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House Dust Mite Allergen *Der f 2*-induced Phospholipase D1 Activation Is Critical for the Production of Interleukin-13 through Activating Transcription Factor-2 Activation in Human Bronchial Epithelial Cells*

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The purpose of this study was to identify the role of phospholipase D1 (PLD1) in *Der f 2*-induced interleukin (IL)-13 production. The major house dust mite allergen, *Der f 2*, increased PLD activity in human bronchial epithelial cells (BEAS-2B), and dominant negative PLD1 or PLD1 siRNA decreased *Der f 2*-induced IL-13 expression and production. Treatment of *Der f 2* activated the phospholipase Cγ (PLCγ)/protein kinase Cα (PKCα)/p38 MAPK pathway. *Der f 2*-induced PLD activation was attenuated by PLCγ inhibitors (U73122 and PAO), PKCα inhibitors (RO320432 and GO6976), and p38 MAPK inhibitors (SB203580 and SB202190). These results indicate that PLCγ, PKCα, and p38 MAPK act as upstream activators of PLD in *Der f 2*-treated BEAS-2B cells. Furthermore, expression and production of IL-13 increased by *Der f 2* were also blocked by inhibition of PLCγ, PKCα, or p38 MAPK, indicating that IL-13 expression and production are related to a PLCγ/PKCα/p38 MAPK pathway. We found that activating transcription factor-2 (ATF-2) was activated by *Der f 2* in BEAS-2B cells and activation of ATF-2 was controlled by PLD1. When ATF-2 activity was blocked with ATF-2 siRNA, *Der f 2*-induced IL-13 expression and production were decreased. Thus, ATF-2 might be one of the transcriptional factors for the expression of IL-13 in *Der f 2*-treated BEAS-2B cells. Taken together, PLD1 acts as an important regulator in *Der f 2*-induced expression and production of IL-13 through activation of ATF-2 in BEAS-2B cells.

House dust mites (HDMs)[3] are the most important source of indoor aeroallergens that contribute to the rising incidence of allergic diseases such as allergic asthma (1, 2). Allergens from two common house dust mite species are *Dermatophagoides farinae* (*Der f*) and *Dermatophagoides pteronyssinus* (*Der p*), which share a similar amino acid sequence (3, 4). Recent data provide evidence that the allergens amplify allergen-induced cytokine expression (5). Indeed, several studies demonstrated that *Der p 1* stimulates release and expression of cytokines in human airway epithelial cells (6), including IL-13 in cord blood mononuclear cells (7) and IL-2 and IL-4 in a cord blood T-cell phenotype with atopic dermatitis (8). Although the release of cytokines by allergen stimulation has been studied, details of the intracellular signaling mechanism, including the secretion of cytokines, remain unclear.

Phospholipase D (PLD) catalyzes the hydrolysis of phospholipids at the terminal phosphodiester bond, thereby producing phosphatidic acid (PA) and releasing the free polar head group. PA can be metabolically converted to diacylglycerol by phosphatidic acid phosphatases type 2A (PPAP2A) or to lysophosphatidic acid (LPA) and arachidonic acid by phospholipase A2 (PLA2G1B) (9). Both of these factors serve as second messengers that contribute to the effects of PLD. Recently, two PLD isozymes (PLD1 and PLD2) have been identified from mammalian cells (10, 11). PLD1 is present throughout the cell, particularly in perinuclear, Golgi, and heavy membrane fractions, whereas PLD2 is located almost exclusively in the plasma membrane of light membrane "lipid raft" fractions (12). The differences in the localization of these PLDs can provide important clues, suggesting their specific roles in various situations and cell types (13). PLD and phospholipase C (PLC) γ play an important regulatory role in cell immune response (14, 15). The activation of PLCγ1 results in the hydrolysis of phosphatidylinositol 4,5-bisphosphates (PIP_2) to generate inositol 1,4,5-

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[3] The abbreviations used are: HDM, house dust mite; IL, interleukin; PLD, phospholipase D; PLC, phospholipase C; PKC, protein kinase C; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinases; JNK, c-Jun N-terminal kinase; ATF-2, activating transcription factor-2; p-ATF-2, phosphorylated ATF-2; siRNA, small interfering RNA; EGFP, enhanced green fluorescent protein; PBt, phosphatidylbutanol; PA, phosphatidic acid; LPA, lysophosphatidic acid; PPAP2A, phosphatidic acid phosphatases type 2A; PLA2G1B, phospholipase A2, group 1B; RT, reverse transcription; ANOVA, analysis of variance; ELISA, enzyme-linked immunosorbent assay; glyceraldehyde-3-phosphate dehydrogenase; DN, double negative; SAPK, stress-activated protein kinase; QIL, quantitative interleukin.

trisphosphates (IP_3) and diacylglycerol (16, 17). These second messengers are responsible for the activation of protein kinase C (PKC) (18). Activation and phosphorylation of PLD1 are regulated by PKCα in phorbol myristate acetate-treated COS-7 cells, also demonstrating the interrelationships between PLD and PKC isoforms in a variety of cell types (19). Previously, we reported that *Der p 1* activates PLD in human peripheral blood mononuclear cells from allergic patients (13) and that *Der f 2* activates PLD in human T lymphocytes from *Der f 2*-specific allergic individuals (1). However, the molecular mechanisms of PLD for the expression of cytokine in the immune response induced by *Der f 2* have not been fully understood.

Recent reports show that mitogen-activated protein kinase (MAPK) activation is involved in the process of allergen-induced cytokine expression (20, 21). There are three distinct MAPK signaling pathways in mammalians: extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs), and p38 MAPK (21, 22). Among the MAPK subtypes, activation of p38 MAPK is critical for the conversion of external stimuli to proinflammatory gene expression (20, 23), and it has also been implicated in the regulation of respiratory burst activity, priming, deregulation, and cytokine production (24, 25). In particular, the p38 MAPK-specific inhibitor pyridinyl imidazole compound SB203580 has facilitated extensive investigation of the role of the p38 MAPK pathway in inflammatory responses (26). Commonly, p38 MAPK phosphorylates activating transcription factor 2 (ATF-2) to augment gene transcription (27). ATF-2 activity is regulated by phosphorylation of Thr-69 and Thr-71 residues in its N-terminal region (28), and the signaling to activate ATF-2 affects the selection of target genes by ATF-2. Therefore, ATF-2 may play a role in the regulation of cytokine gene expression.

IL-13 is produced when cells are exposed to a variety of environmental stimuli such as allergens, pharmacological agents, and infectious stimuli. IL-13 production is highly regulated by a variety of mediators and cytokines, thereby leading to a positive feedback loop which sustains immune responses (29). The importance of IL-13 in allergic disorders has been indicated by consistent associations between IL-13 levels of tissue and genetic variants in the IL-13 gene with asthma and related traits (30). IL-13 production has also been shown to mediate allergic responses through signaling pathways such as PKA, PKC, p38MAPK, ERK1/2, and JAK/STAT in B cells, monocytes/macrophages, eosinophils, basophils, and airway epithelial cells (30–34). However, the mechanisms of *Der f 2*-induced signal transduction and the role of PLD in mediating IL-13 production in human bronchial epithelial cells are not well defined.

In the present study, we investigated *Der f 2*-induced activation of PLD and its regulation by the PLCγ/PKCα/p38 MAPK pathway. This novel mechanism regulated ATF-2-mediated expression and production of IL-13. To the best of our knowledge, this is the first report to show that PLD1 is an important regulator in the production of IL-13 through a *Der f 2*-stimulated PLCγ/PKCα/p38 MAPK pathway in BEAS-2B cells.

EXPERIMENTAL PROCEDURES

Cell Culture—BEAS-2B, a human airway epithelial cell line transformed with adenovirus 12-SV40 virus hybrid, was purchased from the American Type Culture Collection. BEAS-2B cells were cultured in Dulbecco's modified Eagle's medium/F12 with 10% fetal bovine serum, 100 units/ml penicillin, and 100 ng/ml streptomycin (Invitrogen) at 37 °C with 5% CO_2 in humidified air.

Reagents—Fetal bovine serum, penicillin/streptomycin solution, and Dulbecco's modified Eagle's medium/F-12 were purchased from Invitrogen. U73122, PAO, RO320432, GO6976, SB203580, and SB202190 were obtained from Calbiochem, and [^{3}H]palmitic acid was from PerkinElmer Life Sciences. Propranolol, mepacrine, and 1,2-dioctanoly-*sn*-glycerol-3-phosphate sodium salt (PA) were purchased from Sigma-Aldrich. Real-time PCR reagents were from Bio-Rad Laboratories. Antibodies used were as follows: p38 MAPK polyclonal antibody, p-p38 MAPK polyclonal antibody, PKC isozymes, ATF-2 polyclonal antibody, and phosphorylated ATF-2 (p-ATF-2) polyclonal antibody were from Cell Signaling; and GAPDH, integrin α2, PLCγ1, and PKC$\alpha/\beta1/\gamma/\lambda$ were from Santa Cruz Biotechnology. A polyclonal antibody that recognizes both PLD1 and PLD2 was provided by Dr. D. S. Min (Pusan National University, Korea), L-α-phosphatidylbutanol (PBt) and oleoyl-*sn*-glycero-3-phosphate (LPA) were from Avanti Polar Lipids (Alabaster, AL), and the silica gel 60A plates for TLC were purchased from Whatman. All other chemicals were of analytical grade.

Real-time PCR and Reverse Transcription (RT)-PCR—Total cellular RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. For the reverse transcriptase reaction, 5 μg of total RNA was mixed with oligo$(dT)_{16}$ primer and Maloney murine leukemia virus reverse transcriptase (Promega), and the mixture was incubated for 60 min at 42 °C. The transcribed products were mixed with each primer set and *Taq* DNA polymerase (Takara Shuzo Co.) and then amplified. The primer sequences were as follows: IL-13 sense (5′-GAGTGTGTTTGTCACCGTTG-3′) and antisense (5′-TACTCGTTGGTCGAGAGCTG-3′) (PCR product, 275 bp); PLD1 sense (5′-ACTCTGTCCAAAGTTAACATGTCACTG-3′) and antisense (5′-GGCTTTGTCTTGAGCAGCTCTCT-3′) (PCR product, 245 bp); human β-actin sense (5′-AACACCCCAGCCATGTACG-3′) and antisense (5′-ATGTCACGCACGATTTCCC-3′) (PCR product, 293 bp). The PCR products were analyzed in a 1.2% agarose gel. For real-time PCR, an aliquot of 5 μl of the RT reaction was amplified in duplicate in a final volume of 30 μl of iQ™ SYBR® Green Supermix. Thermocycling conditions were 50 °C for 20 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The primer sequences of real-time PCR were as follows: human QIL-13 sense (5′-ACAGCCCTCAGGGAGCTCAT-3′) and antisense (5′-TCAGGTTGA TGCTCCATACCAT-3′); human GAPDH sense (5′-CATGAGAAGTATGACAACAGCCT-3′) and antisense)5′-AGTCCTTCCACGATACCAAAGT-3′).

Transient Transfection with Plasmid DNA in BEAS-2B Cells—BEAS-2B cells were transiently transfected using 5 μg each of pEGFP-C1 (vector), EGFP-PLD1, EGFP-dominant negative PLD1, EGFP-PLD2, and EGFP-dominant negative PLD2 plasmid using Lipofectamine™ reagents (Invitrogen). After 48 h of transfection, the cells were serum-starved for 18 h and then treated with *Der f 2*.

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Preparation of Cytosolic and Membrane Fraction—Serum-starved cells were incubated with *Der f 2* (10 μg/ml) for 1 or 3 min, scraped in ice-cold phosphate-buffered saline, and harvested by microcentrifugation. The cells were then resuspended in a buffer solution (50 mM NaCl, 1 mM $MgCl_2$, 2 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 50 mM HEPES, pH 7.5), disrupted by sonication, and centrifuged at 100,000 × *g* for 1 h. The supernatant was used as the cytosolic fraction, and the pellet (membrane fraction) was resuspended in lysis buffer containing 1% (v/v) Triton X-100.

Western Blot Analysis—Cells were first lysed in 20 mM Tris, pH 7.5, containing 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and 1 mM Na3VO4. Proteins ranging from 10 to 20 μg were subsequently loaded onto SDS-polyacrylamide gels (12%), electrophoresed, and transferred to nitrocellulose membrane (Amersham Biosciences). After being blocked with 5% dried skim milk for 2 h, the membrane was incubated with primary antibodies. The blots were further incubated with horseradish peroxidase-conjugated secondary antibody (1:2000, New England Biolabs, Beverly, MA), and specific bands were detected through ECL (Amersham Biosciences).

Determination of PLD Activity—PLD activity was determined by the formation of PBt, the product of PLD-mediated transphosphatidylation, in the presence of butanol as described previously with a slight modification (35). The cells, which had been incubated for the indicated times in 6-well plates, were radioactively labeled with 2 μCi/ml of [^{3}H]palmitic acid in serum-free medium for 20 h. The cells were then pretreated with 0.3% (v/v) 1-butanol for 15 min before stimulation with *Der f 2*. Also, the cells were preincubated with U73122, PAO, RO320432, GO6976, SB203580, or SB202190 for 30 min after being labeled with [^{3}H]palmitic acid and serum-starved for 20 h. After treatment with *Der f 2* for 30 min, the cells were quickly washed with ice-cold phosphate-buffered saline and suspended in ice-cold methanol. Lipids were extracted according to the method of Bligh and Dyer (36), and PBt was separated by TLC using a acetate/isooctane/acetic acid/water (110:50:20:100,v/v) solvent system. The regions corresponding to the authentic PBt bands were identified with 0.002% (w/v) primulin in 80% (v/v) acetone, scraped, and counted using a liquid scintillation counter.

ELISA—The cell supernants were collected after *Der f 2* treatment for the indicated times, and the levels of IL-13 were quantified by ELISA. The detection limit was 30 pg/ml. Plates were read on a SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, CA) and analyzed with SOFTmax analysis software (Molecular Devices). The means of triplicate ELISA values for each of the dose relationships among PLD1, ATF-2 knockdown, and IL-13 protein expression were calculated by linear regression.

Small Interfering RNAs—The single siRNA for PLD1 was purchased from Dharmacon Research Inc. (Lafayette, IN), and the secondary siRNA oligonucleotide, PLD1 (5′-AAGGUGGGACGACAAUGAGCA-3′), was synthesized by Ambion (Austin, TX). Two kinds of ATF-2 oligonucleotide siRNAs were purchased from Qiagen (Cambridge, MA) and Santa Cruz Biotechnology. Oligonucleotide siRNAs were transfected using the siPORT™ NeoFX (Ambion) reagent according to the manufacturer's instructions. At the indicated intervals following transfection, cell lysates were assayed for gene silencing effects by RT-PCR or Western blotting.

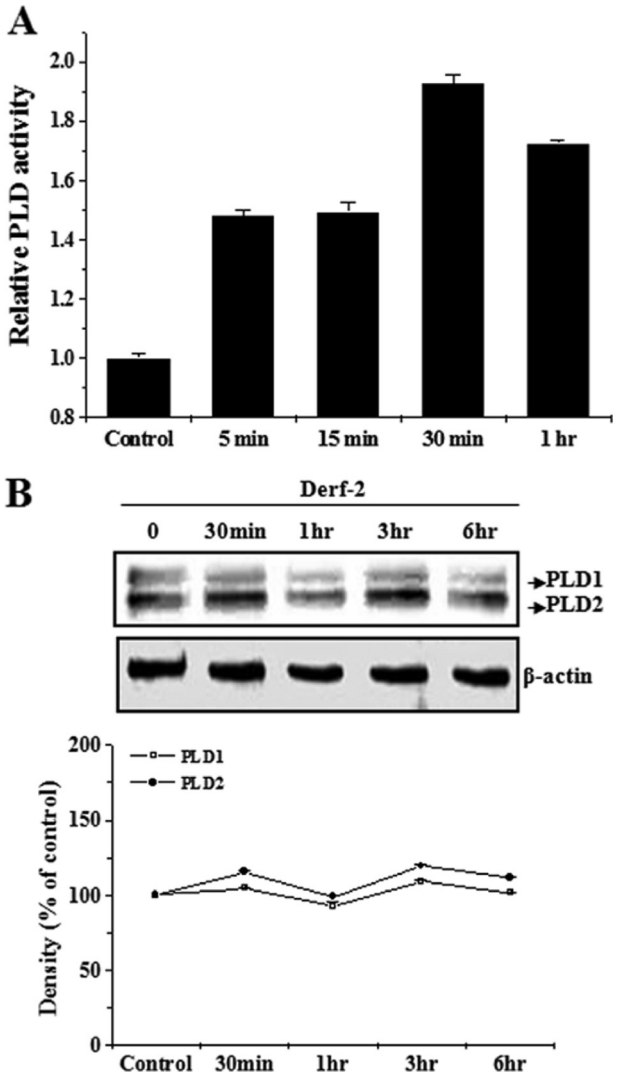

FIGURE 1. **Time-dependent activation of PLD by *Der f 2* and changes in PLD expression level after treatment of BEAS-2B cells with *Der f 2*.** *A*, BEAS-2B cells were labeled with 2 μCi/ml [^{3}H]palmitic acid and then stimulated with *Der f 2* (10 μg/ml) for the indicated times. PLD activities were then determined by estimating the amount of [^{3}H]PBt in the presence of 1-butanol. Results are means ± S.D. from three independent experiments. *B*, cells treated with *Der f 2* (10 μg/ml) for the indicated times were lysed. Each fraction, containing 30 μg of protein, was analyzed by 10% SDS-PAGE and subsequently by Western blotting using anti-PLD polyclonal antibody. Bands were shown by ECL. The intensity of the bands was quantified using Quantity One software (Bio-Rad). All determinations were carried out on three different cell lysates.

Immunocytochemistry—For immunocytochemical tests, BEAS-2B cells were plated on a glass coverslip. Cells were fixed with 4% (w/v) paraformaldehyde, 0.15% (w/v) picric acid in phosphate-buffered saline and then incubated overnight with primary antibody at 4 °C. The antigen fluorescent protein (GFP) monoclonal antibody was used at 1:400 (Roche Molecular Biochemicals). For detection of the primary antibody, fluo-

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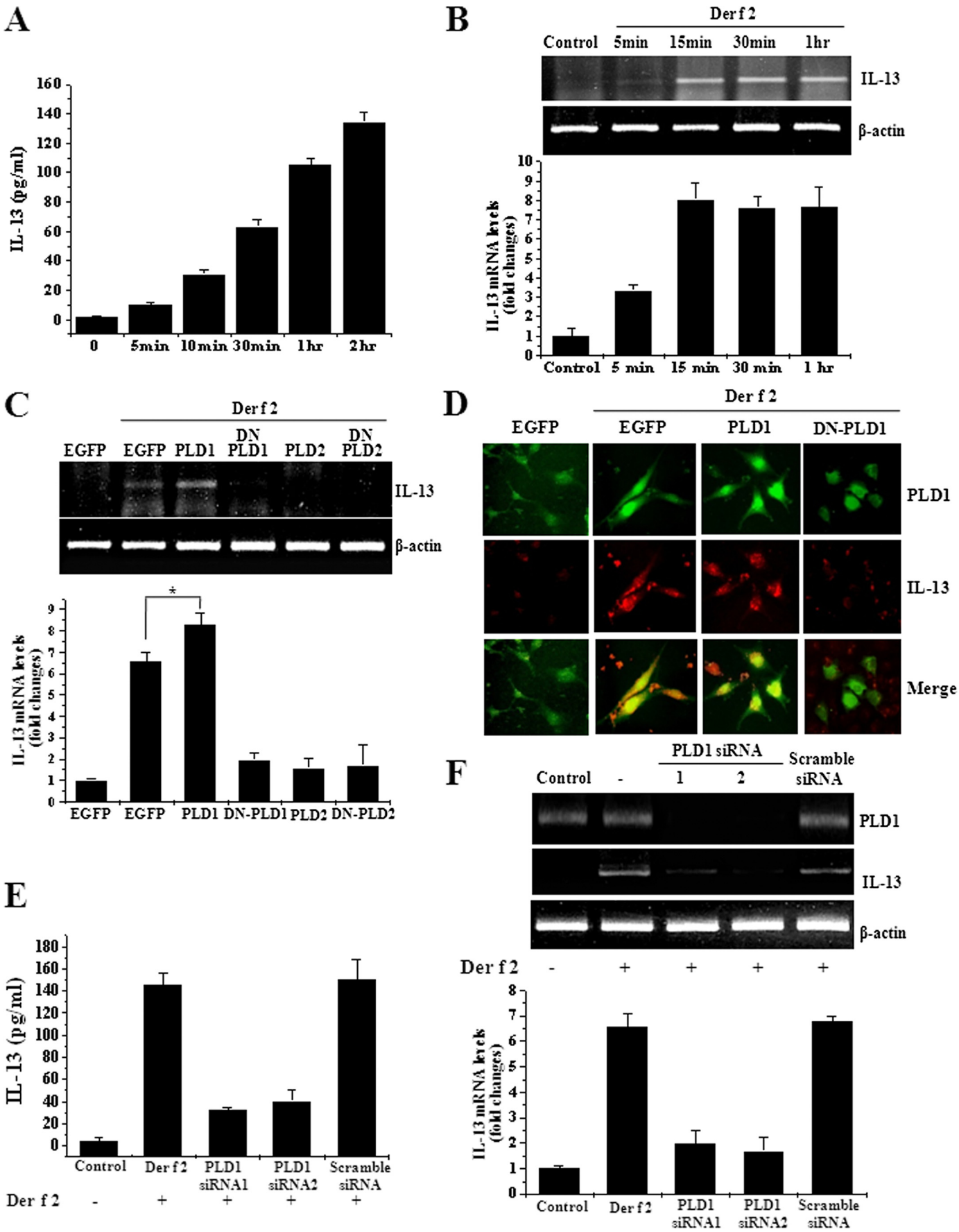

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rescently labeled (Cy3) secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) were used according to the specifications of the manufacturer. Cells were mounted in Vectashield with 4,6-diamidino-2-phenylindole (mounting medium for fluorescence analysis; Vector Laboratories, Burlingame, CA) and photographed using a fluorescent microscope (Nikon).

Statistical Analysis—All experiments were performed at least three to five times, and data analyzed using one-way ANOVA were considered to be significantly different at $p < 0.05$.

RESULTS

Effect of Der f 2 on PLD Activity in Human Bronchial Epithelial Cells—To examine the role of PLD in human bronchial epithelial cells (BEAS-2B), we first measured PLD activity after treating the cells with *Der f 2*. As shown in Fig. 1*A*, when the cells were treated with *Der f 2*, PLD activity was increased by nearly 1.9-fold in 30 min. Subsequently, to examine whether the increase of PLD activity was due to increased *de novo* PLD synthesis, we quantitated the PLD expression by Western blot analysis after treatment of BEAS-2B cells with *Der f 2*. As shown in Fig. 1*B*, PLD1 and PLD2 protein expression levels did not change within 1 h, indicating that up-regulation of PLD activity within 1 h was not due to an increased PLD protein level but rather most likely to the presence of a PLD activation pathway.

PLD1 Regulates Der f 2-induced IL-13 Expression and Production—Many studies have revealed that allergens induce various types of cytokine expression in many mammalian cell lines (37, 38). To determine whether *Der f 2* could enhance IL-13 levels, BEAS-2B cells were cultured with *Der f 2* for the indicated times, and the IL-13 production and expression were then examined by ELISA, RT-PCR, and real-time PCR. *Der f 2*-induced IL-13 production and its expression reached a maximum within 30 min of the treatment (Fig. 2, *A* and *B*).

As PLD stimulates cytokine expression in mammalian cells (39, 40), we investigated which isozymes of PLD in BEAS-2B cells are involved in IL-13 expression when induced by *Der f 2*. Thus, the cells were transfected with wild types and catalytically inactive mutants of PLD1 or PLD2 plasmid constructs. Two days after transfection, cells were treated with *Der f 2* for 15 min. As shown in Fig. 2, *C* and *D*, PLD1 overexpression potentiated IL-13 expression increased by *Der f 2*, whereas knockdown of PLD1 using DN-PLD1 transfection completely abolished IL-13 expression induced by *Der f 2* but not PLD2, indicating that PLD1 is involved in *Der f 2*-induced IL-13 expression. To confirm the role of PLD1 in *Der f 2*-induced production and expression of IL-13, we examined the effects of PLD1 siRNA transfection in BEAS-2B cells. Scrambled siRNA was transfected as a control. Two day after transfection of the cells with PLD1 siRNA or scrambled siRNA, cells were treated with *Der f 2* for 15 min, and the IL-13 and PLD1 expressions were then examined by RT-PCR and real time PCR. As shown in Fig. 2, *E* and *F*, PLD1 siRNA completely abolished both *Der f 2*-induced IL-13 and PLD1 expression and IL-13 production. These data suggest that PLD1 activation is important for *Der f 2*-induced expression and production of IL-13 in BEAS-2B cells.

Der f 2-induced IL-13 Production and Expression Are Regulated by the PLCγ/PKCα/p38 MAPK Pathway in BEAS-2B Cells—The activation of PLC is involved in allergic airway-related signal transduction (41). To investigate how *Der f 2* activates PLD1, we examined PLCγ signaling because there are studies suggesting that PLCγ activation results in increased PLD activity (42). The mammalian PLCγ family has two closely related members, PLCγ1 and PLCγ2. PLCγ1 is ubiquitously expressed, whereas PLCγ2 expression is limited to certain immune cell types (43). PLCγ1 phosphorylation leading to its activation is a key step in intracellular distal signal transduction. Fig. 3*A* shows that PLCγ1 phosphorylation induced by *Der f 2* resulted in translocation of PLCγ1 from cytosol to membrane. To determine whether PLCγ1 affects *Der f 2*-induced PLD activation, cells were pretreated with a specific PLCγ inhibitor, U73122 or PAO, and the effects on *Der f 2*-induced PLD activation and IL-13 production were examined. As shown in Fig. 3*B*, pretreatment with U73122 or PAO for 30 min completely inhibited *Der f 2*-induced PLD activation. Furthermore, when the activity of PLCγ was blocked, a remarkable inhibition of IL-13 production was observed (Fig. 3*C*), indicating that PLCγ1 activation is located upstream of PLD1 and is important for *Der f 2*-induced IL-13 production via PLD activation in BEAS-2B cells.

We had reported earlier that *Der p1* and *Der f 2* can stimulate PLD activity and that PKC activation is involved in this stimulation (1, 13). To assess which PKC isozymes are involved in *Der f 2*-induced IL-13 production, we examined the translocation of PKC isozymes from cytosol to membrane. As shown in Fig. 4*A*, translocation of only PKCα from cytosol to membrane was found when BEAS-2B cells were treated with *Der f 2* for 1 min. Other PKC isozymes such as PKCβ, PKCγ, and PKCλ were not responsive to *Der f 2* in BEAS-2B cells. To determine

FIGURE 2. **Effect of PLD knockdown on *Der f 2*- induced IL-13 production and expression in BEAS-2B cells.** *A*, BEAS-2B cells in 96-well culture plates were treated with *Der f 2* (10 μg/ml) for the indicated times. The results shown are mean values ± S.E. of the amount of IL-13 measured by ELISA for each group of samples ($n = 8$). *B*, cells were treated with *Der f 2* (10 μg/ml) for the indicated times. Total RNA was isolated using TRIzol reagent, and mRNA levels were determined by RT-PCR with primers for IL-13 or β-actin and real-time PCR with primers for QIL-13 or GAPDH. Data are means ± S.D. of three values. *C*, cells were transiently transfected with EGFP, DN-PLD1, PLD1, DN-PLD2, or PLD2 plasmid DNA. After transfection for 24 h, they were treated with *Der f 2* (10 μg/ml) for 15 min. Total RNA was isolated using TRIzol reagent, and mRNA levels were determined by RT-PCR with primers for IL-13 or β-actin and real-time PCR with primers for QIL-13 or GAPDH. *, $p < 0.05$ ANOVA *versus* values for *Der f 2*-treated control. Data are means ± S.D. of three values. *D*, cells were transiently transfected with EGFP, DN-PLD1, or PLD1 plasmid DNA. After transfection for 24 h, they were treated with *Der f 2* for 15 min. Transfected cells are defined by their green or red color under the fluorescence microscope after immunostaining using antibodies against EGFP and IL-13 (original magnification ×200). *E*, BEAS-2B cells on 96-well culture plates were transfected with 100 nM PLD1 siRNAs or scrambled siRNA for 72 h and then stimulated with *Der f 2* (10 μg/ml) for 2 h. The results shown are the mean values ± S.E. of the amount of IL-13 measured by ELISA for each group of samples ($n = 8$). *F*, cells were transiently transfected with 100 nM PLD1 siRNAs or scrambled siRNA for 48 h and then stimulated with *Der f 2* (10 μg/ml) for 15 min. The cells were harvested, and total RNA was isolated using TRIzol reagent. RT-PCR analyses were performed using IL-13 and β-actin primers and real-time PCR with primers for QIL-13 or GAPDH. Data are means ± S.D. of three values.

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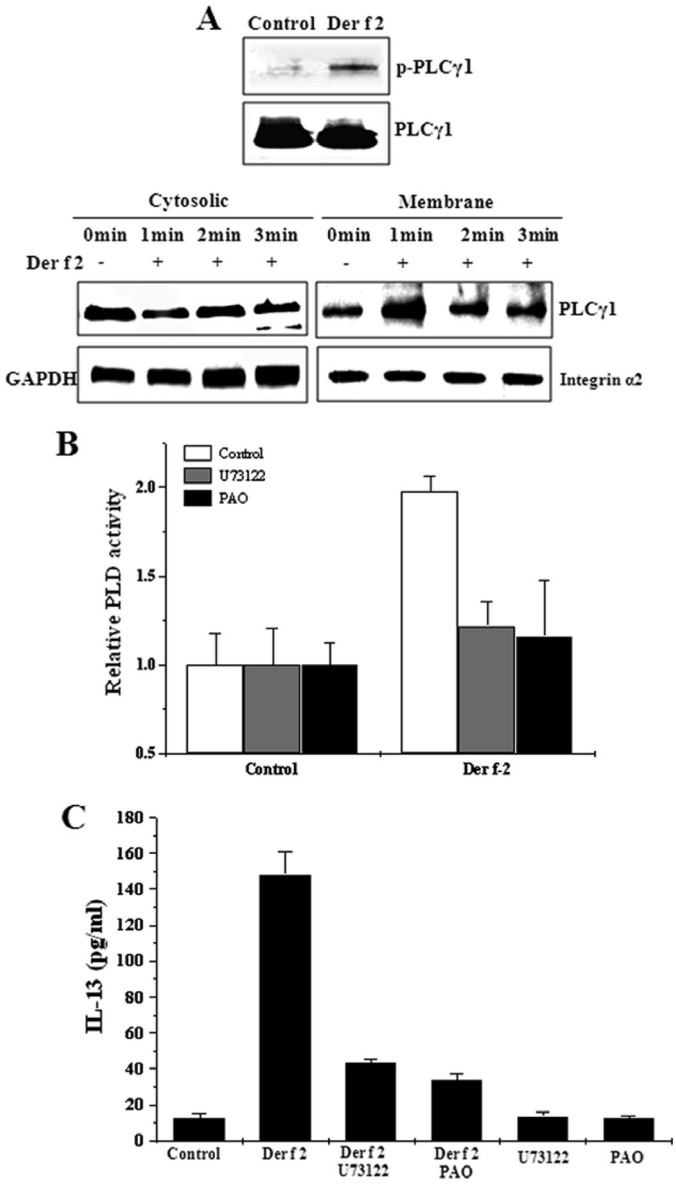

FIGURE 3. **Effect of the PLCγ inhibitors U73122 and PAO on *Der f 2*-induced PLD activation and IL-13 production in BEAS-2B cells.** *A*, BEAS-2B cells were treated with *Der f 2* (10 μg/ml) for 1 min. Cells were harvested, and cell extracts were subjected to immunoblot analysis for active and total PLCγ1 (*upper panel*). Serum-starved cells were stimulated with *Der f 2* (10 μg/ml) for 1, 2, or 3 min. The cells were then lysed and subjected to subcellular fractionation into cytosolic and membrane fractions by centrifugation at 100,000 × *g*, and each fraction containing 30 μg of protein was analyzed by 10% SDS-PAGE and subsequently by Western blotting using anti-PLC γ1 polyclonal antibody (*lower panel*). *B*, cells were labeled with 2 μCi/ml [^{3}H]palmitic acid and then pretreated with 10 μM PLCγ inhibitor U73122 or PAO for 30 min prior to *Der f 2* (10 μg/ml) stimulation. PLD activities were then determined by estimating the amount of [^{3}H]PBt in the presence of 1-butanol. Results are means ± S.D. from three independent experiments. *C*, cells in 96-well culture plates were pretreated with U73122 or PAO (10 μM) for 30 min and then stimulated with *Der f 2* (10 μg/ml) for 2 h. The results shown are mean values ± S.E. of the amount of IL-13 measured by ELISA for each group of samples ($n = 8$).

whether PKCα translocation is related to the PLCγ translocation, we inhibited both PKCα and PLCγ with their specific inhibitors U73122/PAO and RO320432/GO6976, respectively. As shown in Fig. 4*B*, specific PLC inhibitors U73122 and PAO inhibited PKCα translocation stimulated by *Der f 2* (*upper panel*), whereas PKC inhibitors RO320432 and GO6976 could not inhibit PLCγ translocation (*lower panel*). This result showed that PLCγ is located upstream of PKCα in *Der f 2*-stimulated BEAS-2B cells. To further elucidate the role of PKCα in *Der f 2* induced IL-13 production via PLD1 activation, cells were pretreated with RO324032 or GO6976, and the effects of PKC inhibition on *Der f 2*-induced PLD activation and IL-13 production were examined. The pretreatment of the cells with RO320432 or GO6976 for 30 min inhibited *Der f 2*-induced PLD activation (Fig. 4*C*). Furthermore, when the activity of PKCα was blocked, a remarkable inhibition of IL-13 production was observed (Fig. 4*D*). Taken together, these results suggest that PKCα is involved in *Der f 2*-induced PLD activation and is important for *Der f 2*-induced IL-13 production via PLD1 activation in BEAS-2B cells.

One of the targets of *Der f 2* is MAPK (44, 45). We also confirmed that *Der f 2* stimulates the phosphorylation of MAPKs. A time course analysis showed that *Der f 2* induced rapid phosphorylation of p38 MAPK, reaching a maximum in 5 min, whereas the phosphorylation of SAPK/JNK and ERK did not change within 60 min (Fig. 5*A*). To determine whether p38 MAPK phosphorylation has any influence on *Der f 2*-induced PLD activation, p38 MAPK inhibitors SB203580 and SB202190 were used to block the activation of p38 MAPK. When the cells were pretreated with SB203580 or SB202190 for 30 min, *Der f 2*-induced PLD1 activation was abolished (Fig. 5*B*). Furthermore, *Der f-2*-induced IL-13 production was decreased by SB203580 and SB202190 (Fig. 5*C*). These results indicate that p38 MAPK participates in *Der f 2*-stimulated PLD activation and IL-13 production in BEAS-2B cells. Next, we studied whether the PLCγ/PKCα pathway is related to *Der f-2*-stimulated phosphorylation of p38 MAPK. As shown in Fig. 5*D*, phosphorylation of p38 MAPK induced by *Der f 2* treatment was inhibited by U73122, PAO, RO320432, and GO6976, respectively. Furthermore, when treated with U73122, PAO, RO320432, GO6976, SB203580, and SB202190, respectively, IL-13 expression in the cells was significantly reduced compared with the *Der f 2*-treated control (Fig. 5*E*). These results indicate that the PLCγ/PKCα/p38MAPK/PLD1 pathway is critical for *Der f 2*- induced IL-13 production and expression.

Activation of ATF-2 Is Induced by PLD1 Activation via PLCγ/PKCα/p38 MAPK Pathway in Der f 2-treated BEAS-2B Cells—Activation of ATF-2 is known to regulate the production of cytokines in mammalian cell lines as a transcription factor (46, 47). A time course analysis showed that *Der f 2*- induced phosphorylation of ATF-2, reaching a maximum in 5 min (Fig. 6*A*, *upper panel*). When the cells were pretreated with U73122, PAO, RO320432, GO6976, SB203580, or SB202190 for 30 min, *Der f 2*-induced ATF-2 activation was blocked (Fig. 6*A*, *lower panel*), indicating that the PLCγ/PKCα/p38 MAPK pathway is involved in *Der f 2*-induced ATF-2 activation in BEAS-2B cells. Also, we studied the effects of PLD1 on ATF-2 phosphorylation using PLD1 siRNA transfection in BEAS-2B cells. As shown in Fig. 6, *B* and *C*, PLD1 siRNA markedly decreased *Der f 2*-induced ATF-2 activation. To confirm the role of PLD1 in *Der f 2*-induced ATF-2 phosphorylation, the cells were transfected with PLD1 and DN-PLD1, respectively. As shown in Fig. 6*D*, overexpression of PLD1 increased the phosphorylation of

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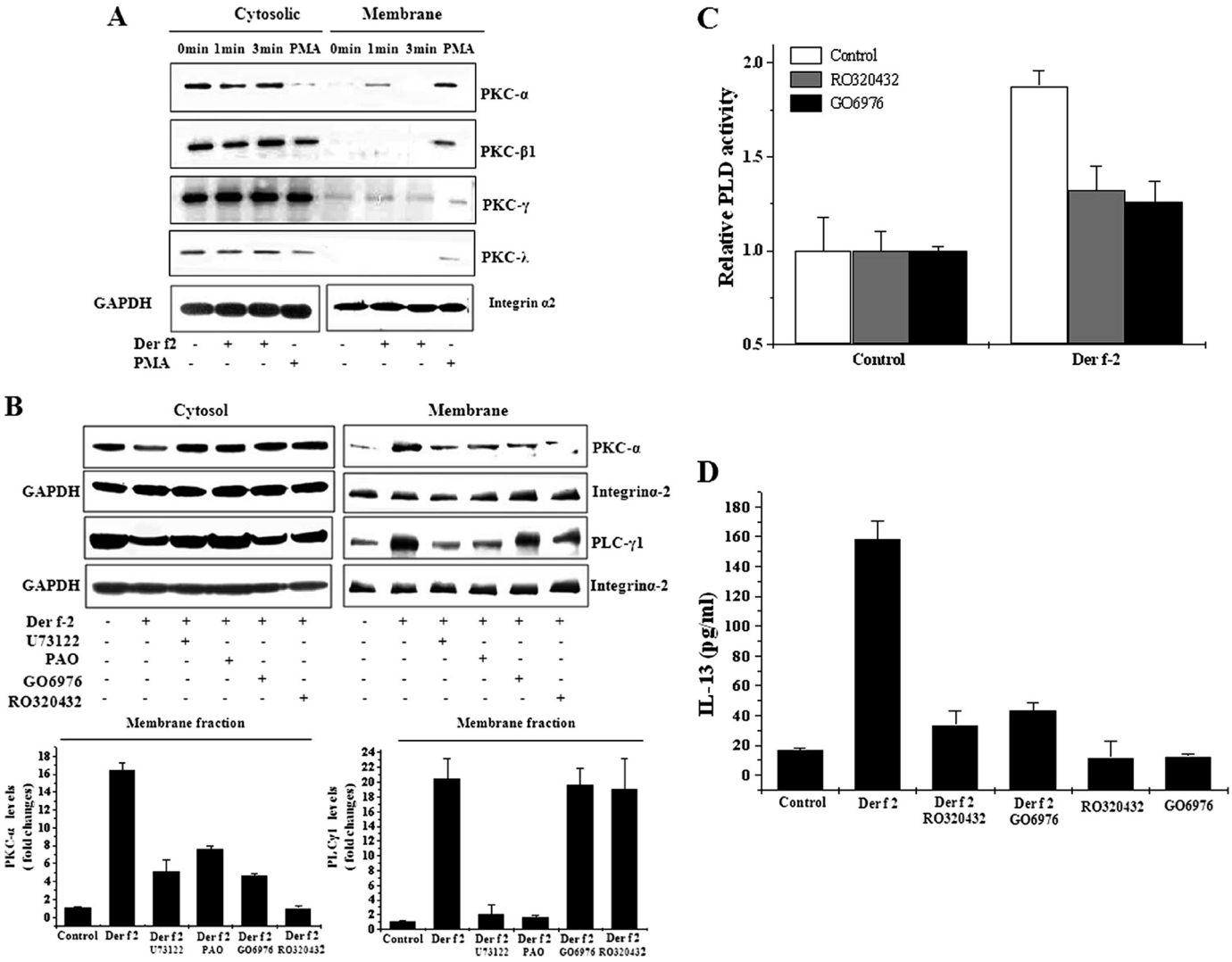

FIGURE 4. **Effect of *Der f 2* on PKCα translocation and effects of PKC down-regulation on *Der f 2*-induced PLD activation and IL-13 production in BEAS-2B cells.** *A*, serum-starved cells were stimulated with *Der f 2* (10 μg/ml) for 1 or 3 min. As a positive control for PKC translocation to the plasma membrane, cells were treated with 200 nM phorbol myristate acetate (*PMA*) for 30 min. The cells were then lysed and subjected to subcellular fractionation into cytosolic and membrane fractions by centrifugation at 100,000 × *g*. Each fraction containing 30 μg of protein was analyzed by 10% SDS-PAGE and subsequently to Western blotting using anti-PKCα, PKCβ1, PKCλ, and PKCγ polyclonal antibody. *B*, serum-starved cells were pretreated with U73122 or PAO (10 μM) for 30 min (*upper panel*) and RO320432 or GO6976 (50 μM) for 30 min (*lower panel*) before stimulation with *Der f 2* (10 μg/ml) for 1 min. The cells were then lysed and subjected to subcellular fractionation into cytosolic and membrane fractions by centrifugation at 100,000 × *g*. Each fraction containing 30 μg of protein was analyzed by 10% SDS-PAGE and subsequently by Western blotting using anti-PKCα polyclonal antibody. The data are expressed as a fold of basal value in membrane fraction and are means ± S.D. of three experiments. *C*, BEAS-2B cells were labeled with 2 μCi/ml [^{3}H]palmitic acid and then pretreated with RO320432 or GO6976 (50 μM) for 30 min before stimulation with *Der f 2* (10 μg/ml) for 30 min. PLD activities were then determined by estimating the amount of [^{3}H]PBt in the presence of 1-butanol. Results are means ± S.D. from three independent experiments. *D*, cells in 96-well culture plates were pretreated with RO320432 or GO6976 (50 μM) for 30 min and then stimulated with *Der f 2* (10 μg/ml) for 2 h. The results shown are mean values ± S.E. of the amount of IL-13 measured by ELISA for each group of samples ($n = 8$).

ATF-2 with no treatment of *Der f 2*, whereas DN-PLD1 transfection completely blocked *Der f 2*-induced ATF-2 phosphorylation. These results not only suggest that ATF-2 activation is associated with PLD1 activation through the PLCγ/PKCα/p38 MAPK pathway triggered by *Der f2* but also show the possibility that ATF-2 activation is able to regulate *Der f 2*-induced IL-13 expression and production in BEAS-2B cells. Furthermore, to confirm the functional role of PLD1 activation, we treated the cells with PA, which is one of the enzymatic products of PLD. When treated with the indicated concentrations of PA for 5 min, ATF-2 activation was increased (Fig. 6*E*, *upper panel*). This result shows the possibility that PA can increase IL-13 expression via ATF-2 activation. However, there are some studies to indicate that ATF-2 activation is controlled directly by p38 MAP kinase activation (27, 48). Therefore, we examined whether p38 MAP kinase is located downstream of PLD1 or not. As shown in Fig. 6*E* (*lower panel*), p38 inhibitors SB203580 and SB202190 could not block ATF-2 activation and IL-13 expression, respectively, induced by exogenously added PA. These results led us to suggest that p38 MAP kinase acts upstream of PLD activation and that PLD is located upstream of ATF-2 activation. PA, an end-product of PLD, is usually degraded to diacylglycerol by PPAP2A or to LPA/arachidonic acid by PLA2G1B. To determine whether PA itself regulates IL-13 expression via ATF-2 phosphorylation, we pretreated the cells with propranolol (a PPAP2A inhibitor) or mepacrine (a

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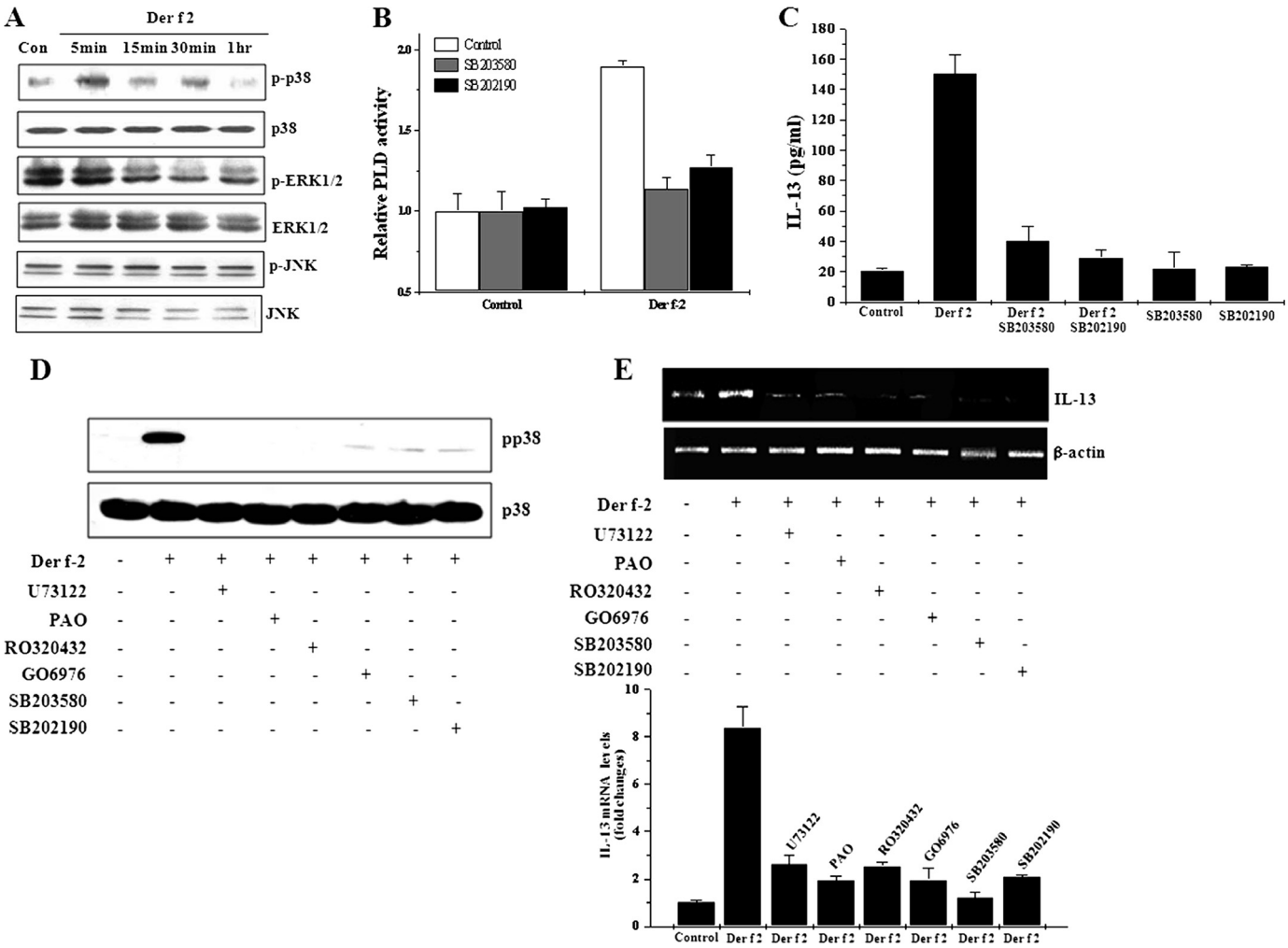

FIGURE 5. **Effect of p38 MAPK inhibition on *Der f 2*-induced PLD activation in BEAS-2B cells.** *A*, BEAS-2B cells treated with *Der f 2* (10 μg/ml) at the indicated times were harvested, and cell extracts were subjected to immunoblot analysis for active and total MAPKs. *B*, cells were pretreated with SB203580 or SB202190 (50 μM) for 30 min and then stimulated with *Der f 2* (10 μg/ml) for 5 min. Phosphorylated p38 MAPK was determined by Western blot analysis. The cells were labeled with 2 μCi/ml [^{3}H]palmitic acid and then pretreated with SB203580 or SB202190 (50 μM) for 30 min before stimulation with *Der f 2* (10 μg/ml) for 30 min. PLD activities were determined by estimating the formation of [^{3}H]PBt in the presence of 1-butanol. Results are means ± S.D. from three independent experiments. *C*, cells in 96-well culture plates were pretreated with SB203580 or SB202190 (50 μM) for 30 min and then stimulated with *Der f 2* (10 μg/ml) for 2 h. The results shown are mean values ± S.E. of the amount of IL-13 measured by ELISA for each group of samples ($n = 8$). *D*, cells were pretreated with U73122(10 μM), PAO (10 μM), RO320432 (50 μM), GO6976 (50 μM), SB203580 (50 μM), or SB202190 (50 μM) for 30 min and then stimulated with *Der f 2* (10 μg/ml) for 5 min. Phosphorylated p38 MAPK was determined by Western blot analysis. The intensity of bands was quantified using Quantity One software (Bio-Rad). Data represent the results of three separate experiments. *E*, cells were pretreated with U73122(10 μM), PAO (10 μM), RO320432 (50 μM), GO6976 (50 μM), SB203580 (50 μM), or SB202190 (50 μM) for 30 min and then stimulated with *Der f 2* (10 μg/ml) for 15 min. Total RNA was isolated using TRIzol reagent. RT-PCR analyses were performed using IL-13 and β-actin primers and real-time RT-PCR with primers for QIL-13 or GAPDH. Data are means ± S.D. of three values.

PLA2G1B inhibitor) for 1 h prior to treatment with PA for 15 min. As shown in Fig. 6*F*, pretreatment with propranolol or mepacrine for 1 h had no effect on IL-13 expression. Moreover, when the cells were treated with LPA for the indicated time (Fig. 6*G*), IL-13 expression was not increased by LPA. These results suggest that PA induces IL-13 expression directly through ATF-2 phosphorylation.

Knockdown of ATF-2 Inhibits Der f 2-induced IL-13 Gene Expression and Production—Finally, we investigated the effect of ATF-2 activation on IL-13 expression and production in *Der f 2*-treated BEAS-2B cells. As shown in Fig. 7, knockdown of ATF-2 using ATF-2 siRNAs transfection decreased *Der f 2*-induced IL-13 expression and production. This result indicates that gene expression and production of IL-13 are regulated by ATF-2 transcriptional activity in BEAS-2B cells.

DISCUSSION

HDMs *Der f* and *Der p* induce expression and production of inflammatory cytokines and also participate in various signaling pathway. The regulation of HDM-induced cytokine production is a key step in immunity-related diseases. Recently, immunotherapy using mite allergen has been tried for the treatment of atopic dermatitis (49). HDMs induce the production of IL-4 and IL-13 in mite-sensitive asthmatic basophils and stimulate IL-8 release in epithelial cells (45, 50, 51). However, *Der f 2*-induced intracellular signaling pathways leading to cytokine expression are still far from clearly understood. In this study, we have provided new insight into the PLD1-regulated signaling pathway for *Der f 2*-induced IL-13 expression and production.

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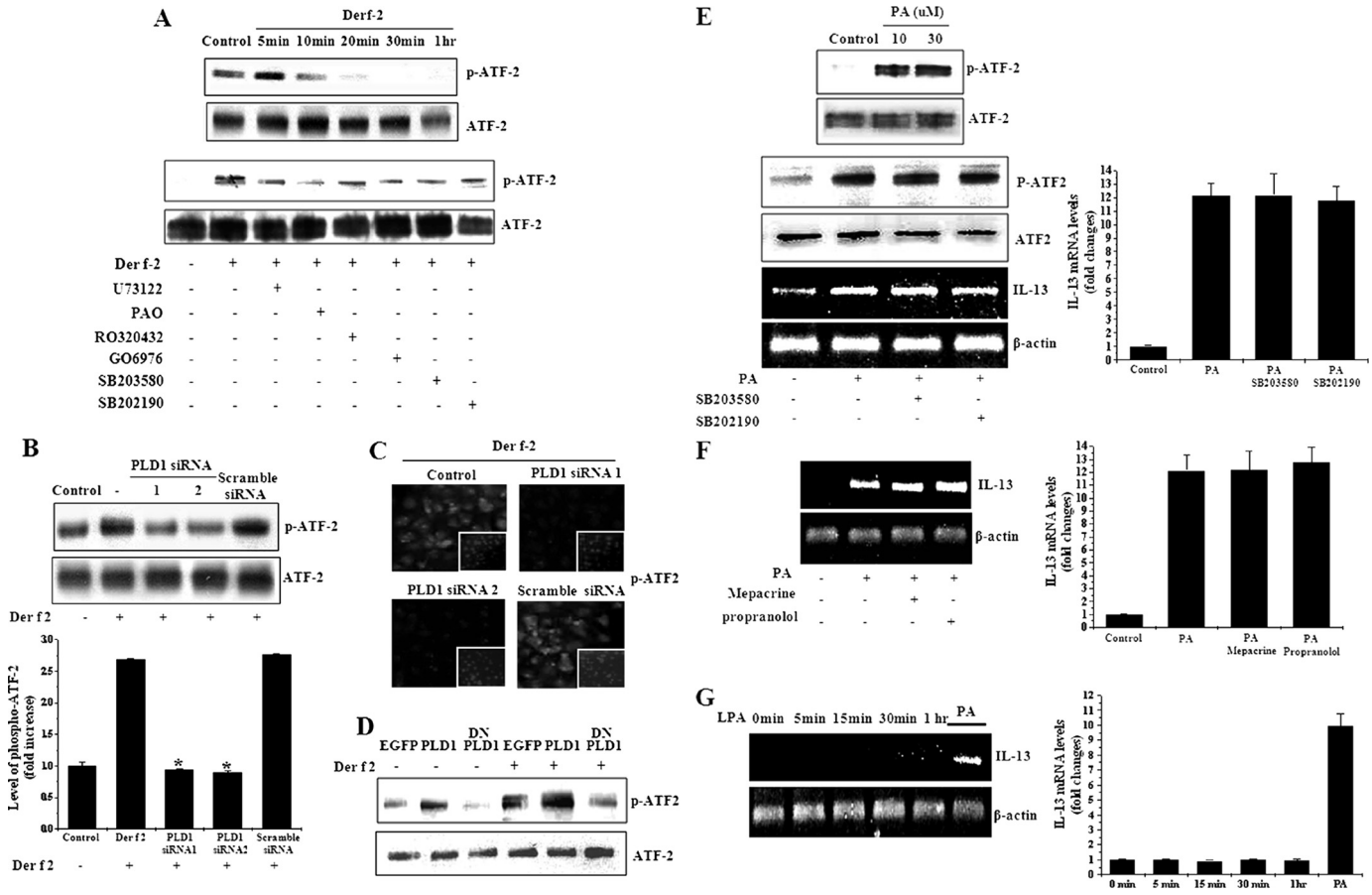

FIGURE 6. **Effect of PLD1 knockdown and exogenous PA treatment on *Der f 2*-induced ATF-2 phosphorylation in BEAS-2B cells.** *A*, BEAS-2B cells were stimulated with *Der f 2* (10 μg/ml) for the indicated time periods, and the amounts of total ATF-2 and phosphorylated ATF-2 (p-ATF-2) were determined by Western blot analysis (*upper panel*). The cells were pretreated with U73122 (10 μM), PAO (10 μM), RO320432 (50 μM), GO6976 (50 μM), SB203580 (50 μM), or SB202190 (50 μM) for 30 min and then stimulated with *Der f 2* (10 μg/ml) for 5 min. Expression levels of ATF-2 and p-ATF-2 were determined by Western blot analysis (*lower panel*). *B* and *C*, cells were transiently transfected with 100 nM PLD1 siRNAs or scrambled siRNA for 72 h and then stimulated with *Der f 2* (10 μg/ml) for 5 min. *B*, expression levels of ATF-2 and p-ATF-2 were determined by Western blot analysis. The intensity of bands was quantified using Quantity One software (Bio-Rad). *, $p < 0.05$ ANOVA *versus* values for *Der f 2*-treated control. Data represent the results of three separate experiments, *C*, p-ATF-2-immunostained cells were observed under a fluorescence microscope and photographed with magnification ×100. *D*, cells were transiently transfected with EGFP, DN-PLD1, or PLD1 plasmid DNA. After transfection for 24 h, the cells were treated with *Der f 2* for 5 min. Expression levels of ATF-2 and p-ATF-2 were determined by Western blot analysis. *E*, cells were stimulated with indicated concentrations of PA for 5 min, and the amounts of total ATF-2 and p-ATF-2 were determined by Western blot analysis (*upper panel*). The cells were pretreated with SB203580 (50 μM) or SB202190 (50 μM) for 30 min and then stimulated with PA (10 μM) for 5 min (for p-ATF-2) and 15 min (for IL-13 mRNA levels). Expression levels of ATF-2 and p-ATF-2 were determined by Western blot analysis. Total RNA was isolated using TRIzol reagent. RT-PCR analyses were performed using IL-13 and β-actin primers (*lower panel*) and real-time RT-PCR with primers for QIL-13 or GAPDH. Data are means ± S.D. of three values. *F*, cells were pretreated with mepacrine (10 μM) or propranolol (50 μM) for 1 h and then stimulated with PA (10 μM) for 15 min. Total RNA was isolated using TRIzol reagent. RT-PCR analyses were performed using IL-13 and β-actin primers and real-time RT-PCR with primers for QIL-13 or GAPDH. Data are means ± S.D. of three values. *G*, cells were stimulated with LPA (10 μM) for the indicated time periods. Total RNA was isolated using TRIzol reagent. RT-PCR analyses were performed using IL-13 and β-actin primers and real-time RT-PCR with primers for QIL-13 or GAPDH. Data are means ± S.D. of three values.

In the present study, *Der f 2* was found to increase PLD activity maximally at 30 min, whereas expressions of PLD1 and -2 were not changed within the indicated time (Fig. 1), indicating that activation of PLD is caused by *Der f 2*-regulated signaling molecules. We also found that *Der f 2* increased both expression and production of IL-13. The production of IL-13 can be triggered by antigen in sensitized hosts as well as by other cytokines under certain circumstances (29). In human bronchial epithelial cells, overexpression of PLD1 or PLD2 potentiates S1P-induced cytokine production, and blockades of PLD activity inhibit cytokine production (40). These studies suggest a possible functional role for PLD in cytokine production and expression. Therefore, to investigate the role of PLD isoforms in *Der f 2*-induced expression and production of IL-13, we transiently overexpressed PLD1, DN-PLD1, PLD2, and DN-PLD2 in BEAS-2B cells with EGFP plasmid vector prior to *Der f 2* stimulation (Fig. 2) and found that PLD1 overexpression increased expression of IL-13 induced by *Der f 2*. On the other hand, overexpression of PLD2 rather down-regulated IL-13 expression induced by *Der f 2*, and overexpression of DN-PLD2 also decreased IL-13 expression induced by *Der f 2*. In the present study, therefore, we have focused on the role of PLD1 in IL-13 expression induced by *Der f 2*. Moreover, we found that DN-PLD1 inhibited the expression of IL-13 induced by *Der f 2*, and PLD1 blockage by PLD1 siRNA also decreased production and expression of IL-13. These results strongly indicate that PLD1 plays an important role in *Der f 2*-induced expression and production of IL-13 in BEAS-2B cells.

Proinflammatory cytokine secretion and inflammatory mediator-induced cytokine expression are dependent on PLC

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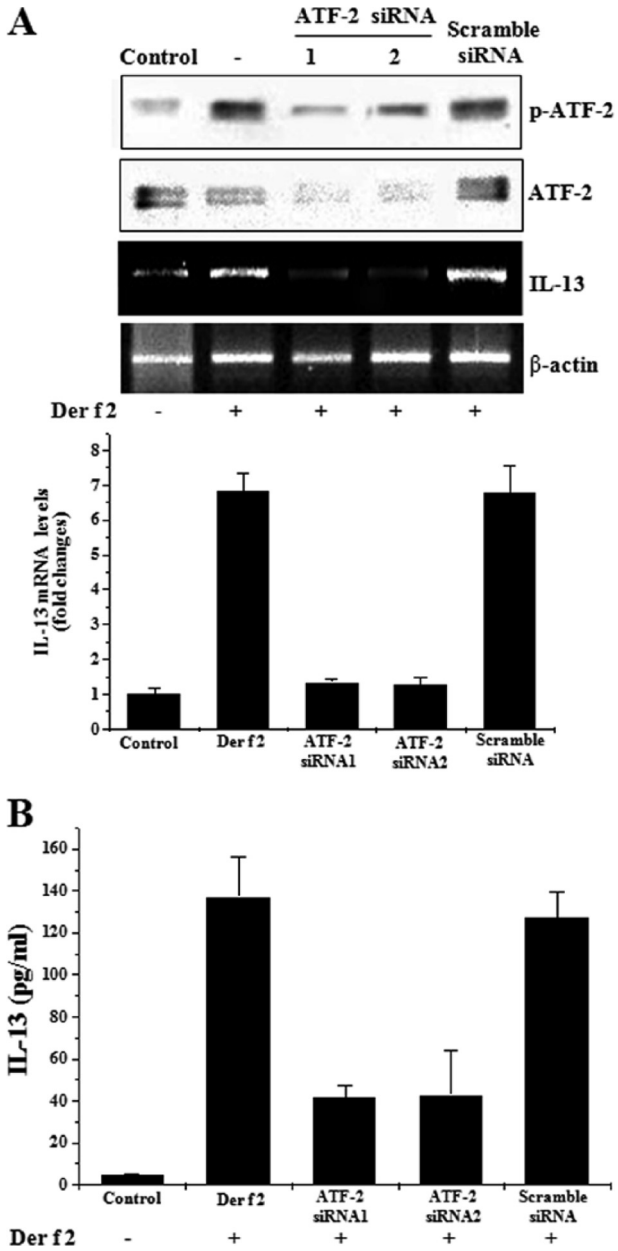

FIGURE 7. **Effect of ATF-2 phosphorylation on IL-13 expression and production induced by *Der f 2* in BEAS-2B cells.** *A*, BEAS-2B cells were transiently transfected with 100 nM ATF-2 siRNAs or scrambled siRNA for 72 h and then stimulated with *Der f 2* (10 μg/ml) for 5 min (for p-ATF-2) and 15 min (for IL-13 mRNA levels). Expression levels of ATF-2 and p-ATF-2 were determined by Western blot analysis (*upper panel*). The cells were harvested and total RNA was isolated using TRIzol reagent. RT-PCR analyses were performed using IL-13 and β-actin primers (*lower panel*) and real-time PCR with primers for QIL-13 or GAPDH. Data are means ± S.D. of three values. *B*, BEAS-2B cells on 96-well culture plates were transfected with 100 nM ATF-2 siRNAs or scrambled siRNA for 72 h and then stimulated with *Der f 2* (10 μg/ml) for 2 h. The result shown are mean values ± S.E. of the amount of IL-13 measured by ELISA for each group of samples ($n = 8$).

in human epithelial and synovial fibroblasts cell, suggesting that activation of PLC is required in signal transduction events, leading to inflammatory response (15, 52). Several studies suggest that PLCγ1 regulates PLD activity in intracellular signal transduction (42, 53, 54). Thus, we tried to determine whether PLCγ signaling pathway is involved in *Der f 2*-induced PLD activation and found that pretreatment with U73122 or PAO, specific PLCγ inhibitors, completely blocked *Der f 2*-induced PLD1 activation and IL-13 production in BEAS-2B cells, suggesting that PLCγ activation is related to PLD activation and IL-13 production.

There are many evidences to indicate that PKC is a major mediator in activating PLD in response to various agonists (19). Moreover, many studies have reported the relationship between PLD and PKC isoforms in a variety of cell types (55–59), suggesting that small molecular weight G proteins such as ARF (ADP-ribosylation factor) and RhoA are involved in agonist-induced PLD activation in several cell types (36); it is not yet clear whether these small molecular weight G proteins may be the regulator of *Der f 2*-induced PLD activation. As we demonstrated earlier that *Der f 2* activates PLD through PKC activation (1, 13), in this study we focused on the PKC-dependent pathway related to *Der f 2*-induced PLD activation. Among PKC isoforms, translocation of only PKCα occurred from cytosol to membrane by treatment with *Der f 2*, and inhibition of PKCα with RO320432 and GO6976 blocked *Der f 2* –induced PLD activation and IL-13 production. Therefore, we suggest that *Der f 2*-induced PLD activation is mediated by PKCα in BEAS-2B cells. Also, we have shown that *Der f 2*-induced PKCα translocation in BEAS-2B cells is dependent on PLC γ1. Although translocation of PLCγ1 to the membrane was not inhibited by PKC inhibitors such as RO320432 and GO6976, PKCα translocation was significantly inhibited by PLCγ inhibitors U73122 and PAO. These results demonstrate that PLCγ acts as an upstream component of *Der f 2*-induced PKCα activation in BEAS-2B cells.

Cross-talk between PKC and MAPKs by agonists has recently been reported in various cell systems (60–62). Specifically, many groups of researchers have demonstrated that the p38 signaling pathway possibly controls inflammatory responses. p38 MAPK plays a role in transducing the mitogenic signal in T-cells in response to IL-2 and IL-7 (63). P38 MAPK also mediates the production of IL-10 and IL-1β in lipopolysaccharide-stimulated monocytes, and it regulates IL-5 synthesis and tumor necrosis factor-α mRNA stability in human T-cells (61). However, these authors did not examine the relationship between p38 MAPK and the PLCγ/PKCα pathway, which regulates PLD activation in immune response. In the present study, we found that *Der f 2* stimulated the activation of p38 MAPK but not SAPK/JNK or ERK1/2. The failure of *Der f 2* to activate ERK1/2 and SAPK/JNK indicates that *Der f 2* specifically activates p38 MAPK. Our observation led us to assume that *Der f 2*-induced p38 MAPK activation is also involved in PLD activation. As expected, the p38 MAPK inhibitors SB203580 and SB202190 attenuated not only *Der f 2*-induced PLD activation but also *Der f 2*-induced IL-13 production, thus raising the possibility that p38 MAPK regulates PLD activation and IL-13 production induced by *Der f 2* in BEAS-2B cells. Furthermore, we have shown that inhibition of PLCγ1 with U73122 and PAO, or of PKCα with RO320432 and GO6976, blocked *Der f 2*-induced p38 MAPK activation. Therefore, it is highly likely that *Der f 2*-induced p38 MAPK activation is dependent on the PLCγ/PKCα pathway in BEAS-2B cells. Next, we tested whether the PLCγ/PKCα/p38 MAPK pathway was involved in *Der f 2*-induced IL-13 expression and found

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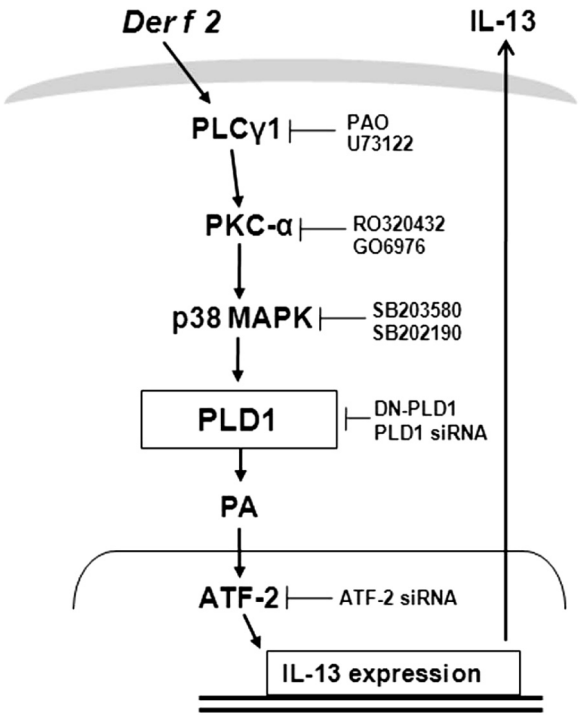

FIGURE 8. **Proposed model for the signaling pathway of *Der f 2-induced* IL-13 expression and production in BEAS-2B cells.** Our model suggests that *Der f 2* induces expression and production of IL-13 via activation of PLD1 through PLCγ/PKCα/p38 MAPK in BEAS-2B cells.

that U73122, PAO, RO320432, GO6976, SB203580, and SB202190 blocked the expression of IL-13, which was elicited by *Der f 2*, suggesting that the PLCγ/PKCα/p38 MAPK pathway is critical for *Der f 2*-induced IL-13 expression. Such an observation appears to be similar to the PLC-dependent pathway in rLZ-induced cytokines in human T-cells (64).

What kind of transcription factor is involved in the expression of IL-13 via PLCγ, PKCα, p38 MAPK, and PLD activation in BEAS-2B cells? Previous studies have indicated that PKC leads to ATF-2 activation and that PKC inhibitors completely block ATF-2 phosphorylation (47). Furthermore, activation of transcription factor ATF-2 in the p38 MAPK pathway for cytokine production has also been reported previously (20, 46). The possibility that PLD might be related to ATF-2 phosphorylation has never been considered before. However, based on the present results, it does not seem to be far-fetched to suggest the possibility of the involvement of PLD in ATF-2 activation, which increases IL-13 expression.

In this study, we have proved that the phosphorylation of ATF-2 is required for *Der f 2*-induced expression and production of IL-13 through PKCα, p38 MAPK, and PLD activation. First, pretreatment with U73122, PAO, RO320432, GO6976, SB203580, or SB202190 decreased the phosphorylation of ATF-2 induced by *Der f 2*. Second, PLD1 siRNAs and DN-PLD1 inhibited *Der f 2*-induced phosphorylation of ATF-2. Third, PA (the functional and enzymatic product of PLD) directly increased the phosphorylation of ATF-2 and IL-13 expression. Fourth, ATF-2 siRNAs reduced the *Der f 2*-triggered elevation of expression and production of IL-13. However, *Der f 2*-induced IL-13 production was not completely reduced by ATF-2 siRNA, whereas ATF-2 siRNA treatment completely blocked IL-13 expression as well as the ATF-2 phosphorylation induced by *Der f 2*. This could be explained by the fact that ATF-2 is not the only transcription factor for IL-13 production in *Der f 2*-treated BEAS-2B cells, and it is quite possible that there are other hitherto unknown transcription factors.

In conclusion, the present study provides evidence that *Der f 2*-induced PLD activation is mediated by the PLCγ/PKCα/p38 MAPK pathway and that activation of PLD1 is critical for the expression and production of IL-13 through activation of ATF-2 by providing PA in BEAS-2B cells (Fig. 8). Further studies, such as the elucidation of molecular mechanisms involved between PLD1 and transcription factors in BEAS-2B cells, are expected to provide deeper insight into the understanding of *Der f 2*-induced PLD activation and cytokine production.

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