

IgE-receptor Activation of Mast Cells Regulates Phosphorylation and Expression of Forkhead and Bcl-2 Family Members

J. Alfredsson*, C. Möller*† & G. Nilsson*†

*Department of Genetics and Pathology, Rudbeck Laboratory, Uppsala University, Uppsala; and †Department of Medicine, Karolinska Institutet, Stockholm, Sweden

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Correspondence to: Dr G. Nilsson, Department of Medicine, Clinical Immunology and Allergy Unit, Karolinska Institutet, KS L2:4, SE-171 76 Stockholm, Sweden. E-mail: Gunnar.P.Nilsson@ki.se

Abstract

Activation of the high-affinity IgE-receptor, FcεRI, expressed on mast cells can result in either enhanced survival or apoptosis depending on the circumstances. In this study, we have analysed signalling pathways involved in the regulation of mast cell survival and apoptosis. FcεRI cross-linking induces phosphorylation of Akt and its downstream target forkhead transcription factors. In addition, Bad, GSK-3β and IκB-α also become phosphorylated. A1, a prosurvival Bcl-2 homologue transcriptionally controlled by NFκB transcription factors, is upregulated upon FcεRI activation. These events have prosurvival effects on the mast cells. Moreover, FcεRI activation upregulates the levels of the proapoptotic protein Bim and induces a rapid, but transient, phosphorylation of Bim. Thus, FcεRI activation of mast cells leads to both prosurvival and proapoptotic signalling events where the outcome most likely depends on the balance between these signals.

Introduction

In both acute and chronic inflammations, mast cells play an important role as effector and regulatory cells. When mast cells become activated, they release granule-stored, preformed inflammatory mediators and/or secrete compounds that regulate the inflammatory response. Examples of substances that can be released or secreted from mast cells are histamine, proteases, prostaglandins, leucotrienes and cytokines (e.g. TNF-α) [1].

Historically, mast cells are best known for their detrimental role in allergy. When a multivalent antigen, such as an allergen, binds to IgE attached to FcεRI on the mast cell membrane, two or more receptors are cross-linked leading to activation and degranulation of the cell. The mediators released from mast cells during an allergic response evoke increased permeability of the blood vessels with leakage of plasma into the tissues, tissue oedema, leucocyte recruitment, bronchoconstriction and inflammation [2].

In healthy individuals, the mast cell numbers are kept at a constant level, but at sites of inflammation, the mast cell numbers are increased [3, 4]. It has previously been demonstrated that allergic activation (IgE-receptor activation) of mast cells can result in either increased survival or increased apoptosis of the cells depending on the conditions [5–8]. We have previously shown that activation-

induced mast cell survival depends on the prosurvival A1, a Bcl-2 family member [6]. In this study, we analysed how cross-linking of the IgE-receptor FcεRI on mast cells affects signalling pathways known to be involved in the regulation of cell survival.

Signalling through Akt has been shown to be an important prosurvival signalling pathway in several cell types [9–13]. Molecules downstream of Akt include the transcription factors forkhead [14–17] and NFκB [18, 19]. The active form of the forkhead transcription factor FOXO3a can induce expression of Bim [20], which is a proapoptotic Bcl-2 family member [21]. Akt exerts its effect on NFκB transcription factors through activation of IKK, which in turn phosphorylates the inhibitor of NFκB, IκB [18, 19], resulting in ubiquitin-induced degradation of IκB. Hence, the NFκB transcription factors become free to translocate into the nucleus where they can activate transcription of genes. The NFκB transcription factors have in B cells and T cells been shown to induce expression of A1, a prosurvival member of the Bcl-2 family [22]. Two additional proteins downstream of Akt are the proapoptotic Bcl-2 family member Bad and the proapoptotic protein GSK-3 [23–25]. Akt can inhibit these proteins by subjecting them to phosphorylation.

The prosurvival Bcl-2 family members include Bcl-2, Bcl-X_L, A1/Bfl-1, Bcl-w and Mcl-1 [26]. The proapoptotic

Bcl-2 family members are further divided into two subgroups, referred to as the Bax/Bak-like group and the BH3-only proteins. The Bax/Bak-like group contains Bax, Bak and Bok. The BH3-only group includes Bad, Bim, Bid, Hrk, Puma, Noxa and Bmf. The proteins in this group are called BH3-only proteins as they, among the Bcl-2 homology (BH) domains, only have the BH3 domain. With the help of the BH3 domain, these proteins can bind to the prosurvival Bcl-2 family members, thereby neutralizing the prosurvival action of these proteins and inducing apoptosis [27]. Data have been presented suggesting that proteins of the BH3-only group initiate cytokine deprivation- and stress-induced apoptosis and that the Bax/Bak-like proteins are also needed for the cell killing but play a role further downstream [28, 29].

The balance between prosurvival and proapoptotic Bcl-2 family members helps determine whether a cell will survive or undergo apoptosis. This balance is not only dependent on the levels of the different proteins but is also affected by whether the proteins are in active or inactive state, the subcellular localization and how the proteins interact with each other [30].

In the present study, we demonstrate that FcεRI cross-linking, such as in allergic activation, results in stimulation of both prosurvival and proapoptotic signalling events. These include phosphorylation of Akt, forkhead, Bad, Bim, GSK-3β and IκB-α, and upregulation of A1, which result in a prosurvival stimulus to the cell. The FcεRI activation also leads to upregulation of Bim, which is a proapoptotic protein.

Materials and methods

Reagents. TNP-BSA (coupling ratio: 9/1) was obtained from Biosearch Technologies (Novato, CA, USA). Anti-mouse antibody (HRP-conjugated) was from Amersham Pharmacia Biotech AB (Uppsala, Sweden). LumiGLO, anti-rabbit antibody (HRP-conjugated), antibodies against phospho-Akt(Ser473), phospho-Akt(Thr308), Akt, phospho-GSK-3β(Ser9), phospho-IκB-α(Ser32), phospho-FKHR(Ser256) and phospho-Bad(Ser112) were all purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies against phospho-FKHRL1(Ser253), phospho-FKHRL1(Thr32) and FKHRL1 were purchased from Upstate Biotechnology (Lake Placid, NY, USA). Anti-Bim antibody was bought from Affinity BioReagents, Inc. (Golden, CO, USA). Anti-actin antibody (HRP-conjugated) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). TriPure isolation reagent was obtained from Boehringer Mannheim (Mannheim, Germany). Foetal bovine serum (FBS) was purchased from Gibco (Paisley, Scotland, UK). Other reagents, including medium, were purchased from Sigma (St Louis, MO, USA).

Mast cell cultures. Bone marrow-derived mast cells (BMMC) were obtained by culturing bone marrow cells from C57BL/6J mice (Bommice, Ry, Denmark) in RPMI-1640 supplemented with 15% v/v WEHI-3 conditioned medium as a source of IL-3, 10% heat-inactivated FBS, 10 mM HEPES buffer, 1× MEM nonessential amino acid solution, 1 mM sodium pyruvate, 50 μM 2-ME, 2 mM L-glutamine, 100 IU/ml of penicillin G and 50 μg/ml of streptomycin. The cells were cultured in 37 °C at 5% CO₂, and the cell medium was replaced once per week. The BMMC were cultured for a minimum of 4 weeks before they were used in experiments. Mast cell differentiation was confirmed by staining with toluidine blue.

Mast cell activation. For activation through cross-linking of FcεRI, BMMC (1 × 10⁶ cells/ml) were incubated for 90 min with a monoclonal mouse anti-TNP IgE-antibody (IgE1-b4, ATCC), used as a 15% v/v hybridoma supernatant. The cells were then washed twice in PBS and resuspended in medium, and the IgE-receptors were cross-linked by addition of 100 ng/ml of TNP-BSA. The cells were incubated for indicated periods of time.

Western blotting. For analysis of Akt, GSK-3β, IκB-α, Bad and forkhead proteins, BMMC were starved for 16 h in medium lacking WEHI, containing 0.1% FBS, before activation, and the FcεRI cross-linking was performed in RPMI-1640 medium supplemented with 0.2% BSA, 2 mM L-glutamine, 100 IU/ml of penicillin G and 50 μg/ml of streptomycin. For the analysis of Bim, the cells were not starved prior to the experiments. The cells were either activated in full medium (see the section about mast cell culturing) or activated in starvation medium (RPMI-1640 medium supplemented with 0.2% BSA, 2 mM L-glutamine, 100 IU/ml of penicillin G and 50 μg/ml of streptomycin). For analysis of proteins, cells were lysed in SDS lysis buffer (62.5 mM Tris-HCL, pH 6.8, 2% w/v SDS, 10% glycerol, 50 mM DTT and 0.01% w/v bromophenol blue) and sonicated on ice. The proteins were size-separated under reducing conditions on NuPAGE Bis-Tris Western gels (NOVEX Electrophoresis GmbH, Frankfurt, Germany) and electrically transferred onto nitrocellulose membranes (Hybond ECL, Amersham Pharmacia Biotech). The membranes were blocked in Tris-buffered saline containing 5% w/v nonfat dry milk and 0.1% Tween-20, followed by incubation with primary antibody overnight at 4 °C, and with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. The proteins were visualized by enhanced chemiluminescence system.

Analysis of RNA expression. BMMC were activated in RPMI-1640 medium supplemented with 0.2% BSA, 2 mM L-glutamine, 100 IU/ml of penicillin G and 50 μg/ml of streptomycin. TriPure isolation reagent was used for isolation of total cellular RNA. RNase protection assay was performed according to the protocol of the

RiboQuant System (PharMingen, San Diego, CA, USA) using the mAPO-2 multiprobe set.

Results and discussion

Activation by FcεRI cross-linking results in phosphorylation of Akt in mast cells

The Akt signalling pathway is considered to be important for regulation of cell survival in several cell types and at different conditions [9–11]. We have therefore analysed the effect of FcεRI cross-linking on Akt phosphorylation in mast cells. Activation of Akt correlates with its phosphorylation at the residues Ser473 and Thr308 [31]. As shown in Fig. 1, FcεRI activation induces a rapid phosphorylation of Akt on both Ser473 and Thr308. Our study results show that Akt is strongly phosphorylated at 5–10 min after cross-linking. Phosphorylation of Akt at Ser473, but not that of Thr308, following FcεRI activation has been analysed previously by others [32], and our study results support those data.

Activation of FcεRI induces phosphorylation of Bad, GSK-3β and IκB-α

Several proapoptotic proteins have been identified as targets for Akt phosphorylation, including the proteins Bad and GSK-3β [23–25]. In this study, we investigated whether FcεRI cross-linking affects the phosphorylation of Bad and GSK-3β. Mast cells were activated through FcεRI, and phosphorylation of Bad at Ser112 was analysed. We observed a rapid phosphorylation of Bad, which was still pronounced after 30 min (Fig. 2). After 2 h, the level of phosphorylation was almost back to background.

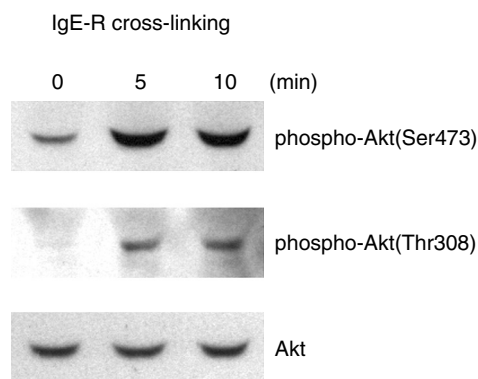


Figure 1 FcεRI activation induces phosphorylation of Akt at Ser473 and Thr308. Bone marrow-derived mast cells (BMMC) were starved for 16 h and then subjected to FcεRI cross-linking for indicated periods of time. Phosphorylated Akt was analysed by Western immunoblotting using phosphospecific antibodies. Probing with anti-Akt antibody was used as a control for the amount of proteins loaded. The results shown are representative of at least three independent experiments. IgE-R, IgE-receptor.

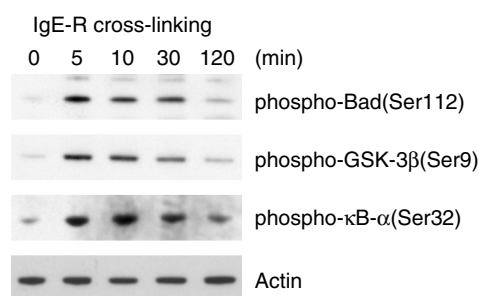


Figure 2 Cross-linking of FcεRI results in phosphorylation/inactivation of Bad, GSK-3β and IκB-α. Following 16-h starvation, bone marrow-derived mast cells (BMMC) were activated through FcεRI for the indicated periods of time, and phosphorylated proteins were analysed by Western immunoblotting. Probing with anti-Actin antibody was used as a control for the amount of proteins loaded. The results shown are representative of at least two independent experiments. IgE-R, IgE-receptor.

It has previously been reported that SCF stimulation can result in Bad phosphorylation in mast cells [33], but this is the first time FcεRI activation has been demonstrated to induce phosphorylation of Bad. It has been shown that phosphorylation of Bad is essential for the pro-survival stimuli mediated by growth factors [34, 35]. Phosphorylation of Bad leads to sequestering of Bad with protein 14-3-3 [36]. This hinders Bad from binding to other Bcl-2 family members, hence restraining Bad from initiating apoptosis.

When analysing GSK-3β, we found that FcεRI cross-linking of BMMC rapidly induces phosphorylation of GSK-3β and that the increase in phosphorylation is transient (Fig. 2). In a previously published study, the phosphorylation of GSK-3β(Ser9) following FcεRI activation was almost invisible [32], but our study results show a prominent phosphorylation of GSK-3β (Fig. 2). GSK-3 has been reported to play a critical role in the regulation of apoptosis. Overexpression of catalytically active GSK-3 induced apoptosis in Rat1 and PC12 cells, and dominant-negative GSK-3 prevented apoptosis following inhibition of PI3-kinase [37]. Akt-mediated phosphorylation of GSK-3β at Ser9 inhibits its catalytic activity [25].

We also analysed whether FcεRI cross-linking has any effect on phosphorylation of IκB-α, an inhibitor of NFκB transcription factors, considered as downstream of Akt [18, 19]. FcεRI cross-linking of BMMC rapidly induced phosphorylation of IκB-α (Fig. 2). In our experiments, the phosphorylation of IκB-α at Ser32 was more prominent than what has been reported previously [32]. Phosphorylation of IκB-α results in activation of the NFκB transcription factor family, and hence induction of expression of genes transcriptionally regulated by NFκB. The NFκB transcription factor family regulates the expression of numerous genes, including genes involved in the regulation of cell survival, such as the prosurvival A1/Bfl-1 [22, 38, 39].

Activation by FcεRI upregulates the prosurvival Bcl-2 homologue A1

It has been shown in B cells and T cells that NFκB regulates the transcription of A1 [22], which is a prosurvival homologue to Bcl-2. As shown in Fig. 3, and also reported by us before [6], FcεRI cross-linking induces expression of A1. It is possible that NFκB transcription factors contribute to the regulation of FcεRI activation-induced transcription of A1 in mast cells, in a similar way as was described for the regulation of A1 expression in other cell types previously studied.

FcεRI cross-linking inhibits forkhead transcription factors in mast cells

The FOXO subgroup belonging to the family of forkhead transcription factors is directly phosphorylated and inactivated by Akt [14–17, 40]. FOXO factors predominantly reside in the nucleus, where they regulate the transcription of several genes involved in apoptosis and cellular proliferation [41]. Phosphorylation of forkhead inhibits its function as transcription factor, as phosphorylation results in nuclear exclusion and inhibition of transcription [17, 40]. We therefore analysed the phosphorylation status of the forkhead proteins FOXO1a and FOXO3a in FcεRI-activated mast cells. We found that both FOXO1a and FOXO3a are rapidly phosphorylated following FcεRI activation (Fig. 4). The phosphorylation of FOXO3a(Thr32) had decreased already after 10 min, whereas the phosphorylation of FOXO3a(Ser253) was more prolonged (Fig. 4).

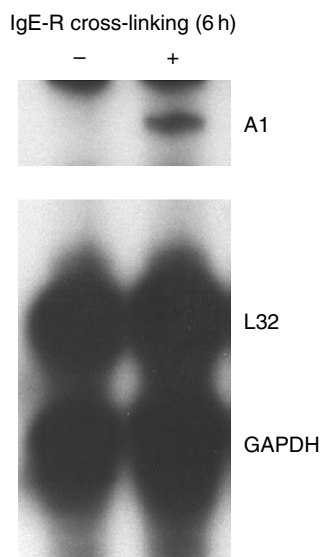


Figure 3 FcεRI cross-linking upregulates the prosurvival A1. Bone marrow-derived mast cells (BMMC) were stimulated by FcεRI cross-linking, and after 6 h, the A1 RNA expression was analysed by RNase protection assay. L32 and GAPDH were also analysed as controls for the amount of RNA loaded. The results shown are representative of at least three independent experiments. IgE-R, IgE-receptor.

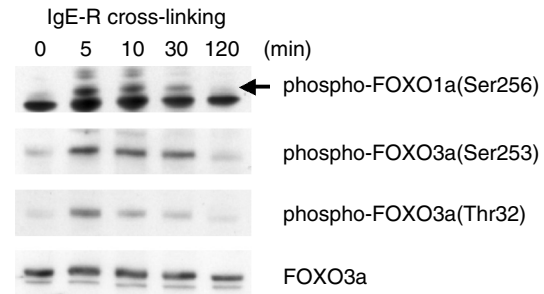


Figure 4 FcεRI activation induces phosphorylation of forkhead transcription factors. Bone marrow-derived mast cells (BMMC) were starved for 16 h and then subjected to FcεRI cross-linking for indicated periods of time. Phosphorylated FOXO1a and FOXO3a were analysed by Western immunoblotting using phosphospecific antibodies. Probing with anti-FOXO3a antibody was used as a control for the amount of proteins loaded. The results shown are representative of at least two independent experiments. IgE-R, IgE-receptor.

Our study results suggest that activation of FcεRI inhibits the expression of genes transcriptionally regulated by FOXO transcription factors. A similar phosphorylation pattern of FOXO1a and FOXO3a in mast cells is achieved by SCF treatment [42]. However, although the FcεRI cross-linking-induced phosphorylation of FOXO1a and FOXO3a in mast cells is as rapid as after SCF stimulation, the phosphorylation is not as prolonged as has been observed after stimulation with SCF.

Activation of FcεRI upregulates the proapoptotic protein Bim in mast cells and induces a rapid phosphorylation of Bim

FOXO3a regulates the transcription of the proapoptotic Bcl-2 family member Bim [20]. There exist at least three different isoforms of Bim: Bim_{EL} (196 aa), Bim_L (149 aa) and Bim_S (110 aa) [21]. Bim proteins have in other cell systems been shown to be upregulated upon withdrawal of growth factors, such as IL-3 for haematopoietic progenitors [43] and NGF for neurones [44]. Similarly, mast cells deprived of growth factors upregulate Bim (Fig. 5A, right panel) [42, 45].

As Bim has been reported to be transcriptionally regulated by FOXO3a, one could expect that the phosphorylation, and hence inactivation, of FOXO3a observed after FcεRI cross-linking of mast cells (Fig. 4) would lead to downregulation of Bim expression. However, when analysing the Bim protein levels in resting and in FcεRI cross-linked mast cells, both Bim_{EL} and Bim_L are upregulated in cells activated by FcεRI cross-linking compared to resting mast cells (Fig. 5A, left panel) [45]. Our study results suggest that also other transcription factor(s) besides FOXO3a are important for regulation of *bim* gene expression in mast cells. Alternative transcription factors for Bim have not been described and have therefore to be explored in future studies.

It has been demonstrated that Bim proteins can be in either phosphorylated or dephosphorylated state and that

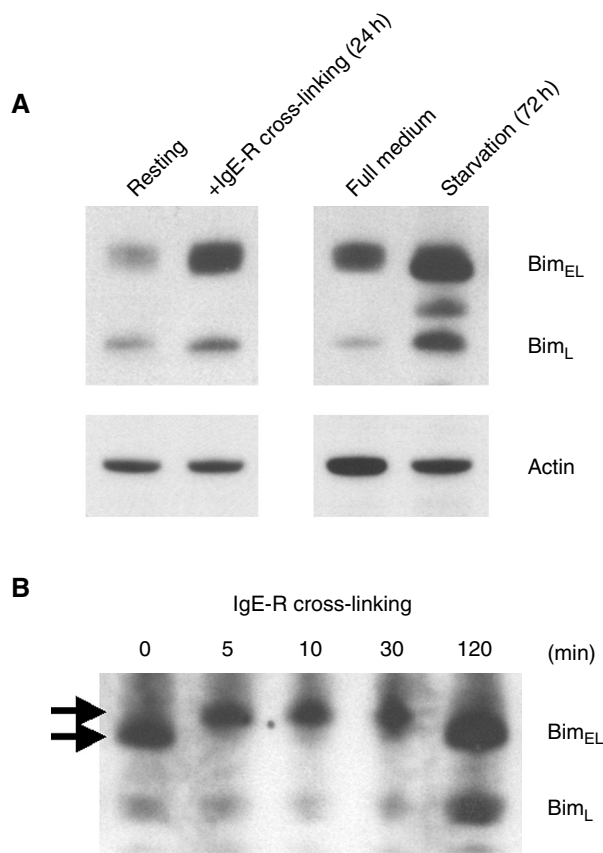


Figure 5 FcεRI cross-linking results in both upregulation and phosphorylation of Bim. (A) Bone marrow-derived mast cells (BMMC) were either activated through FcεRI cross-linking or deprived of growth factor and serum. Bim expression was analysed by Western immunoblotting. Probing with anti-actin antibody was used as a control for the amount of proteins loaded. (B) BMMC were FcεRI-cross-linked, and after the indicated periods of time, Bim protein expression was analysed by Western immunoblotting. Phosphorylated Bim_{EL} was detected by a shift in migration on the electrophoresis gel. The results shown are representative of at least two independent experiments. IgE-R, IgE-receptor.

the phosphorylation state of Bim_{EL} can be easily detected by the migration pattern on SDS-PAGE electrophoresis gels [43, 46–48]. Phosphorylated Bim_{EL} migrates slower on the gel compared to Bim_{EL} that is not phosphorylated. In order to analyse whether activation through FcεRI affects the phosphorylation state of Bim, mast cells were activated by FcεRI cross-linking. The migration pattern of Bim proteins was analysed by gel electrophoresis and Western immunoblotting. We found that Bim_{EL} became rapidly phosphorylated within 5 min after FcεRI activation, and the phosphorylation had declined to background levels after 120 min (Fig. 5B). Phosphorylation of Bim has been reported to induce its degradation through the proteasome pathway [47, 48]. However, as seen in Fig. 5(A), FcεRI cross-linking does not downregulate the Bim levels, as one could expect considering the fact that Bim becomes phosphorylated. Instead, Bim levels are upregulated

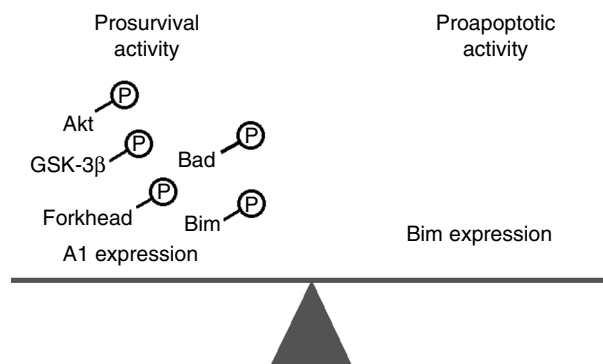


Figure 6 A model describing that activation of FcεRI stimulates both prosurvival and proapoptotic signalling events. The balance between prosurvival and proapoptotic activities might determine whether the mast cell will survive or undergo apoptosis.

following the FcεRI activation. Taken together, the results described in Figs 4 and 5(A,B) suggest that Bim levels are upregulated after FcεRI cross-linking, despite the fact that Bim is rapidly (but transiently) phosphorylated, and furthermore, as FOXO3a is phosphorylated upon FcεRI activation, other transcription factor(s) besides FOXO3a might be responsible for the FcεRI activation-induced upregulation of Bim.

Concluding remarks

Activation of the high-affinity IgE-receptor FcεRI results in stimulation of both prosurvival and proapoptotic signalling events (Fig. 6). This could be a way for the cell to fine-tune the regulation of the fate of the cell after activation. Depending on the circumstances during FcεRI cross-linking, this type of activation can result in either increased survival or increased death of the mast cell [8].

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