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Phosphorylation of SNAP-23 by I κ B Kinase 2 Regulates Mast Cell Degranulation

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SUMMARY

Mast cells are known to play a pivotal role in allergic diseases. Cross-linking of the high-affinity receptor for IgE (Fc ϵ RI) leads to degranulation and allergic inflammation; however, the regulatory mechanisms of IgE-dependent exocytosis remain unknown. We show here that I κ B kinase (IKK) 2 in mast cells plays critical roles in IgE-mediated anaphylaxis *in vivo*, and IgE-mediated degranulation *in vitro*, in an NF- κ B-independent manner. Upon Fc ϵ RI stimulation, IKK2 phosphorylates SNAP-23, the target membrane soluble N-ethylmaleimide-sensitive fusion factor attachment protein receptor (SNARE), and ectopic expression of a phosphomimetic mutant of SNAP-23 partially rescued the impaired IgE-mediated degranulation in IKK2-deficient mast cells. These results suggest that IKK2 phosphorylation of SNAP-23 leads to degranulation and anaphylactic reactions. While this reaction is NF- κ B-independent, we additionally show that IKK2 also regulates late-phase allergic reactions promoted by the release of proinflammatory cytokines in an NF- κ B-dependent manner. The findings suggest that IKK2 is a central player in allergic reactions.

INTRODUCTION

Mast cells are recognized as the major effector cells of the type I hypersensitivity reactions because of their high-affinity receptors for IgE (Fc ϵ RI). They are known to play a pivotal role in allergic diseases (Galli et al., 2005a, 2005b), such as atopic rhinitis, asthma, and atopic dermatitis. Engagement of Fc ϵ RI by IgE, followed by the aggregation of multiple IgE-bearing Fc ϵ RI molecules by poly-valent antigen, leads to degranulation, and release of histamine, LTC₄, and other preformed chemical mediators. Additionally, multiple cytokine genes are transcribed and newly synthesized arachidonic acid metabolites are secreted, all of which trigger allergic inflammation (Galli et al., 2005a, 2005b). It is well established that mast cells mediate the early phase of type I hypersensitivity reactions by releasing granule contents after Fc ϵ RI crosslinking (Bischoff, 2007). However, the precise regulatory mechanisms that lead to IgE-dependent exocytosis in mast cells remain unknown. Furthermore, allergic diseases do not consist only of early-phase reactions, but also of late-phase reactions that occur facultatively following early-phase reactions, and are thought to cause chronic inflammation. Mast cells induce the recruitment of neutrophils by releasing TNF- α and IL-8. In mouse models, mast cell-secreted TNF- α plays an important role in late-phase allergic reactions in an NF- κ B-dependent manner (Klemm et al., 2006). The precise role of I κ B kinase (IKK) 2 in mast cells in late-phase allergic reactions remains unknown.

Soluble N-ethylmaleimide-sensitive fusion factor attachment protein receptor (SNARE) proteins play a central regulatory role in exocytosis by promoting vesicle fusion with the plasma membrane (Jahn and Sudhof, 1999). Degranulation requires the association of v-SNAREs and

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t-SNAREs to form a ternary SNARE complex that catalyzes membrane fusion. The SNARE complex brings the two membranes in apposition, a necessary step in overcoming the energy barrier required for membrane fusion. In mast cells, these include syntaxin 4 (Paumet et al., 2000) and SNAP-23 (Guo et al., 1998) as t-SNAREs, while VAMP-2 (Miesenbock et al., 1998) and VAMP-8 (Paumet et al., 2000) represent candidates for v-SNAREs. The syntaxin binding proteins Munc18b and Munc18c are also essential for exocytosis in mast cells (Martin-Verdeaux et al., 2003). It has been shown that phosphorylation of SNAP-23 modulates exocytosis (Lin and Scheller, 2000; Marash and Gerst, 2001; Hepp et al., 2005). Although protein kinase C can phosphorylate SNAP-23 *in vitro*, it has not been established as to which kinase phosphorylates SNAP-23 *in vivo* and regulates exocytosis in mast cells (Hepp et al., 2005).

Transcription factors of the NF- κ B family regulate hundreds of genes in the context of multiple important biological processes, such as apoptosis, proliferation, innate and adaptive immune responses, and inflammation (Hayden and Ghosh, 2004; Li and Verma, 2002). The IKK complex coordinates the response to most of the external signals leading to the induction of NF- κ B-activated gene transcription. The activity of the IKK complex resides in two catalytic subunits, IKK1 (also called IKK α) and IKK2 (also called IKK β), and two regulatory subunits, NEMO and ELKs (Hayden and Ghosh, 2004; Li and Verma, 2002; Scheidereit, 2006; Perkins, 2007). Interestingly, IKK2 is the major cytokine-responsive I κ B kinase and, thus, is a central component of the intracellular signaling pathway mediating NF- κ B activation (Scheidereit, 2006; Perkins, 2007). We report that IKK2 in mast cells plays a critical role in IgE-mediated anaphylaxis *in vivo* and IgE-mediated degranulation *in vitro* in an NF- κ B-independent manner. IKK2 is recruited into the lipid raft fractions and phosphorylates SNAP-23 at Ser120 and Ser95 upon Fc ϵ RI stimulation, leading to upregulation of IgE-mediated degranulation in mast cells. The late-phase allergic reactions initiated by release of proinflammatory cytokines is also regulated by IKK2 kinase, but requires NF- κ B activity. We conclude that IKK2 kinase regulates both the early- and late-phase allergic reactions.

RESULTS

Severe Impairment of Anaphylactic Responses in the Absence of IKK2

To investigate the role of IKK2 on anaphylactic responses *in vivo*, we used mast cell knock-in mouse model (Galli et al., 2005a, 2005b). FLMCs were generated from Days 11.5–12.5 embryonic liver of wild-type (WT) or IKK2-deficient (IKK2^{-/-}) mice because of the lethality in the IKK2^{-/-} embryos at E14 (Li et al., 1999a). The total number of fetal liver cells was not distinguishable between WT and IKK2^{-/-} mice (WT [4.65 \pm 0.34 \times 10⁵] versus IKK2^{-/-} [4.24 \pm 0.44 \times 10⁵]; n = 10). Liver cells isolated from both genotypes proliferated and expanded normally when cultured in the presence of 100 ng/ml IL-3. After 4 weeks, more than 98% of cells were c-kit positive, and the number of mast cells (c-kit⁺IgE⁺ cells) recovered per mouse was indistinguishable between these mice (see Figure S1A available online). We confirmed the lack of IKK2 expression of fetal liver-derived mast cells (FLMCs) from IKK2^{-/-} mice (Figure S1B). The expression levels of c-kit and Fc ϵ RI α were also indistinguishable (Figure S1C). The WT and IKK2^{-/-} FLMC were morphologically indistinguishable by electron microscopy (Figure S1D). Especially, there was no significant difference in the number of granules (Figure S1D: WT [40.4 \pm 5.4] versus IKK2^{-/-} [41.3 \pm 4.4]; n = 40) and the mean of granule intensity (Figure S1D: WT [93.4 \pm 22.4] versus IKK2^{-/-} [92.1 \pm 18.6]; n = 40) in each mast cell. The number of granules in each mast cell was counted and the density of individual granules was measured and analyzed with Image J software (NIH, Bethesda, MD). Furthermore, most of WT and IKK2^{-/-} FLMCs were annexin-V- and 7-AAD-negative (Figure S1E). IgE-induced Ca²⁺ flux was also normal in IKK2^{-/-} FLMCs (Figure S2). These results indicate that IL-3-induced mast cell development *in vitro* is not dependent on IKK2 activity.

To generate mast cell knock-in mice, cultured FLMCs were transplanted intradermally (i.d.) or intravenously (i.v.) into mast cell-deficient WBB6F1-Kit^{w/Kit^{w-v}} (W/W^v) mice. Four weeks after transplantation, we examined IgE-dependent anaphylactic reactions in mast cell-reconstituted mice. Antigen challenge of mice that had been sensitized previously with monoclonal DNP-specific IgE antibody demonstrated that IKK2^{-/-} FLMC-reconstituted mice exhibited reduced cutaneous anaphylaxis compared with the WT FLMC-reconstituted mice, as assessed by change in ear thickness (Figure 1A) and leakage of injected Evans blue dye in the ear (WT [0.848 ± 0.067] versus IKK2^{-/-} [0.242 ± 0.081], optical density at 620 nm; n = 8; p < 0.01) (Figures 1B and 1C). We also demonstrated similar experiments in the nonreconstituted W/W^v mice and confirmed lack of anaphylactic reactions in these mice as previously described (Galli et al., 2005a, and our unpublished data). Since mast cell numbers in the ear skin were identical between WT FLMC-reconstituted mice and IKK2^{-/-} FLMC-reconstituted mice (WT [14.6 ± 4.4/mm] versus IKK2^{-/-} [15.6 ± 4.6/mm], n = 8), the diminished anaphylaxis in IKK2^{-/-} FLMC-reconstituted mice was most likely due to mast cell hyporeactivity to the antigen. We next examined passive systemic anaphylaxis (PSA) by measuring decrease of body temperature after antigen challenge. Figure 1D shows a decrease in PSA in the IKK2^{-/-} FLMC-reconstituted mice. These data imply a physiological role of IKK2 in mast cells is to induce appropriate mast cell degranulation.

IKK2 Plays Critical Roles in Mast Cell Degranulation

To investigate the cellular basis for the diminished *in vivo* anaphylactic reactions in IKK2^{-/-} FLMC-reconstituted mice, we next compared degranulation (release of β-hexosaminidase and histamine) between FLMCs derived from WT and IKK2^{-/-} mice. Consistent with the defect in anaphylactic responses *in vivo*, IKK2^{-/-} FLMCs showed clear defects in FcεRI-stimulated β-hexosaminidase release (WT [25.0% ± 5.4%] versus IKK2^{-/-} [14.4% ± 5.8%]; n = 5; p < 0.01) (Figure 2A), and percent histamine release (WT [37.7% ± 3.5%] versus IKK2^{-/-} [10.8% ± 1.1%]; n = 5; p < 0.01) (Figure 2B). IKK2^{-/-} FLMCs also showed defects in calcium ionophore A23187-stimulated β-hexosaminidase release (WT [39.8% ± 2.0%] versus IKK2^{-/-} [20.1% ± 0.6%]; n = 5; p < 0.01) (Figure 2C). We next determined the amount of annexin-V specifically bound to mast cell granules exposed after stimulation with FcεRI (Demo et al., 1999). IKK2^{-/-} FLMCs showed reduced annexin-V binding than WT FLMCs (Figure 2D). We also examined the effect of overexpression of IKK2 in the mast cells on FcεRI-stimulated β-hexosaminidase release. We examined FcεRIα expression and apoptosis (annexin-V binding) on RBL-2H3 cells stably expressing GFP or GFP-IKK2 and confirmed no significant difference between those cells (data not shown). RBL-2H3, a rat basophilic cell line stably expressing GFP-IKK2 (Figure 2F), resulted in the increase of FcεRI-stimulated β-hexosaminidase release (mock [43.8% ± 4.0%] versus IKK2 [63.0% ± 4.8%]; n = 5; p < 0.01) (Figure 2E). In order to determine whether kinase activity of IKK2 is required for IKK2-mediated upregulation of FcεRI-stimulated β-hexosaminidase release (Figure 2E), we examined the effect of overexpression of IKK2KM, a kinase-inactive mutant of IKK2 (Mercurio et al., 1997), on FcεRI-stimulated β-hexosaminidase release. Overexpression of IKK2KM (Figure 2F) did not enhance FcεRI-stimulated β-hexosaminidase release (mock [43.8% ± 4.0%] versus IKK2KM [38.1% ± 3.9%]; n = 5) (Figure 2E). These results suggest that IKK2 modulates antigen-induced mast cell degranulation in a kinase activity-dependent manner.

NF-κB Activation Is Not Essential for Mast Cell Degranulation

The seminal event in NF-κB activation is the phosphorylation of IκBs, which is mediated by IKK complex. We therefore examined the role of NF-κB activation in FcεRI-induced degranulation. RBL-2H3 cells stably expressing GFP-tagged IκBα superrepressor (IκBαM) (Karin and Ben-Neriah, 2000) (Figure 3C) and HIV-3x-κB luciferase/β-galactosidase plasmids. Expression of IκBαM in the RBL-2H3 cells resulted in the complete inhibition of

NF- κ B-dependent promoter activation in response to TNF- α (Figure 3A). In contrast, I κ B α M-expressing RBL-2H3 cells showed similar levels of Fc ϵ RI-stimulated β -hexosaminidase release to the control cells (mock [36.2% \pm 4.1%] versus I κ B α M [34.7% \pm 3.4%]; n = 5) (Figure 3B). Figure 3C shows that I κ B α M is expressed in these cells. It thus appears that Fc ϵ RI-induced degranulation is independent of NF- κ B activation pathway. Since the IKK complex contains both IKK1/2 kinases, we next asked if mast cell degranulation requires both kinases. We generated FLMCs from IKK1^{-/-}, IKK2^{-/-}, and IKK1/IKK2^{-/-} double knockout (Li et al., 2000) embryos and assayed for β -hexosaminidase activity following Fc ϵ RI-induced degranulation. Figure 3D shows that release of β -hexosaminidase was impaired in both IKK2^{-/-} and IKK1^{-/-}IKK2^{-/-} FLMCs (WT [25.0% \pm 5.4%] versus IKK1^{-/-}IKK2^{-/-} [13.4% \pm 1.7%]; n = 5; p < 0.01) (Figure 3D) to a much greater extent as compared with IKK1^{-/-} FLMCs (Figure 3D). We thus conclude that IKK2 modulates IgE-induced degranulation by an NF- κ B-independent mechanism.

IKK2 Is Recruited into the Lipid Raft Fractions upon Fc ϵ RI Stimulation and Associates with SNAP-23

Mast cells contain the molecular machinery that drives membrane fusion during granule exocytosis. Essential to this process are soluble NSF (N-ethyl-maleimide-sensitive factor) attachment protein receptor (SNARE) proteins that lie on opposing cellular membranes to form a stable multimeric complex that catalyzes fusion (Jahn and Sudhof, 1999). Mast cells express several plasma membrane-localized SNAREs (Q-SNAREs) that form part of the exocytotic machinery. Both SNAP-23 (Guo et al., 1998) and syntaxin 4 (Paumet et al., 2000) have been implicated in IgE-dependent fusion and are partly localized in lipid rafts, domains of the plasma membrane enriched in sphingolipids, and cholesterol (Puri and Roche, 2006; Kay et al., 2006; Chamberlain and Roche, 2001; Salaun et al., 2005). Furthermore, it has been suggested that, in T cells, IKK1 and IKK2 are recruited to the lipid rafts at the immune synapse upon TCR activation (Thome and Tschopp, 2003; Thome, 2004). Therefore, we first examined the recruitment of IKK2 into the lipid rafts after Fc ϵ RI stimulation. Consistent with previous reports (Puri and Roche, 2006; Kay et al., 2006), SNAP-23 and syntaxin 4 were detected in the membrane raft fractions before and after Fc ϵ RI stimulation (Figure 4A, cosedimentation with CTx in fraction 5). Little or no IKK2 was detected in the lipid raft fractions. In contrast, substantially increased levels of IKK2 were detected in the membrane raft fractions upon Fc ϵ RI stimulation (Figure 4A), suggesting that IKK2 colocalizes with SNAP-23 and syntaxin 4 in the lipid rafts only after stimulation. We next examined whether IKK2 can associate with SNAP-23 or syntaxin 4 by coimmunoprecipitation assay. GFP-tagged SNAP-23 was expressed with or without FLAG-tagged IKK2 in 293T cells and the amount of GFP-tagged SNAP-23 following immunoprecipitation with anti-FLAG mAb was evaluated. Anti-GFP mAb coprecipitated FLAG-tagged IKK2 (Figure 4B). In contrast, myc-tagged syntaxin 4 was not coprecipitated with FLAG-tagged IKK2 (data not shown). Furthermore, we examined whether IKK2 associates with SNAP-23 in the lipid raft fractions before and after Fc ϵ RI stimulation. IKK2 and SNAP-23 coimmunoprecipitated in the raft fractions following Fc ϵ RI stimulation (Figure 4C). These results suggest that IKK2 associates with SNAP-23 in the lipid rafts only after Fc ϵ RI stimulation.

IKK2 Phosphorylates SNAP-23 on Ser120 and Ser95

Phosphorylation of SNAP-23 on Ser120 and Ser95 modulates regulated exocytosis in mast cells (Hepp et al., 2005). We therefore examined whether SNAP-23 is a substrate for IKK2. In vitro kinase assays showed that IKK2 phosphorylated SNAP-23 (Figure 5A). However, when Ser120, Ser95, or both were converted to Ala, phosphorylation of recombinant mutant SNAP-23 (Figure 5B) by IKK2 was substantially attenuated (Figure 5A), indicating that IKK2 can phosphorylate SNAP-23 at both Ser120 and Ser95 in vitro. To assess whether this phosphorylation by IKK2 occurs in vivo, we used phospho-specific antibodies (Hepp et al.,

2005). Consistent with previous report (Hepp et al., 2005), Ser120 and Ser95 were phosphorylated upon FcεRI stimulation in WT FLMCs (Figure 5C). In contrast, little or no phosphorylation was detected in IKK2^{-/-} FLMCs (Figure 5C). These results show that SNAP-23 is phosphorylated by IKK2 at Ser120 and Ser95 in vitro and in vivo, and that IKK2 is required for IgE-induced phosphorylation of SNAP-23. It has been reported that the complex of SNAP-23/syntaxin/VAMP is minimally required for membrane fusion (Weber et al., 1998). Consistent with previous report (Puri and Roche, 2006), syntaxin 4 and VAMP-2 coimmunoprecipitated with SNAP-23 in the lipid raft fractions after FcεRI stimulation in WT, but not IKK2^{-/-} mast cells (Figure 5D). It thus appears that IKK2 regulates the formation of SNARE complex of SNAP-23/syntaxin 4/VAMP-2, resulting in the membrane fusions in the mast cells. We also examined whether VAMP-8 or VAMP-7 is a component of SNARE complex as v-SNAREs. VAMP-8, but not VAMP-7, was coimmunoprecipitated with SNAP-23 in the lipid raft fractions after FcεRI stimulation in WT, but not IKK2^{-/-}, mast cells (Figure 5D). This result suggests that, in addition to VAMP 2, VAMP-8 is also involved as v-SNAREs to mediate mast cell degranulation.

Phosphorylation of SNAP-23 Is Crucial for IKK2-Mediated Degranulation

To examine the effects of SNAP-23 depletion or expression of phosphorylation mutant of SNAP-23 on IKK2-induced upregulation of FcεRI-stimulated β-hexosaminidase release, we generated several RNAi interference (shRNA) constructs capable of silencing the expression of SNAP-23 (Figure S3). We selected RBL-2H3 cells stably expressing shSNAP-23 and consequently lack of SNAP-23 expression (Figure 6A). Figure 6B shows that FcεRI-stimulated β-hexosaminidase release was impaired in shSNAP-23-expressing cells (mock [42.8% ± 3.2%] versus shSNAP-23 [29.1% ± 3.4%]; n = 5, p < 0.01). We next examined the effect of depletion of SNAP-23 on IKK2-induced upregulation of FcεRI-stimulated β-hexosaminidase release. In RBL-2H3 cells stably expressing shSNAP-23 and/or GFP-IKK2 (Figure S4A) expression of IKK2 resulted in the enhancement of FcεRI-stimulated β-hexosaminidase release (U6 + GFP [38.4% ± 2.0%] versus U6 + GFP-IKK2 [59.3% ± 4.2%]; n = 5; p < 0.01) (Figure 6C) in the presence of control shRNA. In contrast, in shSNAP-23-expressing cells, IKK2-induced enhancement of FcεRI-stimulated β-hexosaminidase release was not observed (Figure 6C; shSNAP-23 + GFP versus shSNAP-23 + GFP-IKK2). This result indicates that SNAP-23 is involved in IKK2-mediated degranulation. Since IKK2 phosphorylates SNAP-23 at Ser120 and Ser95 (Figure 5), we next examined the effect of expression of SNAP-23AA (Ser120 and Ser95 mutated to Ala) on IKK2-induced upregulation of FcεRI-stimulated β-hexosaminidase release. We designed (1) shRNA-resistant SNAP23 (R-SNAP23), which has three nucleotide mutations in siRNA target sequence; and (2) SNAP-23 shRNA-resistant SNAP23AA by mutating Ser95 and Ser120 in shRNA-resistant SNAP23 (Figure S4A). Downregulation of FcεRI-stimulated β-hexosaminidase release by depletion of SNAP-23 (U6 + FLAG [41.6% ± 3.4%] versus shSNAP23 + FLAG [28.3% ± 3.0%]; n = 5; p < 0.01) (Figure 6D) was reversed in shRNA-resistant SNAP-23 (shSNAP23 + FLAG [28.3% ± 3.0%] versus shSNAP23 + FLAG-R-SNAP23 [45.6% ± 2.2%]; n = 5; p < 0.01) (Figure 6D), but not SNAP-23 shRNA-resistant SNAP23AA-expressing cells (shSNAP23 + FLAG [28.3% ± 3.0%] versus shSNAP23 + FLAG-R-SNAP23AA [31.2% ± 4.5%]; n = 5) (Figure 6D). We further investigated the effect of SNAP-23AA on IKK2-induced enhancement of FcεRI-stimulated β-hexosaminidase. IKK2-induced enhancement of FcεRI-stimulated β-hexosaminidase was observed in only SNAP-23 shRNA-resistant SNAP23 (Figure 6E; shSNAP23 + GFP + FLAG-R-SNAP23 [43.7% ± 2.3%] versus shSNAP23 + GFP-IKK2 + FLAG-R-SNAP23 [59.2% ± 7.0%]; n = 5; p < 0.01), but not SNAP-23 shRNA-resistant SNAP23AA-expressing cells (Figure S4C) (shSNAP23 + GFP + FLAG-R-SNAP23AA [27.2% ± 4.0%] versus shSNAP23 + GFP-IKK2 + FLAG-R-SNAP23AA [30.9% ± 2.1%]; n = 5; p < 0.01) (Figure 6E). These results suggest that phosphorylation of SNAP-23 on Ser120 and Ser95 is required for IKK2-induced upregulation of FcεRI-stimulated degranulation. To examine the effect of expression of

phosphomimetic mutant of SNAP-23 in $IKK2^{-/-}$ mast cells on Fc ϵ RI-stimulated β -hexosaminidase release and in vivo early-phase passive cutaneous anaphylaxis (PCA) reactions, we generated phosphomimetic mutant SNAP23EE by mutating Ser95 and Ser120. $IKK2^{-/-}$ FLMCs were infected with retroviruses of pMX-puro-FLAG-WT SNAP-23, pMX-puro-FALG-SNAP23EE, or pMX-puro (mock), and, as control, WT FLMCs were infected with mock vectors. All of these cells were selected with puromycin (Figure S5). Ectopic expression of WT SNAP-23 did not rescue the impairment of Fc ϵ RI-induced β -hexosaminidase release in $IKK2^{-/-}$ mast cells (Figure 6F: mock [12.4% \pm 1.44%] versus WT SNAP-23 [10.3% \pm 0.341%]; n = 8; p < 0.01). By contrast, expression of SNAP-23EE partially rescued the impairment of Fc ϵ RI-induced degranulation in $IKK2^{-/-}$ mast cells (Figure 6F: mock [12.4% \pm 1.44%] versus SNAP-23EE [18.9% \pm 1.11%]; n = 8; p < 0.01). We also examined in vivo early-phase PCA reactions in mast cell-reconstituted W/W^v mice. Mice with WT SNAP-23 or mock-transduced $IKK2^{-/-}$ FLMCs exhibited similar levels of reduced cutaneous anaphylaxis (Figure 6G: mock [2.67 \pm 1.03 $\times 10^{-2}$ mm] versus WT SNAP-23 [2.33 \pm 0.82 $\times 10^{-2}$ mm]; n = 6; p < 0.01), in comparison with mice with control WT FLMCs, as assessed by change in ear thickness 1 hr after antigen challenge. By contrast, mice with SNAP-23EE-transduced $IKK2^{-/-}$ FLMCs exhibited increased cutaneous anaphylaxis than mice with mock transduced $IKK2^{-/-}$ FLMCs (Figure 6G: mock [2.67 \pm 1.03 $\times 10^{-2}$ mm] versus SNAP-23EE [5.67 \pm 1.51 $\times 10^{-2}$ mm]; n = 6; p < 0.01). These results suggest that lack of phosphorylation of SNAP-23 on Ser120 and Ser95 is responsible for the impairment of Fc ϵ RI-induced degranulation in $IKK2^{-/-}$ mast cells.

Impairment of Late-Phase Allergic Reactions in the Absence of IKK2

We next examined the late-phase PCA reactions that are promoted by mast cell-derived proinflammatory cytokines. The hapten dinitrofluorobenzene (DNFB) challenge of mice that had been sensitized previously with monoclonal DNP-specific IgE antibody demonstrated that $IKK2^{-/-}$ FLMC-reconstituted mice exhibited reduced cutaneous anaphylaxis in the WT FLMC-reconstituted mice, as assessed by challenge in ear thickness (Figure 7A). Mast cell-secreted TNF- α plays an important role in late-phase allergic reactions. As such, we next examined Fc ϵ RI- or A23187-induced TNF- α secretion from $IKK2^{-/-}$ FLMCs. Consistent with the defect of late-phase allergic reactions in vivo, $IKK2^{-/-}$ FLMCs showed clear defects in Fc ϵ RI-induced (Figure 7B: WT [126.5 \pm 9.0 pg/ml] versus $IKK2^{-/-}$ 24.9 \pm 8.8 pg/ml]; n = 5; p < 0.01), and A23187-induced TNF- α secretion (Figure 7C: WT [273.8 \pm 8.7 pg/ml] versus $IKK2^{-/-}$ [50.8 \pm 7.5 pg/ml]; n = 5; p < 0.01). It has been reported that NF- κ B activation is critical for Fc ϵ RI-induced TNF- α secretion (Klemm et al., 2006). Therefore, we next examined the effect of overexpression of IKK2 or I κ B α M in the mast cells on Fc ϵ RI-stimulated TNF- α secretion. RBL-2H3 stably expressing GFP-IKK2 resulted in the increase of Fc ϵ RI-stimulated TNF- α release (mock [50.8 \pm 7.6 pg/ml] versus GFP-IKK2 [70.9 \pm 5.0 pg/ml]; n = 5; p < 0.01) (Figure 7D). On the other hand, RBL-2H3 stably expressing GFP-I κ B α M resulted in the diminished secretion of TNF- α upon Fc ϵ RI stimulation (mock [50.8 \pm 7.6 pg/ml] versus GFP-I κ B α M [16.4 \pm 3.7 pg/ml]; n = 5; p < 0.01) (Figure 7D). These results indicate that IKK2-NF- κ B signaling pathway plays critical roles in Fc ϵ RI-induced TNF- α secretion from mast cells, resulting in late-phase allergic reactions. It is well known that the exocytotic process is also important to TNF- α secretion. Therefore, our results suggest that IKK2 can influence both SNAP23- (exocytosis) and NF- κ B-dependent (transcription of TNF- α mRNA) pathways to regulate TNF- α release, respectively, during mast cell activation induced by Fc ϵ RI aggregation.

DISCUSSION

We have shown that IKK2 promotes IgE-induced degranulation from mast cells by phosphorylating SNAP-23. First, we found that IKK2 plays a critical role in the induction of anaphylaxis in vivo (Figure 1). Second, we observed that IKK2 also plays a central regulatory

role in IgE-induced degranulation by an NF- κ B-independent mechanism (Figures 2 and 3). Third, we found that IKK2 is recruited into lipid raft fractions upon Fc ϵ RI stimulation (Figure 4A) and phosphorylates SNAP-23 at Ser120 and Ser95 (Figures 5A and 5C). Finally, depletion of SNAP-23 expression by RNAi (shRNA) prevented IKK2-mediated upregulation of IgE-induced degranulation, and expression of the phosphorylation mutant of SNAP-23 (SNAP-23SSAA) also prevented IKK2-mediated upregulation of IgE-induced degranulation (Figure 6). These results indicate that IKK2-induced SNAP-23 phosphorylation is a key event in SNARE complex formation, leading to IgE-induced degranulation in mast cells.

Phosphorylation of I κ B proteins by IKK complex, containing the catalytic kinases IKK1 and IKK2, and the regulatory, nonenzymatic scaffold proteins NEMO and ELKS, is an essential process to activate NF- κ B proteins (NF- κ B1, NF- κ B2, RelA, c-Rel, and RelB). IKK2 is the major cytokine-responsive I κ B kinase, while IKK1 is involved in noncanonical pathway (Hayden and Ghosh, 2004), and may also function as a repressor of NF- κ B signaling in some cells (Lawrence et al., 2005; Li et al., 2005; Correa et al., 2005). We show that Fc ϵ RI-stimulated β -hexosaminidase release is clearly impaired in IKK2^{-/-} FLMCs, but not in IKK1-deficient FLMCs. Consistent with our results, Peng et al. (2005) have demonstrated that inhibition of IKK2 by sulindac, which is a selective inhibitor of NEMO, resulted in the decrease of IgE-stimulated histamine release from mast cells. We were surprised to find that I κ B α M, which is a superrepressor of NF- κ B activation, does not have any significant effect on IgE-induced degranulation (Figure 3B), suggesting that IKK2 modulates IgE-induced degranulation by an NF- κ B-independent mechanism. This is perhaps one of the very few cases where IKK2 functions in an NF- κ B-independent manner, and opens the possibility that IKK2 may have additional substrates.

We have shown that NF- κ B activation is not essential for mast cell degranulation (Figure 3B). Klemm et al. (2006) have also shown the defect of NF- κ B activation and normal degranulation in Bcl10^{-/-} or Malt1^{-/-} mast cells upon Fc ϵ RI stimulation. Their results are consistent with ours, because only IKK complex, but not NF- κ B, is activated in Bcl10^{-/-} T cells upon antigen receptor activation, suggesting that IKK2 can induce degranulation in the absence of NF- κ B activation (Shambharkar et al., 2007). The mechanism of antigen receptor-induced IKK complex activation is, however, still not fully understood, and requires further investigation.

Like TCR (Thome and Tschopp, 2003; Thome, 2004) or BCR (Thome, 2004), IKK2 is recruited into lipid rafts upon Fc ϵ RI stimulation (Figure 4A). However, antigen receptor-induced activation of IKK2 leads to NF- κ B activity, resulting in the induction of the genes that regulate proliferation, cytokine production, and isotype switching (for B cells) (Li and Verma, 2002). In contrast, IKK2 associates with SNAP-23, highly enriched in lipid raft fractions only after Fc ϵ RI stimulation (Figure 4A). More importantly, this association with SNAP-23 in lipid rafts leads to the phosphorylation of SNAP 23 at Ser95 and Ser120, essential for SNARE complex formation and degranulation (Figures 5B and 5C; Hepp et al., 2005). There is increasing evidence that IKK2 regulates substrates other than I κ Bs, NF- κ Bs, or precursors, and mediate additional functions beyond NF- κ B activation (Hu et al., 2004; Lee et al., 2004; Wegener et al., 2006). Most of the NF- κ B-related molecules (I κ B α , I κ B β , I κ B γ , NF- κ B p105) have a putative IKK consensus sequences for phosphorylation (DS κ XXS/T), and phosphorylation of these serine residues by IKK2 is required for subsequent ubiquitination and degradation (Karin and Ben-Neriah, 2000). Like Bcl10 (Wegener et al., 2006) or 14-3-3 β (Gringhuis et al., 2005), SNAP-23 also contains no DS κ XXS/T motif, which is the target for phosphorylation of IKK2 substrates. Phosphorylation of SNAP-23 promotes association with other SNARE proteins (Puri and Roche, 2006) to form the SNARE complex, rather than its degradation, more commonly observed with other IKK2 substrates. In light of these observations, we find it not surprising that phosphorylation sites of SNAP-23 by IKK2 are not identical for a putative IKK consensus sequences for phosphorylation (DS κ XXS/T). We

propose that SNAP-23 is highly enriched in lipid raft fractions before/after stimulation (Figure 5D). In contrast, only small amounts of syntaxin 4 or VAMP-2 are present in lipid raft fractions (Puri and Roche, 2006) (Figure 4A and Figure 5D, lanes 1 and 3). Upon stimulation, IKK2 is recruited into the lipid raft fractions (Figure 4A), phosphorylates SNAP-23 at Ser 95 and Ser120, and now, essentially, most of syntaxin 4 and VAMP-2, the other two proteins in the SNARE complex, are in these raft fractions (Figure 5D, lane 2). In IKK2^{-/-} cells either Syntaxin 4 or VAMP-2 can be found in the rafts (Figure 5D, lane 4). SNAP-23 is anchored in the membrane by palmitoylation, and, following phosphorylation, recruits syntaxin 4 and VAMP-2 to form the SNARE complex. Thus phosphorylation of SNAP-23 by IKK2 is the key event in the formation and promotion of vesicle fusion with the plasma membrane, resulting in the degranulation in mast cells. Other groups have already shown that recombinant syntaxin 4/SNAP-23 and VAMP-2 are capable of driving fusion in vitro (Vicogne et al., 2006; Shen et al., 2007). However, these data do not rule out that SNAP-23 does not require phosphorylation for SNARE complex formation in vivo, because they demonstrated in vitro membrane fusion assay in which recombinant SNAREs were reconstituted on the artificial liposomes. We believe that this assay is suitable to show SNAREpin formation between some SNAREs, but it is inadequate to examine the molecular mechanism of translocation of SNAREs into lipid rafts, because, in many cases, artificial liposome is made without any cholesterol. Our result (Figure 5D) suggests that phosphorylation of SNAP-23 by IKK2 is an essential event in the colocalization of syntaxin 4 and VAMP-2 with SNAP-23 in lipid raft fractions during the exocytotic process. Our data highlight the importance of IKK2 in anaphylactic reactions, but does not rule out other mechanisms.

We have shown the essential role of IKK2 in mast cell degranulation (Figure 2) and early-phase allergic reactions (anaphylactic reactions) (Figure 3). We have also shown that this regulation by IKK2 is dependent on SNAP-23 (Figure 6), but not NF- κ B (Figure 3). On the other hand, we have shown the important role of IKK2 in TNF- α release from mast cells and late-phase allergic reactions (Figure 7). Additionally, we have shown that regulation of TNF- α secretion by IKK2 is partially dependent on NF- κ B activation, consistent with a previous report (Klemm et al., 2006). Taken together, IKK2 in mast cells regulates early-phase allergic reactions in a SNAP-23-dependent manner, and, in late-phase allergic reactions, requires both SNAP-23 and NF- κ B. Therefore, IKK2 is a central modulator in allergic reactions.

EXPERIMENTAL PROCEDURES

Mice

IKK1- (Li et al., 1999b) and IKK2 (Li et al., 1999a) -heterozygous mice were backcrossed for over eight generations onto C57BL/6 mice. Genetically mast cell-deficient mice, WBB6F1-W/W^v mice (W/W^v mice) were purchased from The Jackson Laboratory. Mice were bred and housed in the animal care facility at the Salk Institute. Experimental procedures involving mice followed the guidelines from the National Institutes of Health, and were approved by the Animal Use Committee at the Salk Institute.

Cell Culture

WT, IKK1^{-/-}, IKK2^{-/-}, and IKK1^{-/-}IKK2^{-/-} fetal liver cells from Embryonic Day 11.5–12.5 mice were obtained by removing cells from the liver. Cells were cultured in RPMI 1640 medium containing 10% heat-inactivated FCS, 50 mM β -mercaptoethanol, 2 mM L-glutamine, antibiotics, and 100 ng/ml murine-recombinant IL-3 (R&D Systems). More than 98% of cells obtained after 4 weeks of culture were morphologically mast cells (FLMCs) and positive for c-kit expression. RBL-2H3 cells (CRL-2256 [ATCC]) were grown as monolayer cultures in Eagle's minimum essential medium supplemented with 15% heat-inactivated fetal bovine

serum. 293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FCS and antibiotics.

Reconstitution of W/W^v Mice with FLMCs

For reconstitution of skin mast cells of W/W^v mice, 1×10^6 WT or IKK2^{-/-} FLMCs in 20 μ l of PBS were injected i.d. into the right ear of W/W^v mice; for control, left ears were injected with PBS. For systemic reconstitution of mast cells of W/W^v mice, 1×10^7 WT or IKK2^{-/-} FLMCs, in 100 μ l of PBS, were injected i.v. into W/W^v mice; controls were injected with PBS.

PCA and PSA

To elicit PCA, 4 weeks after FLMC transplantation, mice were first primed with IgE by intradermal injection in the ears, with 100 ng monoclonal mouse dinitrophenol-specific (anti-DNP) IgE (Sigma) in 20 μ l of PBS. After 24 hr, mice were injected i.v. with 200 μ g of DNP-HSA (human serum albumin; Sigma) diluted in sterile 0.9% NaCl. Ear swelling was quantified by three consecutive measurements of ear thickness with a caliper before (baseline) and 1, 2, 3, and 4 hr after antigen challenge. In some experiments, 0.5% Evans blue dye was injected with DNP-HSA. To elicit PSA, 4 weeks later, FLMCs transplantation, mice were first primed with IgE by i.v. injection, with 20 μ g anti-DNP IgE in 100 μ l of PBS. After 24 hr, mice were injected i.v. with 1 μ g of DNP-HSA diluted in sterile 0.9% NaCl. The systemic anaphylactic response was monitored by measuring rectal temperature several times after antigen challenge.

Late-Phase Allergic Reactions

To elicit late-phase PCA reactions, 4 weeks after FLMC transplantation, mice were first primed with IgE by i.v. injection with 20 μ g anti-DNP IgE in 100 μ l of PBS. After 24 hr, mice were challenged by epicutaneous application of 10 μ l of DNFB (0.2% w/v) in acetone:olive oil (4:1) to both sides of both ears. The ear swelling response was assessed by measuring the ear thickness with caliper before (baseline) and 2, 4, 6, 8, 12, 24, and 48 hr after hapten challenge. The increment of ear thickness (postchallenge value - prechallenge baseline value) was expressed as the percent of the baseline value.

Data Analysis

Data are summarized as mean \pm SD. The statistical analysis of the results was performed by the unpaired t test; p values < 0.05 were considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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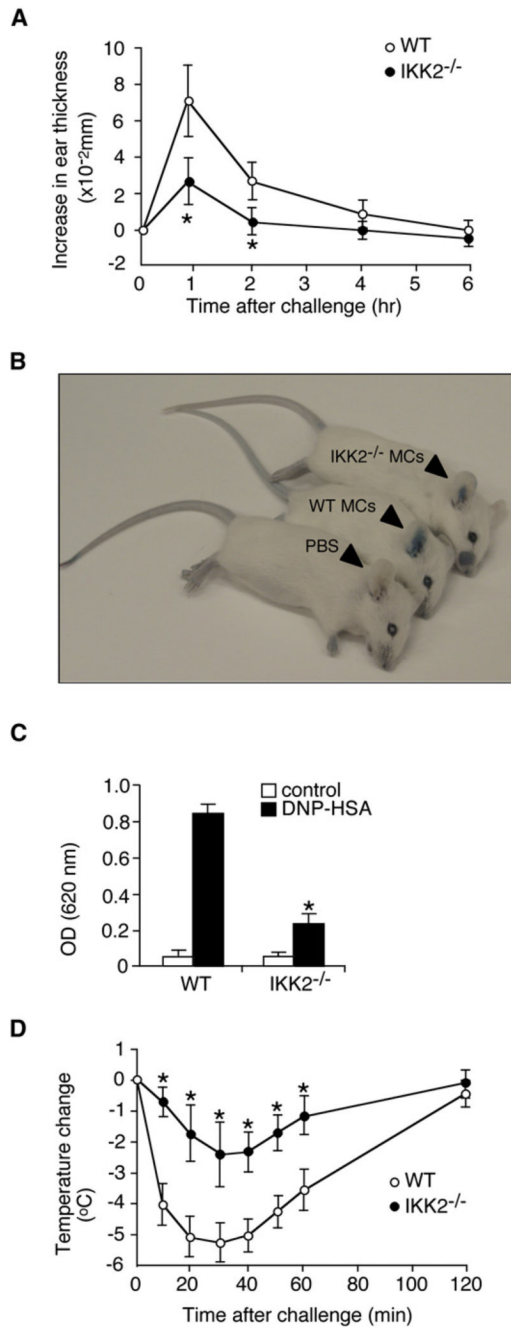


Figure 1. Severe Impairment of Anaphylactic Responses in the Absence of IKK2

(A) PCA in WT or IKK2^{-/-} mast cell-reconstituted W/W^v mice was performed as described in the Experimental Procedures. Ear swelling was quantified by three consecutive measurements of ear thickness with a caliper before (baseline) and 1, 2, 3, and 4 hr after antigen challenge. Data are means \pm SD from eight independent experiments. Results show a severe impairment of anaphylactic shock; * $p < 0.01$.

(B) Upon antigen challenge, leakage of injected Evans blue dye into the ear is severely decreased in IKK2^{-/-} FLMC transplanted mice. Representative data from eight independent experiments are shown.

(C) Vascular leakage assayed by EB extravasation into ear tissues. Inner ear rims were removed 90 min after i.v. challenge with antigen with EB, or EB only, and subsequently extracted in formamide. Plots show mean optical density at 620 nm \pm SD; * $p < 0.01$.

(D) PSA in WT or IKK2^{-/-} mast cell-reconstituted W/W^v mice was performed as described in the Experimental Procedures and monitored by measuring rectal temperature several times following antigen challenge. Data are mean \pm SD from eight independent experiments. Data shown in Figure 1D demonstrate decrease in systemic anaphylaxis in mice injected with IKK2^{-/-} mast cells; * $p < 0.01$.

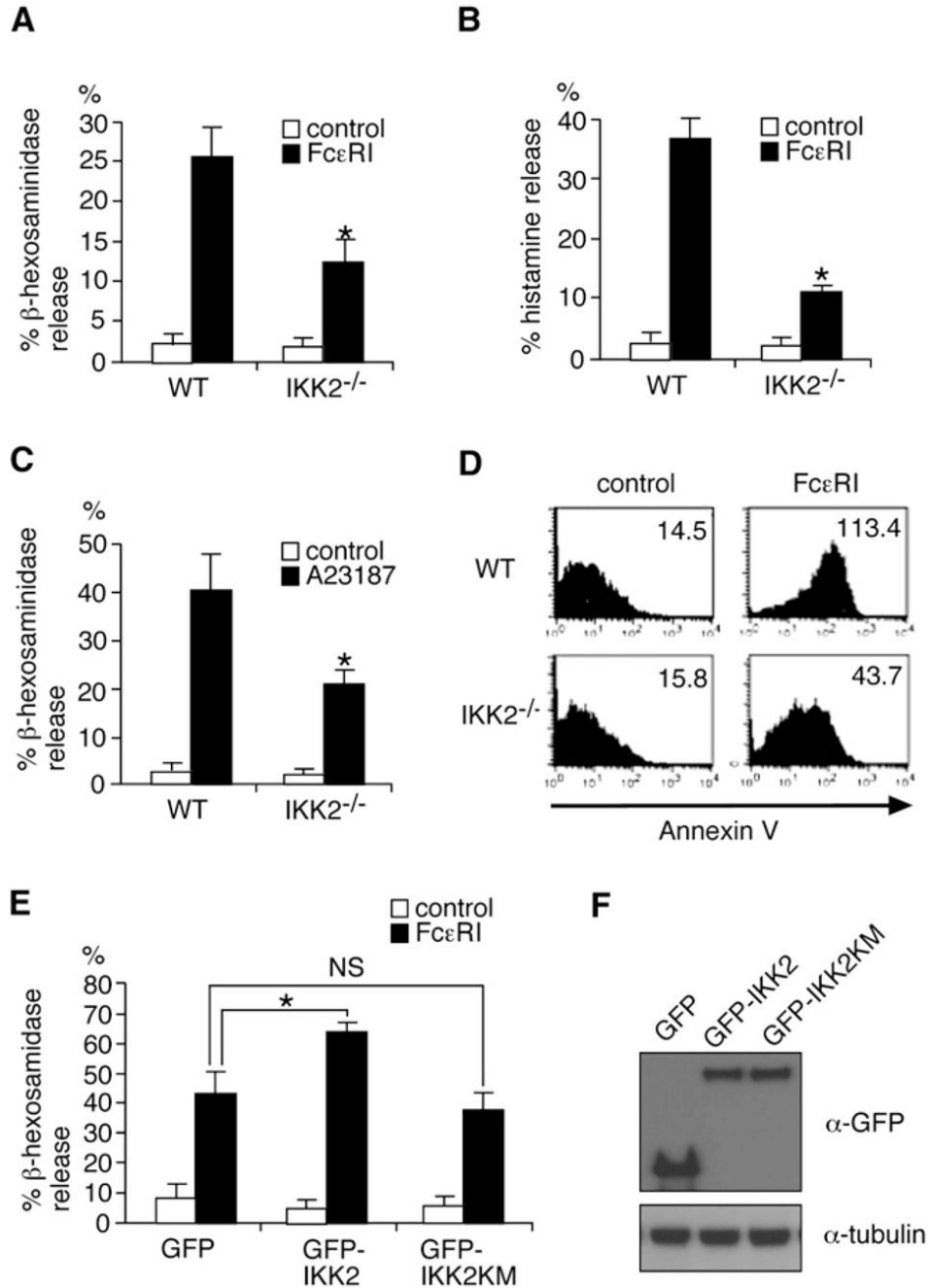


Figure 2. IKK2 Plays Critical Roles in Mast Cell Degranulation

(A) IgE-induced β -hexosaminidase release is impaired in IKK2^{-/-} FLMCs. FLMCs were incubated with anti-TNP IgE for 2 hr and then surface IgE was cross-linked with (black columns) or without (white columns) TNP-BSA. After 30 min, the percentage of specific β -hexosaminidase release was determined as described in the Experimental Procedures. Data are means \pm SD from five independent experiments. *Significantly different from the mean value of control group (WT), * $p < 0.01$.

(B) IgE-induced histamine release is impaired in IKK2^{-/-} FLMCs. FLMCs were incubated with anti-TNP IgE and cross-linked with (black columns) or without (white columns) TNP-BSA. After 30 min, supernatants and cell lysates were recovered. Histamine was measured by

EIA. Data are means \pm SD from five independent experiments. *Significantly different from the mean value of control group (WT), * $p < 0.01$.

(C) A23187-induced β -hexosaminidase release is impaired in IKK2^{-/-} FLMCs. FLMCs were stimulated with (black columns) or without (white columns) A23187 (500 ng/ml). After 30 min, the percentage of specific β -hexosaminidase release was determined as described in the Experimental Procedures. Data are means \pm SD from five independent experiments.

*Significantly different from the mean value of control group (WT), * $p < 0.01$.

(D) IgE-induced annexin-V binding is decreased in IKK2^{-/-} FLMCs. FLMCs were incubated with anti-TNP IgE and cross-linked with or without TNP-BSA. After 30 min, cells were stained with annexin-V and analyzed by flow cytometry. Representative FACS profiles from five independent experiments are shown. (E) Overexpression of IKK2 results in increase of Fc ϵ RI-stimulated β -hexosaminidase release. GFP-IKK2, GFP-IKK2KM, or GFP (as control) stably expressing RBL-2H3 cells were obtained as described in the Experimental Procedures. IgE-induced β -hexosaminidase release was performed as described in Figure 2A. Data are means \pm SD from five independent experiments. *Significantly different from the mean value of control group (mock), * $p < 0.01$. NS, not significant.

(F) Western blots to assess expression of GFP, GFP-IKK2, or GFP-IKK2KM in zeocin-resistant RBL-2H3 cells (Figure 2G). Antitubulin blots were used as loading controls.

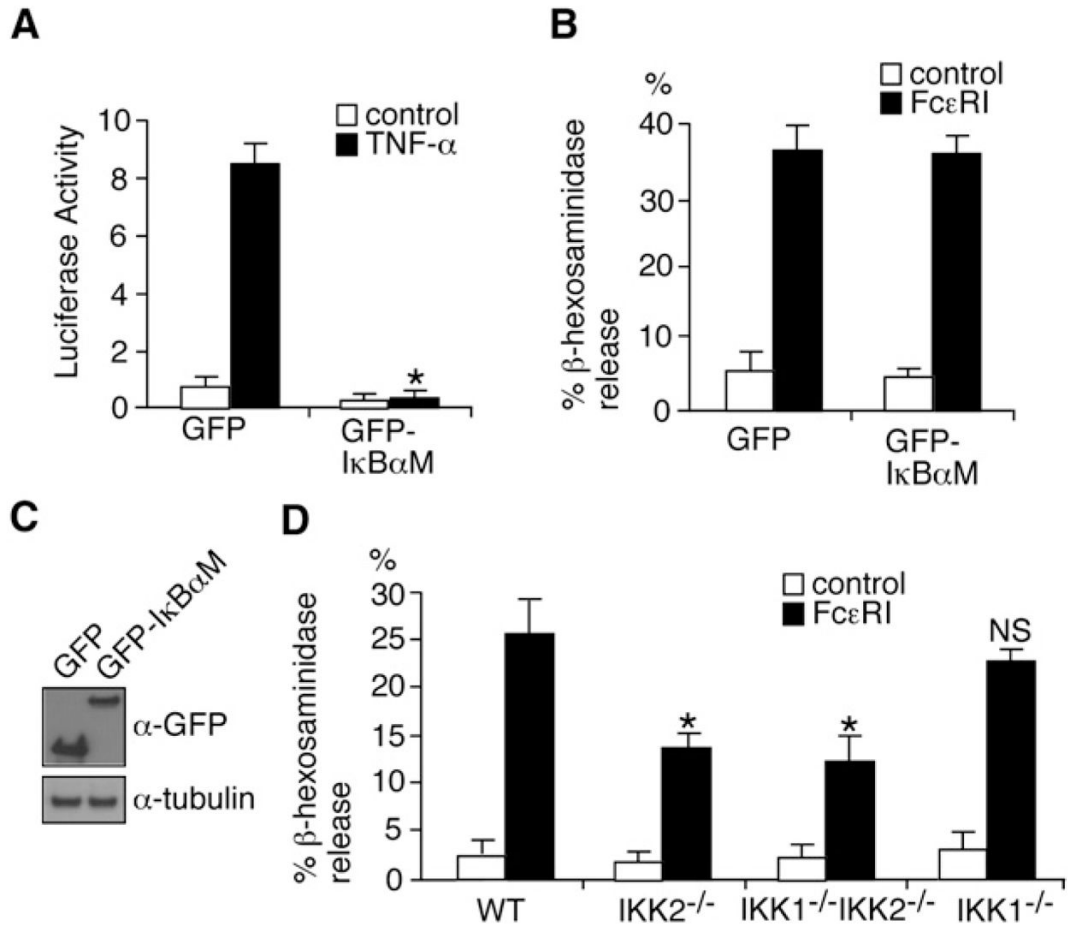


Figure 3. NF- κ B Activation Is Not Essential for Mast Cell Degranulation

(A) I κ B α M stably expressing RBL-2H3 cells were obtained as described in the Experimental Procedures. Cells were transfected with HIV-3 \times - κ B luciferase and β -galactosidase plasmid by electroporation. After 24 hr, cells were stimulated with (black columns) or without (white columns) TNF- α (10 ng/ml); 6 hr later, luciferase activity was measured. Data are means \pm SD from five independent experiments. *Significantly different from the mean value of control group (mock), * $p < 0.01$.

(B) Zeocin-resistant cells were incubated with anti-TNP IgE for 2 hr and then surface IgE was cross-linked with (black columns) or without (white columns) TNP-BSA. After 30 min, the percentage of specific β -hexosaminidase release was determined as described in the Experimental Procedures. Data are means \pm SD from five independent experiments.

(C) Western blots to assess expression of GFP, or GFP-I κ B α M in zeocin-resistant RBL-2H3 cells (Figures 3A and 3B).

(D) IgE-induced β -hexosaminidase release from IKK2^{-/-}, IKK1^{-/-}IKK2^{-/-}, or IKK1^{-/-} FLMCs. IgE-induced β -hexosaminidase release was performed as described in Figure 2A. Data are means \pm SD from five independent experiments; * $p < 0.01$. NS, not significant compared to WT.

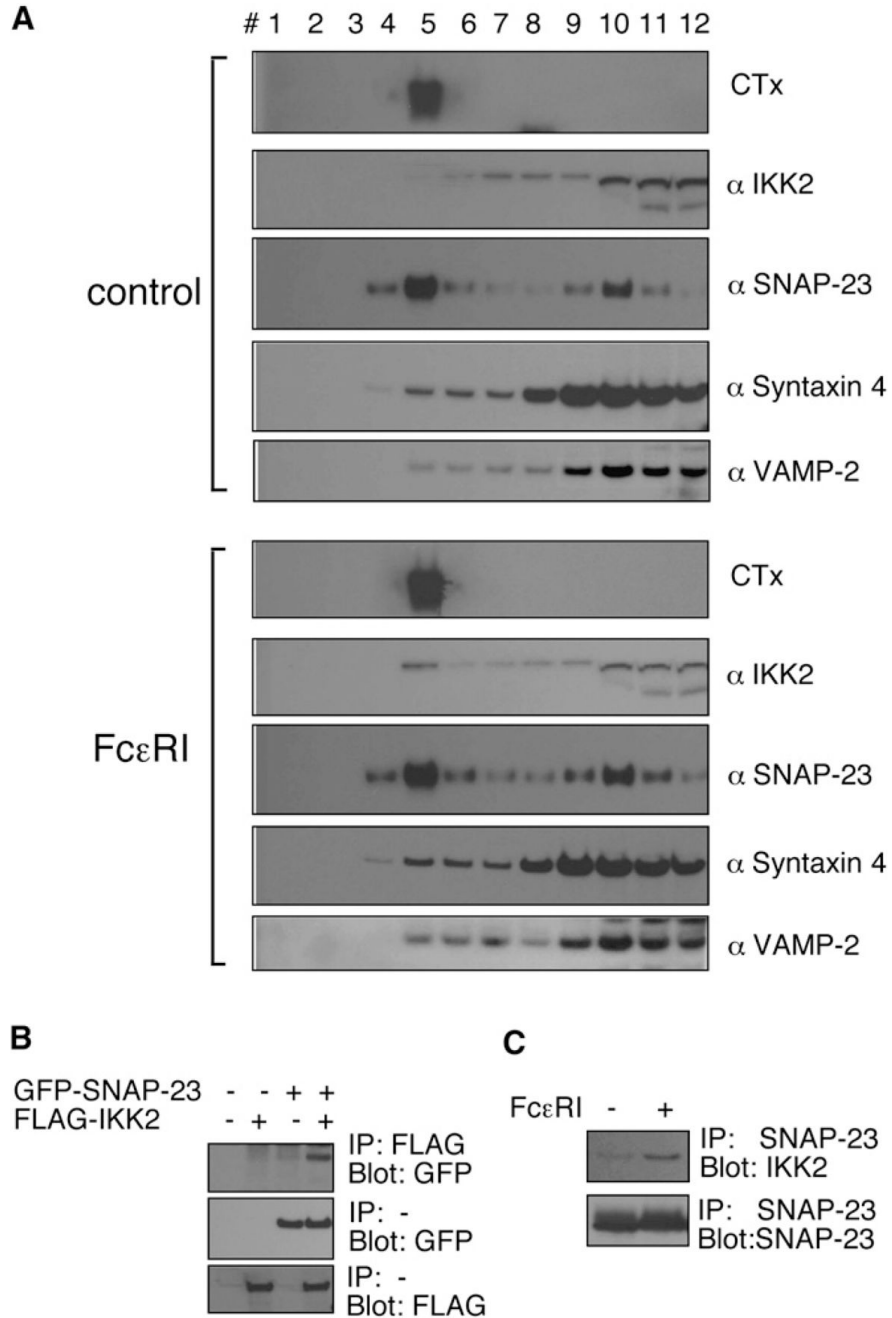


Figure 4. IKK2 Is Recruited to the Lipid Raft Fractions upon FcεRI Stimulation and Associates with SNAP-23
 (A) Localization of GM1 (CTx), IKK2, SNAP-23, syntaxin 4, and VAMP-2 in lipid raft fraction upon FcεRI stimulation. Cell lysates of unstimulated (only IgE) or stimulated (IgE + antigen) RBL-2H3 cells were fractionated by sucrose density gradient ultracentrifugation and blotted with each antibody. Representative data from five independent experiments are shown. (B) IKK2 associates with GFP-SNAP-23 when coexpressed in 293T cells by immunoprecipitation (IP) assay. Representative data from five independent experiments are shown.

(C) IKK2 associates with SNAP-23 in the lipid raft fractions only after FcεRI stimulation. Fraction 5 was solubilized in 1% octylglucoside and subjected to IP with anti-SNAP-23 antibody. Immunoprecipitants were blotted with each antibody.

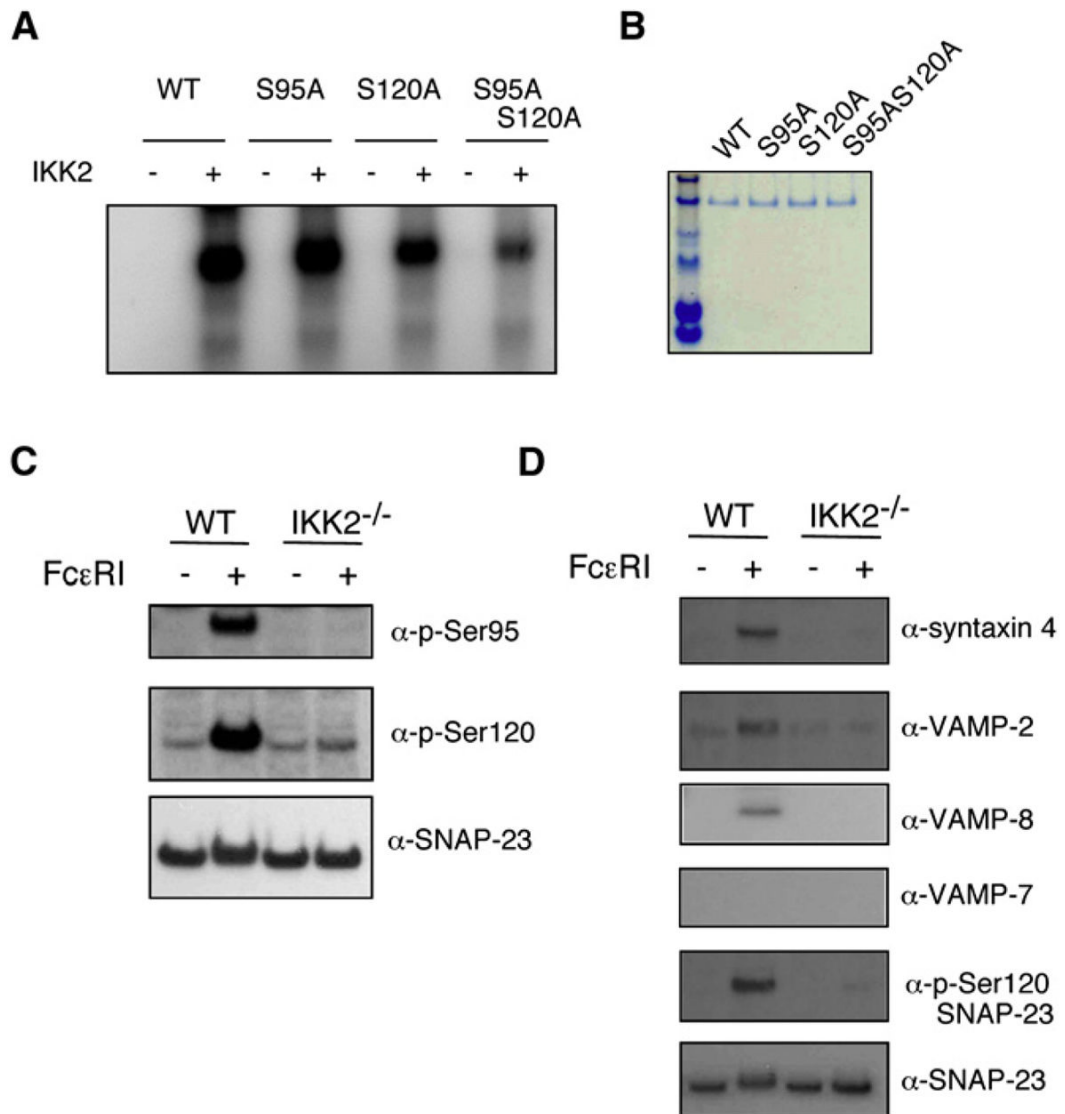
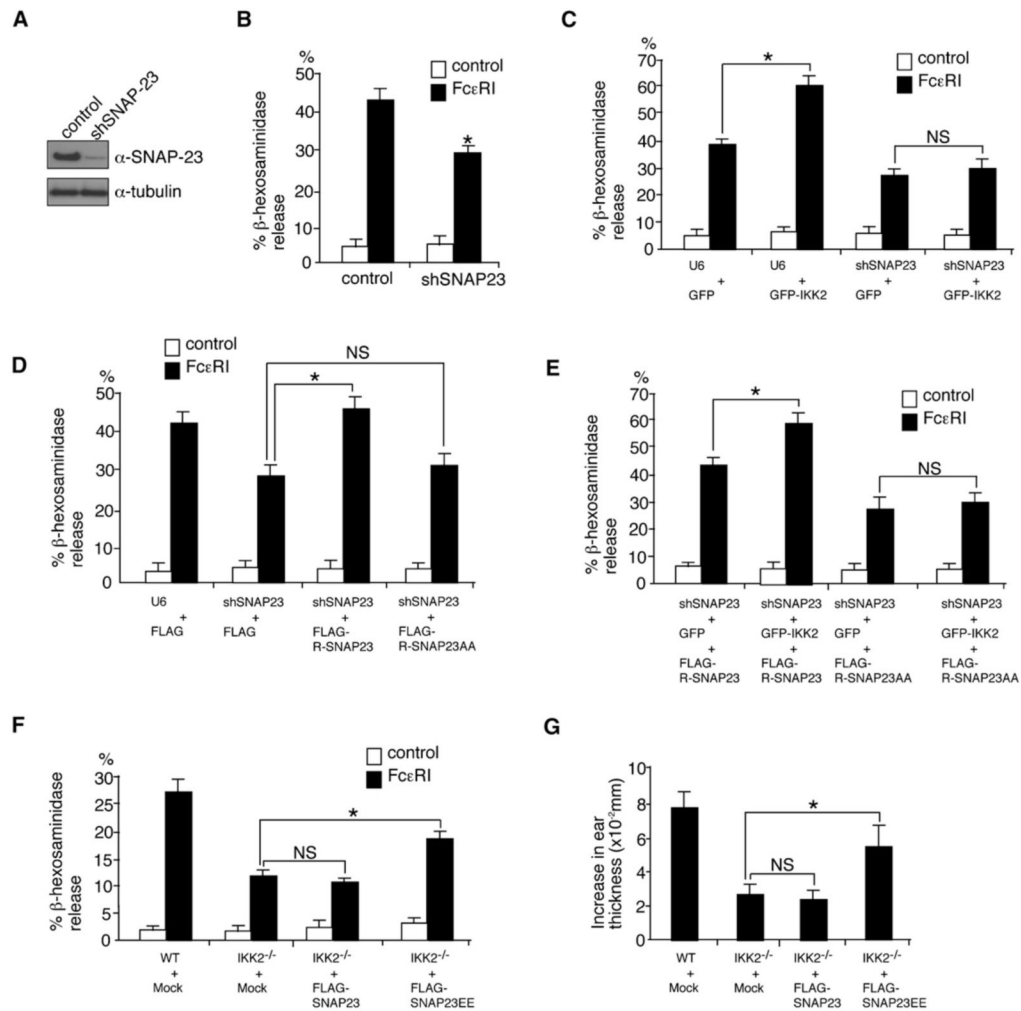


Figure 5. IKK2 Phosphorylates SNAP-23 on Ser120 and Ser95

(A) Equal amounts of purified WT and mutant GST-SNAP-23 proteins were incubated with [γ - 32 P]ATP and IKK2 as described in the Experimental Procedures and the reaction was analyzed by SDS-PAGE and PhosphorImager analysis. Representative data from five independent experiments are shown.

(B) 0.5 μ g of purified GST-fused WT or several mutants of SNAP-23 proteins were submitted to SDS-PAGE and detected with CBB.

(C) IgE-induced phosphorylation of SNAP-23 on Ser120 and Ser95 is impaired in IKK2^{-/-} FLMCs. FLMCs were incubated with anti-TNP IgE and cross-linked with or without TNP-BSA. Fifteen minutes later, cell lysates were recovered and analyzed by immunoblot analysis using phosphorylation site specific antibodies. Representative data from five independent experiments are shown. (D) Fc ϵ RI-induced formation of SNARE complex of SNAP-23/syntaxin 4/VAMP-2 is impaired in IKK2^{-/-} mast cells. Cell lysates of unstimulated (only IgE) or stimulated (IgE + antigen) FLMCs were fractionated by sucrose density gradient ultracentrifugation. Lipid raft fraction 5 was solubilized in 1% octylglucoside and subjected to IP with anti-SNAP-23 antibody. Immunoprecipitants were blotted with each antibody.



(E) Overexpression of IKK2 enhances IgE-induced β -hexosaminidase release in SNAP-23 shRNA and SNAP-23 shRNA-resistant WT SNAP-23, but not SNAP-23 shRNA and SNAP-23 shRNA-resistant SNAP-23AA-expressing mast cells. SNAP-23 shRNA, GFP-IKK2, and FLAG-R-SNAP23 or FLAG-R-SNAP23AA stably expressing RBL-2H3 cells were obtained as described in the Experimental Procedures. GFP indicates control. IgE-induced β -hexosaminidase release was performed as described in Figure 2A. Data are means \pm SD from five independent experiments.

(F) Expression of phosphomimetic mutant of SNAP-23 partially rescue the impairment of Fc ϵ RI-induced β -hexosaminidase release in IKK2^{-/-} mast cells. FLMCs were infected with retroviruses of pMX-puro-FLAG-WT SANP-23, pMX-puro-FLAG-SNAP-23EE, or pMX-puro (mock). After selection with puromycin, IgE-induced β -hexosaminidase release was performed as described in Figure 2A. Data are means \pm SD from five independent experiments; *p < 0.01; NS, not significant compared to mock-infected IKK2^{-/-} FLMCs.

(G) Mock vector transduced WT FLMCs, and mock vector, WT SNAP-23, or SNAP-23EE transduced IKK2^{-/-} FLMCs were injected i.d. in the right ear of W/W^v mice. After 4 weeks, PCA was performed as described in the Experimental Procedures. Ear swelling was quantified by three consecutive measurements of ear thickness with a caliper before (baseline) and 1 hr after challenge. Data are means \pm SD from six independent experiments; *p < 0.01; NS, not significant compared to mock-infected IKK2^{-/-} FLMC-reconstituted mice.

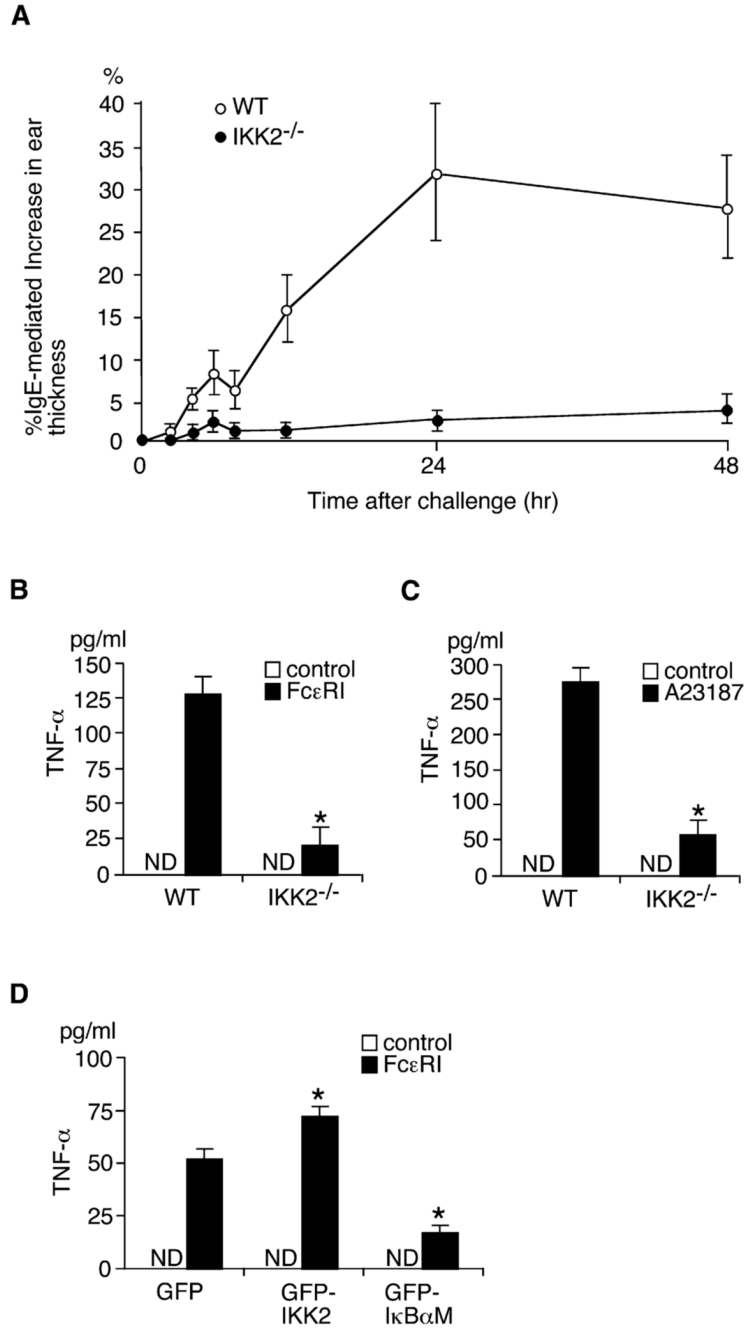


Figure 7. Impairment of Late-Phase Allergic Reactions in the Absence of IKK2

(A) Late-phase PCA reactions in WT or IKK2^{-/-} mast cell-reconstituted W/W^v mice were performed as described in the Experimental Procedures. Ear swelling was quantified by three consecutive measurements of ear thickness with calipers before (baseline) and indicated time after hapten challenge. Data are means ± SD from six independent experiments. Data demonstrate decrease in late-phase PCA reactions in mice injected with IKK2^{-/-} mast cells. (B) IgE-induced TNF-α release is impaired in IKK2^{-/-} FLMCs. FLMCs were incubated with anti-TNP IgE and cross-linked with (black columns) or without (white columns) TNP-BSA; 6 hr later, TNF-α in the supernatants was quantified by ELISA. Data are means ± SD from five

independent experiments. *Significantly different from the mean value of control group (WT), * $p < 0.01$.

(C) A23187-induced TNF- α release is impaired in IKK2^{-/-} FLMCs. FLMCs were stimulated with (black columns) or without (white columns) A23187 (500 ng/ml); 6 hr later, TNF- α in the supernatants was quantified by ELISA. Data are means \pm SD from five independent experiments. *Significantly different from the mean value of control group (WT), * $p < 0.01$.

(D) GFP-IKK2, GFP-I κ B α M, or GFP stably expressing RBL-2H3 cells were obtained as described in the Experimental Procedures. Cells were incubated with anti-TNP IgE, and cross-linked with (black columns) or without (white columns) TNP-BSA; 6 hr later, TNF- α in the supernatants was quantified by ELISA. Data are means \pm SD from five independent experiments. *Significantly different from the mean value of control group (WT), * $p < 0.01$.