

B Cell Lymphoma 10 Is Essential for FcεR-Mediated Degranulation and IL-6 Production in Mast Cells¹

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The adaptor protein B cell lymphoma 10 (Bcl10) plays an essential role in the functions of the AgRs in T and B cells. In this study, we report that Bcl10 also plays an important role in mast cells. Bcl10 is expressed in mast cells. Although Bcl10-deficient mast cells undergo normal development, we demonstrate that Bcl10 is essential for specific functions of FcεR. Although Bcl10-deficient mast cells have normal de novo synthesis and release of the lipid mediator arachidonic acid, the mutant cells possess impaired FcεR-mediated degranulation, indicated by decreased serotonin release, and impaired cytokine production, measured by release of IL-6. In addition, Bcl10-deficient mice display impaired IgE-mediated passive cutaneous anaphylaxis. Moreover, although Bcl10-deficient mast cells have normal FcεR-mediated Ca²⁺ flux, activation of PI3K, and activation of the three types of MAPKs (ERKs, JNK, and p38), the mutant cells have markedly diminished FcεR-mediated activation of NF-κB and decreased activation of AP-1. Thus, Bcl10 is essential for FcεR-induced activation of AP-1, NF-κB, degranulation, and cytokine production in mast cells. *The Journal of Immunology*, 2007, 178: 49–57.

Mast cells function as important effectors in IgE-associated allergic and adaptive immune responses and in Th2-mediated immune responses. They also function as initiators and effectors of innate immunity (1, 2). Mast cells are derived from hemopoietic stem cells, leave the bone marrow as progenitors, and undergo their maturation in peripheral tissues. Mast cells ultimately reside in serosal cavities and all vascularized tissues, particularly at the sites exposed to the environment, such as the skin, airways, and gastrointestinal tract (1, 2). Stem cell factor is essential for survival and maintenance of mast cells in peripheral tissues (1–3). Mice that lack functional stem cell factor or its receptor c-Kit have severe mast cell deficiencies (4, 5). Mast cells are properly positioned to be one of the first cells of the immune system to encounter allergens, Ags, and pathogens.

A variety of stimuli, especially engagement of receptors for the Fc portion of Ig (FcR) on mast cells, can activate these cells. FcRs exist for every Ab class and are exemplified by FcγR binding to IgG, FcαR binding to IgA, and FcεR binding to IgE. High-affinity FcRs are referred to as FcRI and low-affinity FcRs as FcRII/III.

High-affinity FcRs can bind noncomplexed, monomeric Igs while low-affinity FcRs bind aggregated Igs or Abs complexed to multivalent Ags (6, 7). FcRs couple humoral and cellular immunity by directing the interaction of Abs with effector cells (6). FcεRI is expressed primarily by mast cells and basophils (7). Cross-linking of the FcεRI on mast cells induces the release of biologically active mediators: the preformed mediators stored in the cytoplasmic granules, including histamine, serotonin, β-hexosaminidase, and proteoglycans, and the newly synthesized mediators, such as PGs and leukotrienes, many growth factors, cytokines, and chemokines (1, 2).

The FcεR belongs to the Ig receptor superfamily, which also includes TCR and BCR. Like the TCR and BCR, engagement of FcεR initiates activation of several cytoplasmic protein tyrosine kinases, including the Src, Syk, and Tec family of kinases (8, 9). In turn, the activated tyrosine kinases rapidly lead to phosphorylation and recruitment of adaptor molecules, including linker for activation of T cells, Src homology 2-containing leukocyte protein of 76 kDa, non-T cell activation linker, and Grb2-associated binder-like protein 2 (10–13). Subsequently, the receptor complex results in the activation of signaling effectors, including PI3K and phospholipase Cγ (PLCγ).⁴ PI3K phosphorylates the membrane phospholipid phosphatidyl inositol 4,5-bisphosphate (PI-4,5-P₂) to generate the important second messenger, PI-3,4,5-P₃, whereas PLCγ hydrolyzes PI-4,5-P₂ to generate the critical second messengers, diacylglycerol and inositol 1,4,5-trisphosphate (14, 15). Diacylglycerol activates protein kinase C (PKC), while inositol 1,4,5-trisphosphate mediates the mobilization of Ca²⁺ from internal stores, resulting in a transient intracellular Ca²⁺ flux (14). The PLCγ/Ca²⁺/PKC pathway has been shown to be involved in the activation of all types of MAPKs (ERKs, JNKs, and p38 MAPKs) (16–20), although the PKC-independent growth factor receptor-bound protein 2/son of sevenless/ras-activated factor 1 pathway plays a primary role in the activation of MAPKs (17, 21, 22).

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⁴ Abbreviations used in this paper: PLC, phospholipase C; PKC, protein kinase C; HSA, human serum albumin; Bcl10, B cell lymphoma 10; CARD, caspase recruitment domain; CARMA1, CARD membrane-associated guanylate kinase protein 1; MALT, mucosa-associated lymphoid tissue; MALT1, MALT lymphoma translocation protein 1; IKK, IκB kinase; MSCV, murine stem cell virus; IRES, internal ribosomal entry site.

Activated PKC can promote activation of ERK-1 and ERK-2 (16–18). Calcium and PKC also participate in JNK activation (19, 20). In addition, PKC is required for the maximum activation of p38 MAPK (19, 20, 23). Moreover, the Fc ϵ R complex results in activation of the small GTPases, such as Ras, Rac, and Rho. In turn, these GTPases are also involved in regulating the activation of ERK, JNK, and p38 (1, 2, 24). Recently, a mast cell-specific Ras guanine nucleotide-releasing protein, RasGRP4, which contributes to mast cell granule formation, has been identified (25, 26). Ultimately, these signaling cascades lead to the activation of transcription factors, including NF- κ B, AP-1, NFAT, and Elk-1 (27–29).

B cell lymphoma 10 (Bcl10) is an adaptor protein characterized by an N-terminal caspase recruitment domain (CARD) and a C-terminal Ser/Thr-rich region (30, 31). Bcl10 was discovered due to its chromosomal translocation to the genomic locus of the Ig H chain gene in the t(1;14) (p22;q32), which occurs specifically in mucosa-associated lymphoid tissue (MALT) lymphomas (32, 33). Recent studies have demonstrated that Bcl10 is essential for AgR-mediated activation of NF- κ B (34, 35). Bcl10, along with CARD membrane-associated guanylate kinase protein 1 (CARMA1) and MALT lymphoma translocation protein 1 (MALT1), forms a three-component complex, coupling PKC to I κ B kinase (IKK) upon AgR engagement (36, 37). Bcl10 deficiency impairs TCR-induced NF- κ B activation and diminishes TCR-mediated T cell proliferation, IL-2 production, and up-regulation of T cell activation markers (34). In addition, Bcl10 deficiency impairs BCR-induced NF- κ B activation and Bcl10-deficient B cells exhibit a severe defect in BCR-induced proliferation. Bcl10-deficient mice display a complete reduction of the basal levels of all serum Igs and severely impaired immune responses to both T cell-dependent and T cell-independent Ags. Bcl10 deficiency also results in a dramatic reduction in the numbers of mature follicular, marginal zone, and peritoneal B1 B cells (35).

The resemblances between signaling events triggered by Fc ϵ R on mast cells and those by AgRs on lymphocytes (38) and the critical role of Bcl10 in AgR-mediated T and B cell development and function (34, 35), prompted us to examine the role of Bcl10 in Fc ϵ R-mediated mast cell function. A recent study found that Bcl10 is essential for Fc ϵ R-mediated NF- κ B activation and cytokine production in mast cells (39). Our current study uses independently developed Bcl10-deficient mice (35) and shows that Bcl10 is involved in Fc ϵ R signaling in mast cells and is essential for Fc ϵ R-mediated, not only cytokine production, but also serotonin release and IgE-mediated passive anaphylactic reactions.

Materials and Methods

Abs and reagents

Rabbit polyclonal anti-ERK (sc-093), anti-JNK2 (sc-572), anti-p38 (sc-535), anti-phospho-ERK (pThr²⁰²/pTyr²⁰⁴, sc-7383), mouse monoclonal anti-Bcl10 (sc-5273), and goat polyclonal anti-Akt1 (sc-1618) Abs were purchased from Santa Cruz Biotechnology. Rabbit polyclonal anti-phospho-p38 (pThr¹⁸⁰/pTyr¹⁸², no. 9216), anti-phospho-Akt (phospho-Ser⁴⁷³, no. 9271), anti-I κ B α (no. 9242), anti-phospho-PKC (pan. β II Ser⁶⁶⁰, no. 9371), mouse monoclonal anti-phospho-JNK (pThr¹⁸³/pTyr¹⁸⁵, no. 9255), anti-phospho-I κ B α (Ser^{32/36}, no. 9246) Abs were purchased from Cell Signaling Technology. Rabbit anti-mouse IgE Abs (553413), FITC-conjugated anti-mouse IgE Abs (1130-02), anti-trinitrophenyl IgE (03241D), control monoclonal IgG2b (03041D), and the OptEIA mouse IL-6 ELISA kit (550950) were purchased from BD Pharmingen. PE-conjugated anti-mouse c-Kit (no. 12-1171) and PE-conjugated rat IgG2b isotype control (12-4031) Abs were purchased from eBioscience. Mouse monoclonal anti-DNP IgE (SPE-7) Abs and DNP-human serum albumin (DNP-HSA) were purchased from Sigma-Aldrich. PKC α /PKC β inhibitor Go6976 (no. 365250) was purchased from Calbiochem.

Mice

Bcl10-deficient mice were generated as previously described (35). Bcl10-deficient and wild-type control mice were on a mixed C57BL/6 $\times$ 129/Ola genetic background. Heterozygous Bcl10-deficient mice were bred to generate the Bcl10-deficient and wild-type mice. The Bcl10-deficient mice and their wild-type littermates (2–4 mo old) were used for experiments.

Mast cells

Mast cells were derived from bone marrow cells as previously described (40). Briefly, bone marrow cells from wild-type or Bcl10-deficient mice were cultured in RPMI 1640 medium containing 100 U/ml penicillin, 100 μ g/ml streptomycin, 25 U/ml rIL-3 (R&D Systems), and 10% FBS (HyClone) for 4–8 wk, with medium replacement every 3–4 days. For [³H]thymidine incorporation assay, mast cells (1×10^5) were cultured in round-bottom 96-well plates in the presence of indicated concentrations of IL-3 for 48 h. The culture was pulsed with 1 μ Ci/well [³H]thymidine for 20 h. Incorporated [³H]thymidine was determined by a Betaplate Liquid Scintillation Counter.

Flow cytometry analysis

For IgE expression, wild-type and Bcl10-deficient mast cells were first incubated with mouse monoclonal anti-trinitrophenyl IgE or control monoclonal IgG2b, followed by incubation with FITC-conjugated anti-mouse IgE Ab. For c-Kit expression, the mast cells were incubated with PE-conjugated anti-mouse c-kit. The levels of Fc ϵ RI and c-Kit expression were measured by flow cytometry.

IgE-mediated passive cutaneous anaphylaxis

Wild-type and Bcl10-deficient mice were lightly anesthetized and injected intradermally into the ear (250 ng in 10 μ l of PBS) and at the basolateral side of the back (500 ng in 20 μ l of PBS) with monoclonal mouse anti-DNP IgE. Twenty-four hours later, the mice were injected i.v. with 150 μ g of DNP-HSA in 150 μ l of PBS with 0.5% Evans blue dye (Sigma-Aldrich). Sixty minutes later, extravasation was visualized by blue staining of the injection sites at the ear and at the inside of skin sections as an indication of a positive passive cutaneous anaphylaxis reaction (40, 41). The amount of extravasated dye in the ear was measured as previously described (41, 42). Briefly, the ear was dissolved with 0.7 ml of 1 N KOH at 37°C overnight. Subsequently, 9.3 ml of a mixture of 0.6 N H₃PO₄ and acetone (5:13) was added. Following centrifugation, the amount of Evans blue dye in the supernatant was measured colorimetrically at 620 nm.

Calcium fluorometry

Bone marrow-derived mast cells (2×10^6 /ml) were incubated with anti-DNP IgE Abs (10 μ g/ml) in DMEM with 10% FBS at room temperature for 1 h. Indo-1 (10 μ g/ml; Molecular Probes) was added to the cells, followed by further incubation at room temperature for 30 min. The cells were washed and then stimulated with anti-mouse IgE Abs (10 μ g/ml). The calcium concentration was determined by flow cytometry as previously described (40).

Reconstitution of Bcl10 in Bcl10-deficient mast cells

As previously described (43, 44), wild-type Bcl10 was cloned into a bicistronic retrovirus murine stem cell virus (MSCV)-internal ribosomal entry site (IRES)-GFP vector, in which the expression of Bcl10 and GFP (the latter functioning as an indicator of retrovirally transduced cells) are under the control of the murine stem cell virus MSCV promoter. Conditioned media containing high-titer, amphotropic retrovirus particles were derived by cotransfection of 293T cells with the retrovirus vector containing Bcl10 and GFP and a helper plasmid pEQPAM3 containing the required gag, pol, and env retroviral genes. These conditioned media were used to transduce ecotropic packaging cells (GP plus E86), together with 6 μ g/ml polybrene. Cells exhibiting high GFP expression were sorted and subsequently expanded as virus-producing cells. Mast cells were then cocultured on irradiated ecotropic producer cells (GP plus E86) in the presence of IL-3 and polybrene (6 μ g/ml). After 48 h, GFP⁺ cells were sorted out as Bcl10-reconstituted Bcl10-deficient mast cells.

Phosphorylation of MAPKs, PKC, Akt, and I κ B α

Bone marrow-derived mast cells were preloaded with mouse anti-DNP IgE (1 μ g/ml) overnight. Following extensive washing, the cells were stimulated with rabbit anti-mouse IgE Abs (10 μ g/ml) for 0, 2, 5, 10, and 30 min. Cells were lysed in sample buffer and subjected to 10% SDS-PAGE. The protein phosphorylation and expression levels were detected by Western blot with the indicated Abs.

Serotonin release assay

Bone marrow-derived mast cells ($1 \times 10^6/\text{ml}$) were incubated with anti-DNP IgE Abs ($1 \mu\text{g}/\text{ml}$) and $5 \mu\text{Ci}/\text{ml}$ [^3H]serotonin (NEN) for 16 h. After extensive washing, the cells were stimulated with different concentrations of anti-mouse IgE Abs for 1 h. The amount of [^3H]serotonin was measured in duplicate by liquid scintillation in both supernatants and cell pellets. The percentage of serotonin release was calculated using the formula $(S/(S + P)) \times 100$, where S and P are the serotonin contents of the supernatants and pellets, respectively.

Arachidonic acid release assay

Bone marrow-derived mast cells ($1 \times 10^6/\text{ml}$) were incubated with anti-DNP IgE Abs ($1 \mu\text{g}/\text{ml}$) for 3 h, followed by incubation with $2.5 \mu\text{Ci}/\text{ml}$ [^3H]arachidonic acid (PerkinElmer) for 2 h. After extensive washing, the cells were stimulated with different concentrations of anti-mouse IgE Abs for 1 h. The amounts of [^3H]arachidonic acid released into the supernatants and remaining in the cell pellets were quantitated. The percentage of arachidonic acid release was calculated as performed in the serotonin release assay.

IL-6 production assay

Bone marrow-derived mast cells ($1 \times 10^6/\text{ml}$) were incubated with anti-DNP IgE mAb Abs ($1 \mu\text{g}/\text{ml}$) for 3 h and then stimulated with different concentrations of anti-mouse IgE Abs for 24 h. The concentration of IL-6 in the supernatant was measured by ELISA according to the manufacturer's instruction manual.

Gel mobility shift assays

Bone marrow-derived mast cells ($5 \times 10^6/\text{ml}$) were incubated with anti-DNP IgE Abs ($1 \mu\text{g}/\text{ml}$) overnight, followed by stimulation with DNP-HSA ($100 \text{ ng}/\text{ml}$) for the indicated time. Nuclear extracts were prepared for gel mobility shift assays using ^{32}P -labeled probes containing NF- κB - or AP-1-binding sequences (purchased from Promega).

Results

Bcl10 deficiency does not affect development of mast cells

Bcl10 plays a critical role in both TCR and BCR signaling and is essential for TCR- and BCR-mediated lymphocyte functions (34, 35). To study a potential role for Bcl10 in mast cell development and Fc ϵ R-mediated mast cell functions, we first examined the expression of the protein in mast cells derived from mouse bone marrow. Mast cells were lysed and the expression of Bcl10 in the cell lysates was identified in direct Western blot analysis by a Bcl10-specific Ab. Bcl10 was expressed in wild-type mast cells and its expression was abolished in the Bcl10 $^{-/-}$ mast cells (Fig. 1A).

To determine whether Bcl10 deficiency affects mast cell development, the proliferation of the cells and their expression of mature mast cell markers, such as Fc ϵ RI and c-Kit, were examined in wild-type and Bcl10-deficient bone marrow-derived mast cells. Mast cells from wild-type and Bcl10 $^{-/-}$ bone marrow proliferated equally well in vitro in media containing IL-3 (Fig. 1B). In addition, the levels of Fc ϵ RI and c-Kit expression were comparable between wild-type and Bcl10 $^{-/-}$ mast cells (data not shown). Therefore, mast cell proliferation and differentiation were not impaired by the loss of Bcl10.

Bcl10 deficiency impairs Fc ϵ R-mediated degranulation but not de novo synthesis and release of proinflammatory lipid mediators

Cross-linking of the Fc ϵ R on mast cells induces the release of preformed mediators, including serotonin, from the cytoplasmic granules via degranulation as well as the synthesis and release of proinflammatory lipid mediators, such as arachidonic acid metabolites, and many growth factors, cytokines, and chemokines (1, 2, 24). To assess the role of Bcl10 in Fc ϵ R-mediated release of these different biologically active mediators, we examined the ability of Bcl10-deficient mast cells to release serotonin and arachidonic acid metabolites upon Fc ϵ R engagement. Following cross-linking

of Fc ϵ R with increasing doses of anti-IgE Abs, the Fc ϵ R-mediated release of serotonin was impaired in Bcl10-deficient compared with wild-type mast cells (Fig. 2A). In contrast, in response to different doses of anti-IgE Abs, the Fc ϵ R-mediated release of the arachidonic acid metabolites was comparable between Bcl10-deficient and wild-type mast cells (Fig. 2B). These data demonstrate that the release of the preformed mediators and the de novo synthesis and release of the proinflammatory lipid mediators are regulated by different signals emanating from Fc ϵ R. Bcl10 is important for Fc ϵ R-mediated degranulation but not for the synthesis and release of proinflammatory lipid mediators.

Bcl10 deficiency impairs Fc ϵ R-mediated production of IL-6

Cross-linking of the Fc ϵ R on mast cells also induces the synthesis and secretion of multiple cytokines, including IL-6 (1, 2). To explore the role of Bcl10 in Fc ϵ RI-mediated synthesis and secretion of cytokines, we examined the release of IL-6 from Bcl10-deficient mast cells upon Fc ϵ R engagement. Interestingly, the Fc ϵ R-mediated secretion of IL-6 in response to different doses of anti-IgE Abs was dramatically decreased in Bcl10-deficient, relative to wild-type, mast cells (Fig. 2C). Therefore, Bcl10 is essential for the Fc ϵ R-mediated synthesis and release of the cytokine IL-6.

Bcl10-deficient mice have reduced IgE-mediated passive cutaneous anaphylaxis

Fc ϵ R plays an essential role in passive cutaneous anaphylaxis, in which local extravasation, fibrin deposition, and tissue swelling are induced by local injection of Ag-specific IgE followed by i.v. antigenic challenge (45). Thus, the role of Bcl10 in passive cutaneous anaphylaxis in response to IgE-mediated activation was examined. Wild-type and Bcl10-deficient mice were injected intradermally into the ear and at the basolateral side of the back with monoclonal mouse anti-DNP IgE. Twenty-four hours later, the mice were injected i.v. with DNP-HSA and Evans blue dye. One hour thereafter, extravasation was visualized by blue staining of the injection sites at the ear and the inside of the basolateral skin sections. The extravasation of Evans blue dye in the ear seemed comparable between wild-type and Bcl10-deficient mice (Fig. 3A). The total amount of Evans blue dye in the ear was slightly reduced (statistically not significant) in Bcl10-deficient, relative to wild-type, mice (Fig. 3B). However, the extravasation of Evans blue dye at the basolateral side was greatly reduced in Bcl10-deficient, relative to wild-type, mice (Fig. 3C). Therefore, Bcl10 plays a critical role in IgE-mediated passive cutaneous anaphylaxis, consistent with the notion that both Fc ϵ R-mediated degranulation and cytokine production are important for Fc ϵ R-mediated passive cutaneous

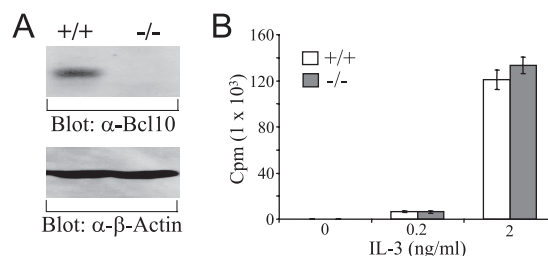


FIGURE 1. Bcl10-deficient mast cells undergo normal development. **A**, Expression of Bcl10 in wild-type but not Bcl10-deficient mast cells. Wild-type (+/+) and Bcl10-deficient (-/-) mast cells were lysed and the cell lysates were subjected to direct Western blot analysis with anti-Bcl10 or anti- β -Actin Abs. **B**, Bcl10-deficient mast cells have normal proliferation. Wild-type and Bcl10-deficient mast cells were cultured in medium containing the indicated concentrations of IL-3 and examined by [^3H]thymidine incorporation.

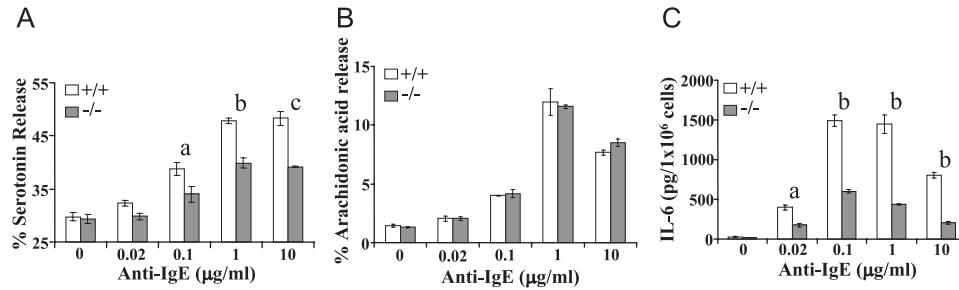


FIGURE 2. FcεR-mediated serotonin, arachidonic acid, and IL-6 release in wild-type and Bcl10-deficient mast cells. *A*, Impaired FcεR-mediated serotonin release in Bcl10-deficient mast cells. Wild-type (+/+) and Bcl10-deficient (-/-) mast cells were incubated with anti-DNP IgE Abs and [³H]serotonin. After exposure to the indicated doses of anti-mouse IgE Abs, the [³H]serotonin released into the supernatants and the [³H]serotonin remaining in the cell pellets were quantitated. The percentage of serotonin release values were calculated. *a*, $p = 0.01$; *b*, $p = 0.02$; *c*, $p < 0.01$. The data shown are representative of three independent analyses. *B*, Normal FcεR-mediated arachidonic acid release in Bcl10-deficient mast cells. Wild-type and Bcl10-deficient mast cells were incubated with anti-DNP IgE Abs, followed by incubation with [³H]arachidonic acid. After exposure to the indicated doses of anti-mouse IgE Abs, the [³H]arachidonic acid released into the supernatants and the [³H]arachidonic acid remaining in the cell pellets were quantitated. The percentage of arachidonic acid release values were calculated. The data are representative of four independent analyses. *C*, Impaired FcεR-mediated secretion of IL-6 in Bcl10-deficient mast cells. Wild-type and Bcl10-deficient mast cells were incubated with anti-DNP IgE Abs. After exposure to the indicated doses of anti-IgE Abs, cell supernatants were collected and subjected to ELISA analysis for detection of IL-6 protein. *a*, $p = 0.02$; *b*, $p < 0.01$. The data are representative of two independent analyses.

anaphylaxis (1, 46) and the findings that Bcl10 is critical for FcεR-mediated degranulation and cytokine production.

Impaired FcεR-mediated functions in Bcl10-deficient mast cells are rescued by reconstitution of Bcl10

The impaired FcεR-mediated degranulation and cytokine production that we observed could potentially be due to alteration of mast cell differentiation due to Bcl10 deficiency or that other genes responsible for the defects were affected during the targeted disruption of the *Bcl10* gene. To exclude these possibilities, we assessed the ability of Bcl10 to rescue FcεR-mediated functions in Bcl10-deficient mast cells. Bcl10 was introduced into Bcl10-deficient mast cells using infectious retrovirus stock produced with a bicis-

tronic MSCV-IRES-GFP vector. Bcl10-transduced Bcl10-deficient mast cells displayed normal FcεR-induced release of serotonin, compared with GFP-transduced wild-type mast cells (Fig. 4A). In contrast, GFP-transduced Bcl10-deficient mast cells still had impaired FcεR-induced release of serotonin (Fig. 4A). Similarly, both Bcl10-transduced Bcl10-deficient mast cells and GFP-transduced wild-type mast cells had normal FcεR-induced IL-6 production whereas GFP-transduced Bcl10-deficient mast cells continued to exhibit impaired FcεR-induced IL-6 production (Fig. 4B). These data demonstrate that reconstitution of Bcl10 rescues FcεR-mediated functions in Bcl10-deficient mast cells. Thus, Bcl10 deficiency directly disrupts FcεR signaling, resulting in impairment of FcεR-mediated functions.

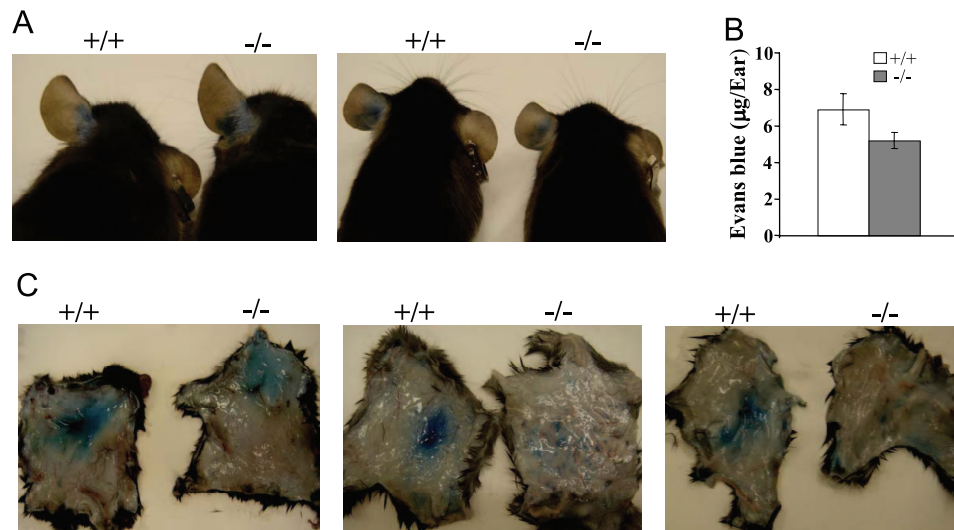


FIGURE 3. Bcl10-deficient mice have reduced IgE-mediated passive cutaneous anaphylaxis. *A*, IgE-mediated passive cutaneous anaphylaxis in the ear of wild-type and Bcl10-deficient mice. Wild-type (+/+) and Bcl10-deficient (-/-) mice were injected intradermally into their ears with monoclonal mouse anti-DNP IgE. Subsequently, the mice were injected i.v. with DNP-HSA with Evans blue dye. Extravasation was visualized by blue staining at the ear. The figure shown is representative of three mice per genotype. *B*, Quantification of IgE-mediated Evans blue extravasation in the ears. The amount of extravasated dye in the ears was measured as described in *Materials and Methods*; $p = 0.13$. *C*, IgE-mediated passive cutaneous anaphylaxis at the basolateral side of wild-type and Bcl10-deficient mice. Wild-type and Bcl10-deficient mice were injected intradermally in their basolateral sides with monoclonal mouse anti-DNP IgE. Subsequently, the mice were injected i.v. with DNP-HSA with Evans blue dye. Extravasation was visualized by blue staining at the injection sites on the inside of skin sections removed from the sites of intradermal injection. The figure shown is representative of six mice per genotype.

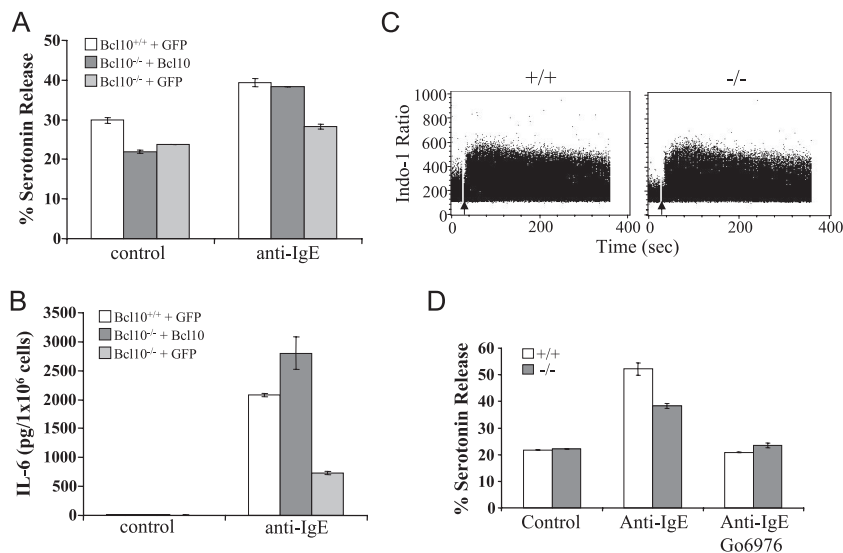


FIGURE 4. Reconstitution of Bcl10-deficient mast cells with Bcl10, as well as FcεR-mediated Ca^{2+} flux and PKC activation and effect of PKC inhibitor Go6976 on FcεR-mediated serotonin release in wild-type and Bcl10-deficient mast cells. **A**, Restoration of FcεR-mediated serotonin release in Bcl10-deficient mast cells by Bcl10. Bcl10 was introduced into Bcl10-deficient mast cells using a bicistronic MSCV-IRES-GFP retrovirus. The GFP⁺-reconstituted mutant mast cells (Bcl10^{-/-} plus Bcl10) were incubated with anti-DNP IgE Abs and [³H]serotonin. After exposure to anti-mouse IgE Abs (1 μg/ml), the [³H]serotonin released into the supernatants and the [³H]serotonin remaining in the cell pellets were quantitated. The percentage of serotonin release values was then calculated. GFP retrovirus-transduced wild-type mast cells (Bcl10^{+/+} plus GFP) were used as a positive control whereas GFP retrovirus-transduced Bcl10-deficient mast cells (Bcl10^{-/-} plus GFP) served as a negative control. The data shown are representative of two independent analyses. **B**, Restoration of FcεR-mediated IL-6 production in Bcl10-deficient mast cells by Bcl10. The above-mentioned mast cells in **A** were incubated with anti-DNP IgE Abs. After exposure to anti-IgE Abs, cell supernatants were collected and subjected to ELISA analysis for detection of IL-6 protein. The data shown are representative of two independent analyses. **C**, Normal FcεR-mediated Ca^{2+} flux in Bcl10-deficient mast cells. Wild-type (+/+) and Bcl10-deficient (-/-) mast cells were incubated with anti-DNP IgE Abs. Induction of intracellular Ca^{2+} concentration was measured by flow cytometry following stimulation of Indo-1-labeled and IgE-coated mast cells with anti-IgE. Anti-IgE was added at the time indicated by the arrow. **D**, Inhibition of FcεR-mediated serotonin release by PKCα/PKCβ inhibitor in wild-type and Bcl10-deficient mast cells. Wild-type and Bcl10-deficient mast cells were incubated with anti-DNP IgE Abs and [³H]serotonin. After exposure to anti-mouse IgE Abs in the absence or presence of the PKCα/PKCβ inhibitor Go6976, the [³H]serotonin released into the supernatants and the [³H]serotonin remaining in the cell pellets was quantitated. The percentage of serotonin release values was then calculated. The data shown are representative of two independent analyses.

Bcl10 deficiency does not affect FcεR-mediated Ca^{2+} flux or activation of PKC

As the expression level of FcεR on Bcl10-deficient mast cells is normal (data not shown), the impaired FcεR-mediated degranulation and cytokine production in Bcl10-deficient mast cells could be due to impaired FcεR signaling. Thus, we examined the effect of Bcl10 deficiency on FcεR signaling. Cross-linking of the FcεR on mast cells leads to activation of PLCγ, resulting in an initial spike in intracellular Ca^{2+} concentration followed by a sustained plateau of intermediate Ca^{2+} concentration that slowly decays to basal levels. PLCγ2 deficiency impairs FcεR-induced degranulation as well as lipid mediator and cytokine production (40). We examined FcεR-induced Ca^{2+} flux in Bcl10-deficient mast cells. Following IgE-mediated FcεR cross-linking, the amplitude and duration of Ca^{2+} elevation were comparable between wild-type and Bcl10-deficient mast cells (Fig. 4C). Thus, Bcl10 deficiency has no effect on FcεR-induced Ca^{2+} flux, consistent with the concept that Bcl10 functions downstream of PLCγ and independent of Ca^{2+} flux in AgR signaling (36, 37).

Activation of PLCγ by FcεR engagement on mast cells also leads to PKC activation (47). Upon AgR engagement in lymphocytes, Bcl10, CARMA1, and MAL1 form a three-component complex, functioning downstream of PKC in activation of IKK (36, 37). We examined FcεR-induced PKC activation in Bcl10-deficient mast cells by immunoblotting with Abs that detect phosphorylation of pan-PKC (48). Following IgE-mediated FcεR cross-linking, the magnitude and kinetics of PKC phosphorylation were comparable between wild-type and Bcl10-deficient mast cells

(data not shown). Thus, Bcl10 deficiency has no effect on FcεR-induced activation of PKC, consistent with the concept that Bcl10 functions downstream of PKC in AgR signaling (36, 37).

Activation of PKC is involved in FcεR-mediated degranulation

Although elevation of intracellular Ca^{2+} is essential for release of the preformed mediators from granules, PKC is suggested to also be involved in this process (47, 49, 50). Importantly, PKCβ-deficient mast cells display impaired FcεR-mediated degranulation (51). We examined the effect of the PKCα/PKCβ inhibitor, Go6976 (52), on FcεR-mediated release of serotonin in wild-type and Bcl10-deficient mast cells. As expected, Go6976 inhibited the FcεR-mediated release of serotonin in wild-type mast cells (Fig. 4D), consistent with the previous findings that PKC is involved in degranulation (47, 50, 51, 53). Interestingly, Go6976 further inhibited the FcεR-mediated release of serotonin in Bcl10-deficient mast cells (Fig. 4D). These data demonstrate that FcεR-mediated degranulation requires activation of PKC and PKC-mediated degranulation is partially impaired by Bcl10 deficiency.

Bcl10 deficiency does not affect FcεR-mediated activation of PI3K or the three types of MAPKs

Engagement of FcεR also results in the activation of PI3K, which leads to the plasma membrane translocation and activation of Akt. Thus, Akt activation is a commonly accepted indicator of PI3K activation (54, 55). Activation of Akt was evaluated by immunoblotting with Abs that detect phosphorylation of Ser⁴⁷³ within Akt, which is known to correlate with its kinase activity (56, 57). As

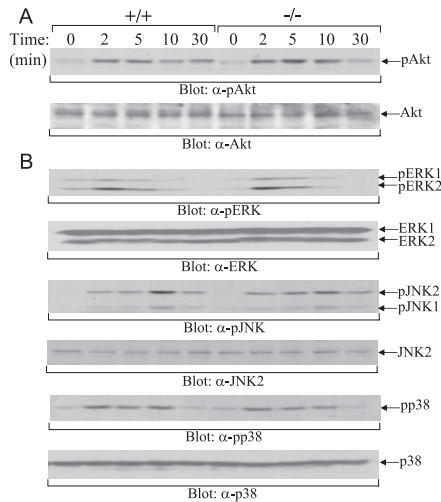


FIGURE 5. FcεR-mediated activation of Akt and MAPK family members ERK, JNK, and p38 in wild-type and Bcl10-deficient mast cells. *A*, Normal FcεR-mediated activation of Akt in Bcl10-deficient mast cells. Wild-type (+/+) and Bcl10-deficient (-/-) mast cells were preincubated with anti-DNP IgE Abs, followed by stimulation with anti-IgE Abs for the indicated times. The cells were lysed and cell lysates were subjected to direct Western blot analysis with anti-phospho-Akt or anti-Akt Abs. The figure shown is representative of two independent analyses. *B*, Normal FcεR-mediated activation of MAPK family members ERK, JNK, and p38 in Bcl10-deficient mast cells. Wild-type and Bcl10-deficient mast cells were preincubated with anti-DNP IgE Abs, followed by stimulation with anti-IgE Abs for the indicated times. The cells were lysed and the cell lysates were subjected to direct Western blot analysis with anti-phospho-ERK1/2, anti-ERK1/2, anti-phospho-JNK1/2, anti-JNK2, anti-phospho-p38, or anti-p38 Abs. The figure shown is representative of two independent analyses.

shown in Fig. 5A, the magnitude and kinetics of Akt phosphorylation were comparable in wild-type and Bcl10-deficient mast cells upon FcεR engagement. Our finding that Bcl10 deficiency has no effect on FcεRI-induced phosphorylation of Akt indicates that Bcl10 is not required for the activation of PI3K following FcεR ligation.

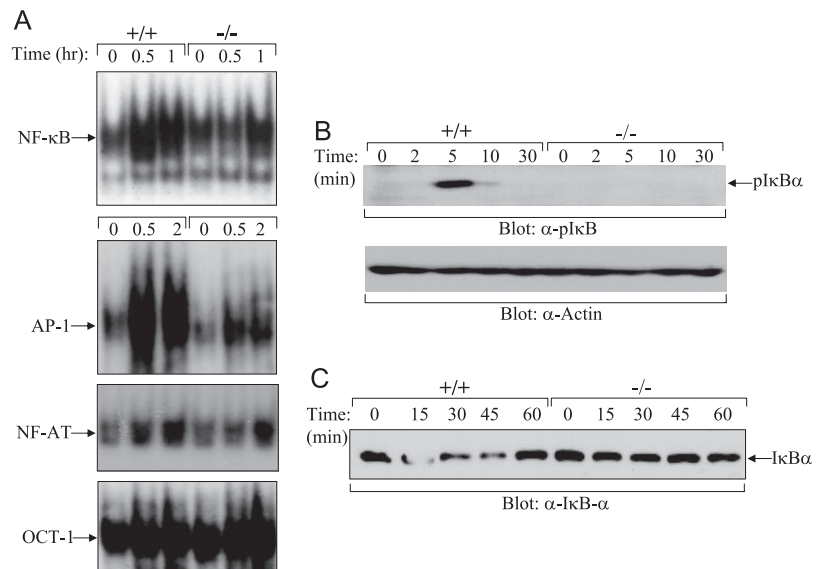
Engagement of FcεR leads to the activation of all types of MAPKs (ERKs, JNKs and p38 MAPKs) via PKC-dependent or the PKC-independent Grb2/SOS/Raf1 pathway (16–22). In addition,

the small GTPases are also involved in the activation of ERK, JNK, and p38 by FcεR (1, 2, 24). To determine the role of Bcl10 in FcεR-mediated activation of MAPKs, we sought to evaluate the extent of ERK, JNK, and p38 MAPK activation in Bcl10-deficient, relative to wild-type, mast cells in response to FcεR cross-linking. Activation of ERK1 and ERK2 was evaluated by immunoblotting with Abs that detect phosphorylation of pThr²⁰²/pTyr²⁰⁴ within ERK1 and pThr¹⁸⁵/pTyr¹⁸⁷ within ERK2 (58, 59). Activation of JNK was evaluated by immunoblotting with Abs that detect phosphorylation of pThr¹⁸³/pTyr¹⁸⁵ within JNK1/2 (60), while p38 activation was evaluated by immunoblotting with Abs that detect phosphorylation of pThr¹⁸⁰/pTyr¹⁸² within p38 (61). Upon FcεR engagement, the magnitude and kinetics of ERK1, ERK2, JNK1, JNK2, and p38 phosphorylation were comparable in Bcl10-deficient and wild-type mast cells (Fig. 5B). Therefore, Bcl10 is not required for FcεR-induced activation of the three types of MAPKs, consistent with our finding that Bcl10-deficient mast cells have normal production of arachidonic acid, which has been shown to be regulated by MAPKs (17, 21, 62).

The activation of NFκB and AP-1 is impaired in Bcl10-deficient mast cells after FcεR engagement

Engagement of FcεR ultimately leads to the activation of transcription factors, including NF-κB (28) and AP-1 (29). Bcl10, along with CARMA1 and MALT1, forms a three-component complex, which couples PKC to IKK upon AgR engagement (36, 37). Bcl10 deficiency impairs BCR- and TCR-mediated activation of NF-κB (34, 35) as well as TCR-mediated activation of AP-1 (34). To determine the role of Bcl10 in FcεR-mediated activation of NF-κB and AP-1, we examined activation of NF-κB and AP-1 in wild-type and Bcl10-deficient mast cells after FcεR ligation. In gel mobility shift assays, FcεR engagement dramatically induced NF-κB complex formation with a peak at 30 min in wild-type mast cells, whereas NF-κB complex formation was severely impaired, although was slightly induced at 60 min, in Bcl10-deficient mast cells (Fig. 6A). In addition, the activation of AP-1 was decreased in Bcl10-deficient relative to wild-type mast cells (Fig. 6A). In contrast, the activation of NF-AT was comparable in both wild-type and Bcl10-deficient mast cells (Fig. 6A). Of note, Oct-1 binding was comparable in every lane, demonstrating equal protein

FIGURE 6. FcεR-mediated activation of NF-κB and AP-1 in wild-type and Bcl10-deficient mast cells. *A*, Impaired FcεR-mediated activation of NF-κB in Bcl10-deficient mast cells. Wild-type (+/+) and Bcl10-deficient (-/-) mast cells were preincubated with anti-DNP IgE Abs, followed by stimulation with anti-IgE Abs for the indicated times. Nuclear extracts were prepared from the cells and gel mobility shift assays were performed using radiolabeled probes containing NF-κB-, AP-1-, NFAT-, or Oct-1-binding sequences. *B*, Impaired FcεR-mediated phosphorylation of IκB in Bcl10-deficient mast cells. Wild-type and Bcl10-deficient mast cells were preincubated with anti-DNP IgE Abs, followed by stimulation with anti-IgE Abs for the indicated times. Cell lysates were subjected to direct Western blot analysis with anti-phospho-IκBα or anti-Actin. *C*, Impaired FcεR-mediated degradation of IκB in Bcl10-deficient mast cells. Wild-type and Bcl10-deficient mast cells were preincubated with anti-DNP IgE Abs, followed by stimulation with anti-IgE Abs for the indicated times. Cell lysates were subjected to direct Western blot analysis with anti-IκBα.



loading for each sample (Fig. 6A). In addition, Fc ϵ R ligation induced the phosphorylation of I κ B α (Fig. 6B), and subsequent degradation of I κ B α (Fig. 6C) (29–31), in wild-type but not in Bcl10-deficient mast cells (Fig. 6, B and C). Therefore, Bcl10 is essential for the Fc ϵ R-mediated activation of NF- κ B and AP-1 in mast cells.

Discussion

Engagement of the Fc ϵ R initiates activation of several cytoplasmic protein tyrosine kinases and recruitment of adaptor molecules (8–13). In turn, the receptor complex results in the activation of multiple signaling cascades, including PI3K/Akt, PLC γ /Ca²⁺/PKC and Grb2/SOS/Raf-1/MAPK pathways (1, 2, 16–24). Ultimately, these signaling cascades lead to the activation of transcription factors, including NF- κ B (27–29). Despite the progress in classifying these mechanisms, signal transduction of the Fc ϵ R in mast cells is far from fully understood. Bcl10 is a newly identified adaptor protein that plays a critical role in B and T cell AgR signaling (34, 35). Bcl10, along with CARMA1 and MALT1, forms a three-component complex, coupling PKC to IKK in AgR-mediated NF- κ B activation (36, 37). Bcl10 deficiency markedly impairs TCR-mediated T cell and BCR-mediated B cell functions (34, 35). Although the signaling events triggered by Fc ϵ R on mast cells resemble those by AgRs on lymphocytes (38), the role of Bcl10 in Fc ϵ R-mediated mast cell function is not known. In the present studies, we find that Bcl10 regulates Fc ϵ R-mediated degranulation, indicated as serotonin release, and cytokine production, measured as release of IL-6, although it is not required for de novo synthesis and release of the lipid mediator, arachidonic acid. Moreover, Bcl10 deficiency does not affect Fc ϵ R-mediated Ca²⁺ flux, activation of PKC β , activation of PI3K, or activation of the three types of MAPKs, ERKs, JNKs and p38 but markedly diminishes Fc ϵ R-mediated I κ B α phosphorylation and abolish subsequent activation of NF- κ B. In addition, Bcl10 deficiency impairs Fc ϵ R-mediated AP-1 activation. Thus, Bcl10 is essential for specific aspects of Fc ϵ R-mediated signaling and functions. A recent study has also demonstrated that Bcl10 deficiency impairs Fc ϵ R-mediated NF- κ B activation, Fc ϵ R-induced cytokine production, and IgE-dependent late-phase anaphylactic reactions (39). However, this study has not observed any effect of Bcl10 deficiency on Fc ϵ R-induced degranulation and immediate phase of IgE-mediated passive cutaneous anaphylaxis, contrary to what we have found. The exact reasons for the discrepancies are not clear. These studies are two independent and concurrent studies and the Bcl10-deficient mice used by the studies were generated independently (34, 35). It is possible there are some differences in the genetic background of the two lines of Bcl10-deficient mice, which might contribute to the discrepancies observed. In addition, the moderate impairment of Fc ϵ R-mediated degranulation might be detected by measuring the release of serotonin in the current study, but not by measuring activity of the granular enzyme β -hexosaminidase in the previous study (39). Of note, we observed a marked difference between the IgE-mediated passive cutaneous anaphylaxis in the ear and basolateral skins. The relative abundances of mast cells in these two areas might contribute to the apparently different reactions. A previous study has shown that skins of mouse ears are relatively rich in mast cells (63). Thus, it is possible that the relatively high number of mast cells in the ears might compensate for the moderately reduced abilities of degranulation of Bcl10-deficient mast cells, obscuring the impairment of Fc ϵ R-mediated passive cutaneous anaphylaxis in the ears. In contrast, the severe reduction of Fc ϵ R-mediated cytokine production caused by Bcl10 deficiency unlikely contributes to the defective IgE-mediated passive cutaneous anaphylactic reactions in the basolateral skins, as the reactions are usually determined by early release of granular contents (1, 46).

It has been shown that a Ras guanine nucleotide-exchange factor, SOS, is recruited to the membrane via the adaptor protein Grb2 to activate Ras upon receptor ligation (64, 65). Subsequently, activated Ras associates with and activates the Raf-1 Ser/Thr kinase, leading to a cascade of kinase activation, which ultimately activates the ERK1 and ERK2 kinases (17, 66, 67). Although the Grb2/SOS/Raf1 pathway is a potent cascade activating MAPKs (17, 21, 22), a number of studies have shown that the PLC γ /PKC/Ca²⁺ pathway also contributes to the activation of all three types of MAPKs (ERK, p38, and JNK) (16–20). Activated PKC can promote activation of ERK1 and ERK2 (16–18), whereas Ca²⁺ and PKC also participate in JNK activation (19, 20). In addition, PKC is required for the maximum activation of p38 MAPK (19, 20, 23). Recent studies have demonstrated that Bcl10, along with CARMA1 and MALT1, functions downstream of PKC in B and T cell AgR-mediated NF- κ B activation (36, 37). However, Bcl10 deficiency has no effect on the activation of any of the three MAPKs, suggesting that activation of MAPKs is independent of the PKC/CARMA1/Bcl10/MALT1 pathway. Our previous studies, which showed that BCR ligation in PLC γ 2-deficient B cells generates severely impaired PKC/Ca²⁺ signals but activates all three MAPKs (68), also support the notion that MAPK activation is independent of PKC signals.

Engagement of the Fc ϵ R induces release of the granule-associated preformed mediators, synthesis and release of proinflammatory lipid mediators, and the production of many growth factors, cytokines, and chemokines (1, 2, 24). Studies have indicated that distinct signaling pathways emanating from Fc ϵ R regulate the production of these three major mediators. Although elevation of intracellular Ca²⁺ is required for Fc ϵ R-mediated degranulation, activation of PKC also plays a critical role in degranulation (47, 50, 53). Fc ϵ R engagement on mast cells leads to PKC β activation via PLC γ (47). Consistent with the fact that Bcl10 functions downstream of PKC in AgR signaling (36, 37), Bcl10-deficient mast cells have normal Fc ϵ R-induced activation of PKC β . However, PKC β deficiency impairs Fc ϵ R-mediated degranulation in mast cells (51). Moreover, our current studies demonstrate that the small molecule PKC inhibitor Go6976 blocks Fc ϵ R-mediated release of serotonin in both wild-type and Bcl10-deficient mast cells. Taken together, Fc ϵ R-mediated degranulation requires not only elevation of intracellular Ca²⁺ but also activation of PKC. PKC-mediated degranulation involves Bcl10 and deficiency of Bcl10 at least partially impairs Fc ϵ R-mediated degranulation in mast cells.

By contrast, the MAPKs are not required for release of the granule-associated mediators but are involved in the production of arachidonic acid and cytokines (17, 21, 62). Here, we demonstrate that Bcl10 deficiency impaired activation of NF- κ B but had no effect on Ca²⁺ flux or activation of the three MAPKs following Fc ϵ R engagement. Bcl10 deficiency resulted in a severely reduced Fc ϵ R-mediated release of serotonin and IL-6, but had no effect on arachidonic acid production. These results demonstrate that the PKC/CARMA1/Bcl10/MALT1 pathway is important for mast cell degranulation and cytokine production, although the Ca²⁺ signal might also be involved in regulating these processes. In addition, these results indicate that activation of MAPKs might be sufficient for production of arachidonic acid but not cytokines. Despite the possibility that release of arachidonic acid may be regulated primarily through MAPKs, PKC might also influence this release (21). These notions are also supported by the previous findings that PLC γ 2 deficiency reduced the amplitude of both PKC and Ca²⁺ signals, resulting in impairment of Fc ϵ R-mediated degranulation, production of arachidonic acid, and secretion of cytokines. NF- κ B activation is essential for Fc ϵ R-mediated cytokine production in mast cells (69). Impairment of Fc ϵ R-mediated NF- κ B activation

could account for the severe reduction of FcεR-induced cytokine production in Bcl10-deficient mast cells. Bcl10 also plays an important role in FcεR-mediated degranulation in mast cells. Bcl10 deficiency impairs FcεR-mediated AP-1 activation, consistent with the observation that TCR-mediated AP-1 activation is impaired in Bcl10-deficient T cells (34). Of note, AP-1 is required for FcεR-mediated degranulation in mast cells. Deficiency of c-Fos, a component of AP-1, markedly inhibits FcεR-induced degranulation (70). Thus, an AP-1-dependent pathway downstream of Bcl10 appears to be involved in FcεR-mediated degranulation in mast cells. Nonetheless, additional studies are required to further address the exact mechanisms by which Bcl10 regulates FcεR-mediated degranulation.

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Disclosures

The authors have no financial conflict of interest.

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