Phosphorylation of SNAP-23 by IkB 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 (FcεRI) leads to degranulation and allergic inflammation; however, the regulatory mechanisms of IgE-dependent exocytosis remain unknown. We show here that IkB kinase (IKK) 2 in mast cells plays critical roles in IgE-mediated anaphylaxis in vivo, and IgE-mediated degranulation in vitro, in an NF-kB-independent manner. Upon FcεRI stimulation, IKK2 phosphorylates SNAP-23, the target membrane soluble N-ethylmaleimide-sensitive fusion factor attachment protein receptor (SNARE), and ectopic expression of a phospho-mimetic 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-kB-independent, we additionally show that IKK2 also regulates late-phase allergic reactions promoted by the release of proinflammatory cytokines in an NF-kB-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 (FcεRI). 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 FcεRI by IgE, followed by the aggregation of multiple IgE-bearing FcεRI molecules by polyvalent 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 FcεRI 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-κB-dependent manner (Klemm et al., 2006). The precise role of IkB 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 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-κB 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-κB-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 IkB kinase and, thus, is a central component of the intracellular signaling pathway mediating NF-κB 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-κB-independent manner. IKK2 is recruited into the lipid raft fractions and...
phosphorylates SNAP-23 at Ser120 and Ser95 upon FcεRI 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-κB 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 ± 0.34 × 10^5] versus IKK2−/− [4.24 ± 0.44 × 10^5]; 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 FcεRIα 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 ± 5.4] versus IKK2−/− [41.3 ± 4.4]; n = 40) and the mean of granule intensity (Figure S1D: WT [93.4 ± 22.4] versus IKK2−/− [92.1 ± 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 Ca^2+ 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-Kitw−/Kitw−v (W/Wv) 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 (Figure 1B). 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.

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 ± SD; *p < 0.01.

PSA in WT or IKK2−/− mast cell-reconstituted W/Wv mice was performed as described in the Experimental Procedures and monitored by measuring rectal temperature several times following antigen challenge. Data are mean ± 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.
Overexpression of IKK2 results in increase of FcεRI-stimulated β-hexosaminidase release. Representative FACS profiles from five independent experiments are shown. FLMCs were incubated with anti-TNP IgE and cross-linked with or without TNP-BSA. After 30 min, supernatants and cell lysates were collected. β-Hexosaminidase release was determined as described in the Experimental Procedures. Data are means ± SD from five independent experiments. *Significantly different from the mean value of control group (WT), p < 0.01.

IKK2 Plays Critical Roles in Mast Cell Degranulation

To investigate the cellular basis for the diminished in vivo anaphylactic reactions in IKK2−/− FMCs, 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 2A). These results suggest that IKK2 modulates mast cell depolarization and histamine release.

IKK2 is required for IKK2-mediated upregulation of FcεRI-stimulated β-hexosaminidase release (Figure 2A). In order to determine whether kinase activity of IKK2 is required for IKK2-mediated upregulation of FcεRI-stimulated β-hexosaminidase release, we examined FcεRI-stimulated β-hexosaminidase release in 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 2A). 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 FcεRI 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 2F). These results suggest that IKK2 modulates mast cell degranulation.

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 ± 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 ± 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 ± 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 FcεRI-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 ± SD from five independent experiments. *Significantly different from the mean value of control group (mock), p < 0.01. NS, not significant.
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 IκBαs, 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 (IκBα-M) (Karin and Ben-Neriah, 2000) (Figure 3C) and HIV-3x-κB luciferase/β-galactosidase plasmids. Expression of IκBα-M in the RBL-2H3 cells resulted in the complete inhibition of NF-κB-dependent promoter activation in response to TNF-α (Figure 3A). In contrast, IκBα-M-expressing RBL-2H3 cells showed similar levels of FcεRI-stimulated β-hexosaminidase release to the control cells (mock [36.2% ± 4.1%] versus IκBα-M [34.7% ± 3.4%]; n = 5) (Figure 3B). Figure 3C shows that IκBα-M is expressed in these cells. It thus appears that FcεRI-induced degranulation is independent of NF-κB 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 FcεRI-induced degranulation. Figure 3D shows that release of β-hexosaminidase was impaired in both IKK2-/- and IKK1-/-/IKK2-/- FLMCs (WT [25.0% ± 5.4%] versus IKK1-/-/IKK2-/- [13.4% ± 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-κB-independent mechanism.

**IKK2 Is Recruited into the Lipid Raft Fractions upon FcεRI 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-ethylmaleimide-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 FcεRI 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 FcεRI 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 FcεRI 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 imunoprecipitation 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 FcεRI stimulation. IKK2 and SNAP-23 coimmunoprecipitated in the raft fractions following FcεRI stimulation (Figure 4C). These results suggest that IKK2 associates with SNAP-23 in the lipid rafts only after FcεRI 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 FcεRI-induced degranulation by an NF-κB-independent mechanism.
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 (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/syntaxin4/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.
Figure 6. Phosphorylation of SNAP-23 on Ser120 and Ser95 Involved in IKK2-Mediated Degranulation

(A) Western blots to assess expression of SNAP-23 in puromycin-resistant RBL-2H3 cells.

(B) IgE-induced β-hexosaminidase release is impaired in SNAP-23 shRNA-expressing mast cells. SNAP-23 shRNA stably expressing RBL-2H3 cells were obtained as described in the Experimental Procedures. IgE-induced β-hexosaminidase release was performed as described for Figure 2A. Data are means ± SD from five independent experiments; *p < 0.01; NS, not significant.

(C) Overexpression of IKK2 does not enhance IgE-induced β-hexosaminidase release in SNAP-23 shRNA-expressing mast cells. SNAP-23 shRNA and/or GFP-IKK2 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 ± SD from five independent experiments; *p < 0.01; NS, not significant.

(D) Transduction of SNAP-23 shRNA-resistant SNAP-23 rescues IgE-induced β-hexosaminidase release in SNAP-23 shRNA-expressing mast cells. SNAP-23 shRNA, and FLAG-R-SNAP23 or FLAG-R-SNAP23AA, 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 ± SD from five independent experiments; *p < 0.01; NS, not significant.

(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. IgE-induced β-hexosaminidase release was performed as described in Figure 2A. Data are means ± SD from five independent experiments; *p < 0.01; NS, not significant.

(F) Expression of phosphomimetic mutant of SNAP-23 partially rescue the impairment of FcεRI-induced β-hexosaminidase release in IKK2−/− mast cells. FLMCs were infected with retroviruses of pMX-puro-FLAG-WT SNAP-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 ± 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/Wv mice. After 4 weeks, PCA was performed as described in the Experimental Procedures. Ear swelling was quantified by three consecutive measurements of ear volume.
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 FcRI-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 silencing the expression of SNAP-23 (Figure S3). We selected several RNAi interference (shRNA) constructs capable of silencing the expression of SNAP-23 (Figure S3). We selected two shRNA constructs capable of silencing the expression of SNAP-23 (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 FcRI-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 degradation. 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 FcRI-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 FcRI-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-expressing cells (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 phosphorylation of SNAP-23 on IKK2-induced enhancement of FcRI-stimulated β-hexosaminidase. IKK2-induced enhancement of FcRI-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 + GPF + FLAG-R-SNAP23AA [27.2% ± 4.0%] versus shSNAP23 + GPF-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 FcRI-stimulated degranulation. To examine the effect of expression of phosphomimetic mutant of SNAP-23 in IKK2−/− mast cells on FcRI-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 FcRI-induced β-hexosaminidase release in IKK2−/− mast cells (Figure 6F; mock [12.4% ± 1.44%] versus WT SNAP-23 [10.3% ± 3.41%]; n = 8; p < 0.01). By contrast, expression of SNAP-23EE partially rescued the impairment of FcRI-induced degranulation in IKK2−/− mast cells (Figure 6F; mock [12.4% ± 1.44%] versus SNAP-23EE [18.9% ± 1.11%]; n = 8; p < 0.01). We also examined in vivo early-phase PCA reactions in mast cell-reconstituted W/Wv mice. Mice with WT SNAP-23 or mock-transduced IKK2−/− FLMCs exhibited similar levels of reduced cutaneous anaphylaxis (Figure 6G: mock [2.67 ± 1.03 × 10−2 mm] versus WT SNAP-23 [2.33 ± 0.82 × 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 ± 1.03 × 10−2 mm] versus SNAP-23EE [5.67 ± 1.51 × 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 FcRI-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 monocular 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 FcRI- 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 FcRI-induced (Figure 7B: WT [126.5 ± 9.0 pg/ml] versus IKK2−/− 24.9 ± 8.8 pg/ml]; n = 5; p < 0.01), and A23187-induced TNF-α secretion (Figure 7C: WT [273.8 ± 8.7 pg/ml] versus IKK2−/− [50.8 ± 7.5 pg/ml]; n = 5; p < 0.01). It has been reported that NFκB activation is critical for FcRI-induced TNF-α secretion (Klemm et al., 2006). Therefore, we next examined the effect of overexpression of IKK2 or IkBβM in the mast cells on FcRI-stimulated TNF-α secretion. RBL-2H3 stably expressing GFP-IKK2 resulted in the increase of FcRI-stimulated TNF-α release (mock [50.8 ± 7.6 pg/ml] versus GFP-IKK2 [70.9 ± 5.0 pg/ml]; n = 5; p < 0.01) (Figure 7D). On the other hand, RBL-2H3 stably expressing GFP-IkBβM resulted in the diminished secretion of TNF-α upon

thickness with a caliper before (baseline) and 1 hr after challenge. Data are means ± 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/Wv 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) A23187 for 6 hr later. TNF-α in the supernatants were 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 ± SD from five independent experiments. *Significantly different from the mean value of control group (WT), *p < 0.01. (D) GFP-IKK2, GFP-IkBαM, 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 ± SD from five independent experiments. *Significantly different from the mean value of control group (WT), *p < 0.01.

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-κB-independent mechanism (Figures 2 and 3). Third, we found that IKK2 is recruited into lipid raft fractions upon FcεRI 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 IκB 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-κB proteins (NF-κB1, NF-κB2, RelA, c-Rel, and RelB). IKK2 is the major cytokine-responsive IκB kinase, while IKK1 is involved in noncanonical pathway (Hayden and Ghosh, 2004), and may also function as a repressor of NF-κB signaling in some cells (Lawrence et al., 2005; Li et al., 2005; Correa et al., 2005). We show that FcεRI-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 IκBαM, which is a superrepressor of NF-κB activation, does not have any significant effect on IgE-induced degranulation (Figure 3B), suggesting that IKK2 modulates IgE-induced degranulation by an NF-κB-independent mechanism. This is perhaps one of the very few cases where IKK2 functions in an NF-κB-independent manner, and opens the possibility that IKK2 may have additional substrates.

We have shown that NF-κB activation is not essential for mast cell degranulation (Figure 3B). Klemm et al. (2006) have also
shown the defect of NF-κB activation and normal degranulation in Bcl10−/− or Malt1−/− mast cells upon FceRI stimulation. Their results are consistent with ours, because only IKK complex, but not NF-κB, is activated in Bcl10−/− T cells upon antigen receptor activation, suggesting that IKK2 can induce degranulation in the absence of NF-κB 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 FceRI stimulation (Figure 4A). However, antigen receptor-induced activation of IKK2 leads to NF-κB 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 FceRI 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 IκBs, NF-κBs, or precursors, and mediates additional functions beyond NF-κB activation (Hu et al., 2004; Lee et al., 2004; Wegener et al., 2006). Most of the NF-κB-related molecules (IκBz, IκBβ, IκBγ, NF-κB p105) have a putative IKK consensus sequences for phosphorylation (DSψXXS/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 14-3-3β (Gringhuis et al., 2005), SNAP-23 also contains no DSψXXS/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 (DSψXXS/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-κB (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-κB 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-κB. 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/Wv mice (W/Wv) 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, modified Eagle’s medium supplemented with 10% FCS and antibiotics. 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/Wv Mice with FLMCs
For reconstitution of skin mast cells of W/Wv mice, 1 × 106 WT or IKK2−/− FLMCs in 20 μl of PBS were injected i.d. into the right ear of W/Wv mice; for control, left ears were injected with PBS. For systemic reconstitution of mast cells of W/Wv mice, 1 × 107 WT or IKK2−/− FLMCs, in 100 μl of PBS, were injected i.v. into W/Wv mice; controls were injected with PBS.

PCA and PSA
To elicited 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 μl of PBS. After 24 hr, mice were injected i.v. with 200 μg 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 injection.
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 μg anti-DNP IgE in 100 μl of PBS. After 24 hr, mice were injected i.v. with 1 μg 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 μg anti-DNP IgE in 100 μl of PBS. After 24 hr, mice were challenged by epicutaneous application of 10 μl of DNFβ (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 ± SD. The statistical analysis of the results was performed by the unpaired t test; p values < 0.05 were considered significant.

**SUPPLEMENTAL DATA**

Supplemental Data include Supplemental Experimental Procedures and five figures and are available with this article online at http://www.cell.com/cgi/content/full/134/3/485/DC1/.

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