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SH2 Domain-Containing Inositol Polyphosphate 5’-Phosphatase Is the Main Mediator of the Inhibitory Action of the Mast Cell Function-Associated Antigen

Rong Xu,* Jakub Abramson,* Mati Fridkin, † and Israel Pecht* *

The mast cell function-associated Ag (MAFA) is a type II membrane glycoprotein originally found on the plasma membrane of rat mucosal-type mast cells (RBL-2H3 line). A C-type lectin domain and an immunoreceptor tyrosine-based inhibitory motif (ITIM) are located in the extracellular and intracellular domains of MAFA, respectively. MAFA clustering has previously been shown to suppress the secretory response of these cells to the FceRI stimulus. Here we show that the tyrosine of the ITIM undergoes phosphorylation, on MAFA clustering, that is markedly enhanced on pervanadate treatment of the cells. Furthermore, the Src homology 3 domain of the protein tyrosine kinase Lyn binds directly to a peptide containing nonphosphorylated MAFA ITIM and PAAP motif. Results of both in vitro and in vivo experiments suggest that Lyn is probably responsible for this ITIM phosphorylation, which increases the Src homology domain 2 (SH2) affinity of Lyn for the peptide. In vitro measurements established that tyrosine-phosphorylated MAFA ITIM peptides also bind the SH2 domains of inositol 5’-phosphatase (SHIP) as well as protein tyrosine phosphatase-2. However, the former single domain is bound 8-fold stronger than both of the latter. Further support for the role of SHIP in the action of MAFA stems from in vivo experiments in which tyrosine-phosphorylated MAFA was found to bind primarily SHIP. In RBL-2H3 cells overexpressing wild-type SHIP, MAFA clustering causes markedly stronger inhibition of the secretory response than in control cells expressing normal SHIP levels or cells overexpressing either wild-type protein tyrosine phosphatase-2 or its dominant negative form. In contrast, on overexpression of the SH2 domain of SHIP, the inhibitory action of MAFA is essentially abolished. Taken together, these results suggest that SHIP is the primary enzyme responsible for mediating the inhibition by MAFA of RBL-2H3 cell response to the FceRI stimulus. The Journal of Immunology, 2001, 167: 6394–6402.

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3 Abbreviations used in this paper: MAFA, mast cell function-associated Ag; RBL-2H3, rat basophilic leukemia 2H3 cells; PTK, protein tyrosine kinase(s); PTp, protein tyrosine phosphatase(s); SH2, SH2 domain-containing protein tyrosine phosphatase-2; SH2 domain-containing protein tyrosine phosphatase-1 (SHIP-1); SHIP, SH2 domain-containing inositol polyphosphate 5’-phosphatase; PLC-γ, phospholipase C-γ; SH2, Src homology 2 domain; SH3, Src homology 3 domain; ITAM, immune receptor-based tyrosine activation motif; ITIM, immune receptor-based tyrosine inhibition motif; SPR, surface plasmon resonance; HBS, HEPES-buffered saline; PIP3, phosphatidylinositol 3,4,5-trisphosphate; DNP11-BSA, BSA conjugated with an average of 11 molecules of 2,4-dinitrobenzene; rSyk, recombinant GST murine Syk; MAFA-IC, intracellular domain of MAFA.

More recently, the genes encoding the human and mouse MAFA homologues were cloned and sequenced. The human homologue was expressed, at least at the RNA level, by NK cells as well as by basophils. It also possesses an intracellular ITIM sequence, VIYSM1, and an extracellular C-lectin-like carbohydrate recognition domain (6). The mouse MAFA homologue was recently renamed as the killer cell lectin-like receptor G1, because it is expressed by NK cells and by activated CD8 T cells but not by mast cells and is up-regulated after expression of class I MHC-encoded molecules (7, 8). An ITIM is also present in the cytoplasmic tail of mouse MAFA with a sequence (SIYSTL) identical with that of rat MAFA. The high degree of sequence conservatism among the three species suggests that MAFA serves an important and evolutionary conserved immunological function that still needs to be resolved.

MAFA clustering on RBL-2H3 cells by its specific mAb (G63) suppresses the secretory response of the cell to the type I FcεRI stimulus (1). Functional studies have shown that MAFA clustering inhibits both the FcεRI-mediated hydrolysis of phosphatidylinositol and the transient rise in the intracellular concentration of free calcium ions ([Ca2+]i) but does not affect degranulation induced by the Ca2+ ionophore A23187. Therefore, it was concluded that MAFA interferes with the FcεRI coupling cascade upstream to phospholipase C-γ (PLC-γ) activation (1). Further studies have shown that in cell lysates, tyrosine-phosphorylated MAFA ITIM peptides bind two Src homology domain 2 (SH2)-containing phosphatases, SH2 domain-containing phosphatases, SH2 domain-containing protein tyrosine phosphatase-2 (SHIP-2) and SH2 domain-containing inositol 5’-phosphatase (SHIP) (9). Thus far, only the role of SHP-2 in the inhibitory function of MAFA has been investigated. Results of these studies suggest that SHP-2 is involved in the action of
MAFA because it causes a reduction in FceRI-induced Syk phosphorylation and activity (10). However, because SHP-2-mediated inhibition accounts only partially for the inhibitory effect of MAFA, it appears that SHP-2 involvement represents only one of the pathways that participate in the inhibitory activity of MAFA. Therefore, we investigated the role of SHP. Evidence was now obtained that MAFA is phosphorylated on its ITIM tyrosine by the protein tyrosine-kinase (PTK) Lyn. Tyrosine phosphorylated MAFA was then found to bind SHIP, thereby recruiting it to the plasma membrane of the cells, proximal to the SHP substrate phosphatidylinositol 3,4,5-trisphosphate (PIP₃). At the plasma membrane, SHIP presumably hydrolyzes PIP₃ and reduces its levels. This interferes with the FceRI stimulus-response-coupling cascade and leads to suppression of the secretory response of the mast cells. Measurements of the inhibitory action of MAFA on the secretory response of RBL-2H3 cells overexpressing either wild-type or mutated SHIP and SHP-2 further support the role of SHP as the primary mediator of the inhibitory function of MAFA.

Materials and Methods

Reagents

BSA conjugated with an average of 11 molecules of 2,4-dinitrobenzene (DNP₃,5-BSA) was prepared in our laboratory from fraction V (Sigma, St. Louis, MO) by reaction with 1-fluoro-2,4-dinitrobenzene (11). Sodium vanadate was purchased from Sigma. Protein G-Sepharose 4 Fast Flow beads and ECL detection reagents were from Amersham Pharmacia Biotech U.K. (Little Chalfont, U.K.). Protease inhibitor cocktail was from Calbiochem (Rishon Le Zion, Israel). The 2,4-dinitrobenzene-specific, IgE class mAb Ab₂ IgE preparation was described previously (12). F(ab')₂ of mAb 6G3 was prepared in our laboratory according to a previously described method (13) and conjugated to CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech) according to supplier’s instruction. Polyclonal Abs specific for phosphorytoine (PY-40) were prepared in our laboratory by rabbit immunization with a mixture of keyhole limpet hemocyanin-phosphotyrosine and tetanus toxoid-phosphotyrosine conjugates and purified as described earlier (14). Rabbit antisemun for a peptide having the 35-aa sequence of the intracellular domain of MAFA (MP35) was prepared in our laboratory by rabbit immunization with a keyhole limpet hemocyanin conjugate of the peptide (see below) prepared using m-maleimidobenzoyl-N-hydroxysuccinimide ester as cross-linker (peptide-protein (w/w) ratio, 1:2). Lyn- and Syk-specific rabbit polyclonal Abs that react with the 1–131 domain of Lyn and the 257–352 linker domain of Syk, respectively, were gifts of Dr. J. Cambier (National Jewish Center, Denver, CO). Polyclonal Abs specific for SHP (SH2 domain) were purchased from Upstate Biotechnology (Lake Placid, NY). The recombinant vaccinia viruses encoding 1) wild-type Lyn or Syk, 2) dominant negative forms of Lyn or Syk (15), 3) wild-type SHP, 4) a deletion mutant of SHP containing only its SH2 domain (16), 5) the wild-type form of SHP-2, and 6) SHP-2 with a point mutation in its catalytic site were kindly provided by Dr. J.-P. Kinet (Harvard University, Boston, MA).

Peptides

MP35, a peptide with a sequence corresponding to the 35-aa of the IC domain of MAFA (MADNSIYSTLELPAAPRVQDDSRWKVKAVLHR PCV), was synthesized by Prof. C. Gilon (The Hebrew University, Jerusalem, Israel). MAFA ITIM peptides nonphosphorylated (MADNSYSTLELC), tyrosine phosphorylated (MADNSYpSTLELC), phosphorylated on both tyrosine and serine (MADNSYpTpSTLELC), as well as phosphorylated only on its Y-2 serine residue (MADNSYpSTpSTLELC) were synthesized and conjugated to beads as previously described (9). For surface plasmon resonance (SPR) studies, the MAFA peptides LDSNYSLELC, LDSNYSpSTLELC, ELPAAPRVQDDSR, and DSNYSIYSTLELC were synthesized and biotinylated at their N termini as described (9).

Cell culture and stimulation

The rat mucosal-type mast cells of the RBL-2H3 line were originally obtained from Dr. R. Siragianian (National Institutes of Health, Bethesda, MD) supplemented with 10% FCS (Life Technologies), 2 mM glutamine, and combined antibiotics (Bio-Lab, Jerusalem, Israel) in a humidified atmosphere with 7% CO₂ at 37°C. Cells were detached by incubation with 10 mM EDTA (in DMEM) for 10 min at 37°C. Then, they were incubated (2 × 10⁶ cells/ml) with 2 µM ascidian fluid containing the monoclonal 2,4-dinitrobenzene-specific IgE (A₂ IgE) for 1–2 h at 37°C in DMEM and washed (three times) with Tyrode’s buffer (130 mM NaCl, 5 mM KCl, 1.4 mM CaCl₂, 1 mM MgCl₂, 5.6 mM glucose, 10 mM HEPES, 0.1% BSA, pH 7.4). MAFA was clustered by incubating the cells with G63 F(ab')₂ conjugated to Sepharose beads (1.5 mg Ab/ml beads); 50 × 10⁶ cells were incubated with 40 µl beads in 1 ml Tyrode’s buffer for 5 min. In controls, the cells were incubated with the same amount of Sepharose beads conjugated with BSA. Then, the cell samples were either stimulated with 100 ng/ml GST-murine Syk recombinant baculovirus (17) or left untreated as control, all at 37°C. Pervanadate used for cell treatment was always freshly prepared by mixing 50 µl 200 mM sodium vanadate with 20 µl 30% hydrogen peroxide in 930 µl deionized water, followed by 5 min incubation at room temperature. Cells (50 × 10⁶/sample) were incubated with 20 µl of this mixture in 1 ml Tyrode’s buffer for the indicated time at 37°C.

Vaccinia virus infection

RBL-2H3 cells were seeded in a 175-cm² flask (1 × 10⁶ cells/flask) or 96-well plates (7 × 10⁵ cells/well) and cultured overnight. Cells were washed once with DMEM, 5% FCS, kept in 5 ml (for flasks) or 50 µl (for plates) DMEM, 5% FCS, and infected with 5 PFU/cell recombinant vaccinia viruses control virus or control for 1.5 h at 37°C, with hand rocking of the flask at 15-min intervals. The medium was removed, 25 µl/flask or 100 µl/well DMEM/10% FCS were added, and culturing was continued for another 6–8 h at 37°C (15, 16).

Immunoprecipitation and immunoblotting

After the indicated treatments, 30 × 10⁶ cells/ml were sedimented by centrifugation and solubilized in lysis buffer (1% Triton X-100, 50 mM HEPES, 100 mM NaF, 10 mM EDTA, 2 mM sodium orthovanadate, 10% glycerol, 10 mM sodium pyrophosphate, 1/200 dilution of protease inhibitor cocktail set III, pH 7.4) on ice for 30 min. Lysates were centrifuged for 15 min at 15,000 × g and 4°C, and after precleaning by incubation with BSA-Sepharose for 1 h at 4°C, the postnuclear supernatants were treated with G63 F(ab')₂-conjugated Sepharose beads or by other specific Abs carried on protein G-Sepharose beads. After 2 h equilibration with rotation at 4°C, the beads were washed (three times) with ice-cold lysis buffer and once with PBS, and the bound proteins were eluted by boiling for 5 min in sample buffer. The eluted proteins were separated by SDS-PAGE, electrotransferred to nitrocellulose membranes, and blocked overnight at 4°C with a 0.1% gelatin solution (only for phosphotyrosine blotting) or 20 min at room temperature with 3% fat milk. This was followed by Western blotting with the specific Abs and detection by ECL.

In the experiments in which MAFA clustering was done by G63 F(ab')₂ conjugated to Sepharose beads, following the indicated treatments, the cells (50 × 10⁶/sample) and Sepharose beads were sedimented together by centrifugation and solubilized in 1% Triton X-100 lysis buffer (3 ml/sample) on ice for 10 min. The Sepharose beads were allowed to sediment by gravity and separated from the lysates. The beads were again suspended in 1 ml lysis buffer, sedimented by gravity and separated from the lysis buffer. The BSA-Sepharose beads were discarded, whereas the G63 F(ab')₂-Sepharose beads were kept for later immunolassociation. This lysis buffer was combined with original cell lysates and kept on ice for another 20 min; then it was centrifuged for 15 min at 15,000 × g and 4°C to separate the nuclear debris. MAFA was immunoinosolated with mAb G63 F(ab')₂-conjugated Sepharose beads from the postnuclear supernatants by 4 h equilibration with rotation at 4°C. To avoid loss of MAFA, in the MAFA aggregated samples, the same Sepharose beads used for aggregation were further used for the immunoinosolation.

In vitro peptide phosphorylation

Lyn or Syk was immunoprecipitated as described above from Ag-stimulated cell lysates (20 × 10⁶/0.7 ml). The beads carrying the proteins were washed (three times) by lysis buffer, once with 0.5 M LiCl, followed by washing once with the kinase assay buffer (20 mM HEPES, 100 mM NaCl, 5 mM MnCl₂, 10 mM MgCl₂, pH 7.5). The in vitro kinase reaction was started by adding 50 µl of the kinase assay buffer containing 10 Ci g⁻¹ cPAP, 4 µM ATP, and 2.5 µg intracellular domain of MAFA (MAFA-IC) peptide (MP35) to the above beads or 0.6 µg recombinant GST murine Syk (rSyk), produced by Spodoptera frugiperda (Sf) insect cells infected by GST-murine Syk recombinant baculovirus (17). The reaction was allowed to proceed for 5 min at 30°C with agitation and stopped by adding sample buffer. The solution was boiled for 5 min, and products were resolved by Tricine SDS-PAGE (16.5% T, 3% C, where T denotes the total percentage concentration of both acrylamide and bisacrylamide and C denotes the percentage concentration of the cross-linker relative to the total...
concentration $T_c$ (18), and the gel was dried and visualized by autoradiography.

**Affinity isolation by ITIM peptides**

RBL-2H3 cell lysates ($30 \times 10^3$ cells/ml sample) were precleared with Sepharose beads and incubated with the peptide-conjugated Sepharose beads ($40 \mu$l/ml lysate) for 2 h at 4°C with shaking. Beads were washed (four times) with ice-cold lysis buffer and once with PBS, and the bound proteins were eluted by boiling for 5 min with reducing SDS-sample buffer.

**SPR analysis**

Biotin or synthetic biotinylated MAFA peptides were immobilized on streptavidin-coated sensor chips (Sensor Chip SA; BIACore, Uppsala, Sweden) at 25°C, at a flow rate of 5 $\mu$lm/min in HEPES-buffered saline (HBS) (10 mM HEPES, 150 mM NaCl, 3.4 mM EDTA, 0.005% surfactant P20, pH 7.4). The concentration (density) of immobilized peptide was determined by the change in refractive index of the hydrogel (expressed in resonance units (RU)). All peptides used were bound at the level of $\sim$200–300 RU and the P-Y-ITIM + PAAP peptide also at the level of ~40RU (because of the high affinity of SHIP). Ligand binding was monitored after injection (250 $\mu$l/sample) of GST fusion proteins (9) (previously dialyzed against HBS) at different concentrations with a flow rate of 20 $\mu$lm/min in HBS running buffer. Dissociation was monitored during subsequent washing of the chip with HBS running buffer for either 1800 s (for SH2 and SH3 domain of Lyn) or 3600 s (SH2 domains of SHP-2 and SHIP) with a flow rate of 20 $\mu$lm/min. Chip regeneration was performed by injection of 10 $\mu$l HBS running buffer supplemented with 0.01% SDS. SPR measurements used the BIACore 2000 apparatus (Pharmacia Biotech, Uppsala, Sweden). All real time curves were best fitted by a two-state reaction model, and all kinetic and affinity parameters were calculated using BIASevaluation 3.0.2 software.

**Secretory response assay**

RBL-2H3 cells were plated in 96-well plates ($7 \times 10^3$/well) and cultured overnight at 37°C. After $6 \text{ h}$ of infection by control vaccinia virus or viruses containing either wild-type SHIP or a deletion mutant containing only its SH2 domain and saturated with A$_{lgE}$ (0.2 $\mu$g/ml), the cells were washed (three times) with Tyrode’s buffer and incubated in 50 $\mu$l Tyrode’s buffer with G63 F(ab$_2$)$_2$ (1 $\times 10^{-5}$M) for 5 min at 37°C. After that, 50 $\mu$l Tyrode’s buffer containing different Ag concentrations were added, and incubation was continued for 50 min at 37°C. Supernatant aliquots (15 $\mu$l) were then transferred to a separate plate, and 40 $\mu$l $\beta$-hexosaminidase substrate solution (1.3 mg/ml p-nitrophenyl-N-acetyl-$\beta$-d-glucosamine in 0.1 M citrate, pH 4.5) was added to the samples. The plate was then incubated for 60 min at 37°C, and the reaction was terminated by addition of 150 $\mu$l 0.2 M glycine solution, pH 10.7. The OD change caused by substrate hydrolysis was measured at 405 nm in an ELISA plate reader. Net secretion was calculated as percent of the total enzyme activity measured in 1% Triton X-100-lysed cells, and all these assays were done at least four times.

**Results**

**MAFA aggregation induces tyrosine phosphorylation of MAFA-ITIM**

It has previously been shown that MAFA isolated from RBL-2H3 cells is phosphorylated at very low levels on both its tyrosine and serine residues as detected by $[^{32}P]$phosphate (5). The tyrosine phosphorylation following MAFA clustering by soluble mAb G63 or its F(ab$_2$)$_2$ was only weakly detected by Western blotting with specific Abs. Therefore, we used mAb G63 F(ab$_2$)$_2$ conjugated to Sepharose beads to achieve more effective aggregation. MAFA was than further immunoisolated by the mAb G63 F(ab$_2$)$_2$-conjugated Sepharose beads from the lysates. The bound proteins were eluted and separated by a reducing SDS-PAGE, electrotransferred to nitrocellulose membranes, and Western blotted with phosphotyrosine-specific polyclonal Abs (PY-40). Fig. 1A shows that tyrosine phosphorylation of MAFA took place in the cells where MAFA was clustered by beads carrying G63 F(ab$_2$)$_2$, whereas Ag stimulation did not cause that change. Similar results were obtained from three independent experiments. The same membranes were further blotted sequentially by Abs specific for Lyn, SHIP, or SHP-2. All three proteins were found to coisolate with tyrosine-phosphorylated MAFA (Fig. 1C). Tyrosine-phosphorylated MAFA was also coisolated when Lyn or SHIP immunoprecipitates were examined by Western blotting with MAFA-specific Abs, although the signals were relatively weak. In addition, Lyn coisolated with the non-tyrosine-phosphorylated MAFA from both untreated and Ag-stimulated cells, suggesting that Lyn also binds to the PAAP motif of MAFA via its SH3 domain, although with considerably lower affinity (see below). On tyrosine phosphorylation of MAFA, this coisolation was markedly enhanced (Fig. 1C).

**Pervanadate treatment markedly increases tyrosine phosphorylation of MAFA-ITIM**

RBL-2H3 cells ($50 \times 10^3$/sample) were either left untreated as control or treated with 200 $\mu$M freshly prepared sodium pervanadate for 5 or 15 min at 37°C. The cells were then lysed, and MAFA was isolated from the lysates by immunoprecipitation using mAb 63 F(ab)$_2$ conjugated to Sepharose beads. The isolated proteins were eluted and analyzed by Western blotting with PY40. Results (Fig. 2A) show that pervanadate treatment markedly increased tyrosine phosphorylation of both the dimeric and monomeric MAFA at 5 min. Significantly, the level of tyrosine-phosphorylated MAFA declined after 15 min. Reblotting the membrane with mAb G63 showed that all three samples contained the same amount of MAFA (Fig. 2B).
Lyn phosphorylates the tyrosine of MAFA-ITIM

To identify the PTK responsible for the tyrosine phosphorylation of MAFA, we have first examined the in vitro tyrosine phosphorylation of a 35-aa-long synthetic peptide with a sequence corresponding to MAFA-IC (MP35). IgE-saturated RBL-2H3 cells (20 × 10^6/sample) were stimulated by Ag and lysed. Lyn or Syk was isolated using polyclonal-specific Abs carried on protein G-Sepharose beads. The in vitro peptide phosphorylation reaction was initiated by adding 2.5 μg MP35 together with 10 μCi [γ-32P]ATP to the reaction mixture containing beads with isolated Lyn or Syk in 50 μl kinase assay buffer (cf. Materials and Methods). Fig. 3A shows that MAFA-IC peptide (molecular mass, ∼3.5 kDa) underwent phosphorylation by both Lyn and Syk isolated from Ag-stimulated cell lysates. However, phosphorylation by Lyn was markedly higher than that caused by Syk. In addition, we tested the phosphorylation capacity of rSyk under the same conditions. Although an increase in rSyk autophosphorylation was observed, only a relatively modest increase in that of the MAFA-IC could be detected. These results indicate that Lyn phosphorylates MAFA-IC much more efficiently than Syk.

Experiments with recombinant vaccinia viruses, which lead to overexpression of either Lyn or Syk when infected in the RBL-2H3 cells, were subsequently conducted. Cells were infected by recombinant vaccinia viruses containing one of the following genes: wild-type Lyn; dominant negative Lyn; wild-type Syk; or dominant negative Syk. Cells infected by unmodifed vaccinia virus were used as control. After viral infection, the cells (50 × 10^6/sample) were either untreated or treated with G63 F(ab')2 conjugated to Sepharose beads as well as by pervanadate for 5 min and lysed. MAFA was isolated by immunoprecipitation using mAb G63 F(ab')2 conjugated to Sepharose beads. The isolated proteins were eluted, separated by SDS-PAGE, transferred to nitrocellulose membranes and Western blotted with anti-MAFA. Pervanadate treatment enhances the tyrosine phosphorylation of MAFA. RBL-2H3 cells (50 × 10^6/sample/ml) were incubated in Tyrode’s buffer with freshly prepared pervanadate (200 μM) for 5 or 15 min or left untreated as control, all at 37°C. Cells were lysed in 1.7 ml of 1% Triton X-100 lysis buffer. After precipitation by BSA-coupled Sepharose beads, MAFA was immunoprecipitated (IP) by mAb G63 F(ab')2 conjugated Sepharose beads. The bound proteins were eluted and analyzed by immunoblotting with the polyclonal phosphotyrosine-specific Abs PY-40 (A) and subsequently reprobed with mAb G63 (B). Detection was done by ECL. kD, kilodaltons.
Tyrosine-phosphorylated MAFA binds SHIP

RBL-2H3 cells (50 x 10^6/sample) were treated by pervanadate for 5 min as before or left untreated as control and then lysed. MAFA was immunoprecipitated, and the isolated proteins were eluted and analyzed as above by Western blotting with SHIP-specific Abs. Fig. 4A shows that the tyrosine-phosphorylated MAFA bound SHIP and rather low amounts of SHP-2, but no SH2 domain-containing protein tyrosine phosphatase-1 (SHIP-1) could be detected (data not shown). Reblotting the membrane by mAb G63 showed that similar amounts of MAFA were isolated from the above samples.

It was reported earlier that the tyrosine-phosphorylated FcεRI β subunit associates in vitro with SHIP (19). Moreover, evidence that some form of association exists between MAFA and the FcεRI has emerged from several different studies (Ref. 20 and unpublished data). Therefore, to examine the possibility that MAFA may indirectly bind SHIP through the FcεRI β subunit, we investigated the association of MAFA and SHIP in an RBL-2H3 subline defective in the expression of the FcεRI (RBL γ−/− cells) (21). FACs analysis using fluorescently labeled IgE or mAb G63 (Fig. 4B) established that there is no FcεRI α expression by the RBL γ−/− cells and therefore none of the other subunits of this receptor. Also shown in the above analysis is a lower MAFA expression by these cells than by the regularly used parental RBL-2H3 cells. Equal numbers (50 x 10^6/sample) of parental RBL and γ−/− cells were either left untreated as controls or treated by pervanadate for 5 min and lysed. MAFA was immunoprecipitated by mAb G63 F(ab′)2-conjugated Sepharose beads from the lysates and analyzed as above. Fig. 4C shows that after pervanadate treatment, MAFA bound SHIP in both cell types. Because the RBL γ−/− cells express only about one-third of MAFA compared with the parental cells (see Fig. 4B), about one-third of the amount of SHIP was monitored by densitometry.

MAFA can bind SHIP and Lyn directly through their SH2 or SH3 domains

To analyze the interactions between MAFA and SHIP or Lyn, peptides with a sequence corresponding to residues 4–12 of the MAFA, phosphorylated on tyrosine (MADNSIYSTL), serine (MADNpSISYTL), or both (MADNSpSISpSTL), were synthesized and conjugated to Sepharose beads. These were then used to isolate proteins from RBL-2H3 cell lysates. The proteins bound to the different peptides were eluted, separated as before, and Western blotted using polyclonal Abs specific for SHIP or Lyn. The results (Fig. 5A) show that whereas the nonphosphorylated ITIM peptide did not bind any of these proteins, the tyrosine-phosphorylated ITIM bound both SHIP and Lyn. The serine phosphorylated ITIM, and in particular the tyrosine- and serine-phosphorylated ITIM peptide, also bound SHIP and Lyn but in considerably lower amounts (Fig. 5A).

To examine whether MAFA pY-ITIM associates directly with SHIP or Lyn and determine the affinity and kinetic parameters of these interactions, the SPR method was used. Soluble GST fusion proteins were prepared with the following: SH2 domain of SHIP; N- or C-terminal SH2 domains of SHP-1; both N- and C-terminal SH2 domains of SHP-2; and the SH2 or SH3 domains of Lyn. The following N terminus biotinylated peptides were used for these measurements: 1) nonphosphorylated MAFA ITIM (LDNSIYSTL EL); 2) MAFA pY-ITIM + PAAP (LDNSpSPSISpSTLpPAAP); 3) MAFA-PAAP motif (ELPAAPRQDQDR); and 4) a peptide containing nonphosphorylated MAFA ITIM and the PAAP motif (ITIM + PAAP: DNSYISTLPAAP). Results of these measurements are illustrated in Fig. 5B and summarized in Table I. These results show that 1) none of the examined proteins binds the...
Nonphosphorylated MAFA ITIM and 2) neither N- nor C-terminal SH2 domains of SHP-1 bound the MAFA pY-ITIM. 3) Although SH2 domain of SHIP and the two SH2 domains of SHP-2 bind the MAFA pY-ITIM peptide, the former (single SH2 domain) binds with considerably higher affinity than the two latter domains ($K_d$ 1.02 and 8.7 nM, respectively) (Fig. 5B and Table I). 4) The SH2 domain of Lyn also binds MAFA pY-ITIM peptide ($K_d$ ~28 nM), whereas its SH3 domain binds the nonphosphorylated peptide containing both ITIM and the PAAP motif ($K_d$ ~55 nM) (Fig. 5B). 5) The peptides containing only the ITIM or the PAAP motif did not bind the SH3 domain of Lyn (Fig. 5B). This suggests that the ITIM may serve as a spacer to facilitate binding of the SH3 domain of Lyn to the PAAP motif or help in maintaining the peptide conformation. This finding is in line with results presented in Fig. 1C which indicates that Lyn also binds, probably via its SH3 domain, to the nonphosphorylated intracellular tail of intact MAFA. On MAFA clustering, this binding may be enhanced and lead to the initial tyrosine phosphorylation of the ITIM, which enables subsequent binding of Lyn and SHIP to the tyrosine-phosphorylated ITIM via their SH2 domains with the noted higher affinity.

**Inhibition by MAFA of the FcεRI secretory response depends on SHIP**

To obtain further evidence for the involvement of SHIP in the MAFA-mediated inhibition of the secretory response to the FcεRI stimulus, we used the recombinant vaccinia virus system to overexpress in RBL-2H3 cells either wild-type SHIP or a mutant containing only its SH2 domain (15, 16). Because SHP-2 is also found to bind to the tyrosine-phosphorylated MAFA, we have performed, in parallel, transfections of the RBL-2H3 cells with vaccinia viruses that led to a similar overexpression of either wt SHP-2 or of its inactive, dominant negative mutant. The secretory response of all these cells was then compared with those of cells infected by the control virus. After 6 h transfection, the densitometric analysis of Western blots revealed that protein expression levels of wild-type SHIP or SHP-2 were almost 7-fold that of control, nontransfected cells. Cells transfected by the mutant SHIP expressed both the regular amount of wild-type SHIP and the mutant containing only its SH2 domain (with an apparent molecular mass of ~25 kDa; data not shown).

The inhibitory action of MAFA on the FcεRI-induced secretion was subsequently assayed over a range of Ag concentrations in cells transfected by these recombinant or control vaccinia viruses. The β-hexosaminidase secretion in response to Ag (DPNP$_1$-BSA) stimulation after MAFA clustering by G63 F(ab$^\prime$)$_2$ was compared with that of cells that were stimulated only by Ag (Fig. 6, A and B). Net Ag-induced secretion by the differently transfected cells was in the range of 40–60% of their β-hexosaminidase activity content. In cells overexpressing wild-type SHIP, MAFA induced a considerably higher inhibition (Fig. 6C) than in control transfected cells (up to 14%). Furthermore, in the cells overexpressing only the SH2 domain of SHIP, the inhibitory action of MAFA was essentially abolished (Fig. 6, A and C). The effect of MAFA clustering

Table I. Affinities and kinetic parameters characterizing the interactions of the SH2 domains of SHIP, SHP-1, SHP-2, and Lyn with MAFA pY-ITIM + PAAP peptide, and of the SH3 domain of Lyn with MAFA ITIM + PAAP peptide

<table>
<thead>
<tr>
<th>Peptide/Protein</th>
<th>$K_d$ (nM)</th>
<th>$k_{on}$ ($10^7$ M$^{-1}$ s$^{-1}$)</th>
<th>$k_{off}$ ($10^{-3}$ s$^{-1}$)</th>
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<tr>
<td>GST-SHIP-SH2</td>
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<td>7.4 ± 1.1</td>
<td>1.9 ± 0.3</td>
<td>1.4 ± 0.2</td>
<td>5.2 ± 0.3</td>
</tr>
<tr>
<td>GST-SHIP-2-SH2(N + C)</td>
<td>8.7 ± 1.2</td>
<td>2.3 ± 1.5</td>
<td>5.7 ± 1.3</td>
<td>2.9 ± 0.5</td>
<td>6.9 ± 2.2</td>
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<tr>
<td>GST-Lyn-SH2</td>
<td>27.5 ± 4.1</td>
<td>4.3 ± 0.6</td>
<td>9.7 ± 1.7</td>
<td>4.1 ± 0.7</td>
<td>49.7 ± 6.7</td>
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<tr>
<td>GST-SHIP-1-SH2(C)</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>ITIM + PAAP/</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>GST-Lyn-SH3</td>
<td>54.6 ± 1.7</td>
<td>7.1 ± 0.6</td>
<td>4.0 ± 0.7</td>
<td>1.8 ± 0.2</td>
<td>17.6 ± 2.5</td>
</tr>
</tbody>
</table>

*Recombinant GST fusion proteins of above domains were used. All constants were calculated from three independent measurements at three concentrations of each GST fusion protein. All data analyses were performed with BIAevaluation 3.0.2 software.
C. Supernatant samples were withdrawn and trans- B, right) for 5 min and then stimulated by the indicated concentrations of clustering MAFA were assayed by measuring the secretory response of the cells to FcRI stimulation. The inhibitory action of MAFA depends on SHIP. The secretion of Ag-stimulated cells transfected with vaccinia viruses (5 PFU/cell) in DMEM, 10% FCS (100 μl/ well) overnight and then transfected by the control or by one of the indicated recombinant vaccinia viruses (5 PFU/cell) in DMEM, 10% FCS (100 μl/ well) for 8 h at 37°C. Transfected cells were either left untreated as control (A, left, and B, left) or treated with G63 F(ab’)2 (1 × 10⁻⁷ M) (A, right, and B, right) for 5 min and then stimulated by the indicated concentrations of Ag for 50 min at 37°C. Supernatant samples were withdrawn and transferred to 96-well plates. After 1 h incubation with β-hexosaminidase substrate, the reaction was stopped, and the OD of the plates was read by an ELISA reader at 415 nm. Secretion was calculated as percent of the total enzyme activity content of the cells measured in 1% Triton X-100-lysed cells. Results shown are the average ± SD (indicated by the size of the data points) of triplicates. A. Cells transfected with vaccinia viruses leading to overexpression of wild-type (wt-SHIP) or of the SH2 domain (SHIP-SH2) of SHIP or transfected by control virus (Control). B. Cells transfected with vaccinia viruses leading to overexpression of wild-type (wt-SHP-2) or by a dominant negative form (dn-SHP-2) of SHP-2 or cells transfected by control virus (Control). C. MAFA-mediated inhibition of the secretory response to FcεRI stimulation was assayed as above in RBL-2H3 cells transfected with vaccinia viruses leading either to overexpression of wild-type (wt) or forms lacking catalytic activity (SHIP-SH2; left) or (SHIP2-SH2; right) or with control transfected virus (both panels). Secretion (D) was calculated as percent of the total enzyme activity measured in 1% Triton on the secretory response of cells overexpressing wt SHP-2 or only its dn mutant was only marginally different from that observed in the cells transfected with the control virus. All the transfected cells exhibited a slightly lower secretion when MAFA was clustered, and those transfected by either two recombinant SHP-2 viruses exhibited essentially the same inhibition as those transfected with the control virus (Fig. 6, B and C (right)) further supporting the limited role of SHP-2 in the action of MAFA. More significantly, these results show that the degree of secretion was markedly lower in cells overexpressing wt SHIP, whereas the overexpression of only the SH2 domain of SHIP led to higher degranulation. These results are consistent with recent findings showing that SHIP has a key role in regulating the secretory response of mast cells (38).

As stated above, RBL-2H3 cell transfection by the vaccinia viruses lowers both the degree of cell secretion as well as its inhibition by MAFA clustering. Although in nontransfected controls the degree of MAFA-mediated inhibition may routinely reach 60%, in cells transfected by control viruses it reaches between 14% and ~20% (Fig. 6C).

Taken together, these results provide functional evidence that SHIP has a major role in the inhibitory effect of MAFA on the response of RBL-2H3 cells to the FcεRI stimulus.

**Discussion**

Results of this study show that MAFA clustering by its specific mAb G63 leads to phosphorylation of its ITIM tyrosine. This phosphorylation is even more pronounced in cells treated with pervanadate. Results of both in vitro and in vivo experiments suggest that Lyn is the protein tyrosine kinase responsible for this phosphorylation. Earlier reports provided evidence that Lyn functions in both positive and negative immunological signaling cascades: Lyn was shown to phosphorylate tyrosine residues present either in the immunoreceptor tyrosine-based activation motifs (ITAM) or in the inhibitory (ITIM) ones. For example, in B cells, Lyn phosphorylates the ITAM tyrosine residues of Igα and Igβ-chains of the B cell Ag receptor, thereby initiating the positive downstream activating signal. However, it also phosphorylates the ITIMs tyrosines of the FcγRIIb, CD22, and PIR-B, initiating inhibitory signaling (22, 23). Similarly, in mast cells, Lyn was shown to phosphorylate the ITAM tyrosines of both FcεRI β and γ subunits as well as the ITIM tyrosine of the FcγRIIB (24, 25). Data emerging from the present biochemical experiments suggest that the tyrosine of the ITIM of MAFA is phosphorylated by Lyn in the rat mucosal type line RBL-2H3. However, when these cells overexpressed a dominant negative form of Lyn, the tyrosine phosphorylation of MAFA was not clearly suppressed (Fig. 3B). This suggests that either the remaining active Lyn in the cells is sufficient for phosphorylating MAFA to the level observed in control cells or that other PTKs such as Syk, especially when overexpressed, may also phosphorylate MAFA.

We further investigated the interactions between Lyn and MAFA by measuring the binding of either SH2 or SH3 domains of Lyn to synthetic MAFA peptides using the SPR method. The results show a considerable affinity (Kₐ 55 nM) between the SH3 domain of Lyn and a peptide containing both the MAFA-ITIM and PAAP motif. Further, the SH2 domain of Lyn binds the tyrosine-phosphorylated ITIM of MAFA with higher affinity (Kₐ 28 nM).

**Figure 6.** The inhibitory action of MAFA depends on SHIP. The secretory response of the cells to FcεRII stimulation and its suppression upon clustering MAFA were assayed by measuring the β-hexosaminidase activity in the supernatants of RBL-2H3 cells transfected by vaccinia viruses overexpressing different forms of SHIP and of SHP-2 or by control virus. Cells were seeded in 96-well plates (7 × 10⁵ cells/well/100 μl medium) overnight and then transfected by the control or by one of the indicated recombinant vaccinia viruses (5 PFU/cell) in DMEM, 10% FCS (100 μl/ well) for 8 h at 37°C. Transfected cells were either left untreated as control (A, left, and B, left) or treated with G63 F(ab’)2 (1 × 10⁻⁷ M) (A, right, and B, right) for 5 min and then stimulated by the indicated concentrations of Ag for 50 min at 37°C. Supernatant samples were withdrawn and transferred to 96-well plates. After 1 h incubation with β-hexosaminidase substrate, the reaction was stopped, and the OD of the plates was read by an ELISA reader at 415 nm. Secretion was calculated as percent of the total enzyme activity content of the cells measured in 1% Triton X-100-lysed cells. Results shown are the average ± SD (indicated by the size of the data points) of triplicates. A. Cells transfected with vaccinia viruses leading to overexpression of wild-type (wt-SHIP) or of the SH2 domain (SHIP-SH2) of SHIP or transfected by control virus (Control). B. Cells transfected with vaccinia viruses leading to overexpression of wild-type (wt-SHP-2) or by a dominant negative form (dn-SHP-2) of SHP-2 or cells transfected by control virus (Control). C. MAFA-mediated inhibition of the secretory response to FcεRI stimulation was assayed as above in RBL-2H3 cells transfected with vaccinia viruses leading either to overexpression of wild-type (wt) or forms lacking catalytic activity (SHIP-SH2; left) or (SHIP2-SH2; right) or with control transfected virus (both panels). Secretion (D) was calculated as percent of the total enzyme activity measured in 1% Triton X-100-lysed cells. Dₓ, Secretion of Ag-stimulated cells; Dₒ, secretion of cells where MAFA was preclustered by G63 F(ab’)2, and then Ag stimulated. The percent inhibition (I) was calculated as [I] = 100 × (Dₒ – Dₓ)/Dₒ. Data are the average ± SD of the average results of at least three experiments. Values are those in which the inhibition reached its maximum.
Results of experiments in the intact cells (Figs. 1C and 3, B and C) resolved in untreated RBL-2H3 cells a basal association between Lyn and non-tyrosine-phosphorylated MAFA. Therefore, the association of MAFA with the SH3 domain of Lyn probably already occurs in untreated cells and MAFA clustering further enhances it. This association may be required for phosphorylation of the ITIM tyrosine of MAFA by Lyn, which then takes place upon MAFA clustering. This tyrosine phosphorylation will then enhance Lyn binding to MAFA via its SH2 domain (Figs. 1C and 3C), and potentially promote phosphorylation of other MAFA molecules in the clusters.

The tyrosine-phosphorylated MAFA bound both SHIP and SHP-2 in the cells (Fig. 1C). It has previously been shown that SHP-2 plays a limited role in the inhibitory action of MAFA by suppressing the activity of Syk (10). The partial inhibition therefore suggested that SHP-2 represents only one pathway of the inhibitory action of MAFA. SHIP has been established as a key negative regulator (“guardian”) (26–32) of the secretory response of mast cells. SHIP activity was proposed to set the threshold for and limit the response to the FcεRI-mediated secretory response. More recently, SHIP was also shown to serve a similar role for the Steel factor-induced signaling in mast cells (26). As reported here, the tyrosine-phosphorylated MAFA ITIM binds SHIP single SH2 domain almost 9-fold stronger than it binds to combined two SH2 domains of SHP-2. Moreover, overexpression of the SH2 domain of SHIP essentially abolished the inhibitory function of MAFA. These results suggest that SHIP represents another, most probably the major, element in the inhibitory action of MAFA.

In addition to its catalytic domain, which hydrolyzes the 5′-phosphate in inositol 1,3,4,5-tetrakisphosphate and PIP3 (27, 28), SHIP contains domains involved in mediating protein-protein interactions. These include the N-terminal SH2 domain, two NPXY motifs, and several proline-rich sequences in its C terminus that resemble SH3 domain binding sites (29). These features suggest that SHIP is able to interact with several signal-coupling molecules and thus also serve as an adaptor. Indeed, SHIP has recently been implicated in pathways that cause both inhibition and feedback regulation of different cell types (30). In particular, it has been shown to inhibit the activation via ITAM-containing receptors of mast and B cells; SHIP was shown to be recruited, via its SH2 domain, to the tyrosine-phosphorylated ITIM of the FcεRIIB and subsequently to inhibit the FcεRI and B cell receptor activation signals (31, 32). The generation of a SHIP knockout mouse opened the way to further investigate its modulatory role. SHIP−/− mast cells were found to have ~4-fold higher secretory response to the FcεRI stimulus than those of SHIP+/− or SHIP+/+ cells. Thus, as already stated, the critical role of SHIP is to establish a threshold for mast cell secretory response (32–38).

Present results of overexpression of either wild-type SHIP or only its SH2 domain in RBL-2H3 cells now provide evidence that SHIP is directly involved in MAFA-mediated inhibition of mast cell degranulation: In the former case, MAFA clustering induced a higher inhibition than that observed in control cells; whereas in the latter, the inhibitory effect of MAFA was essentially abolished. These results are rationalized by the observation that MAFA associates with SHIP via its SH2 domain and that overexpressing only this SH2 domain leads to a competition for the interaction of endogenous SHIP (and probably also of SHP-2) with the tyrosine-phosphorylated MAFA, thus functioning as a dominant negative form of SHIP (34). SHIP recruitment by tyrosine-phosphorylated MAFA most probably leads to translocation of this enzyme from the cytoplasm to the cell membrane. This brings SHIP in proximity to and action on its substrate PIP3 (27), reducing its membranal levels. This in turn, suppresses the PH domain-mediated association of the Bruton’s tyrosine kinase with the membrane, thereby reducing its kinase activity which is responsible for activating PLC-γ (35–37). Therefore, MAFA clustering brings about an increase in the SHIP-catalyzed hydrolysis of PIP3 and thereby also to the observed decrease in PLC-γ activity (1). This, in turn leads to inhibition of the transient rise in [Ca2+]i (1, 26), culminating in suppression of the secretory response of the cells.

Acknowledgments

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References


