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Protein Kinase C-ζ Mediates NF-κB Activation in Human Immunodeficiency Virus-Infected Monocytes

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The molecular mechanisms regulating human immunodeficiency virus (HIV) persistence in a major cell reservoir such as the macrophage remain unknown. NF-κB is a transcription factor involved in the regulation of the HIV long terminal repeat and is selectively activated following HIV infection of human macrophages. Although little information as to what signal transduction pathways mediate NF-κB activation in monocytes-macrophages is available, our previous work indicated that classical protein kinase C (PKC) isoenzymes were not involved in the HIV-mediated NF-κB activation. In this study, we have focused on atypical PKC isoenzymes. PKC-ζ belongs to this family and is known to be an important step in NF-κB activation in other cell systems. Immunoblotting experiments with U937 cells demonstrate that PKC-ζ is present in these cells, and its expression can be downmodulated by antisense oligonucleotides (AO). The HIV-mediated NF-κB activation is selectively reduced by AO to PKC-ζ. In addition, cotransfection of a negative dominant molecule of PKC-ζ (PKC-ζ^{mut}) with NF-κB-dependent reporter genes selectively inhibits the HIV- but not phorbol myristate acetate- or lipopolysaccharide-mediated activation of NF-κB. That PKC-ζ is specific in regulating NF-κB is concluded from the inability of PKC-ζ^{mut} to interfere with the basal or phorbol myristate acetate-inducible CREB- or AP1-dependent transcriptional activity. Lastly, we demonstrate a selective inhibition of p24 production by HIV-infected human macrophages when treated with AO to PKC-ζ. Altogether, these results suggest that atypical PKC isoenzymes, including PKC-ζ, participate in the signal transduction pathways by which HIV infection results in the activation of NF-κB in human monocytic cells and macrophages.

Persistent human immunodeficiency virus (HIV) infection of monocytic cell lines and of human macrophages results in the activation of the transcription factor NF-κB (2, 38, 46, 51, 56). Macrophages are considered to be a major reservoir of persistent HIV replication in infected individuals (19, 20). However, the host cell-virus interaction that regulates viral replication in these cells remains poorly understood. Indirect evidence suggests that virus-induced activation of NF-κB is functionally important. The HIV long terminal repeat (LTR) is regulated by NF-κB alone (41) and in collaboration with other relevant transcription factors such as SP1 (27, 47). HIV provirus deficient in NF-κB DNA binding sites has absent or suboptimal replication in different in vitro models (1, 31, 37, 45, 48), and blocking nuclear translocation of NF-κB or its binding to *cis*-acting sequences results in decreased HIV replication (28, 30, 50). Moreover, Tat, the regulatory viral protein involved in regulating viral replication, interacts directly or indirectly with the LTR NF-κB motif (5, 53, 57). All the above data suggest that HIV infection of macrophages results in the modification of signal transduction pathways and transcription factors to create a favorable environment for its own replication; HIV-induced activation of NF-κB may be one of the mechanisms regulating viral persistence in these host cells.

NF-κB is a heterodimeric protein complex involved in the transcriptional regulation of multiple cellular and viral genes (reviewed in reference 3). One common feature of the Rel family of transcription factors is their anchoring in the cytosol by various inhibitory molecules or IκB (reviewed in reference 35). IκBα, a 35- to 38-kDa protein, anchors the prototype NF-κB (p50/p65) by masking the nuclear localization signal of p65 (4, 18, 25). A large number of stimuli, including soluble cytokines, various second messengers activated in response to an array of cell surface receptors, and viruses, result in post-translational modifications of IκBα and subsequent NF-κB activation (reviewed in references 3 and 35). However, it is still unclear how multiple and unrelated signal transduction pathways target IκBα. Both phosphorylation and subsequent degradation of IκBα following activation of different cells by different stimuli are necessary for the release of NF-κB and its subsequent nuclear translocation (7, 8). In vitro, multiple kinases have been shown to phosphorylate IκBα, including, among others, protein kinase C-α (PKC-α) (22, 54), PKA (22, 54), p38 (24), PKC-ζ-associated kinases (11), and Raf-1 (33). In addition, a variety of other second messengers that function within multiple signal transduction pathways such as $p21^{ras}$, Raf-1, *src*, and PKC-dependent and -independent pathways have been shown to regulate NF-κB activation (9, 15–17, 33, 39).

Human pathogenic viruses other than HIV activate NF-κB. Examples include the Tax protein of human T-lymphotropic virus type 1 and the LMP-1 protein of Epstein-Barr virus (29, 32). Although these two viral proteins target IκBα, it is yet unknown which signal transduction pathway(s) is being activated (26, 55). For HIV, not only are the signal transduction pathways unknown, but in addition what viral protein(s) exerts this action remains to be elucidated. Initial studies by our group demonstrated that HIV infection activates NF-κB in monocytic cells by using classical PKC (cPKC)-independent pathways (46). At least 12 PKC isoforms have been cloned.

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They include the phospholipid-, diacylglycerol-, and Ca^{2+}-dependent PKC or cPKC isoenzymes (α, βI and βII, and γ); the novel PKC (nPKC) isoenzymes (δ, ε, and η), which are Ca^{2+} insensitive; and atypical PKC (aPKC) isoenzymes (ζ), which are insensitive to both Ca^{2+} and diacylglycerol (reviewed in reference 43). Of these PKC isoforms, PKC-ζ clearly participates in the regulation of NF-κB in different cell systems, as demonstrated by the use of antisense oligonucleotides and of negative transdominant molecules that inhibit PKC-ζ function (10, 14, 36). In addition, PKC-ζ is associated with an IκBα kinase activity (36). Although the exact location of PKC-ζ within identified signal transduction pathways is the subject of active study, to date this kinase has been placed downstream of $p21^{ras}$ (12), phosphatidylinositol kinase (PI-3 kinase) (42, 59), phosphatidic acid (34), and ceramide (40). However, the specific substrates and downstream second messengers of PKC-ζ remain unknown.

To continue our investigation of the signal transduction pathways which are upregulated by HIV in monocytic cells and human macrophages to result in the activation of NF-κB and thus test the hypothesis that this virus-induced activation of NF-κB is relevant for its replication and persistence in this host cell, we have focused on the role of aPKC isoenzymes, such as PKC-ζ, in potentially mediating this activation. For this, we have used a model of persistent HIV infection of U937 cells previously shown to contain increased NF-κB activity as a result of posttranslational modifications of IκBα (38). Our studies indicate that PKC-ζ is present in monocytic cells and that its interruption by using different antisense and negative transdominant approaches results in a selective decrease in HIV-induced NF-κB activation and a decrease in HIV replication in human macrophages.

MATERIALS AND METHODS

Cell lines and reagents. U937 cells were purchased from the American Type Culture Collection, Rockville, Md., grown in RPMI supplemented with antibiotics, glutamine, and 5% fetal bovine serum (FBS) (Clontech, Palo Alto, Calif.); and routinely tested to be mycoplasma negative. Phorbol myristate acetate (PMA) was purchased from Sigma Chemicals, St. Louis, Mo., and stored in dimethyl sulfoxide at −20°C. Lipopolysaccharide (LPS) was purchased from GIBCO-BRL, Gaithersburg, Md., and stored at −20°C.

Plasmids, expression vectors, and oligonucleotides. The minimal promoter-reporter construct cona-luc and the three tandem motifs of the HIV LTR NF-κB cloned upstream of cona-luc yielding κB cona-luc, as well as the AP1-luc, CREB-luc, and CMV-luc reporter genes, have been described previously (38). The thymidine kinase–β-galactosidase reporter plasmid was used to normalize for transfection efficacy and was purchased from Clontech. The cytomegalovirus (CMV) expression vector, pcDNA3, referred to in this study as CMV, was purchased from Invitrogen, San Diego, Calif. The PKC-ζ mutant (6, 10) is a kinase-deficient PKC-ζ generated by a substitution of Lys-275 for tryptophan and thus lacks a functional catalytic domain. This gene was cloned downstream of the pcDNA3 to yield CMV–PKC-ζ^{mut}. The phosphorothioate oligonucleotides to PKC-ζ, sense (ATG CCC AGC AGG ACC) and antisense (GGT CCT GCT GGG CAT), have been previously described (13) and are directed to the translation initiation codon. The PKC-γ oligonucleotides are targeted to the translation initiation codon (44), and their sequences are ATG GCT GAC GTT TTC CCG (sense) and CGG GAA AAC GTC AGC CAT (antisense). After purification, oligonucleotides were dissolved in sterile HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)-saline solution, divided into aliquots, and stored at −20°C. For the different experiments, an aliquot was stored at 4°C to avoid cycles of freezing and thawing. Three different batches were used in the different experiments, yielding similar results.

HIV infection and oligonucleotide treatment. Ten million exponentially growing U937 cells were infected as previously described with the LAV_{BRU} strain (38). These cells undergo a phase of cell death at days 7 to 14 postinfection, and the surviving population is refractory to HIV-induced cell death in that a productive persistent infection is established in over 90% of cells as detected by fluorescence-activated cell sorter analysis directed to cytoplasmic p24 (38). U937 cells 21 to 40 days postinfection were used in the experiments. Monocyte-derived human macrophages were generated as previously described (38). Briefly, buffy coats from normal healthy donors from our blood bank were processed through Ficoll-Hypaque gradients. After an extensive washing, 4×10^6 mononuclear cells were placed in 2 ml of RPMI with 10% human sera for 5 days. Thereafter, nonadherent cells were removed by extensive washing and the adherent monocyte-derived macrophages (MDM) were infected with 10^5 cpm of SF_{162} (obtained from the NIH AIDS Research and Reference Reagent Program, Rockville, Md.) in 0.5 ml of RPMI–10% FBS for 18 h. Cell-free virus was then removed by extensive washing, and cells were treated with different oligonucleotides at 1 μM or left untreated and incubated in duplicate wells (per treatment) in 2 ml of RPMI with 10% FBS. Cells were passaged once weekly by removing old culture supernatant and adding RPMI–10% FBS containing or not containing the appropriate oligonucleotide treatment. At each passage, cell supernatant was spun at $1{,}000 \times g$ and the cell-free medium was frozen at −20°C for subsequent p24 determination by using a commercial p24 assay kit (Cellular Products, Buffalo, N.Y.).

Cell transfections. Transfection of U937 cells was performed by using DEAE-dextran as previously described (46). Exponentially growing cells were washed in TS buffer (46) and resuspended at 6×10^6 cells in 600 μl of TS containing the different plasmids and/or oligonucleotides and DEAE-dextran (Pharmacia, Piscataway, N.J.) at a concentration of 0.5 mg/ml for 20 min at 21°C. Cells were then washed in RPMI 1640, and 4×10^6 were resuspended in RPMI 1640–5% FBS at 5×10^5/ml per well in 6-well plates (Costar, Cambridge, Mass.). One additional aliquot of 2×10^6 cells was resuspended in RPMI 1640–5% FBS at 10^6/ml per well in 24-well plates. Stimuli including PMA (20 ng/ml) and LPS (1 μg/ml) were added 36 h after transfection. Twenty-four hours posttransfection, the 2×10^6 aliquot was harvested, washed in phosphate-buffered saline (PBS), and lysed in lysis buffer (Clontech), according to the manufacturer's recommendations to detect β-galactosidase activity. The 4×10^6 aliquot was harvested 48 h posttransfection, washed in PBS, and then lysed in a luciferase lysis buffer according to the manufacturer's recommendations (Promega, Madison, Wis.) to detect luciferase activity. In both aliquots, total protein content was measured by the Bradford technique with the Bio-Rad kit (Bio-Rad, Hercules, Calif.). Transfection results are expressed as relative light units, the ratio of luciferase to β-galactosidase units normalized to total protein. Each set of experiments was done at least three times with different plasmids and oligonucleotide preparations.

Immunoblotting. Forty micrograms of each cell lysate was analyzed by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (SDS–10% PAGE) and then transferred to Immobilon membranes (Millipore, Bedford, Mass.) and immunoblotted with specific antibodies. Anti-PKC isoenzyme antibodies and corresponding peptides were purchased from GIBCO-BRL. Antibody to the p85 subunit of PI-3 kinase was a kind gift from Robert Abraham (Mayo Clinic). Immunoblotting was detected by using the ECL kit (Amersham, Arlington Heights, Ill.).

RESULTS

PKC-ζ is present in U937 cells and is inhibited with specific antisense oligonucleotides. Having previously demonstrated that persistent HIV infection of U937 cells results in the activation of NF-κB in a cPKC-independent fashion (46), we determined whether PKC isoenzymes other than those susceptible to the PKC inhibitor GF (60) that could mediate NF-κB activation were present in these cells. To date, only the aPKC-ζ isoenzyme has been clearly identified to participate in the activation of NF-κB in different cell lines (10, 14, 36). Total cell lysates of U937 cells and rat brain homogenate were analyzed by SDS-PAGE and immunoblotted with polyclonal antibodies directed to different PKC isoenzymes. Within the group of cPKC isoenzymes, only PKC-γ was detected in U937 lysates (Fig. 1, lane 9), while PKC-α, -βI and -βII, and -γ were present in the rat brain homogenate (Fig. 1, lanes 2, 5, and 10). The specificities of the 80-kDa cPKC isoenzymes were determined by preincubating each antibody preparation with the specific peptide prior to immunoblotting (Fig. 1, lanes 3, 4, 7, 8, 11, and 12). We next examined whether nPKC isoenzymes such as PKC-δ and -ε were present in this cell line. Using specific antibodies and competing peptides, we demonstrated that the 80-kDa PKC-δ and the 90-kDa PKC-ε were present in rat brain but not in U937 cells (Fig. 1, lanes 13 and 17). The aPKC isoenzyme, PKC-ζ, with a molecular mass of 65 to 70 kDa was detected in both U937 cell lysates and rat brain homogenate (Fig. 1, lanes 21 and 22). The nature of the double band detected by anti-PKC-ζ antibodies is unclear at this time, although it has been observed by other authors to occur in COS cells (61) and is suggested to be an effect of proteolysis. Alternatively, the anti-PKC-ζ antibody could cross-react with another recently described member of the aPKC isoenzyme

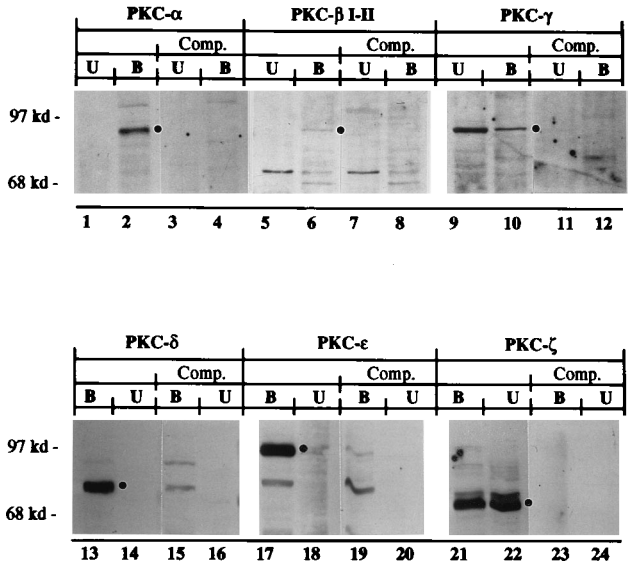


FIG. 1. PKC isoenzymes in U937 cells. Forty micrograms of cell lysates from U937 cells (U) and rat brain homogenate (B) were analyzed by SDS–10% PAGE followed by immunoblotting with specific polyclonal antibodies directed against the different PKC isoenzymes. Antibody was preincubated with a 10-fold (wt/wt) excess amount of peptide prior to immunoblotting (Comp). (Top) cPKC isoenzymes (α, βI and βII, and γ); (bottom) nPKC isoenzymes (δ and ε) and aPKC isoenzymes (ζ). The molecular markers are indicated on the left, and each isoenzyme is marked with a dot.

group (PKC-ι) (52). Thus, the HIV-induced cPKC-independent activation of NF-κB in U937 cells could be potentially mediated by PKC-ζ.

Because of the lack of specific pharmacological inhibitors that target PKC-ζ function, we investigated whether oligonucleotides directed to the translational initiation codon of the RNA of this isoenzyme, previously shown to inhibit its function (13), would be useful to interfere with its function in the U937 cell line. Sense and antisense phosphorothioate oligonucleotides specific for the PKC-ζ sequence were added at different concentrations to exponentially growing U937 cells for 36 h, after which cells were harvested, analyzed for viability, and lysed. Viability and total cell numbers were similar to those for mock-treated cells for the different concentrations of sense and antisense oligonucleotides (data not shown). An equal amount of protein cell lysate from each concentration of oligonucleotide was analyzed by SDS-PAGE followed by immunoblotting with anti-PKC-ζ antibodies. One-hundred-micromolar concentrations of antisense but not sense oligonucleotides to PKC-ζ inhibited the expression of this kinase (Fig. 2). Despite the apparent lack of effect of 100 μM antisense oligonucleotide to PKC-ζ on cell viability and growth of U937, we tested whether the synthesis of cellular proteins unrelated to PKC-ζ could be inhibited nonspecifically. By immunoblotting the same cellular lysates with an anti-p85 antibody that recognizes the p85 subunit of PI-3 kinase, we demonstrated that synthesis of this protein is not inhibited by antisense PKC-ζ oligonucleotides (Fig. 2). In repetitive experiments, the inhibitory dose of PKC-ζ antisense oligonucleotides ranged from 75 to 100 μM.

Antisense oligonucleotides to PKC-ζ interfere with the HIV-induced NF-κB activation in U937 cells. Previous studies have demonstrated that persistently infected U937 cells and human macrophages contain increased NF-κB functional activity as measured by NF-κB-dependent reporter genes (38, 46). To test whether such activity is mediated by PKC-ζ, we developed a model in which oligonucleotides could be transfected concomitantly with reporter genes rather than being added to the transfected cell culture, thus significantly reducing the amount of oligonucleotide required and potential variability in their uptake by cells. Mock- and HIV-infected cells were cotransfected with the minimal conalbumin promoter-luciferase reporter gene (cona-luc) or with the same construct containing three NF-κB tandem motifs of the HIV LTR (3 κB cona-luc) and 10 μM either sense or antisense oligonucleotides to PKC-ζ. As previously reported (38, 46), more NF-κB activity is present in HIV-infected than in mock-infected U937 cells (Fig. 3A, compare "mock" groups for the two cell lines). Cotransfection of PKC-ζ antisense oligonucleotides reduced the NF-κB activity present in HIV-infected U937 cells but not that of uninfected U937 cells. The PKC-ζ sense oligonucleotides did not interfere significantly with NF-κB-dependent function in either cell group. To show specificity of the PKC-ζ antisense oligonucleotides in interfering with the HIV-dependent NF-κB activation, titration of the oligonucleotide concentration was performed with HIV-infected U937 cells. These cells were cotransfected either with the cona-luc or 3 κB cona-luc reporter gene and increasing concentrations of sense or antisense oligonucleotides to PKC-ζ. The NF-κB activity present in HIV-infected cells was reduced with an increasing concentration of antisense but not sense oligonucleotides, with a significant reduction detected around 1 μM (Fig. 3B). In addition, Fig. 3C shows results of three representative experiments which indicate that antisense oligonucleotides to PKC-ζ reproducibly inhibit 60% of the HIV-induced NF-κB activity, with some degree of inhibition, albeit moderate (15%), being observed with the sense oligonucleotides. The lower concentration of oligonucleotides required to inhibit NF-κB-dependent transcriptional activity in HIV-infected cells (10 μM) in comparison with that required to inhibit PKC-ζ expression (100 μM) is possibly due to the introduction of oligonucleotides by transfection techniques in the former situation.

To further control for the specificity of the PKC-ζ antisense oligonucleotides to inhibit HIV-mediated NF-κB activation in U937 cells, we generated oligonucleotides to target another PKC isoenzyme present in these cells, PKC-γ (Fig. 1, lane 9). In experiments similar to those described in the legend to Fig. 3, cotransfection of either sense or antisense oligonucleotides to PKC-γ did not significantly interfere with the HIV-mediated NF-κB activation, in contrast to the inhibitory effect of the antisense oligonucleotide to PKC-ζ (Fig. 4A). To confirm that

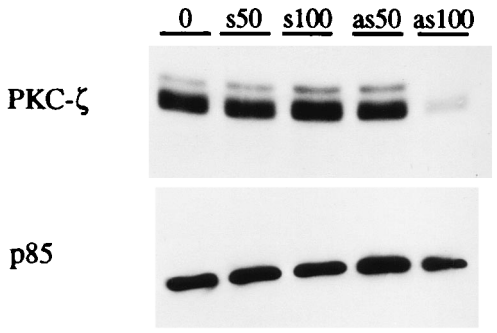
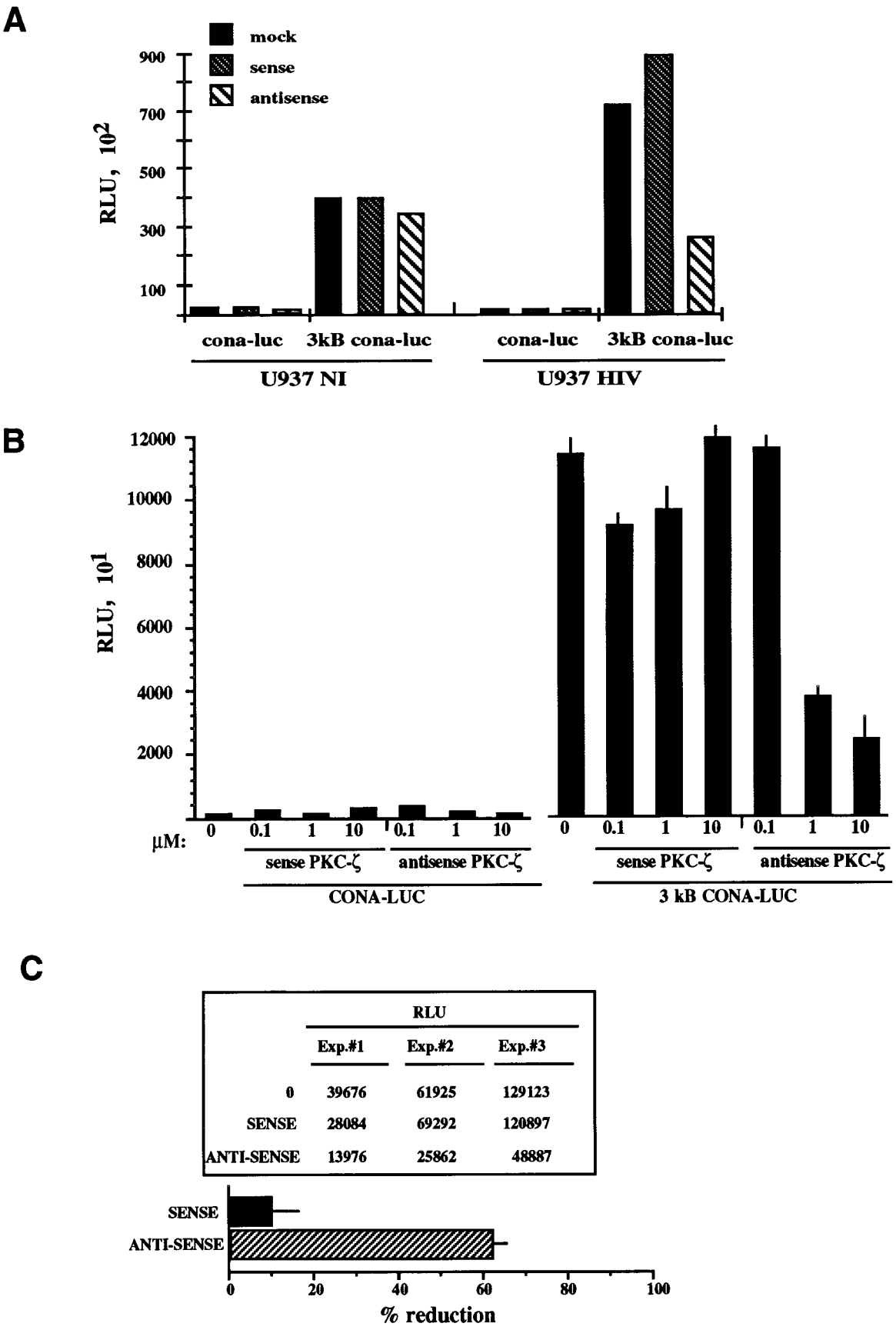


FIG. 2. Antisense oligonucleotide to PKC-ζ inhibits synthesis of PKC-ζ. Cells (5×10^5) were incubated in 2 ml of media containing different micromolar concentrations (indicated above lanes) of the sense (s) or the antisense (as) oligonucleotides directed to PKC-ζ for 36 h. Cells were then counted, lysed, and analyzed by SDS-PAGE followed by immunoblotting with PKC-ζ antibodies. The same amount (40 μg) of cell lysate was loaded in each lane. The membrane was then stripped and reblotted with anti-p85 antibodies.


A
mock
sense
antisense
RLU, 10^2
900
700
500
300
100
cona-luc
3kB cona-luc
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U937 NI
U937 HIV
B
RLU, 10^1
12000
10000
8000
6000
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0
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10
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0.1
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10
sense PKC-ζ
antisense PKC-ζ
sense PKC-ζ
antisense PKC-ζ
CONA-LUC
3 kB CONA-LUC
C
RLU
Exp.#1
Exp.#2
Exp.#3
0 39676 61925 129123
SENSE 28084 69292 120897
ANTI-SENSE 13976 25862 48887
SENSE
ANTI-SENSE
0
20
40
60
80
100
% reduction

FIG. 3. Antisense oligonucleotides to PKC-ζ inhibit the HIV-mediated NF-κB activation in U937 cells. (A) Uninfected (NI) or HIV-infected (HIV) U937 cells were transfected with the 3 κB cona-luc or the control cona-luc reporter gene and mock, sense, or antisense oligonucleotides to PKC-ζ (10 μM). (B) HIV-infected U937 cells were cotransfected with the control cona-luc and the 3 κB cona-luc reporter genes with different micromolar concentrations of sense or antisense PKC-ζ. Experiments were performed in duplicate, and standard deviations are indicated by error bars. (C) Shown are results from three different experiments in which U937 HIV-infected cells were cotransfected with the 3 κB cona-luc and either mock (0), sense (SENSE), or antisense (ANTI-SENSE) (10 μM) oligonucleotides to PKC-ζ following the same experimental design as used for the experiments represented in panels A and B. The mean for the three experiments is expressed as percent reduction from the baseline activity of mock-treated cells. Error bars indicate standard deviations. RLU, relative light units.

antisense oligonucleotides to PKC-γ conveyed a functional inhibitory effect, uninfected U937 cells were cotransfected with a κB-luc reporter gene and sense or antisense oligonucleotides to PKC-γ and PKC-ζ and then subjected to PMA stimulation. As shown in Fig. 4B, the antisense oligonucleotides to PKC-γ but not PKC-ζ partially inhibited the NF-κB activity induced by PMA. These results obtained with antisense oligonucleotides indicate certain specificity of aPKCs such as PKC-ζ in mediating HIV-dependent activation of NF-κB and of the cPKC isoform (PKC-γ) in mediating phorbol ester-dependent NF-κB activation.

PKC-ζ negative dominant molecules selectively interfere with HIV-induced NF-κB activity. Overexpressed functional inactive proteins that lack the catalytic domain of an enzyme compete with the endogenous protein, thus decreasing its function, and behave as negative dominant molecules. A mutant form of PKC-ζ has been previously shown to compete with and inhibit the effects of PKC-ζ in promoting mitogenesis and cell transformation (10, 12). Therefore, to confirm that PKC-ζ is selectively inhibiting the HIV-induced NF-κB activity in U937 cells and to exclude a nonspecific inhibition of other cell functions by oligonucleotide administration, we used the PKC-ζ negative dominant molecule, PKC-ζ^{mut}.

First, we tested whether PKC-ζ^{mut} selectively inhibited the HIV-induced NF-κB activity and not the basal or inducible activity of other transcription factors. Uninfected and HIV-infected U937 cells were transiently cotransfected with AP1-luc or 3 κB-luc and PKC-ζ^{mut}. Confirming the data obtained with the PKC-ζ antisense oligonucleotides, PKC-ζ^{mut} selectively inhibited the HIV-induced NF-κB activity but not the basal activity of an AP1-luc reporter gene (Fig. 5A). Similar results were obtained in different sets of experiments in which transfection of the PKC-ζ^{mut} expression vector did not interfere with basal activity of a CREB-luc reporter plasmid which, as with AP1, was not found to be increased in HIV-infected cells (Fig. 5B). Because transient transfection results in protein expression of the transfected gene in a very low percentage of cells, thus limiting our ability to detect the expression of PKC-ζ^{mut}, and to control for possible differences in expression levels of PKC-ζ between uninfected and HIV-infected cells, the CMV expression vector driving the transcription of PKC-ζ^{mut} was cloned upstream of a luciferase reporter gene (CMV-luc) and transfected in parallel with a 3 κB-luc construct into uninfected and HIV-infected U937 cells. While increased NF-κB-dependent transcriptional activity was present in HIV-infected cells, the basal activities of the CMV-luc reporter genes were similar in the two cell lines (Fig. 5C). This suggests that levels of expression of PKC-ζ^{mut} are similar in the two cell groups.

We next determined whether other stimuli that result in NF-κB activation in U937 cells, such as PMA and LPS, require PKC-ζ. U937 cells were cotransfected with the 3 κB-luc reporter plasmid and either the CMV expression vector or PKC-ζ^{mut} and then stimulated with PMA or LPS. PMA and LPS, respectively, resulted in 3- and 12-fold increases in NF-κB activity which was not inhibited by PKC-ζ^{mut} (Fig. 6A). Moreover, and as observed in additional experiments, PKC-ζ^{mut} cotransfection was associated with increased inducibility of the 3 κB-luc reporter activity by such stimuli. Altogether, these data suggest that PKC-ζ selectively participates in a signal transduction pathway which is HIV induced and independent of those pathways used by other stimuli such as PMA or LPS. Cotransfection of the CMV-, AP1-, or CREB-luc reporter genes and PKC-ζ^{mut} into U937 cells followed by PMA or LPS stimulation resulted in a three- to fourfold increase over the

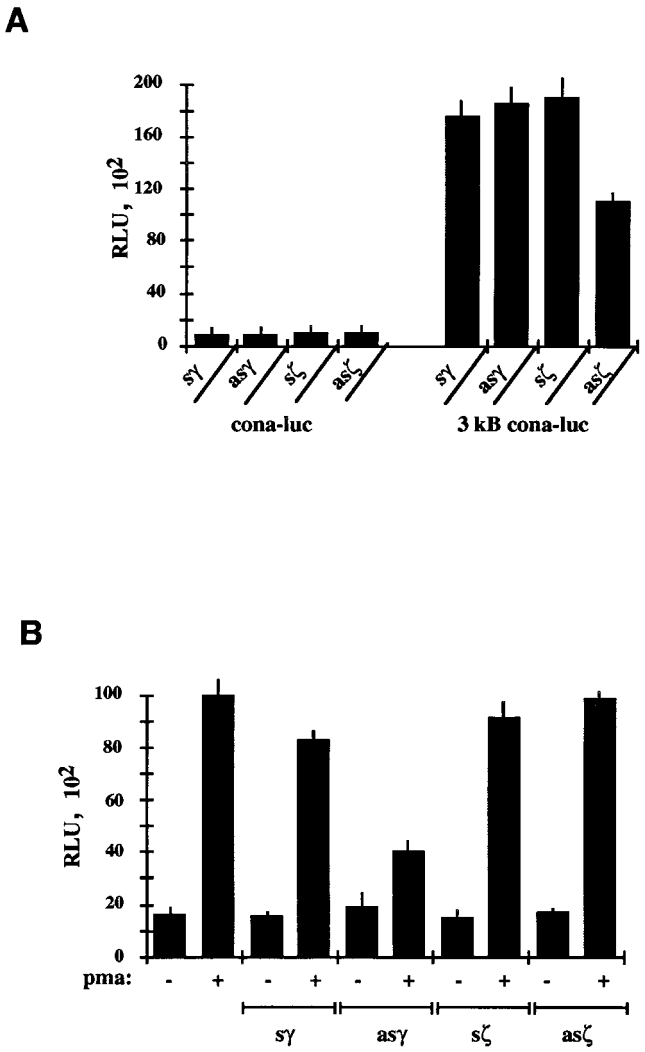

FIG. 4. PKC-γ mediates PMA but not dependent NF-κB activation in U937 cells. (A) HIV-infected U937 cells were cotransfected with the corresponding luciferase reporter gene and 10 μM either sense (s) or antisense (as) oligonucleotides to PKC-γ (γ) or PKC-ζ (ζ) by the methodology described in the legend to Fig. 3. (B) Uninfected U937 cells were cotransfected with an NF-κB reporter plasmid and oligonucleotides as described in the legend for panel A and then subjected to PMA stimulation (pma) (20 ng/ml) for 18 h (+) or left unstimulated (−). Error bars indicate standard deviations. RLU, relative light units.

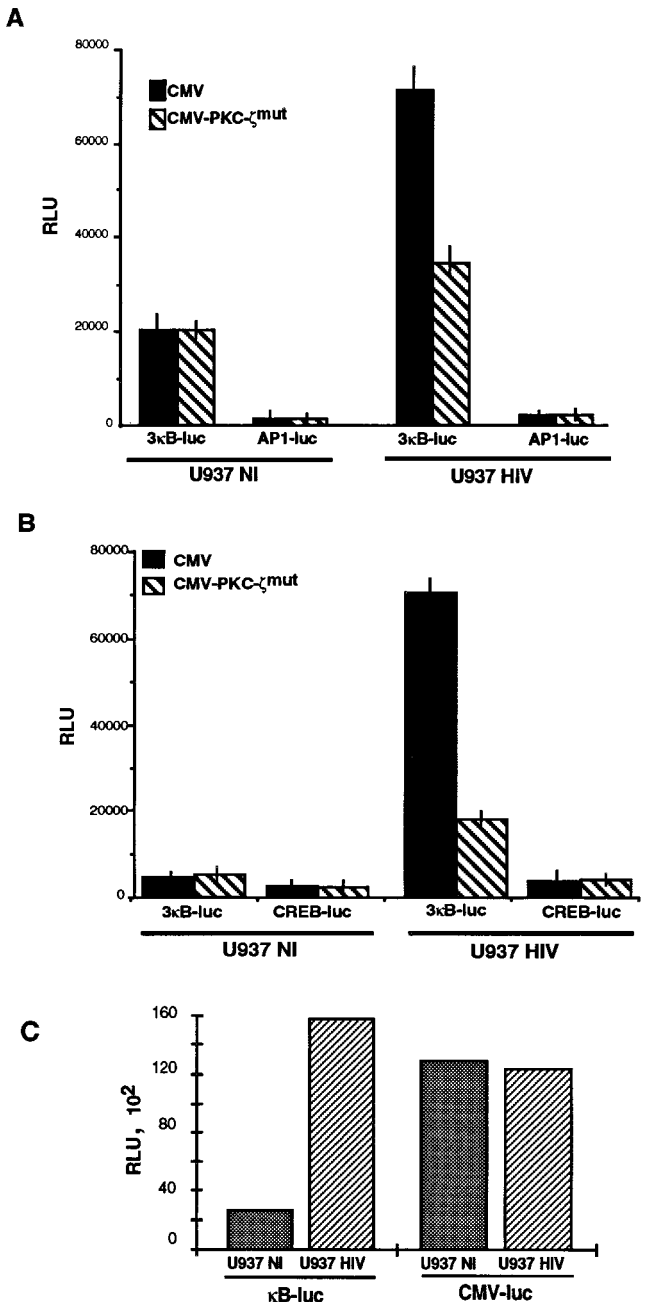

FIG. 5. The negative dominant mutant of PKC-ζ decreases the NF-κB activation induced by HIV in U937 cells. (A) Uninfected (NI) or HIV-infected (HIV) U937 cells were cotransfected with a 3 κB-luc or an AP1-luc reporter gene and the CMV expression vector (CMV) or the CMV promoter (CMV–PKC-ζ^{mut}). Experiments were performed in duplicate, and the standard deviation is represented above each bar. (B) Same as for panel A, except that a CREB-luc reporter gene was used instead of an AP1-luc reporter gene. (C) Uninfected (NI) or HIV-infected (HIV) U937 cells were transfected with either 3 κB cona-luc (κB-luc) or the CMV expression vector used for the experiments represented in panels A and B but cloned upstream of the luciferase gene (CMV-luc). RLU, relative light units.

basal activity of these reporter genes which was not modified by PKC-ζ^{mut} (data not shown).

Previous studies by our group demonstrated that HIV infection upregulates NF-κB activity in U937 cells in a manner independent of PMA-independent pathways (i.e., cPKC) and that PMA and HIV synergize to activate NF-κB (46). To confirm that PKC-ζ participates in the HIV- but not the PMA-induced pathway and to test whether the previously observed synergy between PMA and HIV is reversed by the mutant form of PKC-ζ, we performed the following experiments. Uninfected and HIV-infected U937 cells were cotransfected with the NF-κB-dependent reporter gene and the PKC-ζ^{mut} expression vector and then subjected to PMA stimulation. As shown in previous experiments, the HIV-induced but not the basal or PMA-induced NF-κB activity in uninfected cells was reduced by PKC-ζ^{mut} (Fig. 6B). PMA treatment of HIV-infected U937

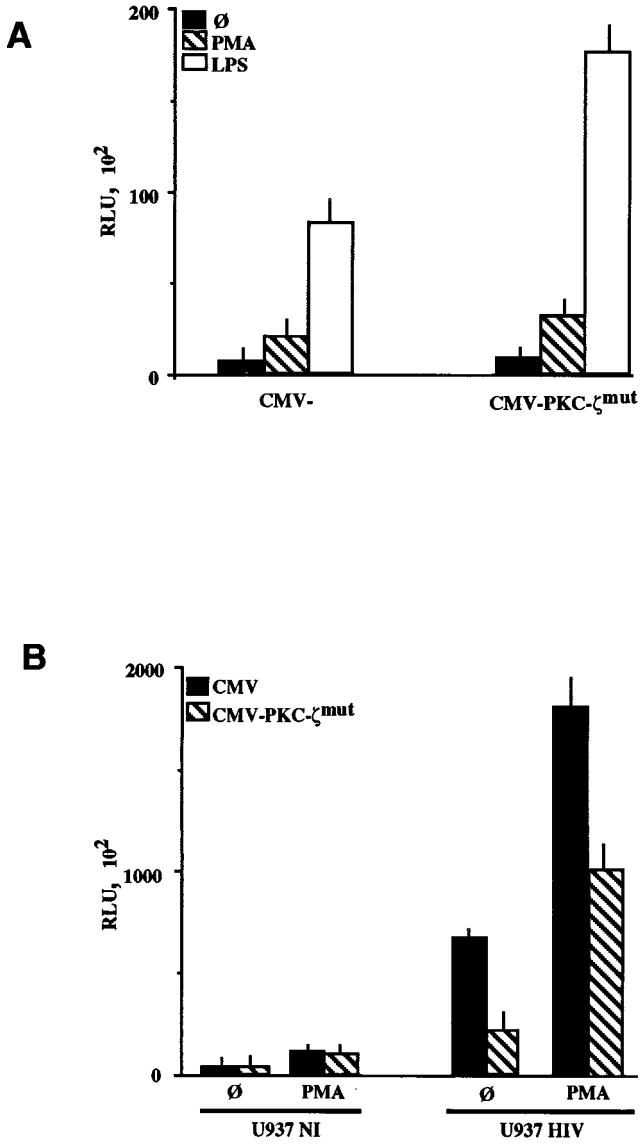

FIG. 6. PKC-ζ^{mut} blocks HIV- but not PMA- or LPS-induced NF-κB activation. (A) Uninfected U937 cells were cotransfected with 3 κB cona-luc and either the CMV expression vector alone (CMV−) or the CMV expression vector driving the PKC-ζ mutant (CMV–PKC-ζ^{mut}). Following transfection, cells were left unstimulated (ø) or stimulated with PMA (20 ng/ml) or LPS (1 μg/ml) for 18 h. The standard deviation is represented above each bar. (B) Same experimental design as for panel A, except that uninfected (NI) and HIV-infected (HIV) cells were cotransfected with 3 κB cona-luc and with the CMV expression vector (CMV) or the CMV expression vector driving the PKC-ζ^{mut} gene (CMV–PKC-ζ^{mut}) and either left unstimulated (ø) or stimulated with PMA (20 ng/ml). RLU, relative light units.

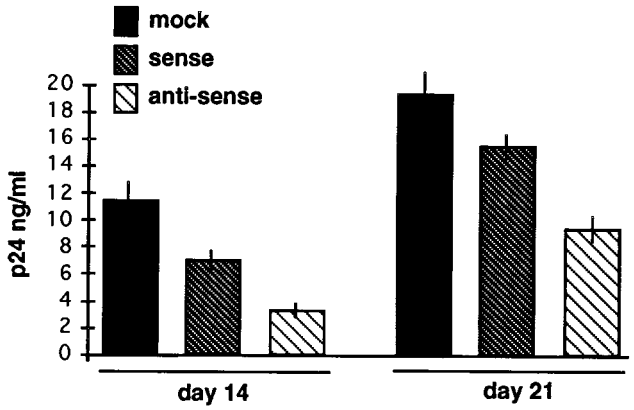

FIG. 7. Antisense oligonucleotides to PKC-ζ decrease HIV replication in HIV-infected human macrophages. Human-derived macrophages were infected with the HIV strain SF_{162} and treated twice weekly with either mock, sense, or antisense oligonucleotides (5 μM). Supernatants were harvested at days 14 and 21 and assayed for p24 content. Experiments were done in duplicate wells, and the standard deviation is indicated by a line on top of each bar.

cells synergistically increased the HIV-induced NF-κB activation, and the effect was decreased by cotransfection of the mutant form of PKC-ζ. Overall, these experiments confirm that HIV infection results in the upregulation of a signal transduction pathway in which PKC-ζ is one of the components and that another pathway(s) or other mechanisms that regulate NF-κB activation, such as those initiated by PMA, can synergize with or complement the HIV–PKC-ζ-dependent pathway.

PKC-ζ antisense oligonucleotides reduce HIV replication in HIV-infected MDM. Having identified a specific second messenger of a signal transduction pathway that appears to be selectively used by HIV infection in monocytic cells to upregulate NF-κB, we tested whether inhibition of PKC-ζ would lead to decreased viral replication and thus establish a correlation between NF-κB activation and persistent viral replication. To test this hypothesis, MDM infected with a monocytotropic strain of HIV were used as a more relevant model of persistent viral replication. HIV-infected MDM were treated with sense or antisense oligonucleotides twice weekly at the time of cell passage, and culture supernatant was then analyzed for p24 content. As shown in Fig. 7, HIV replication was reduced approximately 50 to 60% by the antisense oligonucleotide to PKC-ζ at both 14 and 21 days postinfection. Sense oligonucleotide also interfered with viral replication, albeit to a lesser degree, suggesting a nonspecific effect of oligonucleotides on viral replication. In addition, MDM were monitored morphologically with every culture passage and on day 21 analyzed for cell viability by use of a modified trypan blue analysis. Although cell viabilities were similar among the three treatment groups (mock, sense, and antisense), the decreased p24 levels correlated, in a blinded fashion, with decreased HIV-induced morphological changes (cell enlargement and vacuolization of the apparent infected cells; data not shown).

Taking into account the potential limitations of oligonucleotides in inhibiting HIV replication, these preliminary studies with MDM imply that PKC-ζ is a host cell protein that participates in the regulation of viral replication in MDM. Future studies will need to address the impact of inhibiting this PKC isoenzyme on macrophage function and whether PKC-ζ selectively interferes with NF-κB activation by HIV infection and other physiological stimuli.

DISCUSSION

In search of cellular signal transduction pathways leading to NF-κB activation in HIV-infected cells, our studies indicate that PKC-ζ, a member of the aPKC isoform family, is a second messenger involved in this virus-host cell interaction. Interruption of PKC-ζ function by using different genetic approaches in monocytic cell lines and human macrophages leads to decreased HIV-induced NF-κB activation and viral replication.

Activation of NF-κB by different viruses suggests that this type of virus-host cell interaction may be important for effective viral replication. For HIV, NF-κB has been clearly established as a key transcription factor in modulating the HIV enhancer-promoter region (41, 47), regulating not only reactivation from latency but also viral persistence (1, 5, 28, 30, 31, 37, 45, 47, 50, 53, 56). Since the macrophage is a reservoir of persistent viral replication in infected individuals (19, 20) and since we have shown that infection with monocytotropic viruses increases NF-κB activation in human macrophages (38), we have started to dissect potential signal transduction pathways that regulate NF-κB activation in infected cells. Although little information as to what signal transduction pathways are functional in cells of the monocytic lineage is available, our previous work indicated that PKC isoenzymes regulated by phorbol esters (i.e., cPKC isoforms) were functional in mediating NF-κB activation in monocytic cells (46). However, persistent HIV infection of those cells resulted in NF-κB activation through other PKC isoenzymes and/or PKC-independent pathways. To explore the first option, we have first focused on a PKC isoenzyme that has been classified as atypical (differing from cPKC by the absence of the Ca^{2+}- and diacylglycerol-responsive elements) and shown to mediate NF-κB activation in murine cells and oocytes (10, 14).

The mechanisms by which PKC-ζ results in NF-κB activation are the subject of active study. This transcription factor is regulated at multiple levels and by a variety of inhibitory proteins (reviewed in references 3 and 35). One such inhibitor, IκBα (11), is a target of phosphorylation and degradation following activation of yet-ill-defined signal transduction pathways by multiple extracellular stimuli. Recent studies indicate that PKC-ζ binds a kinase that in vitro leads to the phosphorylation of IκBα, implicating this inhibitor as a potential target of PKC-ζ-dependent steps resulting in NF-κB activation in vivo. Persistent HIV replication in human monocytic cells and macrophages is associated with increased turnover of IκBα, resulting in levels constitutively decreased from those present in uninfected controls (38). Although studies to examine the phosphorylation characteristics of IκBα in infected cells are under way, our preliminary data indicate that IκBα is hyperphosphorylated following HIV infection, probably rendering it more susceptible to degradation, as shown by recent studies (7, 8). Therefore, it is plausible that PKC-ζ-dependent pathways lead to the phosphorylation and subsequent degradation of IκBα in HIV-infected cells, resulting in persistent NF-κB nuclear translocation (38). Further studies are required to evaluate whether PKC-ζ itself or associated kinases are involved in modifying IκBα in HIV-infected cells. Moreover, other NF-κB inhibitory molecules, such as IκBβ, have been recently cloned (58) and shown to be regulated by posttranslational modifications similar to those regulating IκBα; thus, future investigation should address whether NF-κB inhibitor molecules other than IκBα are targets of HIV and/or PKC-ζ.

The fact that HIV infection, but not phorbol esters or LPS, activates NF-κB through this kinase suggests certain specificity for the HIV-induced signal transduction pathways in monocytic cell lines and suggests that this kinase must be upstream

of the converging common pathway that regulates NF-κB by multiple unrelated stimuli. In addition, the function of this kinase may be cell specific. PKC-ζ has been shown to mediate NF-κB activation in mouse fibroblasts and oocytes in response to extracellular stimuli such as phospholipase C (10, 14), but not in T cells in response to different modes of T-cell activation (21, 23). Moreover, interruption of PKC-ζ in other cell systems has not been associated with disruption and other cell functions (10, 14). These observations suggest that disruption of PKC-ζ in monocytes could potentially interfere with HIV-induced NF-κB activation and therefore possibly interfere with viral persistence without altering other physiological functions of the cell and thus target this molecule for future therapeutical intervention.

The exact location of PKC-ζ within known signal transduction pathways is being studied. Recent data point towards an association of this kinase with p21^{ras} (12). This relevant protooncogene is critical in mediating signal transduction signals from a variety of growth factor receptors although dispensable for some cytokine receptor-initiated signals (49). Although the exact relationship between these two second messengers is unknown, PKC-ζ appears to be downstream of p21^{ras} but unrelated to the other well-characterized downstream target of *ras*, *raf-1* kinase (12). PI-3 kinase has been recently shown to be another downstream second messenger of p21^{ras} (49) and to activate PKC-ζ in vitro (42, 59). Moreover, PKC-ζ and *ras* are physically associated following cell activation (12). Altogether, this indicates that p21^{ras} may bifurcate its signalling via the *raf*–mitogen-activated protein kinase or the PI-3 kinase–PKC-ζ pathway. More studies are needed to define whether PKC-ζ can be activated via a p21^{ras}-independent pathway as has been suggested recently for tumor necrosis factor (40).

How this general organization scheme of signal transduction pathways fits with the HIV-induced NF-κB activation in human macrophages is unknown. If PKC-ζ is being activated by p21^{ras} via PI-3 kinase, it is plausible that specific inhibitors of PI-3 kinase, such as wortmannin, should selectively interfere with the HIV-induced NF-κB activation in these cells and thus bypass our current limitation of lacking specific pharmacological inhibitors of this PKC isoenzyme. A new PKC isoform with all the characteristics ascribed to PKC-ζ has been recently cloned (52). This new isoenzyme (PKC-ι), whose function remains unknown, has an extremely high degree of homology (up to 84% in the catalytic domain and carboxy-terminus region) to the better-characterized PKC-ζ. Because many of the reagents utilized to study PKC-ζ (commercial antibodies, which are directed to the C terminus, and dominant negative molecules containing mutations in the catalytic domain) may potentially cross-react with PKC-ι, future studies addressing the role of aPKC-ζ should attempt to determine the specificity of these isoforms in the different models tested thus far.

Understanding the molecular mechanisms regulating the interaction between HIV and host cell proteins that favor effective viral replication and that mediate many of the HIV-induced effects on the infected cell is essential to advance our knowledge of HIV pathogenesis. Systematic dissection of signal transduction pathways upregulated by HIV to result in the activation of transcription factors that are essential for viral replication, such as NF-κB, should identify potential targets for therapeutical intervention.

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