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Regulation of FcεRI Signaling in Mast Cells by G Protein-coupled Receptor Kinase 2 and Its RH Domain*

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Background: Aggregation of high affinity IgE receptors (FcεRI) on mast cells by antigen triggers allergic diseases.
Results: Deletion of GRK2 inhibited antigen-induced mast cell degranulation and abolished cytokine generation. Overexpression of GRK2 or its RH domain in mast cells enhanced antigen-induced responses.
Conclusion: GRK2 is a novel regulator of FcεRI signaling in mast cells.
Significance: Targeting GRK2 may provide a novel therapeutic approach for allergic diseases.

Agonist-induced phosphorylation of G protein-coupled receptors (GPCRs) by GPCR kinases (GRKs) promotes their desensitization and internalization. Here, we sought to determine the role of GRK2 on FcεRI signaling and mediator release in mast cells. The strategies utilized included lentiviral shRNA-mediated GRK2 knockdown, GRK2 gene deletion (GRK2$^{flox/flox}$/cre recombinase) and overexpression of GRK2 and its regulator of G protein signaling homology (RH) domain (GRK2-RH). We found that silencing GRK2 expression caused ~50% decrease in antigen-induced Ca^{2+} mobilization and degranulation but resulted in ablation of cytokine (IL-6 and IL-13) generation. The effect of GRK2 on cytokine generation does not require its catalytic activity but is mediated via the phosphorylation of p38 and Akt. Overexpression of GRK2 or its RH domain (GRK2-RH) enhanced antigen-induced mast cell degranulation and cytokine generation without affecting the expression levels of any of the FcεRI subunits (α, β, and γ). GRK2 or GRK2-RH had no effect on antigen-induced phosphorylation of FcεRIγ or Src but enhanced tyrosine phosphorylation of Syk. These data demonstrate that GRK2 modulates FcεRI signaling in mast cells via at least two mechanisms. One involves GRK2-RH and modulates tyrosine phosphorylation of Syk, and the other is mediated via the phosphorylation of p38 and Akt.

Mast cells are critical regulators of innate immunity and play important roles in allergic and hypersensitive diseases (1). Accordingly, they are abundantly present in mucosal as well as connective tissues such as lung, intestine, and skin (2). It is well established that aggregation of high affinity IgE receptor (FcεRI) on mast cells by allergen/antigen results in the rapid release of inflammatory mediators such as histamine and leukotrienes. This is followed by a delayed phase of mast cell activation resulting in the generation and release of chemokines and cytokines that recruit other immune cells such as eosinophils and lymphocytes leading to sustained inflammation (3). Recent exciting development in allergy research has been the approval by the Food and Drug Administration of a humanized monoclonal anti-IgE antibody, omalizumab, for the treatment of allergic asthma (4). Although omalizumab is extremely expensive and works well only on a subset of allergic patients (5), its utilization provides "proof of concept" that novel therapeutic approaches for allergic diseases can be developed by targeting FcεRI signaling in mast cells.

In addition to FcεRI, mast cells express G protein-coupled receptor (GPCR)[2] for the complement component C3a (C3aR). It has recently been shown that antigen-induced mast cell activation leads to the generation of C3a, which stimulates its cognate GPCR in mast cells to enhance IgE-mediated inflammation (6, 7). These findings suggest that C3aR expressed on mast cells contributes to allergic responses *in vivo*. The focus of our laboratory has been to study the regulation of C3aR signaling in mast cells. It is generally accepted that agonist-induced GPCR phosphorylation by one or more of the G protein-coupled receptor kinases (GRK1–7) is responsible for receptor desensitization (8). We previously showed that overexpression of GRK2 enhances agonist-induced C3aR phosphorylation in mast cells (9). We further demonstrated that knockdown of GRK2 attenuates C3aR desensitization, resulting in enhanced Ca^{2+} mobilization and degranulation (10). However, the role of GRK2 in regulating FcεRI signaling in mast cells has not been reported.

Unlike GPCRs, which are single seven transmembrane domain proteins, FcεRI comprises of an IgE-binding α chain and immunoreceptor tyrosine-based activation (ITAM) motif-containing β and γ chains (11–13). The receptor has no tyrosine kinase activity, and its signaling is dependent on Spleen tyrosine kinase (Syk) (14–16). Following FcεRI aggregation, Syk binds to the ITAM motifs of β and γ chains and becomes phosphorylated by Src kinases and also via its own kinase activity. Phosphorylated Syk then acts as docking site for adapter molecules including LAT, SLP-76, and Vav, which are then

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[2] The abbreviations used are: GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; BMMC, bone marrow-derived mouse mast cells; SCF, stem cell factor; Cre, cre-recombinase.

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phosphorylated by Syk to propagate downstream signaling (15, 17, 18).

GRK2 is an 80 kDa cytosolic protein with a well conserved central catalytic domain (~270 aa), and similar to other serine/threonine kinases, is flanked by an N-terminal domain (~180 aa) and a C-terminal domain (~200 aa) (19). The N-terminal domain is important for GPCR recognition. It also contains an RH domain (regulator of G protein signaling homology domain, GRK2-RH), which can promote GPCR desensitization in the absence of receptor phosphorylation (20, 21). Chen *et al.*, (22) showed that activation of EGF receptor causes phosphorylation of GRK2 at tyrosine residues to increase its catalytic activity. GRK2 phosphorylation by c-Src also regulates its kinase activity (23). It is therefore possible that following antigen stimulation of mast cells, GRK2 undergoes tyrosine phosphorylation by Src or Syk to regulate FcεRI signaling.

We previously used shRNA-mediated gene silencing strategy to study the regulation of C3aR signaling in human mast cells (10, 24, 25). Our goal in this study was to utilize primary murine mast cells to determine the role of GRK2 on FcεRI signaling. Because $GRK2^{-/-}$ mice are embryonic lethal, the strategies we utilized included lentiviral shRNA-mediated GRK2 knockdown, GRK2 gene deletion ($GRK2^{flox/flox}$/cre recombinase) and overexpression of GRK2 and its RH domain (GRK2-RH). Using these combined approaches we demonstrate the novel finding that GRK2 acts as an important regulator of FcεRI signaling in mast cells. We also demonstrate that GRK2 modulates FcεRI signaling in mast cells via at least two mechanisms; one involves its RH domain and modulates tyrosine phosphorylation of Syk, and the other is mediated via the phosphorylation of p38 and Akt.

EXPERIMENTAL PROCEDURES

Mice—C57BL/6 (wild type) and $GRK2^{flox/flox}$ mice were obtained from The Jackson Laboratory (Bar Harbor, ME). All mice were bred and housed under specific pathogen-free conditions and screened regularly for pathogens. Mice were 7–12 weeks old for all experiments. All procedures were approved by the Animal Care and Use Committee of the University of Pennsylvania.

Reagents—All cell culture reagents were purchased from Invitrogen (Gaithersburg, MD). Amaxa transfection kit (Kit V) was purchased from Lonza (Gaithersburg, MD). All recombinant cytokines (mIL-3, mSCF, mIL-33, and hSCF), and murine IL-13, IL-6, and rat CCL2 ELISA kits were purchased from Peprotech (Rocky Hill, NJ). DNP-specific mouse IgE (SPE-7) and NP-specific human IgE antibodies were from Sigma and ABD SeroTech (Raleigh, NC), respectively. DNP-BSA was from Invitrogen and NP-BSA was obtained from Biosearch Technologies (Novato, CA). Allophycocyanin (APC)-conjugated FcεRIα subunit (clone MAR-1) antibody was from eBiosciences (San Diego, CA). Native complement C3a and prostaglandin E_2 (PGE_2) was from Complement Technology (Tyler, TX) and Cayman Chemical (Ann Arbor, MI), respectively. Antibodies for GRK2, β-actin, p-Syk (Tyr-352 for human and 346 for rodents) (26), p-Src, p-ERK, ERK, p-Akt, and p-p38, were obtained from Cell Signaling Technology (Danvers, MA). Antibodies to α- and γ-subunit of FcεRI were obtained from EMD Millipore (Billerica, MA). Antibody to the β subunit of FcεRI was from Santa Cruz Biotechnology (Dallas, TX). Anti-HA antibody (clone 12CA5) was purchased from Roche Diagnostics (Indianapolis, IN). Protein G-agarose beads, SuperSignal® West Femto Maximum Sensitivity Substrate, and HRP-labeled anti-rabbit IgG and anti-mouse IgG were from Thermo Scientific (Rockford, IL).

Cell Culture—Rat basophilic leukemia (RBL-2H3) cells were grown in complete DMEM containing 10% FCS, L-glutamine (2 mM), penicillin (100 IU/ml), and streptomycin (100 μg/ml). Human LAD2 mast cells were maintained in complete StemPro-34 medium (Invitrogen) supplemented with hSCF (100 ng/ml, Peprotech) (27). Mouse bone-marrow-derived mast cells (BMMC) were obtained by flushing bone marrow cells from the femurs of C57BL/6 and $GRK2^{flox/flox}$ mice and culturing the cells for 4–6 weeks in IMDM supplemented with 10% FCS, L-glutamine (2 mM), penicillin (100 IU/ml), and streptomycin (100 μg/ml) and mIL-3 (10 ng/ml) (Peprotech). After 4 weeks in culture, >95% of these cells were mast cells, as judged by morphology and surface expression of FcεRI and cKit.

Lentiviral-mediated Knockdown of GRK2—Human GRK2 targeted shRNA (TRCN0000000559) and a scrambled control non-target shRNA (SHC002) in lentiviral plasmids were purchased from Sigma. Lentivirus generation and GRK2 knockdown in LAD2 mast cells were performed using procedures described previously (28, 29).

Murine GRK2 shRNA in lentiviral plasmid (TRCN0000022807) was from Sigma. Lentivirus generation was performed according to the manufacturer's manual, and the virus was concentrated by ultracentrifugation (30,000 × *g*, 1 h 40 min, 4 °C). The virus pellet was resuspended in 1.5 ml of complete IMDM medium. BMMC (10×10^6) were washed and resuspended in complete IMDM media supplemented with mIL-3 (10 ng/ml) and mSCF (30 ng/ml) 24 h before transduction. Cell transduction was conducted by mixing 1.5 ml of viral supernatant with 3.5 ml of BMMC. Eight hours post-infection, medium was changed to virus-free complete IMDM containing mIL-3 and mSCF, and antibiotic (puromycin, 1.5 μg/ml, Sigma) selection was initiated 16 h later. Cells were analyzed for GRK2 knockdown and used for subsequent assays 10 days following initiation of puromycin selection (30).

Retroviral-mediated Overexpression of GRK2 in BMMC—Bovine GRK2 in pcDNA was a kind gift from Dr. Jeffrey L. Benovic (Thomas Jefferson University). GRK2 was PCR amplified from this construct and was cloned at the HpaI restriction site in the MIGR-eGFP bicistronic retroviral vector. Retrovirus was generated by transfecting Plat-E cells with the GRK2 MIGR-eGFP plasmid using Lipofectamine reagent (Invitrogen). Seventy-two hours later, the supernatant was harvested, and the virus was concentrated by ultracentrifugation (30,000 × *g*, 1 h 40 min, 4 °C) and resuspended in 1.5 ml of complete IMDM medium. Bone marrow cells (10×10^6) isolated from the femurs of C57BL/6 mice were cultured in complete IMDM media supplemented with mIL-3 (10 ng/ml) and mSCF (30 ng/ml) 24 h before transduction. Retrovirus transduction was conducted by mixing 1.5 ml of viral supernatant with 3.5 ml of bone marrow cells. Eight hours post-infection, medium was changed to virus-free complete IMDM containing

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mIL-3 and mSCF. Cells were allowed to mature in this medium to BMMC. Following 3 weeks of culture, eGFP$^+$ cells were sorted using flow cytometry to obtain BMMC overexpressing GRK2.

Deletion of GRK2 in GRK2$^{flox/flox}$ BMMC—Control empty vector and cre-recombinase cloned in pLVX AcGFP lentiviral vector was kindly provided by Dr. Russ P. Carstens (University of Pennsylvania). Lentivirus was generated as described previously (29), concentrated by ultracentrifugation and resuspended in 1.5 ml of complete IMDM medium. BMMC obtained from GRK2$^{flox/flox}$ mice were stably transduced with lentivirus containing the cre-recombinase construct following the procedure described above.

Flow Cytometric Analysis of FcεRIα Expression—BMMC (3×10^5) were washed twice with cold FACS buffer (PBS containing 2% FBS) and stained with APC-conjugated FcεRIα antibody at 4 °C for 30 min. Cells were washed twice with cold FACS buffer and fixed in 250 μl of 1.5% formaldehyde, and samples were acquired and analyzed on BD LSR II flow cytometer (BD Biosciences).

Calcium Mobilization—Ca^{2+} mobilization was determined as described previously (9). Briefly, cells (BMMC, 1×10^6) were loaded with 1 μM indo-1 AM (Invitrogen) for 30 min at room temperature. Cells were washed and resuspended in 1.5 ml of HEPES-buffered saline. Ca^{2+} mobilization was measured in a Hitachi F-2500 spectrophotometer with an excitation wavelength of 355 nm and an emission wavelength of 410 nm. Mean Ca^{2+} ratios were calculated by averaging the ratios at individual time points following stimulation with antigen (DNP-BSA).

Degranulation—Human LAD2 mast cells were sensitized with NP-specific human IgE (1 μg/ml, 18 h). BMMC were sensitized overnight with anti-DNP mouse IgE antibody (1 μg/ml, 18 h) in cytokine-free medium. The cells were then rinsed three times with HEPES buffer containing 0.1% BSA (Sigma) to remove excess IgE. Cells were then resuspended in this buffer at the appropriate cell density for a specific assay. BMMC (5×10^4) and LAD2 cells (5×10^3) were seeded into 96-well plates in a total volume of 50 μl of HEPES buffer containing 0.1% BSA. For RBL-2H3, cells (5×10^4) were seeded into 96-well plates and incubated overnight with anti-DNP IgE antibody (1 μg/ml). The cells were exposed to different concentrations of antigen (DNP-BSA for BMMC or RBL-2H3 and NP-BSA for LAD2) at 37 °C for 30 min. In some assays LAD2 cells were also stimulated with C3a (10 nm). For total β-hexosaminidase release, unstimulated cells were lysed in 50 μl of 0.1% Triton X-100. Aliquots (20 μl) of supernatants or cell lysates were incubated with 20 μl of 1 mM *p*-nitrophenyl-N-acetyl-β-D-glucosamine for 1.5 h at 37 °C. Reaction was stopped by adding 250 μl of a 0.1 M Na_2CO_3/0.1 M $NaHCO_3$ buffer and absorbance was measured at 405 nm. Percent degranulation was calculated by dividing β-hexosaminidase activity in the samples with the total β-hexosaminidase release.

ELISA—Cytokine production was measured as described previously (9). Briefly, BMMC were incubated with DNP-specific mouse IgE (1 μg/ml) overnight in cytokine-free complete IMDM, washed, resuspended in complete IMDM, and then activated with DNP-BSA (1–100 ng/ml) for 6 h. In some assays cells were also stimulated with PGE2 (100 nM) or IL-33 (10 ng/ml). Cytokines were measured from the supernatants using sandwich ELISA kits for IL-13 and IL-6 (Peprotech) as described by the manufacturer. For CCL2 estimation, IgE-sensitized RBL-2H3 cells (2×10^5 cells) were serum starved for 4 h and stimulated with 100 ng/ml of DNP-BSA for 6 h. Supernatants were collected and stored frozen at −80 °C until analysis. CCL2 chemokine levels were quantified by a sandwich ELISA kit (Peprotech) according to the manufacturer's protocol.

Reconstitution of GRK2 in GRK2-deleted Cells—The kinase-dead mutant of GRK2 (GRK2-K220R) construct in MIGR-eGFP vector was generated by site-directed mutagenesis. This was performed by the DNA sequencing core facility at the University of Pennsylvania and the mutation was verified by DNA sequencing. GRK2$^{flox/flox}$ BMMC (5×10^6, stably transduced with either control empty vector or cre-recombinase construct lentivirus) were transfected with 2 μg of plasmids encoding wild type GRK2 or GRK2-K220R mutant using Amaxa nucleofector device and Amaxa kit V (Program T-020). 24 h following transfection, cells were stimulated with IgE/antigen, and the supernatant was assayed for IL-13 as described above.

Overexpression of GRK2 and GRK2-RH in RBL-2H3 Cells—Wild type GRK2 and hemagglutinin (HA)-tagged RH domain (GRK2-RH) were PCR amplified and introduced in the HpaI site of MIGR-eGFP vector. RBL-2H3 cells (1×10^6) were transfected with 2 μg of plasmids encoding wild type GRK2 or GRK2-RH and 0.5 μg of the pcDNA3.1 plasmid using the Amaxa nucleofector device and Amaxa kit V (Program T-030). Stably transfected cells were selected using G-418 (1 mg/ml). GFP$^+$ cells were sorted and expression levels of GRK2 and GRK2-RH were determined by Western blotting using the anti-GRK2 and anti-HA antibodies, respectively. The cells were then used for antigen-induced degranulation and cytokine release experiments.

Immunoprecipitation and Western Blotting—For immunoprecipitation studies IgE-sensitized cells (5×10^6/ml), were either stimulated with DNP-BSA (100 ng/ml) or left unstimulated for the indicated times. Cells were lysed for 40 min at 4 °C with RIPA buffer (150 mM NaCl, 1.0% Nonidet P-40, 0.5% sodium deoxycholate, 0.10% SDS, 50 mM Tris, pH 8.0, 5 mM EDTA, 10 mM NaF, 10 mM Na-pyrophosphate, and protease inhibitor mixture). The lysates were precleared by incubating with 20 μl of protein G-agarose for 1 h at 4 °C. Precleared lysates were centrifuged and transferred into new tubes followed by incubating with FcεRI γ subunit antibody and 30 μl of protein G-agarose overnight at 4 °C. Immunoprecipitates were resolved by SDS-PAGE, transferred on to nitrocellulose membranes and probed with the phospho-Tyr antibody (Cell Signaling Technology) followed by secondary goat anti- rabbit-HRP antibody (Thermo Scientific), and membranes were developed using the SuperSignal® West Femto Maximum Sensitivity chemiluminiscent substrate (Thermo Scientific).

For Western blotting, cells (1×10^6/ml) were washed twice in ice-cold PBS and lysed with RIPA buffer. Protein bands were separated on 10% SDS-PAGE gels and immunoblotted onto nitrocellulose membranes and probed with anti-GRK2 (Cell Signaling Technology), and anti-FcεRI subunit (α, β, and γ) antibodies. Following incubation with the secondary anti-rabbit-HRP or anti-mouse-HRP antibodies, membranes were

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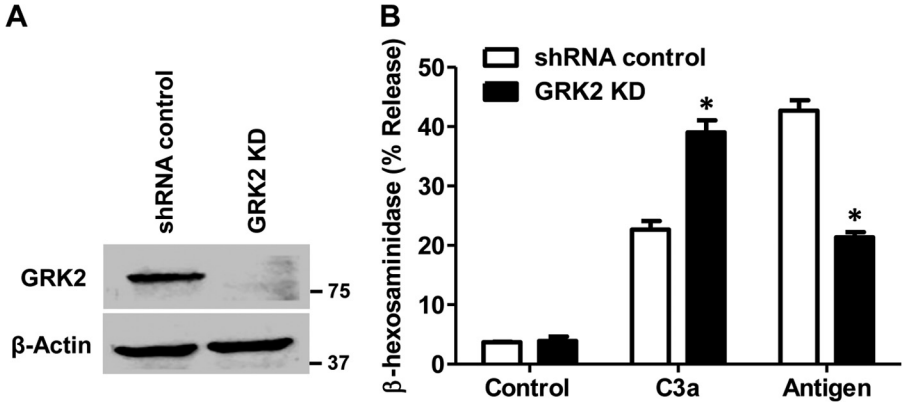

FIGURE 1. **Silencing GRK2 expression inhibits antigen but enhances C3a-induced degranulation in human mast cells.** LAD2 mast cells were stably transduced with scrambled control shRNA lentivirus or shRNA lentivirus targeted against human GRK2. *A*, Western blotting was performed to determine GRK2 expression in shRNA control and GRK2 knockdown (GRK2 KD) cells. *B*, shRNA control and GRK2 KD cells were pretreated with NP specific human IgE (1 μg/ml, 18 h) and stimulated with NP-BSA (*Antigen*, 100 ng/ml) or C3a (10 nM), and percent degranulation (β-hexosaminidase release) was determined. Data are mean ± S.E. of three experiments. Statistical significance was determined by two-way ANOVA with Bonferroni's post test. * indicates $p < 0.01$.

developed using the SuperSignal® West Femto Maximum Sensitivity chemiluminiscent substrate. The membranes were stripped and reprobed with β-Actin antibody (Cell Signaling Technology) followed by anti-rabbit-HRP secondary antibody to confirm equal protein loading. For assays involving activation of signaling proteins following IgE/antigen stimulation, membranes were probed with the phospho-specific antibodies for Syk, Src, p38, Akt, and ERK (Cell Signaling Technology) followed by incubation with the secondary anti-rabbit-HRP antibody. Equal loading was confirmed by stripping and reprobing membranes with ERK or β-actin antibodies (Cell Signaling Technology).

RESULTS

GRK2 Regulates Antigen-induced Responses in Human and Murine Mast Cells—We have previously shown that silencing the expression of GRK2 attenuates C3aR desensitization, resulting in enhanced Ca^{2+} mobilization and degranulation in a human mast cell line, LAD2 (28). We used the same cells to determine the role of GRK2 on antigen-induced degranulation in human mast cells. Western blotting confirmed >90% GRK2 knockdown (Fig. 1*A*). shRNA control and GRK2 knockdown cells were pre-treated with NP-specific human IgE and exposed to NP-BSA and degranulation was determined. As shown in Fig. 1*B*, knockdown of GRK2 resulted in ~50% inhibition of antigen-induced mast cells degranulation (Fig. 1*B*). To examine if this response was specific for antigen, we used C3a as a positive control. Consistent with our previous observation (28), silencing GRK2 expression resulted in enhanced C3a-induced mast cell degranulation (Fig. 1*B*).

To determine the role of GRK2 on antigen-induced responses in primary murine mast cells; we utilized the lentiviral shRNA transduction approach and silenced the expression of GRK2 in BMMCs. A scrambled shRNA that does not bind to any of the known murine mRNAs was used as control. Using this methodology, we achieved a GRK2 knockdown efficiency of >90% as indicated by Western blotting (Fig. 2*A*). Reduced GRK2 expression was associated with a significant reduction in antigen-induced Ca^{2+} mobilization (Fig. 2, *B* and *C*). In addition, consistent with the result obtained with human mast cells (Fig. 1*B*), silencing GRK2 resulted in ~50% reduction in antigen-induced degranulation (Fig. 2*D*). We were surprised to find that antigen-induced cytokine (IL-13 and IL-6) production was almost completely abolished in GRK2-silenced mast cells (Fig. 2, *E* and *F*). This effect was specific for antigen as PGE_2 and IL-33 (not shown)-mediated cytokine production was unchanged in GRK2-silenced cells

To further test the role of GRK2 on antigen-induced responses, we used retrovirus to overexpress GRK2 in murine BMMCs (Fig. 3*A*). Antigen-induced intracellular Ca^{2+} mobilization and degranulation were enhanced by ~50% in BMMCs overexpressing GRK2 as compared with control cells (Fig. 3, *B–D*). By contrast, antigen-induced IL-13 and IL-6 production were increased by up to 10-fold in GRK2-overexpressing cells (Fig. 3, *E* and *F*). This effect was specific for antigen, as PGE_2 and IL-33-induced cytokine production were not modulated by GRK2 overexpression (Fig. 3, *E* and *F*).

To rule out any nonspecific effects of shRNA and retroviral-mediated overexpression, we transduced BMMCs from $GRK2^{flox/flox}$ mice with lentivirus expressing either an empty vector (control) or the cre-recombinase construct to genetically delete GRK2. Following selection with puromycin, cells were analyzed for GRK2 expression by Western blotting. As shown in Fig. 4*A*, this approach resulted in complete deletion of GRK2. Similar to the situation with human mast cells and BMMCs with shRNA-mediated knockdown strategy, we found that antigen-induced intracellular Ca^{2+} mobilization and degranulation were reduced by ~50% in the absence of GRK2 (Fig. 4, *B–D*). Moreover, as with GRK2 shRNA, almost complete inhibition of cytokine production (IL-13/IL-6) was observed in GRK2-deleted cells following antigen stimulation (Fig. 4, *E* and *F*). This was again specific for antigen as IL-33-induced cytokine production was unaffected by GRK2 deletion (Fig. 4, *E* and *F*). Together, these data suggest that GRK2 modulates FcεRI or its downstream signaling pathway to regulate antigen-induced Ca^{2+} mobilization, degranulation, and cytokine production.

Effect of GRK2 on Antigen-induced Cytokine Production Is Independent of Its Catalytic Activity—Because GRK2 knockdown or deletion almost completely abolished antigen-induced

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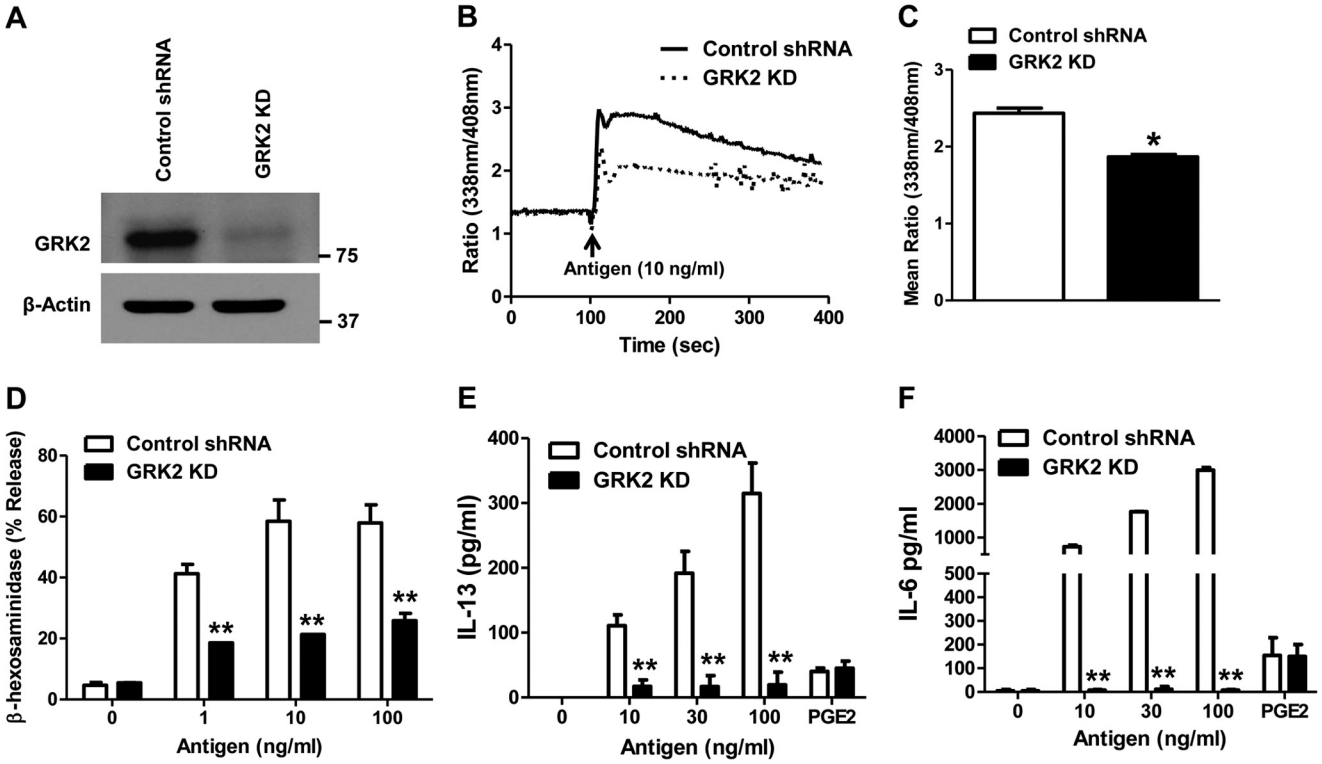

FIGURE 2. **Silencing GRK2 expression inhibits antigen-induced responses in murine BMMC.** Mast cells were stably transduced with scrambled control shRNA lentivirus or lentivirus containing shRNA targeting murine GRK2. *A*, Western blotting was performed to determine GRK2 expression in shRNA control and GRK2 knockdown (GRK2 KD) cells. shRNA control and GRK2 KD BMMCs were pretreated with DNP-specific IgE (1 μg/ml, 18 h) and stimulated with DNP-BSA (*Antigen*) and (*B*, *C*) intracellular Ca^{2+} mobilization, and (*D*) percent degranulation (β-hexosaminidase release) was determined as outlined under "Experimental Procedures." Control or GRK2 KD cells pretreated with IgE were stimulated with DNP-BSA (*Antigen*) or prostaglandin E_2 (*PGE*$_2$, 100 nM) for 6 h and (*E*) IL-13 and (*F*) IL-6 production was determined by ELISA. Data shown are mean ± S.E. of three experiments performed in triplicate. Statistical significance was determined by two-way ANOVA with Bonferroni's post test. * indicates $p < 0.01$, and ** indicates $p < 0.001$.

cytokine generation, we sought to determine if the kinase activity of GRK2 is required for this inhibition. It is well established that a point mutation of GRK2 (K220R) inhibits its kinase activity (31–35). We therefore reconstituted wild-type GRK2 or GRK2-K220R in GRK2-deleted BMMCs using the Amaxa nucleofection method. Empty vector transfection was used as control. Western blotting analysis revealed equivalent reconstitution of GRK2 and GRK2-K220R in GRK2-deleted cells (Fig. 5*A*). As expected, GRK2-deletion resulted in substantial reduction of IL-13 generation in response to antigen stimulation (Fig. 5*B*). Reconstitution of GRK2 restored this response although not completely when compared with control cells. This incomplete restoration suggests that not all cells express the transfected GRK2. Interestingly, reconstitution of GRK2-K220R restored antigen-induced IL-13 production to a similar extent as wild-type GRK2 (Fig. 5*B*). These findings suggest that the effect of GRK2 on antigen-induced cytokine generation is mediated independent of its kinase activity.

Effects of GRK2 on Antigen-induced p38 and Akt Phosphorylation—Protein kinase B (Akt) and p38 are known to be involved in antigen-induced cytokine generation in mast cells (36, 37). We therefore sought to determine the effect of GRK2 deletion on these signaling pathways. ERK1/2 phosphorylation was used for control. As show in Fig. 6*A*, antigen stimulation resulted in a time-dependent increase in p38, Akt and ERK1/2 phosphorylation in control mast cells. However, p38, and Akt phosphorylation were reduced in the GRK2-deleted cells but ERK1/2 phosphorylation was unaffected (Fig. 6*A*). Similar effects were also confirmed in GRK2 knockdown BMMCs (Fig. 6*B*). These findings suggest that p38 and Akt contribute to the effects of GRK2 on antigen-induced cytokine generation in mast cells.

GRK2 and Its RH Domain Enhance Antigen-induced Responses in Mast Cells—Our next goal was to determine the structural components of GRK2 that modulates antigen-induced responses in mast cells. The RH domain of GRK2 (GRK2-RH) has previously been shown to promote GPCR desensitization independent of receptor phosphorylation (20, 38, 39). Given that the effect of GRK2 on antigen-induced mediator release does not require its kinase activity, we sought to determine if GRK2-RH could modulate these responses. We initially attempted to express this domain in murine BMMCs but the expression level was not sufficiently high to study receptor regulation. We therefore utilized an alternative approach and stably expressed GRK2 and GRK2-RH in a rodent mast cell line, RBL-2H3 cells (40). As shown in Fig. 7*A*, both GRK2 and GRK2-RH were expressed in this cell line. Importantly, as for BMMCs, overexpression of GRK2 resulted in enhancement of antigen-induced mast cell degranulation and cytokine (chemokine CCL2) production (Fig. 7, *B* and *C*). Interestingly, we found that the expression of GRK2-RH had the same effect as GRK2 in enhancing antigen-induced responses in mast cells (Fig. 7). These findings suggest that the effects of GRK2 on antigen-induced responses are mediated via its RH domain.

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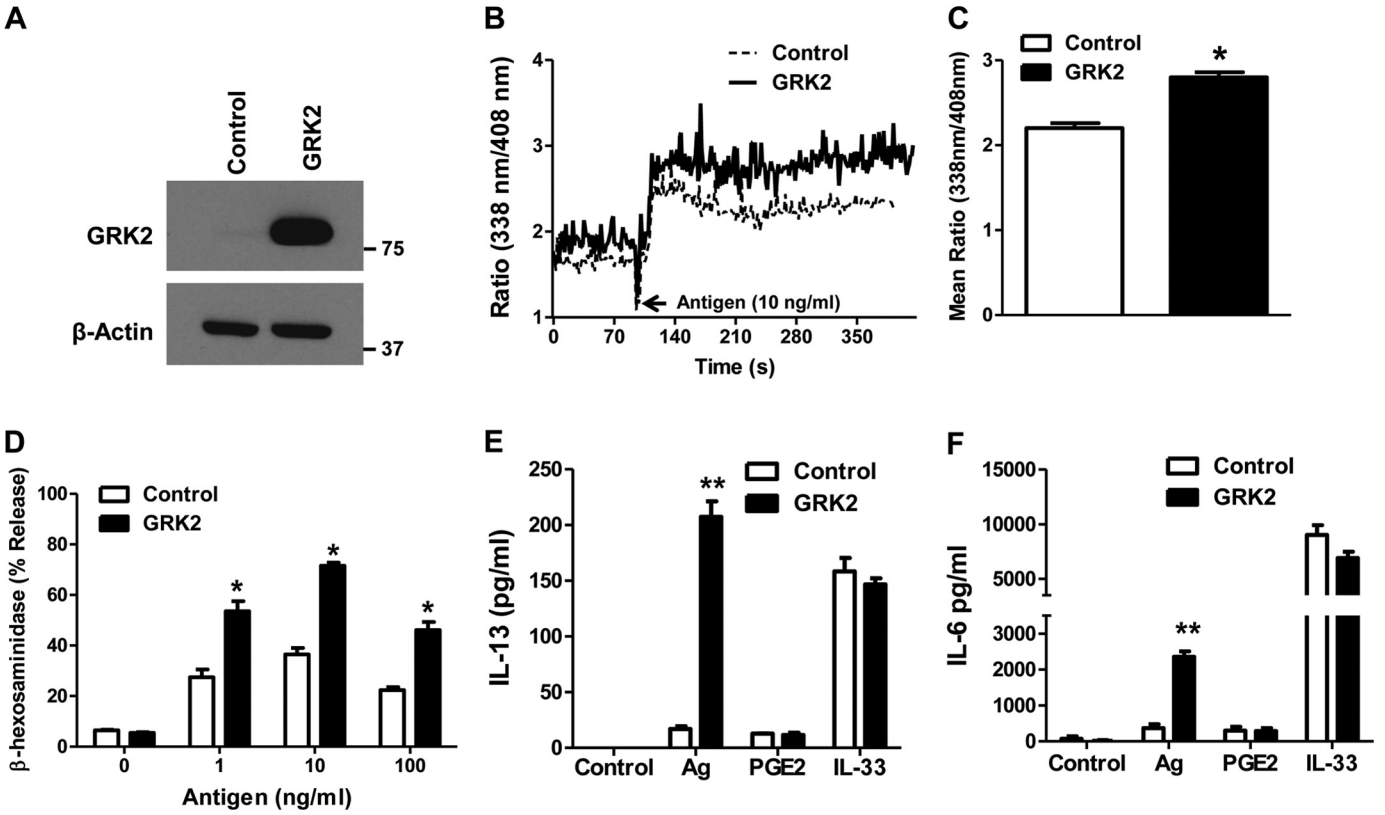

FIGURE 3. **GRK2 overexpression enhances antigen-induced responses in murine BMMC.** Murine bone marrow cells were transduced with retrovirus containing the control MIGR-eGFP or GRK2 MIGR-eGFP vectors. Cells were cultured in mSCF (30 ng/ml) and mIL-3 (10 ng/ml) for 3 weeks to generate BMMCs. eGFP$^+$ BMMCs were sorted using flow cytometry and expanded in culture. *A*, Western blotting was performed to determine GRK2 expression in control MIGR-eGFP (*Control*) and GRK2 MIGR-eGFP (*GRK2*) transduced cells. BMMC were pretreated with DNP-specific IgE (1 μg/ml, 18 h) and stimulated with DNP-BSA (*Antigen*) and (*B*, *C*) intracellular Ca^{2+} mobilization and (*D*) percent degranulation (β-hexosaminidase release) was determined as described under "Experimental Procedures." *E*, IL-13 and *F*, IL-6 generation by control and GRK2-BMMC following treatment with IgE/antigen (*Ag*), prostaglandin E_2 (*PGE*$_2$, 100 nM) or IL-33 (10 ng/ml) was determined by ELISA. Data shown are mean ± S.E. of three experiments performed in triplicate. Statistical significance was determined by two-way ANOVA with Bonferroni's post test. * indicates $p < 0.01$, and ** indicates $p < 0.001$.

Effects of GRK2 on FcεRI Subunit Expression and Phosphorylation of FcεRIγ, Src, and Syk—The data presented above clearly demonstrate that GRK2 utilizes its RH domain to modulate antigen-induced responses in mast cells. The data also show that GRK2 contributes to p38 and Akt phosphorylation for cytokine generation. Since GRK2 regulates early events in FcεRI signaling (*e.g.* Ca^{2+} mobilization), it also likely targets the receptor or the associated tyrosine kinases such as Src and Syk. To determine the effect of GRK2 on the expression of FcεRI subunits (α, β, and γ), we initially performed Western blotting analyses on control and GRK2-deleted BMMCs. We found that deletion of GRK2 had no effect on the expression levels of FcεRI α, β, or γ subunits (Fig. 8*A*). We also examined cell surface expression of the α-subunit (FcεRIα) by flow cytometry. As shown in Fig. 8*B*, cell surface expression of FcεRIα was unaffected in GRK2-deleted BMMCs. Because we found that transfection of GRK2 or GRK2-RH in RBL-2H3 cells enhanced antigen-induced responses, we sought to determine their effects on the expression profiles of FcεRI subunits. As shown in Fig. 8*C*, control RBL-2H3 cells express all three subunits of FcεRI. Furthermore, transfection of GRK2 or GRK2-RH did not affect their expression levels. These findings suggest that GRK2 does not alter FcεRI subunit expression levels.

Our next goal was to determine if GRK2 modulates phosphorylation of the γ subunit of FcεRI (FcεRIγ). This was done by immunoprecipitating FcεRIγ from RBL-2H3 cells expressing GRK2 or GRK2-RH followed by Western blotting using a p-Tyr antibody. IgE/antigen caused rapid tyrosine phosphorylation of FcεRIγ in control cells (Fig. 9*A*). This response was not altered in cells expressing GRK2 or GRK2-RH (Fig. 9*A*). These findings thus suggest that the ability of GRK2 to modulate FcεRI signaling in mast cells likely reflects modulation of signaling downstream of the receptor.

We next sought to determine if GRK2 and GRK2-RH regulates Src and Syk phosphorylation in antigen-stimulated mast cells. This was done by Western blotting with phospho-Src and phospho-Syk antibodies on cell lysates of IgE/antigen-stimulated RBL-2H3 cells. A shown in Fig. 9*B*, although Src phosphorylation was unaltered, phosphorylation of Syk was significantly enhanced in the presence of GRK2 or GRK2-RH.

DISCUSSION

GRK2 is a member of a family of serine/threonine kinases and its role in regulating GPCR function is well documented. GRK2 phosphorylates agonist-occupied GPCRs to promote their desensitization and thus generally acts as a negative regulator of GPCR signaling (8). We have previously shown that GRK2 desensitizes C3aR function in mast cells resulting in the inhibition of both Ca^{2+} mobilization and degranulation (9, 28, 41). In the present study we have uncovered previously unap-

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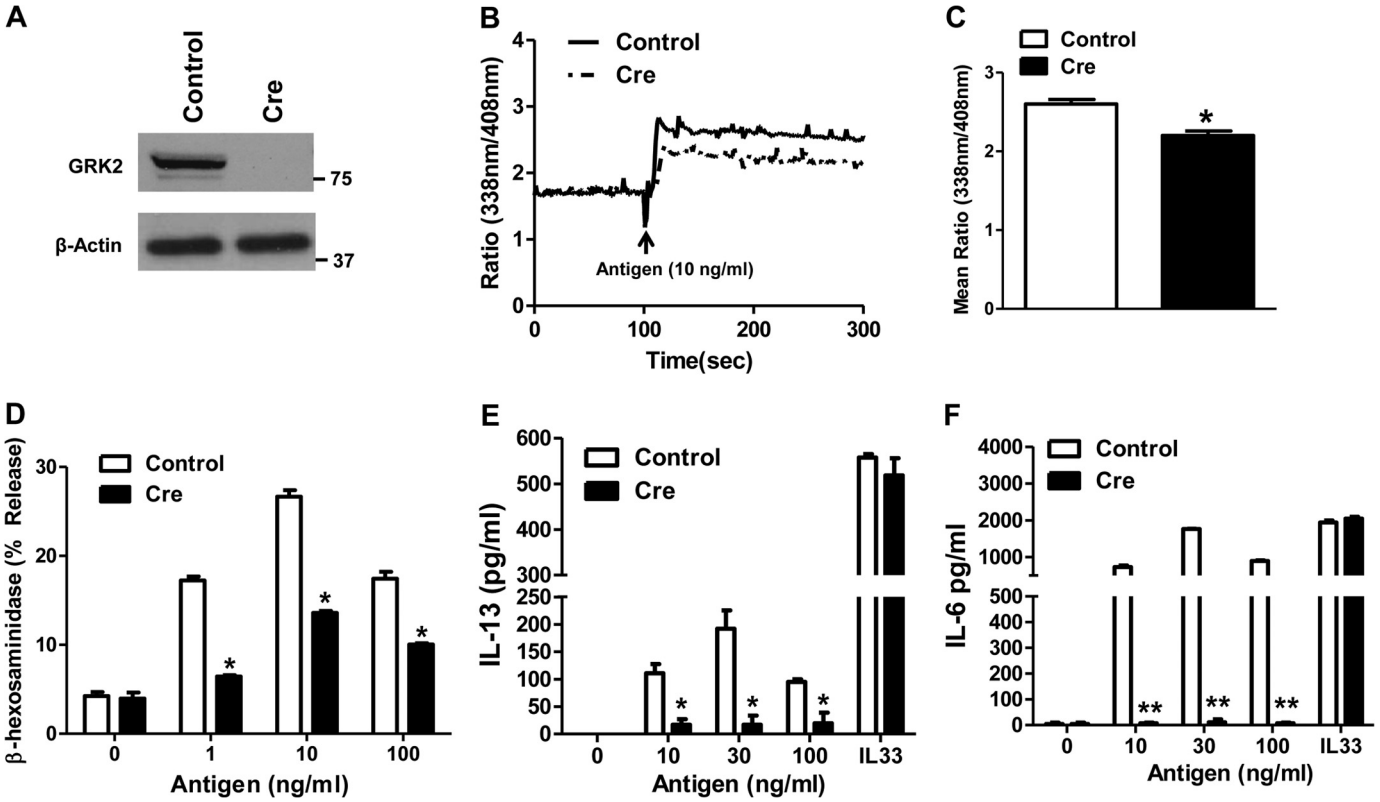

FIGURE 4. **Deletion of GRK2 attenuates antigen-induced responses in murine BMMC.** BMMC from GRK2$^{flox/flox}$ mice were stably transduced with lentivirus containing the control or cre-recombinase construct. *A*, Western blotting was performed to confirm GRK2 deletion in BMMC expressing cre-recombinase (*Cre*). Control and GRK2-deleted (*Cre*) cells were pretreated with DNP-specific IgE (1 μg/ml, 18 h) and stimulated with DNP-BSA (*Antigen*) and (*B*, *C*) intracellular Ca^{2+} mobilization and (*D*) percent degranulation (β-hexosaminidase release) was determined. *E*, IL-13 and *F*, IL-6 generation by BMMC following treatment with IgE/antigen or IL-33 (10 ng/ml) was determined by ELISA. Data shown are mean ± S.E. of three experiments performed in triplicate. Statistical significance was determined by two-way ANOVA with Bonferroni's post test. * indicates $p < 0.01$, and ** indicates $p < 0.001$.

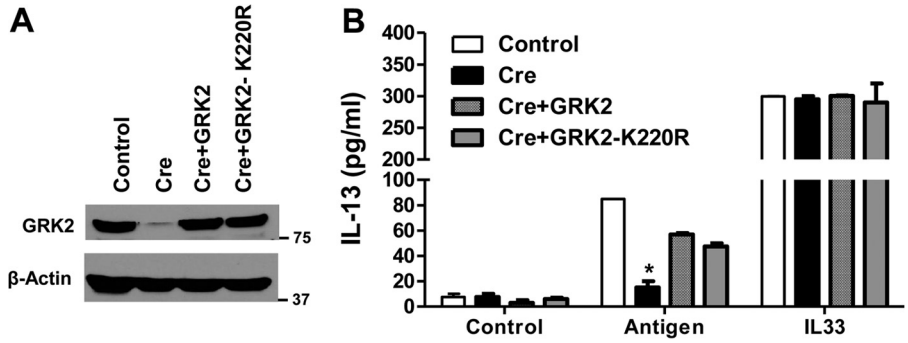

FIGURE 5. **GRK2 regulates antigen-induced cytokine production independent of its kinase activity.** GRK2$^{flox/flox}$ BMMC stably transduced with cre-recombinase lentivirus (*Cre*) were reconstituted with MIGR-eGFP vector containing either wild type (GRK2) or the kinase dead mutant (GRK2-K220R) of GRK2 using the Amaxa nucleofection technique. GRK2 $^{flox/flox}$ BMMC stably transduced with lentivirus containing the empty vector was used as the control. *A*, Western blotting was performed to confirm GRK2 expression in GRK2 or GRK2-K220R BMMC. *B*, cells were pretreated with DNP-specific IgE (1 μg/ml, 18 h) and stimulated with DNP-BSA (*Antigen*, 100 ng/ml) or IL-33 (10 ng/ml) for 6 h, and IL-13 production was determined from the supernatant by ELISA. Data shown are mean ± S.E. of three experiments performed in triplicate. Statistical significance was determined by two-way ANOVA with Bonferroni's post test. * indicates $p < 0.01$.

preciated role of GRK2 as a positive regulator of FcεRI signaling in mast cells. We used multiple strategies including lentiviral shRNA-mediated GRK2 knockdown, GRK2 gene deletion (GRK2$^{flox/flox}$/cre recombinase) and overexpression of GRK2 and GRK2-RH to clearly demonstrate that GRK2 regulates degranulation (early phase) and cytokine production (delayed phase) in mast cells. Furthermore, this study uncovered a novel mechanism for the regulation of FcεRI signaling in mast cells.

Our findings that GRK2 deletion blocked antigen induced Ca^{2+} mobilization whereas its overexpression enhanced this response indicated that this protein regulates FcεRI or its proximal signaling pathway. We found that deletion of GRK2 had no effect on FcεRI α, β, and γ subunit expression in mast cells. Chen *et al.*, (22) have recently shown that activation of EGF receptor causes phosphorylation of GRK2 at tyrosine residues to increase its kinase activity. GRK2 phosphorylation by c-Src also promotes its kinase activity (23). It is therefore possible that following antigen stimulation GRK2 undergoes tyrosine phosphorylation, which subsequently phosphorylates FcεRI (β/γ subunit) or components in its downstream signaling path-

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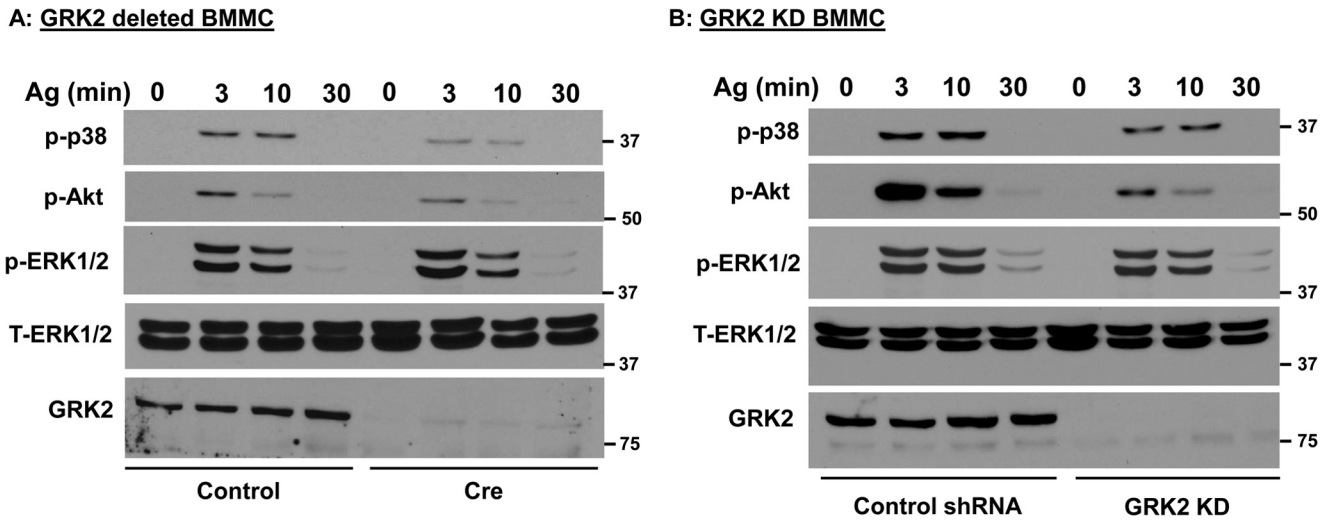

FIGURE 6. **GRK2 regulates antigen-induced p38 and Akt, phosphorylation.** *A*, GRK2$^{flox/flox}$ BMMC stably transduced with empty vector (*Control*) or cre-recombinase lentivirus (*Cre*) and *B*, scrambled shRNA (*shRNA control*) or GRK2 knock down (GRK2 KD) BMMC were stimulated with DNP-specific IgE (1 μg/ml, 18 h) and exposed to DNP-BSA (*Ag*, 100 ng/ml) for indicated time intervals. Total cell lysates were subjected to Western blotting with antibodies specific for phosphorylated p38, Akt, and ERK. The membranes were stripped and reprobed with antibodies for total ERK and GRK2.

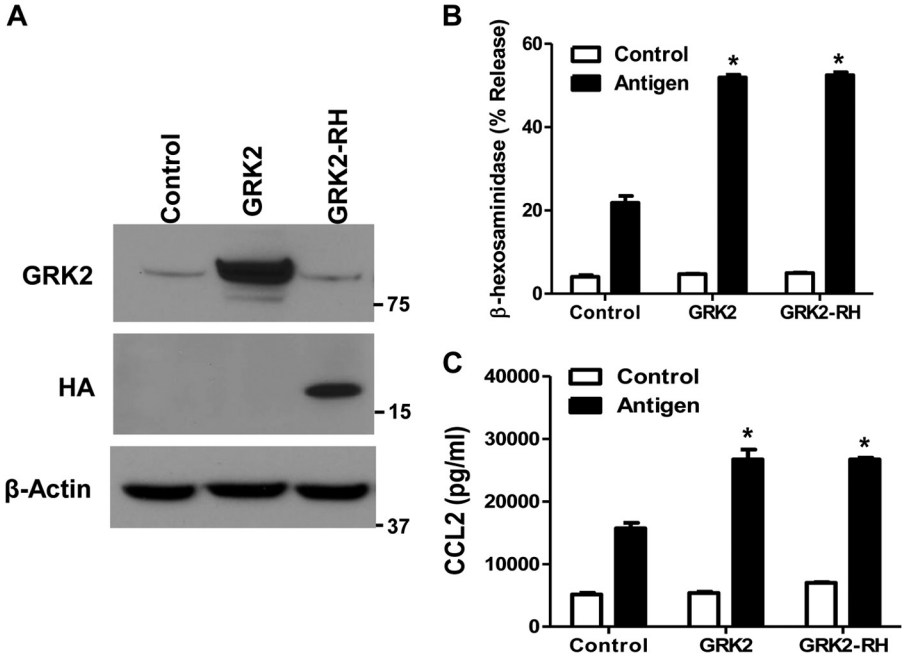

FIGURE 7. **Overexpression of GRK2 and GRK2-RH enhances antigen-induced degranulation and chemokine generation.** RBL-2H3 cells stably expressing the vector (*Control*), wild type (*GRK2*), or HA-GRK2-RH were generated as described under "Experimental Procedures." *A*, Western blotting was performed to confirm GRK2 or GRK2-RH expression using either the GRK2 or the HA antibody and *B*, percent degranulation (β-hexosaminidase release) after stimulation with DNP-specific IgE and DNP-BSA (*Antigen*, 100 ng/ml) was determined. *C*, cells were pretreated with DNP-specific IgE (1 μg/ml, 18 h) and stimulated with DNP-BSA (*Antigen*, 100 ng/ml) for 6 h, and CCL2 production was determined by ELISA. Data shown are mean ± S.E. of three experiments performed in triplicate. Statistical significance was determined by two-way ANOVA with Bonferroni's post test. * indicates $p < 0.01$.

way to promote mediator release. However, this possibility is unlikely as a kinase-dead mutant of GRK2 (K220R) is able to support antigen-induced cytokine generation similar to the wild-type GRK2. These findings suggest that the observed effects of GRK2 on FcεRI signaling are mediated via a mechanism independent of its kinase activity.

GRK2 interacts with Gαq family of G proteins to promote weak GTPase activity (38). Furthermore, GRK2-RH has been shown to inhibit Gαq-mediated phospholipase C activity via a pathway independent of its GTPase activity or receptor phosphorylation (42). It has been proposed that GRK2-RH may function as an antagonist shielding the interaction between G protein and either GPCR or its corresponding effector. Subsequent studies have indeed shown that GRK2-RH inhibits receptor function via mechanisms that include binding to GPCRs and sequestration of Gαq G proteins (20, 39). Given that GRK2 contributes to FcεRI signaling in mast cells, we were interested in determining the effect of GRK2-RH on antigen-induced responses in mast cells. We were quite surprised to find that it enhances antigen-induced degranulation and chemokine generation. This effect does not appear to be mediated via the tyrosine phosphorylation of FcεRIγ or Src but correlates with

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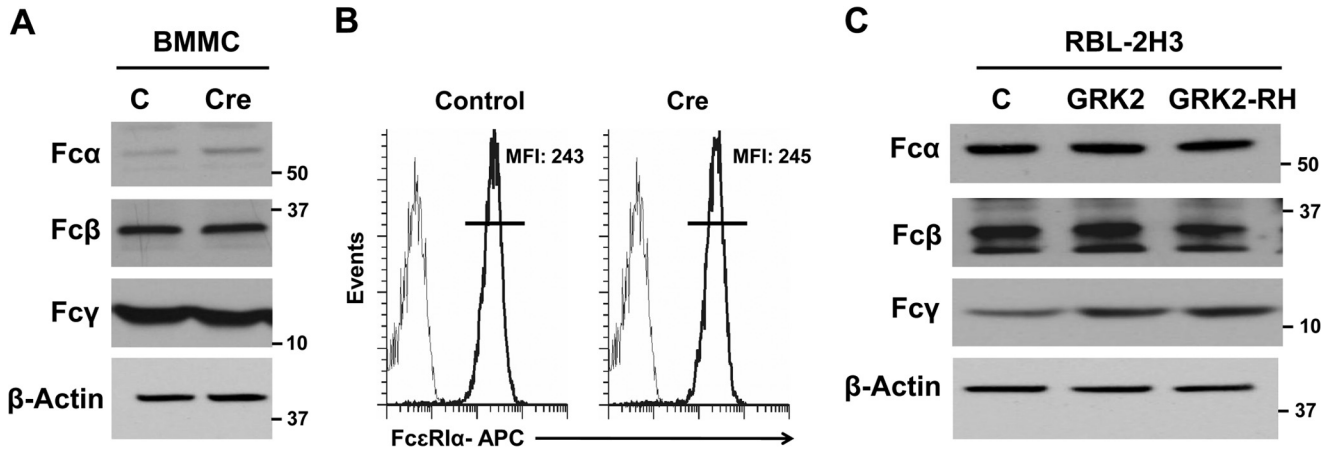

FIGURE 8. **GRK2 does not modulate the expression of FcεRI α, β, and γ subunits in mast cells.** *A*, Western blotting was performed to examine the expression levels of the α, β, and γ subunits of FcεRI in control (*C*) and GRK2-deleted BMMC (*Cre*). A representative blot is shown. *B*, control and GRK2-deleted BMMC (*Cre*) were stained with either the isotype antibody (*thin line*) or the APC conjugated anti-mouse FcεRIα antibody (*thick lines*) and analyzed by flow cytometry. Representative histogram of FcεRIα expression in control and GRK2-deleted BMMC (*Cre*) BMMC is shown. The mean fluorescence intensities (*MFI*) of the individual histograms are also shown in the respective panels. *C*, Western blotting was performed to examine the expression levels of the α, β, and γ subunits of FcεRI in RBL-2H3 cells stably expressing control vector, GRK2 or GRK2-RH. A representative blot is shown. Data shown are representative of three experiments.

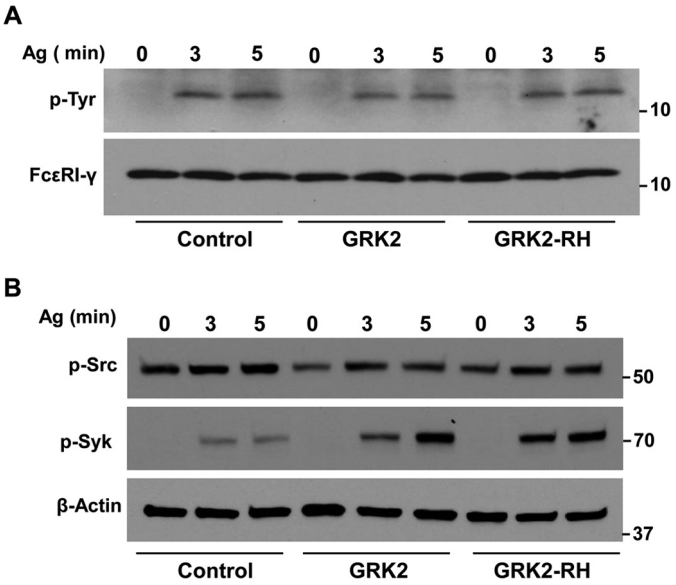

FIGURE 9. **GRK2 and GRK2-RH do not modulate FcεRI γ-subunit or Src phosphorylation but enhance Syk phosphorylation in response to IgE/antigen stimulation.** *A*, RBL-2H3 cells stably expressing the control vector, GRK2, or GRK2-RH were pretreated with DNP-specific IgE (1 μg/ml, 18 h) and stimulated with DNP-BSA (*Ag*, 100 ng/ml) for indicated time intervals. Cell lysates were immunoprecipitated with the FcεRI γ-subunit antibody and protein G-agarose beads followed by Western blotting with an anti-phosphotyrosine (*p-Tyr*) antibody. The blots were stripped and reprobed with FcεRI γ-subunit antibody. A representative blot is shown. *B*, cells were pretreated with DNP-specific IgE (1 μg/ml, 18 h) and stimulated with DNP-BSA (*Ag*, 100 ng/ml), lysed, and subjected to Western blotting with antibodies specific for phosphorylated Src and Syk. β-Actin was used as the loading control. A representative blot is shown. Data shown are representative of three experiments.

enhanced phosphorylation of Syk at Tyr-346. The mechanism via which GRK2 and GRK2-RH enhance Syk phosphorylation in antigen-stimulated mast cells remains unknown. T cell receptor (TCR) is a multimeric complex composed of the polymorphic α and β subunits and the CD3 γ, δ, ε, and ξ chains. It has recently been shown that membrane proximal portion of CD3 ε associates with GRK2 but the function of this interaction is unknown (43). It is therefore possible that one or more subunits of FcεRI associate with GRK2 to enhance Syk phosphorylation following antigen-stimulation in mast cells. This possibility will be the subject of future investigations.

An important and surprising observation of the present study was that while knockdown or deletion of GRK2 led to ~50% decrease in antigen-induced mast cell degranulation cytokine generation was almost completely inhibited. These findings suggest that GRK2 interacts with multiple components of FcεRI signaling pathway. It is well documented that activation of p38 and Akt pathways play important roles in antigen-induced cytokine production in mast cells (36, 37). The data that loss of cytokine generation in GRK2-deleted mast cells is associated with deceases in antigen-induced p38 and Akt phosphorylation suggests that GRK2 modulates these signaling pathways to promote cytokine generation. It has previously been shown that phosphorylation of p38 and Akt by GRK2 results in the inhibition of their function (44, 45). However, our finding that GRK2 contributes to antigen-induced p38 and Akt phosphorylation is opposite to the previous reports in other systems. The molecular mechanism via which GRK2 contributes to antigen-induced p38 and Akt phosphorylation is currently unknown and will be the subject of future investigation.

GRK2 was originally identified as a protein kinase that phosphorylates agonist-occupied GPCRs to promote their desensitization and internalization (46). Emerging evidence suggests that GRK2 interacts with multiple proteins to modulate their function (19). The data presented herein demonstrates the novel finding that GRK2 contributes to antigen-induced mast cell degranulation but it is absolutely essential for cytokine IL-13 and IL-6 generation. These findings suggest that GRK2 modulates multiple components of FcεRI signaling in mast cells. The effect of GRK2 on antigen-induced cytokine generation is independent of its kinase activity and GRK2-RH is able to mimic the effect of GRK2 on antigen-induced mast cell degranulation. Based on these findings we postulate that GRK2 serves as a novel scaffolding molecule to promote FcεRI signaling in

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mast cells. The PH domain of GRK2 has been the target for the development of therapy for heart failure (47–49). Our future studies will also incorporate the PH domain to determine the component of GRK2 that could form the best target to modulate IgE and mast cell-dependent allergic responses *in vivo*.

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