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Protein Kinase C-ζ Regulates Transcription of the Matrix Metalloproteinase-9 Gene Induced by IL-1 and TNF-α in Glioma Cells via NF-κB*

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The regulation of matrix metalloproteinase-9 (MMP-9) expression in glioma cells is one of the key processes in tumor invasion through the brain extracellular matrix. Although some studies have demonstrated the implication of classic protein kinase C (PKC) isoforms in the regulation of MMP-9 production by phorbol esters or lipopolysaccharide, the involvement of specific PKC isoforms in the signaling pathways leading to MMP-9 expression by inflammatory cytokines remains unclear. Here we report that the atypical PKC-ζ isoform participates in the induction of MMP-9 expression by interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) in rat C6 glioma cells. Indeed, zymography and semi-quantitative reverse transcriptase-PCR analysis showed that pretreatment of C6 cells with PKC-ζ pseudosubstrate abolished MMP-9 activity and gene expression induced by IL-1 or TNF-α. Accordingly, IL-1 and TNF-α were able to induce PKC-ζ activity, as demonstrated by *in vitro* kinase assay using immunoprecipitated PKC-ζ. Furthermore, stable C6 clones overexpressing PKC-ζ, but not PKC-ε, displayed an up-regulation of MMP-9 constitutive expression as well as an increase of *mmp-9* promoter activity. These processes were inhibited by an NF-κB-blocking peptide and completely prevented by NF-κB-binding site mutation in the *mmp-9* promoter. Taken together, these results indicate that PKC-ζ plays a key role in the regulation of MMP-9 expression in C6 glioma cells through NF-κB.

Glioma cells have the ability to invade brain tissues by secreting matrix metalloproteinases (MMPs),[1] a family of proteases able to degrade different components of the extracellular matrix including collagen, fibronectin, and proteoglycans. One of these MMPs, MMP-9, has received much attention as its expression correlates with the progression of glioma (1). Furthermore, MMP-9 seems to be essential for the invasiveness of glioma cells, as it was recently reported that the inhibition of MMP-9 expression by antisense gene transfer strongly reduced the invasion of glioblastoma cells *in vitro* and *in vivo* (2). Therefore, understanding the role of the molecules implicated in the signaling pathways leading to *mmp-9* gene expression in glioma cells is important in order to identify new therapeutic targets.

Several studies (3–5) have focused on the implication of protein kinase C (PKCs) in the regulation of *mmp-9* gene expression, most notably by testing the effect of phorbol 12-myristate 13-acetate (PMA) on different types of cells, including human glioma cells. Members of the PKC family are divided into the following three groups of isoenzymes: the conventional PKC isoforms, which are activated by calcium and diacylglycerol (α, βI, βII, and γ); the novel PKCs, which are activated by diacylglycerol but are calcium-insensitive (δ, ε, η, and θ); and the atypical PKCs, which are calcium- and diacylglycerol-insensitive (ζ and ι/λ). Despite the fact that a large number of studies (5–7) have established a link between PKCs and MMP-9 expression using PKC inhibitors, very few studies have addressed the implication of specific PKC isoenzymes in the regulation of MMP-9. Although our group (8) has shown that a dominant-negative form of PKC-α potentiated the secretion of MMP-9 induced by lipopolysaccharide in the mouse macrophage cell line RAW 264.7, others (9) have shown that PKC-β isoform is implicated in PMA-induced *mmp-9* gene expression in human HL-60 myeloid leukemia cells. However, the involvement of specific PKC isoforms in the signaling pathways leading to MMP-9 expression by inflammatory cytokines remains unclear. In a previous study (10), however, we reported that the inflammatory cytokines, interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) but not PMA, were both able to induce MMP-9 expression in the rat C6 glioma cells, raising the possibility that atypical, PMA-independent PKC isoenzymes could be involved. Among the atypical PKC isoforms, PKC-ζ was of potential interest as its activation could be induced by IL-1 and TNF-α (11, 12). Moreover, PKC-ζ plays a critical role in the regulation of gene transcription via nuclear factor-κB (NF-κB) (13), a transcriptional factor required for *mmp-9* gene expression (14).

In this work, we show that induction of MMP-9 expression by IL-1 or TNF-α in C6 glioma cells is inhibited by pretreatment with a PKC-ζ-specific inhibitory peptide (PKC-ζ PS) and that both IL-1 and TNF-α induce the activation of PKC-ζ in C6 cells.

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[1] The abbreviations used are: MMPs, matrix metalloproteinases; PKC, protein kinase C; IL-1, interleukin-1; TNF-α, tumor necrosis factor-α; PKC-ζ PS, PKC-ζ pseudosubstrate; PMA, phorbol 12-myristate 13-acetate; NF-κB, nuclear factor-κB; PMSF, phenylmethylsulfonyl fluoride; AP-1, activating protein-1; PI-3 kinase, phosphatidylinositol 3-kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT, reverse transcriptase; wt, wild type.

Furthermore, stable C6 transfectants overexpressing PKC-ζ isoenzyme, but not clones overexpressing the PKC-ε isoenzyme, display a constitutive expression of MMP-9 at the mRNA and protein levels. Transient transfection experiments using *mmp-9* promoter constructs not only confirmed that PKC-ζ exerts its effect by increasing its transcriptional activity but also implicated NF-κB as a key regulator of MMP-9 expression by PKC-ζ.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Reagents used were obtained from Sigma unless otherwise indicated. SN50 peptide inhibitor of NF-κB translocation and SN50M control peptide were obtained from Calbiochem. The myristoylated PKC-ζ pseudosubstrate peptide was obtained from Quality Controlled Biochemicals. Mouse recombinant IL-1 and TNF-α were purchased from Genzyme. The rabbit polyclonal anti-PKC-ζ antibody (Santa Cruz Biotechnology) or a mouse monoclonal anti-PKC-ε antibody (Transduction Laboratories) was used for Western blot analysis as well as sheep anti-rabbit or anti-mouse antibody conjugated to horseradish peroxidase (ICN Pharmaceuticals).

Cell Lines—The rat C6 glioma cell line was obtained from the American type Culture Collection (ATCC). The cells and stable transfectants were grown in Ham's F-10 medium supplemented with 15% (v/v) horse serum, 2.5% fetal bovine serum, and 10 mM HEPES buffer (complete medium). All tissue culture reagents were purchased from Invitrogen. Routine testing showed the cells to be free of mycoplasma. For stimulation assays with mouse recombinant IL-1α or TNF-α, cells were trypsinized with a solution of 1 mM EDTA, 0.25% (w/v) trypsin, seeded at a density of 10^6 cells/ml in 12-well cluster plates, and incubated for 18 h at 37 °C in 5% CO_2. Monolayers were then washed three times with phosphate-buffered saline and fresh serum-free medium containing the appropriate cytokine added at the indicated concentration. In some experiments, C6 cells or transfectants overexpressing PKC-ζ were pre-incubated for 1 h with a myristoylated PKC-ζ pseudosubstrate or the NF κB inhibitory peptide before IL-1 or TNF-α treatment. Assays using 3,-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were performed to ensure that treatments with these peptides had no effect on cell viability. Unless otherwise indicated, supernatants were harvested after 18 h of stimulation and stored at −20 °C until assayed.

cDNAs and Expression Vectors—Wild type human PKC-ε (15) and wild type mouse PKC-ζ cDNAs (16) were obtained from the ATCC. Both cDNAs were cloned into the *Eco*RI site of the expression vector pCIN-4 (17), and the resulting constructs were designated pCIN-PKC-ε and pCIN-PKC-ζ, respectively.

Transfection—To obtain C6 stable transfectants constitutively overexpressing PKC-ε or PKC-ζ isoforms, transfection was carried out by electroporation with pCIN-PKC-ε or pCIN-PKC-ζ plasmids. Controls were generated using C6 cells transfected with the empty pCIN-4 vector alone. Electroporation was performed using the following parameters: 10 μg of linearized DNA per 4×10^6 cells in Ham's F-10 on ice; 960 microfarads; 300 V/0.4 cm. After 48 h of culture in complete medium, transfected cells were allowed to grow in complete medium containing 400 μg/ml of geneticin (Invitrogen) before individual colonies were picked and expanded into cell lines. Individual clones were then tested for expression of PKC-ε or PKC-ζ by Western blot analysis using antibodies as described below.

Western Blot Analysis—Adherent cells were washed once with phosphate-buffered saline and homogenized in lysis buffer (50 mM Tris-HCl, pH 7.4, 1 mM EGTA, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM NaF, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM Na_3VO_4, and 10% glycerol) containing a mixture of protease inhibitors (Complete™ tablets from Roche Molecular Biochemicals). Protein concentrations were determined using the Bradford assay. Total protein (50 μg) were electrophoresed through a 10% SDS-polyacrylamide gel and were electrotransferred onto a nitrocellulose membrane (Amersham Biosciences), and blots were incubated with a rabbit polyclonal anti-PKC-ζ antibody or with a mouse monoclonal anti-PKC-ε antibody. Sheep anti-rabbit or anti-mouse antibody conjugated to horseradish peroxidase were used as secondary antibodies. The bands were visualized by the ECL system (Amersham Biosciences).

Zymography—Zymography was performed in 10% polyacrylamide gels that had been cast in the presence of gelatin as described previously (10). Briefly, samples (100 μl) were lyophilized, resuspended in loading buffer, and without prior denaturation were run on a 7.5% SDS-polyacrylamide gel containing 0.5 mg/ml gelatin. After electrophoresis, gels were washed to remove SDS and incubated for 18 h at 37 °C in a renaturing buffer (50 mM Tris, 5 mM $CaCl_2$, 0.02% NaN_3, 1% Triton X-100). Gels were subsequently stained with Coomassie Brilliant Blue G-250 and destained in 30% methanol, 10% acetic acid (v/v) to detect gelatinase secretion. Gelatinase activity was measured in arbitrary units by quantitative analysis of negatively stained bands through computerized image analysis (Bio-Rad, model GS-670 densitometer).

RNA Isolation and RT-PCR Analysis—Isolation of total cellular RNA was performed using the Trizol reagent (Invitrogen) according to the manufacturer's instructions. Aliquots of 2 μg of total cellular RNA were used for first strand cDNA synthesis in 20 μl of reaction volume using 100 units of Superscript™ II reverse transcriptase (Invitrogen). Primer pairs for rat MMP-9, mouse β-actin, and GAPDH-specific amplification of cDNA (PCR Core Kit, Roche Molecular Biochemicals) were as follows: (5′) primer 5′-TCCCTCTGAATAAAGTCGACA-3′ and (3′) primer 5′-AGGTGA ACAAGGTGGACCATG-3′ for MMP-9; (5′) primer 5′-CATGGATGACGATATCGCTGCGC-3′ and (3′) primer 5′-GCTGTCGCCACGCTCGGTCAGGATC-3′ for mouse β-actin; (5′) primer 5′-CGGAGTCAACGGATTTGGTCGTAT-3′ and (3′) primer 5′-AGCCTTCTCCATGGTGGTGAA GAC-3′ for GAPDH. The lengths of the MMP-9, β-actin, and GAPDH amplicons were 848, 575, and 306 bp, respectively. PCR amplifications were performed on a MJ Research Thermal Cycler (model PTC-100™) using the following program: step 1, 94 °C for 1 min; step 2, 58 °C for 2 min; step 3, 72 °C for 3 min. Thirty cycles were performed for the amplification of MMP-9 and 20 cycles for β-actin or GAPDH. The amplification for each gene was in the linear curve. PCR products were visualized on 1.5% agarose gels stained by ethidium bromide and UV transillumination. Semiquantitative analysis was conducted using a computerized densitometric imager to obtain MMP-9/GAPDH or MMP-9/β-actin ratios.

Measure of PKC-ζ Activity—Measurement of PKC-ζ activity was performed essentially as described previously (18). Briefly, after stimulation of C6 cells by IL-1 or TNF-α for 10 min, cells were incubated for 30 min at 4 °C in lysis buffer containing 20 mM Tris-HCl, pH 7.5, 0.25 M sucrose, 1.2 mM EGTA, 20 mM β-mercaptoethanol, 1 mM PMSF, 1 mM Na_3VO_4, 1 mM $Na_4P_2O_7$, 1 mM NaF, protease inhibitors (Roche Molecular Biochemicals), 1% Triton X-100, 0.5% Nonidet P-40, and 150 mM NaCl. Aliquots of 500 μg of proteins were then incubated overnight at 4 °C with a PKC-ζ/λ-specific rabbit polyclonal antibody (Santa Cruz Biotechnology), and immunoprecipitates were collected on protein G-Sepharose beads (Amersham Biosciences). The beads were washed three times by low speed centrifugation with the kinase buffer (50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 100 μM Na_3VO_4, 100 μM $Na_4P_2O_7$, 1 mM NaF, and 100 μM PMSF). The immunoprecipitated PKC-ζ was resuspended in 50 μl of kinase buffer, and kinase assay was performed by adding 4 μg of phosphatidylserine, 50 μM ATP, 3 μCi of [γ-^{32}P]ATP (ICN Pharmaceuticals), and 40 μM of ε-peptide (Calbiochem-Novabiochem) for 10 min at 30 °C. Blank values were determined from incubations conducted in the presence of 100 μM PKC-ζ pseudosubstrate and were subtracted from total kinase activity to determine PKC-ζ-specific activity. The reaction was stopped by adding 100 μl of SDS buffer. Samples were boiled for 5 min and separated by SDS-PAGE using an 18% polyacrylamide gel. Dried gels were exposed to x-ray films (Konica Medical Film) at −80 °C. Quantitative measurements of phosphorylation of the ε-peptide was obtained by densitometry through computerized image analysis.

Cloning of mmp-9 Promoter and Transient Transfections—A 737-bp fragment of the 5′-flanking region of the *mmp-9* gene (−681 to +63), containing two Sp1 and one AP-1 and NF-κB-binding sites (all elements necessary for *mmp-9* promoter activity) (14), was obtained by PCR amplification using stringent conditions on a genomic DNA isolated from 164T2 murine lymphoma cells (19), using the following primers: (5′) primer 5′-AGGAAGGATAGTGCTAGCCTGAGAAGGATG-3′ and (3′) primer 5′-CCGAAACTCGAGGAGAGCCAGGAGCAGGG-3′. After sequencing (GenBank™ accession number AF403768), this fragment was cut by *Nhe*I and *Xho*I and subsequently subcloned into pGL-III Basic vector encoding for firefly luciferase (Promega) to generate pGL-MMP-9wt (wild type). Creation of a double-point mutation into the NF-κB-binding site (GGAATTCCCCC to GGAATTGGCCC) to generate pGL-MMP-9 ΔNF-κB was performed using the following (forward) primer: 5′-GGGTTGCCCCGTGGAATTGGCCCAAATCCTGC-3′. The mutant was generated using the Quick Change Site-directed Mutagenesis Kit (Stratagene). Transient transfections were performed using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's instructions. Transfection efficiency was monitored by co-transfection with 0.5 μg of the pSV/β-gal plasmid encoding for β-galactosidase (Promega). Forty eight hours post-transfection, luciferase activity was

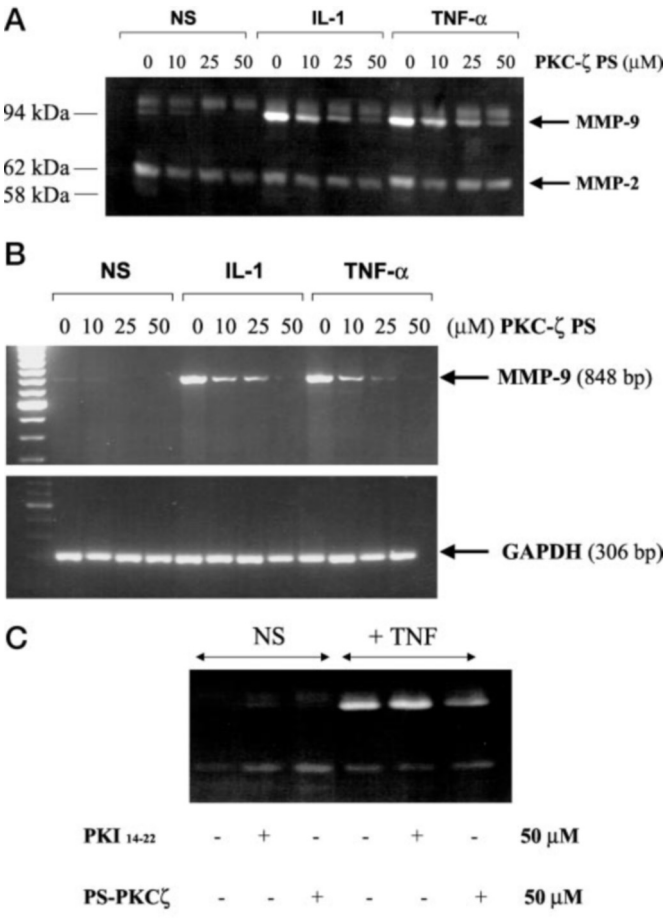

FIG. 1. **Effect of a PKC-ζ pseudosubstrate on the induction of MMP-9 activity and mRNA expression by IL-1 or TNF-α in C6 cells.** Rat C6 cells were treated with the PKC-ζ pseudosubstrate (*PKC-ζ PS*) at the indicated doses for 1 h and either stimulated or not stimulated (*NS*) with murine IL-1 (100 units/ml) or murine TNF-α (100 units/ml). After 18 h, supernatants were collected, lyophilized, and assayed for their gelatinase content by zymography (*A*). Molecular masses (kDa) appear on the *left*. At 10 h, total RNA was extracted. and RT-PCR analysis using MMP-9 and GAPDH-specific primers was performed as described under "Experimental Procedures" (*B*). Molecular weight markers are 100-bp ladder. Results are representative of three independent experiments. *C*, C6 cells were treated at the indicated dose of PKC-ζ pseudosubstrate (*PKC-ζ PS*) or the control peptide (protein kinase inhibitor (PKI_{14-22})) for 1 h before the stimulation with or without (*NS*) murine TNF-α (100 units/ml). After 18 h, supernatants were collected, lyophilized, and assayed for their gelatinase content by zymography. Results are representative of two independent experiments.

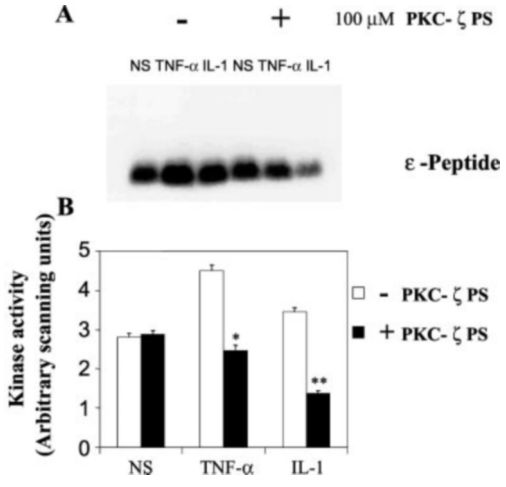

FIG. 2. **Induction of PKC-ζ activity by IL-1 and TNF-α.** *A*, rat C6 cells were either stimulated for 10 min or not stimulated (*NS*) with murine IL-1 (100 units/ml) or murine TNF-α (100 units/ml). After immunoprecipitation of total cell lysates with a PKC-ζ/λ antibody, an *in vitro* kinase assay was performed in the absence (−) or in presence (+) of PKC-ζ PS (100 μM) as indicated under "Experimental Procedures." Phosphorylation of ε-peptide was detected by autoradiography. *B*, quantitative analyses of ε-peptide phosphorylation were performed by imaging densitometry. The *histogram* represents the means of two independent experiments as shown in *A*. Statistical analysis were carried out using Student's *t* test for unpaired samples. *, $p < 0.01$; **, $p < 0.02$.

measured using the Luciferase Assay System protocol (Promega) and a luminometer (Lumat LB 9507, Berthold). The β-galactosidase activity was detected by a colorimetric enzyme assay using *o*-nitrophenyl-β-D-galactopyranoside as a substrate. The ratio of luciferase activity to β-galactosidase activity in each sample served as a measure of normalized luciferase activity.

RESULTS

The Induction of MMP-9 Activity and mRNA Expression by IL-1 or TNF-α Is Inhibited by the PKC-ζ Pseudosubstrate in C6 Glioma Cells—We induced the secretion the 94-kDa precursor form of MMP-9 by IL-1 and TNF-α in the culture supernatant of C6 glioma cells and detected it by zymography (Fig. 1*A*) (10). To determine whether the induction of MMP-9 via these cytokines involved PKC-ζ, we attempted such an induction using a PKC-ζ-specific inhibitory peptide. This peptide, corresponding to the pseudosubstrate (PS) motif of atypical PKCs, suppresses PKC-ζ activity (20) by interacting with the substrate-binding pocket in the catalytic domain (21). Moreover, PKC pseudosubstrates, such as PKC-ζ-PS, specifically block PKC activation by inhibiting their phosphorylation (22, 23). Our results showed that preincubation of C6 cells with increasing doses of the peptide strongly inhibited the induction of MMP-9 by both IL-1 and TNF-α (Fig. 1*A*). The same PKC-ζ inhibitory peptide also reduced constitutive levels of MMP-9. Such a marked dose-dependent inhibition of secretion was specific to MMP-9, as the secretion of MMP-2 was not significantly altered by the incubation with the inhibitory peptide. The level of secretion of an unknown 97-kDa band, occasionally detected in C6 cell supernatants, was not affected by cell treatment with cytokines or upon treatment with the PKC-ζ pseudosubstrate.

To determine whether indeed PKC-ζ was involved in the regulation of MMP-9 at the mRNA level, we next carried out a semi-quantitative RT-PCR. As expected, we found that, although the constitutive levels of MMP-9 mRNA in C6 cells were very low, both IL-1 and TNF-α induced a strong up-regulation of the levels of the same mRNA in these cells. Preincubation with the PKC-ζ-specific inhibitory peptide, however, strongly inhibited, in a dose-dependent manner, the induction of MMP-9 mRNA by these cytokines, whereas GAPDH mRNA levels remained unchanged (Fig. 1*B*). Taken together, these data indicate that PKC-ζ may be involved in the induction, by IL-1 and TNF-α, of *mmp-9* gene expression.

IL-1 and TNF-α Induces PKC-ζ Activity in C6 Glioma Cells—The above results were consistent with the idea that IL-1 and TNF-α are functionally linked with the activation of PKC-ζ. To test for this hypothesis, we investigated whether stimulation of C6 cells via these cytokines induced PKC-ζ enzymatic activity. By using *in vitro* kinase assays on PKC-ζ immunoprecipitates obtained from resting or cytokine-stimulated cells, we first found that stimulation of C6 cells with both cytokines increased the kinase activity associated with the PKC-ζ immunoprecipitates (Fig. 2, *A* and *B*). Most importantly, incubation with the PKC-ζ pseudosubstrate inhibited both TNF-α- and IL-1-induced kinase activity by 50 ($p < 0.01$) and 60% ($p < 0.02$) below the control levels, respectively, although it had no effect on the constitutive levels of unstimulated cells, supporting the hypothesis that IL-1

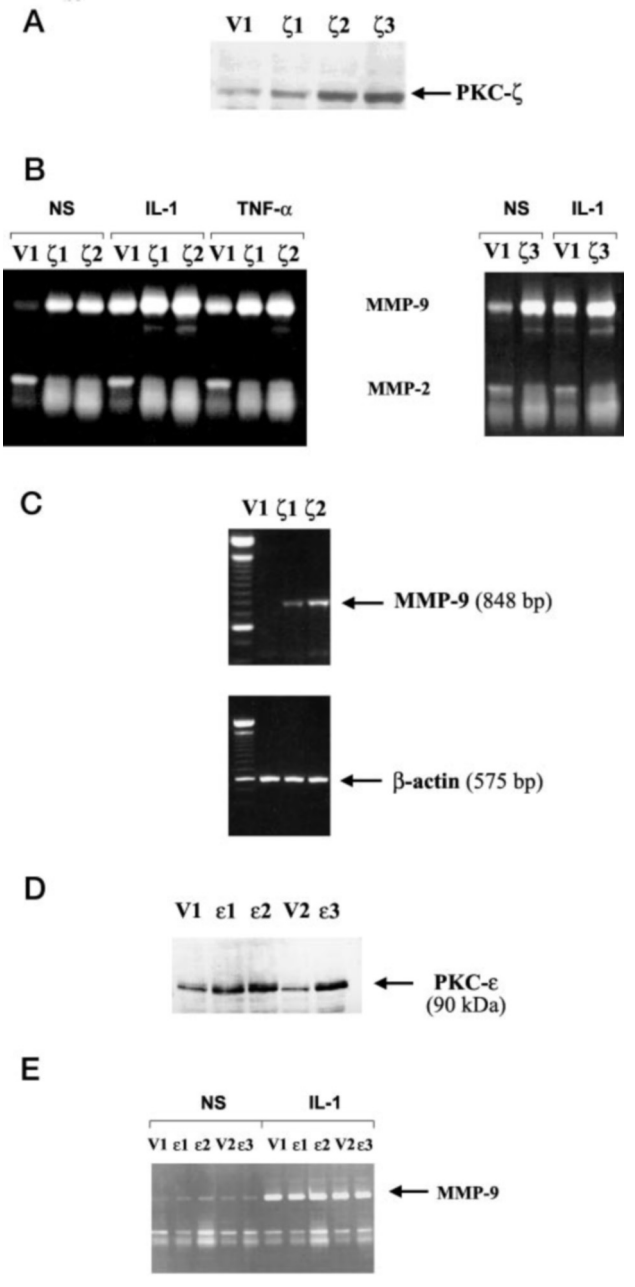

FIG. 3. **Overexpression of PKC-ζ, but not of PKC-ε, induces MMP-9 activity and gene expression.** Stable clones (ζ1, ζ2, and ζ3) overexpressing PKC-ζ (*A*) and PKC-ε (ε1, ε2, and ε3, *D*) were characterized by Western blot analysis compared with C6 cells transfected with the vector alone (V1 for PKC-ζ, and V1, V2 for PKC-ε, respectively). Clones ζ1, ζ2, ζ3 and V1, V2, ε1, ε2, and ε3 were either stimulated or not stimulated (*NS*) with murine IL-1 (100 units/ml) or with murine TNF-α (100 units/ml). After 18 h, supernatants were collected, lyophilized, and assayed for their gelatinase content by zymography (*B* and *E*, respectively). Total RNA isolated from V1, V2, ζ1, and ζ2 clones was assayed for RT-PCR analysis by using MMP-9 and β-actin-specific primers (*C*). Results are representative of three independent experiments.

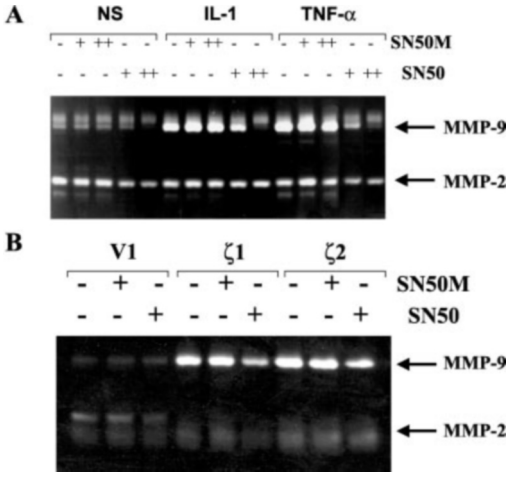

FIG. 4. **MMP-9 induction, either by cytokines or in PKC-ζ clones, is inhibited by the SN50 NF-κB-blocking peptide.** *A*, rat C6 cells were treated with or without (−), 50 (+), or 100 (++) μg/ml of the SN50 or SN50M peptide for 1 h before being stimulated or not (*NS*) by murine IL-1 (100 units/ml) or by murine TNF-α (100 units/ml). After 18 h, supernatants were collected, lyophilized, and assayed for their gelatinase content by zymography. *B*, V1, ζ1, and ζ2 clones were incubated with or without (−) 100 μg/ml of SN50 or of SN50M (+) peptide, and after 18 h, their supernatants were collected, lyophilized, and assayed for their gelatinase content by zymography. Results are representative of three independent experiments.

and TNF-α can both induce PKC-ζ activity.

Overexpression of PKC-ζ, but Not PKC-ε, Up-regulates MMP-9 Expression—As another approach to test the implication of PKC-ζ in the regulation of MMP-9 expression, we transfected C6 cells with a plasmid encoding PKC-ζ, and we selected three independent stable clones (ζ1, ζ2, and ζ3) expressing high levels of this isoform (Fig. 3*A*). Gelatin zymography of conditioned media isolated from clones ζ1, ζ2, and ζ3 showed that overexpression of PKC-ζ in all three clones induced an up-regulation of MMP-9 activity in C6 cells (Fig. 3*B*). No such effect was observed with C6 cells transfected with the empty vector. Overexpression of MMP-9 upon transfection of PKC-ζ was concomitant to the generation of an unidentified 58-kDa gelatinolytic band, corresponding to the activation of the 62-kDa form of MMP-2. MMP-2 was not, however, expressed at the mRNA level in C6 cells or its transfectants (data not shown). Furthermore, the fact that the levels of mRNA in the PKC-ζ transfectants were higher than those found in the control transfectants (Fig. 3*C*) confirmed the idea that the constitutive levels of MMP-9 observed in PKC-ζ transfectants were indeed regulated at the mRNA level.

To determine whether the effect of PKC-ζ on the regulation of MMP-9 was specific, we selected three stable transfectants (ε1, ε2, and ε3) overexpressing PKC-ε (Fig. 3*D*). Two stable clones (V1 and V2) transfected with the empty vector (no cDNA insert) were used as controls. We found that overexpression of PKC-ε had no effect on expression of MMP-9 (Fig. 3*E*), suggesting that the regulation of MMP-9 by PKC-ζ is isoform-specific.

MMP-9 Induction in C6 Cells or in Clones Overexpressing PKC-ζ Is NF-κB-dependent—Up-regulation of *mmp-9* gene expression upon exposure to inflammatory cytokines critically depends on the activation of NF-κB (24, 25). To evaluate the possible implication of this transcription factor in the regulation of MMP-9 by PKC-ζ, we tested the effect of an NF-κB-specific inhibitory peptide, SN50, that blocks its translocation to the nucleus (26). Our results showed that this peptide strongly inhibited, in a dose-dependent manner, the secretion of MMP-9 induced by IL-1 or TNF-α in C6 cells (Fig. 4). In a control experiment, the mutant peptide (SN50M) failed to inhibit the secretion of MMP-9. Furthermore, addition of SN50, but not of the control peptide, decreased the constitutive level of MMP-9 activity in both ζ1 and ζ2 clones (Fig. 4*B*). These results were confirmed by semi-quantitative RT-PCR analysis (data not shown). These data therefore indicate that NF-κB participates in the induction of MMP-9 expression mediated by PKC-ζ.

mmp-9 Promoter Activity Is Up-regulated by PKC-ζ through NF-κB—Finally, to further establish that the up-regulation of

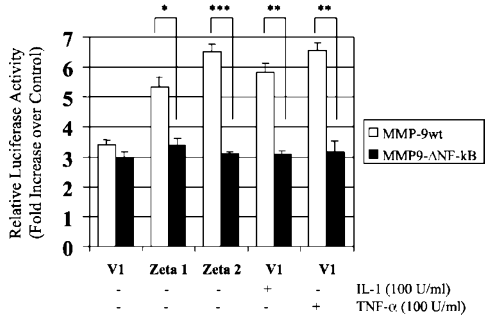

FIG. 5. **Increase of *mmp-9* promoter activity, either by cytokines or in PKC-ζ clones, is inhibited by mutation into the NF-κB-binding site.** V1, ζ1, and ζ2 clones cultured in 6-well plates were cotransfected with 4 μg of pGL-Basic, pGL-MMP-9wt, or pGL-MMP-9-ΔNF-κB, and 0.5 μg of pSV/β-gal. Six hours after transfection, cells were treated with (+) or without (−) 100 units/ml of IL-1 or TNF-α for 48 h. Values of luciferase activities were corrected for transfection efficiencies by assaying for β-galactosidase activity. The *histogram* shows the means ± S.E. of two independent experiments performed in duplicate. Statistical analysis was carried out using Student's *t* test for unpaired samples. *, $p < 0.05$; **; $p < 0.02$, ***; $p < 0.005$.

MMP-9 expression in ζ1 and ζ2 clones depended on its promoter activity through a NF-κB-dependent mechanism, we used reporter gene constructs containing the *mmp-9* promoter harboring a double mutation into the NF-κB-binding site. Transient transfections with pGL-MMP-9wt in V1, ζ1, and ζ2 clones demonstrated a 1.5- and 2-fold increase in MMP-9 transcriptional promoter activity in ζ1 and ζ2 clones, respectively, when compared with the V1 clone (Fig. 5). This induction was completely prevented by the double mutation C → G into the NF-κB-binding site of *mmp-9* promoter. In addition, treatment of V1 clone with IL-1 or TNF-α also induced a 2-fold increase of *mmp-9* promoter activity, which was consistent with the results of Eberhardt *et al.* (24) and was abolished by the mutation into the NF-κB-binding site. Taken together, these data indicate that NF-κB is essential for the up-regulation of *mmp-9* promoter activity by either PKC-ζ or cytokines, IL-1, and TNF-α.

DISCUSSION

In the present study, we have shown that the atypical PKC isoenzyme PKC-ζ plays a critical role in mediating the IL-1- and TNF-α-dependent production of MMP-9 in C6 glioma cells through the downstream activation of NF-κB. Specifically, we showed the following: 1) up-regulation of MMP-9 expression at the mRNA and protein levels by IL-1 and TNF-α is inhibited by a PKC-ζ-specific blocking peptide; 2) both cytokines activates PKC-ζ; 3) overexpression of PKC-ζ, but not of PKC-ε, induces expression of the MMP-9 at the mRNA and protein levels; 4) addition of an NF-κB-blocking peptide, but not of a control peptide, inhibits MMP-9-induced expression in C6 cells or PKC-ζ transfectants; and 5) transient transfection experiments established that the up-regulation of the *mmp-9* promoter activity in cells overexpressing PKC-ζ was completely abolished by mutation in NF-κB-binding site.

In a previous publication (10), we had reported that IL-1 and TNF-α, but not PMA, were able to induce expression of MMP-9 in the rat C6 glioma cells. In the present work, we now identify PKC-ζ, a PMA-insensitive isoenzyme, as a key regulator of *mmp-9* gene expression in C6 cells. Moreover, our data further establish a functional link between PKC-ζ, IL-1, TNF-α, and the activation of NF-κB, a transcription factor necessary for up-regulation of MMP-9 expression (12). This implication of NF-κB and PKC-ζ in the induction of MMP-9 by IL-1 and TNF-α likely results from the activation of IκB kinase by PKC-ζ (27), which in turn induces, upon phosphorylation, the dissociation of the negative regulator IκB-α from NF-κB (28). In addition, PKC-ζ can be involved in the phosphorylation of RelA, a subunit of NF-κB, which leads to an increase of its transcriptional activity (29). In fact, our observation that the induction of MMP-9 by IL-1 or TNF-α was completely abolished by pretreatment with an NF-κB-specific inhibitory peptide, which acts by blocking translocation of NF-κB to the nucleus, strongly supports the idea that MMP-9 induction by these cytokines is mainly NF-κB-dependent. However, this peptide failed to achieve the same level of inhibition with the clones overexpressing PKC-ζ. This could be attributed to the high levels of the activated forms of the isoenzymes. In addition to NF-κB which is necessary but not sufficient to up-regulate MMP-9, the previous demonstration that the activating protein-1 (AP-1) plays an essential role in the transcription of the *mmp-9* gene (12) further supports an association between PKC-ζ and AP-1. Such an increase in the AP-1 binding activity could be the result of an increase of c-*jun* protooncogene mRNA levels, a mechanism previously observed in transfected U937 cells stably overexpressing PKC-ζ (30). Because both AP-1 and NF-κB can be regulated by mitogen-activated protein kinases (31, 32) which can be activated by PKC-ζ (33, 34), it will be of interest to investigate the implication of these molecules in the signaling cascade leading to the up-regulation of MMP-9 in C6 clones overexpressing PKC-ζ. A case in point would be p38, a mitogen-activated protein kinase recently identified as being involved in the regulation of MMP-9 upon stimulation by IL-1 and TNF-α in C6 cells (35).

We found that IL-1 and TNF-α were able to stimulate PKC-ζ activity in C6 glioma cells. Such an activation of PKC-ζ by these cytokines has been reported previously (11, 12) in other cell types, most notably in U937, rhabdomyosarcoma, and bladder-derived carcinoma cells. Among the putative second messengers that could mediate the activation of PKC-ζ upon stimulation with IL-1 and TNF-α are Ras or ceramide (36, 37), both of which having been shown to be potent activator of PKC-ζ. The implication phosphatidylinositol 3-kinase (PI-3K), another activator of PKC-ζ (38), however, is unlikely as treatment of IL-1 or TNF-α-stimulated C6 cells with wortmannin, a PI 3-kinase inhibitor, has been shown to potentiate the induction of MMP-9,[2] suggesting that PI 3-kinase may rather act as a negative regulator of MMP-9. A similar role for this kinase has recently been assigned in the case of the nitric-oxide synthase gene following induction by IL-1 in C6 cells (39), suggesting that both genes are regulated through a common signaling pathway. In support of this notion, C_6-ceramide and $p21^{Ras}$ have been shown to be involved in the induction of nitric-oxide synthase expression by IL-1 or TNF-α through NF-κB in C6 cells and in rat primary astrocytes (40, 41). Further investigations on the early events in the signaling cascade leading the activation of PKC-ζ by IL-1 and TNF-α should clarify this issue.

It is noteworthy that we have observed a low but reproducible decrease of MMP-2 activation upon addition of the SN50 peptide but not the control peptide. Other investigators (42) have also reported SN50 can block activation of MMP-2, most notably in dermal fibroblasts, and attributed its effect to its ability to block the NF-κB signaling pathway that leads to the induction of MT1-MMP. Whether MT1-MMP is implicated in the activation of MMP-2 in glioma cells is under investigation

In conclusion, we showed that PKC-ζ is directly involved in the signaling cascade that controls the transcription of the *mmp-9* gene via the NF-κB-dependent pathway in the C6 glioma cells. Accumulating evidence indicates that MMP-9 contributes not only to tumor invasion but also to the degradation

[2] P. O. Estève and Y. St-Pierre, unpublished observations.

of the blood-brain barrier, to neurodegenerative processes, and to angiogenesis (43–45), three common features associated with glial tumors. *In vivo* studies, using the C6 cells and PKC-ζ-blocking agent, need to be done in order to validate the *in vitro* finding and establish whether PKC antagonists can be envisaged in the therapy of glioma-derived tumors.

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