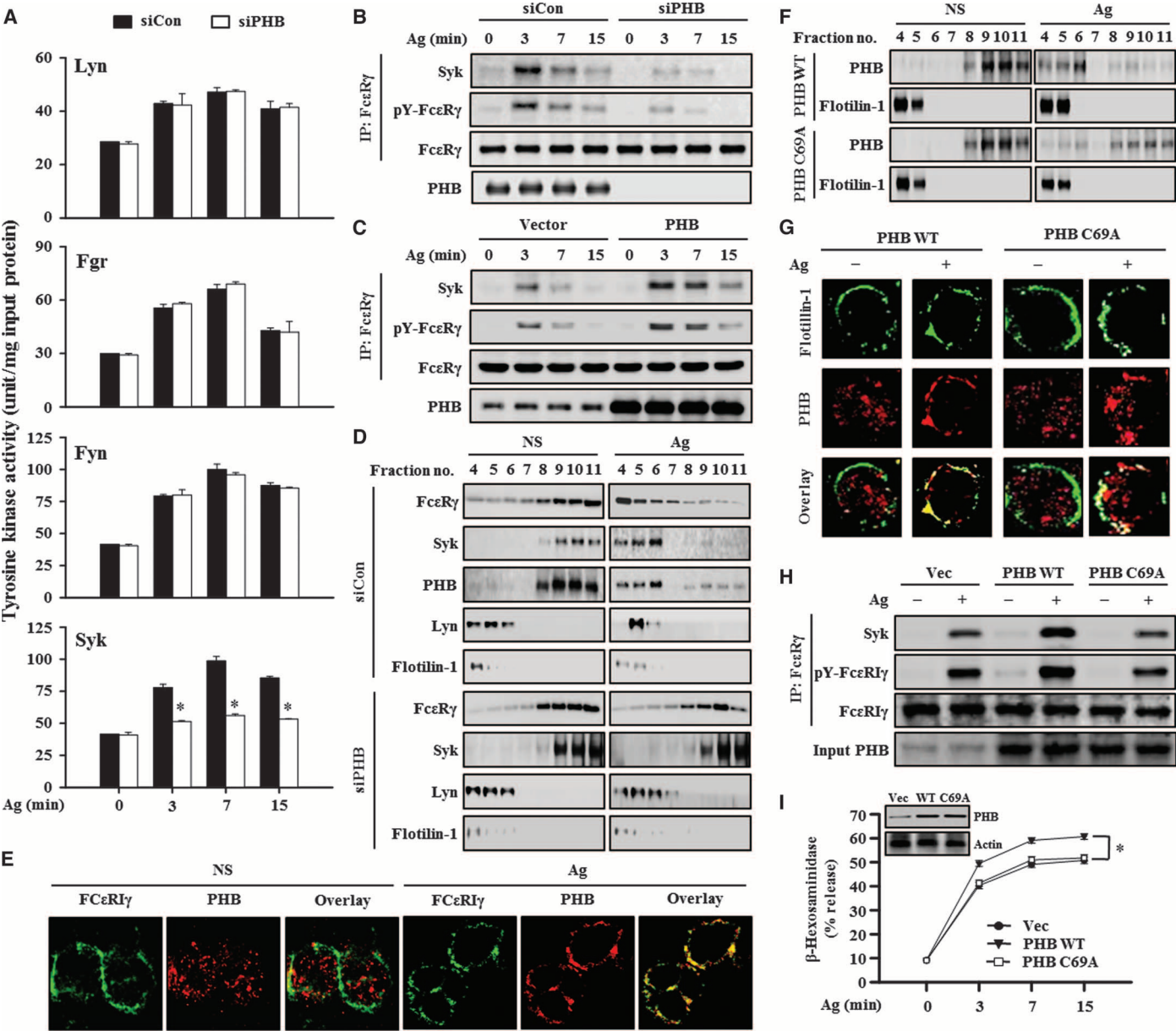


Fig. 3. The translocation of PHB into lipid rafts correlates with the activation of Syk, phosphorylation of FcεRγ, and recruitment of Syk to FcεRγ. (A to D) BMMCs were transfected with PHB-specific siRNAs, plasmid encoding WT PHB, or the appropriate controls before being stimulated with antigen for (A to C) the indicated times or (D) 5 min. (A) Whole-cell lysates (2 mg protein equivalents) were subjected to immunoprecipitation with antibodies against Lyn, Fyn, Fgr, or Syk, and immunoprecipitates were then assayed with an ELISA-based tyrosine kinase assay kit. (B and C) FcεRγ was immunoprecipitated (IP) from whole-cell lysates and then analyzed by Western blotting with antibodies specific for the indicated proteins. (D) Cell lysates from nonstimulated (NS) or antigen-stimulated BMMCs were fractionated by sucrose density gradient centrifugation as described in Materials and Methods. Each fraction was then analyzed by Western blotting with antibodies specific for the indicated proteins. (E) Confocal microscopic analysis of nonstimulated BMMCs and antigen-stimulated BMMCs after 5 min of stimulation with

antigen. Cells were incubated with antibodies specific for FcεRγ (green) and PHB (red). (F to I) BMMCs were transfected with empty vector or with plasmid encoding WT PHB or the PHB C69A mutant before being stimulated with antigen for (F to H) 5 min or (I) the indicated times. (F) Cell lysates were fractionated by sucrose density gradient centrifugation, and each fraction was then analyzed by Western blotting with antibodies specific for PHB or the lipid raft marker flotillin-1. (G) PHB and flotillin-1 were detected by confocal microscopic analysis. (H) FcεRγ was immunoprecipitated from whole-cell lysates, and samples were analyzed by Western blotting with antibodies specific for pY-FcεRγ and Syk. (I) The extent of release of β-hexosaminidase was determined after stimulation of cells with antigen for the indicated times. Inset: Western blotting analysis of the abundance of PHB proteins in transfected cells. Western blots in (B) to (D), (F), (H), and (I) and confocal images in (E) and (G) are representative of three independent experiments. Data in (A) and (I) are means ± SEM from three independent experiments. **P* < 0.05.

its enzymatic activity. Therefore, we hypothesized that the regulatory effect of PHB might be associated with the recruitment of Syk to the phosphorylated ITAMs of FcεRIγ. Indeed, our immunoprecipitation experiments indicated that transfection of BMMCs with PHB-specific siRNAs markedly suppressed the association of Syk with FcεRIγ in antigen-stimulated cells, as well as inhibited the phosphorylation of FcεRIγ (Fig. 3B). Conversely, an increase in PHB abundance in transfected BMMCs enhanced both events in response to antigen stimulation (Fig. 3C).

We next addressed the question of how PHB, which is located largely in granules in unstimulated cells, interacted with Syk and FcεRIγ at the plasma membrane in antigen-stimulated cells. We performed fractionation studies and found that a substantial portion of PHB translocated from the granule fraction into lipid rafts of the plasma membrane (as defined by the marker protein flotillin-1) together with FcεRIγ and Syk after stimulation of BMMCs with antigen (Fig. 3D). Knockdown of PHB with siRNAs markedly suppressed the translocation of FcεRIγ and Syk to the lipid rafts (Fig. 3D). Confocal microscopic analysis confirmed this redistribution of PHB and its enhanced association with FcεRIγ upon stimulation of cells with antigen (Fig. 3E). Cys⁶⁹ of PHB is critical for its translocation to lipid rafts in adipocyte-like 3T3-L1 cells or mouse C2C12 myoblasts (31). In cells transfected with plasmid encoding the PHB C69A mutant, we found that most mutant PHBs failed to translocate to lipid rafts compared with wild-type PHB (Fig. 3F). This failure of the PHB C69A mutant to translocate to lipid rafts was further verified by confocal microscopy (Fig. 3G). Furthermore, in contrast to antigen-stimulated BMMCs transfected with plasmid encoding wild-type PHB, antigen-stimulated BMMCs expressing the PHB C69A mutant did not exhibit enhanced phosphorylation of FcεRIγ, recruitment of Syk to FcεRIγ (Fig. 3H), or degranulation (Fig. 3I) in response to antigen, suggesting that the translocation of PHB to lipid rafts is critical for mast cell degranulation.

Lyn stimulates phosphorylation of tyrosine residues of PHB, leading to the association of PHB with FcεRIγ

PHB is phosphorylated at tyrosine residues in response to insulin (8); however, whether PHB is similarly phosphorylated in antigen-stimulated mast cells is unknown. We investigated this by immunoprecipitation and Western blotting experiments. We found that PHB in BMMCs cells was phosphorylated within 1 min of the addition of antigen, reaching maximal phosphorylation at 3 min, before subsiding at later time points (Fig. 4A). The physical association of PHB and FcεRIγ followed an identical time course (Fig. 4A,

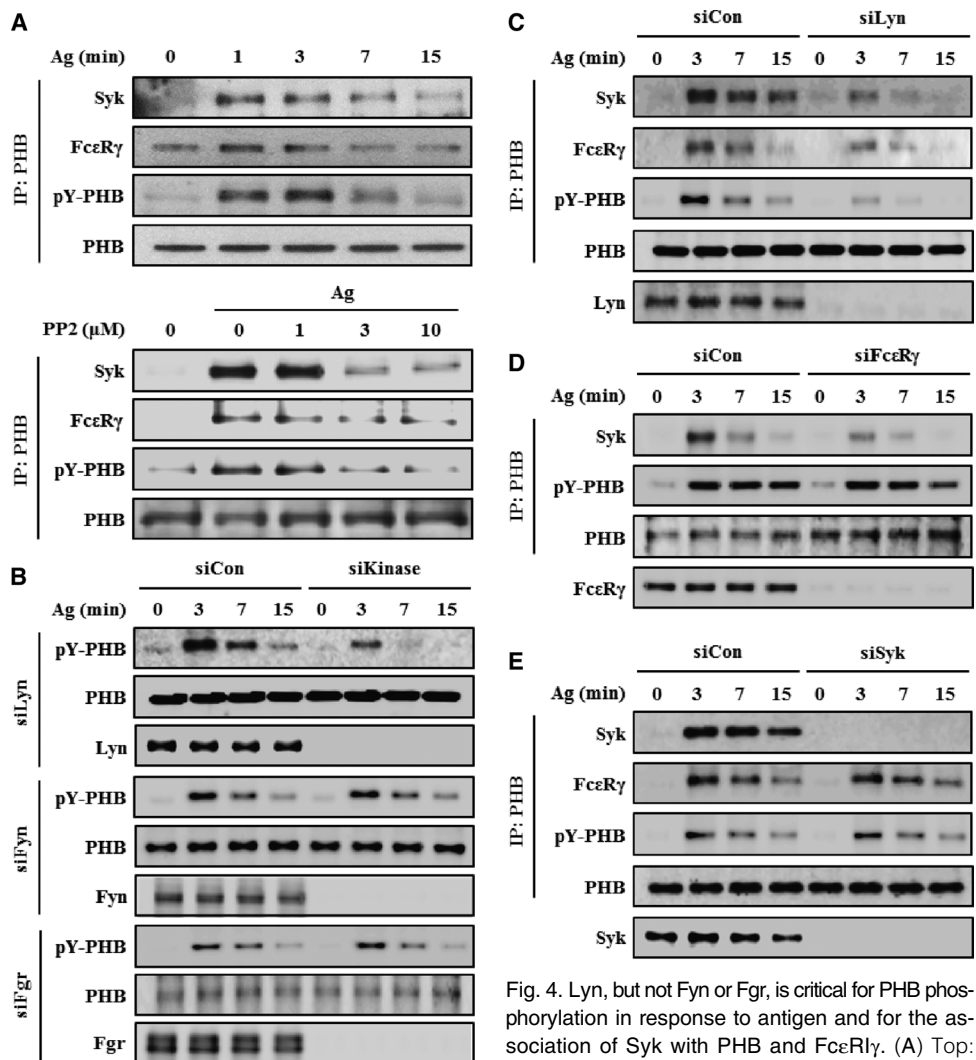


Fig. 4. Lyn, but not Fyn or Fgr, is critical for PHB phosphorylation in response to antigen and for the association of Syk with PHB and FcεRIγ. (A) Top: IgE-primed BMMCs were stimulated with antigen for the indicated times. Bottom: IgE-primed BMMCs were stimulated with antigen for 5 min in the presence or absence of the indicated concentrations of the SFK inhibitor PP2. Whole-cell lysates were subjected to immunoprecipitation with anti-PHB antibody, and samples were analyzed by Western blotting with antibodies specific for the indicated proteins. (B to E) BMMCs were transfected with control siRNAs (siCon) or with the indicated siRNAs, sensitized overnight with IgE, and then stimulated with antigen for the indicated times before being subjected to immunoprecipitation with anti-PHB antibody and analyzed by Western blotting with antibodies specific for the indicated proteins. In (C) to (E), whole-cell lysates were also analyzed by Western blotting to demonstrate effective knockdown of the indicated proteins. All Western blots are representative of three independent experiments.

upper panels), which suggested that this interaction was dependent on the tyrosine phosphorylation of PHB. Moreover, the enhanced association between PHB and FcεRIγ was inhibited by the SFK inhibitor PP2 (Fig. 4A, lower panels), which further suggested that the phosphorylation of PHB was mediated by an FcεRI-proximal SFK.

Although other Src and Tec kinases, such as Hck and Btk, are implicated in intermediate phosphorylation events downstream of FcεRI, the kinases Lyn, Fyn, and Fgr are thought to more closely interact with the receptor (29). Therefore, our studies focused on these three kinases. Of

these, only Lyn appeared to be necessary for the phosphorylation of PHB (Fig. 4B). Because Lyn is critical for the phosphorylation of FcεRIγ and Syk (29), it was notable that Lyn was essential for the association of PHB with FcεRIγ and Syk (Fig. 4C). A further indication of the sequence of events was the finding that whereas in antigen-stimulated cells transfected with FcεRIγ-specific siRNAs, the phosphorylation of PHB was similar to that in cells transfected with control siRNAs, the association between PHB and Syk was impaired (Fig. 4D). Therefore, the phosphorylation of PHB by Lyn was not dependent on the phosphorylation of FcεRIγ or on the association of FcεRIγ with Syk. Similarly, knockdown of Syk by transfecting BMMCs with Syk-specific siRNAs inhibited neither the phosphorylation of PHB nor the association of PHB with FcεRIγ in response to antigen (Fig. 4E). Collectively, these data suggest that the antigen-dependent phosphorylation of PHB by Lyn occurs before the phosphorylation of FcεRIγ and Syk.

Phosphorylation of Tyr¹¹⁴ and Tyr²⁵⁹ of PHB is critical for the association of PHB with FcεRIγ and Syk

To identify which among the five tyrosine residues of PHB were required for its association with FcεRIγ, we prepared mutant PHBs containing individual tyrosine-to-phenylalanine mutations of each of the tyrosines at positions 28, 107, 114, 249, and 259. Only the Tyr¹¹⁴→Phe (Y114F) and Tyr²⁵⁹→Phe (Y259F) mutations impaired the association of PHB with FcεRIγ and Syk, as well as the phosphorylation of Syk, in antigen-stimulated transfected BMMCs (Fig. 5A). Furthermore, expression of the Y114F, Y259F double mutant PHB protein almost completely blocked these events in transfected cells (Fig. 5B), indicating that the phosphorylation of these two tyrosine residues is required for the propagation of FcεRIγ- and Syk-mediated signals. Consistent with this idea, transfection of cells with plasmid encoding wild-type PHB or with plasmids encoding the individual or dual PHB tyrosine mutants respectively enhanced or inhibited antigen-induced degranulation (Fig. 5C). The mutations Y114F, Y259F, and Y114/259F impaired the antigen-induced translocation of the mutant PHB proteins to the plasma membrane (Fig. 5D), but the mutants were still largely observed in the vicinity of plasma membrane.

Knockdown of PHB by siRNAs inhibits passive cutaneous anaphylaxis and passive systemic anaphylaxis reactions in mice

Anaphylaxis is an unusual or exaggerated allergic reaction of an organism to a foreign protein or other substances, and mast cells are one of key effector

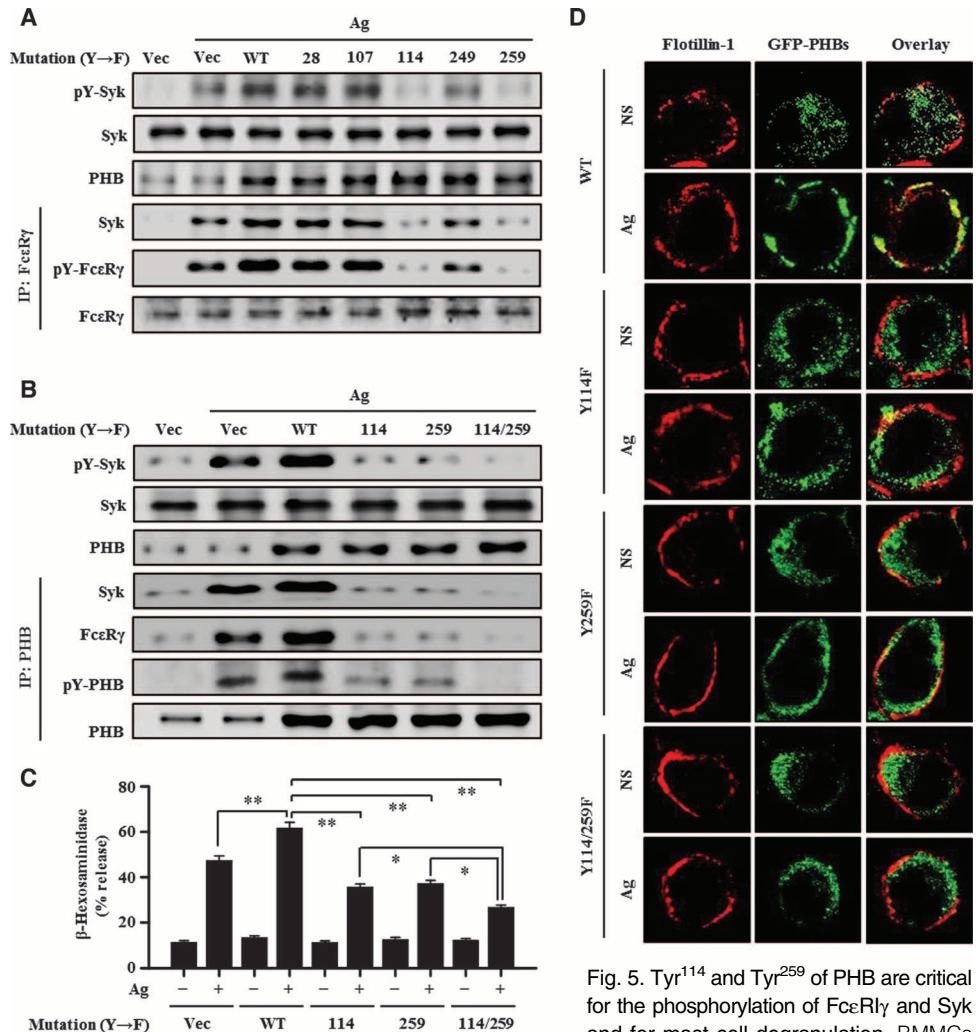


Fig. 5. Tyr¹¹⁴ and Tyr²⁵⁹ of PHB are critical for the phosphorylation of FcεRIγ and Syk and for mast cell degranulation. BMMCs were transfected with 10 μg of empty vector control or with 10 μg of plasmid encoding WT PHB or the indicated Tyr→Phe (Y→F) mutant PHB constructs. Twenty-four hours later, cells were sensitized with IgE and then were left untreated or were stimulated with antigen for 5 min. (A and B) Western blotting analysis was performed on (top) whole-cell lysates and (bottom) samples subjected to immunoprecipitation with antibody specific for (A) FcεRIγ or (B) PHB. Specific antibodies were used to detect the indicated proteins, and an antibody specific for phosphorylated tyrosine residues (4G10) was used to detect tyrosine-phosphorylated proteins. (C) Transfected cells were stimulated with antigen for 15 min before being analyzed to measure the amounts of β-hexosaminidase that were released into the culture medium. Values are means ± SEM from three independent experiments. **P* < 0.05, ***P* < 0.01. (D) Confocal microscopic analysis of BMMCs transfected with plasmids encoding GFP fusions of WT PHB or the indicated PHB mutants that were nonstimulated or were stimulated with antigen for 5 min. Cells were incubated with antibody against flotillin-1. Images in (A), (B), and (D) are representative of three independent experiments. At least 10 cells were imaged for each condition per experiment.

cells of this response (10, 12). We next determined whether PHB had an equally essential role in vivo in experiments in which we induced passive cutaneous anaphylaxis (PCA) or passive systemic anaphylaxis (PSA) in IgE-sensitized mice, given that mast cells are the critical cells in IgE-mediated anaphylaxis in these animals (32). Because genetic loss of PHB in mice leads to embryonic lethality, we used PHB-specific siRNAs to generate PHB-deficient mice. The abundance of PHB, but not of PHB2

or other critical molecules in FcεRI signaling, was reduced in the liver, lung, and ear tissues by the intravenous injection of PHB-specific siRNAs in a dose-dependent manner, with an effective dose of 10 μg per mouse (Fig. 6A). These data indicate that knockdown of PHB by siRNAs was as specific in vivo as it was in BMMCs in vitro.

In the PCA model, we found that injection of PHB-specific siRNAs inhibited the extravasation of Evans blue dye (Fig. 6, B and C) as well as the recruitment and degranulation of mast cells into the antigen-challenged ear tissue (Fig. 6, D and E). We assessed PSA by measurement of rectal temperature, which typically declines after systemic administration of antigen in IgE-sensitized mice. This reduction in temperature was diminished by previous injection of PHB-specific siRNAs (Fig. 6F). In addition, the serum concentrations of histamine and TNF-α were lower in mice treated with PHB-specific siRNA than in mice treated with control siRNA (Fig. 6G). Together, these data suggest that PHB is critical for the activation of mast cells and for IgE-mediated anaphylaxis in mice.

DISCUSSION

PHB is an evolutionarily conserved, ubiquitously expressed, multifunctional protein that serves to maintain tissue homeostasis and suppress inappropriate cell proliferation by regulating cellular senescence, cell cycle, apoptosis, and cellular metabolism (2, 33, 34). These processes may be regulated in part by PHB acting as a scaffold protein upstream of the

PI3K-Akt and Raf-ERK pathways through its interaction with the receptors for insulin, insulin-like growth factor 1 (IGF-1), epidermal growth factor (EGF), and transforming growth factor-β (TGF-β) at the plasma membrane (2). This regulation involves multiple phosphorylation of PHB and its translocation to different cellular components in ways that are not clearly understood. PHB is observed in various intracellular compartments, such as mitochondria, as well as in the cytosol and at the plasma membrane (1, 2, 31). Here, we found that PHB translocated from mast cell granules (Fig. 1, A and B) to lipid raft microdomains of the plasma membrane (Fig. 3D) where it associated with FcεRI (Fig. 3E) upon stimulation of BMMCs with antigen. Furthermore, we found that PHB was also essential for the translocation of FcεRIγ into lipid rafts and for its phosphorylation. The phosphorylation of FcεRIγ enabled the activation of Syk, a pivotal protein tyrosine kinase that mediates FcεRI-dependent mast cell activation and anaphylaxis. Palmitoylation of PHB at Cys⁶⁹ facilitates its membrane translocation for its function (31). We found that the translocation of PHB to lipid rafts in the plasma membrane of mast cells was largely suppressed when Cys⁶⁹ of PHB was mutated to alanine (Fig. 3, F and G). The PHB C69A mutant protein failed to enhance the phosphorylation of FcεRIγ, the recruitment of Syk to FcεRIγ (Fig. 3H), and the degranulation of mast cells in response to antigen stimulation (Fig. 3I). Collectively, these results suggest that the translocation of PHB from intracellular granules to lipid rafts in the plasma membrane is essential for receptor-associated, early signaling events and mast cell activation.

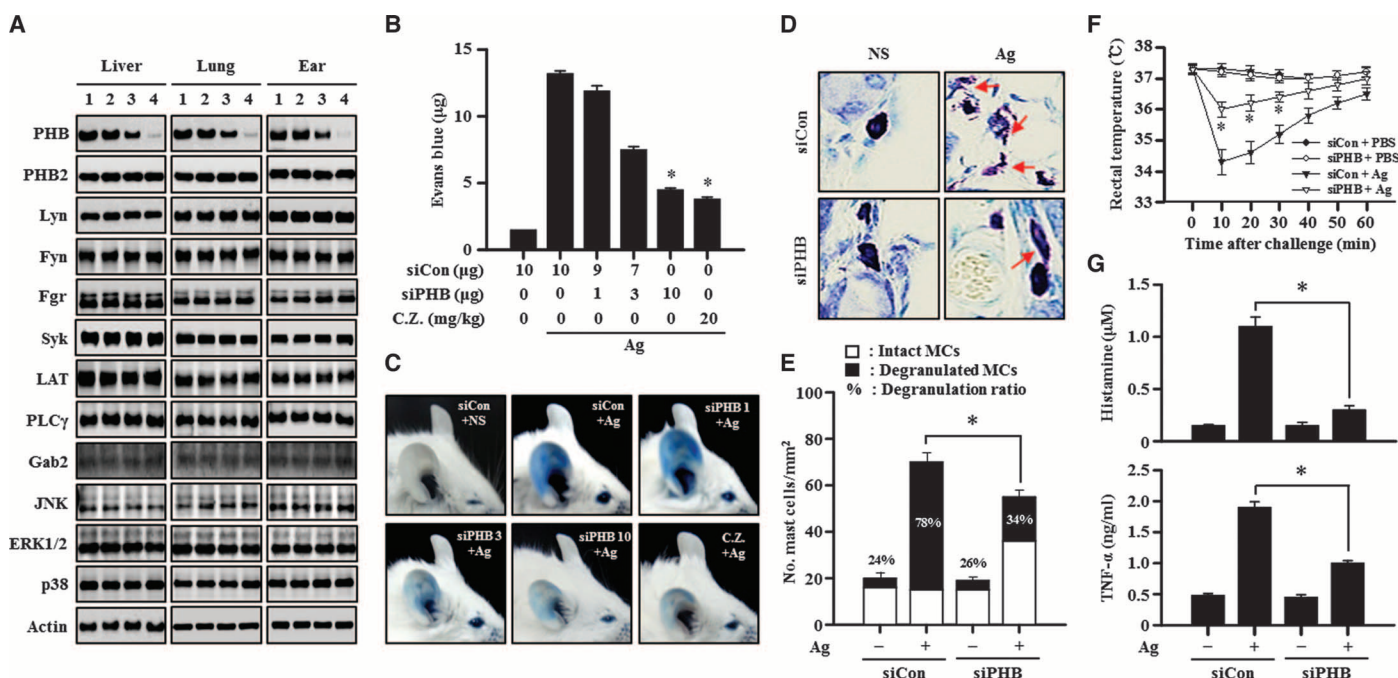


Fig. 6. PHB is critical for IgE-FcεRI-mediated PCA and PSA anaphylaxis in mice. PCA and PSA were induced in mice after they were injected with siRNAs as described in Materials and Methods. (A) Western blotting analysis of extracts from the liver, lungs, and ear of mice that received the following combinations of siRNAs: 1, control siRNAs (siCON, 10 μg); 2, PHB-specific siRNAs (siPHB, 1 μg) and control siRNAs (9 μg); 3, PHB-specific siRNAs (3 μg) and control siRNAs (7 μg); 4, PHB-specific siRNAs (10 μg). (B) Measurement of the amounts of Evans blue dye extracted from IgE-injected ears. Five mice from each group were analyzed. The antihistamine cetirizine (C.Z.) was orally administered 1 hour before the mice were injected with antigen, as a control.

(C) Representative photographs of ears in the PCA-induced mice analyzed in (B). (D) Control or PHB-specific siRNAs (10 μg) were administered to mice as described earlier, and ear tissues were examined histologically after staining with toluidine blue (magnification, ×100). Red arrows indicate degranulated mast cells. (E) Histograms indicating the percentages of the total number of mast cells that were degranulated in individual tissue sections. (F) Rectal temperatures of mice after challenge with antigen for PSA. (G) Serum concentrations of TNF-α and histamine 1 hour after the mice analyzed in (F) were challenged with antigen. Data in (C) and (E) to (G) are means ± SEM from five mice per group. **P* < 0.05.

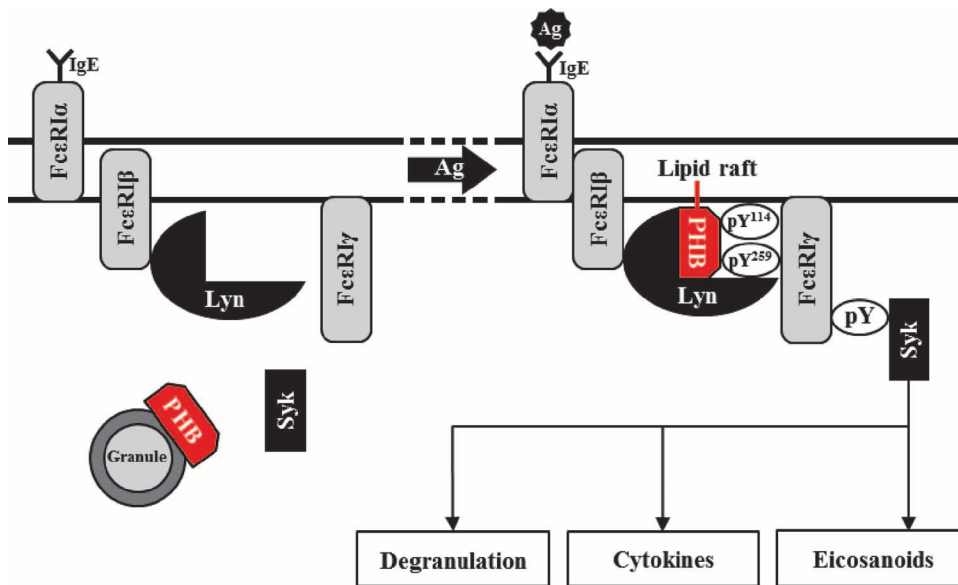


Fig. 7. Suggested model for the interactions of PHB as it acts as an adaptor molecule in the initial FcεRI-mediated signaling events in mast cells. After aggregation of FcεRIs by antigen, Lyn is thought to phosphorylate ITAMs in the β and γ subunits of FcεRI, and the phosphorylated FcεRI ITAMs then provide the docking sites for Syk, which promotes its optimal activation and the subsequent activation of mast cells. On the basis of our results, we propose that Lyn also phosphorylates PHB, which, when phosphorylated, enables the recruitment of FcεRIγ into membrane lipid rafts to form a signaling complex consisting of PHB, FcεRIγ, and Syk. Formation of this complex results in the activation of Syk and of the downstream signals that are necessary for mast cell activation and degranulation.

FcεRI lacks any intrinsic tyrosine kinase activity, and it relies on non-receptor protein tyrosine kinases for cellular activation. The phosphorylation of ITAMs within the FcεRIγ subunit by Lyn (30), or possibly by other SFK members, such as Fyn or Fgr (24, 35), provides a binding site for Syk. Such binding stimulates a conformational change within Syk that increases its enzymatic activity (36). The translocation of PHB to the plasma membrane was likely necessary for the phosphorylation of FcεRIγ and the subsequent binding of Syk to FcεRIγ in lipid rafts (Fig. 3H). Our results suggest that, as with other receptors, PHB at lipid rafts acts as a scaffold protein to facilitate the phosphorylation of FcεRIγ by Lyn.

Lipid rafts are plasma membrane microdomains that are enriched in cholesterol, glycosphingolipid, and sphingolipids. Lipid rafts are insoluble in nonionic detergents (such as Triton X-100), and they contain numerous signaling molecules, such as glycosylphosphatidylinositol-anchored proteins, heterotrimeric guanine nucleotide-binding proteins (G proteins), and SFKs (37, 38). It is generally accepted that lipid rafts function as focal points for the consolidation of receptor signaling components. Our findings fit with this concept, in that PHB, together with FcεRIγ and Syk, translocated into the lipid rafts after mast cells were stimulated with antigen (Fig. 3D). Furthermore, knockdown of PHB resulted in a marked reduction in the amount of FcεRIγ and Syk that translocated to the lipid rafts of the plasma membrane (Fig. 3D). Also, as noted earlier, the incomplete translocation of the PHB C69A mutant to lipid rafts (Fig. 3, F and G) was consistent with the lack of effect of overexpression of the PHB C69A mutant on the phosphorylation of FcεRIγ and the recruitment of Syk to phosphorylated FcεRIγ (Fig. 3H). Therefore, we suggest that PHB serves as a scaffold molecule for the recruitment of FcεRIγ and Syk into lipid rafts to facilitate FcεRI-dependent signaling pathways required for mast cell activation.

The opposing effects of the knockdown and overexpression of PHB in mast cells provided further details on the interactions between PHB, FcεRI, and Syk. That PHB was required for the phosphorylation of FcεRIγ, the association of FcεRIγ with Syk, and the activation of Syk was exemplified by the inhibition of these events in cells in which PHB was knocked down (Fig. 3, A and B), as well as by their enhancement in cells transfected with plasmid encoding PHB (Fig. 3C). Other data suggest the formation of a ternary complex containing PHB, FcεRIγ, and Syk in response to antigen stimulation (Fig. 4A). Point mutation of individual tyrosine residues in PHB also suggested that tyrosine phosphorylation of PHB was a necessary step for complex formation because mutation of either Tyr¹¹⁴ or Tyr²⁵⁹ of PHB prevented its association with FcεRIγ, the phosphorylation of FcεRIγ and Syk, the activation of Syk, as well as mast cell activation (Fig. 5, B and C). Similarly to FcεRIγ (29), PHB was phosphorylated by Lyn (Fig. 4B), and therefore, tyrosine phosphorylation of both molecules by Lyn appears to be necessary for their interaction and for mast cell activation. It is likely that the association of PHB with FcεRIγ occurs before Syk interacts with FcεRIγ because treatment of cells with Syk-specific siRNAs inhibited neither the phosphorylation of PHB nor the association of PHB with FcεRIγ (Fig. 4E). This finding and the fact that the mutation of Tyr¹¹⁴ or Tyr²⁵⁹ of PHB disrupted the recruitment of Syk by FcεRIγ (Fig. 5B) suggest that the prior association of PHB with FcεRIγ is critical for the subsequent recruitment of Syk and for mast cell activation.

Several SFK members, including Lyn, Fyn, and Fgr, are highly localized in the vicinity of FcεRI molecules on the plasma membrane, and they have distinct signaling functions in mast cells. Initially, FcεRI-associated Lyn transphosphorylates tyrosine residues in the ITAMs of the adjacent β and γ chains of FcεRI, and phosphorylated FcεRIγ recruits Syk, leading to its activation (29). Fyn also initiates signals that complement those of Lyn (39). Although the phosphorylation of FcεRIγ is not impaired in Fyn-deficient mast cells, Fyn can phosphorylate Syk (24, 35). Fgr is thought to be critical for the FcεRI-dependent phosphorylation of Syk (35). Here, we identified PHB as an additional tyrosine kinase target because it was phosphorylated at tyrosine residues in a time-dependent manner, which was inhibited by the general SFK inhibitor PP2 (Fig. 4A). PHB was phosphorylated by Lyn, but not by Fyn or Fgr (Fig. 4B), indicating that Lyn phosphorylates PHB as well as the ITAMs of the β and γ subunits of FcεRI.

Of the five tyrosine residues in PHB (Tyr²⁸, Tyr¹⁰⁷, Tyr¹¹⁴, Tyr²⁴⁹, and Tyr²⁵⁹), only Tyr¹¹⁴ and Tyr²⁵⁹ appeared to be critical for the formation of a PHB-FcεRIγ-Syk signaling complex (Fig. 5A) because mutation of either of these sites resulted in a substantial reduction in the extent of phosphorylation of FcεRIγ and Syk and in the association between FcεRIγ and Syk (Fig. 5B). The double point mutation of Tyr¹¹⁴ and Tyr²⁵⁹ augmented these effects (Fig. 5B). Note that degranulation was also substantially suppressed by overexpression of either of the single tyrosine PHB mutants, and more so by expression of the double mutant PHB (Fig. 5C). The exact mechanism of the dominant-negative effect of PHBs mutated

at Tyr¹¹⁴ or Tyr²⁵⁹ is unknown, but possible explanations are that there is competition between mutant and endogenous PHB in binding to the upstream tyrosine kinase Lyn at the plasma membrane, and that the translocation of additional endogenous PHBs to the plasma membrane in response to antigen might be suppressed by PHB mutants that inhibit downstream signaling (Fig. 2B).

In summary, previous studies have shown that after aggregation of FcεRI by antigen in mast cells, Lyn or other SFKs phosphorylate FcεRIγ to provide docking sites for Syk, thus enabling full activation of Syk. Although a role for PHB in this process was unsuspected, we found evidence that PHB was essential for the phosphorylation of FcεRIγ and the recruitment of Syk to FcεRIγ (Fig. 3, B and C). However, knockdown of FcεRIγ had no effect on the phosphorylation of PHB by Lyn, and knockdown of Syk had no effect on the association of PHB with FcεRIγ. These findings suggest that the possible sequence of signaling events might be as follows: First, FcεRI molecules on the mast cell surface are aggregated by antigen. PHB is then phosphorylated by Lyn, which leads to the formation of an initial signaling complex that contains PHB, FcεRIγ, and Lyn. The phosphorylation of FcεRIγ by Lyn leads to the recruitment of Syk to phosphorylated FcεRIγ, the optimal activation of Syk, and the activation of downstream signals for degranulation and cytokine release (Fig. 7). We propose that PHB acts as an adaptor or scaffold protein for the FcεRI-mediated signals required for mast cell activation. The mechanism of the translocation of PHB from intracellular granules to the plasma membrane remains unclear, although we note that some PHB is already associated with FcεRIγ at the cell periphery in resting cells (Fig. 3E). The fusion of granules with the plasma membrane may further facilitate the formation of PHB-FcεRIγ complexes and thus amplify signaling. Our findings add additional insight into proximal FcεRI signaling events and reveal a previously uncharacterized role for PHB after its translocation from intracellular granules to the plasma membrane in mast cells.

MATERIALS AND METHODS

Reagents

Antibodies against phosphotyrosine (pY) residues, FcRγ, and histamine were obtained from Upstate Biotechnology; antibodies against Lyn, Fyn, Fgr, synaptotagmin-1, flotillin-1, Syk, tryptase, and PHB were from Santa Cruz Biotechnology Inc.; and antibodies against phosphorylated forms of Syk, Gab2, PLC-γ1, ERK1/2, p38, JNK, and Akt were purchased from Cell Signaling Technology Inc. Cell culture reagents were from GIBCO/Invitrogen. Dinitrophenyl (DNP)-specific monoclonal IgE and DNP-bovine serum albumin (BSA) were obtained from Sigma. PP2 was from Calbiochem. siGENOME ON-TARGETplus SMARTpool including four specific siRNAs for PHB, Lyn, Fyn, or Fgr and the ON-TARGETplus siCONTROLpool were purchased from Dharmacon Inc.

Culture and stimulation of BMMCs

BMMCs were isolated from male BALB/cJ mice as previously described (40) and were cultured for 4 weeks in 50% enriched medium (RPMI 1640 containing 2 mM L-glutamine, 0.1 mM nonessential amino acids, antibiotics, and 10% BSA) containing IL-3 (10 ng/ml). For each experiment, BMMCs (2×10^5 cells per 1.5-ml tube) were sensitized (or “primed”) with DNP-specific IgE (50 ng/ml) in complete medium overnight, washed twice in phosphate-buffered saline (PBS), resuspended in Tyrode-BSA buffer [20 mM Hepes (pH 7.4), 135 mM NaCl, 5 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5.6 mM glucose, and 0.05% BSA], and then left untreated or stimulated with the antigen DNP-BSA (25 ng/ml) for 10 min, or as indicated otherwise in the figure legends.

Measurement of degranulation and the release of TNF-α and IL-4

Cells were stimulated as described earlier, and the extent of degranulation was determined by measuring the amount of the granule marker β-hexosaminidase that was released into the culture medium (41). The amounts of TNF-α and IL-4 released into the medium 8 hours after cells were stimulated with antigen were measured with specific ELISA kits (Invitrogen-Biosource Cytokine & Signaling) according to the manufacturer's instructions.

Immunoprecipitation and Western blotting analysis

Cells that were sensitized with IgE, as described earlier, were stimulated with antigen for 7 min, or as indicated in the figure legends, washed twice with ice-cold PBS, and then lysed with ice-cold lysis buffer (35). Lysates were kept on ice for 30 min and then centrifuged at 13,000g for 10 min at 4°C. Equal amounts of protein were used for the immunoprecipitation of PHB or FcεRIγ by overnight incubation (at 4°C with gentle rocking) with specific antibodies and, in turn, protein G-agarose. The agarose beads were washed five times with washing buffer (35), resuspended in 2× Laemmli buffer, and then boiled for 5 min. For Western blotting analysis, equal volumes of supernatants were separated by SDS-polyacrylamide gel electrophoresis and then transferred onto polyvinylidene difluoride membranes (Millipore). The membranes were blocked with 5% BSA in tris-buffered saline-Tween 20 (TBS-T) for 1 hour and then incubated with primary antibodies at 4°C overnight. Immunoreactive proteins were detected with horseradish peroxidase-conjugated secondary antibodies.

Transfection of BMMCs with plasmids or siRNAs

BMMCs (5×10^6) were transfected with 100 nM siRNAs against specific targets or with 100 nM control siRNAs with an Amaxa nucleofactor (Lonza Cologne AG) and program T-5 in Dulbecco's modified Eagle's medium containing 20% fetal bovine serum and 50 mM Hepes (pH 7.5). Cells were harvested 48 hours after transfection, and successful gene knockdown was confirmed by Western blotting analysis. To overexpress proteins of interest, BMMCs were transfected with DNA plasmids (10 μg per 5×10^6 cells) with the Amaxa nucleofactor, and cells were used within 48 hours of transfection.

Confocal microscopy

BMMCs were fixed with 4% formaldehyde in PBS for 10 min, washed, and permeabilized with 0.5% Triton X-100 for 15 min. The fixed cells were incubated with 1% BSA in PBS for 60 min and then were incubated overnight with gentle rocking at 4°C with antibodies against PHB, synaptotagmin-1, tryptase, or histamine. Cells were washed five times with 1% BSA and then incubated for 50 min with Rhodamine Red-conjugated goat anti-mouse IgG to detect tryptase, histamine, or synaptotagmin-1, or with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG to detect PHB (Molecular Probes). After coverslips were washed with PBS and mounted with ProLonged Antifade Kit (Molecular Probes), confocal images were obtained with an Olympus FV-1000 confocal laser scanning microscope with an Apochromat 60× objective lens.

Isolation of cellular fractions containing granules, cytosol, or plasma membrane

BMMCs (2×10^7) were sonicated for 15 s in 0.25 M sucrose in 20 mM tris-Hepes (pH 7.4). A stepwise sucrose gradient was created by the sequential addition to centrifuge tubes of 1.6 ml of 40%, 0.8 ml of 15%, and 1.3 ml of 11.5% (w/v) sucrose solution, and finally the sonicated cell lysate. After ultracentrifugation at 27,000g for 2 hours at 4°C, the different fractions were collected, including the cytosolic fraction (top layer), the plasma

membrane fraction (middle opaque fraction), and the granules (pellet at the bottom).

Separation of lipid rafts from the granule fraction

After 5 min of stimulation with antigen, BMMCs (1.5×10^7) were washed twice with ice-cold PBS before being lysed in 2 ml of TNE buffer [50 mM tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 50 mM NaF, 2.5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, leupeptin (1 μ g/ml), and aprotinin (1 μ g/ml)] containing 0.5% Brij 58 for 30 min on ice. Lysates were mixed with an equal volume of 80% (w/v) sucrose in TNE buffer. A sucrose gradient was prepared by the sequential addition of 1.5 ml of 80% sucrose, 2 ml of a 1:1 mixture of 80% sucrose and cell lysate, 4 ml of 35% sucrose, and finally 4 ml of 5% sucrose, which was processed by ultracentrifugation at 27,000g for 18 hours at 4°C. One-milliliter fractions (for a total of 12 fractions) were collected from the top to the bottom of the gradient for Western blot analysis.

In vitro kinase assay

Immunoprecipitates of Lyn, Fyn, Fgr, or Syk from whole-cell lysates (1 mg of protein) were assayed for tyrosine kinase activity with an ELISA-based Universal Tyrosine Kinase Assay Kit (Genway) according to the manufacturer's instructions.

Generation of PHB mutants

Nucleotides encoding tyrosine residues in the complementary DNA (cDNA) encoding mouse PHB in the plasmid pCMV6-XL-4 (Origene Technologies) or pEGFP-N1 (Clontech Laboratories Inc.) were mutated with the QuikChange Site-Directed Mutagenesis Kit (Stratagene) with the following primers: PHB Y28F, 5'-GTGAACCTCTGCTTTGTGTTAATGTGGATGCTGGA-3' (forward) and 5'-TCCAGCATCCACATTAACAAAGCAGAGTTTCAC-3' (reverse); PHB Y107F, 5'-CAGCTTCCTCGTATCTTCACAGCATTGGCGAG-3' (forward) and 5'-CTCGCCAATGCTGGTGAAGATACGAGGAAGCTG-3' (reverse); PHB Y114F, 5'-AGCAITGGCGAGGACTTTGATGAGCGGGTCTCTG-3' (forward) and 5'-CAGGACCCGCTCATCAAAGTCTCGCCAATGCTF-3' (reverse); PHB Y249F, 5'-GCCGAGGACATTGCGTTCAGCTCTCCCGCTCT-3' (forward) and 5'-AGAGCGGGAGAGCTGGAACGCAATGTCTCGGC-3' (reverse); PHB Y259F, 5'-TCTCGGAACATCACCTTCTGCCAGCGGGCCAG-3' (forward) and 5'-CTGGCCCCGTGGCAGGAAGGTGATGTTCCGAGA-3' (reverse); PHB Y114/259F, 5'-CTCTCGGAACATCACCTCCCTG-CCAGCGGGCCAG-3' (forward) and 5'-CTGGCCCCGTGGCAGGAGGTGATGTTCCGAGAG-3' (reverse); PHB C69A, 5'-GAAACCAATTATCTTTGACGCCCGCTCTCGACCACGGAAC-3' (forward) and 5'-GTTCCGTGGTTCGAGAGCGGGCGTCAAAGATAATTGGTTTC-3' (reverse). To make the gain-of-function rat Syk Y623-5F mutant, the cDNA encoding rat Syk in pCMV was mutated with the following primers: forward, 5'-GCGGCGCGCCGAATTCATGGCGGGCAATGCTGTG-3', and reverse, 5'-CGAGGGATACCTAGATTAGTTAACCACGTGCAAGAAGAAATTGCGAAGCCGACTGATTC-3'. The DNA sequence of each plasmid was verified by sequencing.

Knockdown of PHB in mice by siRNAs

BALB/c mice (five mice per group) under mild anesthesia were injected once a day for 3 days in the caudal veins with PHB-specific siRNAs or with control siRNAs (10 μ g in 200 μ l of PBS). Knockdown of PHB was confirmed by Western blotting analysis before each experiment.

Generation of mouse models of PCA and PSA

PCA was induced as described previously (35) by intradermal injection of DNP-specific IgE (0.5 μ g per mouse) into the right ear 6 hours after the

final injection of the siRNAs, as described earlier. The next day, 250 μ g of antigen in 250 μ l of PBS containing 4% Evans blue was injected into the caudal veins of the mice. The mice were euthanized 1 hour later, and the IgE-treated ears were removed to extract extravasated dye by overnight incubation in 700 μ l of formamide at 63°C. The absorbance of the extracted dye was measured at 620 nm. For examination of PSA, siRNA-treated mice were injected into the caudal vein with DNP-specific IgE (200 μ g in 200 μ l of saline per mouse); control mice were injected with saline. The following day, the mice were injected intravenously with 250 μ g of antigen in 250 μ l of PBS, and rectal temperatures were monitored every 10 min with a digital thermometer (Natsume Seisakusyo Co. Ltd.). Sera were collected 60 min after injection of the mice with antigen. Serum concentrations of TNF- α and histamine were measured with ELISA kits from R&D Systems and IBL international, respectively.

Histological analysis

After PCA was induced, ear tissues were fixed in 4% paraformaldehyde in PBS for 24 hours, and 5- μ m paraffin sections were stained with 0.1% toluidine blue and examined with an optical microscope (Olympus DP 70) at $\times 100$ magnification. Mast cells in the ear tissue were counted in three mice (three ear sections per mouse, for a total of nine sections). The percentage of degranulated mast cells in the ear tissue was calculated by assessing the number of cells with >10% extrusion of granules, as previously described (42).

Statistical analysis

The data presented are means $\pm$ SEM from three or more independent experiments. Statistical analysis was performed with one-way analysis of variance (ANOVA) and Dunnett's test. Statistical significance ($*P < 0.05$ and $**P < 0.01$) was determined with SigmaStat software (Systat Software Inc.).

REFERENCES AND NOTES

1. N. Patel, S. K. Chatterjee, V. Vrbanc, I. Chung, C. J. Mu, R. R. Olsen, C. Waghorne, B. R. Zetter, Rescue of paclitaxel sensitivity by repression of Prohibitin1 in drug-resistant cancer cells. *Proc. Natl. Acad. Sci. U.S.A.* **107**, 2503–2508 (2010).
2. S. Mishra, L. C. Murphy, L. J. Murphy, The Prohibitins: Emerging roles in diverse functions. *J. Cell. Mol. Med.* **10**, 353–363 (2006).
3. M. G. Kolonin, P. K. Saha, L. Chan, R. Pasquallini, W. Arap, Reversal of obesity by targeted ablation of adipose tissue. *Nat. Med.* **10**, 625–632 (2004).
4. M. Terashima, K. M. Kim, T. Adachi, P. J. Nielsen, M. Reth, G. Köhler, M. C. Lamers, The IgM antigen receptor of B lymphocytes is associated with prohibitin and a prohibitin-related protein. *EMBO J.* **13**, 3782–3792 (1994).
5. S. Mishra, S. R. Ande, B. L. Nyomba, The role of prohibitin in cell signaling. *FEBS J.* **277**, 3937–3946 (2010).
6. I. C. Morrow, R. G. Parton, Flotillins and the PHB domain protein family: Rafts, worms and anaesthetics. *Traffic* **6**, 725–740 (2005).
7. K. Rajalingam, C. Wunder, V. Brinkmann, Y. Churin, M. Hekman, C. Sievers, U. R. Rapp, T. Rudel, Prohibitin is required for Ras-induced Raf-MEK-ERK activation and epithelial cell migration. *Nat. Cell Biol.* **8**, 837–843 (2005).
8. S. R. Ande, Y. Gu, B. L. Nyomba, S. Mishra, Insulin induced phosphorylation of prohibitin at tyrosine 114 recruits Shp1. *Biochim. Biophys. Acta* **1793**, 1372–1378 (2009).
9. S. R. Ande, S. Mishra, Prohibitin interacts with phosphatidylinositol 3,4,5-trisphosphate (PIP3) and modulates insulin signaling. *Biochem. Biophys. Res. Commun.* **390**, 1023–1028 (2009).
10. M. Metz, M. A. Grimbaldston, S. Nakae, A. M. Piliponsky, M. T. Sai, S. J. Galli, Mast cells in the promotion and limitation of chronic inflammation. *Immunol. Rev.* **217**, 304–328 (2007).
11. S. J. Galli, M. Tsai, A. M. Piliponsky, The development of allergic inflammation. *Nature* **454**, 445–454 (2008).
12. T. Kawakami, T. Ando, M. Kimura, B. S. Wilson, Y. Kawakami, Mast cells in atopic dermatitis. *Curr. Opin. Immunol.* **21**, 666–678 (2009).
13. A. M. Gilfillan, M. A. Beaven, Regulation of mast cell responses in health and disease. *Crit. Rev. Immunol.* **31**, 475–530 (2011).

14. E. Eiseman, J. B. Bolen, Engagement of the high-affinity IgE receptor activates *src* protein-related tyrosine kinases. *Nature* **355**, 78–80 (1992).
15. J. E. Hutchcroft, R. L. Geahlen, G. G. Deanin, J. M. Oliver, FcεRI-mediated tyrosine phosphorylation and activation of the 72-kDa protein-tyrosine kinase, PTK72, in RBL-2H3 rat tumor mast cells. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 9107–9111 (1992).
16. M. H. Jouvin, M. Adamczewski, R. Numerof, O. Letourneur, A. Vallé, J. P. Kinet, Differential control of the tyrosine kinases Lyn and Syk by the two signaling chains of the high affinity immunoglobulin E receptor. *J. Biol. Chem.* **269**, 5918–5925 (1994).
17. A. M. Gilfillan, C. Tkaczyk, Integrated signalling pathways for mast-cell activation. *Nat. Rev. Immunol.* **6**, 218–230 (2006).
18. J. Rivera, A. M. Gilfillan, Molecular regulation of mast cell activation. *J. Allergy Clin. Immunol.* **117**, 1214–1225 (2006).
19. S. Saitoh, R. Arudchandran, T. S. Manetz, W. Zhang, C. L. Sommers, P. E. Love, J. Rivera, L. E. Samelson, LAT is essential for FcεRI-mediated mast cell activation. *Immunity* **12**, 525–535 (2000).
20. H. Turner, J. P. Kinet, Signalling through the high-affinity IgE receptor FcεRI. *Nature* **402**, B24–B30 (1999).
21. W. Li, G. G. Deanin, B. Margolis, J. Schlessinger, J. M. Oliver, FcεRI-mediated tyrosine phosphorylation of multiple proteins, including phospholipase Cγ1 and the receptor βγ2 complex, in RBL-2H3 rat basophilic leukemia cells. *Mol. Cell. Biol.* **12**, 3176–3182 (1992).
22. D. J. Park, H. K. Min, S. G. Rhee, IgE-induced tyrosine phosphorylation of phospholipase C-γ1 in rat basophilic leukemia cells. *J. Biol. Chem.* **266**, 24237–24240 (1991).
23. L. R. Hendricks-Taylor, D. G. Motto, J. Zhang, R. P. Siraganian, G. A. Koretzky, SLP-76 is a substrate of the high affinity IgE receptor stimulated protein tyrosine kinases in rat basophilic leukemia cells. *J. Biol. Chem.* **272**, 1363–1367 (1997).
24. M. Yu, C. A. Lowell, B. G. Neel, H. Gu, Scaffolding adapter Grb2-associated binder 2 requires Syk to transmit signals from FcεRI. *J. Immunol.* **176**, 2421–2429 (2006).
25. S. Iwaki, C. Tkaczyk, A. B. Satterthwaite, K. Halcomb, M. A. Beaven, D. D. Metcalfe, A. M. Gilfillan, Btk plays a crucial role in the amplification of FcεRI-mediated mast cell activation by kit. *J. Biol. Chem.* **280**, 40261–40270 (2005).
26. H. Qiao, M. V. Andrade, F. A. Lisboa, K. Morgan, M. A. Beaven, FcεRI and toll-like receptors mediate synergistic signals to markedly augment production of inflammatory cytokines in murine mast cells. *Blood* **107**, 610–618 (2006).
27. H. T. Ma, M. A. Beaven, Regulators of Ca²⁺ signaling in mast cells: Potential targets for treatment of mast cell-related diseases? *Adv. Exp. Med. Biol.* **716**, 62–90 (2011).
28. S. W. Kim, D. Rai, M. R. McKeller, R. C. T. Aguiar, Rational combined targeting of phosphodiesterase 4B and SYK in DLBCL. *Blood* **113**, 6153–6160 (2009).
29. A. M. Gilfillan, J. Rivera, The tyrosine kinase network regulating mast cell activation. *Immunol. Rev.* **228**, 149–169 (2009).
30. V. S. Pribluda, C. Pribluda, H. Metzger, Transphosphorylation as the mechanism by which the high-affinity receptor for IgE is phosphorylated upon aggregation. *Proc. Natl. Acad. Sci. U.S.A.* **91**, 11246–11250 (1994).
31. S. R. Ande, S. Mishra, Palmitoylation of prohibitin at cysteine 69 facilitates its membrane translocation and interaction with Eps 15 homology domain protein 2 (EHD2). *Biochem. Cell Biol.* **88**, 553–558 (2010).
32. B. K. Wershil, Y. A. Mekori, T. Murakami, S. J. Galli, 125I-fibrin deposition in IgE-dependent immediate hypersensitivity reactions in mouse skin. Demonstration of the role of mast cells using genetically mast cell-deficient mice locally reconstituted with cultured mast cells. *J. Immunol.* **139**, 2605–2614 (1987).
33. J. K. McClung, E. R. Jupe, X. T. Liu, R. T. Dell'Orco, Prohibitin: Potential role in senescence, development, and tumor suppression. *Exp. Gerontol.* **30**, 99–124 (1996).
34. C. F. Chiu, M. Y. Ho, J. M. Peng, S. W. Hung, W. H. Lee, C. M. Liang, S. M. Liang, Raf activation by Ras and promotion of cellular metastasis require phosphorylation of prohibitin in the raft domain of the plasma membrane. *Oncogene* **32**, 777–787 (2013).
35. J. H. Lee, J. W. Kim, D. K. Kim, H. S. Kim, H. J. Park, D. K. Park, A. R. Kim, B. Kim, M. A. Beaven, K. L. Park, Y. M. Kim, W. S. Choi, The Src family kinase Fgr is critical for activation of mast cells and IgE-mediated anaphylaxis in mice. *J. Immunol.* **187**, 1807–1815 (2011).
36. U. Grädler, D. Schwarz, V. Dresing, D. Musil, J. Bomke, M. Frech, H. Greiner, S. Jäkel, T. Rysiok, D. Müller-Pompalla, A. Wegener, Structural and biophysical characterization of the Syk activation switch. *J. Mol. Biol.* **425**, 309–333 (2013).
37. K. A. Field, D. Holowka, B. Baird, FcεRI-mediated recruitment of p53/56^{lyn} to detergent-resistant membrane domains accompanies cellular signaling. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 9201–9205 (1995).
38. X. Qu, K. Sada, S. Kyo, K. Maeno, S. M. Miah, H. Yamamura, Negative regulation of FcεRI-mediated mast cell activation by a ubiquitin-protein ligase Cbl-b. *Blood* **103**, 1779–1786 (2004).
39. V. Parravicini, M. Gadina, N. Kovarova, S. Odom, C. Gonzalez-Espinosa, Y. Furumoto, S. Saitoh, L. E. Samelson, J. J. O'Shea, J. Rivera, Fyn kinase initiates complementary signals required for IgE-dependent mast cell degranulation. *Nat. Immunol.* **3**, 741–748 (2002).
40. B. M. Jensen, E. J. Swindle, S. Iwaki, A. M. Gilfillan, Generation, isolation, and maintenance of rodent mast cells and mast cell lines. *Curr. Protoc. Immunol.* **Chapter 3**, Unit 3.23 (2006).
41. K. Ozawa, Z. Szallasi, M. G. Kazanietz, P. M. Blumberg, H. Mischak, J. F. Mushinski, M. A. Beaven, Ca²⁺-dependent and Ca²⁺-independent isozymes of protein kinase C mediate exocytosis in antigen stimulated rat basophilic RBL-2H3 cells. Reconstitution of secretory responses with Ca²⁺ and purified isozymes in washed permeabilized cells. *J. Biol. Chem.* **268**, 1749–1756 (1993).
42. T. R. Martin, S. J. Galli, I. M. Katona, J. M. Drazen, Role of mast cells in anaphylaxis. Evidence for the importance of mast cells in the cardiopulmonary alterations and death induced by anti-IgE in mice. *J. Clin. Invest.* **83**, 1375–1383 (1989).

Funding: This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MEST) (No. 2011-0028873). M.A.B. was supported by the Intramural Program of the National Heart, Lung, and Blood Institute, NIH. **Author contributions:** W.S.C. and Y.M.K. designed the experiments, analyzed the data, and wrote the paper; D.K.K. and H.S.K. performed most of the experiments; A-R.K. collected and analyzed samples by confocal microscopy; G.H.J. and H.W.K. performed the in vivo experiments; Y.M.P. and Y.H.P. performed all experiments for histological analysis; B.K. contributed reagents, cloned the plasmids, and designed the experiments; and Y.M.K. and M.A.B. provided intellectual input and wrote the paper. **Competing interests:** The authors declare that they have no competing interests.

Submitted 22 February 2013

Accepted 22 August 2013

Final Publication 10 September 2013

10.1126/scisignal.2004098

Citation: D. K. Kim, H. S. Kim, A-R. Kim, G. H. Jang, H. W. Kim, Y. H. Park, B. Kim, Y. M. Park, M. A. Beaven, Y. M. Kim, W. S. Choi, The scaffold protein prohibitin is required for antigen-stimulated signaling in mast cells. *Sci. Signal.* **6**, ra80 (2013).

A Role for Prohibitin in Mast Cell Activation: Location Matters

Hajime Yurugi¹ and Krishnaraj Rajalingam^{2*}

Prohibitin 1 (PHB1) and PHB2 are evolutionarily conserved, ubiquitously expressed, pleiotropic proteins that control various fundamental cellular processes, including proliferation, migration, metabolism, and death. Studies have unveiled a crucial role for plasma membrane-associated PHBs in regulating tumor metastasis, viral entry, and immune cell activation. A study now identifies a role for PHB1 in the activation of mast cells and allergic reactions mediated by immunoglobulin E (IgE). PHB1 was primarily localized in mast cell granules; however, in response to stimulation with antigen, PHB1 translocated to plasma membrane lipid rafts to form a ternary complex with the high-affinity IgE receptor FcεRI γ and the nonreceptor tyrosine kinase Syk. Syk became activated, which led to the activation of downstream signaling that stimulated mast cell degranulation and the secretion of cytokines. PHB1 was phosphorylated by the Src family tyrosine kinase Lyn, and palmitoylation of PHB1 was required for its association with the plasma membrane. These observations unveil a previously uncharacterized facet of prohibitin biology and shed further light on the proximal events that drive the activation of FcεRI by IgE in mast cells during allergic reactions.

Prohibitin (PHB) is the flagship member of a large family of proteins with a distinct, highly conserved stomatin/prohibitin/flotillin/HflK/C (SPFH) domain, which is also called the prohibitin domain. These proteins are often associated with lipid raft microdomains in various cellular membranes, and they contribute to diverse signaling pathways that control cellular processes ranging from proliferation and migration to inflammation and cell death (1). Prohibitin 1 (PHB1) and PHB2 normally exist as a heteromer, and they are also known as B cell receptor (BCR)-associated proteins 32 (BAP32) and (BAP37), because they were identified as interacting partners of the immunoglobulin M (IgM)-BCR complex (2). PHBs are predominantly localized to the mitochondrial inner membrane, where they contribute to cristae morphogenesis and mitochondrial function (3). PHB1 is required for the activation of the kinase CRAF, the central member of the Ras-Raf-mitogen-activated protein kinase (MAPK) cascade at the plasma membrane, and it also interacts with the insulin receptor and phos-

phatidylinositol 3,4,5-triphosphate, as well as modulating activation of the serine and threonine kinase Akt (1, 4). An increase in the abundance of PHB1 associated with lipid rafts correlates with tumor growth and metastasis in cervical carcinoma (5).

Pathogenic viruses such as the Chikungunya virus target plasma membrane-associated PHBs to facilitate their entry into the host cells (6). The Vi capsular polysaccharide, a major virulence antigen of *Salmonella typhi*, targets PHB1 and PHB2 (hereafter, PHB1/2) in intestinal epithelial cells to suppress activation of extracellular signal-regulated kinase 1 (ERK1) and ERK2 (ERK1/2) and early immune responses (7). In B cells, PHBs are associated with CD86, a glycoprotein normally found on the surface of antigen-presenting cells, such as dendritic cells (DCs). In B cells activated (or “primed”) by CD40 ligand (CD40L) and interleukin-4 (IL-4), CD86 signaling requires PHB1 for production of the transcription factor Oct-2, and production of immunoglobulin G1 (IgG1) through the phosphorylation of phospholipase C- γ 2 (PLC- γ 2) and activation of the protein kinase C (PKC) isoforms PKC α and PKC β _{II} (8). In T lymphocytes, the PHB1/2 complex is essential for mitochondrial homeostasis and survival. Furthermore, studies showed that PHBs are exposed at the cell surface in activated T cells, and act as a receptor for Siglec-9, an adhesion molecule found on DCs and macrophages. Cross-linking of

PHBs by antibody to PHB inhibits T cell receptor (TCR)-mediated ERK1/2 signaling (9). Platelets also have PHBs on their surface, where they associate with protease-activated receptor 1 (PAR1). Additionally, antibodies to PHB inhibit PAR1-mediated platelet aggregation, activation of the α IIb β 3 integrin, and secretion of intracellular granules (10). Together, these studies provide mounting evidence for a compelling role for the plasma membrane-associated fraction of PHBs in the regulation of immune responses. Now, Kim *et al.* have uncovered a crucial role for PHB1 in IgE-mediated activation and degranulation of mast cells (11).

These authors investigated the presence of PHB1 in mast cells, the key effector cells in allergic responses mediated by IgE, and they found that PHB1 was primarily localized to the intracellular granules of these cells. FcεRI on the surface of mast cells and basophils serves as a high-affinity receptor for IgE. FcεRI is a tetrameric receptor that consists of an α subunit, which binds to IgE, as well as β and γ subunits, which are responsible for initiating signaling. Binding of antigen to IgE that is associated with FcεRI triggers a cascade of kinase activation and phosphorylation events that lead to mast cell degranulation and the release of histamines, eicosanoids, and cytokines. This cascade starts with the activation of the nonreceptor tyrosine kinase Lyn, which phosphorylates tyrosine residues in the immunoreceptor tyrosine-based activation motifs (ITAMs) of the β and γ subunits of FcεRI. The kinase Syk is recruited to the phosphorylated ITAMs, where it undergoes autophosphorylation and activation. Syk in turn initiates a spectrum of downstream signaling events, including the activation of phospholipase C γ (PLC- γ), ERK1/2, phosphatidylinositol 3-kinase (PI3K)-Akt, Gab2, c-Jun N-terminal kinase (JNK), and p38 mitogen-activated protein kinase (MAPK) pathways, which contribute to inflammation and allergic responses.

Kim *et al.* found that PHB1, but not PHB2, was required for the degranulation of mast cells in response to stimulation with IgE. In most cell types, PHB1 and PHB2 exist together in a complex, and there is also evidence that they are probably functional as heteromers; loss of one isoform often leads to the destabilization of the other. However, in mast cells, the PHB proteins probably function as monomers, because PHB1, but not PHB2, was required for mast cell activation. Kim *et al.* found that upon stimulation

¹Department of Molecular Biosciences, Faculty of Life Sciences, Kyoto Sangyo University, Kamigamo-Motoyama, Kitaku, Kyoto 603-8555, Japan. ²Cell Death Signaling Group, Institute of Biochemistry II, Goethe University Medical School, Goethe University, Theodor Stern Kai 7, 60590 Frankfurt, Germany.

*Corresponding author. E-mail: krishna@biochem2.de

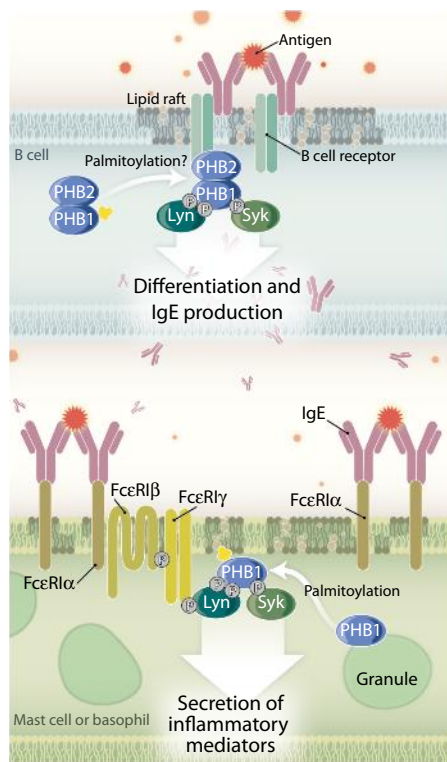


Fig. 1. Proposed model for the role of PHB1 in the initiation of allergic reactions. (Top) The nonreceptor tyrosine kinase Lyn is a key mediator of BCR-mediated signaling. PHB1 and PHB2 are associated with IgM BCRs and might contribute to Lyn-Syk signaling in B cells. Furthermore, PHB interacts with phosphorylated Syk in B cells. (Bottom) In mast cells and basophils, PHB1 mediates Lyn-Syk signaling upon ligation of the FcεRI with a complex of antigen and IgE. PHB1 is localized within granules in mast cells, and it translocates to the plasma membrane in a palmitoylation-dependent manner. PHB1 functions as a scaffold at plasma-membrane lipid rafts, leading to the formation of a ternary complex consisting of the γ subunit of FcεRI, PHB1, and Syk. Complex formation leads to activation of Syk, which in turn stimulates a cascade of kinases, resulting in mast cell degranulation and the release of proinflammatory factors. These findings suggest that PHB1 is a critical regulator of allergic responses in these cells.

of mast cells with antigen, PHB1 was translocated from the intracellular granules to the plasma membrane, where it accumulated in lipid raft fractions. Under nonstimulated conditions, most of the PHB1 was associated with the granules, and its translocation to the plasma membrane upon stimulation was facilitated by palmitoylation at its only cysteine residue (Cys⁶⁹).

An earlier study from Morrow *et al.* showed that palmitoylation of the conserved cysteine residue in the PHB domain is required for the trafficking of flotillins to lipid rafts in the plasma membrane (12). Prohibitin undergoes various posttranslational modifications, including phosphorylation, ubiquitylation, transamidation, tyrosine nitrosylation, and O-GlcNAc modification, which contribute to various functions (1). Several laboratories have demonstrated that PHB1 can be phosphorylated (for example, at Thr²⁵⁸ by Akt) in response to insulin and other growth factors. A study by Ande and Mishra provided direct evidence for the palmitoylation of PHB1 at Cys⁶⁹, which leads to its translocation to the plasma membrane, tyrosine phosphorylation, and interaction with Eps 15 homology domain protein 2 (13). Consistent with these observations, Kim *et al.* found that mutation of Cys⁶⁹ prevented the translocation of PHB1 to the plasma membrane in mast cells, thus preventing activation and degranulation. These data suggest the biological relevance of the palmitoylation of PHB1 to immune responses. Whether this lipid modification is responsible for the association of PHB1 with the plasma membrane in other cell types, such as T cells and migrating tumor cells, deserves further investigations.

Kim *et al.* found that loss of PHB1 prevented the translocation of the FcεRI γ subunit to lipid rafts, thus preventing its phosphorylation by the tyrosine kinase Lyn. Consequently, Syk was not recruited to the receptor-containing complex, even when cells were stimulated with antigen. These data suggest that PHB1 might function as a scaffold in lipid rafts to stabilize the complex formed between FcεRI γ , Lyn, and Syk. Whether PHB1 directly interacts with FcεRI γ is currently unclear; however, PHB1 is also phosphorylated by Lyn (at Tyr¹¹⁴ and Tyr²⁵⁹), and these phosphorylation events are required for the formation of a ternary complex consisting of PHB1, FcεRI γ , and Syk. Apparently, loss of FcεRI γ did not affect the phosphorylation of PHB1 by Lyn. Thus, the phosphorylation of PHB1 is likely proximal to the phosphorylation of FcεRI γ and the recruitment of Syk to this receptor subunit. In this regard, it is worth noting that phosphorylated Syk binds to PHB1 in response to clustering of the BCR (14). Further, Thr²⁵⁸ is recognized as a target site for the O-GlcNAcylation of PHB1; thus, it would be interesting to investigate whether there is any crosstalk between

these two posttranslational modifications in the regulation of mast cell activation. Basophils also have FcεRI on their cell surface, and, upon activation, they undergo degranulation and they secrete inflammatory mediators, similarly to mast cells. Thus, it is tempting to propose that PHB1 in basophils may also contribute to FcεRI-Lyn-Syk signaling.

Together, these findings suggest that PHB1 may play a key role in the allergic response by mediating Lyn-Syk signaling in mast cells, basophils, and B lymphocytes (Fig. 1). Indeed, Kim *et al.* further presented evidence that depletion of PHB1 with siRNAs reduced the extent of IgE-mediated anaphylaxis in mice. By using IgE-sensitized mice and by inducing both cutaneous and systemic anaphylaxis, the authors demonstrated that PHB1 was required for mast cell recruitment, degranulation, and TNF- α secretion. These interesting observations should stimulate an investigation of the role of plasma membrane-associated prohibitins in other settings, and further work is needed to investigate whether PHB1 may be targeted therapeutically to combat allergic reactions.

References and Notes

1. S. Mishra, S. R. Ande, B. L. Nyomba, The role of prohibitin in cell signaling. *FEBS J.* **277**, 3937–3946 (2010).
2. M. Terashima, K. M. Kim, T. Adachi, P. J. Nielsen, M. Reth, G. Köhler, M. C. Lamers, The IgM antigen receptor of B lymphocytes is associated with prohibitin and a prohibitin-related protein. *EMBO J.* **13**, 3782–3792 (1994).
3. C. Merkwirth, S. Dargazanli, T. Tatsuta, S. Geimer, B. Löwer, F. T. Wunderlich, J. C. von Kleist-Retzow, A. Waisman, B. Westermann, T. Langer, Prohibitins control cell proliferation and apoptosis by regulating OPA1-dependent cristae morphogenesis in mitochondria. *Genes Dev.* **22**, 476–488 (2008).
4. K. Rajalingam, C. Wunder, V. Brinkmann, Y. Churin, M. Hekman, C. Sievers, U. R. Rapp, T. Rudel, Prohibitin is required for Ras-induced Raf-MEK-ERK activation and epithelial cell migration. *Nat. Cell Biol.* **7**, 837–843 (2005).
5. C. F. Chiu, M. Y. Ho, J. M. Peng, S. W. Hung, W. H. Lee, C. M. Liang, S. M. Liang, Raf activation by Ras and promotion of cellular metastasis require phosphorylation of prohibitin in the raft domain of the plasma membrane. *Oncogene* **32**, 777–787 (2013).
6. P. Wintachai, N. Wikan, A. Kuadkitkan, T. Jaimipuk, S. Ubol, R. Pulmanasahakul, P. Auewarakul, W. Kasinrer, W. Y. Weng, M. Panyasrivani, A. Paemanee, S. Kittisenachai, S. Roytrakul, D. R. Smith, Identification of prohibitin as a Chikungunya virus receptor protein. *J. Med. Virol.* **84**, 1757–1770 (2012).
7. A. Sharma, A. Qadri, Vi polysaccharide of *Salmonella typhi* targets the prohibitin family of molecules in intestinal epithelial cells and suppresses early inflammatory responses. *Proc. Natl. Acad. Sci. U.S.A.* **101**, 17492–17497 (2004).

8. C. R. Lucas, H. M. Cordero-Nieves, R. S. Erbe, J. W. McAlees, S. Bhatia, R. J. Hodes, K. S. Campbell, V. M. Sanders, Prohibitins and the cytoplasmic domain of CD86 cooperate to mediate CD86 signaling in B lymphocytes. *J. Immunol.* **190**, 723–736 (2013).
9. H. Yurugi, S. Tanida, A. Ishida, K. Akita, M. Toda, M. Inoue, H. Nakada, Expression of prohibitins on the surface of activated T cells. *Biochem. Biophys. Res. Commun.* **420**, 275–280 (2012).
10. Y. Zhang, Y. Wang, Y. Xiang, W. Lee, Y. Zhang, Prohibitins are involved in protease-activated receptor 1-mediated platelet aggregation. *J. Thromb. Haemost.* **10**, 411–418 (2012).
11. D. K. Kim, H. S. Kim, A.-R. Kim, G. H. Jang, H. W. Kim, Y. H. Park, B. Kim, Y. M. Park, M. A. Beaven, Y. M. Kim, W. S. Choi, The scaffold protein prohibitin is required for antigen-stimulated signaling in mast cells. *Sci. Signal.* **6**, ra80 (2013).
12. I. C. Morrow, S. Rea, S. Martin, I. A. Prior, R. Prohaska, J. F. Hancock, D. E. James, R. G. Parton, Flotillin-1/reggie-2 traffics to surface raft domains via a novel golgi-independent pathway: Identification of a novel membrane targeting domain and a role for palmitoylation. *J. Biol. Chem.* **277**, 48834–48841 (2002).
13. S. R. Ande, S. Mishra, Palmitoylation of prohibitin at cysteine 69 facilitates its membrane translocation and interaction with Eps 15 homology domain protein 2 (EHD2). *Biochem. Cell Biol.* **88**, 553–558 (2010).
14. L. L. Paris, J. Hu, J. Galan, S. S. Ong, V. A. Martin, H. Ma, W. A. Tao, M. L. Harrison, R. L. Geahlen, Regulation of Syk by phosphorylation on serine in the linker insert. *J. Biol. Chem.* **285**, 39844–39854 (2010).

Funding: K.R. is a PLUS3 Fellow of the Boehringer Ingelheim foundation, and work in the Rajalingam laboratory is also funded through grants from the Deutsche Forschungs Gemeinschaft (DFG) (RA1739/1-1, RA1739/3-1, and INST 247/685-1).

10.1126/scisignal.2004646

Citation: H. Yurugi, K. Rajalingam, A role for prohibitin in mast cell activation: Location matters. *Sci. Signal.* **6**, pe29 (2013).