The Adaptor 3BP2 Is Required for Early and Late Events in Fc \( \varepsilon \)RI 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 Cγ1, 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 MC 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 Cγ1, 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: 2727–2734.
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 Tyr183, 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 FceRI-dependent degranulation in the rat basophilic leukemia cell line RBL-2H3 (22), evidence for a role for endogenous 3BP2 in human MC 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 FceRI 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 MC activation through FceRI. Altogether our data show an important role for this adaptor in MC function through one of the major MC receptors.

Materials and Methods

**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 serum-free (2 mM), penicillin (100 U/ml), streptomycin (100 µg/ml), and 100 ng/ml recombinant stem cell factor (SCF) (Amgen, Thousand Oaks, CA). The LUV 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; Institute of Allergy and Infectious Diseases Institutional Review Board and according to the manufacturer’s instructions).

**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)) × 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/pool were loaded with 5 µM 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 Bio-systems (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 µM 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 Bio-tech, 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 prepared by SDS-PAGE gel and electroblotted 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 FceRI-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 anti-pP38 Thr180/Tyr182, anti–p-ERK Thr202/Tyr204, and anti–p-JNK Thr183/Y185/Tyr185. Images were analyzed using MetaMorph software (Molecular Devices).
IgG1 isotype control (1 μg/ml) at 4˚C for 1 h. After washing with ice-cold PBS, cells were incubated with 1 μg/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 μg/ml) for 6 h. Supernatants were collected and analyzed by ELISA.

Statistical data analysis

All results are expressed as mean ± 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 FceRI 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 FceRI-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 knockdown cells (Supplemental Fig. 1). Moreover, 3BP2 relocates mainly at the plasma membrane after early FceRI 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 FceRI aggregation.

3BP2 recruits essential signaling mediators in huMCs

We next analyzed 3BP2 phosphorylation and signalosome assembly after FceRI 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 FceRI signaling molecules Lyn, Syk, and PLCγ 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 PLCγ (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 3BP2 could play a central role in the FceRI-anchored signalosome, we investigated the consequences of 3BP2 depletion to FceRI signaling. We first analyzed the efficiency of lentiviral-encoded shRNA sequences for their ability to silence 3BP2, as compared with nontransfected 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 FceRI expression (Fig. 3C) in NT and 3BP2-silenced LAD2 and CD34+–derived huMCs. Our results show that both FceRI and Lyn expression were unaffected by 3BP2 knockdown.

3BP2 is required for FceRI-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 FceRI activation (Fig. 2). Syk has an essential role in early and late MC responses (30), binding the phosphorylated FceRIγ-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 FceRI 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 FceRI stimulation (Supplemental Fig. 3).
3BP2 is required for FcεRI-dependent LAT and PLCγ phosphorylation and calcium signaling

Activated Syk phosphorylates LAT, which acts as a scaffold for a multimolecular signaling complex that includes PLCγ among other molecules (31). Because 3BP2 expression modulates Syk phosphorylation, we expected that LAT and PLCγ 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 FcεRI stimulation, and we found a decrease in LAT and PLCγ1 phosphorylation when 3BP2 expression was reduced (Fig. 5A). PLCγ activation and phosphorylation facilitated by LAT are key steps in Ca2+ release from intracellular stores (29, 32). Hence, we measured calcium fluxes following FcεRI 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 FcεRI 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 2. 3BP2 coprecipitates with Lyn, Syk, and PLCγ 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-PLCγ, 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.

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 FcεRI expression (C) were similar in 3BP2 shRNA and control-transduced huMCs and LAD2 cells. FcεRI expression was determined by FACS using PE-conjugated anti-FcεRI. The isotype control is gray filled. Data are representative of three experiments.
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 in huMC degranulation. Thus, we examined β2-hexosaminidase release in 3BP2 shRNA- versus NT shRNA-transduced 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 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 domain (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...
phosphorylated and activated Syk after AgR engagement of B cells (15). Syk also binds to the tyrosine-phosphorylated γ ITAM of the FcεRI through its SH2 domains (35) and is essential for all known FcεRI-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 PLCγ, which regulates intracellular calcium release and protein kinase C activation (31, 35). We observed that 3BP2 can bind PLCγ when the adaptor is phosphorylated. Indeed, it has been described that Syk can also induce phosphorylation of Y183 in 3BP2 (18), which then binds PLCγ1, 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-PLCγ 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 PLCγ Y738 phosphorylation. Pharmacological inhibitors of Syk kinase catalytic activity bearing therapeutic potential have been 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 FcεRI 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 PLCγ2 and Vav1 (18).

In this work, we show that in MCs, 3BP2 regulates and is necessary for GM-CSF production following FcεRI 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, PLCγ2, and MAPK activation.

**FIGURE 7.** 3BP2 silencing impairs FcεRI-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 released as mean ± SE. Data represent the mean of three independent experiments. Statistical significance (**p < 0.0001) is relative to NT shRNA.
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 will be consistent with the observation of wild-type sequence of 3BP2 in 3 of 12 families with cherubism, a disease where 3BP2 has a redundant function of 3BP2. The presence of a 3BP2 in SH3BP2 “cherubism” mice. Cell 128: 71–83.


