p66Shc Is a Negative Regulator of FcεRI-Dependent Signaling in Mast Cells

Cristina Ulivieri, Daniela Fanigliulo, Giulia Masi, Maria Teresa Savino, Alessandra Gamberucci, Pier Giuseppe Pelicci and Cosima T. Baldari

*J Immunol* 2011; 186:5095-5106; Prepublished online 23 March 2011; doi: 10.4049/jimmunol.1001391

http://www.jimmunol.org/content/186/9/5095

**Supplementary Material**

http://www.jimmunol.org/content/suppl/2011/03/21/jimmunol.1001391.DC1

**References**

This article cites 46 articles, 25 of which you can access for free at:

http://www.jimmunol.org/content/186/9/5095.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:

http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:

http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:

http://jimmunol.org/alerts
p66Shc Is a Negative Regulator of FcεRI-Dependent Signaling in Mast Cells

Cristina Ulivieri,*1 Daniela Faniglilo,*1 Giulia Masi,* Maria Teresa Savino,* Alessandra Gamberucci,† Pier Giuseppe Pelicci,‡ and Cosima T. Baldari*

Aggregation of FcεRI on mast cells activates signaling pathways, resulting in degranulation and cytokine release. Release of mast cell-derived inflammatory mediators is tightly regulated by the interplay of positive and negative signals largely orchestrated by adapter proteins. Among these, the Shc family adapter p52Shc, which couples immunoreceptors to Ras activation, positively regulates FcεRI-dependent signaling. Conversely, p66Shc was shown to uncouple the TCR for the Ras–MAPK pathway and prime T cells to undergo apoptotic death. Loss of p66Shc in mice results in breaking of autoimmune disease, which includes alopecia among its pathological manifestations. The presence of numerous activated mast cells in alopecic skin areas suggests a role for this adapter in mast cells. In this study, we addressed the involvement of p66Shc in FcεRI-dependent mast cell activation. We showed that p66Shc is expressed in mast cells and that mast cells from p66Shc−/− mice exhibit enhanced responses following Ag stimulation of FcεRI. Furthermore, using RBL-2H3 cell transfectants, we showed that aggregation of FcεRI resulted in the recruitment of a p66Shc–SHIP1 complex to linker for activation of T cells. Collectively, our data identified p66Shc as a negative regulator of mast cell activation. 

The Journal of Immunology, 2011, 186: 5095–5106.

Abbreviations used in this article: BCS, bovine calf serum; BMMC, bone marrow-derived mast cells; CH1, collagen homology 1; DNP- ALB, albumin-conjugated DNP; IKK, IκB kinase; LAT, linker for activation of T cells; PH, pleckstrin homology; PI3,4,5P3, phosphatidylinositol 3,4,5-trisphosphate; FTK, protein tyrosine kinase; RBL, rat basophilic leukemia; WT, wild-type.

†Department of Evolutionary Biology, University of Siena, 53100 Siena, Italy; 2Department of Physiopathology, University of Siena, 53100 Siena, Italy; and 3Department of Molecular Oncology, European Institute of Oncology, 20139 Milan, Italy

© 2011 by The American Association of Immunologists, Inc. 0022-1767/11/$16.00

Address correspondence and reprint requests to Dr. Cristina Ulivieri, Department of Evolutionary Biology, Via Aldo Moro 2, 53100 Siena, Italy. E-mail address: ulivieri@unisi.it

Copyright © 2011 by The American Association of Immunologists, Inc. 0022-1767/11/1S16/00

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1001391

Received for publication April 27, 2010. Accepted for publication February 22, 2011.

This work was supported by grants from Associazione Italiana Ricerca sul Cancro, Istituto Tumori Regione Toscana, and Progetti di Ricerca di Interesse Nazionale (to C.T.B.) and Piano di Ateneo per la Ricerca 2006 (to C.U.).

Abstract address and reprint requests to Dr. Cristina Ulivieri, Department of Evolutionary Biology, Via Aldo Moro 2, 53100 Siena, Italy. E-mail address: ulivieri@unisi.it

The online version of this article contains supplemental material.

Mast cells have long been identified as critical effector cells in allergic disorders. Mast cells, which are activated by several inflammatory signals, such as microorganisms, complement components, immune complexes, and T cell interaction, release a number of mediators, including cytokines, chemokines, proteolytic enzymes, and lipid-derived and vasoactive mediators, which, in turn, may influence the development, magnitude, and duration of the inflammatory response. In addition, mast cells residing in tissues near the site of Ag entry are now considered central players in protective and pathological immune responses (1–3).

Aggregation of FcεRI on mast cells, through binding of allergen-specific IgE molecules and subsequent cross-linking by cognate allergen, is central to mast cell activation and results in the immediate release of proinflammatory mediators. FcεRI is an immune receptor composed of a recognition module responsible for binding IgE, as well as a signal-transducing module, characterized by the presence of ITAMs, which are phosphorylated by the Src family protein tyrosine kinase (PTK) Lyn in response to Ag and, as such, recruit the PTK Syk to the plasma membrane. Syk and Lyn then phosphorylate multiple intracellular proteins. Among the substrates of these PTKs are several adapters that promote the assembly of intracellular signaling complexes. The transmembrane adapters linker for activation of T cells (LAT) and non-T cell activation linker have been identified as sites for nucleation of proximal signaling complexes whose composition is determinant to the fine-tuning of mast cell responses (4). The principal pathway of FcεRI signaling is orchestrated by the lipid raft resident LAT, which was recently proposed to control mast cell responses positively and negatively (5). Allergen-induced LAT phosphorylation results in the recruitment of signaling mediators, such as PLCγ, Gads/SLP-76/Val, and Grb/Shc/Sos, leading to degranulation, as well as to cytokine and eicosanoid production (4). Moreover, LAT is responsible for recruitment of the lipid phosphatase SHIP1, the major negative regulator of FcεRI signaling (5). Consistent with a view of adapters as key players in coordinating FcεRI signaling, mice lacking LAT (6), non-T cell activation linker (7), SLP-76 (8), or Gab2 (9) are resistant to IgE-mediated passive anaphylaxis.

The Shc adapter family is composed of four members, Shc/ShcA, ShcB/Shi, ShcC/Shi/RFaN-Shc, and ShcD/RaLP, which differ in expression and function. All family members participate in signaling by a variety of cell surface receptors and share a highly conserved modular organization consisting of an N-terminal phosphotyrosine-binding domain, a central proline-rich collagen homology (CH1) domain, and a C-terminal SH2 domain. In some isoforms, the phosphotyrosine-binding domain is preceded by a CH2 domain (10). ShcA is expressed as three isoforms of 52, 46, and 66 kDa. Of these, p52Shc has been implicated in signaling by immunoreceptors. Following ligand binding, p52Shc is recruited to the activated receptor and is phosphorylated in the central CH1 domain on conserved Tyr residues. In this state, p52Shc couples immunoreceptors to Ras activation through the association with Grb2–Sos complexes (11). p52Shc also associates with other signaling mediators, including Syk, Cbl, Gab1, SLP-76, and SHIP, thus participating in additional pathways triggered by immunoreceptors (12).

The Journal of Immunology, 2011, 186: 5095–5106.
In mast cells, p52Shc is recruited to the β-chain of FcεRI directly through its SH2 domain and indirectly through its interaction with Grb2; as such, it activates the Ras–MAPK pathway, thereby acting as a positive regulator of FcεRI signaling (13, 14). In addition, p52Shc was shown to positively regulate FcεRI signaling by preventing the interaction of negative regulators, such as SHIP1 and PKCδ, with the activated receptors (15). However, p52Shc was also implicated in the negative regulation of IL-3R signaling in mast cells through the recruitment of SHIP1 to the IL-3R complex, where it hydrolyzes phosphatidylinositol-3,4,5-trisphosphate (P13,4,5P3), thereby impairing P13,4,5P3-mediated activation of multiple pathways (16).

Opposite to the positive role exerted by p52Shc in Ag-receptor signaling, we recently showed that the 66-kDa isoform, which is expressed in lymphocytes and monocytes, albeit at lower levels compared with p52/p46Shc, inhibits TCR/BCR-dependent activation of the Ras–MAPK pathway and promotes T and B cell apoptosis (17, 18). Furthermore, we demonstrated that ablation of the p66shc gene in mice leads to spontaneous lymphocyte activation and development of a lupus-like autoimmune disease, indicating a key role for p66Shc in controlling lymphocyte homeostasis (19). Interestingly, histological analysis of alopecic skin areas from p66shc−/− mice revealed the presence of numerous activated mast cells (19), suggesting a potential role for this protein in mast cell activation.

The expression and the function of p66Shc in mast cells have not been investigated. In this study, we showed that p66Shc is expressed in mast cells and that p66Shc−/− mast cells exhibit increased responses in the absence of stimulation and following FcεRI engagement by IgE/Agal. Furthermore, using a panel of RBL-2H3 cell transfectants expressing p66Shc or mutants thereof, we provide evidence that p66Shc limits mast cell degranulation and proinflammatory cytokine production by promoting SHIP1 recruitment to LAT. The data identified p66Shc as a novel negative regulator of mast cell activation.

Materials and Methods
Mice, cells, and plasmids
The work was carried out on p66Shc−/− (20) and 129xw mice colonies bred in the animal facility at the University of Siena, where only specific pathogen-free animals are admitted. Animals were housed and used in agreement with the Guiding Principles for Research Involving Animals and Human Beings and approved by the local ethical committees. Bone marrow-derived mast cells (BMMC) were prepared from femurs of 4–8 wk-old mice, as described elsewhere (21). Briefer, femurs of mice were flushed with RPMI 1640 (Sigma Aldrich, Milan Italy). Bone marrow cells were then cultured in RPMI 1640 medium supplemented with 10% bovine calf serum (BCS) (cat No. SH30072.03; HyClone) and 30% WEHI-3B-conditioned medium, as described elsewhere (21). Briefly, femurs of mice were flushed with RPMI 1640 (Sigma Aldrich, Milan Italy). Bone marrow cells were then cultured in RPMI 1640 medium supplemented with 10% bovine calf serum (BCS) (cat No. SH30072.03; HyClone) and 30% WEHI-3B–conditioned medium. After 4–6 wk of culture, cells were stained for cell-surface expression of FcεRI and c-Kit, as well as with toluidine blue. Peritoneal cells were collected from sacrificed mice by injecting 5 ml RPMI 1640 medium into the peritoneum. The mixed peritoneal cells were stained immediately for cell-surface expression of c-Kit or cultured in RPMI 1640 medium supplemented with 10% BCS and 30% WEHI-3B–conditioned medium for 48 h for degranulation assays. The rat basophilic leukemia (RBL) cell line (RBL-2H3) was used for the generation of stable transfectants. The WEHI-3B and RBL-2H3 cell lines were kindly provided by M. De Bernard (Department of Biology, University of Padua, Padua, Italy). The expression constructs encoding p66Shc, p66Shc(3F) and p66Shc(3A) were described elsewhere (17). The construct encoding the GFP-tagged pleckstrin homology (PH) domain of Akt/PKB was a kind gift of D. Cantrell (University of Dundee, Dundee, U.K.).

Abs for immunoprecipitation and immunoblot assays
Anti-Shc Abs include monoclonal (Santa Cruz Biotechnology Santa Cruz, CA) or polyclonal anti-SH2 Abs (Upstate Biotechnology, Boston MA). p66Shc isoform and mutants thereof were immunoprecipitated using a rabbit polyclonal antiserum specific for the CH2 domain (20). A mAb recognizing phosphorylated S36 on p66Shc was purchased from Alexis Biochemicals (San Diego, CA). Phosphospecific Abs recognizing the active forms of Erk1/2 (Thr 202/Tyr204), Merk 1/2 (Ser217/221), p38 (Thr180/Tyr182), SAPK/Jnk (Thr183/Tyr185), IKKα (Ser180)/IKKβ (Ser181), Akt (Thr308), and Syk (Tyr525/526), as well as control anti-Ig Abs, were from Cell Signaling Technology (Beverly, MA); mouse anti-phosphotyrosine, rabbit anti-LAT, anti-Gab1, anti-FcεRI, and anti-Syk Abs were purchased from Upstate Biotechnology (Boston MA). Mouse anti-Syk and anti-Crk-2, rabbit anti-SHIP1, anti-Crk-2, and anti-Erk Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). A mouse anti-actin mAb was obtained from Chemicon (Temecula, CA).

Stable transfectants
Expression constructs encoding wild-type (WT) or mutated versions of p66Shc were introduced by electroporation into RBL-2H3 cells. Expression constructs included pcDNA3-p66Shc encoding WT p66Shc, pBabe-p66Shc(3F) encoding a p66Shc mutant carrying a serine-to-alanine mutation at position 36 (p66S36A), and Pogo-p66Shc encoding a p66Shc mutant carrying tyrosine-to-phenylalanine substitutions at position 234/290 and p66Shc (17). Stably transfected cells were selected in medium containing 1 mg/ml G418 (pcDNA3-p66Shc; Life Technologies, BRL, Life Technologies, Milan, Italy) or 1 μg/ml puromycin (Pogo-p66Shc and pBabe-p66Shc; Sigma, Milan, Italy). Transfectants were screened based on the level of p66Shc expression by immunoblotting of total cell lysates with anti-Shc Abs. The FcεRI expression level was assessed as described in Flow cytometry and Ca++ flux analysis.

Flow cytometry and Ca++ flux analysis
The stable RBL transfectants were transiently transfected with the plasmid encoding the GFP-tagged PH domain of Akt/PKB using a modification of the DEAE/dextran procedure, as described (22). Four hours post-transfection, cells were primed with mouse anti-DNP IgE mAb (SPE-7) (1 μg/ml) and allowed to recover for 18 h before activation. Activation was carried out using albumin-conjugated DNP (DNP-ALB) (1 μg/ml). Five to ten minutes after activation, cells were washed twice with PBS and analyzed. Confocal laser scanning microscopy was carried out on a Zeiss LSM700 (Zeiss, Germany) using a 63 × objective. Detectors were set to detect an optimal signal below the saturation limits. Images were processed with Adobe Photoshop Elements 8.

BMMC were nucleofected with the pEGFP-C1 vector, alone or in combination with the pcDNA3-p66Shc expression construct using the Amaxa mouse T-cell nucleofector kit (Amaza Biosystems Cologne, Germany) in the Amaxa nucleofector device (program U-023). After transfection, BMMC were allowed to recover for 48 h. The transfection efficiency was measured by flow cytometry and immunoblot analysis. Degranulation assays were subjected to measurement of degranulation or Erk activation by flow cytometry using PE-labeled Annexin V (Immunostep, S.L. Salamanca, Spain) or anti-Erk phosphospecific Abs, respectively, gating live on GFP+ cells.

Degranulation assays
BMMC and RBL-2H3 degranulation was determined by β-hexosaminidase activity assay. A total of 0.2 × 10^6 cells/sample was left untreated or sensitized with anti-DNP IgE (1 μg/ml) overnight at 37˚C, washed, and challenged for 20 min with DNP-ALB (20–2000 ng/ml) or treated with A23187 (500 ng/ml), ATP (3 mM), and thapsigargin (2 μM) (Sigma). The β-hexosaminidase content of supernatants and cells was determined, as described (23), and calculated as follows: β-hexosaminidase released/β-hexosaminidase in supernatants + β-hexosaminidase in cells) × 100. Alternatively, measurement of degranulation by Annexin V binding was performed on peritoneal cells, as described (24). Briefly, 1 × 10^6 peritoneal cells collected in WEHI-3B–conditioned medium were sensitized with anti-DNP IgE (1 μg/ml) for 3 h at room temperature, washed, and challenged with DNP-ALB (20 ng/ml) for 30 s. The reaction was stopped by adding ice-cold Annexin V-binding buffer and transferring the tube on ice. Phosphatidylserine exposure to the cell surface was quantitated using FITC-labeled Annexin V (eBioscience, San Diego, CA).

Activation, immunoprecipitation, in vitro binding, and immunoblot assays
BMMC or RBL transfectants were left untreated or sensitized with anti-DNP IgE (1 μg/ml) overnight at 37˚C, washed, and challenged at 37˚C for the indicated times with DNP-ALB (20–100 ng/ml) or 1 μg/ml. Alternatively, BMMC were treated with rIL-3 (5 ng/ml) (Immunosciences, Padua, Italy) for 5 min at 37˚C. Cells (2 × 10^6 cells/sample for analysis of total cell lysates or 50 × 10^6 cells/samples for immunoprecipitation and
in vitro binding assays) were lysed in 3 or 1% (v/v) Triton X-100 in 20 mM Tris-HCl (pH 8.0), 150 mM NaCl (in the presence of 0.2 mg/ml Na orthovanadate; 1 μg/ml pepstatin, leupeptin, and aprotinin; 10 mM phenyl methyl sulfonyl fluoride; and 50 mM NaF), resolved by SDS-PAGE, and transferred to nitrocellulose. Alternatively, postnuclear supernatants were immunoprecipitated using the indicated polyclonal Abs and protein A-Sepharose (Amersham Biosciences). p66Shc immunodepletion was carried out using an anti-CH2 Ab as described (17). In vitro binding assays were carried out, as described, using agarose-bound GST or an agarose-bound GST fusion protein containing the p66Shc CH2 domain (GST-CH2) (17). The GST-CH2 fusion protein was phosphorylated in vitro in 50 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 2 mM EGTA, 0.1% (v/v) Triton X-100, 1 mM DTT, 100 μM Na orthovanadate, and 100 μM ATP for 20 min at 37°C using 50 ng GST–Erk-1/Erk-2/Erk-3 (Pierce, Rockford, IL). Immunoblots were scanned using a laser densitometer (Duoscan T2500 Agfa, Milan, Italy) and quantified using ImageJ software.

p66Shc (66 kDa) has a lower electrophoretic mobility compared with Syk (72 kDa) in RBL-2H3 immunoprecipitation assays, probably resulting from substantial S36 and tyrosine phosphorylation, even in the absence of stimulation. A slightly lower mobility for p66Shc compared with ZAP-70 in low-density fraction were extracted and concentrated, as described (25).

Flow cytometry and Ca2+ flux analysis

For flow cytometric analyses, 2×105 cells/sample were analyzed using Flow cytometry (Becton Dickinson, San Jose, CA). Cells were stained for FcεRI expression with 2 μg/ml anti-DNP IgE, followed by FITC-conjugated anti-mouse Ig (Dako) or with PE-conjugated anti-CD117 mAb (BD Biosciences, San Jose, CA) to assess c-Kit expression. Mass cell proliferation was measured by flow cytometric analysis of CFSE-labeled cells, as described (17). Cells were resuspended at 1×106 cells/ml in PBS and loaded with 10 μM CFSE (Molecular Probes/Invitrogen Life Technologies, CA) for 5 min at room temperature. Cells were subsequently washed twice in RPMI 1640 7.5% BCS and cultured with WEHI-3B–conditioned medium. Cell division was determined by monitoring cell fluorescence dilution using a FACScan flow cytometer (Becton Dickinson, San Jose, CA).

Intracellular Ca2+ concentrations were determined in cells loaded with 3 μM Fura-2 (Molecular Probes) in 140 mM NaCl, 5.4 mM KCl, 1 mM MgCl2, 0.2 mM EGTA, and 15 mM HEPES (pH 7.4) in the presence of 1 mM Ca2+, as described (26). Loaded cells were sensitized with anti-DNP IgE (1 μg/ml) and stimulated with DNP-ALB (1 μg/ml or 20 ng/ml), thapsigargin (2 mM), or ATP (3 mM). Fluorescence was measured using a Varian Cary Eclipse fluorescence spectrophotometer (Palo Alto, CA) (excitation wavelengths, 340 and 380 nm; emission, 510 nm). Cells were subsequently washed twice in RPMI 1640 7.5% BCS and cultured with WEHI-3B–conditioned medium. Cell division was determined by monitoring cell fluorescence dilution using a FACScan flow cytometer (Becton Dickinson, San Jose, CA).

Intracellular Ca2+ concentrations were determined in cells loaded with 3 μM Fura-2 (Molecular Probes) in 140 mM NaCl, 5.4 mM KCl, 1 mM MgCl2, 0.2 mM EGTA, and 15 mM HEPES (pH 7.4) in the presence of 1 mM Ca2+, as described (26). Loaded cells were sensitized with anti-DNP IgE (1 μg/ml) and stimulated with DNP-ALB (1 μg/ml or 20 ng/ml), thapsigargin (2 mM), or ATP (3 mM). Fluorescence was measured using a Varian Cary Eclipse fluorescence spectrophotometer (Palo Alto, CA) (excitation wavelengths, 340 and 380 nm; emission, 510 nm) equipped with magnetic stirring and temperature control. At the end of each incubation, digitonin (50 μg/ml) and EGTA (20 mM) (Sigma) were added. Intracellular Ca2+ concentrations were determined using a Ca2+–specific fluorophore, Fura-2, which exhibits two excitation maxima at 340 and 380 nm. The fluorescence at 380 excitation under Ca2+-free conditions, and Sb380 is the fluorescence at 380 excitation under Ca2+-saturating conditions.

RNA purification, reverse transcription, and real-time quantitative PCR

Total RNA was extracted from BMCC or RBL transfectants using Tri Reagent (Ambion, Austin, TX). RT-PCR was carried out on 5 μg total RNA using Invitrogen-MultiPCR reverse transcriptase and oligo-dT (Promega Italia srl, Milan, Italy) as first-strand primer, whereas pairs of specific primers were used for CDNA amplification. Real-time quantitative PCR was performed using SYBR Green I SensMix dT Kit (Quantace, Watford, U.K.), according to the manufacturer’s instructions, in an Opticon 2 Continuous Fluorescence Detection System (MJ Research, Bio-Rad Laboratories, Waltham, MA). All samples were run in duplicate on 96-well optical PCR plates (Roche Diagnostics, Milan, Italy). The cDNA fragments corresponding to mouse and rat TNF-α, IL-6, and GAPDH were amplified using specific pairs of primers after cDNA amplification for 10 min at 95°C, denaturation for the subsequent 50 cycles was performed for 15 s at 95°C, followed by 15 s primer annealing at 60°C and final extension at 72°C for 30 s. The starting copy number of the unknown samples was determined using the comparative ΔΔCt method, as previously described (27). Levels of different transcripts were normalized to GAPDH, used as housekeeping gene.

Cytokine measurements

For detection of TNF-α, mouse BMCC were incubated with 1 μg/ml anti-DNP IgE for 3 h and stimulated for 4 or 24 h with 20 ng/ml DNP-ALB. Supernatants were collected, and secreted TNF-α was determined using a murine-specific TNF-α ELISA detection kit (Biosource, Camarillo, CA).

Statistical analysis

Mean values, SD values, and the Student t test (unpaired) were calculated using the Microsoft Excel application. The p < 0.05 were considered statistically significant.

Results

p66Shc deficiency does not affect BMCC development

The expression of p66Shc was evaluated in total cell lysates of BMCC established from 129/sv and p66Shc−/− mice by immunoblot analysis with an anti-Shc Ab that recognizes all three ShcA isoforms. p66Shc was found to be constitutively expressed in BMCC, albeit at lower levels compared with p52/p46Shc. The abundance of the latter was not affected by the lack of p66Shc, consistent with their expression from independent promoters (28) (Fig. 1A). No difference in the expression levels of FceRI and c-Kit or in the percentage of FceRI and c-Kit+ cells in primary cultures was observed between p66Shc−/− and control BMCC, as assessed by flow cytometry (Fig. 1A). By contrast, using toluidine blue staining to assess granule content, we observed enlarged granules in p66Shc−/− BMCC compared with control cells (Fig. 1B, left panel). Because BMCC development is dependent on IL-3, we asked whether the morphological differences between p66Shc−/− and control BMCC could be the result of the modulation of IL-3 signaling by p66Shc. IL-3–dependent Erk and Akt phosphorylation in response to IL-3 were comparable between WT and p66Shc−/− BMCC (Fig. 1B, middle and right panels). Moreover, the growth and proliferation of BMCC generated from WT and p66Shc−/− mice following culture in IL-3–containing medium were similar to those of control cells (Fig. 1C), suggesting that IL-3 signaling is not affected by p66Shc during BMCC development. To further address this issue on in vivo-differentiated mast cells, we analyzed peritoneal mast cells from p66Shc−/− mice. The percentage of peritoneal mast cells was comparable between WT and p66Shc−/− mice, as assessed by flow cytometric analysis of surface c-Kit (Fig. 2A), indicating that, similar to BMCC, the differentiation and maturation of peritoneal mast cells are not affected by the lack of p66Shc.

p66Shc-deficient mast cells show enhanced responses to FceRI stimulation by IgE/Ago

To investigate the role of p66Shc in FceRI-mediated signaling, we compared BMCC generated from WT and p66Shc−/− mice for β-hexosaminidase release in response to FceRI stimulation by IgE/Ago. p66Shc−/− and control BMCC were sensitized overnight with two concentrations of IgE (0.15 μg/ml or 1 μg/ml) and stimulated with increasing concentrations of Ag (20, 200, or 2000 ng/ml) for 20 min. The release of β-hexosaminidase in p66Shc−/− and control BMCC followed the typical bell-shaped dose–response curve (29). However, the degranulation of p66Shc−/− BMCC induced by stimulation with 20 ng/ml Ag was significantly enhanced compared with control BMCC at both IgE concentrations (Fig. 3A, left panel). Moreover, degranulation induced by 0.15 μg/ml IgE in combination with a high concentration of Ag (200 ng/ml) was still significantly greater in p66Shc−/− BMCC compared with control cells (Fig. 3A, left panel), suggesting that the lack of p66Shc lowers the activation threshold of FceRI. In support of a negative role for p66Shc in FceRI signaling, reconstitution of its expression...
in p66Shc−/− BMMC by nucleofection resulted in a reduction of Ag-induced degranulation (Fig. 3A, left panel).

Consistent with the data obtained on BMMC, p66Shc−/− peritoneal mast cells showed a significant increase in degranulation in response to IgE/Ag compared with WT cells (Fig. 2B), further supporting the notion that p66Shc is a negative regulator of mast cell degranulation.

Interestingly, p66Shc−/− BMMC (Fig. 3A, left panel), as well as peritoneal mast cells (Fig. 2B), showed substantial spontaneous degranulation compared with WT controls. Treatment of p66Shc−/− BMMC with IgE in the absence of Ag reduced this spontaneous degranulation to control levels (Fig. 3A, left panel). This effect may have resulted from enhancement in the cortical F-actin ring, which was recently shown to be induced by IgE sensitization of mast cells (30). Notwithstanding the enhancement in spontaneous degranulation, p66Shc−/− BMMC display an apparently normal complement of granules, which appear enlarged compared with controls (Fig. 1B). The total cellular content of β-hexosaminidase in BMMC from control and p66Shc−/− mice was comparable (data not shown), suggesting that the rate of synthesis of this enzyme might be higher in p66Shc−/− BMMC to replenish the intracellular pool, which is likely to be continuously depleted as the result of the spontaneous degranulation. It should be emphasized that the degranulation response to other agonists, including ATP, A23187, or thapsigargin, was also enhanced in p66Shc−/− BMMC compared with controls (Supplemental Fig. 1).

FIGURE 1. IL-3–dependent mast cell growth and proliferation do not require p66Shc. A, Immunoblot analysis with anti-Shc Abs of BMMC from control (+/+) or p66Shc−/− (−/−) mice (left panel). A short and long exposure are shown. Expression of FcεRI and c-Kit on BMMC from control (+/+) or p66Shc−/− (−/−) mice cultured in WEHI-3B–conditioned medium for 4 wk (middle panel). Cells were treated with anti-DNP IgE mAb (1 μg/ml) and then incubated with FITC-conjugated goat anti-mouse Ig; c-Kit was quantitated using PE-conjugated anti–c-Kit Abs. Control staining was with FITC-conjugated goat anti-mouse Ig. Cells were analyzed by flow cytometry, and the results are expressed as the log_{10} relative fluorescence versus the number of cells. Graphs show the percentage of FcεRI or c-Kit+ cells in primary BMMC from control (+/+) or p66Shc−/− (−/−) mice at day 21 or 30 of culture (right panel). Data are presented as mean ± SD (n = 15 individual BMMC cultures from control or from p66Shc−/− mice). B, Toluidine blue staining of BMMC from control (+/+) or p66Shc−/− (−/−) mice (left panel). Twenty cells/sample were scored, and the percentage of cells harboring large granules versus total cells (mean ± SD) was 28 ± 8.6% for control (+/+) and 54 ± 12% for p66Shc−/− (−/−) BMMC (n = 2; four mice/experiment). p = 0.012. The diameter of 10 granules/cells was measured using ImageJ software. The average diameter was 0.44 ± 0.07 μm in control BMMC and 0.87 ± 0.1 μm in p66Shc−/− BMMC. BMMC were left untreated (−) or treated with rIL-3 (5 ng/ml) for 5 min at 37˚C (IL-3), and total cell lysates were analyzed by immunoblot with anti–P-Akt (middle panel) or anti–P-Erk Abs (right panel). The relative decrease in CFSE staining was monitored by flow cytometry. The graphs show the mean ± SD of the fluorescence intensity with the mean fluorescence intensity at day 0 taken as 100% (n = 3; six independent sets of cultures from control or p66Shc−/− mice). Representative fluorescence graphs from one experiment are also shown.
were comparable between p66Shc enhanced spontaneous activation highlighted by degranulation data, compared with control cells (Fig. 3). The enhanced degranulation was paralleled by a strong increase in p66Shc-dependent and -independent responses were significantly enhanced and IL-6. The levels of TNF-α and IL-6 mRNA were greater in p66Shc−/− BMMC compared with controls, even in the absence of stimulation. The enhancing effect of p66Shc deletion on spontaneous and FcεRI-dependent mast cell activation strongly supports an essential role for p66Shc in mast cell homeostasis and Ag-induced responses. Aggregation of FcεRI induces tyrosine and serine phosphorylation of p66Shc in RBL-2H3 cells To mechanistically address the role of p66Shc in FcεRI signaling and to overcome the problem of the low expression of p66Shc in BMMC, all subsequent analyses were performed on stable mast cell transfectants expressing WT or mutated versions of p66Shc. Specifically, RBL-2H3 mast cells, which do not express p66Shc, were stably transfected with empty vector (pc), vector encoding p66Shc (p66), or mutants thereof. The mutated versions of p66Shc included a mutant lacking S36 (p66S36A) or a mutant lacking YY239/240 and Y317 (p66S36Y317A). Bulk transfectants were analyzed by immunoblotting to determine p66Shc expression (Fig. 4A) and by flow cytometry to assess IgER expression. No differences in surface FcεRI were observed among transfectants (data not shown).

In T cells, tyrosine and serine 36 phosphorylation were shown to be essential for the function of p66Shc (17). To examine whether p66Shc is involved in the FcεRI-signaling pathway, we first analyzed the state of p66Shc phosphorylation on tyrosine residues or S36 in response to FcεRI stimulation with IgE/Ag. Cell lysates were immunoprecipitated with anti-p66Shc Ab, and the immunoprecipitates were analyzed by immunoblotting with phospho-S36 or phosphotyrosine-specific Abs. Aggregation of FcεRI by Ag induced tyrosine and S36 phosphorylation on p66Shc, indicating that p66Shc may be involved in FcεRI-signaling pathway. FcεRI-dependent S36 phosphorylation was also observed in the transfectants expressing the mutant lacking YY239/240 and Y317 (p66S36Y317A) (Fig. 4B). Of note, p66Shc was found to be basally tyrosine phosphorylated in RBL-2H3 cells, as previously demonstrated for the p52 isoform (31), as well as on S36.

Overexpression of p66Shc suppresses FcεRI-mediated Ca2+ mobilization and degranulation To determine whether the observed differences in Ag-induced responses between p66Shc−/− and control BMMC could be accounted for by a negative regulation of FcεRI signaling by p66Shc, we first measured FcεRI-induced β-hexosaminidase release in the RBL-2H3 transfectants. Cells were stimulated by IgE/Ag or the Ca2+ ionophore A23187. Ag-induced β-hexosaminidase release was suppressed in p66Shc-expressing cells compared with control cells. The suppression of Ag-induced β-hexosaminidase release was reversed by substitution of the S36 residue on p66Shc (Fig. 4C). A23187-induced β-hexosaminidase release was comparable among all of these cell lines, suggesting a specific role for p66Shc on FcεRI-mediated degranulation. Consistent with a critical role for Ca2+ elevation in mast cell degranulation and in agreement with the results obtained in p66Shc−/− BMMC, Ag stimulation of p66Shc-expressing RBL-2H3 cells resulted in a lower Ca2+ increase compared with control cells and cells expressing the p66ShcS36A mutant (Fig. 4D). These results demonstrated that p66Shc acts as a negative regulator of FcεRI-mediated degranulation and Ca2+ mobilization and that phosphorylation of serine 36 is required for this activity.

FcεRI-dependent activation of MAPKs and cytokine production are inhibited by p66Shc To evaluate the impact of p66Shc on FcεRI-mediated MAPK activation, we analyzed Erk, p38, and JNK activation using the levels of TNF-α and IL-6 mRNA were greater in p66Shc−/− BMMC compared with controls, even in the absence of stimulation.

FIGURE 2. Normal numbers but enhanced degranulation of p66Shc−/− peritoneal mast cells. A. Percentage of mast cells in the peritoneum of control (−/−) or p66Shc−/− (−/−) mice, as assessed by flow cytometry using PE-conjugated anti-c-Kit Abs. Data are presented as mean ± SD (n = 4; six mice/experiment). Representative fluorescence bar graphs of surface c-Kit are also shown. B. Peritoneal cells were obtained from control (+/+), −/+ or p66Shc−/− (−/−) mice and cultured in 30% WEHI-3B-conditioned medium for 48 h. Cells were untreated (−) or treated with anti-DNP IgE (1 μg/ml) for 3 h and then stimulated with 20 ng/ml DNP-ALB. Ag-induced degranulation was determined by flow cytometry, as described in Materials and Methods. Data are presented as fold increase ± SD of mean fluorescence of p66Shc−/− versus control cells, with the mean fluorescence of untreated control cells taken as 1 (n = 3; six mice/experiment). *p < 0.01.
FIGURE 3. Enhanced Ag-induced responses in p66Shc\(^{-/-}\) BMMC. A, Analysis of FcεRI-mediated β-hexosaminidase release in supernatants of BMMC from control (+/+) or p66Shc\(^{-/-}\) (\(2/2\)) mice (left panel). Cells were left unstimulated (-) or sensitized with anti-DNP IgE (0.15 or 1\(\mu\)g/ml) and then stimulated with the indicated concentrations of DNP-ALB (Ag). Data are presented as percentage ± SD of β-hexosaminidase activity in supernatants versus total activity (n = 3; eight mice/experiment, n = 4 from control [+/-] or n = 4 from p66Shc\(^{-/-}\) [\(2/2\)] mice). *p < 0.05, **p < 0.01, ***p < 0.001 versus control BMMC. Analysis of FcεRI-mediated degranulation of BMMC from control or p66Shc\(^{-/-}\) mice nucleofected with pEGFP-C1 vector alone (WT GFP, knockout [KO] GFP) or cotransfected with pEGFP-C1 vector and the pcDNA3-p66Shc expression construct (KO p66) (middle and right panels). Immunoblot analysis with anti-Shc Abs of control or p66Shc\(^{-/-}\) BMMC that were nucleofected (WT GFP, KO GFP, KO p66) or not (WT, KO) (middle panel). Cells were sensitized with anti-DNP IgE (1\(\mu\)g/ml) and then stimulated with 20 ng/ml of DNP-ALB (Ag). Degranulation was measured by flow cytometry using PE-labeled Annexin V (right panel). Data are mean ± SD of PE fluorescence intensity of live GFP+ cells with the mean fluorescence intensity of Ag-stimulated control cells (WT GFP) taken as 100% (n = 2). *p < 0.05, **p < 0.001 versus WT GFP. B, Ca\(^{2+}\) mobilization in control (+/+) and p66Shc\(^{-/-}\) (\(2/2\)) BMMC following Ag stimulation. Intracellular Ca\(^{2+}\) concentration was determined in Fura-2–loaded BMMC sensitized with anti-DNP IgE (1\(\mu\)g/ml). A representative experiment is shown (left panel). The arrows indicate the time of addition of DNP-ALB (1\(\mu\)g/ml) (Ag) or thapsigargin (2\(\mu\)M) (TG) (n = 3; four mice/experiment). The bar graphs show the intracellular Ca\(^{2+}\) concentration in BMMC from control (+/+) or p66Shc\(^{-/-}\) (\(2/2\)) mice treated with IgE alone (IgE) or treated with IgE+DNP-ALB (1\(\mu\)g/ml) (Ag) (right panel). Intracellular Ca\(^{2+}\) concentrations were calculated using Fura-2 software, as described in Materials and Methods. Data are mean ± SD of PE fluorescence intensity of live GFP+ cells with the mean fluorescence intensity of Ag-stimulated control cells (WT GFP) taken as 100% (n = 2). **p < 0.01, ***p < 0.001 versus WT GFP. B, Ca\(^{2+}\) mobilization in control (+/+) and p66Shc\(^{-/-}\) (\(2/2\)) BMMC following Ag stimulation. Intracellular Ca\(^{2+}\) concentration was determined in Fura-2–loaded BMMC sensitized with anti-DNP IgE (1\(\mu\)g/ml). A representative experiment is shown (left panel). The arrows indicate the time of addition of DNP-ALB (1\(\mu\)g/ml) (Ag) or thapsigargin (2\(\mu\)M) (TG) (n = 3; four mice/experiment). The bar graphs show the intracellular Ca\(^{2+}\) concentration in BMMC from control (+/+) or p66Shc\(^{-/-}\) (\(2/2\)) mice treated with IgE alone (IgE) or treated with IgE+DNP-ALB (1\(\mu\)g/ml) (Ag) (right panel). Intracellular Ca\(^{2+}\) concentrations were calculated using CA Cricket Graph III software, as described in Materials and Methods. Data are mean ± SD of PE fluorescence intensity of live GFP+ cells with the mean fluorescence intensity of Ag-stimulated control cells (WT GFP) taken as 100% (n = 2). **p < 0.01, ***p < 0.001 versus WT GFP. C, BMMC from control (+/+) or p66Shc\(^{-/-}\) (\(2/2\)) mice were left untreated (-) or were sensitized with IgE-anti-DNP (1\(\mu\)g/ml) overnight at 37˚C and challenged with DNP-ALB (100 ng/ml) for 5 min at 37˚C (Ag). Total cell lysates were analyzed by immunoblot with anti-P-Akt or anti-P-Syk Abs. Alternatively, postnuclear supernatants were immunoprecipitated using anti-LAT Ab and analyzed by immunoblot with anti–P-Tyr Ab. Stripped filters were immunoblotted with anti-Akt, anti-Akt, or anti-LAT Abs as loading controls. Densitometric analysis of Akt, Syk, and LAT phosphorylation was performed, and the relative protein phosphorylation is indicated (n = 2). P-Akt, p = 0.04 +/+/Ag versus -/-Ag; P-Syk, p = 0.23 +/+/Ag versus -/-Ag; P-LAT, p = 0.01 +/+/Ag versus -/-Ag. D, BMMC from control (+/+) or p66Shc\(^{-/-}\) (\(2/2\)) mice were treated as in C, and total cell lysates were analyzed by immunoblot with anti-P-Erk, anti-P-Jnk, or anti-P-p38 Abs. Stripped filters were immunoblotted with anti-Erk, anti-p38, or anti-Jnk Abs.
phosphospecific Abs. Overexpression of p66Shc correlated with a significant decrease in Ag-induced Mek, Erk, JNK, and p38 activation. This activity is largely dependent on S36 residue, because no inhibition was observed in cells expressing the p66ShcS36A mutant (Fig. 4E).

NF-κB activity is regulated by IκBα, which, following Ag stimulation, is phosphorylated by the activated IκB kinase (IKK) and targeted for proteosomal degradation, allowing NF-κB translocation to the nucleus. Because NF-κB regulates the expression of many proinflammatory cytokines, such as TNF-α and IL-6, in mast cells (32), we assessed the activity of IKK in the RBL-2H3 transfectants stimulated with IgE/Ag, using phosphospecific Abs. Moreover, we quantified the mRNA levels of TNF-α and IL-6 by real-time RT-PCR. We found that IKK was not activated/phosphorylated in the p66Shc transfectant (Fig. 4F). Furthermore, the levels of TNF-α and IL-6 mRNA were significantly lower in p66Shc-expressing cells compared with control cells and cells expressing the p66ShcS36A mutant (Fig. 4G). These results indicated that p66Shc is likely to suppress expression of various cytokines by inhibiting FceRI activation of the MAP family kinases and NF-κB.

p66Shc forms a complex with LAT and SHIP-1 in response to FcεRI engagement by IgE–Ag complexes

To determine the mechanism responsible for the inhibitory effects of p66Shc on FcεRI signaling observed in RBL-2H3 transfectants, we examined the proteins that associate with p66Shc following Ag stimulation of FcεRI. RBL transfectants were left untreated or were treated with IgE/Ag, and total cell lysates were immunoprecipitated with anti-p66Shc specific Ab. The immunoprecipitates were analyzed by immunoblotting with anti–phosphotyrosine Ab. p66Shc was found to associate with a number of phosphoproteins before and following IgE/Ag stimulation (Fig. 5A, left panel). The phosphoproteins of ~130, 72, and 37 kDa (arrows) were subsequently identified by immunoblot analysis of the stripped filters with specific Abs, such as SHIP1, Syk, and LAT, respectively (Fig. 5A). Ag stimulation induced a strong increase in the phosphorylation status of SHIP1, Syk, and LAT associated with p66Shc (Fig. 5A). The association of p66Shc with SHIP1 and LAT was confirmed in reciprocal coimmunoprecipitation experiments (Fig. 5B). Interestingly, the Ag-dependent phosphorylation of LAT and SHIP1, but not of Syk, was reduced in p66Shc-expressing cells compared with control transfectants (Fig. 5C), suggesting that p66Shc dampens FcεRI signaling downstream of Syk. Because SHIP1 is known to associate with p52Shc in response to Ag stimulation (33), we asked whether SHIP1 preferentially associates with p66Shc or p52Shc. To address this point, all Shc isoforms were immunoprecipitated from control or p66Shc-expressing cells; the latter were previously immunodepleted or not of p66Shc. The association of SHIP-1 with Shc was drastically reduced in cells previously immunodepleted of p66Shc (Fig. 5D). Consistent with this finding, the levels of p52Shc found in SHIP1-specific immunoprecipitates were reduced in p66Shc-expressing cells compared with control transfectants (Supplemental Fig. 2). Together, these data indicated a preferential interaction of SHIP1 with p66Shc.

In addition to SHIP1 and Syk, p52Shc was reported to positively regulate multiple-receptor signaling by interacting with Gab-1 and Grb2 (34). To understand whether the two isoforms might compete for binding with these signaling mediators, we analyzed the interaction of p52Shc with Gab-1 and Grb2 following Ag stimulation. The presence of p66Shc decreased the interaction of p52Shc with Gab-1 (Supplemental Fig. 2), suggesting that p66Shc may affect the Fyn–Gab–PI3K-amplification pathway, in addition to the principal pathway orchestrated by LAT. Hence, p66Shc has the ability to prevent the interaction of p52Shc with key signaling mediators, thereby attenuating downstream signals.

Interestingly, as opposed to lymphocytes, we found that p66Shc did not alter p52Shc binding to Grb2 (Supplemental Fig. 2), suggesting that the impairment in Erk activation observed in p66Shc-expressing cells involves a mechanism other than competitive inhibition of Grb2 recruitment by p52Shc.

Assembly of the p66Shc–SHIP1–LAT complex is dependent on p66Shc phosphorylation on YY239/240 and Y317

To investigate whether phosphorylation of p66Shc on YY239/240 and Y317 or on S36 is required for the formation of the p66Shc–SHIP1–LAT complex, we examined the proteins that associate with p66ShcYY239/240F or p66ShcSA following Ag stimulation of FcεRI. A weak association between p66ShcYY239/240F and SHIP1 and no association between p66ShcSA and Syk or phospho-LAT was observed, indicating that phosphorylation of these residues is required for formation of the complex (Fig. 5A). As opposed to the tyrosine residues, the S36 residue was dispensable for the formation of the complex, because the p66ShcSA mutant was found to associate with SHIP1, LAT, and Syk (Fig. 5A), similar to p66Shc. Furthermore, using a GST fusion protein containing the CH2 domain of p66Shc, as such or phosphorylated on the S36 residue, we found that the association of p66Shc with Syk involves the CH2 domain and is independent of S36 phosphorylation (Supplemental Fig. 3). Hence, formation of the p66Shc–SHIP1–LAT complex depends on the presence of phosphorylated YY239/240 and Y317, but not on the presence of phospho-S36, on p66Shc.

Recruitment of SHIP1 to the plasma membrane induced by p66Shc results in impairment of FcεRI signaling

SHIP1 functions as a negative regulator of cytokine and receptor signaling in immune cells by controlling the levels of PI3,4,5P3; as such, it blocks the recruitment and activation of PI-containing proteins (e.g., Akt/PKB, PDK-1, and Btk) (35). Moreover, SHIP-
FIGURE 4. p66Shc overexpression results in impaired FcεRI-mediated responses in RBL-2H3 cells. A, Immunoblot analysis using anti-Shc Abs of total cell lysates from RBL-2H3 cells stably transfected with the vector pcDNA3 (pc) or expression vectors encoding WT p66Shc (p66), a p66Shc mutant carrying a serine-to-alanine mutation at position 36 (p66SA), or a p66Shc mutant carrying tyrosine-to-phenylalanine substitutions at position 239/240 and 317 (p663F). Immunoblot analysis of total cell lysates from control (+/+ or p66Shc−/− (−/−)) BMMC is also shown. B, Immunoblot analysis of tyrosine or S36 phosphorylation on p66Shc-specific immunoprecipitates of RBL-2H3 cells expressing p66Shc or p66Shc3F. Cells were left untreated (−) or were primed overnight with anti-DNP IgE (1 μg/ml) and then stimulated with DNP-ALB (1 μg/ml) for 3 min at 37°C (Ag). C, Analysis of FcεRI-mediated β-hexosaminidase release in supernatants of transfectants. RBL-2H3 cells transfectants were left untreated (−) or were cultured overnight in the presence of anti-DNP IgE (200 ng/ml) and then stimulated with 20 ng/ml DNP-ALB (Ag) or A23187 (500 ng/ml). Data are percentage ± SD of β-hexosaminidase activity in supernatants versus total activity, with the release induced by A231187 taken as 100% (n = 6). *p < 0.001. D, Ca2+ mobilization in control cells (pc) and in cells expressing p66Shc (p66) or the S36 mutant (p66SA), stimulated as above. Intracellular Ca2+ concentrations were determined in Fura-2-loaded cells. The arrows indicate the time of addition of DNP-ALB (Ag; 20 ng/ml) or thapsigargin (TG; 2 μM). A representative experiment is shown (n = 3). E, MAPK activation following Ag stimulation. Control cells (pc) and cells expressing p66Shc (p66) or S36 mutant (p66SA), were left untreated (−) or were primed overnight with anti-DNP IgE (1 μg/ml) and then stimulated with DNP-ALB (1 μg/ml) at 37°C for 3–5 min (Ag). Total cell lysates were analyzed by immunoblotting with anti–phospho-Erk (P-Erk), anti–phospho-Mek (P-Mek), anti–phospho-Jnk (P-Jnk), or anti–phospho-p38 (P-p38) Abs. Stripped filters were immunoblotted with anti-Erk or anti-actin Abs as loading controls. The bar graphs represent the quantitation, by laser densitometry, of the relative levels of Erk1/2, Mek, Jnk, and p38 phosphorylation at 3 min of stimulation (n = 3). F, Total cell lysates of control cells (pc) and cells expressing p66Shc (p66) or S36 mutant (p66SA), and stimulated as above were analyzed by immunoblotting with anti–phospho-IKK (P-IKK) Abs. Stripped filters were immunoblotted with anti-Erk or anti-actin Abs as loading controls. The bar graphs represent the quantitation, by laser densitometry, of the relative levels of IKK phosphorylation at 3 min of stimulation (n = 3). G, Quantification, by real-time RT-PCR, of the levels of IL-6 or TNF-α mRNA in control cells (pc) and cells expressing p66Shc (p66) or S36 mutant (p66SA), sensitized as in E, and stimulated for 1 h with DNP-ALB (1 μg/ml) at 37°C (Ag). Transcript levels...
1 negatively regulates immunoreceptor signaling independently of its phosphatase activity by antagonizing Grb2 and Ras/MAPK activation by p52Shc (36), as well as by recruiting Dok-1 and RasGAP (37, 38). In BMMC, SHIP1 modulates stem cell factor- and IgE/Ag-induced degranulation, Ca²⁺ influx, and Akt/PKB activation (39, 40). Interestingly, it was proposed that SHIP1 plays a key role in setting the threshold for degranulation, because BMMC derived from SHIP1−/− mice undergo massive degranulation when stimulated with IgE alone (39). Because BMMC lacking p66Shc are reminiscent of BMMC from SHIP1−/− mice, and p66Shc forms a complex with SHIP1, we asked whether p66Shc stabilizes/activates SHIP1 at the plasma membrane following FcεRI engagement, thereby inhibiting receptor signaling.

FIGURE 5. p66Shc interacts with SHIP-1 and LAT in response to aggregation of FcεRI. Control cells (pc) and cells expressing p66Shc (p66) or its mutated versions (p66SA and p663F) were left untreated (−) or were primed overnight with anti-DNP IgE (1 µg/ml) and then treated with DNP-ALB (1 µg/ml) for 3 min at 37°C (Ag). Cells were lysed in 3% Triton X-100 lysis buffer and immunoprecipitated with Abs that recognize p66Shc only (anti-p66Shc) (A), all ShcA isoforms (anti-panShcA) (D), or anti-LAT, anti-SHIP1, and antiphosphotyrosine (P-Tyr) Abs (B, C). Alternatively, total cell lysates were analyzed by immunoblot with anti–P-Syk Abs (C). The immunoblot analysis was performed using antiphosphotyrosine (P-Tyr), anti-SHIP1, anti-LAT, anti-Syk, or anti-Shc Abs. Representative experiments are shown (n = 4). In D, lysates of p66Shc-expressing cells were immunodepleted (pre anti-p66Shc) or not of p66Shc with an anti-p66Shc Ab before performing anti-panShcA–specific immunoprecipitates. Note that anti-SH2 Abs immunoprecipitate all Shc isoforms (p46, p52, and p66). Lysates from p66Shc-expressing cells left untreated or treated with IgE/Ag were immunoprecipitated with control IgG. IgG did not pull down Shc, LAT, SHIP1, or protein phosphorylated on tyrosine residue, as assessed by immunoblotting with anti–P-Tyr, anti-Shc, anti-LAT, or anti-SHIP1 Abs (data not shown).
distribution of the GFP-PH domain was cytosolic in unstimulated cells. FceRI stimulation by IgE/Ag induced a robust membrane localization of GFP-PH in control and in p66ShcS36A cells but not in p66Shc-expressing cells (Fig. 6A), indicating that p66Shc promotes SHIP1 activation. In agreement with this finding, Ag-induced Akt phosphorylation was significantly decreased in p66Shc-expressing cells compared with control cells. This activity of p66Shc was found to depend on S36 phosphorylation, because p66ShcS36A mutant was comparable to control cells (Fig. 6B), indicating that p66Shc promotes SHIP1 activation.

Therefore, we analyzed membrane fractions prepared from p66Shc or control RBL-transfectants for the presence of SHIP1. At variance with control cells, in which SHIP1 was lost from rafts in response to Ag, membranes from p66Shc-expressing cells were still enriched in SHIP1 following Ag stimulation, supporting the notion that p66Shc might stabilize SHIP1 at the plasma membrane (Supplemental Fig. 4A). Of note, p66Shc did not affect the interaction of SHIP1 with FceRI (Supplemental Fig. 4B). Hence, p66Shc suppresses FceRI signaling, at least in part, by promoting the recruitment (through its phosphorylated YY239/240 and Y317 residues) of SHIP1 close to the membrane near its substrate and its phosphorylation/activation, a function that is dependent on S36 phosphorylation (Fig. 7).

**Discussion**

The results of this study showed that p66Shc is expressed in BMMCs and that, when tyrosine and serine phosphorylated in response to FceRI stimulation, it plays a critical role in allergen-mediated activation of mast cells. Comparing p66Shc−/− with control BMMCs, we found that p66Shc−/− BMMC display enhanced allergen-induced degranulation, which was paralleled by increased Ca2+ mobilization, MAPK activation, and cytokine expression, suggesting a role for p66Shc in setting the FceRI activation threshold and in limiting Ag-dependent mast cell activation. Of note, mast cell development and expansion, which crucially depend on signals emanating from IL-3R, are not affected by p66Shc deficiency, indicating a specific role for p66Shc as a negative regulator of FceRI signaling. Consistent with these findings, p66Shc−/− BMMC seem to be basally activated, as indicated by the enlarged granules, the spontaneous degranulation, and the enhanced basal expression of TNF-α and IL-6, which are likely to result from enhanced tonic signaling by FceRI. Interestingly, the enhanced degranulation displayed by p66Shc−/− BMMC is not unique to FceRI; it was also observed in response to other agonists, suggesting the interesting possibility that, in addition to its activity as attenuator of FceRI signaling, p66Shc might be a novel regulator in the pathway that controls the degranulation process.

The function of p66Shc as a negative regulator of mast cell activation is consistent with our finding that p66Shc−/− mice spontaneously developed lupus-like autoimmune disease characterized by elevated numbers of activated mast cells in the dermis of alopecic skin areas (19). Interestingly, p66Shc−/− mice share some phenotypic characteristics with SHIP1−/− mice, including splenomegaly, extramedullary hematopoiesis, and mast cell hyperplasia in the skin (41, 42). The systemic mast cell hyperplasia described in SHIP1−/− mice was shown to correlate with high levels of IL-6 and TNF-α secreted by mast cells in vivo (42), which is consistent with the negative role of SHIP1 in the regulation of IgE/Ag-induced IL-6 and TNF-α production in mast cells in vitro (43). Our finding that p66Shc−/− BMMC have elevated levels of IL-6 and TNF-α mRNA, even in the absence of stimulation, suggests that, similar to SHIP1, p66Shc is implicated in the control of mast cell homeostasis.

It is generally accepted that mast cells can influence adaptive immunity through the release of inflammatory mediators (44). Recently, mast cell-derived particles containing TNF-α were identified and suggested to function as extracellular chaperones responsible for carrying signaling molecules from the periphery to the draining lymph nodes, where the interaction between lymphocytes and APCs occurs (45). In addition, evidence of a role for mast cells in Ag presentation was provided by Kambayashi et al. (46). In this study, mast cells were shown to be able to act as an Ag reservoir for T cells after Ag capture through the FceRI receptors. These results, in addition to the documented expression of TLRs and MHC molecules on mast cells (44), identified a key role for mast cells in connecting innate immunity with Ag-specific adaptive immunity. It is tempting to speculate that hyperactive mast cells would contribute to promote and/or support the documented basal activation of T and B lymphocytes in p66Shc−/− mice. Further studies are required to assess whether mast cells contribute to the autoimmune disease observed in p66Shc−/− mice.

To gain further insight into the molecular mechanism governing the negative role of p66Shc in mast cell activation, we overexpressed...
it in the RBL-2H3 mast cell line. Consistent with its role as a negative regulator of Ag-induced responses in BMMC, over-expression of p66Shc suppressed Ag-induced degranulation, Ca²⁺ mobilization, MAPK activation, and cytokine expression. These effects were found to be dependent on S36 phosphorylation, because they were not reproduced by the p66Shc mutant lacking this residue. In T cells, the mechanism underlying the antimitogenic function of p66Shc has been related to the ability of p66Shc to competitively inhibit the recruitment of p52Shc to the activated TCR, as a result of its higher affinity for ZAP-70, and to sequester the Grb2–Sos complexes in a nonfunctional state, thereby inhibiting Ras/MAPK activation. The S36 residue, as well as the three tyrosine residues in the CH1 domain, were found to be essential for these functions of p66Shc (17). In mast cells, p66Shc does not competitively inhibit the interaction of p52Shc with Grb2 in response to FcεRI engagement, while inhibiting, albeit to a limited extent, the interaction of p52Shc with SHIP1, Gab1, and Syk (data not shown, Supplemental Fig. 2). The competition of p66Shc with p52Shc for Grb2 in response to FceRI engagement, while inhibiting, albeit to a limited extent, the interaction of p52Shc with SHIP1, Gab1, and Syk (data not shown, Supplemental Fig. 2). The competition of p66Shc with p52Shc for Grb2 in response to FceRI engagement, while inhibiting, albeit to a limited extent, the interaction of p52Shc with SHIP1, Gab1, and Syk (data not shown, Supplemental Fig. 2). The competition of p66Shc with p52Shc for Grb2 in response to FceRI engagement, while inhibiting, albeit to a limited extent, the interaction of p52Shc with SHIP1, Gab1, and Syk (data not shown, Supplemental Fig. 2).

We found that p66Shc interacts with SHIP1, LAT, and Syk, even in the absence of Ag, and that phosphorylation of these molecules is further enhanced following Ag stimulation, suggesting that p66Shc may favor the recruitment and/or the stabilization/activation of SHIP1 into the FceRI signaling complex. In mast cells, it was reported that SHIP1 is recruited by LAT following FceRI engagement (5) and that it negatively regulates FceRI signaling by controlling the levels of PI3,4,5P₃ and, independently of its phosphatase activity, by antagonizing Grb2 and p52Shc signaling to Ras/MAPK (5, 36), as well as by recruiting Dok-1 and RasGAP (37, 38). Although SHIP1 becomes tyrosine phosphorylated in response to FceRI stimulation, its phosphatase activity does not seem to be regulated by phosphorylation but rather by its localization at the plasma membrane (35). In this study, we showed that p66Shc, in addition to interacting with SHIP1, interacts with LAT and Syk, suggesting that it may function as an adapter to maintain SHIP1 close to the membrane near its lipid substrates. Interestingly, the three phosphorylatable tyrosine residues in the CH1 domain of p66Shc are required for SHIP1 and Syk recruitment. In contrast, the S36 residue, although dispensable for formation of the p66Shc–LAT–Syk–SHIP1 complex, is required for SHIP1 activation, because Ag-induced Akt phosphorylation and cellular localization of the GFP-tagged PH domain of Akt/PKB were comparable in control cells and in cells expressing the p66SA mutant. Furthermore, because LAT and SHIP1 Ag phosphorylation are reduced in p66Shc-expressing cells compared with control cells and cells expressing the p66SA mutant, it can be hypothesized that, when phosphorylated on S36, p66Shc may fold in a conformation that prevents effective LAT and SHIP1 phosphorylation, thereby attenuating downstream signals. Our results suggest a model in which, following FceRI stimulation, p66Shc interacts through its CH2 domain with Syk, which, in turn, is recruited to the activated receptor, and through its phosphorylated CH1 domain with SHIP1 which is bound to phospho-LAT, thus connecting the LAT and FceRI signaling complexes. Our results suggest that the interaction between p66Shc and SHIP1 might stabilize and/or assist the proper localization of the phosphatase into the complex, which would result in inhibition of PIP₃-dependent signaling. Moreover, SHIP1 could impair PIP₃-independent activation of MAPKs through its adaptor function (Fig. 7).

Acknowledgments
We thank Marina De Bernard, Doreen Cantrell, Maurizio Orlandini, and Andreas Wack for the generous gift of valuable reagents, Eugenio Paccagnini for advice on toluidine blue staining of BMMC, Francesca Finetti for...
help with confocal microscopy, and Sonia Grassini for technical assistance. Giulia De Falco is also gratefully acknowledged.

Disclosures

The authors have no financial conflicts of interest.

References

Supplementary figure 1. Enhanced FcεRI-independent degranulation but not Ca\textsuperscript{2+} mobilization in p66Shc BMMC

A. Analysis of β-hexosaminidase release in supernatants of BMMC from control (+/+, white bars) or p66Shc\textsuperscript{-/-} (-/-, black bars) mice. Cells were untreated (-) or treated with A23187 (500 ng/ml), ATP (3 mM) or Thapsigargin (TG; 2 μM) for 20 min 37°C. Data are presented as percentage ± SD of β-hexosaminidase activity in supernatants versus total activity (n=2, >4 mice/experiment, ***P≤0.001).

B. Analysis of Ca\textsuperscript{2+} mobilization in control (+/+, white bars) or p66Shc\textsuperscript{-/-} (-/-, black bars) BMMC. Cells were treated with A23187 (500 ng/ml), ATP (3 mM) or thapsigargin (2 μM) (TG) at 37°C for 10 min. Data are presented as mean of peak value ± SD of intracellular Ca\textsuperscript{2+} concentration (n=5, > 4 mice/experiment).

Supplementary figure 2. p66Shc compete with p52Shc for the interaction with SHIP1 and Gab1, but not with Grb2.

Control cells (pc) and cells expressing either p66Shc (p66) or its mutated versions (p66\textsuperscript{SA}) were either left untreated (-) or primed overnight with anti-DNP IgE (1 μg/ml) and then treated with DNP-ALB (1 μg/ml) for 3 min at 37°C (Ag). Cells were lysed in 1% Triton lysis buffer and postnuclear supernatants immunoprecipitated with anti-Grb-2, anti-SHIP1 and anti-Gab1 antibodies. The immunoblot analysis was performed using anti-Shc antibodies. The histograms show the results of the densitometric analysis of p52Shc associated with Grb2, SHIP1 or Gab1 following antigen stimulation.

Supplementary figure 3. The CH2 domain of p66Shc mediates the association with Syk independently of S36 phosphorylation.
Immunoblot analysis with anti-Syk, anti-phospho-S36 and anti-CH2 antibodies of the proteins recovered from in vitro binding assays of a control cell lysate using a CH2-GST fusion protein (37kDa). The CH2-GST fusion protein was either unphosphorylated (\(\sim\)) or phosphorylated (P) in vitro using recombinant Erk (n>3). The GST-fusion protein input was checked by immunoblot with anti-CH2 antibody.

**Supplementary figure 4. p66Shc stabilizes SHIP1 at the plasma membrane**

A. Immunoblot analysis with anti-SHIP1 antibodies of low-density rafts enriched fractions. A total cell lysate prepared from unstimulated p66 cells is also shown. Control cells (pc) and cells overexpressing p66Shc (p66) were either left untreated (-), or primed overnight with anti-DNP IgE (1 \(\mu\)g/ml) and stimulated with DNP-ALB (1 \(\mu\)g/ml) at 37°C for 3 min (Ag). The low-density rafts enriched fractions were analyzed by anti-SHIP1 and anti-Shc immunoblotting. After stripping the filter was reprobed with anti-Thy-1 (a lipid raft resident protein) antibodies as loading control. B. Control cells (pc) and cells expressing either p66Shc (p66) or its mutated versions (p66\(^{SA}\)) were stimulated as in A and lysed in 1% (v/v) Triton X-100. Postnuclear supernatants were immunoprecipitated using anti-Fc\(\varepsilon\)RI\(\gamma\) antibodies. The immunoblot analysis was performed using anti-SHIP1 antibodies. Stripped filters were immunoblotted with anti-Fc\(\varepsilon\)RI\(\gamma\) antibody as loading control.
Figure S1
Figure S2
GST-CH2

/  P

64—

WB anti-Syk

37—

WB anti-P-Ser36

37—

WB anti-CH2

Figure S3
Figure S4

A

Lipid rafts

<table>
<thead>
<tr>
<th>Lys</th>
<th>pc</th>
<th>Ag</th>
<th>p66</th>
<th>Ag</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td></td>
<td></td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

WB anti-SHIP1

WB anti-Shc

WB anti-Thy1

B

IP anti FcεRIγ

<table>
<thead>
<tr>
<th>pc</th>
<th>p66</th>
<th>p66SA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>Ag</td>
<td>Ag</td>
</tr>
</tbody>
</table>

WB anti-SHIP1

WB anti-FcεRIγ

Figure S4