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PKCβII Augments NF-κB-Dependent Transcription at the CCL11 Promoter via p300/CBP-Associated Factor Recruitment and Histone H4 Acetylation[1]

Deborah L. Clarke, Amy Sutcliffe, Karl Deacon, Dawn Bradbury, Lisa Corbett, and Alan J. Knox[2]

The transcription factor NF-κB plays a pivotal role in regulating inflammatory gene expression. Its effects are optimized by various coactivators, including histone acetyltransferases (HATs) such as CREB-binding protein/p300 and p300/CBP-associated factor (p/CAF). The molecular mechanisms regulating cofactor recruitment are poorly understood. In this study, we describe a novel role for protein kinase C (PKC) βII in augmenting NF-κB-mediated TNF-α-induced transcription of the target gene CCL11 in human airway smooth muscle cells by phosphorylating the HAT p/CAF. Studies using reporters, overexpression strategies, kinase-dead and HAT-defective mutants, and chromatin immunoprecipitation showed that PKCβII activation was not involved in NF-κB translocation, but facilitated NF-κB-mediated CCL11 transcription by colocalizing with and phosphorylating p/CAF, and thereby acetylating histone H4 and promoting p65 association with the CCL11 promoter. The effect was dependent on p/CAF's HAT activity. Furthermore, mouse embryonic fibroblasts from PKCβ knockout mice showed markedly reduced TNF-α-induced CCL11 expression and NF-κB reporter activity that was restored on PKCβII overexpression, suggesting a critical role for this pathway. These data suggest a novel important biological role for PKCβII in NF-κB-mediated CCL11 transcription by p/CAF activation and histone H4 acetylation. *The Journal of Immunology,* **2008, 181: 3503–3514.**

Nuclear factor κB is a ubiquitously expressed transcription factor, made up of hetero- and homodimers of members of the DNA-binding Rel family of proteins. NF-κB regulates the transcription of multiple target genes, including inflammatory cytokines, chemokines, adhesion molecules, and other transcription factors, many of which have been implicated in the pathogenesis of asthma and chronic obstructive pulmonary disease. Due to this, inhibition of NF-κB is an attractive target for the treatment of lung disease, and, as such, has generated much interest (1–3). In resting cells, NF-κB is sequestered in the cytoplasm as an inactive heterodimer anchored to its inhibitor IκBα. This is regulated by the IκB kinase (IKK)[3] complex, consisting of the kinases IKKα and IKKβ and the structural protein IKKγ. Activation of NF-κB by proinflammatory cytokines such as IL-1β and TNF-α involves phosphorylation, ubiquitination, and proteasome-mediated degradation of IκBα and NF-κB translocation to the nucleus, where it binds to recognition elements in gene promoters (1).

Nuclear *trans* activation by NF-κB requires several cofactors, including CREB-binding protein (CBP), p300, and p300/CBP-associated factor (p/CAF) (4), all of which are histone acetyltransferases (HATs) (5, 6). In quiescent cells, DNA is tightly packaged in chromatin, which must unravel to allow access of basal transcription factors and RNA polymerase II. Chromatin consists of four core histones, an H3-H4 tetramer and two H2A-H2B dimers that undergo covalent modifications (acetylation, phosphorylation, methylation), which control the unraveling process and thereby gene transcription. Histone acetylation increases gene transcription through DNA unwinding, thereby increasing accessibility to the transcriptional machinery. In contrast, histone deacetylases (HDACs) silence genes (7, 8). Our previous studies of CCL11, IL-8, and MCP-1 (9–11) have identified important transcription factors for these genes and, moreover, identified histone H4 acetylation as a key event in inflammatory gene transcription (9–10, 12).

Several studies demonstrate the importance of cofactor recruitment as a key optimization event in switching on gene transcription. For example, NF-κB p65 and p/CAF have been shown to be recruited to the promoters of TNF-α and cyclooxygenase-2 (COX-2) under diabetic conditions, resulting in histone H4 acetylation and subsequent transcription (13). p/CAF is also recruited to the COX-2 promoter after PMA, IL-1β, or LPS stimulation in fibroblasts (14). The signaling pathway(s) regulating recruitment of cofactors such as p/CAF to inflammatory gene promoters is poorly described, but is potentially very important, because these cofactors are essential to optimize NF-κB-mediated gene transcription.

In this study, we tested the hypothesis that members of the protein kinase C (PKC) family play an important role in this regulatory process. The transmission of cellular signals usually proceeds

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[3] Abbreviations used in this paper: IKK, IκB kinase; Bis I, bisindolylmaleimide I; CBP, CREB-binding protein; ChIP, chromatin immunoprecipitation; COX-2, cyclooxygenase-2; EGR-1, early growth response-1; HASM, human airway smooth muscle; HAT, histone acetyltransferase; HDAC, histone deacetylase; MEF, mouse embryonic fibroblast; p/CAF, p300/CBP-associated factor; PKC, protein kinase C; WT, wild type.

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through multiprotein complexes involving enzymes positioned in close proximity to their upstream activators and downstream substrates. PKC is a family of isozymes that differ in function, tissue, and cell distribution and Ca^{2+} dependence. Group A, the classic isozymes, PKC-α, -βI, -βII, and -γ, are Ca^{2+} dependent and phorbol ester responsive; group B, the novel PKC isozymes, PKC-δ, -ε, -η, and -θ, are Ca^{2+} independent, but phorbol ester responsive; and group C, the atypical PKC isozymes, PKC-ζ, -μ, and -ι (-λ), are Ca^{2+} independent and phorbol ester unresponsive. When unstimulated, PKC is mainly cytosolic and, on stimulation, translocates to the plasma membrane (15). Because PKCβII can also translocate to the nucleus (16, 17), we postulated that it might regulate gene transcription events.

We therefore investigated the regulation of the eosinophil chemoattractant NF-κB-dependent gene CCL11 by specific PKC isoforms and their signal transduction pathways in primary human airway smooth muscle (HASM) cells and used embryonic fibroblasts from genetically modified mice to examine the molecular mechanism in detail. Studies in HASM cells using pharmacological PKCβ inhibitors and in wild-type (WT) and PKCβ knockout (KO) mouse embryonic fibroblasts (MEFs) show a marked reduction in NF-κB reporter activation accompanied by virtual abolition of the CCL11 response, which was restored in PKCβ KO MEFs by overexpressing PKCβII. We demonstrate that PKCβII, when activated by TNF-α, translocates from the cell membrane to the nucleus of HASM cells, and show for the first time that it associates with and phosphorylates p/CAF, promoting its recruitment to the CCL11 promoter, altering histone H4 acetylation and CCL11 transcription. These data define a novel role for PKCβII in NF-κB-dependent transcription of the CCL11 gene. The marked effects of inhibiting this pathway suggest an important biological role. In view of the importance of NF-κB, in inflammatory biology this phenomenon may have wider biological significance.

Materials and Methods

Reagents and plasmids

Polyclonal anti-human PKC Abs (PKCα SC-208, PKCβI SC-209, PKCβII SC-210, PKCγ SC-211), lamin A/C (SC-20681), p65 (SC-372), p300 (SC-585), and p/CAF (SC-8999) were purchased from Santa Cruz Biotechnology; anti-IκBα and phosphorylated p65 Abs from Cell Signaling Technology; GAPDH from Biogenesis; bisindolylmaleimide I (Bis I), Go6976, LY333531, Rottlerin, and MG-132 from Calbiochem; FuGene 6 from Roche; the dual-luciferase reporter assay system, *Renilla* luciferase pRL-SV40, and the PepTag Assay from Promega; and SYBR Green and Excite Mastermix for real-time PCR from Biogene. Primers were from MWG Biotec; ChIP-IT kits from Active Motif; human and murine CCL11 ELISA duosets, and human and murine rTNF-α from R&D Systems; purified p/CAF from Calbiochem; active PKCβII and histone H1 from Upstate Biotechnoloy; and [γ-^{32}P]ATP from Amersham Pharmacia. All other reagents were purchased from Sigma-Aldrich.

The NF-κB-dependent luciferase reporter, 6NFκBtkluc (18, 19), was obtained from R. Newton (University of Calgary, Calgary, Canada). Human WT PKCβII, catalytically inactive PKCβII (PKCβII K371D), and hemagglutinin-WT PKCβI were from A. Newton (University of California, San Diego, CA); CBP and p300 expression vectors were from D. Chakravarti (University of Pennsylvania, Philadelphia, PA). p/CAF and δ HAT p/CAF were from J. Freeman (University of Texas, San Antonio, TX) and T. Kouzarides (Cambridge University, Cambridge, U.K.). In all experiments, empty vector experiments were performed that had little or no significant effect on luciferase activity (data not shown). PKCβI and PKCβII KO, and WT MEFs were obtained from M. Leitges (University of Oslo, Oslo, Norway) (20).

Cell culture

Primary cultures of HASM cells were prepared from explants from postmortem individuals (3 female, 4 male, age range: 21–59 years; median age: 31.5 years) and cultured, as previously reported (12, 21–24). This protocol was approved by the Nottingham City Hospital Research Ethics Committee. Passage 6–7 cells were used for all experiments. HASM cells were cultured in 24-well culture plates for cytokine release and real-time PCR, 6-well plates for Western blotting, 90-mm petri dishes for nuclear cytosol separation, and EMSA and 75-cm^2 cell culture flasks for chromatin immunoprecipitation (ChIP). HASM cells used for luciferase reporter assays were seeded at 3×10^4 cells/ml and cultured to 50–60% confluence. All cells were growth arrested in serum-free medium for 24 h before experiments. Each experiment was performed in at least three different donors.

WT and PKCβ KO MEFs were used at passage 7–10. Twenty-four-well culture plates were used for cytokine release, and six-well plates for Western blotting, and cells were seeded at 3×10^4 cells/ml and cultured to 50–60% confluence for transfection experiments. Cells were growth arrested in serum-free medium for 24 h before experiments.

CCL11 assay

ELISA was used to measure human and murine CCL11 (R&D Systems), according to the manufacturer's instructions.

Western blotting

Western blotting was performed to determine PKC localization, p65 nuclear translocation, and phosphorylation (Ser^{276}, Ser^{438}, and Ser^{536}), as well as IκBα degradation. Nuclear and cytosolic fractions were prepared using NuClear extraction kit (Sigma-Aldrich), following the manufacturer's protocol. For a detailed Western blotting method, see Clarke et al. (25).

Immunofluorescent staining protocol for PKCβII

HASM cells were seeded at a concentration of 25,000 cells/well in an eight-well chamber slide in complete medium. After serum deprivation for 24 h, cells were left unstimulated or stimulated with TNF-α for 30 min and then fixed for 30 min in 100% methanol (ice cold). Permeabilization was performed with 0.2% (v/v) Triton X-100 in PBS plus 0.1% (w/v) BSA for 15 min at room temperature. Cells were incubated with 10% normal goat serum in the working buffer for 20 min at room temperature and incubated overnight at 4°C with diluted primary Ab (anti-PKCβII Ab), and then washed (3×5 min) in buffer. Bound primary Ab was detected using goat-raised anti-rabbit secondary Ab conjugated to AlexaFluor488 (Molecular Probes; 1:200). Slides were then washed thoroughly in PBS (5×5 min). After thorough washing with buffer, nuclei were counterstained with 4′,6-diamidino-2-phenylindole, mounted using glycerol/PBS, and stored in the dark. Confocal microscopy was performed using a Zeiss Axiovert 100 inverted microscope, with a Zeiss LSM510 *UV* combi confocal attached.

PKC activity assay

PKC activity was determined, as previously described (26), using a PepTag Assay for nonradioactive detection of PKC (Promega), as per the manufacturer's instructions.

RNA isolation and reverse transcription

Cells were pretreated (30 min) with PKC inhibitors and stimulated for 6 h with TNF-α (1 ng/ml). Total RNA was isolated by using the RNeasy mini kit (Qiagen), as per the manufacturer's protocol. A quantity amounting to 1 μg of total RNA was reverse transcribed in a total volume of 20 μl, including 200 U of Moloney murine leukemia virus reverse transcriptase, 25 U of RNase inhibitor, 0.5 μg of oligo(dT)$_{15}$ primer, 0.5 mM each dNTPs, and 1× first-strand buffer provided by Promega. The reaction was incubated at 42°C for 90 min. Reverse-transcriptase product aliquots were used for quantitative real-time RT-PCR.

Quantitative real-time RT-PCR

Human CCL11 expression was determined using the following primer sequences: sense, 5′-AGGAGAATCACCAGTGGCAAA-3′ and antisense, 5′-GGAATCCTGCACCCACTTCTT-3′. β_2-Microglobulin was used as the housekeeping gene (27). For studies using MEFs from PKCβ WT and null mice, the following primers were used for CCL11: sense, 5′-GATC TTCTTACTGGTCATGATAAAGCA-3′ and antisense, 5′-TGTCTCCC TCCACCATGCA-3′. β-actin was used as the housekeeping gene: sense, 5′-AAA TCG TGC GTG ACA TCA AA-3′ and antisense, 5′-AAG GAA GGC TGG AAA AGA GC-3′. One nanogram of reverse-transcribed cDNA was subjected to real-time PCR using Excite Real Time Mastermix with SYBR Green (Biogene) and the ABI Prism 7700 detection system (Applied Biosystems), as described (9, 28). CCL11 expression was normalized to the housekeeping gene by dividing the mean of the CCL11 triplicate by the mean of the β_2-microglobulin triplicate value for all human cell experiments and by the β-actin triplicate value for murine cell experiments.

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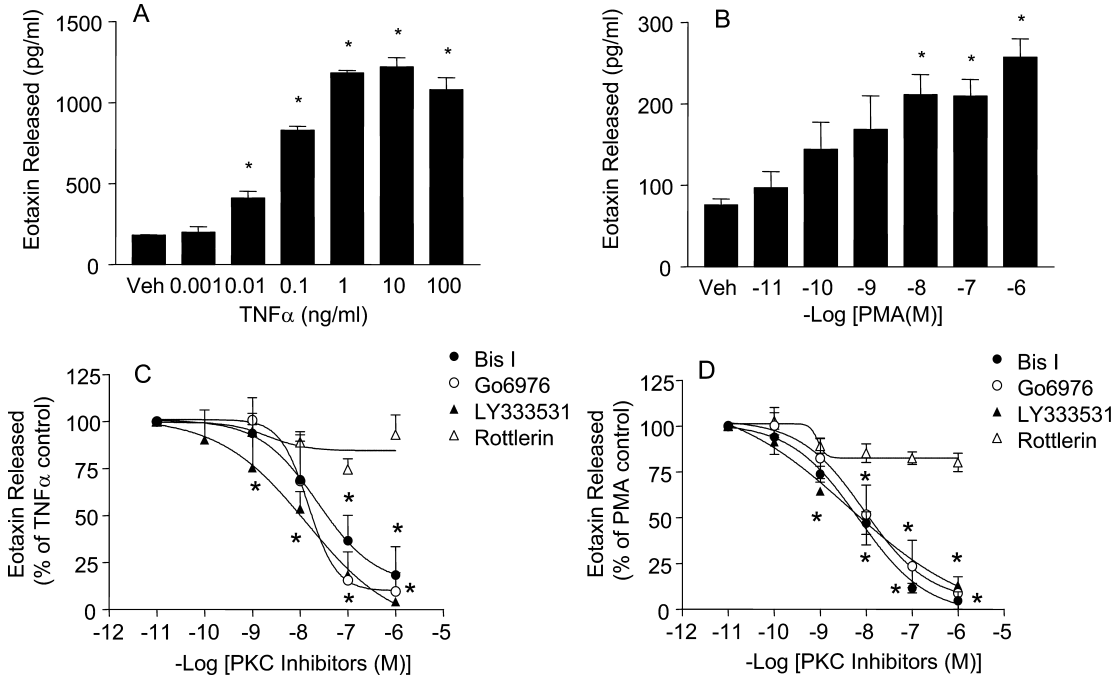

FIGURE 1. TNF-α and the direct PKC activator PMA induce CCL11 release, which is inhibited by PKC inhibitors. *A* and *B*, HASM cells were growth arrested for 24 h, and then treated with increasing concentrations of TNF-α or PMA for 24 h. *C* and *D*, HASM cells were pretreated for 30 min with PKC inhibitors before TNF-α (1 ng/ml) or PMA (10^{-7} M) for 24 h. CCL11 release was quantified by ELISA. Data are the mean ± SEM of three independent determinations using cells from different donors. A p value of less than 0.05 was deemed to be significant, and indicated with ∗.

Cell viability

Drug and vehicles toxicity was determined by MTT assay, as previously described (25). None of the drugs/vehicles altered cell viability (data not shown).

DNA transfections and luciferase assays

Transient transfections were performed, as described previously, using FuGene 6 (12, 23, 28). Cells were growth arrested for 8 h and transiently transfected for 24 h. After transfection, cells were treated with or without the drugs for 30 min before TNF-α or PMA incubation for 6 h, firefly and *Renilla* luciferase activities were measured, and relative luciferase activity was calculated. The NF-κB reporter 6κbtk.luc and CXCL-10 were transfected at 0.2 μg/well; CXCL-8 was used at 0.4 μg/well; the overexpression plasmids for PKCβ were transfected at 0.5 μg/well; and p300, CBP, p/CAF, and p/CAF$_{HAT}^-$ were transfected at 0.05 μg/well. All results are normalized to *Renilla* (0.4 ng/well).

EMSA

The in vitro DNA binding of transcription factors was analyzed, as described previously, by EMSA (12, 28), using ^{32}P-labeled or unlabeled human NF-κB consensus oligonucleotides 5′-GGCTTCCCTGGAATCTC CCACA-3′ (Sigma-Aldrich). In competition assays, 50× unlabeled competitors were added at the same time as probe addition.

ChIP

ChIP was performed using the ChIP-IT kit (catalogue 53001) from Active Motif, as per the manufacturers' instructions, as previously described (28). Eighty-90% confluent and serum-deprived HASM cells in 75-cm^2 dishes were pretreated with or without drugs for 30 min and then incubated with or without TNF-α (1 ng/ml) for 1 h. For negative controls, we performed immunoprecipitations in the absence of Ab or with normal mouse IgG. Quantitative real-time PCR was performed with 10 μl of DNA sample using the following specific CCL11 primer pairs spanning promoter regions that contain major regulatory elements, such as NF-κB: forward, 5′-CTTCATGTTGGAGGCTGAAG-3′ and reverse, 5′-GGATCTGGAATCTGGTCAGC-3′.

In vitro kinase assay

Kinase assays were performed in a final volume of 40 μl of kinase buffer. Protein kinase reactions were initiated by addition of appropriate recombinant substrate proteins (4 μg each of p/CAF and histone H1) and kinase buffer containing 250 μM ATP and 5 μCi of [γ-^{32}P]ATP. Purified PKCβII was used. Reactions were incubated for 20 min at 30°C and terminated by addition of an equal volume of Western sample buffer. Samples were boiled for 5 min, and proteins were resolved by SDS-PAGE. Gels were dried under vacuum, and phosphorylated proteins were visualized by autoradiography.

Coimmunoprecipitation of p/CAF and PKCβ

Confluent growth-arrested HASM cells were stimulated with TNF-α for 0–1 h, and then lysed in ice-cold modified radioimmunoprecipitation assay buffer (50 mM (pH 7.4) Tris-HCl; 1% Nonidet P-40; 0.25% sodium deoxycholate; 150 mM sodium chloride; 1 mM EDTA; 1 mM PMSF; aprotinin, leupeptin, and pepstatin each at 1 μg/ml; 1 mM sodium orthovanidate; and 1 mM sodium fluoride). After preclearing for 1 h, 4°C with 100 μl of protein A-agarose, immunoprecipitation was performed with 4 μg of p/CAF Ab overnight at 4°C. The immunocomplex was captured by adding 100 μl of protein A-agarose beads at 4°C for 2 h. Precipitates were washed four times with lysis buffer, and after boiling in Western sample buffer, samples were subjected to SDS-PAGE and Western blot analysis.

Data analysis

All of the values in the figures and text are expressed as mean ± SE of n observations. Impacts of cytokine exposure and single drug concentrations were compared using Student's t test with a Mann-Whitney U posttest for unpaired data. Statistical analyses of multiple comparisons were made using the Kruskal-Wallis test, followed by a Dunn's posttest. Differences were deemed significant when $p < 0.05$.

Results

TNF-α and the direct PKC activator PMA promote CCL11 release from HASM cells

We investigated the role of PKC in the induction of CCL11, a chemokine induced by several cytokines in HASM cells (22, 29–31). TNF-α increased CCL11 release from HASM cells in a concentration-dependent manner (Fig. 1*A*; $n = 3$). PMA, a classical and novel PKC activator, had a similar effect (Fig. 1*B*; $n = 3$).

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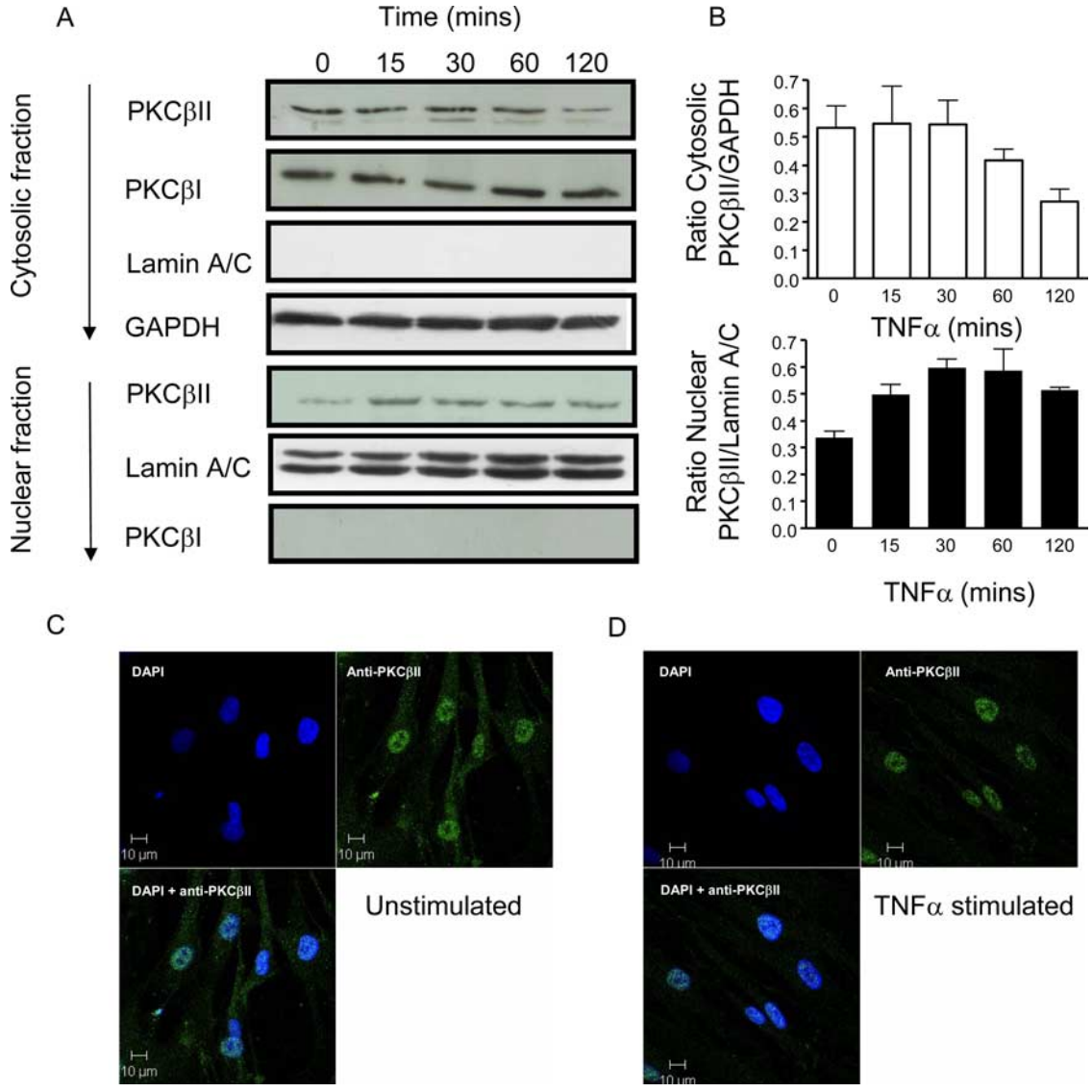

FIGURE 2. TNF-α induces nuclear translocation of PKCβ. Serum-deprived HASM cells were incubated with TNF-α (1 ng/ml) for 0–120 min. Cytosolic and nuclear (*A*) proteins were prepared, and PKC isoform expression was analyzed by Western blot using isoform-specific Abs. Nuclear lamin A/C staining shows lack of cytosolic contamination in the nucleus. Densitometry has been performed, and cytosolic PKCβII was normalized to GAPDH in *B* (*upper panel*) and lamin A/C for the nuclear fraction (*B*, *lower panel*). Blots are representative of three experiments. *C* and *D*, Immunofluorescence confocal microscopy of PKCβII nuclear translocation. HASM cells were treated with an anti-PKCβII Ab for localization of PKCβII (green fluorescence). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (blue fluorescence), and slides were visualized using confocal microscopy. Cells were stimulated with TNF-α for 30 min (10 ng/ml). Merged images of the green and blue fluorescence are also shown (*lower panels*). The images are representative of three independent preparations.

PKC isozymes are implicated in TNF-α-induced CCL11 release from HASM cells

We next determined whether PMA- and TNF-α-induced CCL11 release involved was PKC dependent. The broad spectrum PKC inhibitor Bis I (10^{-6} M) and the classical PKC inhibitor Go6976 (10^{-6} M) inhibited TNF-α-stimulated CCL11 release in a concentration-dependent manner. The PKC-δ-selective inhibitor rottlerin (10^{-6} M; $n = 3$) had no effect (Fig. 1*C*). Similar inhibition was seen in PMA-stimulated cells (Fig. 1*D*).

These data suggest that a classical isoform (PKCα, βI, βII, or γ) regulates TNF-α-induced CCL11 release. Upon further investigation, we found that the selective PKCβ inhibitor LY333531 (32) mimicked the effect of Bis I and Go6976 (Fig. 1, *C* and *D*), suggesting PKCβ involvement. None of the agents used inhibited p38 or ERK activation (data not shown).

TNF-α induces nuclear translocation of PKCβII

To investigate TNF-α-induced PKC activation, we used a nonradioactive fluorescent peptide substrate. PKC activation was increased 4-fold by TNF-α (data not shown). Given the pharmacological data indicating a role for PKCβ, we next determined whether TNF-α induces PKCβI and PKCβII translocation to the nucleus. PKCβI was only found in the cytoplasmic fraction (which includes the cell membrane portion) of resting or TNF-α-stimulated cells. However, PKCβII was detected in both the cytosol/cell membrane fraction and the nucleus, with TNF-α treatment resulting in an increased nuclear level determined by Western blot (Fig. 2*A*). Fig. 2*B* shows densitometry from cytosolic fractions probed for PKCβII and normalized to GAPDH, and nuclear fraction probed for PKCβII and the nuclear marker protein lamin A/C. Fig. 2, *C* and *D*, demonstrates

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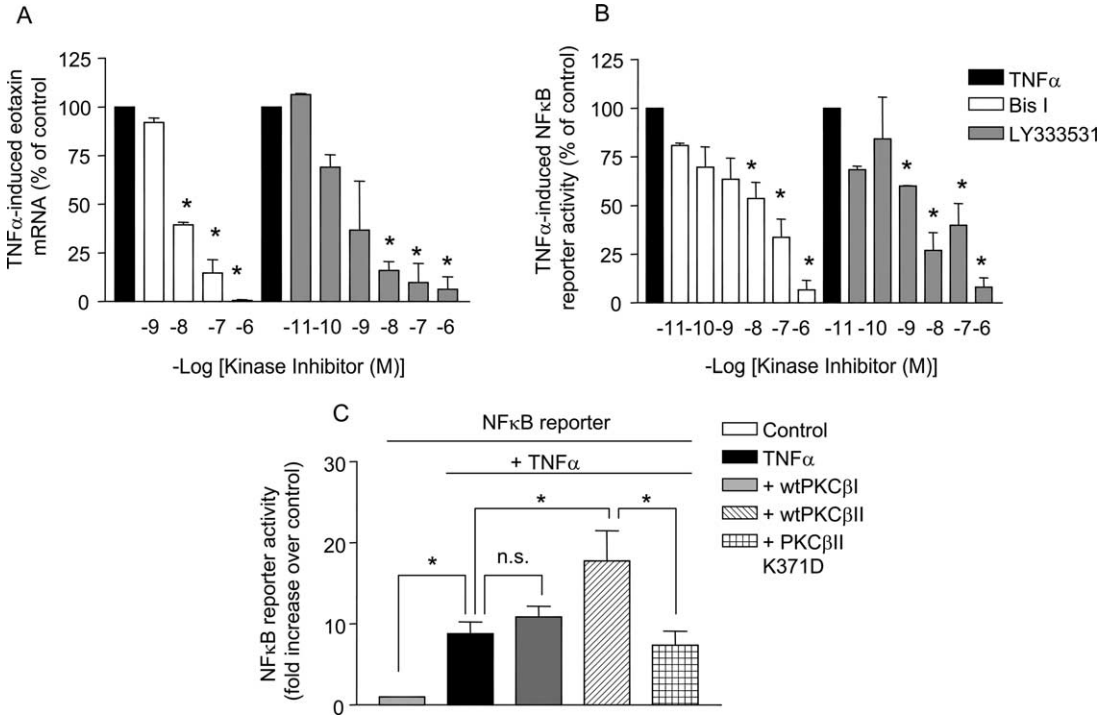

FIGURE 3. PKCβ transcriptionally regulates CCL11 gene expression and NF-κB reporter activity. *A*, HASM cells were pretreated (30 min) with PKC inhibitors before TNF-α (6 h). β_2-Microglobulin and CCL11 mRNA were measured by quantitative real-time RT-PCR. Results are expressed as percentage inhibition from TNF-α-stimulated cells. Each point represents the mean ± SEM of three determinations using cells from different donors. *B* and *C*, HASM cells were transiently transfected with the NF-κB reporter (0.2 μg/well) either alone (*B*) or in the presence of PKC overexpression constructs (0.5 μg/well; *C*). In the case of *B*, cells were pretreated with inhibitors (30 min) before TNF-α (1 ng/ml) for 6 h. Firefly and *Renilla* luciferase activity was measured, and results were normalized to *Renilla*. The figure represents the mean and SEM of three to four experiments performed in sixes. A p value of less than 0.05 was deemed to be significant, and indicated with ∗.

confocal microscopy performed after TNF-α stimulation (Fig. 2, *C* and *D*).

PKCβ acts transcriptionally to inhibit TNF-α-induced CCL11 mRNA production and NF-κB promoter activity in HASM cells

Our previous studies have shown that NF-κB is the key transcription factor mediating TNF-α-induced CCL11 gene transcription in HASM cells with no contribution from STAT6 or AP-1 (9). We therefore determined whether PKCβ regulated CCL11 transcriptionally via NF-κB.

TNF-α increased CCL11 mRNA 5.6 ± 1.7-fold. This increase was concentration dependently inhibited by the broad spectrum inhibitor Bis I (Fig. 3*A*; $-\log EC_{50}$, 8.2 ± 0.2; $n = 4$) and the PKCβ selective inhibitor LY333531 (Fig. 3*A*; $-\log EC_{50}$, 9.2 ± 0.3; $n = 4$).

TNF-α stimulated NF-κB-dependent reporter luciferase activity time dependently between 1 and 24 h (data not shown). For additional experiments, a 6-h time point was chosen. Bis I and LY333531 concentration dependently inhibited TNF-α-induced NF-κB reporter activity (Fig. 3*B*; $-\log EC_{50}$ values of 7.6 ± 0.2 and 8.4 ± 0.3, respectively; $n = 4$), which suggests that PKCβ acts transcriptionally to regulate NF-κB-mediated CCL11 expression.

PKCβII overexpression augments TNF-α-stimulated NF-κB reporter activity in HASM cells

To further establish the role of PKCβ, we cotransfected HASM cells with the NF-κB reporter and WT PKCβI, PKCβII, and kinase-dead PKCβII (PKCβII K371D) constructs, before stimulation with TNF-α (1 ng/ml) for 6 h. TNF-α significantly increased NF-κB reporter activity compared with control (8.78 ± 1.45-fold increase; raw values, 0.29 ± 0.04 to 2.55 ± 0.45) (Fig. 3*C*). WTPKCβI overexpression had a slight, nonsignificant effect (0.29 ± 0.04 to 3.21 ± 0.49); however, PKCβII overexpression enhanced NF-κB reporter activity significantly 17.78 ± 3.7-fold above control (0.29 ± 0.04 to 4.82 ± 0.65). The kinase-dead mutant PKCβII K371D construct had no effect (0.29 ± 0.04 to 2.31 ± 0.58; Fig. 3*C*). These data further support a role for PKCβII in NF-κB activation and NF-κB-mediated CCL11 release.

TNF-α-induced CCL11 release is abolished in PKCβ KO mice and abrogated in WT cells after PKCβ inhibition

We next studied MEF cells from PKCβI and PKCβII (PKCβ KO) KO and WT mice (20). This approach is technically less demanding than knocking down target genes in primary cells. Western blots confirmed PKCβII's presence in WT, but not PKCβ KO MEFs (Fig. 4*A*).

Cells from WT mice released low basal CCL11 levels. TNF-α (10 ng/ml for 24 h) increased CCL11 4-fold (40.3 ± 4.5 to 176.6 ± 11.4 pg/ml). MEFs from PKCβ KO mice released undetectable CCL11 basally or after TNF-α (Fig. 4*B*). Additionally, use of the pharmacological inhibitors of PKCβ (Bis I and LY333531 both at 10^{-6} M) resulted in abrogation of TNF-α-induced CCL11 release from the WT MEFs (Fig. 4*C*). The regulation of CCL11 at the mRNA level was also regulated by PKC in WT MEFs. TNF-α induced an 88.7 ± 14.6-fold increase in CCL11 expressed, which was inhibited after treatment with Bis I and LY333531 (both at 10^{-6} M; 38.8 ± 23.9 and 0.7 ± 0.03, respectively; Fig. 4*D*).

PKCβ KO cells have reduced NF-κB activation

MEFs from WT and PKCβ KO mice were transiently transfected with the NF-κB reporter. TNF-α (10 ng/ml)-induced NF-κB reporter activity was reduced from 6.28 ± 1.66-fold over control

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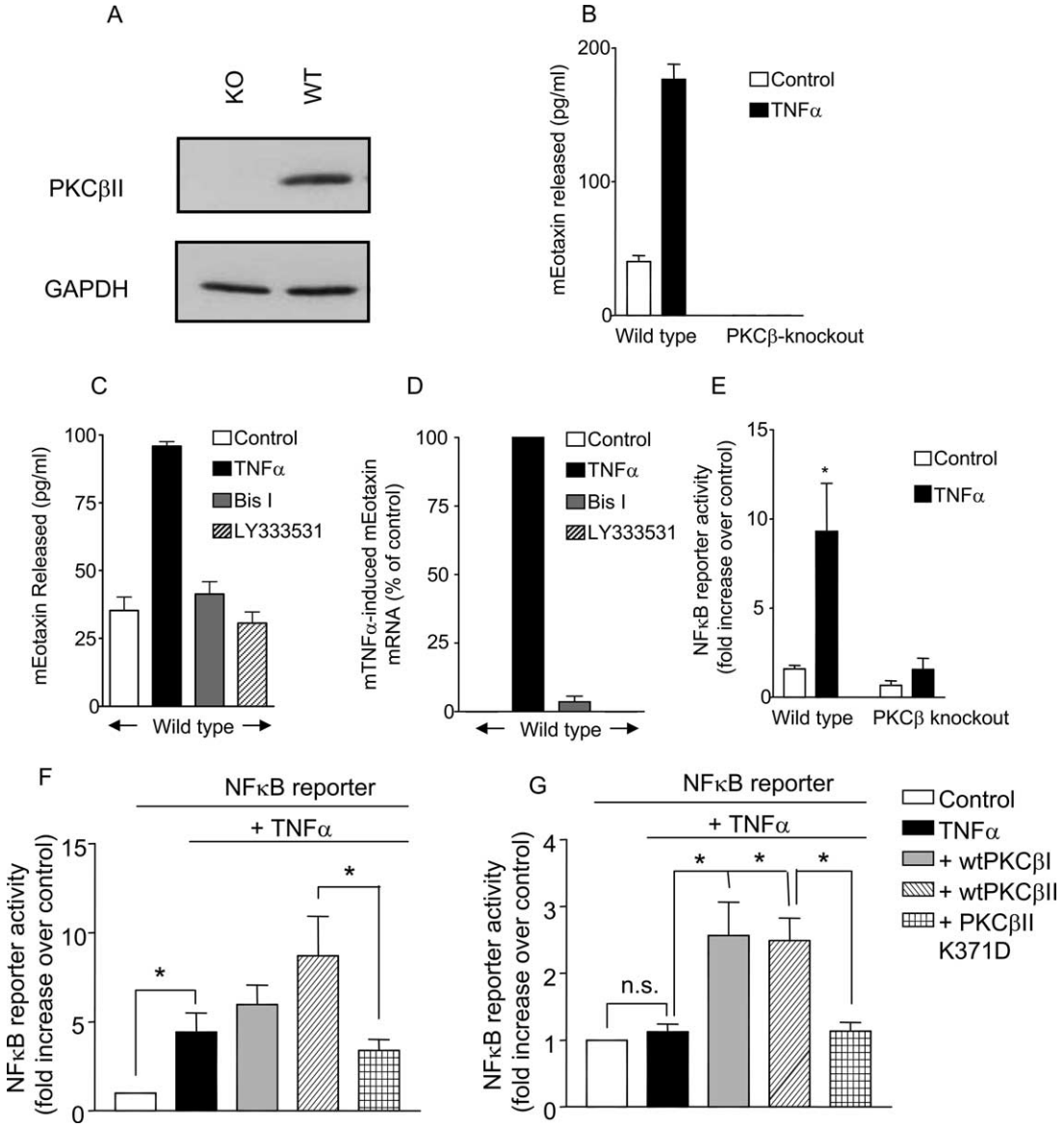

FIGURE 4. TNF-α-induced CCL11 release and NF-κB reporter activity is abolished in PKCβ KO mice, and restored with PKCβII overexpression. MEFs from WT and PKCβ KO mice were cultured to confluence, growth arrested for 24 h, and either protein extracted to assess expression of PKCβII by Western blot (*A*) or stimulated with murine TNF-α alone (*B*) or in the absence and presence of Bis I (10^{-6} M) or LY333531 (10^{-6} M) for 24 h (*C*) for cytokine release and 6-h (*D*) mRNA expression studies. *B*, Murine TNF-α-induced CCL11 release was quantified by ELISA, and murine eotaxin mRNA expression was analyzed by real-time PCR (*D*). Each point represents the mean ± SEM of three independent observations. *E*, WT and PKCβ KO MEFs were transiently transfected for 24 h with a NF-κB reporter (0.5 μg/well) and stimulated for 6 h with murine TNF-α. WT (*F*) and PKCβ KO (*G*) cells were transiently transfected with NF-κB (0.2 μg/well) in the absence and presence of PKC overexpression constructs (0.5 μg/well) before murine TNF-α for 6 h. Firefly luciferase values were normalized to *Renilla*. Results are the mean and SEM of three to four experiments. A p value of less than 0.05 was deemed to be significant, and indicated with ∗.

(1.59 ± 0.19 to 9.31 ± 2.68; Fig. 4*D*) in WT MEFs to a 2.54 ± 0.63-fold increase (0.67 ± 0.24 to 1.55 ± 0.64; Fig. 4*D*) in PKCβ KO MEFs.

PKCβII overexpression augments TNF-α-stimulated NF-κB reporter activity in WT MEFs and re-establishes TNF-α responses in PKCβ KO cells

WT PKCβI overexpression had little effect on TNF-α-induced NF-κB reporter activity in WT MEFs (4.44 ± 1.06-fold increase with TNF-α alone, increased to 5.98 ± 1.08-fold increase in the presence of WT PKCβI; $n = 3$; Fig. 4*F*). WT PKCβII overexpression augmented the response (4.44 ± 1.06 to 8.69 ± 2.14-fold increase over control; $n = 3$), although this effect did not reach statistical significance. The observed effect was dependent on kinase activity because it was not seen with the PKCβII K371D construct (raw values: control, 1.98 ± 0.47; TNF-α, 6.48 ± 1.18; TNF-α plus WT PKCβI, 8.77 ± 1.06; TNF-α plus WT PKCβII, 12.44 ± 2.51; TNF-α plus PKCβII K371D, 5.48 ± 1.31; Fig. 4*F*).

Interestingly, in PKCβ KO MEFs, TNF-α did not increase NF-κB reporter activity (Fig. 4*G*), but overexpression of either WT PKCβI or WT PKCβII, but not PKCβII K371D, re-established the TNF-α response (raw values: control, 0.59 ± 0.12; TNF-α, 0.62 ± 0.09; TNF-α plus WT PKCβI, 1.36 ± 0.22; TNF-α plus WT PKCβII, 1.38 ± 0.18; TNF-α plus PKCβII K371D, 0.65 ± 0.11; Fig. 4*G*). Whereas PKCβI overexpression augmented NF-κB reporter activity in PKCβ KO MEFs, it had no

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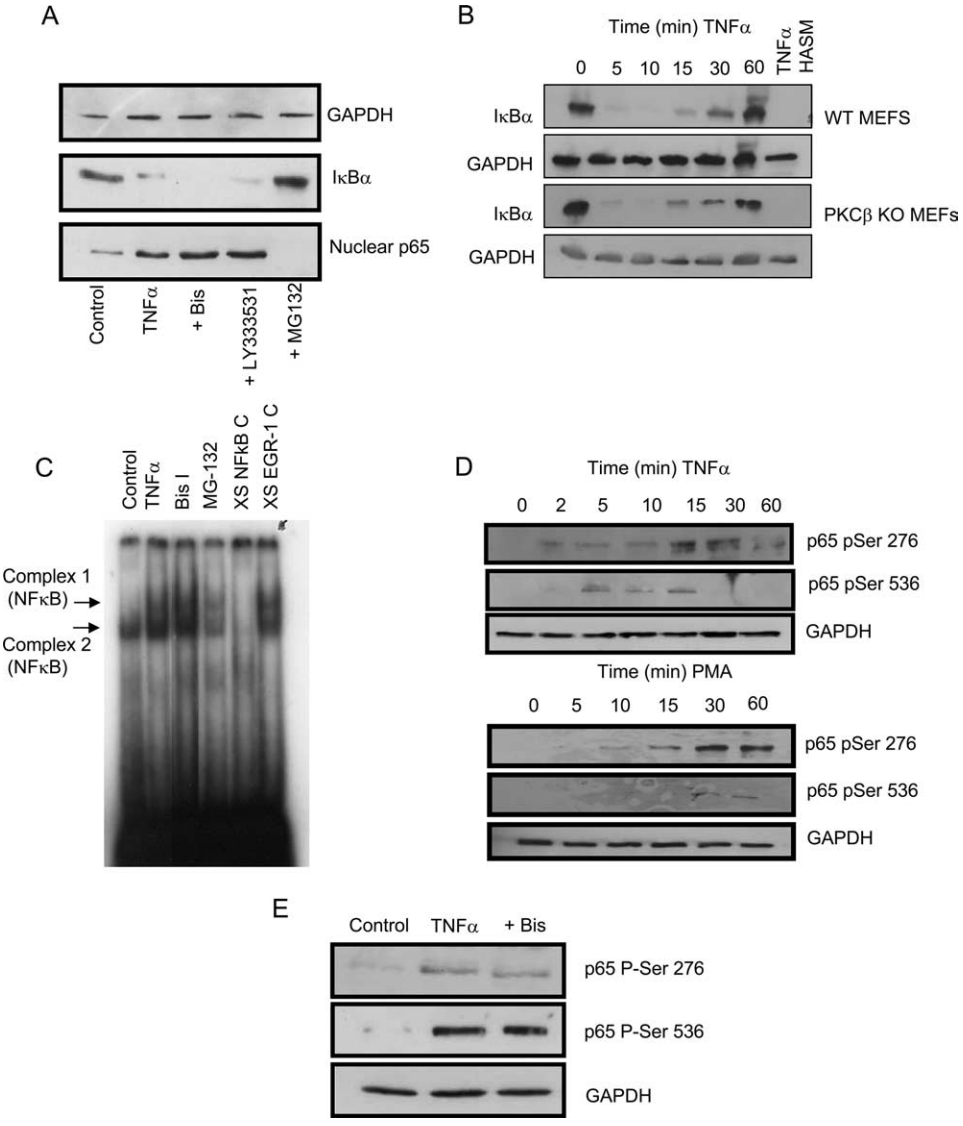

FIGURE 5. PKC isoforms do not affect IκBα degradation, p65 translocation, phosphorylation, or NF-κB DNA binding in vitro. HASM cells either were treated with TNF-α alone or pretreated with Bis I (10^{-6} M), LY333531 (10^{-6} M), or MG-132 (3×10^{-5} M) for 30 min before TNF-α (1 ng/ml) for 10 min (*A* and *E*), 1 h (*C*), or times indicated in the figure. Additionally, WT and PKCβKO MEFs were treated with murine TNF-α for the times indicated in *B*. Cytosolic and nuclear proteins were prepared and separated on a 7.5% SDS-PAGE gel, transferred to nitrocellulose, and probed for IκBα (*A* and *B*), GAPDH (*A*, *B*, *D*, *E*), p65 (*A*), or phosphorylated p65 at Ser^{276} and Ser^{536} (*D* and *E*), or subject to NF-κB DNA binding analyzed by EMSA (*C*). Binding was specific, as shown by competitive binding (*C*). Nuclear extracts from TNF-α-treated cells were incubated with labeled NF-κB oligonucleotides (hot NF-κB) in the presence of a 50-fold excess of unlabelled NF-κB oligonucleotides (cold NF-κB) or unlabelled EGR-1 (cold EGR-1). The figures are representative of three experiments. SDS-PAGE and gel shift assays are representative of three experiments in three different donors.

effect on TNF-α-induced NF-κB reporter activity in WT MEFs (Fig. 4*F*), or primary HASM cells (Fig. 3*C*).

PKCβ signaling is not involved in p65 translocation, in vitro NF-κB binding, nor p65 phosphorylation

Collectively, the studies in HASM and MEFs strongly implicate PKCβ in TNF-α-induced NF-κB activation and CCL11 production. We then investigated the effects of PKC inhibitors on several aspects of NF-κB function, i.e., p65 translocation, in vitro NF-κB binding by EMSA, and p65 phosphorylation. None of these processes was altered by the PKC inhibitors (Fig. 5).

TNF-α-induced p65 nuclear translocation

TNF-α-induced cytoplasmic IκBα degradation and p65 nuclear translocation in HASM cells were unaffected by Bis I or LY333531. MG-132 (proteasome inhibitor), a positive control, inhibited both IκBα degradation and p65 nuclear translocation (Fig. 5*A*). These experiments were performed at multiple time points to confirm these results (15, 30, 60, and 120 min; data not shown). Similar results were seen in PKCβ WT and KO MEFs (Fig. 5*B*).

TNF-α-induced NF-κB binding in vitro

TNF-α induced NF-κB binding using probes to consensus NF-κB oligonucleotides in EMSAs, which were not affected by Bis I (Fig. 5*C*). Competition studies using 50-fold excess unlabeled NF-κB oligonucleotides blocked transcription factor binding, whereas excess early growth response-1 (EGR-1) oligonucleotides did not block NF-κB transcription factor binding, demonstrating that binding was specific (Fig. 5*C*). Previously, we have identified the NF-κB-DNA complex by supershift assay using specific Abs (9).

TNF-α-induced p65 phosphorylation

TNF-α induced phosphorylation of Ser^{276} (Fig. 5*D*) and Ser^{536} (Fig. 5*D*), but not Ser^{468} (data not shown), using Western blotting Abs directed against Ser^{276}, Ser^{468}, and Ser^{536}. PMA also phosphorylated p65 at Ser^{276} and Ser^{536} (Fig. 5*D*). However, Bis I had no effect on TNF-α-induced phosphorylation of Ser^{536} or Ser^{276} (Fig. 5*E*).

Overexpression of p300 and p/CAF augments TNF-α-induced NF-κB transcription

We then postulated that rather than being a direct effect on NF-κB, PKCβ may have an indirect effect by modifying the chromatin environment that regulates NF-κB access to the promoter, specifically by regulating coactivator HATs. Several cofactors with HAT activity can associate with NF-κB, including CBP, p300, and p/CAF (4, 33, 34). We first confirmed a p65 and CBP interaction in HASM cells by overexpressing CBP with the NF-κB reporter,

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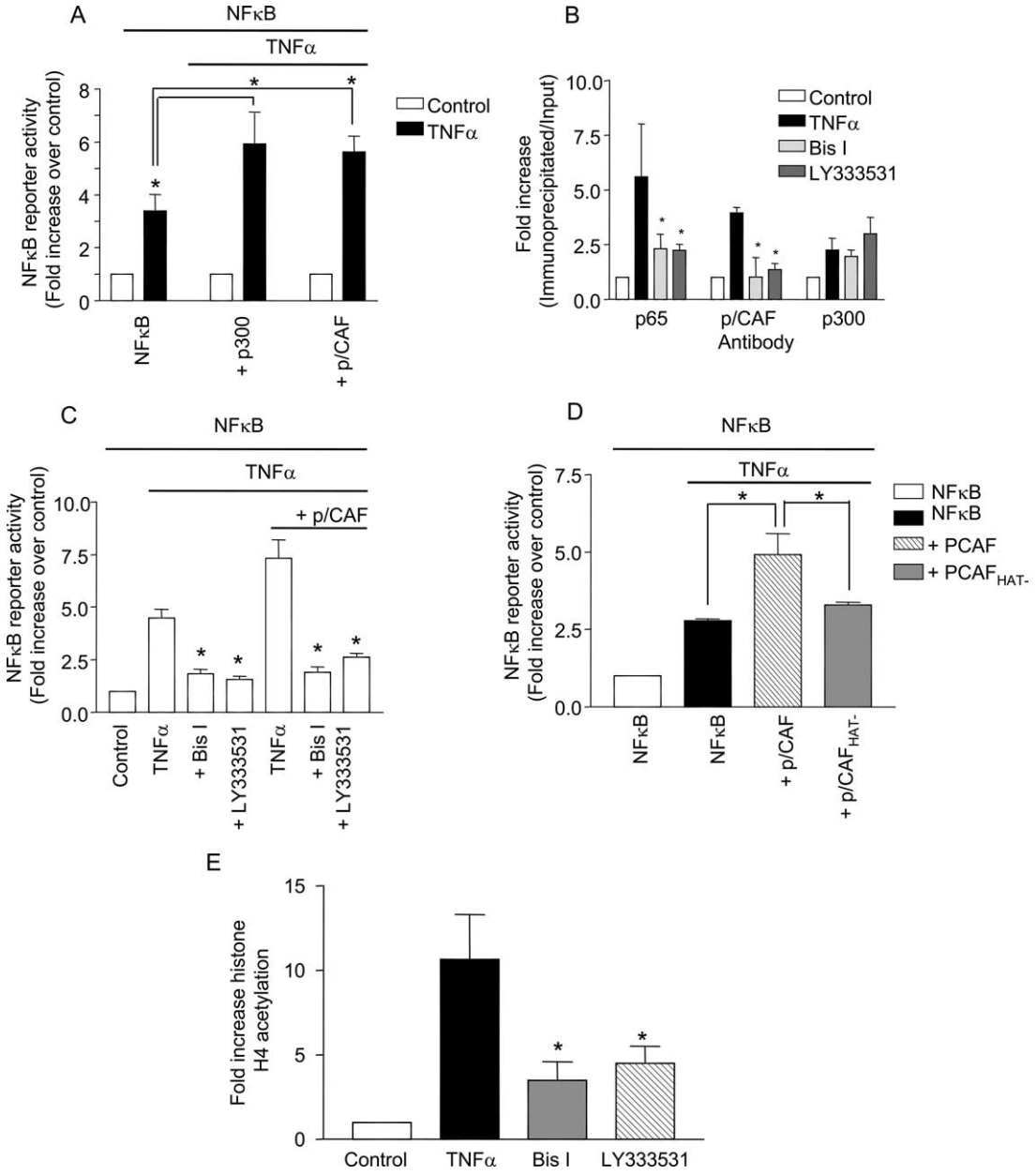

FIGURE 6. p/CAF overexpression augments TNF-α-induced NF-κB transcription and promotes p/CAF binding to the CCL11 promoter in vivo via a PKCβ- and HAT-dependent mechanism. *A*, *C*, and *D*, HASM cells (50–60% confluent) were cotransfected with 0.2 μg/well NF-κB reporter construct, 0.4 ng/well *Renilla* luciferase, and 0.05 μg/well p300 (*A*), p/CAF (*A*, *C*, and *D*), or p/CAF$_{HAT}^{-}$ (*D*) using FuGene 6 for 24 h. Cells were then stimulated with TNF-α (1 ng/ml) for 6 h. In *C*, cells were pretreated with Bis I or LY333531 (10^{-6} M; 30 min). Data represent $n = 3$ independent experiments performed in sixes. *B*, *D*, and *E*, HASM cells in T 75-cm^2 flasks were pretreated with Bis I and LY333531 (10^{-6} M) before TNF-α (1 ng/ml) for 1 h. The in vivo p65, p300, and p/CAF binding to the CCL11 promoter and histone H4 acetylation at the CCL11 promoter was analyzed by ChIP assay with specific anti-p65, anti-p300 anti-p/CAF, and anti-histone H4 acetylation Abs. The real-time PCR primers amplified the –136 to +61 region of the CCL11 promoter. Results were normalized by dividing the mean of the triplicate CCL11 result by the mean of the corresponding input result, and are expressed as a fold increase over unstimulated cells. Data represent $n = 4$ independent experiments performed in duplicate. A p value of less than 0.05 was deemed to be significant, and indicated with ∗.

which augmented the response (data not shown). We next performed experiments examining whether other cofactors such as p300 and p/CAF regulate NF-κB transcription and its binding at the CCL11 promoter.

We cotransfected the cells with p300 and p/CAF together with the NF-κB reporter, and stimulated with TNF-α. NF-κB reporter activity was increased 3.4 ± 0.6-fold with TNF-α stimulation, as previously shown (raw firefly/*Renilla* values, 5.7 ± 2.1 to 21.9 ± 11.3) (Fig. 6*A*). When cells were cotransfected with p300 or p/CAF, TNF-α-induced NF-κB reporter activity was increased to 5.9 ± 1.1 (raw values, 1.5 ± 0.5 to 10.6 ± 4.2) and 5.6 ± 0.6-fold, respectively (raw values, 1.9 ± 0.5 to 10.9 ± 3.6).

PKCβ is involved in the in vivo recruitment of p/CAF to the CCL11 promoter

ChIP assay showed a time-dependent increase of TNF-α-induced in vivo p65 association with the CCL11 promoter DNA in HASM cells, as previously reported (9). At 1 h, p65, p300, and p/CAF recruitment to the CCL11 promoter was increased 5.6 ± 2.4-fold,

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3.9 ± 0.3-fold, and 2.3 ± 0.5-fold, respectively (raw immunoprecipitated/input values: p65 from 0.7 ± 0.3 to 4.7 ± 3.3; p300 from 0.8 ± 0.4 to 2.0 ± 1.4; p/CAF from 0.5 ± 0.3 to 2.2 ± 1.2; Fig. 6*B*). Control experiments were performed where no Ab was added to the reaction, and no significant effects were observed of TNF-α on p65, p300, or p/CAF recruitment to the CCL11 promoter, as expected.

Both Bis I and LY333531 inhibited TNF-α-induced recruitment of p/CAF to the CCL11 promoter (raw values: from 2.2 ± 1.2 to 0.3 ± 0.2 for Bis I, and 0.8 ± 0.5 for LY333531), but had no effect on p300 (Fig. 6*B*). This was associated with inhibition of p65 recruitment to the CCL11 promoter (from 4.7 ± 3.3 to 1.8 ± 1.1 for Bis I, and 1.68 ± 0.8 with LY333531; Fig. 6*B*). These results indicate that the inhibitory effect of PKCβ on CCL11 release is dependent on chromatin given the earlier EMSA results showing a lack of effect using Bis I on p65 binding in vitro, in the absence of chromatin. These data suggest that PKCβ plays a role in the recruitment of p/CAF to the CCL11 promoter.

p/CAF augments TNF-α-induced NF-κB transcription via PKCβ

To confirm the involvement of PKCβ in p/CAF recruitment to the CCL11 promoter, we overexpressed p/CAF with the NF-κB reporter in the absence and presence of Bis I. TNF-α augmented NF-κB activity by 4.48 ± 0.42-fold. This was inhibited to 1.84 ± 0.2-fold increase with Bis I and 1.57 ± 0.15 with LY333531. Cotransfection with p/CAF augmented the TNF-α response to 7.32 ± 0.89-fold above control values. Pretreatment of cells cotransfected with p/CAF/NF-κB with Bis I, and LY333531 reduced levels to near basal (1.91 ± 0.26 and 2.63 ± 0.18, respectively; Fig. 6*C*).

p/CAF's effect is dependent on its HAT activity

To determine whether HAT activity was required for p/CAF's effect, we transiently transfected a mutant p/CAF lacking HAT activity (35) into HASM cells with the NF-κB reporter and stimulated with TNF-α. TNF-α stimulated NF-κB reporter activity 2.77 ± 0.06-fold above control levels (raw values from 2.7 ± 0.3 to 7.6 ± 0.6). Cotransfection with p/CAF augmented this to 4.92 ± 0.68 (raw value, 12.5 ± 2.2). In contrast, transfection with the HAT-mutated p/CAF construct showed no augmentation (3.29 ± 0.09-fold above control; raw value, 7.96 ± 0.04; Fig. 6*D*).

TNF-α signals through PKCβ to alter histone H4 acetylation at the CCL11 promoter

Because these data suggest that the effect of p/CAF is HAT dependent, we next measured TNF-α effects on histone H4 acetylation at the CCL11 promoter in HASM cells using an Ab directed against acetylated histone H4 by ChIP assay. TNF-α increased histone H4 acetylation 10.67 ± 2.63-fold at 1 h in keeping with previous reports (raw immunoprecipitated/input values: control, 0.1 ± 0.04; TNF-α, 1.0 ± 0.2). Bis I inhibited this to 3.5 ± 1.1-fold above basal (0.4 ± 0.3 immunoprecipitated/input value), and LY333531 reduced this to 4.5 ± 1.0-fold above basal (0.4 ± 0.1 immunoprecipitated/input) (Fig. 6*E*).

These data suggest that PKCβ increases CCL11 transcription by recruiting p/CAF and increasing histone H4 acetylation at the promoter.

PKCβII phosphorylates p/CAF in vitro, and PKCβII and p/CAF colocalize

To assess whether PKCβII can directly phosphorylate p/CAF, an in vitro kinase assay was performed. PKCβII phosphorylated purified p/CAF in vitro, depicted by a band at 19 kDa (Fig. 7*A*). Histone H1, a known PKC target (36), was included as a positive control (32-kDa band) (Fig. 7*A*).

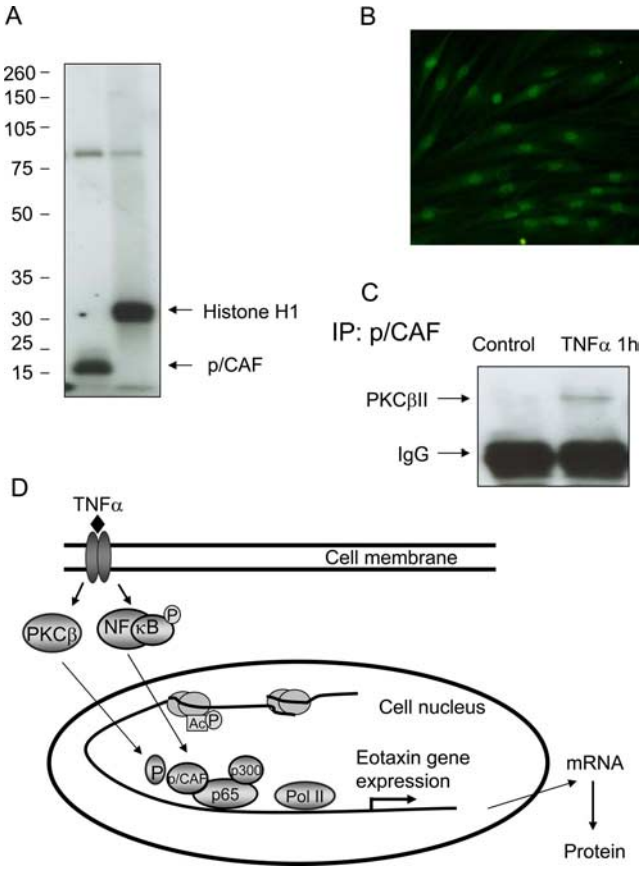

FIGURE 7. PKCβ phosphorylates p/CAF in vitro, and p/CAF and PKCβ colocalize in HASM cells. Model for PKCβ function in TNF-α-mediated CCL11 production from HASM cells. *A*, Protein kinase reactions were initiated by adding 4 μg of p/CAF or histone H1 and kinase buffer containing 250 μM ATP and 5 μCi of [γ-^{32}P]ATP, using purified PKCβII. Reactions were incubated for 20 min at 30°C and terminated by adding an equal volume of Western sample buffer. Samples were boiled for 5 min, and proteins were resolved by SDS-PAGE. Gels were dried under vacuum, and phosphorylated proteins were visualized by autoradiography. *B*, Confocal microscopy showing p/CAF expression in HASM cells. *C*, Cells were stimulated for 1 h with TNF-α (1 ng/ml) and protein extracted, and cells were precleared for 1 h at 4°C with 100 μl of protein A-agarose. Immunoprecipitation was performed with 4 μg of p/CAF Ab overnight at 4°C, and the immunocomplex was captured by adding 100 μl of protein A-agarose beads at 4°C for 2 h. Precipitates were washed four times with lysis buffer, boiled in Western sample buffer, and subjected to SDS-PAGE and Western blot analysis. Blot is representative of two independent experiments in cells from different donors. *D*, In response to TNF-α, PKCβ is activated and translocates to the nucleus, whereby it activates the binding of the cofactor p/CAF to the CCL11 promoter, an effect dependent on its HAT activity and resultant histone H4 acetylation. This switches on NF-κB (p65)-mediated CCL11 transcription.

TNF-α caused PKCβII translocation to the nucleus (Fig. 2), where p/CAF is localized (Fig. 7*B*). To determine whether PKCβ and p/CAF colocalize, native coimmunoprecipitations were performed whereby p/CAF was immunoprecipitated and the precipitates were probed for PKCβII. A PKCβII band was visualized from HASM cells treated with TNF-α, but not unstimulated cells (Fig. 7*C*). These data support the existence of a signal-induced interaction between PKCβ and p/CAF.

Discussion

This study demonstrates a novel role for PKCβII in regulating NF-κB-mediated gene transcription (Fig. 7*D*). This effect was mediated by PKCβII translocation to the cell nucleus; association

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with the cofactor p/CAF, which in turn promotes downstream acetylation of histone H4 at the CCL11 promoter; and promotion of gene transcription. We studied NF-κB gene regulation with particular emphasis on CCL11 because this is an important chemokine relevant to asthma and other allergic disease (37), which we have shown previously is primarily NF-κB dependent under TNF-α-stimulated conditions (9). We used HASM cells because they are a good model system for studying inflammatory gene transcription in primary human cells, and used embryonic fibroblasts from genetically modified mice to allow us to fully characterize the mechanism of the effect.

Our initial studies looking at PKC isoform activation and translocation, the effects of PMA, and pharmacological PKCβ inhibitors strongly suggested that PKCβ was playing a key role in TNF-α-stimulated CCL11 production in primary HASM cells, and we went on to confirm this using suitable molecular tools. Studies using NF-κB reporter constructs indicated that PKCβ was involved in the transcriptional control of CCL11, which we have shown previously is driven by NF-κB (9). Overexpression of PKCβII, but not its kinase-dead mutant or PKCβI, augmented the NF-κB reporter activity. These data suggest that PKCβII is the isoform involved and that its kinase activity was responsible for the effect. We then characterized the molecular mechanism whereby PKCβ regulates NF-κB activity. Previous studies have shown that NF-κB-mediated transcription can be regulated at several levels. Early studies focused on phosphorylation, ubiquitination, and proteasome-mediated degradation of IκBα and resultant NF-κB translocation (18, 19). We clearly showed that whereas IκBα and resultant NF-κB translocation occurred in response to TNF-α, these were not regulated by PKCβ, because PKCβ inhibition had no effect on NF-κB translocation. Another level of control is by modification of NF-κB by phosphorylation (38, 39). In our studies, although we were able to demonstrate that TNF-α induced phosphorylation of Ser^{276} and Ser^{536}, again PKCβ inhibition had no effect on this mechanism.

The other major control point of NF-κB transcription is by covalent modification of the chromatin environment, which regulates its access to gene promoters. This control is achieved by recruitment of protein complexes that alter chromatin structure via enzymatic modifications of histone tails and/or nucleosome remodelling. NF-κB activation requires several cofactor HATs, including CBP, p300, p/CAF, and SRC-1, of which p/CAF appeared to be relatively more important (4). Consistent with this, we demonstrate that in HASM cells, overexpression of p300 and p/CAF enhances TNF-α-induced NF-κB activity. Furthermore, the binding of p/CAF, but not p300, to the CCL11 promoter was dependent on PKCβ. The action of p/CAF was dependent on its HAT activity, because its enhancing effect on NF-κB reporter activity was not seen with a HAT-defective mutant. Furthermore, ChIP studies showed that p/CAF association with the CCL11 promoter was associated with acetylation of histone H4.

Although no previous studies have looked at p300 and p/CAF binding at the CCL11 promoter, these cofactors augment NF-κB-dependent transcription of other genes such as COX-2 (13, 14). Our data suggest that the recruitment of p/CAF to the CCL11 promoter alters the chromatin environment via its intrinsic HAT activity, opening up the DNA and allowing transcription factors to bind and promote gene transcription. In resting cells, DNA is packed as chromatin. The fundamental subunit of chromatin is the nucleosome, which consists of 146 bp of DNA wrapped twice around an octamer core of four histones (two molecules each of histones H2A, H2B, H3, and H4) (40). This nucleosome prevents accession of transcription factors and RNA polymerase II to their respective recognition sequences and the initiation of transcription (41). One of the chromatin modifications associated with inflammatory gene transcription is histone 4 acetylation on the highly conserved lysines at the N-terminal tail, and we have previously demonstrated that TNF-α increases histone H4 acetylation at the CCL11 promoter, but did not identify the HATs responsible (9). The current studies suggest a key role for p/CAF.

Having provided data in primary human cells implicating PKCβII in regulating TNF-α-induced NF-κB activity and CCL11 production, we used WT and PKCβ KO MEFs to obtain further proof. These studies showed a striking abolition of CCL11 production in response to TNF-α in KO cells compared with WT, accompanied by a marked reduction in the activity of a transiently transfected NF-κB reporter construct. Furthermore, NF-κB reporter activity was restored with PKCβII overexpression. Overexpression of PKCβI also partially restored activity in KO MEFs; however, our studies in HASM cells suggested that PKCβI was not involved, because its overexpression did not augment NF-κB activity in these cells. Furthermore, PKCβI did not translocate to the nucleus in HASM cells. Finally, we determined whether p/CAF was a substrate for PKCβII in vitro and whether these molecules interacted in vivo. In vitro phosphorylation assays confirmed that p/CAF was phosphorylated by PKCβII in vitro, and coimmunoprecipitation experiments provided evidence of their interaction in vivo. These findings are strengthened by the ChIP studies that show that PKCβ inhibition prevented the binding of p/CAF to the CCL11 promoter in vivo.

p/CAF is a coactivator molecule with intrinsic HAT activity involved in the reversible acetylation of various transcriptional regulators, and is implicated in many cellular processes, such as transcription, differentiation, proliferation, and apoptosis (42, 43). Acetylation of core histones, thereby reducing their charge, allows the chromatin structure to transform from the resting closed conformation to an activated open form (43), thus allowing binding of TATA box-binding protein, TATA box-binding protein-associated factors, and, finally, RNA polymerase II, which initiates gene transcription (6). This molecular mechanism is probably common to all genes, including those involved in differentiation, proliferation, and activation of cells. p/CAF is regulated/recruited to gene promoters under diabetic conditions (13) and after LPS, PMA, and IL-1β stimulation (14). p/CAF can be acetylated by homeodomain-interacting protein-kinase 2, a nuclear serine/threonine kinase, resulting in the induction of p53-dependent apoptosis (44). In addition, p/CAF holds intrinsic ubiquitination activity, which controls the stability of the oncoprotein Hdm2 (45). We have demonstrated that PKCβII is a key signaling kinase involved in the regulation of p/CAF recruitment; hence, pharmacological manipulation may be of therapeutic benefit.

There has been some previous interest in the role of PKC isoforms in NF-κB-dependent transcription. For example, PKCζ has been demonstrated to directly activate IKKβ in vitro, suggesting that it may function as an IKK (46) and PKCζ phosphorylates RelA/p65 (47, 48). One study has shown an association between PKC and p/CAF via the phosphorylation and activation of the global gene regulator SATB1, although the exact isoform was not determined (49). This may be a potential mechanism whereby PKCβII can selectively target p/CAF to the CCL11 promoter.

Our present experiments show that inhibition of PKCβ abrogates TNF-α-induced NF-κB promoter activity in transient transfection. Although the actual chromatin status of the transfected promoter is unclear, it is likely to be a loose and disorganized structure and, therefore, is generally more open and accessible than repressed cellular chromatin structure of endogenous gene promoters (50). In this sense, it is unlikely that histone acetylation is involved in the effects of the drugs. There is good evidence that

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HAT and HDAC regulate NF-κB-dependent gene expression not only through histone acetylation and deacetylation, but also through site-specific acetylation and deacetylation of NF-κB p65 (51, 52). Acetylation of p65 lysine 221 enhances DNA binding and impairs IκBα assembly, whereas acetylation of lysine 310 is required for full transcriptional activity of NF-κB in the absence of effects on DNA and IκBα binding (53, 54). HDAC inhibitors such as trichostatin and sodium butyrate have been shown to potentiate TNF-α-induced expression of several natural NF-κB-driven promoters, such as CXCL-8, IL-6, and ICAM-1, in transient assay (55), and β_2-agonists and steroids have been shown to affect CCL11 promoter activity (9), strongly suggesting that HAT and HDAC are involved in the transcriptional activity of transiently transfected promoters, probably through the acetylation and deacetylation of NF-κB rather than histones. Because we have demonstrated in this study that PKC inhibitors inhibit histone H4 acetylation, it is reasonable to speculate that they may also inhibit NF-κB acetylation, leading to the inhibition on TNF-α-induced promoter activity in transient assay, whereas the inhibition of acetylation of both NF-κB and histone H4 may account for their inhibition of eotaxin gene transcription in vivo. However, other effects cannot be excluded, and further studies are required to understand the precise mechanisms. However, our studies and others (9) clearly show that important transcriptional mechanisms cannot be identified using in vitro methods such as EMSA alone. ChIP offers the advantage of studying gene transcription in vivo with the chromatin environment still intact.

PKCβII is one of the classic Ca^{2+}-dependent and phorbol ester-responsive PKC isoforms. Because TNF-α treatment can increase myocyte activation due to altered Ca^{2+} influx pathways (56), this is the likely mechanism of PKCβII activation. Consistent with this, we found that TNF-α-induced CCL11 release could be inhibited by the Ca^{2+} chelator BAPTA-AM (data not shown).

The PKCβ inhibitor LY333531 (ruboxistaurin) is currently being trialed in the clinic for the treatment of diabetic retinopathy. Ruboxistaurin is orally bioavailable, and in controlled, randomized clinical trials, ruboxistaurin had an adverse event profile comparable to placebo, and was well tolerated (57, 58). Our studies suggest it may have a role in the treatment of chronic inflammatory disorders, particularly those associated with eosinophil influx.

In conclusion, we have described a novel mechanism whereby PKCβII amplifies NF-κB-mediated transcriptional activation of an inflammatory gene (CCL11) by translocating to the nucleus and activating the binding of the cofactor p/CAF. This effect of p/CAF was dependent on its HAT activity and resultant acetylation of histone H4. It will be interesting in future studies to characterize the phosphorylation sites. We have begun to determine whether this effect regulates other inflammatory response genes. Our preliminary data on CXCL-10, another NF-κB-related chemokine, show a similar inhibitory effect of PKCβ inhibition, although there appears to be little effect on the regulation of CXCL-8, which can be regulated by AP-1 and NF-IL-6 (Fig. 4). These studies provide important new insight into NF-κB signaling.

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Disclosures

The authors have no financial conflict of interest.

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