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Calcineurin–Rcan1 Interaction Contributes to Stem Cell Factor–Mediated Mast Cell Activation

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The receptor for stem cell factor (SCF) is expressed on mast cells and hematopoietic progenitors. SCF-induced signaling pathways remain incompletely defined. In this study, we identified calcineurin and regulator of calcineurin 1 (Rcan1) as novel components in SCF signaling. Calcineurin activity was induced in SCF-stimulated primary mouse and human mast cells. NFAT was activated by SCF in bone marrow–derived mast cells (BMMCs) and mouse bone marrow cells, which contain hematopoietic progenitors. SCF-mediated activation also induced expression of Rcan1 in BMMCs. Rcan1-deficient BMMCs showed increased calcineurin activity and enhanced transcriptional activity of NF-κB and NFAT, resulting in increased IL-6 and TNF production following SCF stimulation. These results suggest that Rcan1 suppresses SCF-induced activation of calcineurin and NF-κB. We further demonstrated that SCF-induced Rcan1 expression is dependent on the transcription factor early growth response 1 (Egr1). Interestingly, SCF-induced Egr1 was also suppressed by Rcan1, suggesting a negative regulatory loop between Egr1 and Rcan1. Together, our findings revealed that calcineurin contributes to SCF-induced signaling, leading to NFAT activation, which, together with NF-κB and Egr1, is suppressed by Rcan1. Considering the wide range of biological functions of SCF, these novel regulatory mechanisms in SCF signaling may have broad implications. The Journal of Immunology, 2013, 191: 5885–5894.
regulatory-feedback loop, in which Egr1 drives Rcan1 expression, which, in turn, feeds back to inhibit Egr1 expression, extends to SCF-mediated signaling events.

**Materials and Methods**

**Animals**

Rcan1-deficient mice were generated as previously described (23). The protocols were approved by the University Committee on Laboratory Animals, Dalhousie University, in accordance with the guidelines of the Canadian Council on Animal Care.

**Abs and reagents**

Abs to p38 MAPK and actin and HRP-linked secondary Abs were purchased from PeproTech (Rock Hill, NJ). Cycloheximide and cyclosporine A were purchased from Sigma-Aldrich. rh-IL-3 and rh-IL-6 were purchased from eBioscience (San Diego, CA). FITC-conjugated annexin V and 7-aminominoacyclinomycin D (7-AAD) were purchased from BD. FITC-conjugated rat anti-mouse CD117 (c-Kit) mAb and FITC-rat IgG2a were purchased from Cedarlane Laboratories. Other Abs were purchased from Cell Signaling Technology (Beverly, MA).

**Cell culture and activation**

Wild-type and Rcan1-deficient BMMCs were cultured as previously described (24). BMMCs were activated by addition of SCF (100 ng/ml) or other indicated concentration) for various times. For Egr-mediated activation, BMMCs were passively sensitized overnight with trinitrophenyl (TNP)-specific IgE from TIB-141 cells (American Type Culture Collection); the following day, unbound IgE was removed by washing cells with RPMI 1640 before resuspending them in RPMI 1640 media supplemented with 10% FBS and 50 U/ml each of penicillin and streptomycin (Invitrogen). Cells were then activated by stimulation with 10 ng/ml TNF-BSA (Biosearch Technologies, Novato, CA). Human mast cell line HMC-1 SC6 was maintained in IMDM in 5% CO2 in a humidified atmosphere at 37°C. Culture medium was supplemented with 10% FBS (Sigma-Aldrich) and 50 U/ml each of penicillin and streptomycin (Invitrogen). Bone marrow from C57BL/6 mice was isolated, and a single-cell suspension was obtained and incubated in RPMI 1640 supplemented with 10% FBS and 50 U/ml each of penicillin and streptomycin (Invitrogen). For preparation of calcineurin inhibition samples, cells were preincubated with Abs for NFAT (c1 and c2) to block protein–probe interaction, and then the calcineurin activity was assayed using ImageJ software. The specificity of the NFAT EMSA probe was confirmed using 50× unlabeled probes or mutant probes or by preincubating with Abs for NFAT (c1 and c2) to block protein–probe interaction.

**Luciferase assay**

Luciferase assay was performed as previously described (24). Briefly, pNFAT-Luc or pNF-kB–Luc (Agilent Technologies) plasmid and plRE–TK control plasmid (Promega) were cotransfected into BMMCs that were plated in culture medium and allowed to recover for 24 h. Subsequently, cells were incubated with 100 ng/ml SCF for 5 h. Firefly and Renilla activities were quantified using a dual-luciferase reporter assay system (Promega).

**Calcineurin-activity assay**

Masst cells were incubated with 100 ng/ml SCF for 6 h. Lysates were prepared, and phosphatase activity was measured using a calcineurin-activity assay (Biomed International-Enzo Life Sciences, Plymouth Meeting, PA), according to the manufacturer’s instructions.

**Real-time quantitative PCR**

The mRNA levels of various genes were quantified using TaqMan MGB probes and TaqMan Master Mix on a sequence detection system (ABI Prism 7000; Applied Biosystems). GAPDH was used as an endogenous reference. Data were analyzed using the relative standard curve method, according to the manufacturer’s protocol. A mean value of each gene after GAPDH normalization at the time point showing highest expression was used as a calibrator to determine the relative levels of Rcan1 or Egr1 under different conditions. In addition, PCR products were resolved and visualized on a 2% agarose gel and stained with ethidium bromide.

**Proliferation assay**

Proliferation assays were performed as previously described (9, 10). Briefly, BMMCs were washed twice with RPMI 1640 and starved in RPMI 1640 supplemented with 0.2% BSA and 50 U/ml each of penicillin and streptomycin for 4 h. For each sample, 5 × 105 cells were seeded in a 96-well plate in 200 μl RPMI 1640 supplemented with 10% FBS and 50 U/ml each of penicillin and streptomycin and 0.2% BSA. For each sample, 100 ng/ml SCF was added into the plate and incubated at 37°C for 48 h. Cells were cultured for 48 h and subsequently pulsed with 1.0 μCi [3H] thymidine for the final 16 h in culture. Cells were harvested, and thymidine incorporation was determined as cpm on a Wallac scintillation counter.

**Cell-survival assay**

BMMCs were washed twice and starved in RPMI 1640 containing 0.2% BSA and 50 U/ml each of penicillin and streptomycin for 4 h, and a total of 2 × 106 cells/well was seeded in a 96-well plate in the absence or presence of SCF (0–100 ng/ml). After 48 h of culture, cells were washed with PBS and stained with FITC–annexin V and 7-AAD before FACS analysis (FACSArray; BD) to determine the percentage of live and apoptotic cells. Alternatively, 2 × 105 cells/well were seeded in a 96-well plate in BMMC complete medium without IL-3, and cell survival was tested using a WST-1 assay (Clontech, Mountain View, CA) or by counting cell numbers on days 1–4 in culture.

### Western blotting

For preparation of calcineurin-inhibition samples, cells were preincubated with 1 μg/ml cyclosporine A at 37°C for 20 min, and SCF was added to a final concentration of 100 ng/ml for 5 min. Cells were lysed and subjected to electrophoresis. Separated proteins were transferred to polyvinyldene difluoride membrane, probed with primary and secondary Abs, and detected by an ECL-detection system (Western Lightning Plus-ECL, PerkinElmer). Blots were scanned and quantified using ImageJ software.

**EMSA**

Nuclear protein extracts were obtained using a nuclear extract kit (Active Motif, Carlsbad, CA), according to the manufacturer’s protocol. HMC-1 cells were treated with tyrosine kinase inhibitors, PKC412 (final concentration 1 μM; Cayman Chemical, Ann Arbor, MI) and/or dasatinib (final concentration 1 μM; Synkinease, San Diego, CA) for 4 h, and then nuclear protein was extracted. EMSA was performed as previously described (27). The following synthesized double-stranded oligonucleotides (Sigma-Aldrich) were used: NFAT binding consensus sequence, 5′-AAGGTGTTTTCCCAAGCCTTTTCCC-3′; Egr1 binding consensus sequence, 5′-GGATCCAGCGGGGCGAAGCGGGGGG-3′; and NF-κB binding consensus sequence, 5′-TATCAAATGGGAGTTTCCCAF-3′. Results were scanned and quantified using ImageJ software. The specificity of the NFAT EMSA probe was confirmed using 50× unlabeled probes or mutant probes or by preincubating with Abs for NFAT (c1 and c2) to block protein–probe interaction.
Migration assay

Migration of mast cells was evaluated using a Transwell migration assay (28). Briefly, a total of $1 \times 10^6$ BMMCs in 100 μl of RPMI 1640 containing 1% FBS was loaded onto each Transwell filter (8-μm pore filter Transwell, 24-well cell clusters; Costar, Boston, MA). Filters were placed in wells containing 600 μl RPMI 1640 containing 1% FBS, with or without 10 ng/ml SCF. After 4 h of incubation at 37°C in 5% CO2, the upper chamber was carefully removed, and the cells in the bottom chamber were resuspended and counted.

Statistics

Data are presented as mean ± SE of the indicated number of experiments. Statistical significance was determined by assessing means with a paired t test. Differences were considered significant at $p < 0.05$.

Results

Calcineurin-NFAT signaling is activated following SCF–c-Kit activation in a SCF dose-dependent manner

Calcineurin-NFAT signaling is essential in various biological responses (17–20). However, to the best of our knowledge, direct evidence of calcineurin activation in the SCF–c-Kit–signaling pathway has not been demonstrated previously. To determine whether calcineurin-NFAT is activated in c-Kit signaling, we tested calcineurin activity in BMMCs stimulated with various doses of SCF for 6 h. Treated BMMCs showed significantly enhanced calcineurin activity in a dose-dependent manner, peaking at 50 ng/ml SCF (Fig. 1A). To determine the effect of this SCF-induced enhancement of calcineurin activity on NFAT transcription factor activity, luciferase and EMRAs were used. NFAT activity was also significantly enhanced in BMMCs in a SCF dose-dependent manner (Fig. 1B, 1C, 1F). These results demonstrate that calcineurin-NFAT signaling is induced by SCF-mediated mast cell activation in a dose-dependent manner.

To examine whether SCF-mediated activation of the calcineurin-NFAT pathway is limited to mast cells or also occurs in other cells, we used bone marrow cells that contain progenitor cells that also express c-Kit (1). Bone marrow cells from C57BL/6 mice were incubated or not with SCF for 1 h. Nuclear protein was analyzed by EMRAs. NFAT activity was significantly enhanced in bone marrow cells in a SCF dose-dependent manner (Fig. 1D, 1G). These data suggest that SCF-mediated induction of calcineurin-NFAT signaling is not restricted to mature mast cells, but can also be achieved in bone marrow cells. The specificity of the NFAT EMRAs probe was confirmed using a series of standard competition assays (Fig. 1E).

To determine whether the SCF–calcineurin–NFAT pathway is limited to murine cells, the human mast cell line HMC-1 and primary CBMCs were used in this study. In the c-Kit D816V–mutated mastocytosis cell line HMC-1, c-Kit is constitutively phosphorylated and activated, rendering it insensitive to c-Kit–dependent stimulation (29). As expected, NFAT activity was higher in basal conditions and was unaffected in these cells following SCF stimulation, as determined using a luciferase reporter assay and EMRAs (Fig. 2A, 2B). However, when HMC-1 cells were treated with tyrosine kinase inhibitors PKC412 and/or dasatinib for 4 h, NFAT activity was reduced (Fig. 2C). Furthermore, when primary human mast cells (CBMCs) were stimulated with SCF, calcineurin activity (Fig. 2D) and NFAT activity (Fig. 2E) were significantly enhanced.

These data demonstrate that a previously unrecognized c-Kit–calcineurin–NFAT–signaling cascade exists that is activated by SCF-mediated c-Kit signaling. Importantly, this pathway is not limited to mast cells and appears to be more broadly activated in additional c-Kit–expressing cells.

Rcan1 deficiency leads to increased NFAT activity in response to SCF-dependent mast cell activation

Next, we determined how the c-Kit–calcineurin–NFAT pathway is regulated. The duration of SCF-mediated signaling is influenced by the phosphorylation, internalization, and degradation kinetics of c-Kit (12). The role of Rcan1 in these processes has not been examined previously. BMMCs from wild-type and Rcan1-deficient mice were starved for 2 h before incubation with cycloheximide for an additional 2 h to inhibit new protein synthesis. Cells were incubated or not with SCF (0–60 min), and whole-cell lysates were analyzed by Western blotting for phosphorylated or total c-Kit (Fig. 3A). In addition, a FITC-conjugated c-Kit Ab was used to detect cell surface expression of c-Kit following SCF activation (Fig. 3B).

No Rcan1-dependent effect on the phosphorylation state or internalization kinetics of c-Kit was observed. These results suggest that Rcan1 has no effect on SCF signaling at the c-Kit level.

To examine whether SCF-mediated activation of the calcineurin-NFAT pathway is regulated by Rcan1, Rcan1-deficient and wild-type BMMCs were stimulated with SCF for 6 h and then subjected to analysis for calcineurin activity. Although no differences were detected between samples from untreated wild-type and Rcan1-deficient cells, Rcan1-deficient cells displayed significantly increased calcineurin activity following stimulation with SCF (Fig. 3C), suggesting that Rcan1 is a negative regulator of calcineurin in the context of c-Kit activation in mast cells.

Calcineurin is a central regulator of NFAT nuclear translocation and transcriptional activation (30). To determine the effect of Rcan1 deficiency on NFAT activity, EMRAs and luciferase reporter assays were used. Nuclear protein from SCF-stimulated wild-type and Rcan1-deficient BMMCs analyzed by EMRAs showed that NFAT activity was significantly increased from 20 min to 1 h following SCF stimulation, after which it began to decrease in wild-type cells. However, the level of NFAT activation was significantly enhanced in Rcan1-deficient cells starting 60 min poststimulation, and it remained relatively elevated at later time points (Fig. 3D, 3E). To further confirm this effect on NFAT activation, Rcan1-deficient and wild-type BMMCs were transfected with an NFAT activity–reporting luciferase plasmid and were left untreated or stimulated with SCF for 5 h, at which point luciferase activity was assessed. Although no differences were observed between untreated wild-type and Rcan1-deficient cells following SCF stimulation, a significant induction of NFAT activity was detectable, and the level of induced NFAT activity was significantly higher in Rcan1-deficient cells compared with wild-type cells (Fig. 3F). Together, these results demonstrate that NFAT activation occurs in mast cells in response to SCF stimulation and that this activation is negatively regulated by Rcan1.

Increased activation of the NF-κB pathway in Rcan1-deficient BMMCs in response to SCF stimulation

In addition to NFAT, NF-κB is a major transcription factor that regulates mast cell function (31). To examine the effect of Rcan1 deficiency on the NF-κB pathway following SCF stimulation, we analyzed the phosphorylation levels of IkBα, a potent inhibitor of NF-κB activity. Upon activation, IkBα is targeted for degradation following phosphorylation, freeing NF-κB for nuclear translocation. Western blot analysis of SCF-stimulated wild-type and Rcan1-deficient BMMCs showed that IkBα is phosphorylated within 5 min of SCF stimulation and degraded within 20 min poststimulation in Rcan1-deficient mast cells, whereas weaker phosphorylated IkBα can be detected at the same time point in wild-type BMMCs (Fig. 4A, 4B). IkBα levels rebounded very quickly, returning to baseline levels as early as 40 min following SCF stimulation.

Previous reports indicated that calcineurin may regulate NF-κB activity at the level of NF-κB–IkB interactions (32). Thus, Rcan1 may regulate NF-κB via a calcineurin-dependent mechanism. Conversely, Rcan1 was shown to regulate the IkB–NF-κB pathway via a calcineurin-independent mechanism (33). To better understand whether the observed effects on IkB kinetics in Rcan1-deficient cells
were calcineurin dependent or independent, wild-type and Rcan1-deficient cells were treated with cyclosporine A, a potent inhibitor of calcineurin activity. BMMCs were preincubated or not with cyclosporine A for 20 min and then treated with SCF for 5 min, because this was the time point at which maximal IκB phosphorylation was observed previously. Interestingly, IκBα phosphorylation was strongly blocked by cyclosporine A, suggesting that Rcan1 regulates IκB–NF-κB activation primarily through a calcineurin-dependent mechanism (Fig. 4C).

We next assessed the impact of IκBα hyperphosphorylation following SCF stimulation in Rcan1-deficient cells on NF-κB transcriptional activity. Nuclear proteins from BMMCs, which were stimulated or not with SCF for various times (0–360 min), were subjected to EMSA using a 32P-labeled NF-κB probe (Fig. 4D, 4E). Wild-type BMMCs did not show significant changes in NF-κB activation in response to SCF stimulation, suggesting that there is little or no activation of NF-κB in response to SCF stimulation in mast cells. However, in the case of Rcan1-deficient cells, NF-κB activation was observed at 20 min and reached a maximum at 60 min poststimulation. NF-κB activity was further confirmed in Rcan1-deficient and wild-type BMMCs using a luciferase reporter assay. Wild-type and Rcan1-deficient BMMCs were transfected with an NF-κB luciferase reporter plasmid and an internal control plasmid. Cells were left untreated or were activated with SCF for 5 h, and luciferase activity was assessed. No differences were observed between untreated wild-type and Rcan1-deficient cells. However, following SCF stimulation there was significantly more NF-κB activity detected in Rcan1-deficient cells compared to untreated cells, suggesting that Rcan1 regulates NF-κB activation through a calcineurin-dependent mechanism.
cells compared with wild-type cells, which did not show a significant induction of NF-κB activity compared with untreated controls (Fig. 4F). Together, these results suggest that Rcan1 is a negative regulator of NF-κB activation in response to SCF stimulation in BMMCs. SCF-induced NF-κB activation in Rcan1-deficient cells is aberrant, because NF-κB is not significantly activated by SCF in wild-type mast cells.

**Rcan1 deficiency does not affect MAPK-signaling pathways in SCF-mediated mast cell activation**

The MAPK-signaling pathways are activated in response to SCF stimulation (7). To assess the role of Rcan1 during SCF-mediated MAPK signaling, cell lysates from SCF-stimulated BMMCs were subjected to Western blotting for phospho-JNK, phospho-p38, and phospho-ERK1/2, as well as their total protein levels. As seen in Fig. 4G, SCF stimulation induced an increase in the phosphorylation of ERK1/2, p38, and JNK within 5–20 min, which diminished thereafter. The activation of the MAPK pathways was unaltered between wild-type and Rcan1-deficient BMMCs following SCF stimulation (Fig. 4G). PI3K pathway activation was also examined via Western blot analysis for phospho-Akt, an indicator of PI3K activation. Akt was rapidly phosphorylated following SCF stimulation and remained activated throughout the course of the experiment. However, no differences were observed between wild-type and Rcan1-deficient BMMCs (Fig. 4G, Supplemental Fig. 1). These results suggest that SCF-mediated activation of the MAPK and PI3K pathways occurs in an Rcan1-calcineurin-independent manner.

**FIGURE 2.** SCF–c-Kit–calcineurin–NFAT signaling exists in human mast cells. (A) HMC-1 (5C6) cells were cotransfected with pNFAT-Luc and the control reporter plasmid pRL-TK. After transfection (24 h), cells were either left untreated or stimulated with the indicated concentration of SCF for 5 h. Firefly and Renilla activities were quantified sequentially using a dual-luciferase reporter assay system. Data are mean ± SEM (n = 4 independent experiments). (B, C, and E) NFAT binding consensus sequence was labeled with [32P] to create a probe for EMSA. HMC-1 (5C6) cells were either stimulated with the indicated concentrations of SCF or not treated (NT) (B) or were treated with tyrosine kinase inhibitors PKC412 and/or Dasatinib (C). (E) CBMCs were either stimulated with 100 ng/ml SCF or not treated (NT). Nuclear proteins were isolated and subjected to EMSA. Shown is a representative exposure from three independent experiments. (D) CBMCs were treated with 100 ng/ml SCF for 6 h or left untreated. Cells were lysed, and calcineurin activity was analyzed using a calcineurin-activity assay.
Rcan1 deficiency affects c-Kit–mediated mast cell IL-6 and TNF production

To examine whether Rcan1 regulates SCF-mediated cytokine production, BMMCs from wild-type and Rcan1-deficient mice were stimulated with SCF (1–24 h) or left untreated. Cell-free supernatants were subjected to analysis for IL-6 (Fig. 5A) and TNF (Fig. 5B) by ELISA. Production of both cytokines was significantly enhanced in Rcan1-deficient cells compared with wild-type BMMCs. These results suggest that SCF-induced mast cell activation is modulated negatively by Rcan1.

According to the study by Iwaki et al. (13), SCF–c-Kit signaling can induce cytokine production alone or it can enhance Ag-IgE–mediated cytokine production through a BTK-dependent pathway. To determine whether the c-Kit/IgE–BTK–cytokine pathway is regulated by Rcan1, wild-type and Rcan1-deficient BMMCs were stimulated with SCF and/or TNP-BSA. Cytokine production and BTK phosphorylation were examined. Increased production of IL-6 and TNF was detectable by BMMCs stimulated through both receptors (Supplemental Fig. 2). BTK was rapidly phosphorylated following SCF stimulation alone or in combination with Ag-IgE activation. However, no differences were observed between wild-type and Rcan1-deficient BMMCs. These data suggest that Rcan1-regulated cytokine production is BTK independent.

A major biological function of SCF is to promote mast cell survival and proliferation (4). To confirm that the SCF-induced enhancement of IL-6 and TNF production in Rcan1-deficient BMMCs was directly due to Rcan1-mediated c-Kit–signaling events, and not due to differences in proliferation, we set out to study the effect of Rcan1 deficiency on c-Kit–induced mast cell proliferation and survival. To this end, BMMCs were starved for 4 h and then incubated in RPMI 1640 containing 0.2% BSA, with or without SCF, for an additional 48 h. Cell survival was examined by FACS-based FITC–annexin V and 7-AAD staining (Supplemental Fig. 4A). Alternatively, BMMCs were cultured in BMMC complete medium without IL-3. Cell survival was tested by counting the total cell number (Supplemental Fig. 4B) or using the WST-1 method (Supplemental Fig. 4C) from culture days 1–4. To examine proliferation, BMMCs were starved for 4 h, and cells were seeded in RPMI 1640 supplemented with 10% FBS in the absence or presence of SCF (0–100 ng/ml) for an additional 48 h. Cell proliferation was tested by [3H]thymidine incorporation (Supplemental Fig. 4D). No differences in SCF-mediated cell survival or proliferation were observed between wild-type and Rcan1-deficient
BMMCs, indicating that Rcan1 is not essential in these contexts and supporting a role for SCF–c-Kit–mediated Rcan1 signaling as a direct negative regulator of IL-6 and TNF production in SCF-stimulated BMMCs.

SCF is a potent migration factor. To determine whether SCF-induced mast cell migration is regulated by Rcan1, migration was assayed in a Transwell chamber device, with a pore size of 8 μm, in medium containing SCF as a chemoattractant. Migrated cells were assessed after 4 h (Supplemental Fig. 4E). No differences in SCF-mediated cell migration were observed between wild-type and Rcan1-deficient BMMCs, indicating that Rcan1 is not essential in SCF-induced mast cell migration.

**FIGURE 4.** Rcan1 deficiency induces increased SCF-mediated IκBα phosphorylation and NF-κB activation. (A) BMMCs were stimulated with SCF (100 ng/ml) for various times. Using Western blot analysis, increased IκBα phosphorylation in Rcan1-deficient BMMCs was found at 5 min after SCF stimulation compared with wild-type cells. Similar results were observed in three independent experiments. (B) Densitometry analysis of phosphorylated IκBα normalized to total IκBα. Data are expressed as mean ± SEM (n = 3). (C) BMMCs were preincubated or not with 1 μg/ml cyclosporine A for 20 min at 37°C and then treated with 100 ng/ml SCF for 5 min. Cell lysates were analyzed by Western blot for IκBα phosphorylation. Cyclosporine A suppressed IκBα phosphorylation. Similar results were observed in two independent experiments. (D) NF-κB–binding consensus sequence was labeled with [32P] for use as a probe in EMSA. BMMCs were either stimulated with SCF (100 ng/ml) for the indicated times or not treated (NT). Nuclear proteins were isolated and analyzed by EMSA. Shown is a representative from three independent experiments. (E) Densitometry analysis of NF-κB activation by EMSA was performed based on three experiments. (F) BMMCs were cotransfected with pNF-κB–Luc and the control reporter plasmid pRL-TK. After transfection (24 h), cells were either left untreated (NT) or stimulated with SCF (100 ng/ml) for 5 h. Firefly and Renilla activities were quantified sequentially using a dual-luciferase reporter–assay system. Data are mean ± SEM (n = 5 independent experiments). (G) Rcan1 deficiency does not affect MAPK or Akt signaling. BMMCs were stimulated with SCF (100 ng/ml) for various times. Total cell lysates were analyzed by Western blot for the indicated phosphorylated and total proteins. The pattern of SCF-induced phosphorylation of p38, JNK, ERK, and Akt is similar between wild-type and Rcan1-deficient BMMCs. Shown is a representative from three independent experiments. *p < 0.05, **p < 0.01.
The Egr1-Rcan1 regulatory-feedback interaction is a feature of SCF signaling in mast cells

Our previous research suggests that IgE-mediated Egr1 activation promotes Rcan1 expression (24) and that Egr1 is strongly induced in response to SCF stimulation (34). Thus, we hypothesize that SCF-induced Egr1 regulates Rcan1. To examine the expression kinetics of Egr1 and Rcan1 following SCF stimulation, wild-type BMMCs were stimulated with SCF for various times. Egr1 and Rcan1 expression was examined by real-time quantitative PCR (qPCR). Egr1 expression peaked at 30 min following SCF stimulation, whereas Rcan1 expression followed Egr1 expression, peaking at 60 min poststimulation (Fig. 6A, 6B). The sequential expression of Egr1 and Rcan1 suggests that SCF-induced Egr1 may be used to drive Rcan1 expression. To confirm that SCF-mediated Egr1 regulates Rcan1 expression, mRNA isolated from SCF-stimulated wild-type and Egr1-deficient BMMCs was used in qPCR analysis (Fig. 6C). Rcan1 expression was significantly decreased, but not ablated, in Egr1-deficient BMMCs following SCF stimulation. These data suggest that Egr1 is required for SCF-induced Rcan1 expression.

It has not been reported whether Rcan1 regulates Egr1 expression. We examined Egr1 expression in SCF-stimulated wild-type or Rcan1-deficient BMMCs by qPCR or EMSA. Interestingly, SCF-induced Egr1 mRNA expression was significantly increased in Rcan1-deficient BMMCs (Fig. 7A). SCF-induced Egr family transcription factor activity was enhanced in the nucleus in Rcan1-deficient BMMCs (Fig. 7B, 7C). These data suggest that Rcan1 is an inhibitor of Egr1 expression in mast cells. Together, our results suggests that, in the context of mast cell SCF signaling, Egr1 and Rcan1 form a feedback loop through which expression of Egr1 induces Rcan1 expression, which then feeds back to inhibit transcription of Egr1 (Fig. 8).

Discussion

The SCF receptor, c-Kit, is expressed on many nondifferentiated hematopoietic cells, including myeloblasts, promonoblasts, and terminally differentiated mast cells (1). Activation of c-Kit by SCF is essential for cell development and function. SCF-induced activating signals are turned off by subsequent inhibitory signals (6). These SCF-induced activating and subsequent inhibiting signals are highly ordered sequential molecular events critical for maintaining cellular homeostasis (6). Despite intense investigations, the number of molecules implicated in turning off c-Kit signaling is limited. Protein kinase C (35, 36) and SHP1 (37) are involved in the negative-feedback loop of c-Kit signaling through direct phosphorylation or dephosphorylation of c-Kit, respectively. SOCS-1 is induced by SCF and binds to c-Kit, leading to selective suppression of SCF-stimulated cell proliferation and enhancement of cell survival (38). Similarly, SPRED-1 suppresses SCF-mediated cell pro-
performed based on three experiments. *Experiments. (B) BMMCs were either stimulated with SCF (100 ng/ml) for the indicated times or not treated (NT). Nuclear proteins were isolated and analyzed by EMSA. BMMCs were either stimulated with SCF (100 ng/ml) for 1 h. RNA was isolated and analyzed by qPCR for GAPDH. The data are expressed as relative mRNA levels compared with the mean expression level in wild-type BMMCs treated with SCF for 60 min (= 1). Error bars represent SEM from four independent experiments. (C) Egr1-binding consensus sequence was labeled with \[^{32}P\] for EMSA. BMMCs were either stimulated with SCF (100 ng/ml) for the indicated times or not treated (NT). Nuclear proteins were isolated and subjected to EMSA. Shown is a representative from three independent experiments. (D) Densitometry analysis of Egr1 activation by EMSA was performed based on three experiments. *p < 0.05.

**FIGURE 7.** Rcan1 suppresses Egr1 expression in response to SCF-mediated BMMC activation. (A) BMMCs were either not treated (NT) or stimulated with SCF (100 ng/ml) for 1 h. RNA was isolated and analyzed by qPCR for Egr1. Egr1 expression was normalized to endogenous control (GAPDH). The data are expressed as relative mRNA levels compared with the mean expression level in wild-type BMMCs treated with SCF for 60 min (= 1). Error bars represent SEM from four independent experiments. (B) Egr1-binding consensus sequence was labeled with \[^{32}P\] for EMSA. BMMCs were either stimulated with SCF (100 ng/ml) for the indicated times or not treated (NT). Nuclear proteins were isolated and subjected to EMSA. Shown is a representative from three independent experiments. (C) Densitometry analysis of Egr1 activation by EMSA was performed based on three experiments. *p < 0.05.

Liberation via inhibition of ERK signaling (39). In contrast, Rab10 enhances SCF-induced cell proliferation and suppresses cell survival, likely through inhibition of Ras activation (12). Rab10 does not affect SCF-induced cell migration or adhesion (12). It appears that distinct inhibitory molecules of the SCF-induced signaling pathway are associated with specific biological functions via specific mechanisms, yet the scope of our understanding of these specific mediators remains limited. In this study, we report that Rcan1 is a novel regulator of SCF-mediated mast cell activation through inhibition of a novel pathway involving c-Kit–calcineurin signaling.

SCF mediates a wide range of biological events through activation of c-Kit in hematopoietic progenitor cells, mast cells, and cancer cells. However, prior to this study, there was no direct evidence suggesting that calcineurin is involved in SCF–c-Kit–mediated signaling events. In this study, we demonstrated that, following SCF-mediated activation of mast cells, calcineurin activity is significantly enhanced and results in increased NFAT activity. We further demonstrated that SCF also stimulates the calcineurin-NFAT activity in bone marrow cells. The mastocytosis cell line HMC-1 harbors c-Kit V560G and D816V mutations, leading to constitutive phosphorylation and activation of the c-Kit pathway (29). We found that HMC-1 cells showed constitutive NFAT pathway activation. Thus, it is likely that the SCF-mediated calcineurin-NFAT–activation pathway is a universal mechanism in SCF signaling. Considering the broad biological and pathological roles of SCF, the significance of the SCF-mediated calcineurin-NFAT–signaling pathway identified in this study may extend beyond mast cells.

Cyclosporine A and FK506 are calcineurin inhibitors and have been used as tools for the study of calcineurin in a range of cells, including mast cells (40). Previously, it was reported that cyclosporine A did not affect SCF-induced MAPK activation in mast cells (40). Consistent with this finding, we reported in this article that Rcan1 deficiency does not affect SCF-induced MAPK activation. Although we (24) and other investigators (40) showed a role for calcineurin in IgE-mediated signaling in mast cells, and IgE-mediated calcineurin is downregulated by Rcan1 (24), a role for Rcan1 in SCF signaling was not reported previously. In the current study, we demonstrate that, following SCF–c-Kit stimulation in mast cells, Rcan1 is rapidly activated by 15 min post-stimulation, with peak expression occurring at 60 min. Deficiency of Rcan1 enhanced SCF-induced calcineurin activity, as well as NFAT and IκBα–NF-κB pathway activation. Thus, we propose that SCF-induced Rcan1 expression, in turn, suppresses calcineurin activity, inhibits NFAT activation, and stabilizes the inhibitory IκBα–NF-κB complex, preventing activation of NF-κB following SCF stimulation. Hence, Rcan1 coordinates a negative-feedback loop to turn off SCF-mediated activating signals.

We further investigated mechanisms involved in the regulation of Rcan1 in mast cells. Egr1-deficient mast cells showed impaired Rcan1 expression following SCF stimulation, suggesting that Egr1 promotes Rcan1 expression in the context of c-Kit signaling. In contrast, SCF-induced Egr1 expression and activity are enhanced in Rcan1-deficient mast cells, suggesting that Rcan1 suppresses Egr1

**FIGURE 8.** A working model of Rcan1-dependent downstream signaling in SCF-mediated mast cell activation. Mast cells are activated by SCF cross-linking of c-Kit receptor on the plasma membrane (PM). Subsequent rapid activation of calcineurin is required for IκBα–NF-κB, NFAT, and Egr1 activation. These activated transcription factors regulate cytokine production, and both NFAT and Egr1 can regulate Rcan1 expression. Finally, Rcan1 binds and inhibits calcineurin activity, completing the regulatory-feedback loop. NM, Nuclear membrane.
It is a known fact that activation of the c-Kit receptor by its ligand, stem cell factor (SCF), induces a signaling cascade that leads to the activation of multiple downstream pathways, including the PI3K/Akt and MAPK/ERK pathways. The phosphorylation of the c-Kit receptor by SCF leads to the recruitment of Grb2/SOS, which in turn activates the Ras/MAPK pathway. Additionally, the binding of SCF to c-Kit recruits the lipid kinase PI3K, leading to the activation of the PI3K/Akt pathway. These pathways are essential for the proliferation, survival, and differentiation of hematopoietic cells.

In the context of hematopoiesis, the c-Kit receptor is expressed on the surface of hematopoietic stem cells and progenitor cells. The ligand SCF binds to c-Kit with high affinity, leading to the activation of the receptor and the subsequent activation of signaling pathways. The activation of these pathways results in the proliferation, differentiation, and survival of hematopoietic cells.

The signaling pathways downstream of c-Kit activation are highly conserved among different hematopoietic cell types. For example, the activation of the PI3K/Akt pathway promotes cell survival and proliferation, while the activation of the MAPK/ERK pathway regulates cell cycle progression and differentiation.

In conclusion, the activation of c-Kit by SCF results in the recruitment of multiple signaling pathways, including PI3K/Akt and MAPK/ERK, which are essential for the proliferation, survival, and differentiation of hematopoietic cells. These pathways are conserved among different hematopoietic cell types and are crucial for the maintenance of hematopoiesis.

References: