Original Article

Coaggregation of FcεRI with FcγRIIB Inhibits Degranulation but Not Induction of Bcl-2 Family Members A1 and Bim in Mast Cells

Maria Ekoff, MSc; Christine Möller, PhD; Zou Xiang, PhD; Gunnar Nilsson, PhD

Abstract

The aggregation of high-affinity immunoglobulin E (IgE) receptors (FcεRI) on mast cells is a critical event in the initiation of an allergic reaction. Coengagement of FcεRI with immunoglobulin G (IgG) low-affinity receptor FcγRIIB/CD32 inhibits degranulation and the release of inflammatory mediators from mast cells and has therefore been proposed as a new therapeutic approach for the treatment of allergies. In this study, we investigated whether FcγRIIB, besides inhibiting degranulation, negatively regulates other signalling pathways downstream of FcεRI. For this, we determined the phosphorylation and/or expression of proteins involved in the regulation of mast-cell apoptosis. Coaggregation led to an attenuation of Akt phosphorylation but did not inhibit phosphorylation of transcription factor Foxo3a or its proapoptotic target, Bim. Similarly, FcεRI-dependent expression of the prosurvival gene A1 was not affected by coaggregation. Our data demonstrate that coengagement of FcεRI and FcγRIIB inhibits degranulation but not the signalling pathways regulating Bcl-2 family members Bim and A1.

Mast cells are critical effector cells mediating immunoglobulin E (IgE)–dependent allergic responses. Binding of an allergen to IgE, already bound to its high-affinity receptor FcεRI on mast cells, leads to aggregation and subsequent activation. This initiates signalling events that typically result in degranulation, changes in gene expression, and the release of inflammatory mediators, contributing to acute and late-phase allergic responses.[1–3] FcεRI consists of a tetrameric protein complex, the IgE-binding amplifying α chain, a signalling β chain, and two γ chains.[4] The β and γ subunits of the FcεRI each contain an immunoreceptor tyrosine-based activation motif (ITAM), which is phosphorylated upon FcεRI aggregation and which is both necessary and sufficient for receptor-induced signal transduction.[5]

Mast cells also express other Fc receptors, either constitutively or upon stimulation; among these, FcγRI (CD64), FcγRIIB (CD32), and FcγRIII (CD16) are receptors for immunoglobulin G (IgG). FcγRI (high-affinity IgG receptor) and FcγRIII (low-affinity IgG receptor) are activating receptors, both containing ITAM, that initiate signalling upon aggregation.[6,7] FcγRIIB is a low-affinity receptor containing an immunoreceptor tyrosine-based inhibitory motif (ITIM),[8] which negatively regulates the activating signal when coaggregated with activating receptors bearing an ITAM.[9] The coaggregation results in the

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recruitment of the inhibitory signalling molecule SHIP, leading to the abrogation of the ITAM-induced activation.[2,10,11]

IgE-induced mast cell activation (ie, FcεRI aggregation) is negatively regulated by coaggregation of FcεRI with FcγRIIB.[9,12] The release of mediators and cytokines is inhibited in a process in which FcεRI contributes to the ITIM-dependent inhibition of its own intracellular signalling. This is achieved by the FcεRI-associated tyrosine kinase Lyn, which phosphorylates the FcγRIIB ITIM that recruits SHIP1, thus leading to FcεRI signal abrogation.[11,13,14] The receptors interact with the F-actin skeleton that enables FcγRIIB to recruit SHIP1, which is provided by filamin-1. FcγRIIB is believed to negatively regulate FcεRI signalling in two ways: by facilitating the translocation of FcεRI into the F-actin skeleton but also by concentrating SHIP1 at the site close to FcεRI.[15] Investigations of the mechanism by which SHIP mediates its FcγRIIB inhibitory function have also suggested $p62^{dok}$ as a possible mediator of FcγRIIB inhibition of FcεRI signalling downstream of SHIP in mast cells.[16]

FcεRI-mediated degranulation and release of mediators are inhibited when FcεRI is coaggregated with FcγRIIB.[12] In addition to elucidating the impact of coaggregation on mast-cell degranulation, this study has elucidated the effect on the activation of downstream signalling pathways involved in the regulation of mast-cell survival. The aggregation of FcεRI induces rapid but transient phosphorylation of the signalling protein Akt and the forkhead transcription factor Foxo3a, known to regulate *Bim* expression at the transcriptional level.[17] Phosphorylated Akt phosphorylates and thereby inactivates Foxo3a, which in its unphosphorylated state is located in the nucleus and acts as a transcription factor for Bim. Bim is a proapoptotic protein of the Bcl-2 family, involved in the regulation of mast-cell apoptosis.[18,19] Another Bcl-2 family member of crucial importance for FcεRI-mediated activation-induced mast-cell survival is A1.[20] Mast cells lacking A1 do not survive IgE receptor aggregation.[20]

In this study, we investigated if FcεRI-mediated activation/expression of Akt, Foxo3a, Bim, and A1 are inhibited when FcεRI is coengaged with FcγRIIB. We report here that although mast-cell degranulation is inhibited and the phosphorylation of Akt is attenuated by the coaggregation of FcεRI with FcγRIIB, Foxo3a and Bim are still phosphorylated and up-regulated, respectively. We also demonstrate that the level of A1 messenger ribonucleic acid (mRNA) induced by FcεRI is not significantly altered upon coaggregation with FcγRIIB. Altogether, this indicates that only certain signalling pathways are affected by the coaggregation of FcεRI with FcγRIIB whereas others, closely related to cell survival, remain largely unaffected.

Materials and Methods

Mast-Cell Cultures

The murine mast cell line C57[21] (kindly provided by Dr. S.J. Galli, Stanford University, Stanford, CA) was cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM of L-glutamine, 100 μg/mL of penicillin/streptomycin, and 50 μM of 2-mercaptoethanol. All culture reagents were obtained from Sigma Chemical Co. (St. Louis, MO). The C57 mast cell line has previously been characterized for FcγRII/FcγRIII expression.[22]

Antibodies and Reagents

AffiniPure rabbit anti-mouse IgG (RAM IgG), AffiniPure RAM IgG $F(ab')_2$ fragment (RAM $F(ab')_2$), and AffiniPure mouse anti-rat IgG (H+L) $F(ab')_2$ fragment (MAR $F(ab')_2$) were all purchased from Jackson ImmunoResearch Laboratories, Inc., Baltimore, MD. Purified RAM CD16/CD32 (FcγIII/II receptor) monoclonal antibody (2.4G2 rat Ab) was obtained from BD Biosciences, Heidelberg, Germany. Anti-rabbit IgE horseradish peroxidase–linked donkey anti-rabbit antibody was obtained from Amersham Biosciences, Uppsala, Sweden. LumiGLO reagent and peroxide, 10× cell lysis buffer, antibodies directed against phospho-Akt (serine [Ser] 473 and threonine [Thr] 308), and Akt were purchased from Cell Signaling Technology, Beverly, MA. Antibodies directed against phospho-Foxo3a (Thr 32 and Ser 253) and Foxo3a were

obtained from Upstate Biotechnology, Lake Placid, NY. Anti-Bim antibody was purchased from Affinity Bioreagents, Inc., Golden, CO. 4× NuPAGE LDS Sample Buffer and 10× NuPAGE Sample Reducing Agent were obtained from Invitrogen, Carlsbad, CA; TriPure Isolation Reagent was purchased from Boehringer Mannheim, Mannheim, Germany; and Tween 20 was obtained from Merck Schuchardt, Hohenbrunn, Germany. All other reagents were purchased from Sigma Chemical Co.

Antibody Conjugation

MAR F(ab´)2 was trinitrophenylated by incubation for 2 hours at room temperature with picrylsulfonic acid (2,4,6-trinitrobenzene sulfonic acid in borate-buffered saline, pH 8.0). The TNP_7-F(ab´)2 MAR obtained was purified on a prepacked disposable PD-10 column containing Sephadex G-25 medium (Amersham Biosciences).

Mast-Cell Activation

Mast _cells to be used for ribonuclease (RNAse) protection assay and α-hexosaminidase release assay were resuspended in RPMI-1640 medium supplemented with 0.2% bovine serum albumin, 2 mM of L-glutamine, and 100 μg/mL of penicillin/streptomycin. The cells were sensitized for 90 minutes at 37°C by the addition of 0.1 μg/mL of monoclonal anti-dinitrophenyl (anti-DNP) clone SPE-7 IgE mouse antibody (anti-DNP IgE). After washing, the cells were activated by the addition of either 45 μg/mL of RAM IgG (coaggregation of FcεRI with FcγRIIB) or 30 μg/mL of RAM $F(ab´)_2$ (aggregation of FcεRI) at 37°C for the time periods indicated. Mast cells to be used for Western blot analysis were resuspended in the previously mentioned medium. The cells were sensitized for 90 minutes at 37°C by the addition of 0.1 μg/mL of the same IgE as previously mentioned or 0.1 μg/mL of the same IgE together with 5 μg/mL of 2.4G2 rat Ab. After being washed, the cells were activated by the addition of 10 μg/mL of TNP7-$F(ab´)_2$ mouse anti-rabbit (MAR) at 37°C, causing either coaggregation of FcγRIIB with FcεRI or aggregation of FcεRI, for the time periods indicated. The conjugated antibody, TNP7-MAR $F(ab´)_2$, functions as a multivalent antigen recognized by the FcεRI-bound IgE but also recognizing bound 2.4G2 rat Ab.[13] Aggregation with 2.4G2 rat Ab together with TNP7-MAR $F(ab´)_2$ does not cause degranulation, which indicates that expression of FcγRIII (an activating low-affinity receptor for IgG) on C57 cells does not interfere with our system (data not shown). In experiments in which the phosphorylation pattern of Akt and Foxo3a as well as the total amount of these two proteins were measured, the mast cells were starved for approximately 24 hours at 37°C in RPMI-1640 medium supplemented with 0.5% FBS before sensitization and activation. For Bim expression experiments, the mast cells were resuspended in RPMI-1640 medium supplemented with 10% filtered FBS, 2 mM of L-glutamine, 100 μg/mL of penicillin/streptomycin, and 50 μM of 2-mercaptoethanol during both sensitization and activation, which lasted for 24 hours.

N-Acetyl-β-D-Hexosaminidase Release Assay

For detection of the granular enzyme β-hexosaminidase, an enzymatic colorimetric assay was used.[23] After 30 minutes of activation, 60 μL of supernatant were transferred to a 96-well plate and mixed with an equal volume of substrate solution (7.5 mM of p-nitrophenyl-N-acetyl-β-D-glucosaminide dissolved in 80 mM of citric acid, pH 4.5). The mixture was incubated on a rocker platform for 2 hours at 37°C. After incubation, 120 μL of glycine (0.2 M, pH 10.7) was added to each well, and the absorbance was measured with an Emax Precision Microplate Reader (Molecular Devices, Sunnyvale, CA).

Western Blot Analysis

The cells were lysed in SDS sample buffer (125 mM of tris-hydrochloric acid [pH 6.8], 4% w/v SDS, 20% glycerol, 0.02% w/v bromphenol blue, and 50 mM of dithiothreitol, added just before use) or in cell lysis buffer (1× cell lysis buffer, 1 mM of phenylmethylsulfonyl fluoride [PMSF]) before

being sonicated on ice. The phosphorylation and/or the total amount of proteins of interest were studied by Western blot with a NuPAGE Bis-Tris Western gel (Invitrogen, Carlsbad, CA). After electrophoresis, the proteins were electrically transferred to a nitrocellulose membrane (Hybond ECL, Amersham Biosciences). All was performed according to the manufacturers´ instructions. The membrane was incubated overnight at 4°C with the primary antibody and thereafter incubated with horseradish peroxidase–conjugated secondary antibody for 1 hour at room temperature. The proteins were visualized with an enhanced chemiluminescence system (LumiGLO) and exposure to a Hybond ECL film.

RNAse Protection Assay

TriPure isolation reagent was used for isolation of total cellular ribonucleic acid (RNA). An RNAse protection assay (RPA) was performed (according to RiboQuant System protocol) with an mAPO-2 multiprobe set (PharMingen, San Diego, CA). The gel was dried and exposed on Kodak film (Eastman Kodak Company, Rochester, NY) with intensifying screens at –70°C. Expression of RNA was detected with a phosphoimager device, and levels of expression were quantified with *MacBAS 2.2* software (Fuji Photo Film Co., Ltd., Tokyo, Japan).

Statistical Analysis

We used an analysis of variance, followed by multiple comparison with the Wilcoxon matched-pairs test.

Results

Coaggregation of FcεRI with FcγRIIB Inhibits IgE-Dependent Mast-Cell Degranulation

To analyze the effect of FcγRIIB-mediated inhibition of mast-cell activation, we used murine C57 mast cells known to express the receptors FcεRI and FcγRIIB. C57 cells were sensitized with murine IgE and challenged with polyclonal RAM $F(ab´)_2$ to aggregate FcεRI or with RAM IgG to coaggregate FcεRI and FcγRIIB. The RAM $F(ab´)_2$ induced activation of mast cells, leading to degranulation as measured by β-hexosaminidase release (Figure 1A). When FcεRI was coaggregated with FcγRIIB by the addition of RAM IgG, the release of β-hexosaminidase was inhibited (see Figure 1A).

In addition to the activation system whereby RAM IgG or RAM $F(ab´)_2$ was added, we also used another system for Western blot analysis, one by which each receptor can be aggregated separately or coaggregated. C57 cells were sensitized with murine anti-DNP IgE and incubated with or without 2.4G2 rat Ab before challenge with TNP-MAR $F(ab´)_2$, TNP-conjugated $F(ab´)_2$ fragments of mouse anti-rat IgG. The conjugated antibody, TNP7-MAR $F(ab´)_2$, functions as a multivalent antigen recognized by the FcεRI-bound IgE but also recognizing FcγRII-bound 2.4G2 rat Ab.[13] The addition of TNP7-MAR $F(ab´)_2$ will aggregate FcεRI in cells sensitized with IgE, aggregate FcγRIIB in cells sensitized with 2.4G2 rat Ab, and (as a consequence) coaggregate FcεRI and FcγRIIB in cells sensitized with both IgE and 2.4G2 rat Ab. Since aggregation using 2.4G2 rat Ab together with TNP7-MAR $F(ab´)_2$ does not cause degranulation, this indicates that expression of FcγRIII (an activating low-affinity receptor for IgG) on C57 cells does not interfere with our system (data not shown). Although not as sufficient as the other system for causing degranulation, this system induced the activation of mast cells, causing degranulation, and showed inhibition upon coaggregation of FcεRI with FcγRIIB (see Figure 1B).

Phosphorylation of Akt Is Attenuated by Coaggregation of FcεRI with FcγRIIB

To assess the effects of coaggregating FcεRI with FcγRIIB on signals transduced downstream of FcεRI, the phosphorylation pattern of Akt protein was investigated. Akt is a signal-transducing protein downstream of PI3-kinase, involved in a variety of cellular functions such as survival and

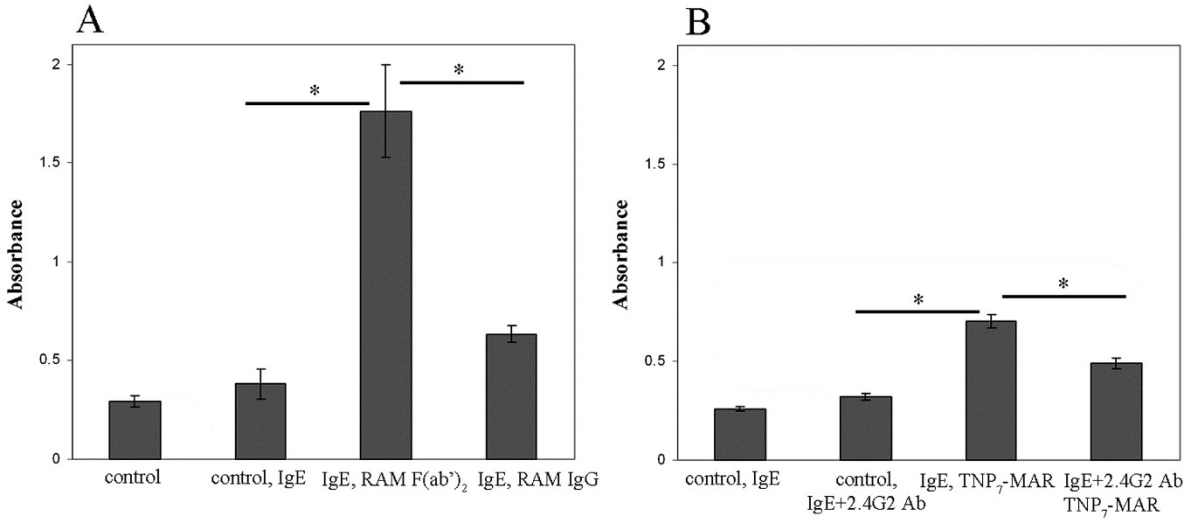

Figure 1 Inhibition of IgE-mediated mast-cell degranulation by FcγRIIB. *A,* Mast cells were sensitized with 0.1 μg/mL of monoclonal anti-dinitrophenyl (anti-DNP) clone SPE-7 immunoglobulin E (IgE) mouse antibody (anti-DNP IgE) before being challenged with 30μg/mL of rabbit anti-mouse (RAM) $F(ab')_2$ or 45μg/mL of RAM immunoglobulin G (IgG) for 30 minutes. Resting cells or cells incubated with 0.1 μg/mL of anti-DNP IgE served as a background control. The figure represents mast-cell activation estimated by β-hexosaminidase release as measured by absorbance. *B*, Mast cells were sensitized with 0.1 μg/mL of monoclonal anti-DNP clone SPE-7 IgE mouse antibody (anti-DNP IgE) and preincubated with or without 5 μg/mL of RAM CD16/CD32 (FcγIII/II receptor) monoclonal antibody (2.4G2 rat Ab), before challenge with 10 μg/mL of TNP-$F(ab')_2$ mouse anti-rat (MAR) for 30 minutes. Cells incubated with 0.1 μg/mL of anti-DNP IgE or 0.1 μg/mL of anti-DNP IgE and 5 μg/mL of 2.4G2 rat Ab served as a background control ($n = 6$; results are presented as mean ± standard error of the mean). *$p < .05$.

metabolism.[24,25] Via 3-phosphoinositide–dependent protein kinases, the PI3-kinase can activate Akt by phosphorylation at three different sites, two of which—threonine 308 (Thr 308) and serine 473 (Ser 473)—were investigated in this report. We compared the pattern of Akt phosphorylation at the Thr 308 and Ser 473 sites in cell lysates after FcεRI aggregation or coaggregation of FcεRI with FcγRIIB. FcεRI aggregation induced rapid phosphorylation of Akt at Thr 308; the maximum phosphorylation stage was reached within 1 minute, and phosphorylation decreased at 5 minutes. The phosphorylation of Akt after FcεRI aggregation at Ser 473 was achieved within 1 minute and remained at a comparable level for 10 minutes before decreasing at 30 minutes (Figure 2). Considerable reductions of Akt phosphorylation at Thr 308 and Ser 473 were observed as early as 1 minute after coaggregation of FcεRI with FcγRIIB (see Figure 2).

Coaggregation of FcεRI with FcγRIIB Does Not Affect the Phosphorylation of Transcription Factor Foxo3a

Phosphorylated Akt phosphorylates and thereby inactivates the forkhead protein Foxo3a.[26] The phosphorylation of Foxo3a prevents its translocation into the nucleus, where it acts as a transcription factor for certain genes. We investigated the phosphorylation of Foxo3a at sites Ser 253 and Thr 32. Phosphorylation of Foxo3a at Ser 253 occurred within 1 minute but reached background phosphorylation level again after 30 minutes (Figure 3). However, after rapid phosphorylation at site Thr 32 within 1 minute after FcεRI aggregation, phosphorylation remained constant until 30 minutes had elapsed (see Figure 3). In contrast to the effect on Akt phosphorylation, coengagement of FcεRI with FcγRIIB did not affect either the levels of phosphorylation or the duration of the FcεRI-induced Foxo3a phosphorylation (see Figure 3).

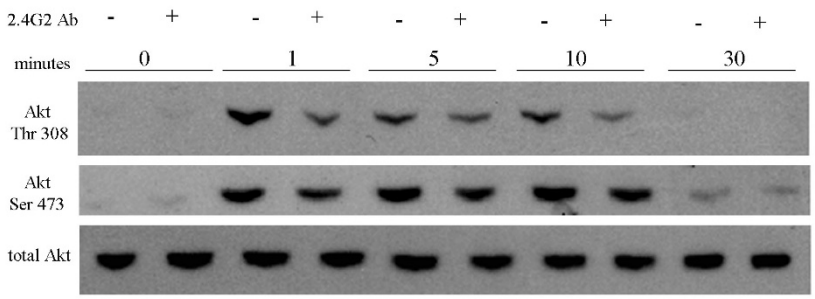

Figure 2 Reduction of immunoglobulin E (IgE)–dependent phosphorylation of Akt by FcγRIIB. C57 mast cells were activated as in Figure 1B for the indicated periods of time. Cell lysates were prepared, and the phosphorylation of Akt was analyzed by Western blot with the indicated antibodies. The result is representative of three independent experiments. Ser = serine; Thr = threonine; 2.4G2 Ab = anti-mouse CD16/CD32 (FcγIII/II receptor) monoclonal antibody.

FcεRI-Induced Expression of Bim Is Not Inhibited by Coaggregation with FcγRIIB

A key regulator of apoptosis is the Bcl-2 family of proteins, which consists of proapoptotic and antiapoptotic proteins. Bim, one of the proapoptotic members, is transcriptionally regulated by Foxo3a,[27] and we recently showed that Bim is involved in the regulation of mast-cell apoptosis.[18,19] Furthermore, Bim is induced upon aggregation of FcεRI.[18] Therefore, we next investigated if coaggregation of FcεRI with FcγRIIB would have an effect on Bim expression. After FcεRI aggregation and coaggregation of FcεRI and FcγRIIB, respectively, the two isoforms of Bim (Bim_{EL} and Bim_{L}) were up-regulated to similar levels (Figure 4). Bim_{EL} consisted of two bands, owing to a shift in band motility; this shift of the Bim_{EL} band is probably the result of phosphorylation.[19,28] The results herein demonstrate that Bim induced by FcεRI aggregation is not affected by coaggregation with FcγRIIB (see Figure 4).

Coaggregation of FcεRI with FcγRIIB Does Not Affect the Induction of A1

Apoptosis is regulated by members of the Bcl-2 family. A1, one of the antiapoptotic Bcl-2 family members, is described as being important for the survival of mast cells during allergic reactions.[20] To determine whether the coaggregation of FcεRI with FcγRIIB affects the induced transcriptional regu-

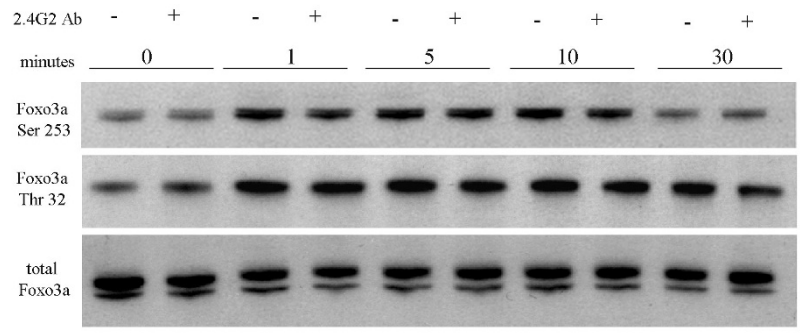

Figure 3 Phosphorylation of Foxo3a induced by FcεRI and FcγRIIB or by FcεRI alone. C57 mast cells were activated as in Figure 1B for the indicated periods of time. Cell lysates were prepared, and the phosphorylation of Foxo3a was analyzed by Western blot with the indicated antibodies. The result is representative of three independent experiments. Ser = serine; Thr = threonine; 2.4G2 Ab = anti-mouse CD16/CD32 (FcγIII/II receptor) monoclonal antibody.

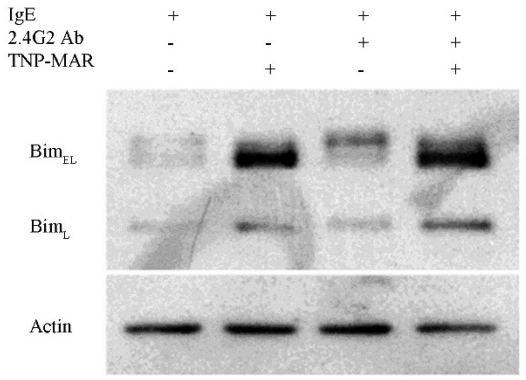

Figure 4 Expression of Bim induced by FcεRI and FcγRIIB or by FcεRI alone. C57 mast cells were activated as in Figure 1B for 24 hours. Cells sensitized only with 0.1 μg/mL of anti-dinitrophenyl immunoglobulin E (lgE) and incubated with or without 5 μg/mL of rabbit anti-mouse CD16/CD32 (FcγIII/II receptor) monoclonal antibody (2.4G2 rat Ab) were used as controls. Cell lysates were prepared, and the induction of Bim was analyzed by Western blot with the indicated antibodies. The result is representative of three independent experiments. MAR = mouse anti-rat; TNP = trinitrophenyl. .

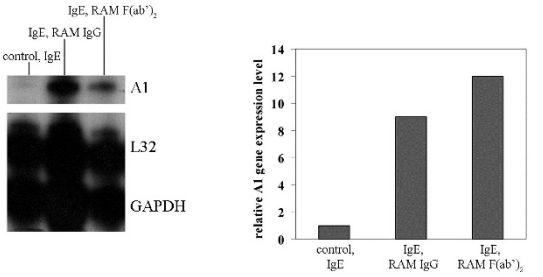

Figure 5 A1 expression induced by FcεRI and FcγRIIB or by FcεRI alone. A ribonuclease protection assay was performed on ribonucleic acid isolated from C57 cells activated as in Figure 1A for 6 hours, and quantifications of A1 transcript levels are shown relative to their corresponding levels GAPDH housekeeping gene. Data shown are representative of three separate experiments. IgE = immunoglobulin E; IgG = immunoglobulin G; RAM = rabbit anti-mouse.

lation of A1, an RPA was performed. A1 was absent in cells incubated only with IgE but was substantially up-regulated after FcεRI aggregation, as well as in cells where FcεRI had been coaggregated with FcγRIIB for 6 hours (Figure 5). The A1 mRNA level in cells activated by FcεRI aggregation had increased 12-fold, and coaggregation of FcγRIIB with FcεRI led to a ninefold increase when the signal was compared to control cells incubated with IgE alone (see Figure 5). Thus, although A1 up-regulation is slightly reduced after the coaggregation of FcεRI with FcγRIIB when compared to FcεRI aggregation, the induction of A1 in cells after either coaggregation of FcεRI with FcγRIIB or FcεRI aggregation (as compared to resting cells) was consistent in several experiments.

Discussion

Although coaggregation of FcεRI with FcγRI is known to inhibit mast-cell degranulation, the effect of coaggregation on other signalling pathways in mast cells has not been investigated previously. In this study, we found that even though coaggregation of FcεRI with FcγRIIB inhibits degranulation and decreases the phosphorylation of Akt, we observed no effect on Foxo3a phosphorylation or Bim expression (see Figures 2, 3, and 4). Results from RPAs showed that the mRNA of A1 (an antiapoptotic Bcl-2 family member) was up-regulated both when mast cells were activated through FcεRI aggregation and when they were activated through coaggregation of FcεRI with FcγRIIB (see Figure 5). Thus, FcγRIIB inhibits some but not all signalling pathways downstream of FcεRI.

One pathway affected by FcεRI aggregation is the PI3-K pathway, where PI3-K is phosphorylated and thereby activated.[5] Activated PI3-K can, via 3-phosphoinositide–dependent protein kinases or specific lipid products, phosphorylate the protein Akt.[29,30] Phosphorylation of Ser 473 and/or Thr 308 enables Akt to carry out its multifunctional activities, which are involved in a variety of cellular functions such as survival and metabolism.[24,25,31,32] Akt became rapidly phosphorylated at the two sites that were investigated after FcεRI aggregation. The phosphorylation at Thr 308 was clearly diminished already after 5 minutes whereas the phosphorylation of Ser 473 remained for at least 20 minutes. This difference in phosphorylation between the two sites might reflect a strict regulation of phosphorylation of Akt.

After coaggregation of FcεRI with FcγRIIB, the phosphorylation of Akt was attenuated when compared to FcεRI aggregation. Akt is more heavily phosphorylated after FcεRI aggregation, but the duration of the phosphorylation does not change after coaggregation of FcεRI with FcγRIIB. These data are in line with data from earlier studies showing that coaggregation of FcγRIIB and the B-cell receptor (as well as coaggregation with the receptor for stem-cell factor Kit, present on mast cells) affects the PI3-K pathway and thereby inhibits the activation of Akt.[33,34]

Members of the transcription factor forkhead family, such as Foxo3a, can be inactivated through phosphorylation by activated Akt.[26] We found that FcεRI aggregation and FcεRI coaggregation with FcγRII result in the same phosphorylation pattern of Foxo3a. This is an interesting observation because one might expect the phosphorylation of Foxo3a to decrease in response to less phosphorylated Akt being available. A possible explanation is that because the phosphorylation of Akt is not totally abrogated, there might still be enough to phosphorylate Foxo3a to the same extent. Another interesting feature is that phosphorylated Foxo3a is present in cells that are not activated by either FcεRI aggregation or coaggregation of FcγRIIB with FcεRI. This suggests a natural equilibrium between phosphorylated and unphosphorylated Foxo3a in the cells, which is shifted toward phosphorylation upon activation. Akt is a major effector protein, and although the phosphorylation of Foxo3a by Akt does not seem to be affected, a pathway (or pathways) other than the one investigated might be where the inhibition of Akt phosphorylation plays a more crucial role.

A protein known to be under the transcriptional control of the forkhead transcription factor Foxo3a is Bim.[27] We previously found Bim to be strongly increased upon FcεRI aggregation.[18] After coaggregation of FcγRIIB with FcεRI or after FcεRI aggregation, the two isoforms of Bim (BimEL and Bim_L) were up-regulated in comparison to unactivated control cells. The results demonstrate that FcεRI-induced Bim up-regulation is not affected upon coaggregation with FcγRIIB. Bim_{EL} consisted of two bands, probably due to phosphorylation. We have previously seen that stem-cell factor (SCF) promotes the survival of mast cells through inactivation of Foxo3a, preventing the up-regulation of Bim and leading to increased phosphorylation of Bim. Those results show that inhibition of Foxo3a and (consequently) Bim provides an important mechanism by which SCF acts to prevent apoptosis in mast cells.[19]

Antiapoptotic members of the Bcl-2 family are needed for cell survival. One of the murine prosurvival Bcl-2 family members is A1, which plays a prominent role in preventing apoptosis in a variety of cell systems.[35,36] Previously, we demonstrated that mRNA levels for A1 are increased after FcεRI aggregation and that A1 is critical for the activation-induced survival of mast cells.[20] Similarly, the human homologue bfl-1 is up-regulated in human mast cells upon FcεRI aggregation.[37] We examined the mRNA induction of the antiapoptotic A1 protein after coaggregation of FcεRI with FcγRIIB; we found that A1 mRNA was up-regulated both when mast cells are activated through FcεRI aggregation and when FcεRI is coaggregated with FcγRIIB. Our finding that both antiapoptotic A1 and proapoptotic Bim proteins are up-regulated as a result of FcεRI aggregation could be an explanation of why this activation results in cell death or survival in some experimental settings, since the fate of cells is likely to be influenced by the relative balance of these molecules.

The only treatment of allergic diseases that leads to long-lasting effects is allergen-specific immunotherapy. The immunologic mechanisms responsible for a successful treatment are still not fully defined. One hypothesis is that the antigen-specific IgG that increases in serum during treatment blocks antibodies,[38] leading to possible coaggregation of FcεRI with FcγRIIB. The finding that allergic activity is inhibited by coaggregating FcεRI with FcγRIIB by using a human Fcγ-Fcε fusion protein highlights a new promising therapeutic approach to immunomodulation.[39] The fusion protein showed antiallergic effects both in vitro and in vivo and was shown to inhibit IgE-mediated activation of blood basophils and cord blood–derived mast cells.[40]

Furthermore, evidence for negative regulation of allergic responses by FcγRIIB has been

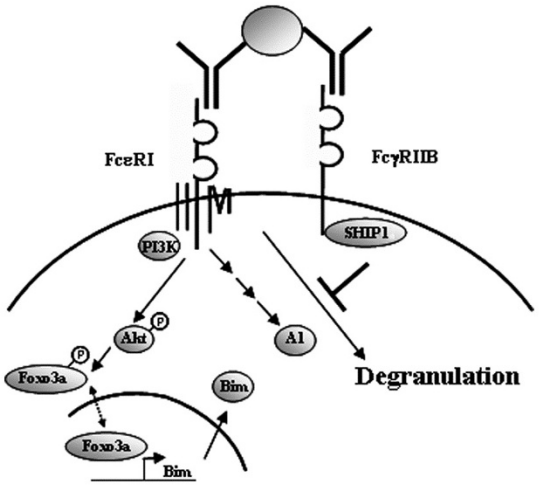

Figure 6 Schematic diagram showing the effect of coengagement of FcεRI with FcγRIIB. Coaggregation of FcεRI with FcγRIIB inhibits degranulation but not the induction of Bim and A1.

demonstrated by the use of FcγRIIB-deficient mice. These mice produce more immunoglobulin than wild-type mice in response to immunization,[41] in which this increase is partly due to the increase in IgG1. The negative regulation of IgG production by FcγRIIB probably decreases the production of IgE. This would work in favour of reduced FcεRI expression on the cells and less IgE being available for activation.[42,43] FcγRIIB-deficient mice also display more vascular permeability in the IgG-dependent passive cutaneous anaphylaxis reaction than do wild-type mice, indicating mast-cell activation of a greater extent than that seen in wild-type mice.[41] During IgE- and IgG-dependent passive systemic anaphylaxis, the FcγRIIB-deficient mice undergo increased hypothermia and death.[44] These findings indicate an important role for FcγRIIB on mast cells in down-regulating immediate hypersensitivity reactions as a result of anaphylactic mast-cell activation.

This report shows that although mast-cell degranulation is inhibited by coaggregation of FcεRI with FcγRIIB, other downstream signalling proteins that are closely related to cell survival remain largely unaffected. Figure 6 presents a schematic overview of how these processes could be separated in the cell. Our previous finding that both proapoptotic and antiapoptotic proteins are up-regulated as a result of FcεRI aggregation suggests that the fate of cells is likely to be based on the balance between these proteins.[17]

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