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*J Immunol* 2012; 188:5665-5673; Prepublished online 25 April 2012;
doi: 10.4049/jimmunol.1102306
http://www.jimmunol.org/content/188/11/5665

Supplementary Material
http://www.jimmunol.org/content/suppl/2012/04/25/jimmunol.110230
6.DC1

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Regulation of Cytosolic Phospholipase A2 Phosphorylation by Proteolytic Cleavage of Annexin A1 in Activated Mast Cells

Joon Hyun Kwon,* Jea Hwang Lee,* Ki Soon Kim,* Youn Wook Chung,† and Ick Young Kim*

Annexin A1 (ANXA1) is cleaved at the N terminal in some activated cells, such as macrophages, neutrophils, and epithelial cells. We previously observed that ANXA1 was proteolytically cleaved in lung extracts prepared from a murine OVA-induced asthma model. However, the cleavage and regulatory mechanisms of ANXA1 in the allergic response remain unclear. In this study, we found that ANXA1 was cleaved in both Ag-induced activated rat basophilic leukemia 2H3 (RBL-2H3) cells and bone marrow-derived mast cells. This cleavage event was inhibited when intracellular Ca2+ signaling was blocked. ANXA1-knockdown RBL-2H3 cells produced a greater amount of eicosanoids with simultaneous upregulation of cytosolic phospholipase A2 (cPLA2) activity. However, there were no changes in degranulation activity or cytokine production in the knockdown cells. We also found that cPLA2 interacted with either full-length or cleaved ANXA1 in activated mast cells. cPLA2 mainly interacted with full-length ANXA1 in the cytosol and cleaved ANXA1 in the membrane fraction. In addition, introduction of a cleavage-resistant ANXA1 mutant had inhibitory effects on both the phosphorylation of cPLA2 and release of eicosanoids during the activation of RBL-2H3 cells and bone marrow-derived mast cells. These data suggest that cleavage of ANXA1 causes proinflammatory reactions by increasing the phosphorylation of cPLA2 and production of eicosanoids during mast-cell activation. The Journal of Immunology, 2012, 188: 5665–5673.

Mast cells are considered central effector cells in allergic diseases, such as asthma, allergic rhinitis, and food allergies, and mast-cell–derived proinflammatory mediators play a key role in these pathologies (1–3). Ag-mediated activation of mast cells is regulated by a complex series of intracellular signaling processes that are initiated by FcεRI aggregation. Activated mast cells show three biological characteristics: degranulation (secretion of mediators stored in intracellular granules), the production of cytokines and chemokines, and the release of newly synthesized lipid mediators (4–7). Most studies on FcεRI-mediated signaling have been carried out using rat basophilic leukemia 2H3 (RBL-2H3) cells and mutated sublines (8). Although a number of studies have described the mast-cell activation pathway, it is still not fully understood.

Annexin A1 (ANXA1) is a member of the ANX family of Ca2+- and phospholipid-binding proteins, and plays different roles under various biological conditions, for example, membrane organization, apoptosis, and inflammation (9, 10). ANXA1 consists of two domains: an N-terminal tail and a C-terminal core domain. The C-terminal core domain consists of four repeats of a highly conserved amino acid sequence responsible for binding with Ca2+ and phospholipids (9). The N-terminal peptide is known to be cleaved by a number of proteases, including elastase, calpain, plasmin, cathepsin D, and proteinase 3 (11–14), and is also phosphorylated to regulate the activity of the protein (15, 16). N-terminal truncated ANXA1 has a proinflammatory effect and promotes neutrophil transendothelial migration (17). Truncated ANXA1 has also been linked to an epidermal growth factor (EGF)-triggered signaling pathway involving cytosolic phospholipase A2 (cPLA2) in normal and malignant squamous epithelial cells (14). In activated polymorphonuclear (PMN) leukocytes, ANXA1 is cleaved and then translocates to the extracellular surface (13). Mutant ANXA1, which is resistant to cleavage by proteases isolated from PMN cell extracts, was previously shown to have anti-inflammatory activities in the inflamed microcirculation of mice and in a skin trafficking model (18).

We previously demonstrated the proteolytic cleavage of ANXA1 and activation of cPLA2 in a murine OVA-induced asthma model (19). cPLA2 plays a key role in the release of arachidonic acid (AA) for the production of lipid inflammatory mediators, such as leukotrienes and PGs. In mast cells, the aggregation of IgE-loaded FcεRI induced by polyvalent Ag leads to the phosphorylation of ERK and cPLA2, followed by release of AA, which is then further converted to leukotrienes and PGs by 5-lipoxygenase (5-LO) and cyclooxygenase (COX), respectively (20). Cysteinyl leukotrienes (cysLTs) are involved in the pathogenesis of asthma and are determinants of the severity of asthma (21). cPLA2 activity is regulated by intracellular Ca2+ concentrations and phosphorylation (22). Increased intracellular Ca2+ concentrations promote the translocation of cPLA2 from the cytosol to the membrane (23, 24). The catalytic domain of cPLA2 is phosphorylated by MAPKs (Ser505) and MAPK-interaction kinases (Ser175) (25–27). Furthermore, cPLA2 activity is inhibited by direct interaction with...
ANXA1 (28, 29). However, the inhibitory mechanisms of ANXA1 remain unclear. In particular, the functional significance of ANXA1 cleavage in cPLA2 activity during mast-cell activation remains unknown. In this study, we demonstrate that cleavage of ANXA1 activates cPLA2 by phosphorylation, stimulating a proinflammatory reaction in activated mast cells.

Materials and Methods

Reagents

Monoclonal anti-DNP Clone SPE-7, A23187 (a calcium ionophore), BAPTA-AM, 2-aminoethoxydiphenyl borate (2-APB), thapsigargin (TG), HEPES, sodium pyruvate, sodium orthovanadate, and PMSF were obtained from Sigma-Aldrich. DNP-BSA and p-nitrophenyl-2-acetyl-β-D-glucosaminide were purchased from Calbiochem. Recombinant mouse IL-3 and stem cell factor were obtained from R&D Systems. Abs against p-ERK1/2, ERK1/2, and p-cPLA2 (Ser505) were purchased from Cell Signaling Technology. ANXA1 Abs were obtained from Zymed, and cPLA2 (4-4B-3C) Abs were purchased from Santa Cruz Biotechnology. Abs against α-tubulin were purchased from LabFrontier (Seoul, Korea). Negative control (NC) stealth small interfering RNAs (siRNAs), ANXA1 stealth siRNAs, and Neon transfection kit (MP100) were obtained from Invitrogen.

Mast-cell culture and activation

Bone marrow-derived mast cells (BMMCs) were isolated from the femurs of C57BL/6 mice (8- to 12-wk-old) as described previously (30). The isolated BMMCs were cultured in RPMI 1640 medium (Life Technologies) supplemented with 10% FBS, 2 mM t-glutamine, 1% nonessential amino acids (Invitrogen), 50 μM 2-ME, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, 25 mM HEPES, 10 ng/ml stem cell factor, and 5 ng/ml IL-3 for 4–8 wk at 37°C and 5% CO2. Purity, measured by flow cytometry analysis using FITC-conjugated rat anti-mouse c-Kit mAbs (BD Biosciences) at 4 wk, was >97%.

RBL-2H3 cells were maintained as a monolayer in DMEM medium (Life Technologies) supplemented with 15% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C and 5% CO2.

RBL-2H3 cells (1 × 106 cells in a 60-mm dish) and BMMCs (5 × 106 cells/ml) were sensitized with or without monoclonal anti-DNP IgE (500 ng/ml) for 12 h in cytokine-free medium and serum starved for 6 h with DMEM or RPMI 1640 containing 0.5% FBS. Serum-starved cells were activated with various stimuli for the indicated times.

Assay for β-hexosaminidase release

β-Hexosaminidase release was used as a measure of degranulation activity as described previously (31). In brief, IgE-sensitized and serum-starved RBL-2H3 cells and BMMCs were washed twice with Tyroid buffer (135 mM NaCl, 5 mM CaCl2, 1 mM MgCl2, 5.6 mM glucose, 1 mg/ml BSA, and 20 mM HEPES [pH 7.4]) and then stimulated with DNP-BSA (1 μg/ml) or A23187 (1 μM) at 37°C in Tyroid buffer for the indicated times. The incubation buffer and cell lysates, prepared by the addition of identical volumes of Tyroid buffer containing 1% Triton X-100, were then recovered for quantitation of β-hexosaminidase release.

Assay for cysLTs and PGE2 release

Release of cysLTs and PGE2 was measured using an enzyme immunoassay system (Amersham), according to the manufacturer’s instructions. IgE-sensitized and serum-starved RBL-2H3 cells and BMMCs were stimulated with DNP-BSA (1 μg/ml), A23187 (1 μM), or TG (1 μM) at 37°C for the indicated times. When necessary, BAPTA-AM (30 μM) and 2-APB (50 μM) were added to the cells for 30 min before activation with DNP-BSA. Then, 100 μl culture medium was concentrated by freeze-drying overnight, followed by reconstitution in assay buffer. Samples and standard cysLTs or PGE2 were incubated with antiserum in a 96-well plate for 2 or 3 h, followed by cysLT or PGE2-peroxidase conjugates for 3 or 1 h, respectively, at 4°C. To remove unbound ligand, we washed the wells five times with wash buffer. Then the amount of bound peroxidase-labeled cysLTs and PGE2 was determined by the addition of substrate for 30 min.

FIGURE 1. Time-dependent activation of RBL-2H3 cells and proteolytic cleavage of ANXA1. IgE-sensitized RBL-2H3 cells were serum starved and activated with DNP-BSA (1 μg/ml) for the indicated times. (A) The incubation buffer and cell lysates were recovered for quantitative analysis of β-hexosaminidase release as a measure of degranulation activity. (B) Total RNA isolated from activated cells was subjected to RT-PCR analysis. The expression of TNF-α, IL-4, and IL-6 mRNA was determined for measurement of cytokine production. GAPDH was used as a loading control. (C) The levels of cysLTs and PGE2 secreted from the activated cells were analyzed using an enzyme immunoassay system. (D and E) Whole-cell lysates prepared from the cells activated by DNP-BSA for the indicated times were subjected to immunoblotting with Abs against (D) ERK1/2, p-ERK1/2, cPLA2, p-cPLA2, (E) ANXA1, and β-actin. (F) Total RNA isolated from activated cells was subjected to RT-PCR analysis for measurement of ANXA1 and GAPDH mRNA. (A and C) Data are expressed as the means ± SE of three independent experiments. (B, D–F) Results are representative of at least three independent experiments.
RNA isolation and RT-PCR

Total RNA was isolated from RBL-2H3 cells with TRizol reagent (Invitrogen), and first-strand cDNA was synthesized by reverse transcription using oligo(dT) and SuperScript III reverse transcriptase (Invitrogen). PCR analysis of the resulting cDNA was performed using a PCR premix, Sapphire (Super Bio, Seoul, Korea), and the following forward and reverse primers: ANXA1, 5'-GCG GAG CAT CTA TGG TCA GAA TAC TTC CTC-3' and 5'-GGG GGC GCG TCG ACT TAG TTT CCT CCA CAA AGA GCC AC-3', TNF-α, 5'-CAA GGA GAA GGA GAA GAA GAA TGG TCT CCC AA-3' and 5'-GGG ACT CGG TGA TGT CTA AG-3'; IL-4, 5'-ACC TGT CTG TCA CCC TGT TC-3' and 5'-TGG TTA GCG TGG TCG ACT TC-3'; IL-6, 5'-GAA ATG ATG GAT GCT TCC AAA CTG G-3' and 5'-GGA TAT ATT TTC TGA CCA TCG TGA GG-3'; GAPDH, 5'-AGG TCG TGA TCG CAT GAT TT-3' and 5'-AGG TGG AGG AGT GGG TGT CG-3'. The PCR products were resolved on a 1 or 1.5% agarose gel and visualized by staining with ethidium bromide.

Subcellular fractionation

The subcellular fractionation of DNP-BSA or A23187-stimulated RBL-2H3 cells was performed using a ProteoJET membrane protein extraction kit (Fermentas). In brief, activated cells were washed with cell wash solution, scraped with cell permeabilization buffer containing protease inhibitors, and then incubated on ice for 10 min. Permeabilized cells were centrifuged at 16,000 g for 15 min at 4°C. Supernatants were collected for isolation of the cytosolic fraction. Membrane protein extraction buffer was added to the pellet, which was then incubated on ice for 30 min and centrifuged at 16,000 g for 15 min at 4°C. After centrifugation again, supernatants were collected for isolation of the membrane fraction. These cytosolic and membrane fractions were used for immunoblotting and immunoprecipitation assays.

Immunoprecipitation and immunoblot analysis

Activated RBL-2H3 cells and BMMCs were washed twice with cold PBS and lysed in lysis buffer containing 10 mM HEPES (pH 7.4), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.5 mM PMSF, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 0.5% Nonidet P-40, 1 mM NaF, and 1 mM Na3VO4. After centrifugation at 16,000 × g for 15 min at 4°C, supernatants containing the whole cell lysates were collected. The protein concentrations of the whole-cell lysates and subcellular fractions were determined using Bradford reagent (Sigma). For immunoprecipitation, 500 μg protein was precleared with protein G-agarose (GE Healthcare) for 1 h at 4°C, followed by incubation with 1 μg cPLA2 or GFP Abs overnight at 4°C. Immune complexes were further incubated with protein G-agarose for 2 h at 4°C and then washed with lysis buffer three times. For immunoblotting, proteins prepared by whole-cell lysis, subcellular fractionation, and immunoprecipitation were boiled with SDS-PAGE sample buffer for 5 min and then separated by electrophoresis on 8% (for cPLA2, p-cPLA2, ERK, p-ERK, GAPDH, and β-actin) or 10% (for ANXA1) SDS-polyacrylamide gels. The separated proteins were then transferred to a polyvinylidene fluoride membrane, and the membrane was then blocked in 5% skim milk and incubated overnight with primary Ab at 4°C. After incubation with HRP-conjugated secondary Ab for 1 h at room temperature, immunoreactive bands were visualized using a West Pico enhanced ECL detection kit (Pierce). Band intensities were determined densitometrically using ImageJ software.

Construction of ANXA1 expression vector and site-directed mutagenesis

Total RNA and first-strand cDNA were obtained from HEK293 cells as described earlier. ANXA1 cDNA was generated by RT-PCR using specific primers (forward, 5'-CGG CTC GAG ATG CCA ATG GTA TCA GAA TTC CTC-3' and reverse, 5'-AGG TGG AGG AGT GGG TGT CTA G-3'). PCR products were cloned into XhoI and BamHI sites of the pEGFP-N1 vector. A cleavage-resistant ANXA1 mutant (A11R/V22K/V36K) was constructed by sequential site-directed mutagenesis reactions using QuikChange site-directed mutagenesis (Stratagene), according to the manufacturer’s instructions.

Transfection of siRNA and plasmid DNA

Transfections were carried out using the Neon transfection system, according to the manufacturer’s instructions. In brief, RBL-2H3 cells (1 × 10⁶ cells/ml) and BMMCs (2 × 10⁶ cells/ml) were washed with PBS and resuspended in R buffer containing 100 pmol ANXA1 siRNAs for RBL-2H3 cells (siRNA-1, 5'-CCA GCA GAT CCA GAG ACC AUA CUU A-3'; siRNA-2, 5'-CAG AUG AAG ACA CUC UUG AGA U-3') or 5 μg plasmid DNA for RBL-2H3 cells and BMMCs. Resuspended cells were then transferred into a gold tip and electroporated by 1 pulse at 1600 V for 30 ms, followed by incubation overnight in growth media without antibiotics.

Results

Proteolytic cleavage of ANXA1 in activated mast cells

We previously confirmed the proteolytic cleavage of ANXA1 in lung extracts prepared from a murine asthma model (19). To further

FIGURE 2. Effects of ANXA1 knockdown on RBL-2H3 cell activation. NC and siANXA1 were transfected into RBL-2H3 cells by electroporation. Cells were incubated with IgE overnight, serum starved for 6 h, and then activated with DNP-BSA for 30 min. (A) Total RNAs isolated from NC- and siANXA1-transfected cells were subjected to RT-PCR analysis for measurement of ANXA1 and GAPDH mRNA. (B) Degranulation activity. (C) Production of cytokines, and (D) release of cysLTs and PGE₂ from NC- and siANXA1-transfected cells were measured as described in Fig. 1. (E) Lysates from NC- and siANXA1-transfected cells were subjected to immunoblot analysis with Abs against ANXA1, ERK1/2, p-ERK1/2, cPLA2, and β-actin. (A, C, and E) Results are representative of at least three experiments. (B and D) Data are expressed as the means ± SE of three independent experiments. Significant differences were determined by comparison of siANXA1 with the NC in activated cells (*p < 0.05).
elucidate the regulatory mechanisms of ANXA1 cleavage and their roles in inflammation, we activated both RBL-2H3 cells and BMMCs with DNP-BSA. For activation, IgE-sensitized cells were serum starved and then stimulated with DNP-BSA (1 μg/ml) for the indicated times. RBL-2H3 cell activation was determined by measuring degranulation, cytokine production, and eicosanoid generation. Release of β-hexosaminidase from cytosolic granules to extracellular spaces was used as a measure of degranulation activity. The activity reached a maximum at 30 min after stimulation (Fig. 1A). Production of cytokines was determined by RT-PCR analysis of TNF-α, IL-4, and IL-6 mRNA levels. Expression of both TNF-α and IL-4 was rapidly induced by DNP-BSA treatment, whereas IL-6 production was induced only after ≥30 min of treatment (Fig. 1B). The generation of eicosanoids was represented by the presence of cysLTs and PGE2 in cell-free supernatants (Fig. 1C). Like degranulation activity, the production of eicosanoids also reached a maximum at 30 min. ERK and cPLA2 were differentially phosphorylated on DNP-BSA treatment in RBL-2H3 cells (Fig. 1D). ERK was rapidly phosphorylated, and cPLA2 phosphorylation was detected at later time points. ANXA1 was cleaved without any changes in mRNA expression (Fig. 1E, 1F).

BMMC activation was also determined by measuring degranulation and eicosanoid generation. Release of β-hexosaminidase and production of eicosanoids were similar to those of RBL-2H3 cells (Supplemental Fig. 1A, 1B). Similar to our results in RBL-2H3 cells, cleavage of ANXA1 and phosphorylation of ERK and cPLA2 were also detected in BMMCs (Supplemental Fig. 1C).

Release of eicosanoids and phosphorylation of cPLA2 are increased in ANXA1-knockdown RBL-2H3 cells

To investigate the role of ANXA1 in mast-cell activation, we transfected ANXA1 siRNAs into RBL-2H3 cells (Fig. 2A). There was no difference in either degranulation activity or cytokine production between the NC and ANXA1 siRNA-transfected cells (Fig. 2B, 2C). However, the release of cysLTs and PGE2 was significantly increased in ANXA1-knockdown cells (Fig. 2D). To understand the increased production of eicosanoids, we examined the phosphorylation of ERK and cPLA2. Whereas ANXA1 siRNA transfection did not affect ERK phosphorylation, cPLA2 phosphorylation was significantly increased (Fig. 2E). We also found that expression of COX-2 and translocation of 5-LO were not changed in ANXA1-knockdown RBL-2H3 cells (Supplemental Fig. 2). These results suggest that cPLA2 phosphorylation is associated with ANXA1 during mast-cell activation.

**Intracellular Ca2+-dependent translocation of ANXA1 and cPLA2 from the cytosolic to membrane fraction in activated mast cells**

ANXA1 and cPLA2 are known to translocate from the cytosol to the membrane on increase in intracellular Ca2+ concentrations (9, 32). Similar to DNP-BSA stimulation, both A23187, a calcium ionophore, and TG, which increases cytosolic Ca2+ levels, activated RBL-2H3 cells and BMMCs (Fig. 3). Thus, ANXA1 cleavage and cPLA2 phosphorylation were measured on treatment with A23187 and TG. CysLTs and PGE2 were also produced by A23187 and TG treatment. When the cells were pretreated with...
BAPTA-AM, a Ca²⁺ chelator, or 2-APB, a store-operated Ca²⁺ channel inhibitor, ANXA1 cleavage and the production of cysLTs and PGE₂ significantly decreased (Fig. 3A, 3B). We also observed that full-length ANXA1 was localized to both the cytosolic and membrane fractions of nonactivated RBL-2H3 cells. When the cells were activated by DNP-BSA or A23187, ANXA1 was cleaved, and the amount of full-length ANXA1 in the cytosol decreased. In the membrane fraction, the level of full-length ANXA1 did not differ from that of control cells, although cleavage of ANXA1 increased (Fig. 4A, 4B). Thus, in response to cell activation, cleaved ANXA1 translocated mainly to the membrane. Furthermore, both cPLA₂ and p-cPLA₂ translocated from the cytosol to the membrane in activated RBL-2H3 cells (Fig. 4A, 4C). However, ANXA1 knockdown did not affect the translocation of either cPLA₂ or p-cPLA₂ (Fig. 4D).

Both full-length and cleaved ANXA1 interact with cPLA₂

To elucidate the interactions between ANXA1 and cPLA₂ during mast-cell activation, we performed coimmunoprecipitations of the proteins from whole-cell lysates and subcellular fractions. In the whole-cell lysates, cPLA₂ interacted with either full-length or cleaved ANXA1 (Fig. 5A). However, coimmunoprecipitation of proteins from the subcellular fractions showed that cPLA₂ mainly interacted with full-length ANXA1 in the cytosol and with cleaved ANXA1 in the membrane fraction (Fig. 5B). A cleavage-resistant ANXA1 mutant inhibits phosphorylation of cPLA₂

It was previously reported that cleavage of ANXA1 occurred at three sites (Ala¹¹, Val²², and Val³⁶) in the N-terminal region during the activation of neutrophils (13). The ANXA1 mutant (A11R/V22K/V36K), which is resistant to cleavage, controls inflammation in the microvasculature (18). In this study, we investigated whether mutant ANXA1 regulates cPLA₂ activity during RBL-2H3 cell and BMMC activation. GFP-tagged expression vectors containing wild-type ANXA1 (wtANXA1) or triple-mutant ANXA1 (mtANXA1) were constructed; then these vectors were introduced into RBL-2H3 cells and BMCCs as described in the Materials and Methods. We confirmed that mtANXA1 was not cleaved by DNP-BSA stimulation, whereas both endogenous ANXA1 and wtANXA1 were cleaved (Fig. 6A, Supplemental Fig. 3A). We further observed that phosphorylation of cPLA₂ was significantly decreased in both activated RBL-2H3 cells and BMCCs expressing mtANXA1, whereas ERK phosphorylation was not affected (Fig. 6B, Supplemental Fig. 3B). We also found that both wtANXA1 and mtANXA1 interacted with cPLA₂, p-cPLA₂ was not detected in mtANXA1-expressing RBL-2H3 cells and BMCCs under activation conditions (Fig. 6C, Supplemental Fig. 3C).
ANXA1 cleavage regulates production of eicosanoids in mast-cell activation

Next, we determined the effects of ANXA1 cleavage on the production of proinflammatory mediators in both RBL-2H3 cells and BMMCs expressing wtANXA1 or mtANXA1. Compared with control or wtANXA1, mtANXA1-expressing cells did not show any difference in either degranulation activity or cytokine production (Fig. 7A, 7B). However, release of cysLTs and PGE2 was significantly decreased in mtANXA1-expressing RBL-2H3 cells and BMMCs (Fig. 7C).

Discussion

In this study, we confirmed that ANXA1 was cleaved in activated mast cells, and this cleavage was required for the phosphorylation of cPLA2 and subsequent inflammatory reactions, such as the production of eicosanoids. In resting RBL-2H3 cells, full-length ANXA1 interacted with cPLA2 in the cytosol. However, when cells were activated by DNP-BSA or A23187, both ANXA1 cleavage and cPLA2 phosphorylation were observed. Furthermore, cleaved ANXA1 and phosphorylated cPLA2 interacted and colocalized mainly to the membrane fractions of activated mast cell. We also found that mutant ANXA1, which is resistant to cleavage in activated mast cells, exhibited inhibitory effects on both the phosphorylation of cPLA2 and production of eicosanoids.

Different degrees of ANXA1 expression and susceptibility to modulation by allergic stimuli have been observed in connective tissue and mucosal mast cells (33). ANXA1-null mice have shown resistance to the anti-inflammatory actions of glucocorticoids in several experimental models of inflammation (34–36). Mast cells from ANXA1-null mice are more prone to release of histamine and PGD2 than those of wild-type mice in response to compound 48/80 and zymosan, respectively (37). Moreover, ANXA1-derived peptide Ac2-26 and antiinflammin, nonapeptide fragments of lipocortin I, also inhibited mast-cell degranulation (38, 39). These inhibitory effects were thought to be mediated by formyl peptide receptor in mast cells. In this study, we found that ANXA1-knockdown RBL-2H3 cells released more eicosanoids and exhibited increased phosphorylation of cPLA2. These effects were mediated through endogenous ANXA1, not formyl peptide receptor downstream signaling. The release of β-hexosaminidase from RBL-2H3 cells did not change with DNP-BSA treatment, as has been shown with release of histamine by compound 48/80 treatment (Fig. 2). ERK phosphorylation is known to be necessary for the generation of inflammatory mediators in mast-cell activation. Activated ERK has been shown to phosphorylate cPLA2 for AA release (40). To determine whether the increased phosphorylation of cPLA2 in ANXA1-knockdown cells was due to changes in ERK phosphorylation, we measured ERK phosphorylation in RBL-2H3 cells transfected with ANXA1 siRNA. In this experiment, we found that the level of ERK phosphorylation in ANXA1-knockdown RBL-2H3 cells was the same as that in control cells. However, cPLA2 phosphorylation was markedly increased in the knockdown cells (Fig. 2E). These data suggest that ANXA1 may regulate ERK-mediated cPLA2 phosphorylation during mast-cell activation.

Eicosanoids are produced predominantly by inflammatory cells, such as PMN cells, macrophages, and mast cells. The FcεRI-mediated mast-cell activation signal pathway for eicosanoid production is regulated by a complex series of signaling molecules. In particular, cPLA2 activation by ERK and intracellular Ca2⁺ is
imported for the production of eicosanoids (27, 41). cPLA₂ is located mainly in the cytosol in resting mast cells and undergoes translocation to the nuclear envelope and endoplasmic reticulum in response to various stimuli (23). Phosphorylation of cPLA₂ and increased intracellular Ca²⁺ synergistically promote the full activation of cPLA₂ for AA release (42, 43). AAs are converted to leukotrienes and PGs by 5-LO and COX, respectively (20). Membrane translocation of 5-LO and increased levels of COX-2 without changes in COX-1, 5-LO, and cPLA₂ expression were observed in Ag-stimulated mast cells (44, 45). In this study, we demonstrated the expression of COX-2 and translocation of 5-LO in ANXA1-knockdown RBL-2H3 cells (Supplemental Fig. 2). COX-2 was expressed when both control and ANXA1-knockdown RBL-2H3 cells were activated. 5-LO translocated from the cytosol to the membrane in activated RBL-2H3 cells and ANXA1-knockdown cells. Production of cysLTs and PGE₂ also had similar patterns in activated RBL-2H3 cells and BMMCs. These data indicated that ANXA1 did not affect COX or 5-LO activity.

Previous studies have demonstrated that the phosphorylation of cPLA₂ at Ser²⁰⁵ increases its catalytic activity and phospholipid-binding affinity at low Ca²⁺ levels both in vitro and in vivo (25). However, the S505A mutant cPLA₂ also translocated to the membrane in response to a calcium ionophore, although it released less AA (46). In addition, a recent study revealed that phosphorylation of cPLA₂ acts to regulate catalytic activity, but not calcium-dependent membrane binding (47). We found that increased intracellular Ca²⁺ concentrations caused by treatment with DNP-BSA, A23187, and TG regulated not only the cleavage of ANXA1, but also the phosphorylation of cPLA₂ and release of eicosanoids. In contrast, when intracellular Ca²⁺ levels were decreased by pretreatment with BAPTA-AM and 2-APB, ANXA1 cleavage, cPLA₂ phosphorylation, and eicosanoid production were all inhibited (Fig. 3). We also observed that cPLA₂ translocated from the cytosol to the membrane in activated RBL-2H3 cells, but downregulation of ANXA1, did not affect the translocation of cPLA₂. However, cPLA₂ phosphorylation was increased in the membrane fraction of ANXA1-knockdown cells (Fig. 4D). These data indicate that ANXA1 regulates cPLA₂ phosphorylation, but not cPLA₂ translocation.

ANXA1 is present in both the nucleus and cytoplasm of rat mast cells (48). In this study, we also investigated the intracellular localization of ANXA1 in activated RBL-2H3 cells. The level of full-length ANXA1 decreased in the cytosolic fraction, whereas ANXA1 cleavage increased in the membrane fraction on activation of RBL-2H3 cells with DNP-BSA or A23187 (Fig. 4A, 4B, 5B). However, the level of full-length ANXA1 in the membrane fraction did not change during activation. Together with the data on cPLA₂ translocation described earlier, these results indicate that both cleaved ANXA1 and cPLA₂ translocate from the cytosol to the membrane in activated mast cells.

ANXA1 is a cPLA₂-binding protein that negatively regulates cPLA₂ activity both in vitro (28, 49) and in vivo (14, 50). Interactions of cPLA₂ with either full-length or cleaved ANXA1 were observed in whole-cell lysates (Fig. 5A). Using subcellular fractions, we found that cPLA₂ mainly interacted with full-length ANXA1 in the cytosol and cleaved ANXA1 in the membrane (Fig. 5B). Furthermore, phosphorylated cPLA₂ was mainly observed in the membrane fraction of activated RBL-2H3 cells.

The N-terminal peptide of ANXA1 is cleaved by several proteases, including elastase, calpain, plasmin, cathepsin D, and proteinase 3 (11–14). ANXA1 cleavage promotes neutrophil transendothelial migration (17) and regulates EGF-triggered signaling and AA production in normal and malignant squamous epithelial cells (14). Recently, an ANXA1 mutant (A11V/V22K/V36K) was reported to be resistant to cleavage by all proteases present in PMN cell extracts (18). In this study, we tested the effects of

**FIGURE 7.** A cleavage-resistant ANXA1 mutant inhibited the production of eicosanoids in DNP-BSA-stimulated mast cells. GFP-, wtANXA1-, or mtANXA1-overexpressing RBL-2H3 cells and BMMCs were activated with DNP-BSA for 30 min. (A) Incubation buffer and cell lysates were recovered for comparative analysis of β-hexosaminidase release as a measure of RBL-2H3 cell degranulation activity. Data are expressed as means ± SE of three independent experiments. (B) Total RNA isolated from activated RBL-2H3 cells was subjected to RT-PCR analysis. The expression of TNF-α, IL-4, and IL-6 mRNA was determined for measurement of cytokine production. GAPDH was used as a loading control. Results shown are representative of at least three independent experiments. (C) The levels of secreted cysLTs from activated RBL-2H3 cells and BMMCs were analyzed by an enzyme immunoassay system. Data are expressed as means ± SE of three independent experiments. Significant differences were determined by comparison of wtANXA1 or mtANXA1 constructs with GFP (*p < 0.05).
N-terminal cleavage on inflammatory reactions using RBL-2H3 cells and BMMCs expressing mtANXA1. Expression of this mtANXA1 protein blocked the phosphorylation of cPLA2 in activated RBL-2H3 cells and BMMCs, but ERK phosphorylation was not affected (Fig. 6B, 6C, Supplemental Fig. 3B, 3C). The release of cysLTs and PGE2 was inhibited in mtANXA1-expressing RBL-2H3 cells and BMMCs; however, there were no changes in degranulation activity or cytokine production (Fig. 7). This could be attributed to the inhibition of cPLA2 phosphorylation by the mutant full-length ANXA1.

In summary, we suggest that ANXA1 cleavage is a necessary process for inflammatory reactions in mast cells. cPLA2, which constitutively interacts with ANXA1, is phosphorylated by cleavage of ANXA1 during mast-cell activation. Phosphorylated cPLA2 then produces AA, which is converted into various eicosanoids. Cleavage of ANXA1 may result in the loss of ANXA1’s inhibitory effect on cPLA2 phosphorylation and may promote eicosanoid production during mast-cell activation. Although the mechanism of ANXA1 cleavage has not been fully elucidated, we found that intracellular Ca2+ concentration is one of the most important regulatory factors for ANXA1 cleavage in mast-cell activation.

Acknowledgments
We thank Drs. Jae-Hong Kim and Kyung-Jin Cho (College of Life Sciences and Biotechnology, Korea University) for BMMC culture technique.

Disclosures
The authors have no financial conflicts of interest.

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