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The Adaptor 3BP2 Is Required for Early and Late Events in FcεRI Signaling in Human Mast Cells

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Adaptor molecules are essential in organizing signaling molecules and in coordinating and compartmentalizing their activity. SH3-binding protein 2 (3BP2) is a cytoplasmic adaptor protein mainly expressed by hematopoietic cells that has been shown to act as a positive regulator in T, B, and NK cell signal transduction. 3BP2 is an important regulator of cytotoxic granule release in NK cells. Mast cells (MCs) similarly degranulate following Ag-dependent aggregation of the FcεRI on the cell surface. Activation of these cells induces the release of preformed inflammatory mediators and the de novo synthesis and secretion of cytokines and chemokines. Thus, MCs participate in both innate and acquired responses. We observed that 3BP2 is expressed in human MCs (huMCs) from diverse origins. Moreover, 3BP2 coimmunoprecipitates with essential MC signaling mediators such as Lyn, Syk, and phospholipase C γ; thus, a role for this adaptor in MC function was postulated. In the present work, we used the short hairpin RNA lentiviral targeting approach to silence 3BP2 expression in huMCs. Our findings point to a requirement for 3BP2 in optimal immediate and late MCs responses such as degranulation and IL-8 or GM-CSF secretion. 3BP2 was determined to be necessary for optimal phosphorylation of Syk, linker for activation of T cells, and phospholipase C γ₁, critical signals for calcium release from intracellular stores. Taken together, our results show that by participating in FcεRI-mediated signal transduction 3BP2 is an important regulator of huMC activation. Thus, 3BP2 could be a potential therapeutic target for IgE-dependent MC-mediated inflammatory disease. *The Journal of Immunology*, 2012, 189: 000–000.

Mast cells (MCs) are key effector cells in IgE-dependent hypersensitivity, as well as in allergic and inflammatory disorders. Ligation of the FcεRI, constitutively expressed on MCs, promotes cell activation and immediate release and production of proinflammatory mediators (1, 2). FcεRI-mediated MC activation can be dramatically enhanced by concurrent activation of the tyrosine kinase type III KIT (CD117), which plays

a role in cell survival, proliferation, and differentiation (3, 4). The signals generated as a consequence of Ag interaction with specific IgE bound to FcεRI is a highly ordered process where adaptor molecules are essential in organizing, coordinating, and compartmentalizing signaling molecules to direct specific cellular responses. Adaptors display several motifs and domains that coordinate protein–protein and protein–lipid interactions and can be transmembrane or cytoplasmic (5). Src homology (SH)3-binding protein 2 (3BP2) is a cytoplasmic adaptor originally identified as a protein interacting with the SH3 domain of the protein tyrosine kinase (PTK) Abl (6). The 3BP2 encoding gene is located on human chromosome 4 (4p16.3 region). Human 3BP2 is a 561-aa protein containing an N-terminal pleckstrin homology (PH) domain, SH3-binding proline-rich regions, and a C-terminal SH2 domain. The proline-rich region (PR1, PR2, and PR3) are known to associate to Src family PTKs (Fyn, Lyn, and Lck) (7, 8). The SH2 domain of 3BP2 interacts with Syk family PTKs Syk and ZAP-70 (7). 3BP2 is known to regulate bone homeostasis through osteoclast activation and osteoblast differentiation and function (9). Mutations in the proline-rich region of 3BP2 are responsible for the cherubism autosomal dominant inherited disorder characterized by excessive bone degradation of the upper and lower jaws, which results in facial swelling (10). It has been recently shown that these mutations uncouple 3BP2 from the poly(ADP-ribose) polymerase Tankyrase-reducing ADP ribosylation and subsequent destruction of 3BP2 via ubiquitylation. Tankyrase ankyrin repeats bind 3BP2 RxxPDG hexapeptide, and disruption of such interaction stabilizes 3BP2 protein (11). Consequently, elevated 3BP2 protein levels result in increased Src, Syk, and Vav activity and osteoclasts hyperactivation, which leads to cherubism (12). 3BP2 is preferentially expressed in hematopoietic tissues where it contributes to the regulation of immune responses (13). This molecule regulates transcriptional activities via calcineurin- and Ras-dependent pathways in T lymphocytes (7). 3BP2-mediated

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Abbreviations used in this article: 3BP2, Src homology 3-binding protein 2; huMC, human mast cell; LAT, linker for activation of T cells; MC, mast cell; NT, nontarget; PH, pleckstrin homology; PLC, phospholipase C; PTK, protein tyrosine kinase; SA, streptavidin; SCF, stem cell factor; SH, Src homology; shRNA, short hairpin RNA.

signals are regulated on T cells via Src homology region 2 domain-containing phosphatase-1 tyrosine phosphatase (SHP-1), which acts as negatively regulated by dephosphorylation of the adaptor (14). A positive regulatory role for 3BP2 in BCR function has been described previously (15) wherein 3BP2-deficient mice show impaired optimal B cell activation and thymus-independent humoral responses (16, 17). Regarding 3BP2 phosphorylation, in B cells, 3BP2 gets tyrosine phosphorylated in a Syk-dependent manner after BCR stimulation (18). 3BP2 also plays an important function in NK cells, where it regulates cell-mediated cytotoxicity via its PH, SH2, and proline-rich regions (19). Moreover, phosphorylation of 3BP2 Tyr 183, which mediates the interaction with Vav-1 and phospholipase C (PLC)- γ , is critical for the ability of 3BP2 to positively regulate NK cell-mediated killing (19). In NK cells, 3BP2 was described to directly associate with the signaling lymphocytic activation molecule family member CD244 (2B4) and to regulate CD244-mediated cytotoxicity through SLAM-associated protein (SAP) and protein kinase C-dependent mechanism, which does not affect IFN- γ secretion (20, 21). Although it has been described that the overexpression of 3BP2 SH2 domain impairs Fc ϵ RI-dependent degranulation in the rat basophilic leukemia cell line RBL-2H3 (22), evidence for a role for endogenous 3BP2 in human MCs (huMCs) function has remained elusive. Furthermore, as overexpression of dominant-negative mutant constructs can cause off-target effects by interfering with other SH2 domain-containing proteins and/or may have incomplete effects on their specific targets, how 3BP2 regulates MC function in general is not clear. Thus, in this work, we investigated the role of endogenous 3BP2 on Fc ϵ RI signaling and function in huMCs using a short hairpin RNA (shRNA) knockdown approach. Our data indicate that 3BP2 is important for Syk phosphorylation and, consequently, for short- and long-term events in MCs activation through Fc ϵ RI. Altogether our data show an important role for this adaptor in MC function through one of the major MC receptors.

Materials and Methods

Cells and Abs

The LAD2 huMC line, provided by Drs. A. Kirshenbaum and D.D. Metcalfe (National Institutes of Health, Bethesda, MD), was grown in StemPro-34 serum-free medium (Invitrogen Life Technologies, Carlsbad, CA), supplemented with StemPro-34 Nutrient and with L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μ g/ml), and 100 ng/ml recombinant stem cell factor (SCF) (Amgen, Thousand Oaks, CA). The LUVA huMC line, provided by Dr. J. Steinke (University of Virginia, Charlottesville, VA), was grown as indicated above without SCF. Primary huMCs derived from CD34-positive peripheral blood cells were obtained from healthy donors following informed consent on a protocol (98-I-0027; principal investigator: Dr. A. Kirshenbaum) approved by the National Institute of Allergy and Infectious Diseases Institutional Review Board and differentiated in vitro for 8 wk in the presence of 100 ng/ml IL-6 and 100 ng/ml SCF as described previously (23). Mouse anti-3BP2 (clone 470) and rabbit anti-PLC γ Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-Syk was obtained from BioLegend (San Diego, CA). Anti-phosphotyrosine monoclonal and anti-total ERK Abs were from Zymed Laboratories (Invitrogen). Anti-linker for activation of T cells (LAT), anti-pLAT Y191, anti-pSyk Y352, anti-pPLC γ 1Y783, anti-pP38 Thr¹⁸⁰/Tyr¹⁸², anti-p-ERK Thr²⁰²/Tyr²⁰⁴, and anti-p-JNK Thr¹⁸³/Tyr¹⁸⁵ were purchased from Cell Signaling Technology (Danvers, MA). Biotinylated human IgE was obtained from Abbiotec (San Diego, CA).

shRNA silencing by lentiviral infection

Lentiviral particles to silence the 3BP2 gene expression were generated using Mission shRNA technology, according to the manufacturer's instructions (Sigma-Aldrich, St. Louis, MO). The 3BP2-specific shRNA sequence selected was 5'-CCG GGC GAA GTG GAA AGG TTG TTC ACT CGA GTG AAC AAC CTT TCC ACT TCG CTT TTT G-3'. Non-target (NT) shRNA was used as a control. Virus preparations were done by transfection of 293LTV (Cell Biolabs, San Diego, CA) cells and harvesting

of supernatant 3 d posttransfection as described previously (24). Briefly, supernatants were concentrated 35 times by centrifugation and used to infect huMCs. A total of 5×10^6 of 6-wk huMCs per shRNA preparation were infected. Selection was started 1 wk later with 0.2 μ g/ml puromycin (Sigma-Aldrich), and the extent of 3BP2 knockdown was determined by Western blot analysis 1 wk after starting selection. All experiments in huMCs were performed at weeks 8 and 9 of cell culture.

RNA extraction and retrotranscription

Total RNA was extracted with RNAeasy Mini Kit (Qiagen, Hilden, Germany) from 2×10^6 nontransduced, NT control and 3BP2 knockdown MCs sensitized with biotinylated IgE and challenged with streptavidin (SA) (Sigma-Aldrich). cDNA was generated from mRNA High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions.

Real-time PCR

Real-time PCR for IL-8 was performed using TaqMan Gene Expression Assay (Applied Biosystems) on an ABI-Prism 7300 Sequence Detector (Applied Biosystems). 18S RNA amplification control was used for cycle normalization. Data were analyzed with 7500 SDS Software (Applied Biosystems). All PCRs were set up in triplicates.

Degranulation assays

huMCs were sensitized overnight with 100 ng/ml biotinylated IgE in StemPro-34 media with SCF and IL-6. After washing, cells were stimulated with 100 ng/ml SA at 37°C for 30 min in Tyrode's buffer (10 mM HEPES, 137 mM NaCl, 2.7 mM KCl, 0.4 mM NaH₂PO₄, 1.8 mM CaCl₂, 1.3 mM MgSO₄, 5.6 mM glucose, and 0.025% BSA) for 30 min at 37°C. IgE-dependent MC degranulation was monitored by β -hexosaminidase release as described previously (25). The resulting β -hexosaminidase activity was expressed as the percentage of total cell content (samples lysed with Triton X-100): percentage of β -hexosaminidase release = (sample release minus spontaneous release/maximum release minus spontaneous release) $\times$ 100.

Calcium mobilization

Calcium mobilization in CD34⁺-derived huMCs and LAD2 cells was followed by fluorimetric analysis of free calcium with Fluo-4-AM fluorescent dye (Molecular Probes, Invitrogen). A total of 0.2×10^6 cells/point were loaded with 5 μ M Fluo-4-AM for 30 min at 4°C in the dark, washed twice with Tyrode's buffer, and resuspended. Fluorimetric measurements were done in a Modulus II Microplate Multimode Reader, Turner Biosystems (Promega, San Luis Obispo, CA), according to the manufacturer's instructions.

Cell activation, immunoprecipitation, and immunoblotting

Cells were sensitized overnight with biotinylated IgE (100 ng/ml) in culture media. The following day, the cells were stimulated with 100 ng/ml SA in the Tyrode's buffer for the indicated times. Whole-cell lysate preparations were obtained as described elsewhere (26). For immunoprecipitation experiments, cells were washed twice with ice-cold PBS and solubilized in lysis buffer (1% Triton X-100, 50 mM Tris [pH 7.4], 150 mM NaCl, 20 mM octyl- β -glucoside, 100 mM NaF, 1 mM Na₃VO₄, 1 mM PMSF, 1 mM sodium pyrophosphate, and protease inhibitor mixture [Roche Molecular Biochemical, Indianapolis, IN]). Cell lysates were precleared by centrifugation. Precleared lysates were first incubated with the indicated primary Abs and subsequently with protein A-Sepharose beads (Pharmacia Biotech, Uppsala, Sweden). After rotation for 3 h at 4°C, the beads were washed three times with lysis buffer, and immunoprecipitated proteins were eluted by heat treatment at 100°C for 5 min with 3-fold times concentrated sampling buffer. The immunoprecipitates and total cell lysates were separated by SDS-PAGE and electrotransferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). Blots were probed with the indicated Abs. In all blots, proteins were visualized by ECL (Santa Cruz Biotechnology).

Immunofluorescence microscopy

Unstimulated and Fc ϵ RI-activated LAD2 cells at various times (1×10^6 cells of each), or unstimulated NT shRNA and 3BP2 shRNA-infected LAD2 cells (Supplemental Fig. 1), were fixed with 4% paraformaldehyde in PBS and permeabilized with 0.05% saponin in PBS. After washing with PBS and incubated for 1 h with blocking buffer (0.2% nonfat dry milk, 2% FCS, 1% BSA, 20% rabbit serum, 0.01% NaN₃, and 0.01% Triton X-100), cells were labeled in suspension with anti-human 3BP2 or mouse

IgG1 isotype control (1 $\mu\text{g/ml}$) at 4°C for 1 h. After washing with ice-cold PBS, cells were incubated with 1 $\mu\text{g/ml}$ anti-mouse Cy3 (Jackson ImmunoResearch Laboratories) at 4°C (control) for 30 min. For Supplemental Fig. 2, anti-pSyk (Cell Signaling Technology) was incubated for 1 h at 4°C and developed with Alexa 488-conjugated anti-rabbit Ab (Molecular Probes). Cells were then washed twice with ice-cold PBS and immobilized on polylysine-treated coverslips at 4°C for 30 min. Samples were washed and mounted in Fluoromount-G (Southern Biotechnology Associates). Fluorescence images were obtained using an Axio Observer.Z1 microscope (Zeiss) or a confocal microscope (TCS NT; Leica) for Supplemental Fig. 2. Digital images were processed using Adobe Photoshop 8.0 (Adobe Systems, San Jose, CA) programs.

GM-CSF and IL-8 production

IgE/Ag-dependent GM-CSF and IL-8 production and release were measured by ELISA with the DuoSet ELISA Development System as described by the manufacturer (R&D Systems, Minneapolis, MN). Briefly, after overnight sensitization with biotinylated IgE (100 ng/ml), cells were incubated with SA (1 $\mu\text{g/ml}$) for 6 h. Supernatants were collected and analyzed by ELISA.

Statistical data analysis

All results are expressed as mean $\pm$ SD of the mean (SD). A Student *t* test was used to determine significant differences (*p* value) between two experimental groups after determination of normal distribution of the sample and variance analysis.

Results

3BP2 is expressed and relocalized toward membrane upon FcεRI activation

To elucidate the role of the 3BP2 adaptor in huMCs, we first determined its expression in primary huMCs as well as in LAD2, LUVA, and tissue-derived lung MCs by Western blot analysis (Fig. 1A). We also examined its cellular distribution in resting and FcεRI-dependent activated huMCs by fluorescent microscopy (Fig. 1B). 3BP2 shows a cytoplasmic staining pattern in huMCs in resting conditions. Interestingly, the protein distribution in the cytoplasm is not diffuse but shows a punctate expression pattern. Similar punctuated pattern was observed in NT shRNA-infected cells, whereas no 3BP2 staining was observed in 3BP2 knock-down cells (Supplemental Fig. 1). Moreover, 3BP2 relocalizes mainly at the plasma membrane after early FcεRI activation, where it colocalizes with phosphorylated Syk kinase (Supplemental Fig. 2). Ten minutes following activation, the 3BP2 distribution resembles the basal situation. These data suggest a rapid and transient translocation of the adaptor to the plasma membrane induced by FcεRI aggregation.

3BP2 recruits essential signaling mediators in huMCs

We next analyzed 3BP2 phosphorylation and signalosome assembly after FcεRI signaling. Biotinylated IgE-sensitized LAD2 cells were stimulated with SA for various times, and 3BP2 was immunoprecipitated. As shown in Fig. 2, 3BP2 is phosphorylated as early as 5 min after receptor ligation and coimmunoprecipitates with several phosphoproteins. The essential FcεRI signaling molecules Lyn, Syk, and PLC γ were identified as 3BP2 binding partners in huMCs. After the interaction of cell-bound IgE with cognate Ag, Lyn kinase phosphorylates the β - and γ -chain ITAMs of proximal receptors (27). These phosphorylated ITAMs become docking sites for Syk kinase (28), which propagates downstream signals by phosphorylation of several mediators including LAT and PLC γ (29). In our experiments, 3BP2 consistently coimmunoprecipitated with a 35- to 40-kDa phosphoprotein with apparent characteristics of LAT; however, we failed to identify this interaction by immunoblots with the anti-LAT available Abs. Nevertheless, others have reported that LAT associates with the SH2 domain of 3BP2 (22). Given that these findings suggested that

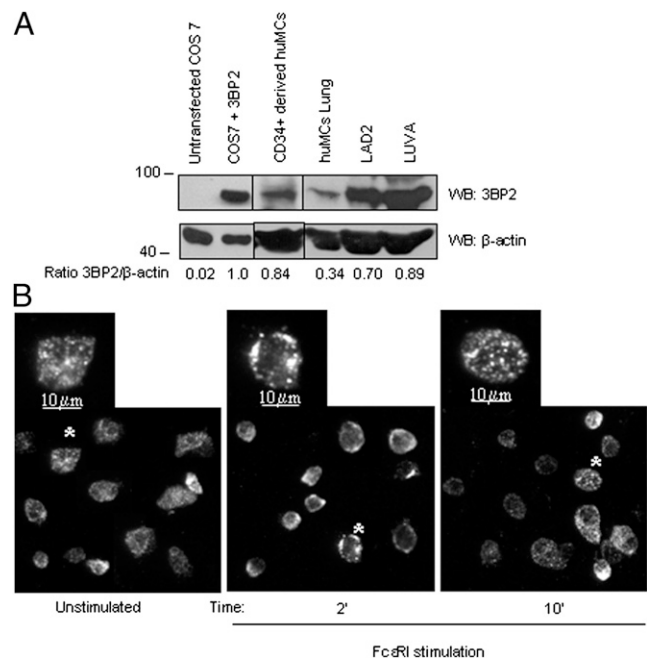


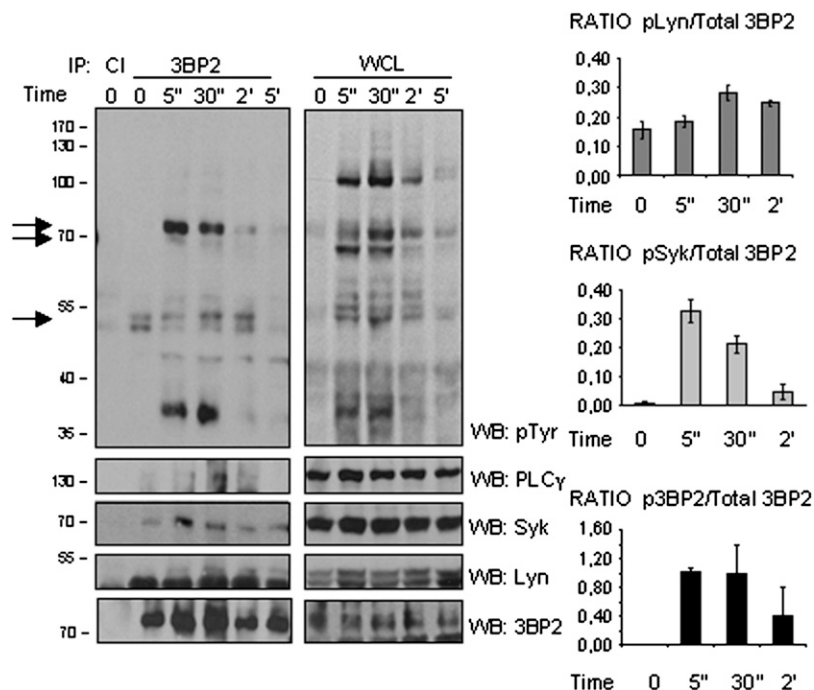
FIGURE 1. 3BP2 is expressed in huMCs. Anti-3BP2 Western blot analysis was performed on nontransfected and 3BP2-transfected COS-7, LAD2, CD34⁺ peripheral blood-derived huMCs, LUVA, and lung MCs whole cell lysates. Membrane was probed with anti- β -actin to calculate 3BP2/total protein ratio and to check loading levels per lane (A). IgE-bound LAD2 cells were activated with SA at 2 and 10 min. Nonstimulated and activated cells were fixed, permeabilized, and stained with anti-3BP2 and isotype control Abs (data not shown) and further stained with anti-mouse-Cy3 as indicated in *Materials and Methods* (B). Scale bars, 10 μm .

3BP2 could play a central role in the FcεRI-anchored signalosome, we investigated the consequences of 3BP2 depletion to FcεRI signaling. We first analyzed the efficiency of lentiviral-encoded shRNA sequences for their ability to silence 3BP2, as compared with nontransduced cells or control/NT shRNA-transduced cells. As shown in Fig. 3A, we identified shRNA sequences that could effectively suppress 3BP2 expression in huMC lines and CD34⁺-derived huMCs. We evaluated Lyn (Fig. 3B) as well as FcεRI expression (Fig. 3C) in NT and 3BP2-silenced LAD2 and CD34⁺-derived huMCs. Our results show that both FcεRI and Lyn expression were unaffected by 3BP2 knockdown.

3BP2 is required for FcεRI-dependent Syk phosphorylation

3BP2 was first identified as a Syk binding partner in a two-hybrid screening (7). This interaction involves the 3BP2 SH2 domain and Syk, suggesting that the 3BP2 SH2 domain associates with autophosphorylated Tyr residues in the Syk linker or kinase domain (7). In our work, we found that 3BP2 interacts with Syk in huMCs after FcεRI activation (Fig. 2). Syk has an essential role in early and late MC responses (30), binding the phosphorylated FcεRI γ -chain ITAMs after receptor aggregation, resulting in a conformational change that increases its enzymatic activity (29), which leads to downstream propagation of signals. To evaluate whether 3BP2 participates in the regulation of Syk activation, we examined the kinase phosphorylation in 3BP2-silenced versus NT shRNA-transduced huMCs following FcεRI stimulation. We found reduced Syk phosphorylation in Tyr³⁵² when 3BP2 expression is reduced both in LAD2 and huMCs cells, whereas total levels of the kinase remain unaffected (Fig. 4). Reduction in Syk phosphorylation in 3BP2 knockdown cells occurs as early as 5 min after FcεRI stimulation (Supplemental Fig. 3).

FIGURE 2. 3BP2 coprecipitates with Lyn, Syk, and PLC γ in huMCs. Biotinylated IgE-bound LAD2 cells (25 million cells/point) were activated with SA at the indicated times and lysed as discussed in *Materials and Methods*. The lysates were immunoprecipitated using control Ig (CI) and 3BP2 Abs. Western blot analysis was performed with the following Abs: anti-phosphotyrosine, anti-PLC γ , anti-Lyn, and anti-Syk. The immunoprecipitates (IP) and whole-cell lysates (WCL) (1.5 million) were loaded in the same gel. The experiment has been performed various times, and the graphics comparing phosphorylation of Lyn, Syk, and 3BP2 from 3BP2 immunoprecipitation versus total 3BP2 immunoprecipitated are the mean of three independent experiments.



3BP2 is required for Fc ϵ RI-dependent LAT and PLC γ phosphorylation and calcium signaling

Activated Syk phosphorylates LAT, which acts as a scaffold for a multimolecular signaling complex that includes PLC γ among other molecules (31). Because 3BP2 expression modulates Syk phosphorylation, we expected that LAT and PLC γ phosphorylation could also be negatively affected after 3BP2 silencing. Thus, we examined phosphorylation of these molecules in 3BP2-silenced versus NT shRNA-transduced huMCs following Fc ϵ RI stimulation, and we found a decrease in LAT and PLC γ phosphorylation when 3BP2 expression was reduced (Fig. 5A). PLC γ activation and phosphorylation facilitated by LAT are key steps in Ca²⁺ release from intracellular stores (29, 32). Hence, we measured calcium fluxes following Fc ϵ RI aggregation in 3BP2-silenced huMCs and LAD2 cells and compared these responses to those obtained in the NT shRNA-transduced cells. Our analysis showed that the initial calcium rise was similar in both control and 3BP2-silenced

huMCs, but calcium mobilization in the 3BP2-silenced cells was not equally sustained with time (Fig. 5B). Conversely, the calcium mobilization was equal in both control and 3BP2-silenced cells after ionomycin treatment (Fig. 5C). Importantly, the maintenance of high calcium concentration is mediated through the influx of calcium from the extracellular environment and is a requirement for optimal MC degranulation (33). Thus, our findings indicate that 3BP2 is an important regulator of calcium influx after Fc ϵ RI stimulation.

3BP2 silencing affects ERK1/2 and p38 phosphorylation

Once phosphorylated, LAT recruits a number of signaling intermediaries that include Gab2, guanosine triphosphate exchangers, and Son of sevenless (SOS) that further activate small GTPases such as Ras, resulting in the stimulation of the MAPK pathways. Previously, we have reported that overexpression of 3BP2 increases ERK1/2 phosphorylation, without affecting p38

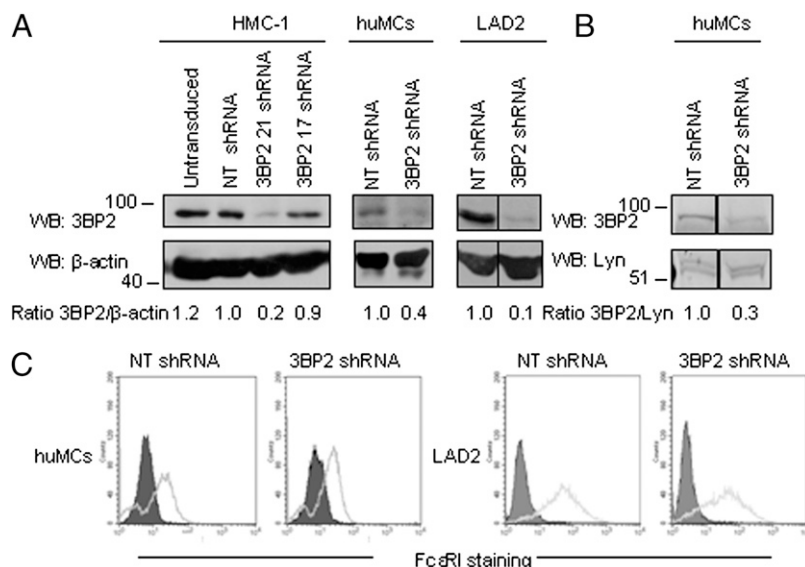


FIGURE 3. Selective knockdown of 3BP2 in huMCs. 3BP2 expression was analyzed in HMC-1 cells by Western blot following treatment of the cells with control (NT shRNA) and two different 3BP2-targeting shRNAs. The sequence corresponding to shRNA clone 21 was chosen for further assays in CD34⁺ peripheral blood-derived huMCs and LAD2 cells (A). Quantitation of the band intensity was performed by densitometry. Lyn (B) and Fc ϵ RI expression (C) were similar in 3BP2 shRNA and control-transduced huMCs and LAD2 cells. Fc ϵ RI expression was determined by FACS using PE-conjugated anti-Fc ϵ RI. The isotype control is gray filled. Data are representative of three experiments.

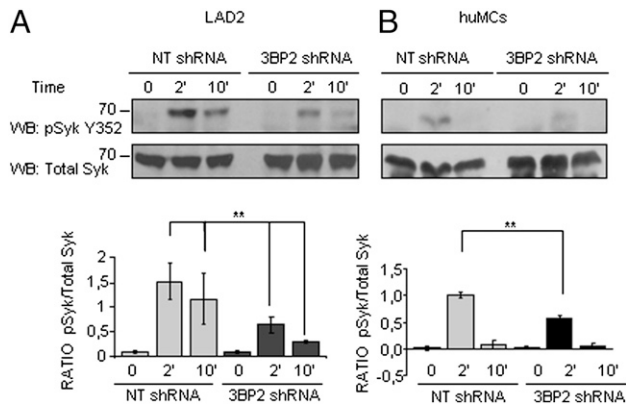


FIGURE 4. Silencing of 3BP2 reduces FcεRI-induced Syk_{Y352} phosphorylation. Specific Abs recognizing phosphorylated Syk_{Y352} and total Syk were used to immunoprobe for these proteins in huMC lysates of 3BP2 shRNA- and NT shRNA-transduced cells following biotinylated IgE cross-linking with 100 ng/ml SA at indicated times. Western blots correspond to LAD2 cells (**A**) and CD34⁺ peripheral blood-derived huMCs (**B**). The band intensity quantitation was performed by densitometry. Densitometric analysis and phosphorylated/total ratio for each molecule from three independent experiments is represented in the bar charts on the bottom. Statistical significance (***p* < 0.001) is relative to NT shRNA.

phosphorylation, in NK cells after CD244 triggering, which correlate with enhancement of cytotoxicity (20). In this work, we studied how these pathways were altered after 3BP2 silencing in huMCs and LAD2 cells. Our data show that FcεRI-mediated ERK1/2 and p38 phosphorylation were decreased following 3BP2 knockdown (Fig. 6), supporting the idea of an important role for 3BP2 in signal propagation from the FcεRI.

3BP2 is a positive regulator of MC degranulation and cytokine responses

Given the relationship between calcium mobilization and MC activation, we explored the effect of 3BP2 silencing on huMC degranulation. Thus, we examined β-hexosaminidase release in 3BP2 shRNA- versus NT-infected cells. Reduced levels of 3BP2 expression in huMCs resulted in reduced FcεRI-mediated degranulation compared with that observed in NT-transduced cells. When the nonimmunologic stimulus PMA plus ionomycin was added, 3BP2 silencing did not affect granule release, indicating that 3BP2 acts specifically downstream of FcεRI and upstream of late events (Fig. 7). Late responses after IgE stimulation on MCs include the de novo synthesis and secretion of cytokines and chemokines (34). IL-8 and GM-CSF constitute two of the most abundant of such mediators; hence, we evaluated the role of 3BP2 in their production. Biotinylated IgE-sensitized, 3BP2-silenced, and NT shRNA-transduced huMCs were stimulated with SA for 6 h, and IL-8 and GM-CSF contents were measured in cell culture supernatants (Fig. 8A, 8B). In addition, in the case of IL-8, 3BP2 was required for optimal production at the transcriptional level, as evidenced by a marked reduction in IL-8 mRNA levels after 3BP2 silencing (Fig. 8C). Collectively, the findings demonstrate that the 3BP2 adaptor molecule is necessary for optimal triggering of early and late FcεRI signaling events.

Discussion

MCs are involved in both innate and adaptive immunity, and they induce the release of preformed and the de novo synthesis of inflammatory mediators after activation. The final outcome will depend on the balance of engaging inhibitory and activating cell surface receptors as well as the positive and negative intracellular

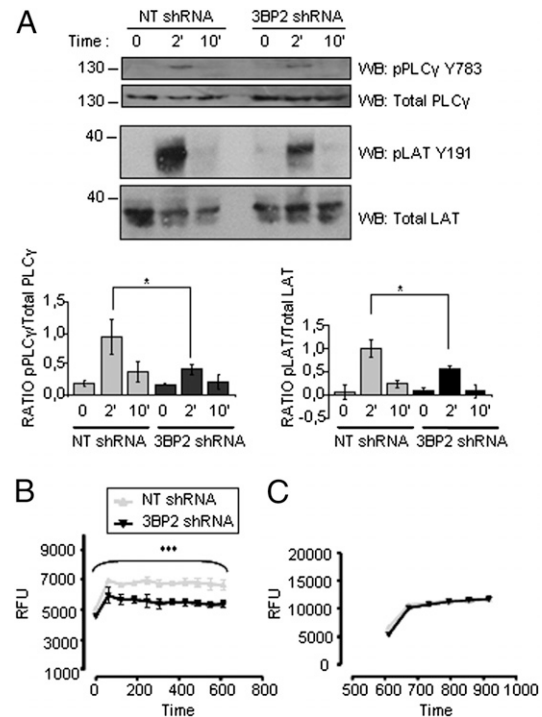


FIGURE 5. FcεRI-mediated phosphorylation of PLCγ and LAT and resulting calcium signal are reduced in 3BP2 shRNA-silenced cells. Specific anti-phosphorylated LAT_{Y191} and PLCγ_{Y783} Abs were used to blot membranes with 3BP2 knockdown versus control shRNA huMCs cell lysates upon biotinylated IgE cross-linking with 100 ng/ml SA for various times as indicated. Densitometric analysis and phosphorylated/total ratio for each molecule from three independent experiments is represented in the bar charts next to the blot (**A**). Calcium fluxes following FcεRI stimulation were measured in biotinylated IgE-sensitized 3BP2, shRNA-silenced, and NT shRNA-transduced LAD2 cells loaded with Fluo-4-AM. Cells were stimulated with 100 ng/ml SA and analyzed by fluorimetric measure of free calcium concentrations. Results are expressed as mean ± SD of triplicates, and are representative of six independent experiments (**B**). 3BP2 shRNA- and NT shRNA-transduced LAD2 cells responses to ionomycin, respectively, are shown in (**C**). Statistical significance (**p* < 0.005, ****p* < 0.0001) is relative to NT shRNA. RFU, Relative fluorescence unit.

molecular events that involve kinases and phosphatases as well as other protein and lipid mediators with adaptor functions. In this study, to our knowledge, we report for the first time that 3BP2 is expressed in huMCs and that it is required for FcεRI signaling.

3BP2 was tyrosine phosphorylated at early times after FcεRI stimulation and consequentially was observed to coimmunoprecipitate with the src kinase Lyn, implicating this adaptor in the organization of early events in MC signaling. Lyn is constitutively associated with the β subunit of the FcεRI and activated upon Ag-mediated FcεRI aggregation (35). Following Ag stimulation, 3BP2 was recruited to the plasma membrane, presumably by its PH domain. Once in close proximity to the plasma membrane, 3BP2 could interact by its proline-rich domain with the Lyn SH3 domain and be a target for phosphorylation by this Src kinase. This view is supported by experiments in COS-7 cells showing that 3BP2 interacts with, and is a substrate for, Lyn. In addition, phosphorylation of Y446 in 3BP2 can create a binding site for the Lyn SH2, thereby increasing Lyn autophosphorylation (8). Our data show that 3BP2 also binds Syk in huMCs after FcεRI stimulation. Both 3BP2 and phosphorylated Syk are translocated to the membrane where they colocalize following receptor cross-linking. 3BP2 (via its SH2 domain) has been shown to interact with

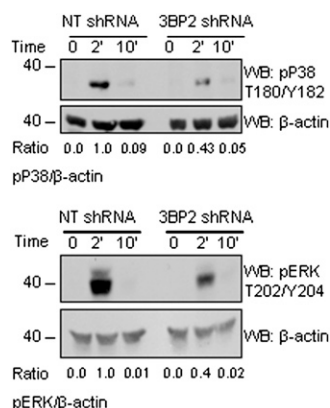


FIGURE 6. ERK and p38 MAPK phosphorylation is reduced in 3BP2-silenced huMCs. Biotinylated IgE-sensitized 3BP2 shRNA- and NT shRNA-transduced huMCs were stimulated with 100 ng/ml SA for 2 and 10 min. Cells were lysed, and total cell lysates were analyzed for ERK1/2 and p38 MAPK phosphorylation. Actin was used as loading control. Band intensity quantitation for each blot was performed by densitometry. The experiment is representative of three independent assays.

phosphorylated and activated Syk after AgR engagement of B cells (15). Syk also binds to the tyrosine-phosphorylated γ ITAM of the Fc ϵ RI through its SH2 domains (35) and is essential for all known Fc ϵ RI-mediated responses (36). 3BP2 also undergoes rapid dephosphorylation within 2 min upon stimulation, which could be mediated by Src homology region 2 domain-containing phosphatase-1 phosphatase, which has been described as a 3BP2 substrate in T cells (14). The aforementioned early phosphorylation events lead to the recruitment of molecules such as LAT and to the activation of enzymes such as PLC γ , which regulates intracellular calcium release and protein kinase C activation (31, 35). We observed that 3BP2 can bind PLC γ when the adaptor is phosphorylated. Indeed, it has been described that Syk can also induce phosphorylation of Y183 in 3BP2 (18), which then binds PLC γ , stabilizing the interaction of this enzyme with LAT and contributing to optimal phosphorylation of these molecules in B cells (18). The above interactions of 3BP2 would likely constitute an amplification mechanism that promotes Lyn-Syk-LAT-PLC γ signaling through stabilizing this complex. Reduction in 3BP2 expression did not completely ablate signaling, but we observed weaker phosphorylation of Syk Y352 as early as 5 min after stimulation and, downstream of this, reduced LAT Y191 and PLC γ Y738 phosphorylation. Pharmacological inhibitors of Syk kinase catalytic activity bearing therapeutic potential have been

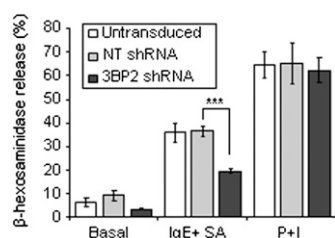


FIGURE 7. 3BP2 silencing impairs Fc ϵ RI-mediated degranulation in huMCs. Biotinylated IgE-sensitized CD34 $^{+}$ peripheral blood-derived huMCs with normal or reduced levels of 3BP2 (shRNA silenced) were stimulated with 100 ng/ml SA for 30 min. β -Hexosaminidase release was measured in collected supernatants. Results are expressed as a percentage of total β -hexosaminidase content reported as mean $\pm$ SE. Data represent the mean of three independent experiments. Statistical significance (***) is relative to NT shRNA.

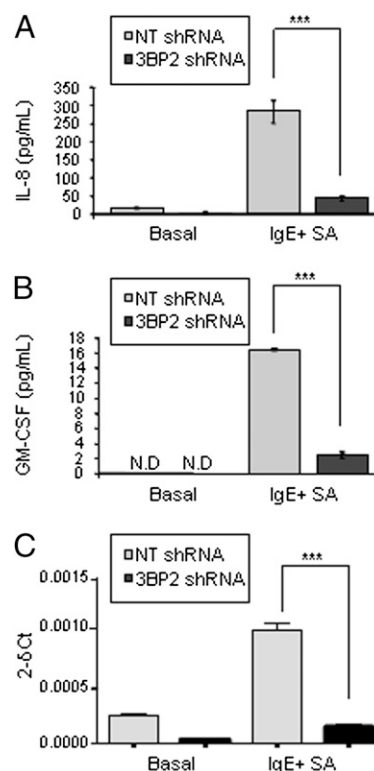


FIGURE 8. 3BP2 is required for normal huMC cytokine production. CD34 $^{+}$ peripheral blood-derived huMCs with normal or reduced levels of 3BP2 (NT shRNA and 3BP2 shRNA, respectively) were sensitized overnight with biotinylated IgE and stimulated for 6 h with 1 μ g/ml SA. IL-8 and GM-CSF production were measured by ELISA (A, B) and IL-8 transcripts relative to a control RNA were measured by real-time PCR (C). Results are expressed as the mean $\pm$ SD. Significant (***) difference was found between NT shRNA and 3BP2 shRNA. Data are the mean of triplicates, and are representative of three independent experiments from three different donors. N.D., Not detectable.

developed (37). However, Syk is widely distributed in different cell types, and inhibiting its catalytic activity could result in unwanted consequences. Inhibition of Syk interactions with its cellular partners, even while maintaining its kinase activity, might well result in the dampening of cellular responses and could be useful as a strategy with therapeutic potential (38). Because 3BP2 is a Syk binding protein it constitutes an interesting candidate for potential intervention in Syk-mediated signals. In addition to participating in early signaling events, 3BP2 also affects other downstream signaling molecules, which would account for the observed reduction in cytokine production following 3BP2 knockdown. Our findings show that ERK1 and 2 and p38 MAPKs are less active in 3BP2-silenced cells. Optimal activation of MAPKs requires LAT function as revealed in LAT-deficient bone marrow-derived mast cells (39, 40). Thus, the contribution of 3BP2 in achieving complete phosphorylation of LAT could explain the observed effects in late Fc ϵ RI responses, including MAPKs and transcription factor activity. In support of this view, 3BP2 has been reported to contribute to BCR-dependent activation of transcription factors such as NFAT, creating a multiprotein signalosome with the SH2 domain(s) of PLC γ 2 and Vav1 (18).

In this work, we show that in MCs, 3BP2 regulates and is necessary for GM-CSF production following Fc ϵ RI stimulation. Transcription of GM-CSF is known to be NFAT dependent (41). The two murine models in which the 3BP2 gene is deleted have generated some controversy as to whether there is a role for 3BP2 in cell viability and in Syk, PLC γ 2, and MAPK activation.

However, it is quite clear that B cell proliferation and BCR signaling are impaired, whereas there is normal T cell development, proliferation, cytokine secretion, and signaling in response to TCR ligation in 3BP2-deficient mice (16, 17). These findings suggest that 3BP2 is critical for BCR and humoral responses but not for TCR signaling. T cells could still have a homologous molecule that has a redundant function of 3BP2. The presence of a 3BP2 homolog is consistent with the observation of wild-type sequence of 3BP2 in 3 of 12 families with cherubism, a disease where mutation of 3BP2 results in molecule's gain of function (42).

Cherubism involves a chronic noninfectious inflammation of the bone, which occurs in the absence of a detectable adaptive immune response. Cherubism mice exhibit increased myeloid responses to M-CSF and RANKL, leading to the activation of osteoclast. As we learn more about the immunological features of this disease, it appears that TNF- α -secreting myeloid cells are emerging as primary players (9, 43). Although such cells are likely to be important players, MC involvement cannot be excluded. Recently, it has been demonstrated that cherubism mutations disrupt the interaction between 3BP2 and Tankyrase, a poly(ADP-ribose) polymerase, resulting in higher 3BP2 levels because increased protein stability, leading to hyperactive Src, Syk, and Vav pathways in osteoclasts (12). Structural evidence that deregulation of substrate recognition by Tankyrase is indeed the underlying mechanistic basis of cherubism has been as well provided (11). Conversely, it was published that overexpression of the cherubism 3BP2 mutants suppress Vav and MAPK activation attenuating the Fc ϵ RI-mediated activation in RBL-2H3 cells, suggesting that point mutations of 3BP2 cause dysfunction of 3BP2 in vivo (44). These data revealed the importance of elucidating the role of the protein in the most appropriate and physiological context.

In conclusion, in this study, we present evidence for the expression and critical role of 3BP2 in huMCs activation. This function is related to its essential role as an adaptor molecule for both early and late signal transduction responses required for, respectively, degranulation and the generation and release of cytokine. Increasing our knowledge on how 3BP2 functions will be crucial toward further understanding its role in the regulation of Fc ϵ RI signaling, which may also open new avenues of research with therapeutic promise in MC-derived diseases.

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Disclosures

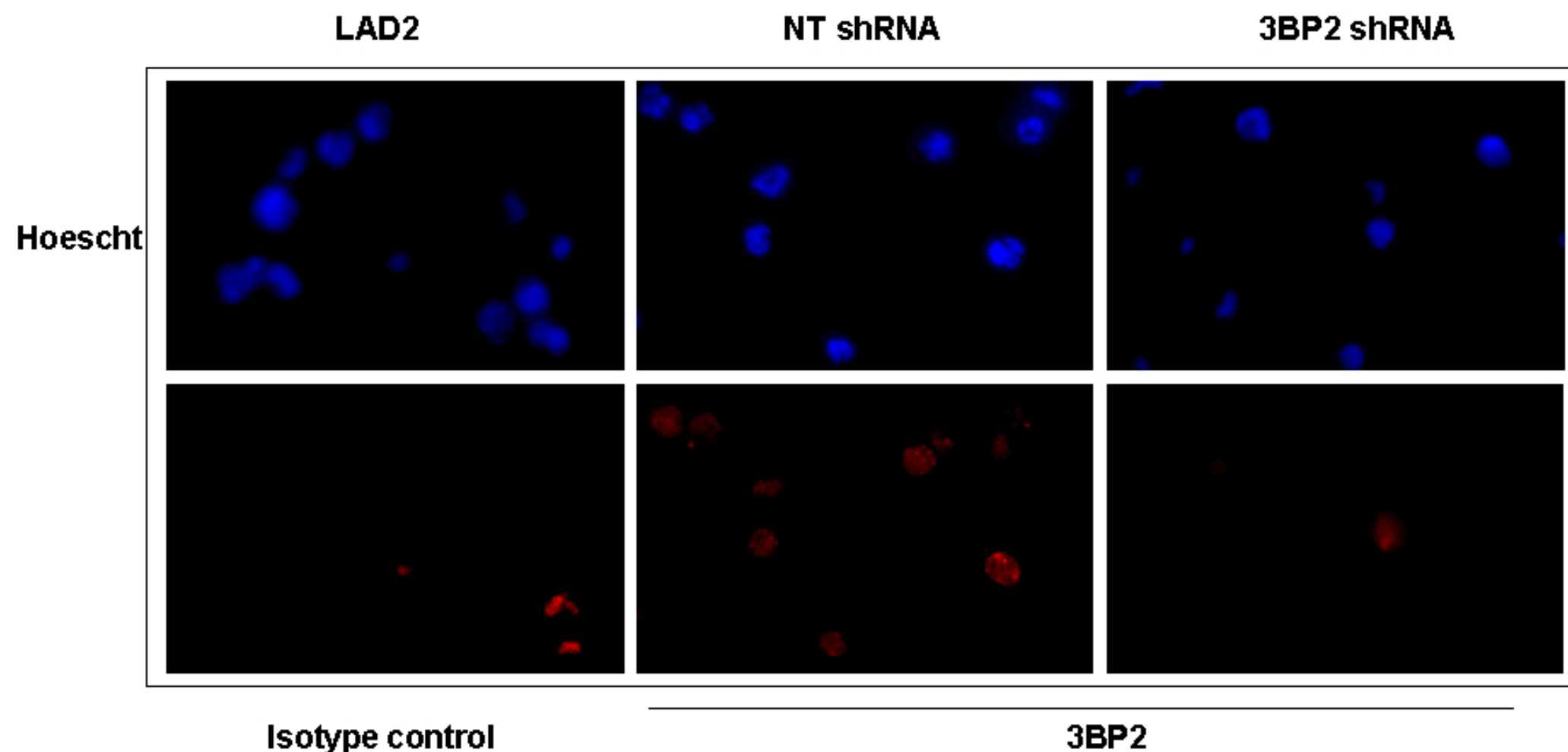
The authors have no financial conflicts of interest.

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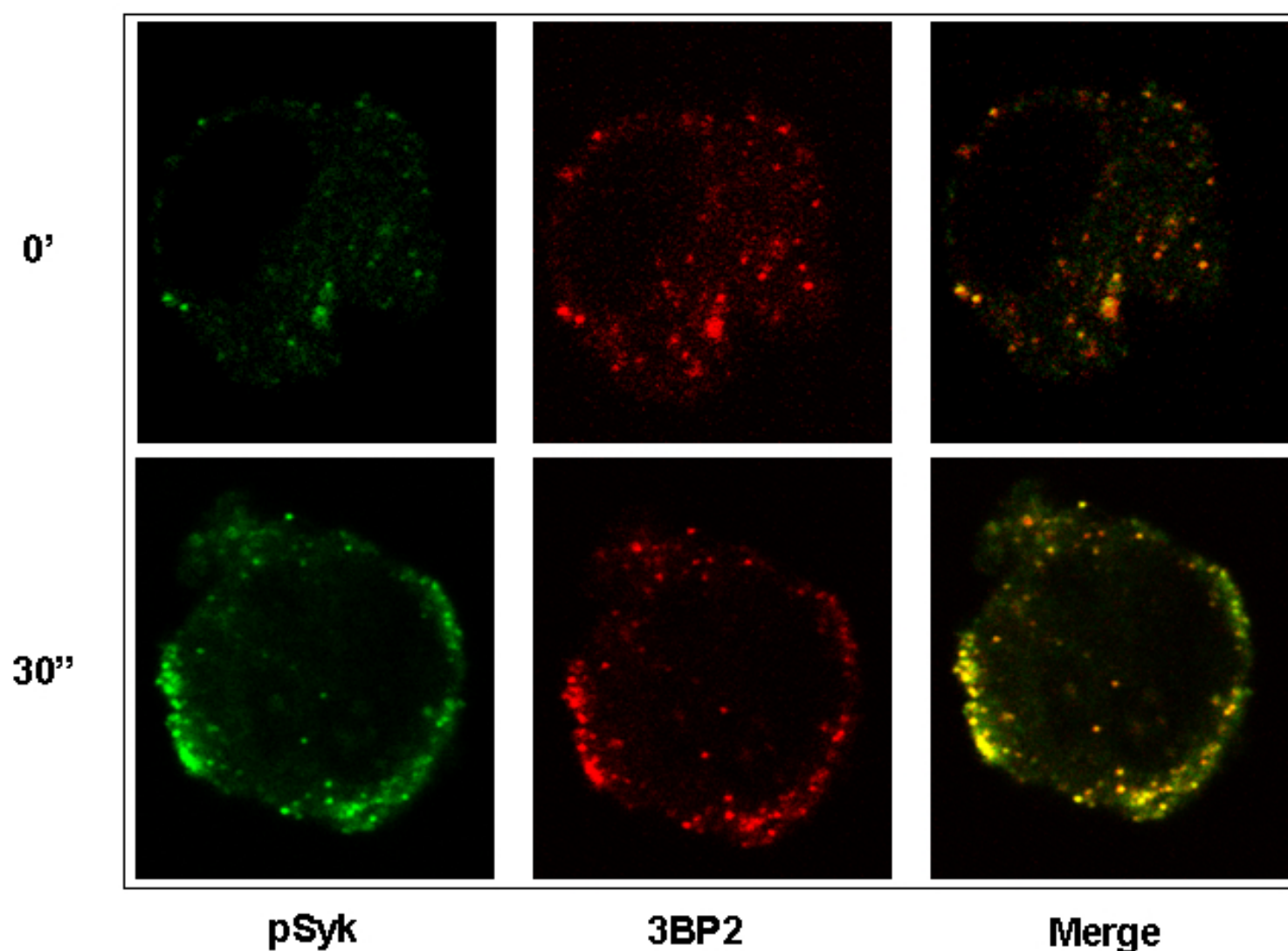
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Supplemental Figure 1



Supplemental Figure 1. 3BP2 intracellular punctuated pattern. Non target shRNA infected, 3BP2 shRNA infected and uninfected LAD2 cells were fixed, permeabilized and stained with anti-3BP2 (bottom middle and bottom right panels) and isotype control antibodies (bottom left panel) and further stained with Cy3 conjugated anti-mouse secondary antibodies as indicated in Materials and Methods section . Nuclei were visualized with Hoescht staining (upper panels).

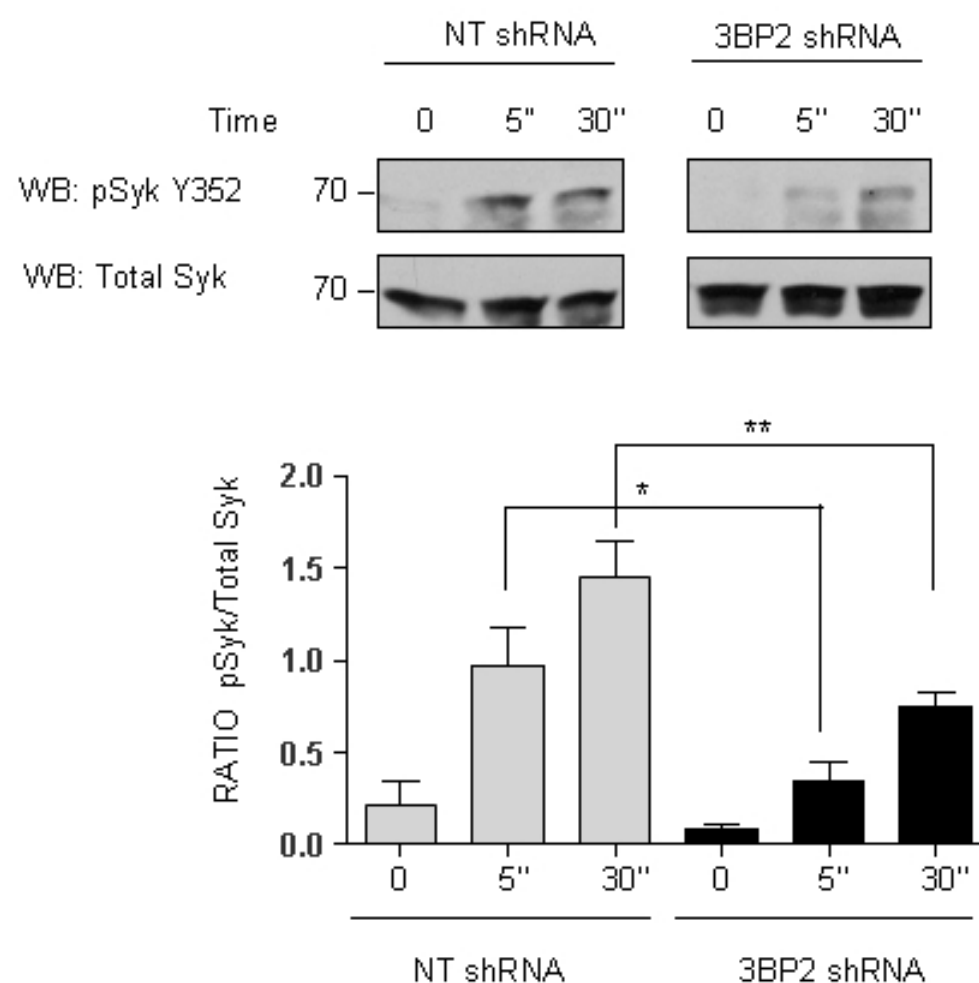
Supplemental Figure 2



Supplemental Figure 2. 3BP2 relocates at the plasma membrane and co-localizes with phospho Syk kinase.

Unstimulated and FcεRI-activated LAD2 cells at 30 seconds were fixed, permeabilized and stained against phospho-Syk and 3BP2 antibodies as indicated in Material and Methods Section. Fluorescence images were obtained using confocal microscope (TCS NT; Leica).

Supplemental Figure 3



Supplemental Figure 3. Silencing of 3BP2 reduces FcεRI-induced Syk Y352 phosphorylation at early times

Specific antibodies recognizing phosphorylated Syk Y352 and total Syk were used to immunoprobe these proteins in 3BP2 shRNA- and NT shRNA- transduced LAD2 cells following biotinylated IgE crosslinking with 100 ng/ml SA at indicated times. Densitometric analysis and phosphorylated/total ratio for Syk from 3 independent experiments is represented in the bar charts on the bottom. Statistical significance (* $p < 0.05$, ** $p < 0.001$,) is relative to NT shRNA.