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Swiprosin-1 Is Expressed in Mast Cells and Up-Regulated Through the Protein Kinase CβI/η Pathway

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ABSTRACT

Swiprosin-1 exhibits the highest expression in $CD8^+$ T cells and immature B cells and has been thought to play a role in lymphocyte physiology. Here we report that swiprosin-1 is also expressed in mast cells and up-regulated in both in vitro cultured mast cells by phorbol ester and in vivo model tissues of passive cutaneous anaphylaxis and atopic dermatitis. Targeted inhibition of the specific protein kinase C (PKC) isotypes by siRNA revealed that PKC-βI/η are involved in the expression of swiprosin-1 in the human mast cell line HMC-1. In contrast, down-regulation of swiprosin-1 by A23187 or ionomycin suggests that calcium-signaling plays a negative role. The ectopic expression of swiprosin-1 augmented PMA/A23187-induced NF-κB promoter activity, and resulted in increased expression of cytokines. Moreover, knock-down of swiprosin-1 attenuated PMA/A23187-induced cytokine expression. Collectively, these results suggest that swiprosin-1 is a PKC-βI/η-inducible gene and it modulates mast cell activation through NF-κB-dependent pathway. J. Cell. Biochem. 108: 705–715, 2009.

KEY WORDS: MAST CELLS; SWIPROSIN-1; PROTEIN KINASE C; CALCIUM

Swiprosin-1 was first identified in human lymphocytes, predominantly in $CD8^+$ lymphocytes [Vuadens et al., 2004] and later in immature B cells, resting and activated B cells, and non-lymphoid tissue, especially in the brain [Avramidou et al., 2007]. Database mining revealed that swiprosin-1 putatively contains four myristylation sites, three binding sites for SH3 domain containing proteins, two potential EF-hand domains, and a coiled-coil domain at the C-terminus, and therefore, may have a role as a small adaptor protein involved in calcium signaling [Vuadens et al., 2004]. In accordance with this prediction, swiprosin-1 was implicated in phosphotyrosine-based signaling events involved in the cellular stimulation of early growth factor (EGF) and in actin rearrangement [Blagoev et al., 2004].

However, the functions of swiprosin-1 are still largely unknown. The only reported functions are that swiprosin-1 is associated with lipid rafts in the immature B-cell line WEHI231 and that it participates in enhancement of BCR signals and contributes to BCR-induced apoptosis [Mielenz et al., 2005; Avramidou et al., 2007]. Regulation of swiprosin-1 expression and potential signaling pathways involved in swiprosin-1 expression also have not been determined yet. Avramidou et al. [2007] reported that swiprosin-1 is expressed throughout B-cell differentiation, with the highest expression in immature bone marrow B cells. However, they demonstrated that the level of swiprosin-1 is not changed during B-cell activation when stimulated with IgM $F(ab)_2$/IL-4, LPS, or anti-CD40/IL-4 [Avramidou et al., 2007].

Mast cells are broadly distributed throughout mammalian tissues and play a critical role in a variety of biological responses [Metzger et al., 1986; Kemp and Lockey, 2002; Rivera and Gilfillan, 2006]. Typically, mast cells are considered in association with immediate-type hypersensitivity [Kemp and Lockey, 2002]. However, several recent reports have provided evidence for the possible participation of mast cells in more persistent, and even in chronic, inflammatory and immunological, responses [Church and Levi-Schaffer, 1997; Metcalfe et al., 1997]. Of note, a variety of cytokines including IL-3, IL-4, IL-5, IL-6, IL-8, TNF-α, and IFN-γ [Plaut et al., 1989; Wodnar-Filipowicz et al., 1989; Gordon and Galli, 1990] are produced in mast cells and play an important role in immunological processes other

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than IgE-mediated hypersensitivity reactions. Until recently, however, only a limited number of reports have examined the regulatory mechanism of cytokine expression in mast cells.

As part of genome-wide approaches to finding novel genes that may be involved in mast cell activation, we performed high-density oligonucleotide microarrays and, interestingly, found that swiprosin-1 is over-induced in the human mast cell line HMC-1 stimulated with PMA/A23187 (Fig. 1). This result led us to examine whether swiprosin-1 plays a role in mast cell biology. In the present study, we therefore first examined whether swiprosin-1 is expressed in vitro in cultured mast cells and in vivo in model tissues of passive cutaneous anaphylaxis and atopic dermatitis. Second, we investigated whether and how swiprosin-1 expression is regulated in mast cells. A variety of pharmacologic agents and small interfering RNAs (siRNA) were employed to specifically determine which intracellular signaling pathways are involved in regulation of swiprosin-1 expression in mast cells. Finally, we asked whether swiprosin-1 potentially modulates mast cell activation.

HMC-1 is a cell line that was established from a patient with mast cell leukemia, and HMC-1 cells exhibit many characteristics of immature mast cells [Butterfield et al., 1988]. Although these cells do not express cell surface FcεRI and, therefore, cannot be activated by antigen, they can be activated by treatment with phorbol esters and calcium ionophore [Alizadeh et al., 1986; Burd et al., 1989]. These cells can, therefore, serve as a useful system for examining mast cell expression of human cytokines or chemokines. The results presented here strongly demonstrate that swiprosin-1 is expressed in human mast cells and potentially acts as a regulator for cytokine expression.

MATERIALS AND METHODS

ANTIBODIES AND REAGENTS

Goat polyclonal antibody to swiprosin-1 was from Imgenex (San Diego, CA). Antibodies to protein kinase C (PKC)-α, PKC-βI, PKC-η, PKC-ζ, actin, and I-κBα were from Santa Cruz Biotechnology, Inc.

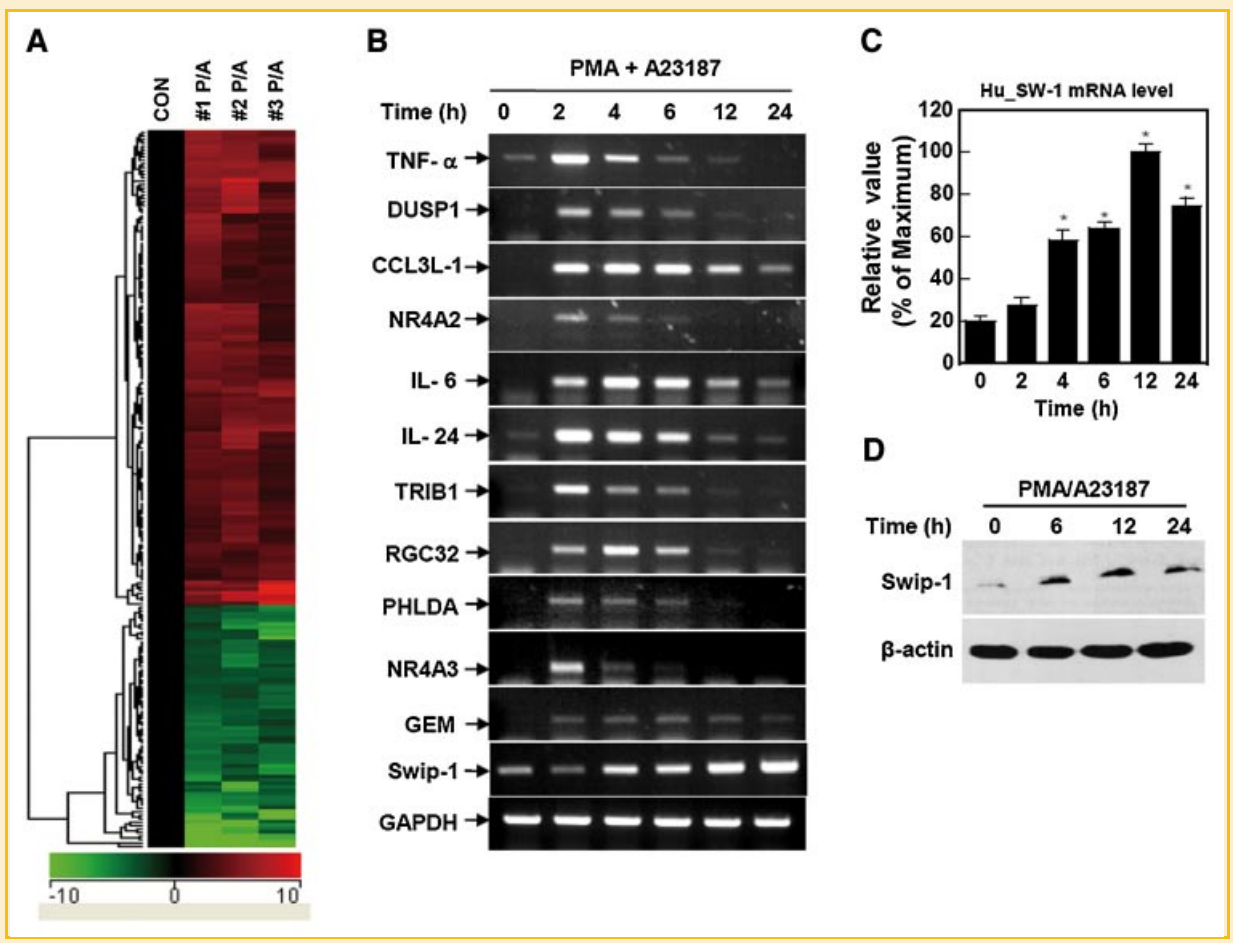

Fig. 1. Dendrogram of gene expression profiles and results of RT-PCR and Western blot. A: HMC-1 cells (1×10^7) were stimulated for 4 h with or without PMA (200 nM)/A23187 (1 μM). Microarray and data analysis were carried out as described in Materials and Methods Section. CON indicates the mean value of triplicate experiments; #1–3 P/A, triplicates of PMA/A23187-treated cells. The green blocks represent down-regulated genes and the red blocks represent up-regulated genes. B: HMC-1 cells (1×10^6) were stimulated with PMA (200 nM)/A23187 (1 μM) at various time points, and then mRNA levels of indicated genes were analyzed by RT-PCR. Amplification of GAPDH was used as an internal control. Data represent two separate experiments. C,D: The expression of swiprosin-1 was also determined by quantitative RT-PCR (C) and Western blot (D). The data shown in the bar graphs represent the mean ± SD of three independent experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

(Santa Cruz, CA). Antibodies to PKC-δ, and PKC-θ were from Cell Signaling Technology, Inc. (Beverly, MA). HRP-conjugated anti-goat, anti-rabbit, and anti-mouse IgGs were from GE Healthcare (Chalfont St. Giles, United Kingdom). Gö6983, Gö6976, rottlerin, and staurosporin were purchased from Calbiochem-Behring (La Jolla, CA). Total RNA isolation reagent was from WelPrep™ Join Bio Innovation (Daegu, South Korea). Maxime RT Premix (oligo dT primer), Maxime PCR PreMix, and a plasmid purification kit were from iNtRON Biotechnology (Daejon, South Korea). SYBR premix Ex Taq was from Takara Bio Inc. (Shiga, Japan). The dual-luciferase reporter assay system was from Promega Corporation (Madison, WI). Small interfering RNA (siRNA) targeting swiprosin-1, PKC isotypes, and a scrambled siRNA were obtained as a pool of four or more siRNA duplexes from Dharmacon (Chicago, IL). All other reagents used in this study were purchased from Sigma Chemical Co. (St. Louis, MO).

CELL CULTURE

HMC-1 cells were cultured in IMDM medium supplemented with 10% heat inactivated FBS, 100 units/ml penicillin G, and 100 μg/ml streptomycin.

DNA MICROARRAY ANALYSIS

dUTP-labeled cDNA was amplified from total RNA samples (5 μg/sample) by RT-IVT Labeling Reagents (Applied Biosystems, Boston, MA). Fifty micrograms of labeled cDNA were hybridized on a microarray chip containing oligonucleotide probes for 33,096 human genes (Applied Biosystems) and further processed according to the Applied Biosystems protocol.

Microarray images were acquired with an AB 1700 microarray analyzer (Applied Biosystems). Signal quantification and data processing were performed using AVADIS (Strand Genomics, Bengaluru, India). Each experiment was repeated three times to reduce the risk of false-positive or false-negative results. Significant differences in gene expression between the data obtained from the two different groups (control, PMA/A23187) were identified using the one-way ANOVA Kruskal–Wallis Test with a significance threshold of $P < 0.05$. The K-means clustering method was used to cluster those genes selected by the above test. To estimate fold induction, the ratios of treated- to untreated-signal intensities were calculated. The log 2 of each ratio was determined to equalize the magnitude of deflection of up-regulated and down-regulated genes, and the differences in gene expression were ranked based on absolute values. Among the 1,467 genes that were filtered by a significant test ($P < 0.05$), 208 were differentially expressed at least threefold in response to treatment with PMA/A23187 and displayed by dendrogram (Fig. 1A). The green blocks represent the down-regulated genes, the red blocks represent up-regulated genes, and the black blocks represent the genes that were not altered relative to the control. Semiquantitative RT-PCR was performed to validate the gene expression data from microarray analysis. Eleven genes that are not well characterized but were highly expressed in mast cells by PMA/A23187 are shown in Figure 1B. Amplification of GAPDH and hTNF-α were used as an internal control and an activated mast cell marker, respectively.

ANIMALS

The original stock of male ICR mice and Balb/c J mice (6–8 weeks old) were purchased from the Dae-Han Experimental Animal Center (Daejeon, Korea) and SLC Co (Hamamatsu, Japan), respectively. The animals were housed 5–10 per cage in a laminar air flow room (conventional conditions) and maintained at a temperature of $22 \pm 2^{\circ}C$ with a relative humidity of $55 \pm 5\%$ throughout the study. The care and treatment of the mice were in accordance with the guidelines established by the Public Health Service Policy on the Humane Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee in GIST.

PASSIVE CUTANEOUS ANAPHYLAXIS (PCA) REACTION

An IgE-dependent cutaneous reaction was examined as previously described [Kim et al., 2005b]. Briefly, the mice were injected intradermally with 0.5 μg of anti-DNP IgE. After 48 h, each mouse was injected via the tail vein at indicated time intervals with 1 μg of DNP-HSA containing 4% Evans blue (1:4). Control mice were injected with 1× PBS. After indicated times of challenge, the mice were killed; the dorsal skin was removed and used for the evaluation of swiprosin-1 mRNA expression.

INDUCTION OF ATOPIC ECZEMA/DERMATITIS SYNDROME (AEDS)-LIKE SKIN LESIONS

Induction of AEDS-like skin lesions using mite antigen was performed according to the previously established method [Kang et al., 2006] with several modifications. Briefly the dorsal surfaces of both ears (Balb/c J mice) were tape-stripped three times; 30 min later, 20 μl of 1% di-nitro-chlorobenzene (DNCB) dissolved in acetone/olive oil solution (acetone:olive oil = 1:3) was painted onto the surface of each ear. After 4 days of DNCB painting, 20 μl of 10 mg/ml of mite extracts (Yonsei University, Seoul, Korea) was re-painted onto the surface of each tape-stripped ear. The tape-stripping, and DNCB and mite antigen painting were conducted one time per week for 4 weeks. The PBS solution was painted on instead of the DNCB and mite antigen solution in control mice. On day 21, mice were sacrificed; the ears were removed and used for the evaluation of swiprosin-1 mRNA expression and histological observations.

HISTOPATHOLOGY

To assess the PCA, images of the dorsal skin, stained with Evans blue dye, were taken with a simple camera. To assess AEDS-like skin lesions, the ears were fixed with 10% formaldehyde, embedded in paraffin, and thin sections (10–20 μm) were cut. The skin sections were stained with hematoxylin and eosin, and images were taken under the microscope (Olympus; magnification, 200×).

RECOMBINANT DNA CONSTRUCTS

To generate the swiprosin-1/pEGFP-C1 construct, the human swiprosin-1 clone coding for the full-length open reading frame of swiprosin-1 in the pOTB7 vector was purchased from RZPD German Resource Center (Berlin, Germany). This was used as a template, and a PCR amplification was performed using the primers: sense 5′-AAGAATTCTATGGCCACGGACGAGCTGGCCACC-3′ containing the *Eco*RI restriction site and anti-sense 5′-TTTGGATCCC-

TACTTAAAGGTGGACTGCAGCTC-3′ containing the *Bam* HI restriction site. The PCR product was subcloned as a *Eco*RI/*Bam* HI fragment into a pEGFP-C1 vector (Clontech Laboratory, Inc.) of a neomycin resistant gene, resulting in an in-frame fusion of swiprosin-1 to the COOH terminus of GFP. The amino acid sequences of a linker polypeptide between EGFP and swiprosin-1 were SGLRSRAQAS (10 aa). To generate wild-type swiprosin-1 in a pHJ-1 vector (lentiviral vector), swiprosin-1 cDNA from the pOTB7 vector was transferred into the pHJ-1 vector by Not I restriction digestions and confirmed by automated sequencing.

ESTABLISHMENT OF STABLE CELL LINES

Stable HMC-1 transfectants were established using the Nucleofector device and corresponding kits (Amaxa, Cologne, Germany) by introducing pEGFP-C1 or swiprosin-1/pEGFP-C1 cDNA followed by selection with 1 mg/ml geneticin (Invitrogen). The cells were then subjected to a FACS and sorted to selectively obtain GFP-positive cells. They were then cultured in complete medium supplemented with the same concentrations of antibiotics. The stable cells that expressed EGFP or EGFP fused with swiprosin-1 were designated as H-GFP cells and H-swip-1_GFP cells, respectively.

LENTIVIRAL INFECTION

Lentiviral vector (10 μg of pHJ-1 or swiprosin-1/pHJ-1) with the appropriate insert (1 μg of pHDM-Hgpm2, 1 μg of pRC/CMV-Rev1b, and 3 μg of pHDM.G) were transfected into 293-T cells using the lipofectamine 2000 (Invitrogen). The culture supernatants were collected 48 h after transfection. The supernatants were pooled, concentrated by ultracentrifugation, and stored at −80°C until use. For lentiviral infection, virus particles were mixed with HMC-1 cells (2×10^5/500 μl) in a 15-ml centrifuge tube in the presence of 8 μg/ml polybrene. Following centrifugation at 2,000 rpm for 1 h, the cells were transferred into a 60-mm culture dish and infection efficiency was checked by Western blot after 48 h of infection.

RNA ISOLATION AND RT-PCR

Cells from the tissue samples or in vitro cultures were harvested and total RNA was isolated using the WelPrep™ JBI method (iNtRON Biotechnology) according to the manufacturer's instructions. Reverse transcription of the RNA was performed using oligo dT primer Maxime RT-PCR PreMix (iNtRON Biotechnology). Two micrograms of RNA was transferred to an oligo dT primer mixture tube. The reaction volume was 20 μl. cDNA synthesis was performed at 45°C for 60 min, followed by RT inactivation at 95°C for 5 min. Thereafter, the RT-generated DNA was diluted to 40 μl volume with distilled water. The diluted RT-generated DNA (2 μl) was amplified using Maxime PCR PreMix (iNtRON Biotechnology). The primers used for cDNA amplification were as follows: Swip-1, sense 5′-ATCTTCCGCAAGGCGGCGGCCGGGGAG-3′ and antisense 5′-GACTGCAGCTCCTTGAAGGCCGCTTTC-3′; hIL-3, sense 5′-CTTTGCCTTTGCTGGACTTC-3′ and antisense 5′-CGAGGCTCAAAGTCGTCTG-3′; hIL-8, sense 5′-GTGCAGTTTTGCCAAGGAGT-3′ and antisense 5′-CTCTGCACCCAGTTTTCCTT-3′; GAPDH, sense 5′-CGGAGTCAACGGATTTGGTCGTAT-3′ and antisense 5′-AGCCTTCTCCATGGTGGTGAAGAC-3′. Amplification conditions were denaturation at 94°C for 30 s, annealing at 58–68°C for 20 s, and extension at 72°C for 40 s for 30–35 cycles. The PCR products were resolved and visualized on a 1 or 1.5% agarose gel and stained with ethidium bromide.

REAL-TIME QUANTITATIVE RT-PCR

In all the experiments, the expression levels of the examined genes were evaluated by real-time RT-PCR, unless otherwise indicated. PCR amplification was performed in DNA Engine Opticon for a continuous fluorescence detection system (MJ Research, Waltham, MA) in a total volume of 20 μl containing 2 μl of cDNA/control and gene specific primers using the SYBR premix Ex Taq kit (Takara, Shiga, Japan). The PCR was performed under the following conditions: 94°C for 30 s, 58–68°C for 30 s, 72°C for 30 s, plate read (detection of fluorescent product) for 40 cycles, followed by 7 min of extension at 72°C. A melting curve analysis was done to characterize the dsDNA product by slowly raising the temperature (0.2°C/s) from 65 to 95°C with fluorescence data collected at 0.2°C intervals. The levels of expression (of swiprosin-1, IL-3, and IL-8) that were normalized by GAPDH were expressed as a relative value (i.e., percentage) of the maximum. The result of the maximum level in each experiment was considered as 100%.

CELL EXTRACT PREPARATION AND WESTERN BLOT ANALYSIS

For the analysis of swiprosin-1, PKC isoforms (including PKC-α, PKC-βI, PKC-θ, PKC-δ, PKC-η and PKC-ζ, and the I-κB, HMC-1 cells (parent or transfected cells) were rinsed twice with ice-cold PBS and then lysed in ice-cold lysis buffer (10 mM Tris-HCl, pH 7.4, containing 50 mM NaCl, 1% Triton X-100, and a protease inhibitor cocktail tablet). Cell lysates were centrifuged at 14,000 rpm for 20 min at 4°C and equal amounts of protein supernatant were mixed with a one-fourth volume of 4× SDS sample buffer, boiled for 5 min, and then separated through an 8% or 10% sodium dodecyl sulfate–polyacrylamide gel. After electrophoresis, proteins were transferred to a nitrocellulose membrane by means of the Trans-Blot SD semidry transfer cell (Bio-Rad, Hercules, CA). The membrane was blocked in 5% skim milk (1 h), rinsed, and incubated overnight at 4°C with primary antibodies in TBS containing 0.1% Tween 20 (TBS-T) and 3% skim milk. Excess primary Ab was then removed by washing the membrane three times in TBS-T, and the membrane was incubated with 0.1 μg/ml horseradish peroxidase-labeled secondary Ab (against goat, rabbit, or mouse) for 2 h. Following three washes in TBS-T, bands were visualized by ECL Western blotting detection reagents and exposed to X-ray film.

NF-κB LUCIFERASE ASSAY

HMC-1 cells (1.5×10^6) were transfected with 100 μl of Amaxa's Nucleofector solution (Amaxa, Cologne, Germany) containing 1 μg of swiprosin-1/pEGFP-C1 or pEGFP-C1 with 1 μg of pRL-TK renilla and 1 μg of pGL3/NF-κB, and then the cells were immediately transferred to 2.0 ml of complete medium and cultured in 6-well plates at 37°C. After 24 h of transfection, the medium was replaced with IMDM medium containing 10% FBS and antibiotics. Cells were stimulated as described above and incubated at 37°C for 12 h. Cell lysates were prepared and assayed for luciferase activity using the Dual Luciferase Assay System (Promega, Madison, WI), according to the manufacturer's instructions.

STATISTICAL ANALYSIS

The mean values were calculated from data taken from at least three (usually four or more) separate experiments conducted on separate days. Where significance testing was performed, an independent *t*-test (Student's; two populations) was used. A *P*-value of less than 0.05 was considered an indicator of statistical significance.

RESULTS

SWIPROSIN-1 IS EXPRESSED IN MAST CELLS AND UP-REGULATED BOTH IN VITRO AND IN VIVO

Genetic chip technology is characterized by high communication, low consumption and miniaturization [Lockhart et al., 1996; Schena et al., 1996], thus providing a technological platform to discover novel genes behind essential biological processes in various model systems. As part of genome-wide approaches to understanding mast cell activation at the molecular level, we performed high-density oligonucleotide microarrays with mRNAs prepared from HMC-1 cells stimulated with PMA/A23187 (Fig. 1). Using this approach, we found several meaningful genes that have not been studied in mast cells associated with cytokine regulation. Among these candidate genes, swiprosin-1 was particularly of interest because this novel gene was not investigated in mast cells and, more interestingly, in contrast to the previous report in B-cell activation [Avramidou et al., 2007], this gene was over-induced in mast cells during the activation process (Fig. 1A,B). With two more different methods, including real-time quantitative PCR and Western blot, we confirmed that swiprosin-1 is significantly over-induced in HMC-1 cells when stimulated with PMA (100 nM)/A23187 (1 μM) (Fig. 1C,D). A time-dependent experiment revealed that maximal induction is observed after 12–24 h of stimulation in HMC-1 cells (Fig. 1C,D).

The PCA reaction is one of the most well characterized in vivo models of local allergic reaction [Vaz and Prouvost-Danon, 1969; Galli et al., 1984]. We further examined the expression of swiprosin-1 in tissue samples prepared from the dorsal skin after the PCA reaction. The blue color represents Evans blue as an indicator of the PCA reaction (Fig. 2A). Interestingly, swiprosin-1 was significantly

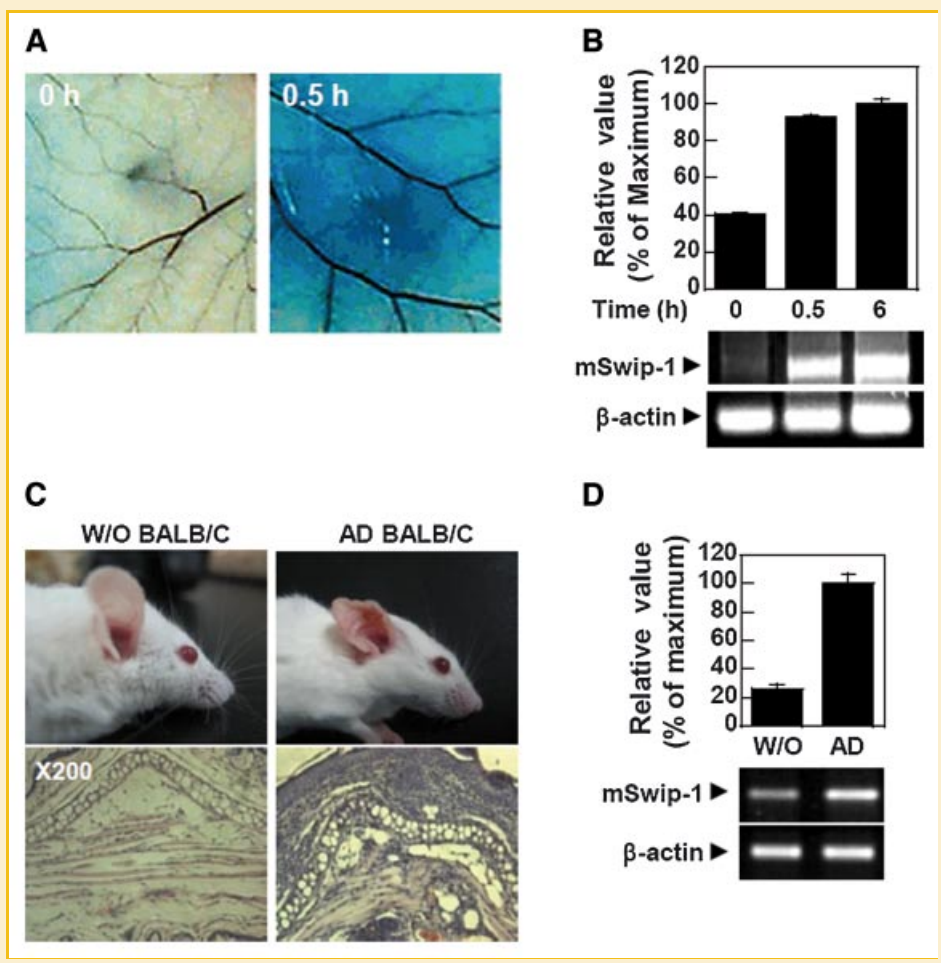

Fig. 2. Swiprosin-1 is up-regulated in skin tissues of PCA and AEDS-like models. A: Mouse dorsal skins before (0 h) and after PCA reaction (0.5 h). B: After PCA reaction, as described in Materials and Methods Section, total RNAs were extracted at the indicated time points (0–6 h), and swiprosin-1 levels were determined by RT-PCR (top) and quantitative RT-PCR (bottom). C: Histological status of control and AEDS-like mouse ear skins. D: Swiprosin-1 levels were determined by RT-PCR (top) and quantitative RT-PCR (bottom). β-Actin was used as an internal control. Gel data are representative of two separate experiments. The data shown in the bar graphs represent mean ± SD values of triplicate experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

induced in tissue samples of the PCA reaction (Fig. 2B). We also employed a mouse model of atopic dermatitis, which is a common chronic type of eczema (an inflammatory skin disease) [Groneberg et al., 2003] and found that swiprosin-1 is highly expressed in the samples from atopic dermatitis but not in control Balb/c mouse (Fig. 2C,D). Taken together, these results strongly suggest that swiprosin-1 expression is also transcriptionally controlled in vivo in situations such as acute cutaneous anaphylaxis and chronic atopic dermatitis.

SWIPROSIN-1 EXPRESSION IS INDUCED THROUGH PKC-βI/η-DEPENDENT PATHWAYS BUT IS DOWN-REGULATED BY CALCIUM SIGNALING

The calcium and PKC pathways are important and essential for the activation of a variety of cells as well as for various cellular processes [Wodnar-Filipowicz and Moroni, 1990; Kawakami et al., 1998; Jeong et al., 2002]. As swiprosin-1 was over-induced in HMC-1 cells by simple co-treatment of PMA with A2387, we examined the effect of each treatment singly, to further identify the corresponding signaling pathway responsible for the swiprosin-1 expression. To our surprise, swiprosin-1 was only induced in HMC-1 cells treated with PMA (Fig. 3A,B). Treatment with calcium ionophore A23187 or ionomycin had no effect on swiprosin-1 expression alone; rather, they partially inhibited PMA-induced swiprosin-1 expression in HMC-1 cells (Fig. 3A,B), thereby suggesting that a calcium signal plays a negative role on swiprosin-1 expression in mast cells.

As PKCs are primary targets for PMA, we next examined which PKC-isoforms could be responsible for swiprosin-1 expression in HMC-1 cells. Initially, we used pharmacologic agents that can modulate specific PKC isoforms. To this end, HMC-1 cells were pretreated with various concentrations of diverse PKC inhibitors, and then the cells were stimulated with PMA. As shown in Figure 4A,B, Gö6976 (inhibitor of PKCα and PKCβI) and rottlerin (inhibitor of PKCδ and PKCι) revealed no effects on PMA-induced swiprosin-1 expression. Gö6983 (inhibitor of PKCs α, βI, βII, δ, and ζ) showed only minimal blocking effect. In contrast, staurosporine, a non-specific PKC inhibitor, significantly blocked PMA-induced swiprosin-1 expression (Fig. 4A,B). The data to this point demonstrated a requirement for PKC(s) in PMA-induced swiprosin-1 expression, but did not establish which PKC isoform(s) is (are) specifically involved in this process. We therefore used siRNAs to knockdown target PKC isoforms. Western blot analysis revealed that mast cells express PKCα, βI, θ, δ, η, and ζ forms (Fig. 5A) and that transfection of siRNA significantly down-regulated each target PKC in a time-dependent manner, showing maximal inhibition at 72 h of transfection (Fig. 5B). As shown in Figure 5C,D, among the six PKC isoforms examined, targeted knockdown of PKC-βI and PKC-η slightly but significantly inhibited PMA-induced swiprosin-1 expression, suggesting that both PKC-βI and PKC-η play a major function in regulating swiprosin-1 expression. In accordance with this assumption, knockdown of both PKC-βI/η by siRNAs dramatically inhibited swiprosin-1 expression, while knockdown of PKC-α/δ showed no effect (Fig. 5E,F).

SWIPROSIN-1 ENHANCES MAST CELL ACTIVATION BY PHORBOL ESTER AND CALCIUM IONOPHORE

Induction of swiprosin-1 during mast cell activation, coupled with the fact that swiprosin-1 has a potential for a novel signaling adaptor protein [Blagoev et al., 2004; Vuadens et al., 2004; Mielenz et al., 2005; Avramidou et al., 2007], suggests that swiprosin-1 may be involved in mast cell activation as a signal adaptor molecule. Since a central event in mast cell activation is the pathway involving the nuclear factors, we therefore tested whether swiprosin-1 activates the transcriptional activity of the NF-κB. To this end, we generated stable HMC-1 cells that over-express either GFP

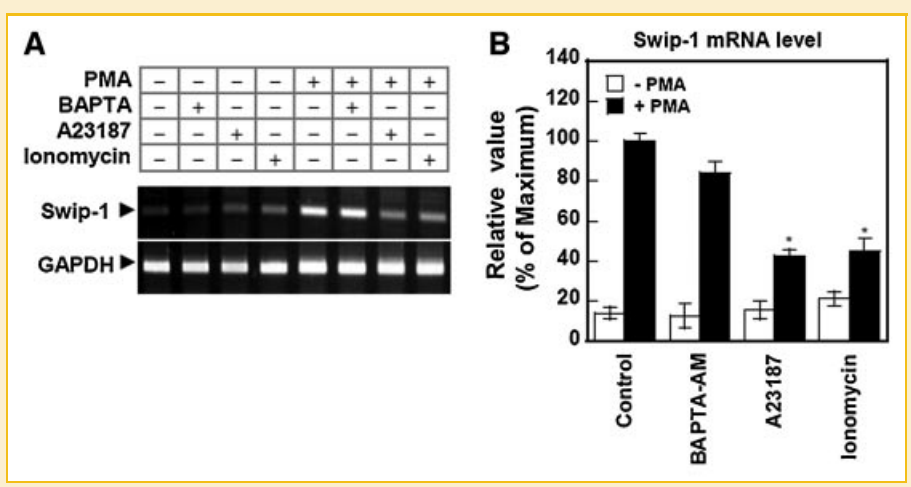

Fig. 3. Swiprosin-1 is induced by phorbol ester but down-regulated by calcium activators. HMC-1 cells were pretreated for 30 min with or without calcium regulators including BAPTA-AM (50 μM), A23187 (1 μM), and ionomycin (1 μM). The cells were further stimulated for 6 h with or without PMA (200 nM). The expression of swiprosin-1 was determined by RT-PCR (A) and quantitative RT-PCR (B). Amplification of GAPDH was used as an internal control. Data represent three separate experiments. The data shown in the bar graphs represent mean ± SD values of triplicate experiments.

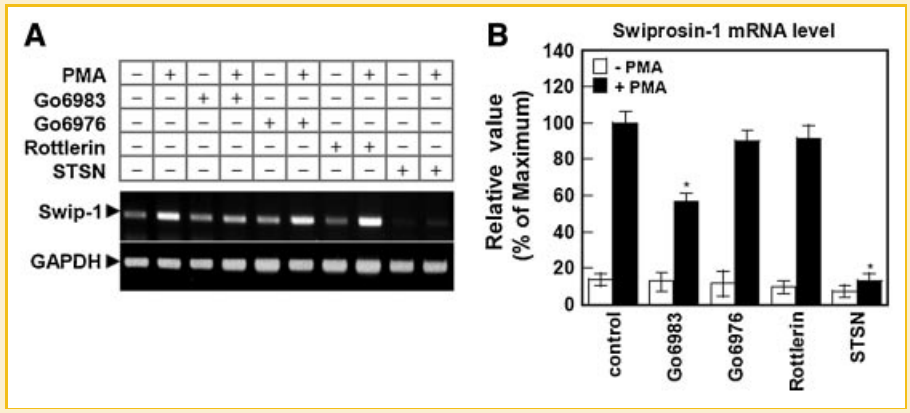

Fig. 4. Swiprosin-1 expression is down-regulated by PKC inhibitors. HMC-1 cells were pretreated for 30 min with or without PKC inhibitors including Gö6983 (100 nM), Gö6976 (100 nM), rottlerin (10 μM), and staurosporin (500 nM). The cells were further stimulated for 6 h with or without PMA (200 nM). The expression of swiprosin-1 was determined by RT-PCR (A) and quantitative RT-PCR (B). Amplification of GAPDH was used as an internal control. Data represent three separate experiments. The data shown in the bar graphs represent mean ± SD values of triplicate experiments.

(GFP cells) alone or GFP fused to swiprosin-1 (Swip-1_GFP cells) (Fig. 6A,B), and then the cells were transiently transfected with NF-κB reporter vector. As shown in Figure 6C, I-κBα degradation was significantly enhanced in Swip-1_GFP cells. Accordingly, transcriptional activity of NF-κB in response to the PMA/A23187 was also enhanced in these cells (Fig. 6D).

To eliminate a potential influence of GFP in mast cell activation, we also generated HMC-1 cells that over-express wild-type

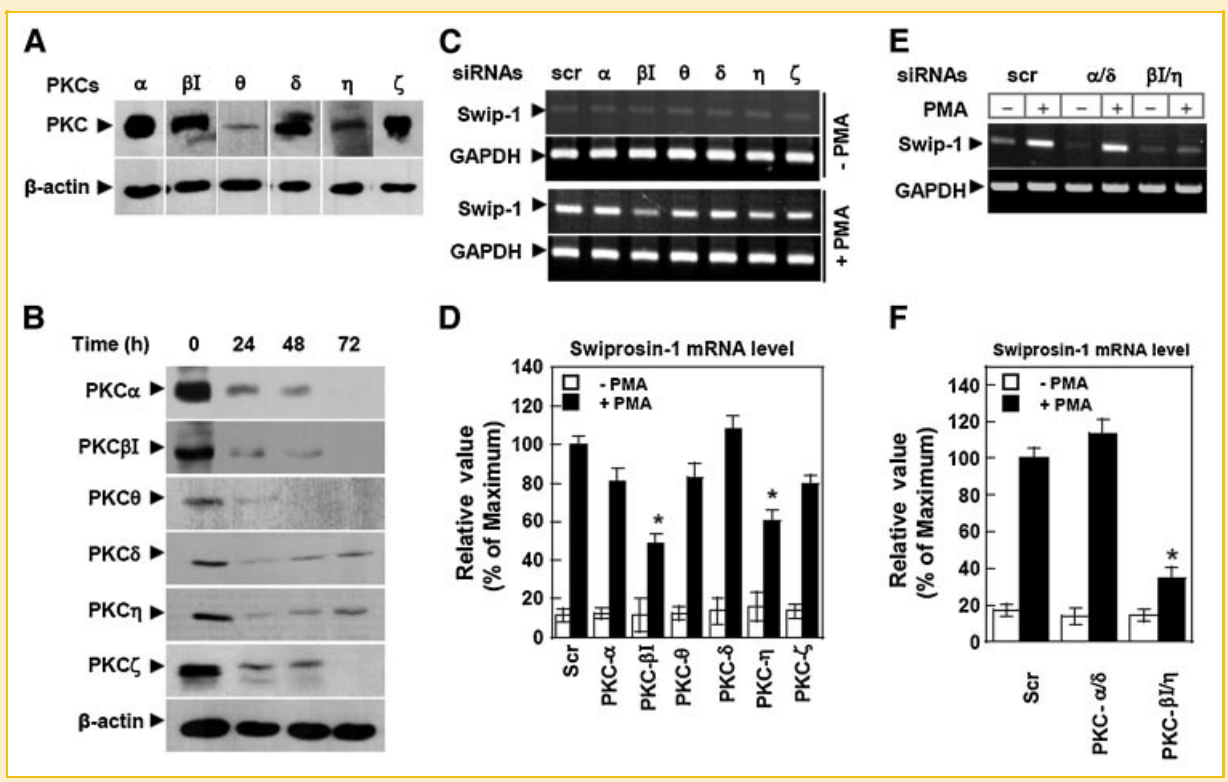

Fig. 5. Swiprosin-1 expression is dependent on PKC-βI/η pathways in mast cells. A: Detection of PKC isoforms by Western blot in HMC-1 cells. B: Knock-down of PKC isoforms by specific siRNA. HMC-1 cells were transfected with siRNA targeting specific PKC isoforms. At the indicated time points, the expression of PKC isoforms was analyzed by Western blot. Blots were also probed with antibody to β-actin to confirm an equal loading. C–F: Knock-down of PKC-βI/η inhibits phorbol ester-induced swiprosin-1 expression in HMC-1 cells. After 72 h of siRNA transfection against indicated PKC isoforms, cells were treated with or without PMA (200 nM) for 6 h and the expression of swiprosin-1 was analyzed by RT-PCR (C,E) and quantitative RT-PCR (D,F). Amplification of GAPDH was used as an internal control. Data represent three separate experiments. The data shown in the bar graphs represent mean ± SD values of triplicate experiments.

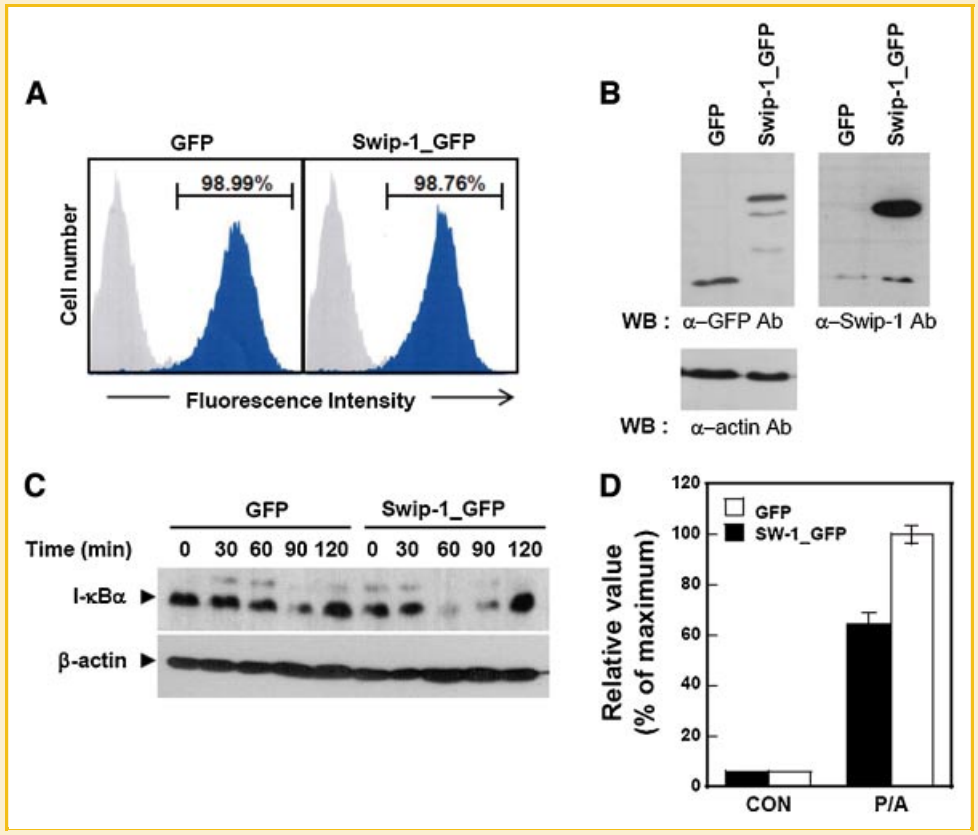

Fig. 6. Ectopic expression of swiprosin-1 enhances NF-κB activity induced by phorbol ester and calcium ionophore. A,B: Establishment of stable cell lines that express GFP or swip-1_GFP. GFP or swip-1_GFP cells were established as described in Materials and Methods Section, and then the expression of swiprosin-1 was determined by flow cytometry (A) and Western blot (B). *Note*: Over 95% of GFP-positive cells were used for this study. C: GFP or swip-1_GFP cells were treated with PMA (200 nM)/A23187 (1 μM). At the indicated time points, the cells were lysed and I-κBα degradation was determined by Western blot. D: GFP or swip-1_GFP cells were transiently transfected with NF-κB luciferase reporter construct. After 24 h, the cells were treated with PMA/A23187 and incubated for 12 h. NF-κB-dependent transcriptional activity was determined by luciferase activity assay. Data are representative of three separate experiments. The data shown in bar graphs represent mean ± SD values of triplicate experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

swiprosin-1 without GFP, by using a lentiviral vector containing swiprosin-1 cDNA (Fig. 7A). We found that over-expression of swiprosin-1 significantly enhanced PMA/A23187-induced cytokine expression (IL-3 and IL-8; Fig. 7B). Further, this result suggests that GFP has little effect on mast cell activation. We next tested whether silencing swiprosin-1 can alter the cytokine expression in HMC-1 cells. To this end, we transfected HMC-1 cells with siRNA targeting swiprosin-1, and then evaluated the cells for cytokine expression. As shown in Figure 7C,D, knockdown of swiprosin-1 showed a modest but significant inhibitory effect on PMA/A23187-induced IL-3 and IL-8 mRNA expression in HMC-1 cells.

DISCUSSION

Genetic chip technology is one of the powerful ways to hunt for novel genes behind essential biological processes in various model systems [Lockhart et al., 1996; Schena et al., 1996]. Using this approach, we found that a novel gene, swiprosin-1, is expressed in human mast cells and over-induced during mast cell activation. The expression of swiprosin-1 was also up-regulated both in vitro in cultured mast cells by phorbol ester and in vivo in tissue models of PCA and atopic dermatitis. An siRNA approach targeting PKC isotypes demonstrated that the PKCs-βI/η are primary targets for phorbol ester-mediated induction of swiprosin-1 in HMC-1 cells. We also found that calcium signaling decreases swiprosin-1 expression. Molecules produced by mast cells also can activate mast cells in an autocrine manner. For example, TNF-α is one of the major cytokines that autocrinely activates HMC-1 cells [Wong et al., 2006]. In a similar manner, ectopic expression of swiprosin-1 augmented PMA/A23187-induced NF-κB promoter activity as well as cytokine expression.

A previous study demonstrated by Northern blot analysis that swiprosin-1 is in spleen, lung, liver, and, most abundantly, in brain [Avramidou et al., 2007]. In accordance with these data, the authors showed that swiprosin-1 is expressed throughout B-cell differentiation, with the highest expression in immature bone marrow B cells [Avramidou et al., 2007]. In addition, a very recent report demonstrated that swiprosin-1 is associated with tau protein, a known causative of the neurodegenerative disease tauopathy in human [Vega et al., 2008]; thereby suggesting that swiprosin-1 may play an important physiological and/or pathological role. However, whether the expression of swiprosin-1 is regulated in vitro or in vivo in models of physiological/pathological conditions has not been

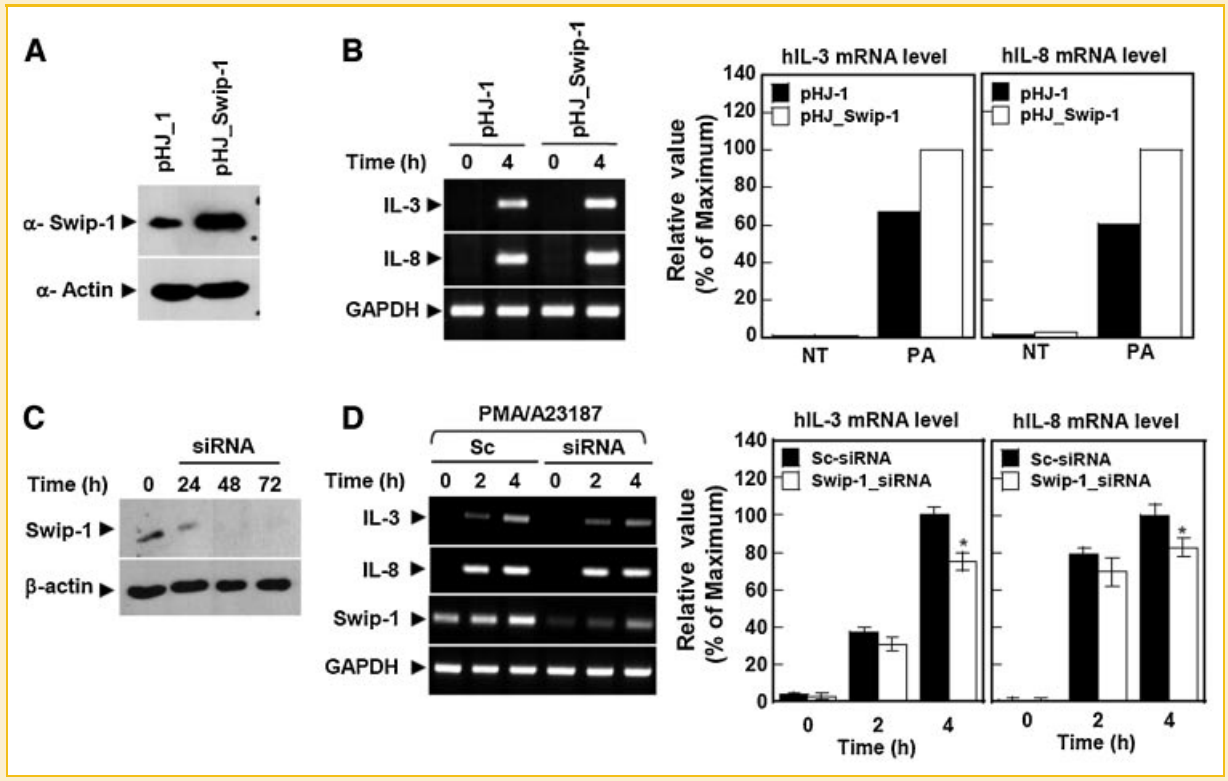

Fig. 7. Swiprosin-1 involves in cytokine expression in HMC-1 cells. A,B: Ectopic expression of swiprosin-1 enhances mast cell activation by phorbol ester and calcium ionophore. A: Over-expression of swiprosin-1 by lentiviral infection in HMC-1 cells. The expression of swiprosin-1 was determined by Western blot. B: Over-expression of swiprosin-1 enhanced PMA/A23187-induced cytokine expression in HMC-1 cells. HMC-1 cells described in (A) were treated with PMA (200 nM)/A23187 (1 μM) for 4 h, and then cytokine expression (IL-3 and IL-8) was determined by RT-PCR (left) and quantitative RT-PCR (right). Amplification of GAPDH was used as an internal control. The data shown in the bar graphs are representative of one of the duplicate experiments. C,D: Knock-down of swiprosin-1 slightly inhibits cytokine expression in HMC-1 cells. C: Targeted knock-down of swiprosin-1 by siRNA. HMC-1 cells were transfected with siRNA targeting swiprosin-1. At the indicated time points, the expression of swiprosin-1 was analyzed by Western blot. D: After 72 h of siRNA transfection, cells were treated with PMA/A23187 for various times (0–4 h), and the cytokine expression (IL-3 and IL-8) was analyzed by RT-PCR (left) and quantitative RT-PCR (right). Amplification of GAPDH was used as an internal control. Data represent three separate experiments. The data shown in the bar graphs represent mean ± SD values of four separate experiments.

known. Actually, the former authors reported that swiprosin-1 expression is not changed during B-cell activation by IgM/IL-4, LPS, or anti-CD40/IL-4 [Avramidou et al., 2007]. In contrast to the previous study, however, we unambiguously demonstrate here that swiprosin-1 expression is transcriptionally regulated both in vitro in a model of phorbol ester-induced activation and in vivo in pathological models. These results strongly suggest that swiprosin-1 may be importantly involved in physiological and/or pathological processes. Furthermore, increased expression of swiprosin-1 in tissue models of PCA and atopic dermatitis suggests a potential role for swiprosin-1 in inflammatory responses. Therefore, further studies are now in progress to elucidate the role of swiprosin-1 in vivo by making transgenic mice that hyper-express swiprosin-1 in specific cell types.

Members of the PKC family are expressed in many cell types, where they are known to regulate a wide variety of cellular processes that affect cell growth and differentiation, cytoskeletal remodeling, and gene expression in response to diverse stimuli [Tan and Parker, 2003]. PKC-regulated signaling pathways play an especially significant role in many aspects of immune responses, from development, differentiation, activation, and survival of lymphocytes to macrophage activation [Tan and Parker, 2003]. The activation of PKC is also clearly required for both the early and delayed responses of the mast cell-mediated inflammatory process [Nechushtan and Razin, 2001]. In this sense, induction of swiprosin-1 by the PKC-βI/η pathway also suggests that swiprosin-1 is involved in the inflammatory response of mast cells. However, our unpublished results demonstrated that swiprosin-1 expression induced by PKC is not restricted to mast cells but occurs in other immune cells, including human T cells. These results suggest that swiprosin-1 is a PKC-responsible gene in various cell types, although whether it requires specific PKC isoforms in different cell types is currently unknown.

Calcium is important in initiating a signaling cascade leading to degranulation of mast cells and the release of inflammatory mediators [Neher, 1991; Barker et al., 1999]. Cross-linking of FcεRI by IgE-Ag results in a sustained influx of extracellular calcium across the plasma membrane and leads to activation of many transcription factors, thereby inducing various pre- or pro-inflammatory cytokine genes [White et al., 1984; Barker et al., 1999; Kim et al., 2005a; Gwack et al., 2007]. In the present study, however, it was surprising that calcium increase by A23187 or

ionomycin induces down-regulation of swiprosin-1 rather than induction. Therefore, analysis of promoter regions over the swiprosin-1 genes will be critical to understand why or how calcium signaling inhibits swiprosin-1 expression in HMC-1 cells. It will also be interesting to examine whether this phenomenon is present in other immune cells such as lymphocytes, given its pivotal action on lymphocyte activation [Macian, 2005; Savignac et al., 2007]. One expectation is that swiprosin-1 has two EF-hand motifs effectively binding to calcium [Vega et al., 2008]. This substantial property of swiprosin-1 may modulate intracellular calcium signals or levels, and therefore, it can be inversely assumed that the calcium signal must regulate swiprosin-1 level in cells which, in turn, is important for maintaining intracellular calcium homeostasis as well as calcium signaling evoked by outside stimuli.

The amplifying effect of swiprosin-1 on PMA/A23187-induced HMC-1 cell activation in terms of cytokine expression is intriguing, as this result demonstrates that swiprosin-1 lies on the signaling pathways that regulate activation of HMC-1 cells. In this sense, it is important to examine whether swiprosin-1 also participates in high affinity IgE-mediated degranulation and cytokine expression. Therefore, further study will be necessarily required in other model systems such as bone marrow-derived mast cells (BMMC) and RBL-2H3 cells. Nevertheless, the HMC-1 model adopted in the current study is valuable because this cell has many characteristics of immature human mast cells [Butterfield et al., 1988], and notably, these cells can still be activated by treatment with phorbol esters and calcium ionophores, as can normal or transformed $Fc\varepsilon R^{+}$ mast cell lines [Burd et al., 1989].

The mast cell is a central player in allergy and asthma. Activation of these cells induces the release of preformed inflammatory mediators localized in specialized granules and the de novo synthesis and secretion of cytokines, chemokines, and eicosanoids [Rivera and Gilfillan, 2006]. Upon stimulation, mast cell response can be regulated by the balance of both positive and negative intracellular molecular events [Rivera and Gilfillan, 2006]. Here, we suggest that swiprosin-1 is a novel regulator for the positive feedback activation of mast cells. The induction of swiprosin-1 is restricted in the pathway of PKC-βI/η. In pathological situations, this protein may regulate local allergic or inflammatory responses by amplifying signals that are required for mast cell activation.

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