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$G\alpha_{12}$ Specifically Regulates COX-2 Induction by Sphingosine 1-Phosphate

ROLE FOR JNK-DEPENDENT UBIQUITINATION AND DEGRADATION OF IκBα[*][S]

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Cyclooxygenase-2 (COX-2) plays a critical role in vasodilatation and local inflammatory responses during platelet aggregation and thrombosis. Sphingosine 1-phosphate (S1P), a sphingolipid released from activated platelets, stimulates COX-2 induction and activates G-protein-coupled receptors coupled to Gα family members. In this study, we investigated whether $G\alpha_{12}$ family regulates COX-2 induction by S1P and investigated the molecular basis of this COX-2 regulation. Gene knock-out and chemical inhibitor experiments revealed that the S1P induction of COX-2 requires $G\alpha_{12}$ but not $G\alpha_{13}$, $G\alpha_q$, or $G\alpha_{i/o}$. The specific role of $G\alpha_{12}$ in COX-2 induction by S1P was verified by promoter luciferase assay, $G\alpha_{12}$ transfection, and knockdown experiments. Experiments using siRNAs specifically directed against $S1P_{1-5}$ showed that $S1P_1$, $S1P_3$, and $S1P_5$ are necessary for the full activation of COX-2 induction. Gel shift, immunocytochemistry, chromatin immunoprecipitation, and NF-κB site mutation analyses revealed the role of NF-κB in *COX-2* gene transcription by S1P. $G\alpha_{12}$ deficiency did not affect S1P-mediated IκBα phosphorylation but abrogated IκBα ubiquitination and degradation. Moreover, the inhibition of S1P activation of JNK abolished IκBα ubiquitination. Consistently, JNK transfection restored the ability of S1P to degrade IκBα during $G\alpha_{12}$ deficiency. S1P injection induced COX-2 in the lungs and livers of mice and increased plasma prostaglandin E_2, and these effects were prevented by $G\alpha_{12}$ deficiency. Our data indicate that, of the Gα proteins coupled to S1P receptors, $G\alpha_{12}$ specifically regulates NF-κB-mediated COX-2 induction by S1P downstream of $S1P_1$, $S1P_3$, and $S1P_5$, in a process mediated by the JNK-dependent ubiquitination and degradation of IκBα.

Prostaglandins (PGs)[2] play important roles in the regulation of vasorelaxation and platelet aggregation (1). Moreover, PGs are produced by members of the cyclooxygenase (COX) family, and whereas COX-1 is constitutively expressed in most mammalian tissues, COX-2 induction is restricted to some pathological lesions (*e.g.* atherosclerosis, myocardial infarction, and cancerous tissues) (2). Transcription of the *COX-2* gene is promoted by transcription factors encoded by immediate early genes and can be up-regulated by various proinflammatory agents, such as bacterial lipopolysaccharide, cytokines, and mitogens. Moreover, PGs produced by inducible COX-2 participate in the vasodilatation associated with thrombosis and amplify inflammatory responses (3).

A number of studies have shown the involvement of lipid factors in diverse cellular responses. In particular, sphingosine 1-phosphate (S1P) is a bioactive lipid mediator that exerts a wide range of physiological activities (4). S1P enhances vascular smooth muscle cell proliferation and migration and thus serves as a factor that stimulates coronary artery disease, atherosclerosis, and an abnormal vascular tone during aging (5, 6). The most well known actions of S1P are mediated by its binding to a family of S1P G-protein-coupled receptors (GPCRs) (7). Moreover, S1P receptors couple to a variety of G-proteins and thus regulate cell migration, angiogenesis, vascular maturation, cardiac development, neuronal survival, and immunity (4, 8). Recent studies have implicated sphingolipids in the regulation of the *COX-2* gene and in those of other target genes, whose products mediate vascular inflammatory responses (9). Nevertheless, the functions of sphingolipids in the regulation of the *COX-2* gene and their relations with the promotion of inflammatory processes are not completely understood. However, the observation that the down-regulation of sphingosine kinase-1 prevents cytokines from inducing COX-2 suggests that an S1P-mediated cell signaling pathway is required for COX-2 induction (9, 10).

G-proteins interact with GPCRs, and then activated G-proteins transmit signals to regulate physiological responses. Heterotrimeric G-proteins are defined by their α subunits, which are classified as $G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_q$, and $G\alpha_{12}$. Of the G-protein families, $G\alpha_{12}$ members are activated by sphingolipids, TXA_2, or lysophosphatidic acid (11). $G\alpha_{12}$ and $G\alpha_{13}$ appear to differ in

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1 and S2.

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[2] The abbreviations used are: PG, prostaglandin; COX, cyclooxygenase; $G\alpha_{12}$W, wild-type $G\alpha_{12}$; $G\alpha_{12}^{-/-}$, $G\alpha_{12}$-null cells; $G\alpha_{13}^{-/-}$, $G\alpha_{13}$-null cells; $G\alpha_{12/13}^{-/-}$, $G\alpha_{12/13}$-null cells; GPCR, G-protein-coupled receptor; $RK^{-/-}$, rhodopsin kinase-null cells; S1P, sphingosine 1-phosphate; MEF, mouse embryonic fibroblast; siRNA, small interfering RNA; C/EBP, CCAAT/enhancer-binding protein; CREB, cAMP-response element/E-box; WT, wild type; PTX, pertussis toxin; RT, reverse transcription; JNK, c-Jun N-terminal kinase; E3, ubiquitin-protein isopeptide ligase; ERK, extracellular signal-regulated kinase.

terms of their abilities to couple to different ligands and to recruit different signaling pathways as physiological effectors (11, 12). Gene knock-out experiments revealed that $G\alpha_{13}$ knock-out ($G\alpha_{13}^{-/-}$) mice have impaired angiogenesis and intrauterine death, whereas $G\alpha_{12}$-deficient mice survived (12).

In this study, we investigated the regulatory roles of $G\alpha_{12}$ on *COX-2* gene induction by S1P, a sphingolipid released from activated platelets. We examined whether COX-2 induction by S1P occurs via pathways involving $G\alpha_{12}$, $G\alpha_{13}$, $G\alpha_q$, or $G\alpha_{i/o}$. Based on our previous finding that $G\alpha_{12}$ members regulate NF-κB activation by thrombin (13), we further examined whether NF-κB regulation is linked with G-protein-mediated signaling for COX-2 induction by S1P. We also explored the molecular basis of $G\alpha_{12}$ function in the S1P-induced IκBα degradation necessary for NF-κB activation, with particular reference to its JNK-dependent IκBα ubiquitination. In addition, we verified the physiological role of $G\alpha_{12}$ on COX-2 induction by S1P in an animal model.

EXPERIMENTAL PROCEDURES

Materials—Anti-p65, anti-IκBα, anti-$G\alpha_{12}$ antibodies, and $G\alpha_{12}$ siRNA were supplied by Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-COX-2 antibody was obtained from Cayman (Ann Arbor, MI). S1P was purchased from Merck. Bay117082 was supplied by Alexis (Tokyo, Japan). SP600125 (JNKI, JNK inhibitor) was obtained from Calbiochem. $S1P_{1-5}$ siRNA were provided by Dharmacon (Chicago, IL). Other reagents were purchased from Sigma.

Cell Culture—Mouse embryonic fibroblast (MEF) cells generated from genetically engineered mice (14) that contained gene knockouts for rhodopsin kinase (RK) (15), and $G\alpha_{12}/G\alpha_{13}$ (16) were supplied by Dr. M. Simon (Caltech, Pasadena, CA). The MEF cell lines were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 50 units/ml penicillin, and 50 μg/ml streptomycin at 37 °C in humidified atmosphere with 5% CO_2. The cells were plated at a density of 5×10^6/dish (10-cm diameter) and preincubated for 24 h at 37 °C. For all experiments, cells were grown to 80–90% confluence. The cells were scraped, transferred to microtubes, and allowed to swell by adding lysis buffer. Nuclear extracts and total lysates were prepared, as described previously (13, 17).

PCR Analysis—S1P receptor and COX-2 transcripts were analyzed by semiquantitative reverse transcription or real time PCR. RNA was isolated from cells by using an RNeasy mini kit (Qiagen). Total RNA (2 μg) was reverse-transcribed using an $oligo(dT)_{16}$ primers to obtain cDNA. The cDNA was amplified by PCR. The sequences of the primers used were as follows: $S1P_1$ receptor (197 bp), 5′-GATGCGCCGGGCCTTCAT-3′ (sense) and 5′-AGGAAGAAGAACTGACGTTTCCA-3′ (antisense); $S1P_2$ receptor (205 bp), 5′-ACGTGGCGTAGCCGGGAC-3′ (sense) and 5′-CATTTTCCCTTCAGACCACTG-3′ (antisense); $S1P_3$ receptor (209 bp), 5′-TCTTCCGGTTGGTGTGCGG-3′ (sense) and 5′-CTTGCAGAGGACCCCGTTCT-3′ (antisense); $S1P_4$ receptor (321 bp), 5′-GCTGCCCCTCTACTCCAA-3′ (sense) and 5′-ATTAATGGCTGAGTTGAACAC-3′ (antisense); $S1P_5$ receptor (167 bp), 5′-CCAACAGCTTGCAGCGATC-3′ (sense) and 5′-GGTTGCTACTCCAGGACTG-3′ (antisense); COX-2 (624 bp), 5′-TCTCCAACCTCTCCTACTAC-3′ (sense) and 5′-GCACGTAGTCTTCGATCACT-3′ (antisense).

Immunoblot Analysis—Immunoblot analyses were performed according to the previously published procedures (13, 17). Proteins of interest in lysates or nuclear fractions were resolved using a 7.5% gel and developed using an ECL chemiluminescence system (Amersham Biosciences).

Gel Shift Assay—Double-stranded DNA probes for the consensus sequences of CCAAT/enhancer-binding protein (C/EBP) (5′-TGCAGATTGCGCAATCTGCA-3′), cAMP-response element/E-box (CREB) (5′-AGAGATTGCCTGACGTCAGAGAGCTAG-3′), and NF-κB (5′-AGTTGAGGGGACTTTCCCAGGC-3′) were used for gel shift analyses after end labeling of each probe with [γ-^{32}P]ATP and T_4 polynucleotide kinase. Nuclear extracts were prepared by modification of the procedure published previously (13, 17).

Immunocytochemistry of p65—Cells were grown on Lab-TEK chamber slides® (Nalge Nunc International Corp.) and incubated in serum-free medium for 24 h. A standard immunocytochemical method was used as described previously (17). Counterstaining with propidium iodide verified the location and integrity of nuclei. Stained cells were examined using a laser-scanning confocal microscope (Leica TCS NT, Leica Microsystems, Wetzlar, Germany).

Transient Transfection—Cells were plated at a density of 1×10^5 cells/well in a 6-well dish and transfected the following day. Briefly, the cells were incubated with a minigene construct expressing C-terminal peptide of $G\alpha_{12}$ or $G\alpha_{13}$ that serves as a blocker of the specific site of the GPCR ($G\alpha_{12}$ or $G\alpha_{13}$ minigene) and 3 μl of Lipofectamine® reagent (Invitrogen) in 1 ml of antibiotics-free minimal essential medium for 3 h. Culture medium was changed with serum-free minimal essential medium with antibiotics, and the cells were further incubated for 24 h. In some experiments, cells were transfected with the wild type $G\alpha_{12}$ plasmid.

Knockdown Experiment Using siRNA—Cells were transfected with control siRNA or siRNA directed against $G\alpha_{12}$ (100 pmol) using Lipofectamine 2000 according to the manufacturer's instructions. COX-2 was immunoblotted in the lysates of cells incubated with S1P for 3 h. The target sequences of a commercially available $G\alpha_{12}$ siRNA mixture were 5′-CCAGUAAGCAAGACAUCCU-3′, 5′-GCAUCACAUCUAUCCUGUU-3′, and 5′-CUCUGCUGUUGAUCUGUAA-3′ (Santa Cruz Biotechnology).

Immunoprecipitation—To assess ubiquitinated IκBα, cells were transfected with the plasmid encoding His-tagged ubiquitin. Cell lysates (250 μg/ml) were incubated with anti-His antibody overnight at 4 °C. The antigen-antibody complex was immunoprecipitated after incubation for 2 h at 4 °C with protein G-agarose. Immune complexes were solubilized in 2× Laemmli buffer. Protein samples were resolved and immunoblotted with anti-IκBα antibody.

COX-2 Promoter-Luciferase Assay—Genomic DNA was isolated from ICR mouse tail. To generate pGL-mCOX2-724, a COX-2 promoter-luciferase construct, the COX-2 promoter region from −724 to + 7 bp was amplified and then ligated into KpnI/XhoI sites of pGL3-basic plasmid (Promega). Mutation constructs to the NF-κB and E-box sites of COX-2 promoter

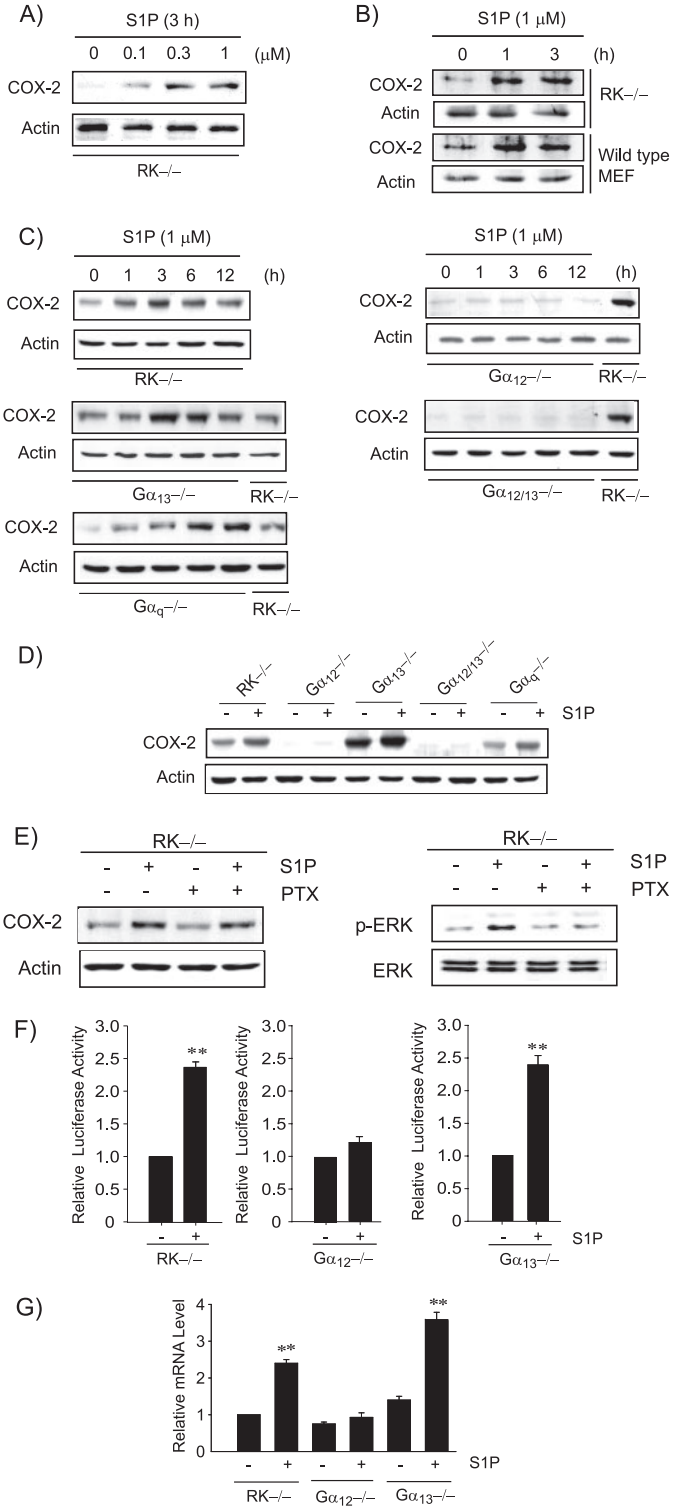

FIGURE 1. **The effects of $G\alpha_{12}$ or $G\alpha_{13}$ deficiency on *COX-2* gene expression by S1P.** *A*, the effect of varying concentrations of S1P (0.1–1 μM, 3 h) on COX-2 expression in $RK^{-/-}$ cells. *B*, comparison of COX-2 induction by 1 μM S1P in $RK^{-/-}$ and wild type MEF cells. *C*, the time courses of COX-2 expression in cells treated with S1P (1 μM). *D*, comparison of the levels of COX-2 in cells treated with vehicle or S1P (1 μM, 3 h). *E*, the effect of PTX. Cells were treated with S1P (1 μM, 3 h) in the presence or absence of PTX (0.1 ng/ml, 1-h pretreatment). Equal loading of proteins was verified by probing the replicate blots for actin. Each lane contained 20 μg of lysate proteins. *F*, repression by $G\alpha_{12}$ deficiency of pGL-mCOX-2-724 transactivation by S1P (1 μM, 3 h). The values represented the mean ± S.E. with four separate experiments (significant as compared with the respective control; **, $p < 0.01$; luciferase expression in $RK^{-/-}$ cells transfected with pGL-mCOX-2-724, *1.0*). *G*, real time PCR analyses of COX-2 mRNA transcripts after S1P treatment (1 h). The values represented the mean ± S.E. with three separate experiments (significant as compared with the respective control; **, $p < 0.01$; COX-2 mRNA in $RK^{-/-}$ cells, *1.0*).

were produced by using a QuikChange site-directed mutagenesis kit (Stratagene). The oligonucleotides used for mutagenesis were 5′-GGGGAGAGGTGAGGGccTTCCCTTAGTTAGGACC-3′ and 5′-GTCACCACTACGTCACGcaGAGTCCGCTTTACAGAC-3′, respectively (lowercase letters indicate mutant nucleotides) (3, 18). All constructs were verified by DNA sequencing (ABI7700). The COX-2 luciferase (or mutant) construct and pCMV-LacZ were co-transfected to $RK^{-/-}$ and/or $G\alpha_{12}{}^{-/-}$ cells and then incubated with 1 μM S1P for 3 h. To determine the COX-2 promoter activity, we used the luciferase reporter assay system (Promega, Madison, WI). The activity of β-galactosidase was measured to normalize transfection efficiency using *o*-nitrophenyl-β-D-galactopyranoside as a substrate at 420 nm.

Chromatin Immunoprecipitation Assays—Cells were treated with S1P for 1 h, and then formaldehyde was added to the cells to a final concentration of 1% for cross-linking of chromatin. Chromatin immunoprecipitation assays were conducted as described previously (17). PCR was performed with the specific primers flanking the NF-κB region of the *COX-2* gene (sense, 5′-ATGTGGACCCTGACAGAGGA-3′; antisense, 5′-TCTCCGGTTTCCTCCCAGTC-3′, 222 bp).

Knock-out Animals—$G\alpha_{12}$ knock-out mice generated as described previously (11) were supplied by Dr. M. Simon. After they were anesthetized with ketamine, wild type (WT) or $G\alpha_{12}$ knock-out mice were infused with S1P for 30 min via the femoral vein or intraperitoneally injected with lipopolysaccharide (1 mg/kg). Animals were sacrificed 3 h after treatment. COX-2 was immunoblotted in the lung or liver homogenates. Prostaglandin E_2 contents in plasma were measured by enzyme-linked immunosorbent assay (Amersham Biosciences).

Statistical Analysis—One-way analysis of variance procedures were used to assess significant differences among treatment groups. For each significant effect of treatment, the Newman-Keuls test was used for comparisons of multiple group means. The criterion for statistical significance was set at $p < 0.01$.

RESULTS

COX-2 Gene Repression by $G\alpha_{12}$ Deficiency—S1P treatment at 0.3 or 1 μM notably induced COX-2 in $RK^{-/-}$ MEF cells. In this study, $RK^{-/-}$ cells were used as a knock-out control, because the physiological function of rhodopsin kinase is restricted to phototransduction (19) and is not relevant to the function of endogenous G-protein in MEFs. S1P at 0.1 μM, which is equivalent to the plasma concentration observed in healthy control animals (20), weakly enhanced COX-2 expression (Fig. 1*A*), demonstrating that S1P at submicromolar concentrations has a threshold effect on COX-2 induction. Considering the micromolar range of S1P observed in plasma (*i.e.* 1–5 μM) (20) after platelet activation, the concentrations of S1P used in this study appropriately represent pathophysiological situations. We confirmed that the ability of S1P to induce COX-2 in $RK^{-/-}$ cells was the same as that in wild type MEF

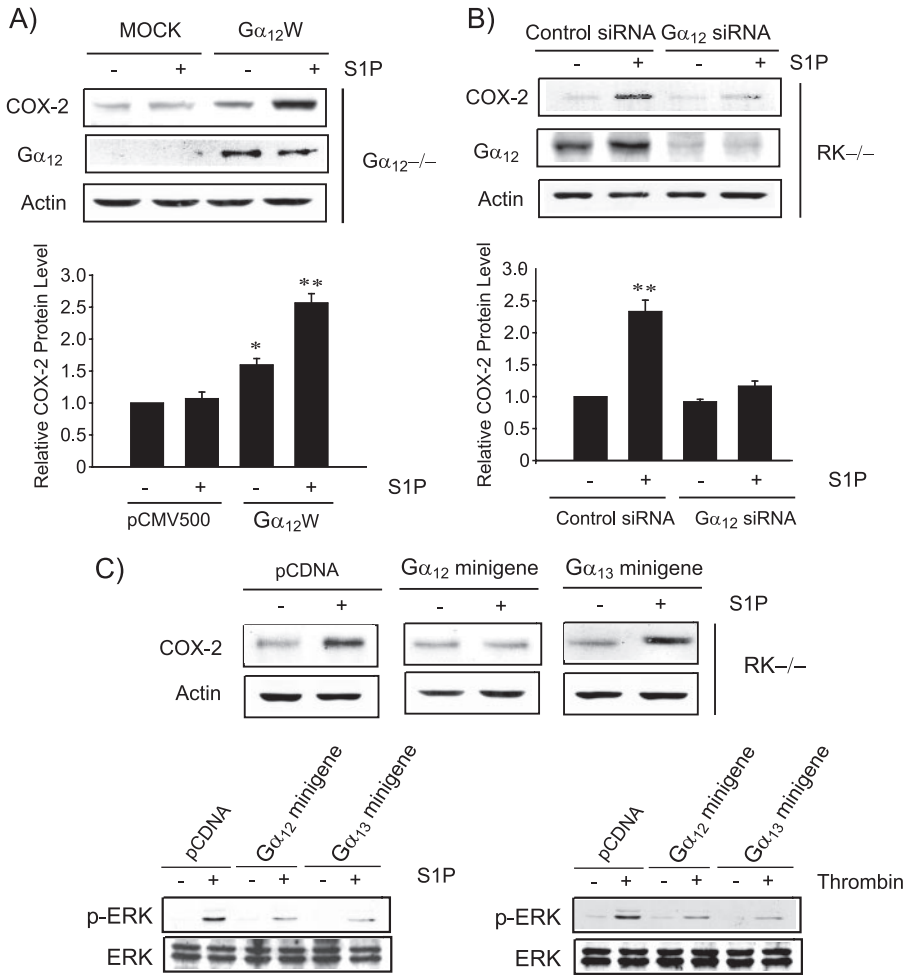

FIGURE 2. **Role of $G\alpha_{12}$ in COX-2 induction by S1P.** *A*, the effect of $G\alpha_{12}$W. COX-2 was immunoblotted in the lysates of $G\alpha_{12}{}^{-/-}$ cells transfected with pCMV500 or $G\alpha_{12}$W (1 μg each) and incubated with or without S1P (1 μM, 3 h) (significant as compared with vehicle-treated mock-transfected cells; *, $p < 0.05$; **, $p < 0.01$; vehicle-treated mock-transfection, *1.0*). *B*, the effect of $G\alpha_{12}$ knockdown on S1P-mediated COX-2 induction. Cells were transfected with control or $G\alpha_{12}$ siRNA (100 pmol). Cells serum-starved for 3 h were stimulated with vehicle or S1P, and total lysates were subjected to immunoblottings. Results were confirmed by repeated experiments. The values represented the mean ± S.E. with three separate experiments (significant as compared with control siRNA-transfected cells; **, $p < 0.01$; control siRNA transfection, *1.0*). *C*, the effect of minigene transfection on COX-2 induction by S1P. $RK^{-/-}$ cells were transfected with pCDNA or a minigene construct (1 μg) expressing the C-terminal peptide of $G\alpha_{12}$ or $G\alpha_{13}$, cultured in the medium containing 1% fetal bovine serum for 12 h, and further incubated with S1P for 3 h. Inhibition by $G\alpha_{12}$ or $G\alpha_{13}$ minigene of S1P- or thrombin-induced ERK phosphorylation validated the minigene system and the transfection conditions. Equal loading of proteins was verified by probing the replicate blots for actin or ERK. Each lane contained 20 μg of lysate proteins.

cells (Fig. 1*B*). In subsequent experiments, we determined whether deficiencies of specific Gα proteins affected the induction of COX-2 by S1P. Whereas COX-2 expression increased in $RK^{-/-}$, $G\alpha_{13}{}^{-/-}$, or $G\alpha_q{}^{-/-}$ cells 1–12 h after S1P treatment, $G\alpha_{12}$ deficiency completely blocked the ability of S1P to induce COX-2 (Fig. 1, *C* and *D*). Moreover, COX-2 was minimally expressed in untreated $G\alpha_{12}{}^{-/-}$ or $G\alpha_{12/13}{}^{-/-}$ cells compared with control or $G\alpha_q{}^{-/-}$ cells, which suggests that the constitutive expression of COX-2 requires $G\alpha_{12}$ (Fig. 1*D*).

Activated $S1P_{1-5}$ couple with $G\alpha_i$ proteins and thus inhibit S1P action (21). To exclude the involvement of $G\alpha_{i/o}$ in the induction of COX-2 by S1P, COX-2 was immunochemically monitored in cells incubated with 1 μM S1P after pretreatment with 0.1 ng/ml PTX, an inhibitor of $G\alpha_{i/o}$ proteins. Our results demonstrated that PTX treatment failed to alter the ability of S1P to induce COX-2 (Fig. 1*E*), which indicates that COX-2 induction by S1P is unaffected by the $G\alpha_{i/o}$ pathway. A previous study showed that S1P promotes ERK1/2 phosphorylation via a $G\alpha_{i/o}$-dependent pathway (22). Data showing that PTX inhibited ERK1/2 phosphorylation by S1P confirmed the effectiveness of PTX.

We next determined whether the lack of COX-2 induction by S1P in $G\alpha_{12}{}^{-/-}$ cells is the result of the down-regulation of *COX-2* gene transcription. Reporter gene assays revealed that the luciferase activity of pGL-mCOX-2-724, containing the promoter region of the human *COX-2* gene, was significantly increased by S1P (1 μM, 3 h) in $RK^{-/-}$ cells (Fig. 1*F*). Moreover, $G\alpha_{12}$ deficiency completely inhibited the ability of S1P to induce luciferase expression. On the other hand, a lack of $G\alpha_{13}$ did not change *COX-2* gene induction. Real time PCR analysis revealed that S1P treatment enhanced the level of endogenous COX-2 mRNA in $RK^{-/-}$ cells but not in $G\alpha_{12}{}^{-/-}$ cells (Fig. 1*G*). In $G\alpha_{13}{}^{-/-}$ cells, S1P also increased COX-2 mRNA levels. Our data indicate that $G\alpha_{12}$, but not $G\alpha_{13}$, plays a critical role in COX-2 induction by S1P, which results from the transcriptional activation of the COX-2 gene. Given the specific role of $G\alpha_{12}$ in the induction of COX-2, we focused subsequently on the role of $G\alpha_{12}$ in the gene regulation.

Transfection and siRNA Knockdown Experiments—To confirm the regulatory role of $G\alpha_{12}$ on COX-2 induction by S1P, we examined the effect of wild-type $G\alpha_{12}$ ($G\alpha_{12}$W) overexpression in $G\alpha_{12}{}^{-/-}$ cells. $G\alpha_{12}$W transfection enabled these cells to respond to S1P with respect to COX-2 induction (Fig. 2*A*). To further verify the role of $G\alpha_{12}$ on S1P-mediated COX-2 induction, we measured COX-2 expression levels under knockdown conditions. Fig. 2*B* shows that $G\alpha_{12}$ knockdown by siRNA decreased S1P-dependent COX-2 induction; this knockdown of $G\alpha_{12}$ was confirmed by immunoblotting. These data confirm that $G\alpha_{12}$ indeed mediates the induction of COX-2 by S1P. Consistently, the induction of COX-2 by S1P was completely abrogated by transfection with the minigene vector of $G\alpha_{12}$ but not by that of $G\alpha_{13}$ (C-terminal peptide of $G\alpha_{12}$ or $G\alpha_{13}$ blocks GPCR) in control cells (Fig. 2*C*, *top*). $G\alpha_{12}$ or $G\alpha_{13}$ minigene transfection inhibited S1P- or thrombin-inducible ERK phosphorylation, as was previously observed (23), which validated our assay conditions (Fig. 2*C*,

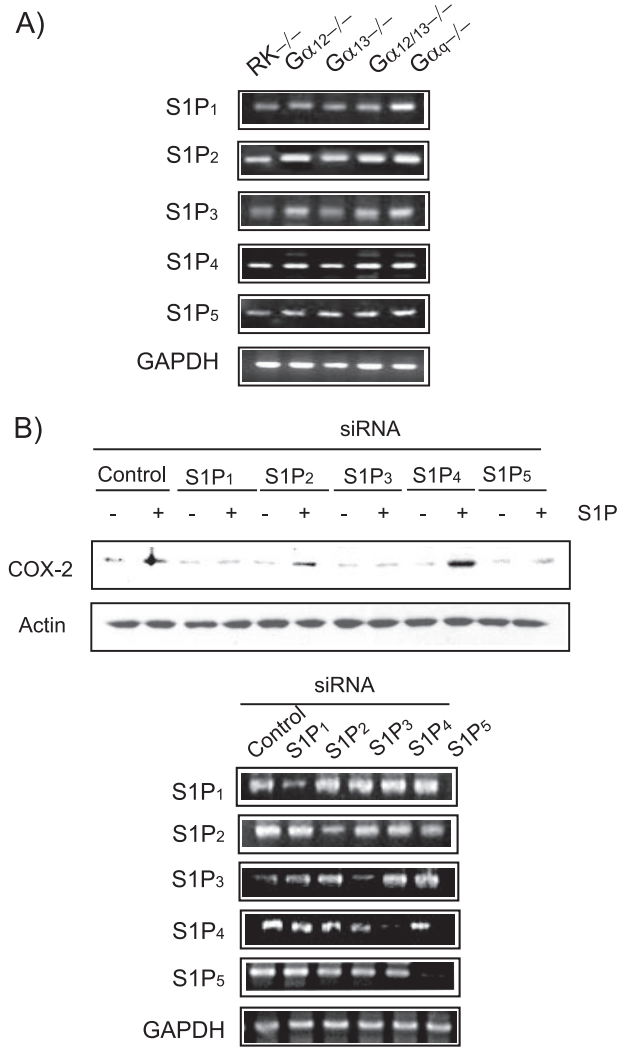

FIGURE 3. **Involvement of $S1P_1$, $S1P_3$ and $S1P_5$ in COX-2 induction by S1P.** *A*, the mRNA transcript levels of $S1P_{1-5}$ in the MEF cells. The levels of S1P receptor transcripts were measured by semiquantitative RT-PCR analyses. Shown above are the representative RT-PCR analyses. *B*, the effects of S1P receptor knockdown on S1P-mediated COX-2 induction. Cells were transfected with control siRNA or siRNA directed against $S1P_1$–$S1P_5$ (100 pmol of each) and treated as described in the legend to Fig. 2*B*. Specific knockdown of S1P receptor was confirmed by RT-PCR analyses. *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

bottom). This finding suggests the involvement of $G\alpha_{12}$ coupling to GPCR during the induction of COX-2 by S1P.

Identification of S1P Receptors for COX-2 Induction—To determine whether S1P receptors are required for COX-2 induction and to identify the responsible S1P receptor isoforms, we performed an experiment with siRNAs specifically directed against $S1P_{1-5}$. First, the presence of S1P receptor subtypes in MEF cells was examined by RT-PCR of $S1P_{1-5}$ gene products (Fig. 3*A*). The intensities of bands were comparable with each other in the MEF cells, indicating that the transcripts for S1P receptors are comparable except for specific Gα-protein knockouts. Subsequently, transfection with siRNAs directed against $S1P_1$, $S1P_3$, or $S1P_5$ notably suppressed S1P-mediated COX-2 induction, whereas transfection with siRNAs against $S1P_2$ or $S1P_4$ failed to do so (Fig. 3*B*, *top*). RT-PCR control experiments confirmed specific knockdown of S1P receptors by siRNAs (Fig. 3*B*, *bottom*). Our results suggest that S1P requires $S1P_1$, $S1P_3$, and $S1P_5$ for full activation of COX-2 induction.

Role of NF-κB Activation in $G\alpha_{12}$-mediated COX-2 Induction—NF-κB, C/EBP, and CREB proteins are critical transcription factors and interact with the upstream region of the *COX-2* gene (24). To determine whether COX-2 induction by S1P was accompanied by the activations of transcription factors, we first performed electrophoretic mobility shift assays. Treatment of control cells with S1P (1 μM, 30 min to 3 h) resulted in increases in the band intensities of NF-κB DNA binding (Fig. 4*A*). Immunocompetition assays using anti-p65 antibody confirmed the specificity of NF-κB DNA binding (data not shown). In contrast, the bindings of C/EBP and of CREB to their respective consensus oligonucleotides were not increased by S1P treatment. Moreover, a deficiency of $G\alpha_{12}$ completely abolished the formation of NF-κB DNA complex, indicating that $G\alpha_{12}$ regulates NF-κB activation in response to S1P.

Next, to explore the functional role of NF-κB activation by S1P in *COX-2* gene induction, we performed chromatin immunoprecipitation analysis. Genomic DNA-protein complexes were immunoprecipitated with anti-p65 (a major component of NF-κB) antibody, and this was followed by the reversal of cross-linking and PCR amplification using primers flanking the proximal and distal regions of the DNA corresponding to the NF-κB binding site in the *COX-2* gene promoter. In control cells treated with S1P, PCR product intensity was significantly higher than in untreated cells, and increases in the band intensity of NF-κB DNA complex were prevented by the absence of $G\alpha_{12}$ (Fig. 4*B*). We next tested whether mutation of the NF-κB binding site present in the promoter region of the *COX-2* gene suppressed NF-κB-mediated gene transcription by S1P. Consistent with the above results, mutation of the NF-κB binding site abolished an increase in COX-2 reporter activity by S1P (Fig. 4*C*). A previous study showed that mutation of the E-box in the promoter region of COX-2 did not change lipopolysaccharide-induced reporter activity (3). Consistent with this report, COX-2 induction by S1P was unaffected by specific mutation of the E-box consensus sequence, which supports the specific role of NF-κB. These data indicate that NF-κB activation by S1P depends on $G\alpha_{12}$ and contributes to *COX-2* gene induction.

Nuclear Translocation of p65—Activated NF-κB, which consists of p65 and p50, translocates into the nucleus after being relieved from its IκBα binding. Immunocytochemistry showed that p65 was located mainly in the cytoplasm of MEF cells (Fig. 5*A*). S1P treatment (1 μM, 1 h) allowed p65 to translocate into the nucleus in control cells. On the contrary, the nuclear translocation of p65 was blocked by the absence of $G\alpha_{12}$. As was expected, the absence of $G\alpha_{13}$ did not inhibit p65 nuclear localization, confirming the specific role of $G\alpha_{12}$ in NF-κB activation. The nuclear integrity was confirmed by propidium iodide staining of the MEF cells. In addition, immunoblot analyses were performed to compare the nuclear levels of p65 as a function of time. S1P treatment (30 min to 3 h) increased p65 levels in the nuclear fractions of control or $G\alpha_{13}{}^{-/-}$ cells but not in those of $G\alpha_{12}{}^{-/-}$ cells (Fig. 5*B*), thus verifying that the lack of $G\alpha_{12}$ inhibits NF-κB nuclear translocation.

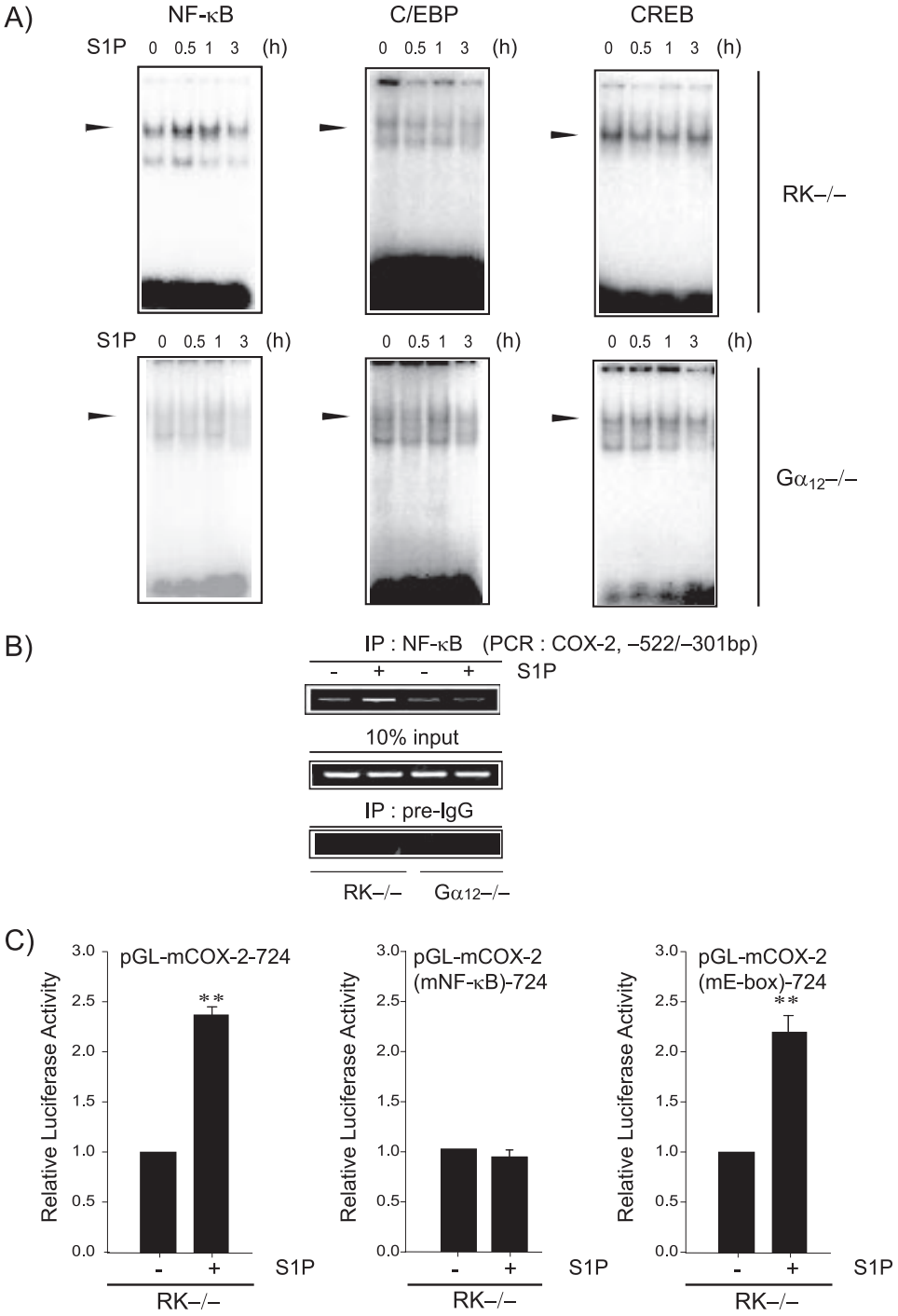

FIGURE 4. **Role of $G\alpha_{12}$ in NF-κB activation and NF-κB-mediated gene transcription.** *A*, gel shift analyses. Nuclear extracts were prepared from cells cultured with S1P (1 μM, 0.5–3 h). All lanes contained 5 μg of nuclear extract and 5 ng of labeled NF-κB, C/EBP, or CREB binding oligonucleotide. *B*, chromatin immunoprecipitation assays. DNA-protein complexes in cells treated with S1P (1 μM, 1 h) were immunoprecipitated with anti-p65 antibody or pre-IgG. Samples were then PCR-amplified using the primers flanking the proximal and distal regions of the DNA comprising the NF-κB binding site in the *COX-2* promoter. *IP*, immunoprecipitation. *C*, the effects of NF-κB binding site mutation (pGL-mCOX-2(mNF-κB)-724) on luciferase induction. Luciferase reporter assays were performed in the lysates of cells transfected with pGL-mCOX-2-724 or pGL-mCOX-2(mNF-κB)-724, in which the NF-κB binding site was mutated by deletion and treated with vehicle or S1P (1 μM, 3 h). pGL-mCOX-2(mE-box)-724 mutant construct was used as a negative control. Luciferase activity was calculated as a relative change compared with that of β-galactosidase. Values represented the mean ± S.E. for four separate experiments (significant as compared with control; **, $p < 0.01$).

IκBα Degradation by $G\alpha_{12}$—Activation of NF-κB is initiated by extracellular stimuli that lead to the activation of IKK complex, which phosphorylates IκBα proteins. To address whether NF-κB-mediated COX-2 induction by S1P involves IκBα phosphorylation, COX-2 expression was monitored in $RK^{-/-}$ cells incubated with Bay117082 (a specific IKK inhibitor) prior to S1P treatment (25). Bay117082 pretreatment (5 μM) was found to inhibit COX-2 induction by S1P (Fig. 6*A*), thus supporting the notion that S1P induces IκBα phosphorylation presumably via IKK.

Given the fact that the phosphorylated IκBα subunit is proteolytically degraded during the process of NF-κB activation, we sought to determine whether S1P treatment elicits the degradation of IκBα following its phosphorylation and, if so, whether $G\alpha_{12}$ regulates these processes. Fig. 6*B* shows that IκBα was phosphorylated in $RK^{-/-}$ cells treated with S1P (1 μM, 1 h) and thereby degraded. $G\alpha_{12}$ deficiency did not alter S1P-induced IκBα phosphorylation. However, IκBα degradation induced by S1P was inhibited by $G\alpha_{12}$ knock-out or siRNA knockdown (Fig. 6, *B* and *C*), establishing the role of $G\alpha_{12}$ in the process of IκBα degradation. Moreover, inhibition of S1P-induced IκBα degradation by $G\alpha_{12}$ minigene reconstitution additionally confirmed the role of $G\alpha_{12}$ (supplemental Fig. S1).

JNK-mediated IκBα Ubiquitination Downstream of $G\alpha_{12}$—Phosphorylated IκBα was multiply ubiquitinated before its degradation in the 26 S proteasome system (26). To understand the molecular mechanism underlying IκBα degradation, we tested the possibility that $G\alpha_{12}$ mediates a signal that leads to IκBα ubiquitination. The extent of IκBα ubiquitination was monitored by ubiquitin immunoprecipitation and by immunoblot analysis for IκBα in cells transfected with plasmid encoding His-tagged ubiquitin and subsequently treated with S1P. Ubiquitination of IκBα was strongly increased by S1P in $RK^{-/-}$ cells compared with untreated controls. However, S1P treatment failed to enhance the intensity of the ubiquitinated IκBα band in $G\alpha_{12}{}^{-/-}$ cells (Fig. 7*A*), indicating that the lack of $G\alpha_{12}$ prevents IκBα ubiquitination. Given the JNK-mediated proteaso-

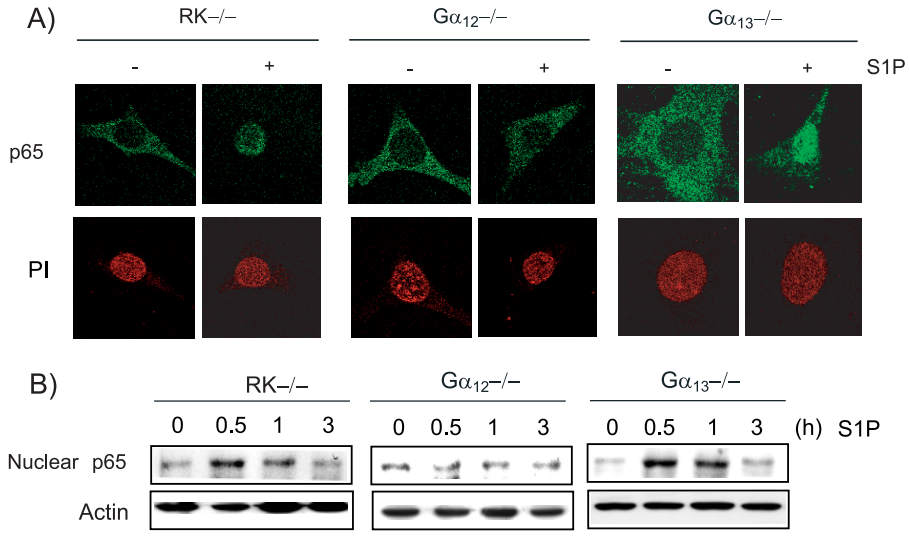

FIGURE 5. **Inhibition of S1P-induced p65 nuclear translocation by $G\alpha_{12}$ deficiency.** *A*, immunocytochemistry. Cells were treated with vehicle or S1P (1 h) and subjected to immunocytochemistry for p65. The same fields were counterstained with propidium iodide (*PI*) to locate the nuclei. *B*, p65 levels in nuclear fractions. p65 was immunoblotted in the nuclear fractions of the cells treated with S1P (1 μM, 0.5–3 h). Equal loading of proteins was verified by probing the replicate blots for actin. Each lane contained 20 μg of nuclear proteins.

mal degradation of certain transcription factors downstream of $G\alpha_{12}$ (27), we next assessed the role of JNK in IκBα ubiquitination by S1P. S1P treatment resulted in the activation of JNK in control or $G\alpha_{13}^{-/-}$ cells but not in $G\alpha_{12}^{-/-}$ cells (Fig. 7*B*). In $RK^{-/-}$ cells, chemical inhibition of JNK using JNKI (a chemical inhibitor of JNK) completely prevented the ubiquitination of IκBα elicited by S1P (Fig. 7*C*). Moreover, JNK transfection consistently restored the ability of S1P to degrade IκBα in the absence of $G\alpha_{12}$ (Fig. 7*D*). This observation suggests that the lack of IκBα degradation under conditions of $G\alpha_{12}$ deficiency may be associated with defective JNK activation in response to S1P. Collectively, it appears that the signal pathway involving $G\alpha_{12}$ regulates the IκBα ubiquitination and thereby its degradation but not the phosphorylation of IκBα.

Lack of COX-2 Induction by $G\alpha_{12}$ Knock-out in Mice—Finally, we explored whether targeted disruption of the $G\alpha_{12}$ gene abrogated S1P induction of COX-2 in mice. PCR DNA amplification confirmed specific disruption of the $G\alpha_{12}$ gene in mice (supplemental Fig. S2*A*). Next, COX-2 expression levels were monitored in the lungs of WT or $G\alpha_{12}$ knock-out mice 3 h after infusing vehicle or a single dose of 0.1 mg/kg S1P over 30 min into a femoral vein. This infusion of S1P notably increased COX-2 levels in the lung tissues of WT mice (Fig. 8*A*), although this increase was smaller than that observed in animals intraperitoneally injected with 1 mg/kg lipopolysaccharide (3 h post treatment) (supplemental Fig. S2*B*). In $G\alpha_{12}$ knock-out mice, constitutive COX-2 expression in lung tissue notably decreased, and more importantly, the ability of S1P to induce COX-2 was abolished. Immunohistochemistry verified COX-2 induction by S1P in the lungs of WT mice and the lack of COX-2 induction in $G\alpha_{12}$ knock-out mice (Fig. 8*B*). Examinations of hematoxylin and eosin-stained replicate lung tissue sections suggested that COX-2 was prominently induced in areas containing large vessels, in bronchial smooth muscle, and in scattered cells of alveolar septa in WT animals administered S1P. In addition, S1P induced COX-2 in the livers of WT mice but not in $G\alpha_{12}$ knock-out mice (supplemental Fig. S2*C*). Finally, an enzyme-linked immunosorbent assay established the ability of S1P to specifically increase plasma prostaglandin E_2 contents in WT but not in $G\alpha_{12}$ knock-out animals (Fig. 8*C*), thus corroborating $G\alpha_{12}$-mediated COX-2 induction *in vivo* by S1P.

DISCUSSION

COX-2 plays an important role in inflammatory processes and serves as an important therapeutic target (28). Despite reports on COX-2 induction by phospholipids (9, 10), information on the effects of S1P on enzyme expression, the responsible transcription factors, and the mechanistic bases of COX-2 gene transactivation are limited, especially in association with the activation of phospholipid-activated GPCRs.

The majority of S1P functions appear to be mediated through the activation of $S1P_{1-5}$. Moreover, fundamental differences in signaling through S1P receptors relate primarily to variations in G-protein coupling. It has been shown that $S1P_1$ couples to $G\alpha_i$, whereas $S1P_3$ and $S1P_2$ couple to $G\alpha_i$, $G\alpha_q$, and $G\alpha_{12/13}$ (29, 30). $S1P_4$ receptor was later reported to associate with $G\alpha_i$ (31) and more recently with $G\alpha_{12/13}$ (32), whereas $S1P_5$ couples to $G\alpha_i$ and $G\alpha_{12}$ (33). The present study shows that the major S1P receptors are comparably expressed except for specific $G\alpha$ knockouts. Hence, we can exclude the possibility that $G\alpha$ proteins autostimulate S1P receptor expression and that S1P binding to certain receptors elicits differential signals in knock-out cells. Moreover, our results showing that siRNA knockdowns of $S1P_1$, $S1P_3$, or $S1P_5$ inhibit the ability of S1P to induce COX-2 suggest that all three receptor isoforms are necessary for the full activation of COX-2 induction. Moreover, our data are in line with previous reports on this issue and show the inhibition of COX-2 induction by $S1P_1$- or $S1P_3$-specific antisense oligonucleotide transfection (34).

Previous studies have shown that S1P induces COX-2 and that this has growth-regulating properties (10). In the present study, we demonstrate for the first time that the cell signaling pathway coupled with $G\alpha_{12}$, but not with $G\alpha_{13}$, plays an essential role in the regulation of COX-2 expression by S1P. Moreover, $G\alpha_{12}$ deficiency completely blocked the ability of S1P to induce COX-2, as evidenced by a lack of constitutive or inducible COX-2 expression in $G\alpha_{12}^{-/-}$ or $G\alpha_{12/13}^{-/-}$ cells and by no induction of COX-2 in $G\alpha_{12}$ knockdown $RK^{-/-}$ cells. Also, the lack of COX-2 induction by S1P in $G\alpha_{12/13}^{-/-}$ cells suggests that the two G-proteins do not act antagonistically toward each other. Efficacious COX-2 induction by S1P in $G\alpha_{13}^{-/-}$ cells, shown in the present study, supports the notion that $G\alpha_{13}$ might not be necessary for S1P-mediated cell signaling, which

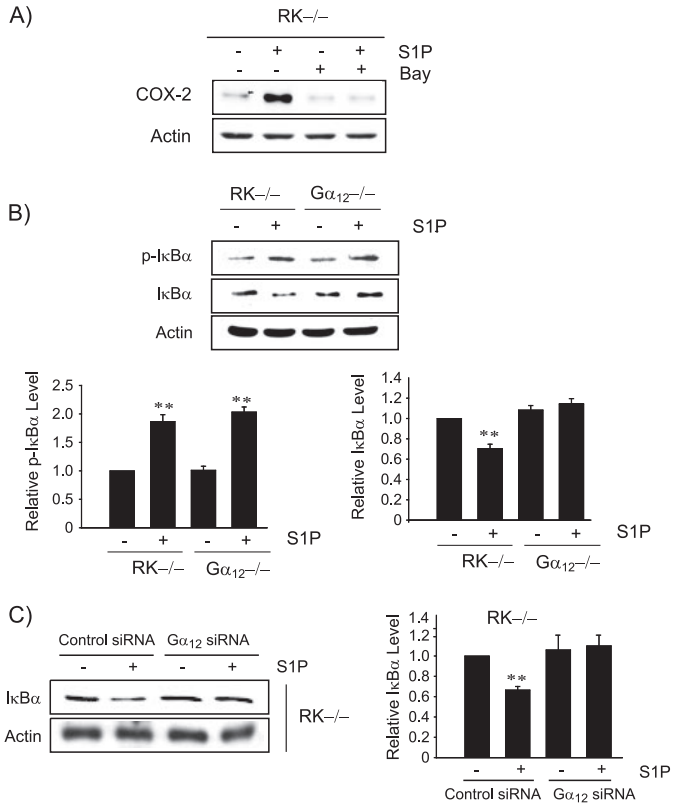

FIGURE 6. **Role of $G\alpha_{12}$ in IκBα ubiquitination.** *A*, the effect of Bay117082 (Bay) on COX-2 induction by S1P. Cells were treated with 5 μM Bay and then exposed to 1 μM S1P for 3 h in the continuing presence of Bay. A representative immunoblot shows the levels of COX-2. *B*, phosphorylation and degradation of IκBα. Phosphorylated or total IκBα was immunoblotted in the lysates of cells treated with S1P for 1 h. *C*, the effect of $G\alpha_{12}$ knockdown on S1P-mediated IκBα degradation. Cells transfected with siRNA, as described in the legend to Fig. 2*B*, were treated with S1P for 1 h. Values represented the mean ± S.E. for three separate experiments (significant as compared with control; **, $p < 0.01$). Each lane contained 20 μg of lysate proteins. Results were confirmed by repeated experiments.

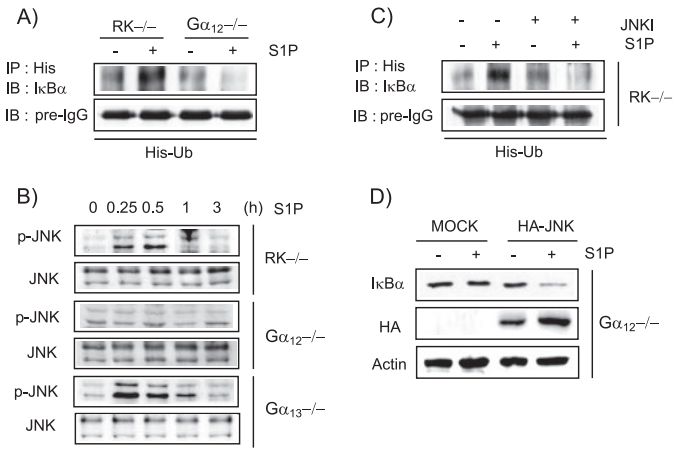

FIGURE 7. **Role of $G\alpha_{12}$ in JNK-dependent IκBα ubiquitination.** *A*, immunoblot analysis (*IB*) of ubiquitinated IκBα. Ubiquitinated proteins were immunoprecipitated (*IP*) with anti-His antibody in the lysates of cells that had been transfected with the plasmid encoding His-tagged ubiquitin and exposed to S1P (1 μM, 1 h) and subjected to immunoblot analysis for p65. *B*, the levels of phosphorylated JNK in MEF cells. *C*, the effect of JNKI on IκBα ubiquitination by S1P. *D*, the effect of HA-JNK expression on the level of IκBα in $G\alpha_{12}^{-/-}$ cells treated with S1P. Each lane contained 20 μg of lysate proteins. *HA*, hemagglutinin.

differs from the finding of a previous report that showed COX-2 induction by a constitutively active mutant of $G\alpha_{13}$ (35). This discrepancy may have resulted from differences between the experimental approaches and the use of GPCR ligand (*i.e.* physiological activation of $G\alpha_{13}$ by S1P *versus* persistent activation of $G\alpha_{13}$ by an activated mutant). A supplemental experiment showed that COX-2 induction by thrombin (another ligand of GPCR coupled to $G\alpha_{12/13}$) was inhibited only by $G\alpha_{12}$ and $G\alpha_{13}$ double knock-out, which raised the possibility that $G\alpha_{12}$ members differentially coupled to different GPCRs depending on ligand specificity. Moreover, we observed that $G\alpha_{12}$ deficiency did not inhibit the induction of COX-2 by lipopolysaccharide or by tumor necrosis factor-α, non-GPCR ligands (data not shown), which adds support to the specificity of $G\alpha_{12}$ for S1P GPCRs.

Real time PCR analysis for endogenous COX-2 transcripts demonstrating the inability of S1P to increase COX-2 mRNA during $G\alpha_{12}$ deficiency was paralleled by the result of our pGL-COX-2 reporter gene experiment. Further, recovery of the ability of S1P to induce COX-2 in $G\alpha_{12}^{-/-}$ cells after transfection with wild type of $G\alpha_{12}$ further supported the crucial role of $G\alpha_{12}$. Our data provide compelling evidence that the lack of COX-2 induction by S1P during $G\alpha_{12}$ deficiency results from defective transcriptional activation, whereas $G\alpha_{13}$ deficiency did not inhibit *COX-2* induction by S1P. These results indicate that the S1P induction of COX-2 is regulated only by $G\alpha_{12}$. Our minigene reconstitution experiment further supported the specific role of $G\alpha_{12}$ and the importance of $G\alpha_{12}$ interaction with ligand-bound S1P receptors, because the minigene product competitively binds to the G-protein-interacting cytoplasmic face of activated receptors (23). Therefore, it is likely that $G\alpha_{12}$ activity conveys information from ligand-bound S1P receptors for *COX-2* gene induction. These observations demonstrate that the induction of COX-2 by S1P is transcriptionally regulated by a signaling pathway involving $G\alpha_{12}$ and its coupling to ligand-activated GPCRs.

The production of PGs as a result of COX-2 induction is stimulated by transcriptional activation of the COX-2 gene (3). The cis-acting elements identified to act at the promoter region of *COX-2* include NF-κB, C/EBP, and CREB (36). NF-κB is a pleiotropic gene regulator and is primarily involved in immune and inflammatory responses (26). In this report, we provide evidence that S1P selectively activates NF-κB to transactivate *COX-2* and that NF-κB plays a critical role in S1P-induced gene expression, which is consistent with the observation that S1P activates NF-κB (29). Immunoblot and immunocytochemical assays confirmed that the nuclear translocation of p65 is inhibited in the absence of $G\alpha_{12}$. The requirement for activated NF-κB for *COX-2* induction was strengthened by the observed $G\alpha_{12}$-dependent increase in NF-κB binding to its binding site located in the *COX-2* promoter and by mutation analysis of the NF-κB binding site.

The pathways of NF-κB-inducing kinase and MEKK1 regulate I-κBα phosphorylation via IKK (37). In general, IκBα phosphorylation precedes its degradation. Phosphorylation of the serine residues of IκBα by IKK targets this inhibitory protein for degradation by the ubiquitin-proteasome system, and the release of phosphorylated IκBα from NF-κB complex results in the formation of an active p65/p50 heterodimer and the nuclear

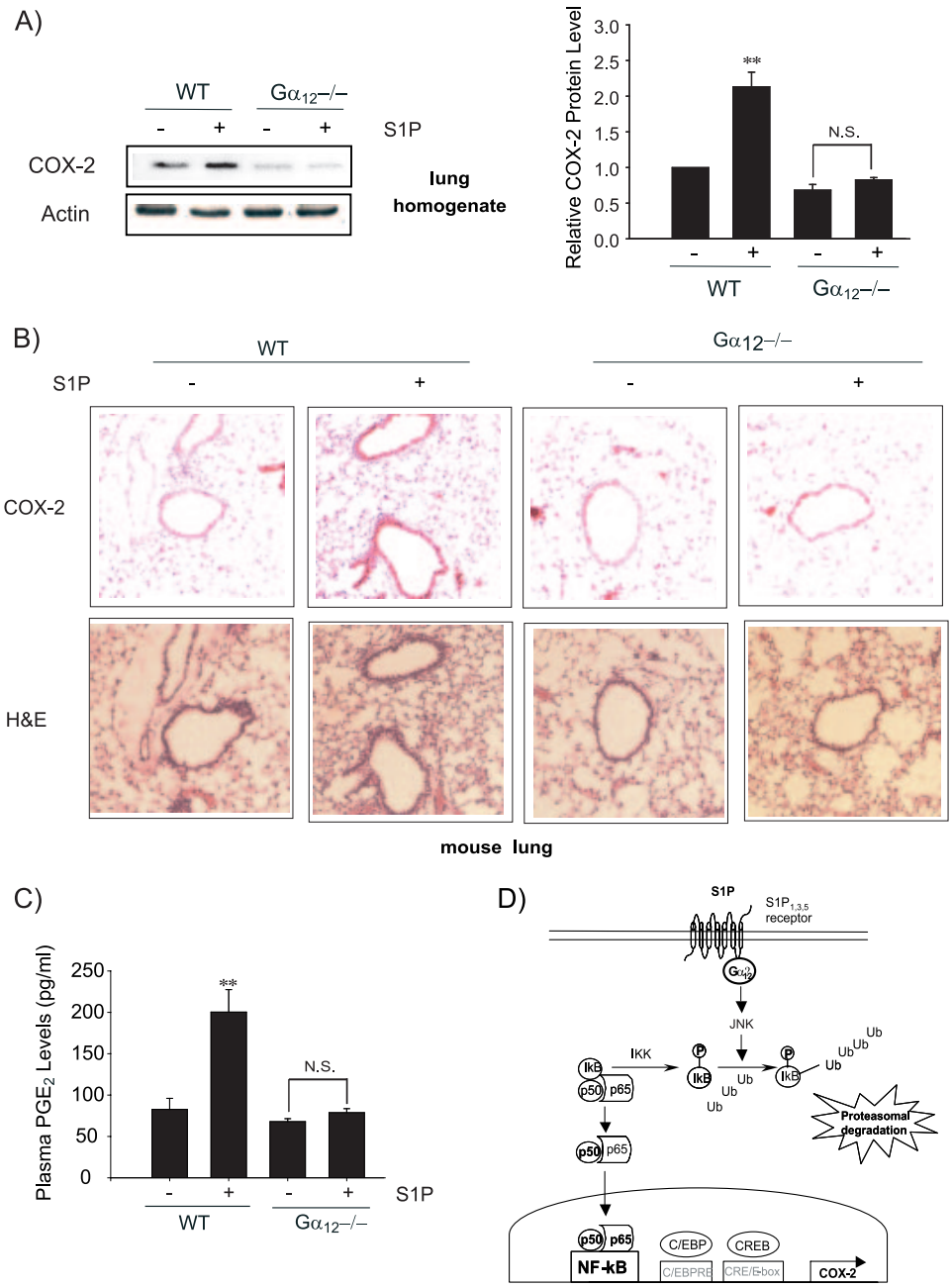

FIGURE 8. **Role of $G\alpha_{12}$ in the induction of COX-2 by S1P in mice.** *A*, immunoblot analysis for COX-2 in lung homogenates. Each lane contained 20 μg of lung homogenate proteins. The values represented the mean ± S.E. with at least three separate experiments (significant as compared with vehicle-treated WT mice; **, $p < 0.01$; vehicle-treated WT mice, *1.0*). *B*, immunohistochemistry of COX-2 and hematoxylin and eosin staining (*H&E*). COX-2 expression was immunohistochemically monitored in the lungs of WT or $G\alpha_{12}$ knock-out mice treated with vehicle or S1P (100×). *C*, prostaglandin E_2 contents. *D*, schematic diagram illustrating the mechanism by which $G\alpha_{12}$ regulates COX-2 expression. *N.S.*, not significant.

translocation of p65. The observation that Bay117082 completely abolished COX-2 induction by S1P supports the role of IκBα in the $G\alpha_{12}$-mediated signal transduction conveyed by S1P-mediated receptor activation. In this study, we found using knock-out, knockdown, and minigene experiments that S1P failed to degrade IκBα when $G\alpha_{12}$ was deficient but that IκBα phosphorylation was unaffected. Thus, our results demonstrate that the S1P receptor-$G\alpha_{12}$ transduction pathway regulates the degradation of IκBα but not its IKK-mediated phosphorylation. The ubiquitin-proteasome system is responsible for degrading phosphorylated IκBα (26), and previously, we showed that $G\alpha_{12/13}$ contributes to the JNK-dependent induction of the iNOS gene by thrombin, which is also regulated by NF-κB (13). In this previous study, we found that JNK downstream of $G\alpha_{12}$ regulated the cell signaling necessary for IκBα degradation. Also, another study suggested that JNK participates in the proteasomal degradation of retinoid X receptor (27). Our finding of phosphorylated IκBα accumulation, failure of IκBα ubiquitination, and IκBα degradation in the presence of $G\alpha_{12}$ deficiency characterize $G\alpha_{12}$ control of the pathway involving IκBα ubiquitination. Of the Gα proteins coupled to S1P receptors, $G\alpha_{12}$ specifically regulates NF-κB for COX-2 induction by S1P. The most novel findings of the present study are $G\alpha_{12}$ regulation of JNK in response to S1P and the critical role of $G\alpha_{12}$ in the ubiquitination of IκBα. It has been shown that JNK activation increases E3 ligase activity, which enhances the ubiquitination of target proteins, such as c-Jun (38) and c-FLIP (39). Moreover, JNK-mediated phosphorylation enhances c-Jun degradation by allowing its recognition by E3 ligase Fbw7-containing Skp-Cullin-F-box protein complex (38). Another study suggested that IκBα ubiquitination is carried out by E3 ligase βTrCP (40, 41), and because JNK induces βTrCP accumulation, which increases the ubiquitination and degradation of IκBα (42), it is likely that $G\alpha_{12}$ deficiency does not allow JNK to be activated in response to S1P and consequently decreases IκBα ubiquitination.

Our observations that a single infusion of S1P led to COX-2 induction in the lungs and livers of wild type mice but not in $G\alpha_{12}$ knock-out animals and that these inductions occurred in parallel with simultaneous increases in plasma prostaglandin E_2 level, together with the finding that basal COX-2 expression was lower in $G\alpha_{12}$ knock-out mice than in wild type mice, confirm the *in vivo* regulation of COX-2 by $G\alpha_{12}$. The findings presented provide insight into the S1P-mediated cell signaling pathways required for COX-2 gene regulation (Fig. 8*D*) and may be of assistance in the understanding of the physiology of inflammation and cell proliferation during vascular pathogenesis.

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