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The Lipophosphoglycan of *Leishmania donovani* Up-Regulates HIV-1 Transcription in T Cells Through the Nuclear Factor-κB Elements[1]

Richard Bernier, Benoît Barbeau, Michel J. Tremblay,[2] and Martin Olivier[2]

We have recently demonstrated that the parasite *Leishmania donovani* and its surface molecule, lipophosphoglycan (LPG), can activate HIV-1 replication in monocytoid cells. Our present interest was to determine whether LPG could also up-regulate HIV-1 transcription in T cells. Using a CD4-positive human lymphoid T cell line (1G5) containing a stably integrated HIV-1 long terminal repeat (LTR)-luciferase construct, we found that LPG is a potent inducer of HIV-1 LTR activity. Treatment of 1G5 cells with signaling antagonists revealed that protein tyrosine kinase- and protein kinase A-dependent pathways were actively participating in the LPG-induced enhancement of HIV-1 LTR-driven activity. Transfection of Jurkat E6.1 cells with plasmids containing wild-type and nuclear factor-κB (NF-κB)-mutated HIV-1 LTR-luciferase constructs has suggested a role for NF-κB binding sites in the LPG-mediated induction of HIV-1 LTR activity. An LPG-induced binding factor specific to the NF-κB consensus sequences could be observed using electrophoretic mobility shift assay. Finally, transfection experiments performed with a vector containing HIV-1 κB binding sites only showed similar LPG-mediated induction, which was abrogated by sodium salicylate, a known NF-κB inhibitor. We thus demonstrate that the LPG-mediated induction of HIV-1 LTR activity in T cells involves several second messengers culminating in activation of HIV-1 LTR-driven transcription via NF-κB-binding consensus sequences. In conclusion, these results reinforce the idea that *L. donovani* is a putative cofactor in HIV-1 pathogenesis. ***The Journal of Immunology*****, 1998, 160: 2881–2888.**

The etiologic role of HIV-1, previously known as lymphadenopathy-associated virus (1) or human T cell lymphotropic virus type III (2), in the pathogenesis of AIDS is now well established (1, 2). This virus has been demonstrated to be mainly tropic for cells bearing the surface CD4 molecule, which acts as the primary cellular receptor for virus entry into susceptible cells (3–5). In the infected human host, HIV-1 may replicate at low levels for long periods without evoking clinical disease. Indeed, one landmark of HIV-1 infection is clinically characterized by a long latency period and the presence of opportunistic infections resulting from the induced state of immunodeficiency, the time of infection, and the onset of AIDS (6, 7). Elucidation of the mechanism(s) that governs the spread and progression of HIV-1 infection may thus have important clinical implications. Of particular interest are stimuli that can activate the regulatory elements of HIV-1 located within the long terminal repeat (LTR)[3] sequences. Such stimuli have been hypothesized to induce HIV-1 gene expression from latent proviruses and increase viral replication and disease progression (8). Indeed, many patients infected with HIV-1 become subsequently coinfected with a variety of others microorganisms as their immune function deteriorates. Therefore, the possibility exists that some of these opportunistic pathogens can act as cofactors that stimulate the expression of HIV-1, thereby causing clinically apparent diseases.

Previous studies have demonstrated that the HIV-1 LTR is activated by a vast array of heterologous micro-organisms that are frequently seen in AIDS patients. For example, herpes simplex type 1, EBV, CMV, papovaviruses, hepatitis B virus, and human herpes virus type 6 have all been reported to positively modulate HIV-1 expression (9–14). In addition, tetanus toxin and bacterial immunomodulators such as mycobacterial trehalose and detoxified endotoxin have been reported to up-regulate HIV-1 expression (15, 16). The same is true for *Mycobacterium tuberculosis* and its major antigenic determinant of the cell wall, the lipoarabinomannan, which have been shown to promote HIV-1 gene expression based on clinical studies and in vitro experiments (17–21).

Our knowledge about the putative role played by protozoan parasites in the progression of AIDS is minimal. However, we have recently hypothesized that the protozoan parasites of the genus *Leishmania* should be considered as putative cofactors in the pathogenesis of HIV-1 infection (22). This postulate was based on several important observations. First, the presence of both agents is already overlapping in several countries. For example, several patients coinfected with *Leishmania donovani* and HIV-1 have been

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[3] Abbreviations used in this paper: LTR, long terminal repeat; LPG, lipophosphoglycan; core-PI, core phosphatidylinositol; PKC, protein kinase C; NF-κB, nuclear factor-κB; TS, transfection solution; NaB, sodium butyrate; PTK, protein tyrosine kinases; PKA, protein kinase A; PKG, protein kinase G; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*, *N*, *N′*, *N′*-tetraacetic acid.

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reported in southern Europe (France, Spain, and Italy) (23, 24). Second, *Leishmania* is now considered an opportunistic organism in immunosuppressed AIDS patients (25, 26). Indeed, *Leishmania* parasites have a worldwide distribution and are seen as a major public health problem in Asia, Latin America, Africa, India, and southern Europe (27–30). Third, we have demonstrated that *L. donovani* and the lipophosphoglycan (LPG) can induce HIV-1 replication in monocytoid cells chronically infected with HIV-1 (31). It should be noted that LPG is a glycoconjugate that is considered one of the major constituents expressed on the surface of *Leishmania* promastigotes transmitted to the host by the bite of the sandfly vector. Once engulfed by the macrophages and surrounded by the phagolysosome, the parasite will differentiate into the aflagellated amastigote form. At this stage, the core phosphatidylinositol (core-PI), an intramembrane structural component of LPG, can be found on the surface of the parasite. In addition, these surface molecules have been reported to promote intracellular survival of *Leishmania* parasite (32). In fact, LPG protects *L. donovani* against destruction within macrophage phagolysosomes (33) by attenuation of several macrophage functions and modulation of cell signaling through effects on protein kinase C (PKC) (32).

In this study we demonstrate for the first time that *L. donovani* LPG is a potent inducer of HIV-1 LTR transcription in T cells. We further determine that the effect of *L. donovani* LPG on HIV-1 was acting via NF-κB binding sequences and culminates in positive modulatory action on HIV-1 regulatory sequences.

Materials and Methods

Cell lines and plasmids

1G5 is a clonal cell line derived from Jurkat E6.1 cells stably transfected with the luciferase reporter gene driven by the HIV-1_{SF2} LTR (34). The lymphoid T cell line Jurkat E6.1 is CD4 positive and has been widely used to study signal transduction pathways mediated via the TCR/CD3 complex (35). MT-2 is a human T cell leukemia virus type 1-producing T cell line that has been shown to be highly susceptible to HIV-1-induced cytopathic effects (36). OM-10.1 has been derived from HL-60 promyelocytic cells latently infected with HIV-1 (37). U937 is a premonocytoid cell line that can be induced to differentiate into macrophage by exposure to PMA (38). The promonocytic cell line U1 is a U937 derivative that carries two integrated HIV-1 copies per cell (39). All cell lines were grown in complete culture medium made of RPMI 1640 supplemented with 10% FBS (HyClone Laboratories, Logan, UT), glutamine (2 mM), penicillin G (100 U/ml), and streptomycin (100 μg/ml). These cell lines were provided by the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health (Bethesda, MD). The molecular constructs pLTRLUC (HIV-1 LTR from strain HXB2) and mutated NF-κB pmκBLTRLUC have been used in our studies and were provided by Dr. Calame (Columbia University, New York, NY). These eukaryotic expression vectors contain the luciferase reporter gene under the control of wild-type (GGGACTTTCC) or NF-κB-mutated (CTCACTTTCC) HIV-1 LTR (40). The pκB-TATA-LUC contains the minimal HIV-1 κB region and a TATA box placed upstream of the luciferase reporter gene (41). This vector was supplied by Dr. W. C. Greene (The J. Gladstone Institute, San Francisco, CA).

LPG and core-PI fragment

LPG molecules from *L. donovani* promastigotes were purified by solvent extraction and column chromatography before quantitation by a colorimetric carbohydrate assay as described previously (42). Preparation of core-PI was performed by cleaving LPG with a mild acid hydrolysis (0.02 N HCl, 5 min, 100°C). Thereafter, generated products were separated by chromatography on a column made of phenyl-coupled Sepharose. The core-PI was eluted from the column with water/ethanol/ether/pyridine/NH_4OH (1/15/5/1/0.017), dried under a stream of N_2, and resuspended in the appropriate buffer. Purified LPG and core-PI were supplied by Dr. Turco (University of Kentucky, Lexington, KY).

Transfection, cell treatments, and luciferase assay

Jurkat E6.1 cells (5–10 × 10^6) were washed once in transfection solution (TS; 137 mM NaCl, 25 mM Tris-HCl (pH 7.4), 5 mM KCl, 0.6 mM Na_2HPO_4, 0.5 mM $MgCl_2$, and 0.7 mM $CaCl_2$) and resuspended in 1 ml of TS containing 15 μg of the indicated plasmid (pLTRLUC, pmκ BLTRLUC, or pκB-TATA-LUC) and 500 μg/ml of DEAE-dextran (final concentration). The cell/TS/plasmid/DEAE-dextran mix was incubated for 25 min at room temperature. Thereafter, cells were diluted at a concentration of 1 × 10^6/ml using complete culture medium supplemented with 100 μM chloroquine (Sigma Chemical Co., St. Louis, MO). After 45 min of incubation at 37°C, cells were centrifuged and resuspended in complete culture medium. Transiently transfected Jurkat E6.1 and stably transfected 1G5 cells were seeded in 96-well flat-bottom plates at a density of 10^5 cells/well (100 μl). In one set of experiments, transfected cells were either pretreated or not with 2.5 mM sodium salicylate (Sigma) for 1 h at 37°C. Cells were left untreated or were treated with LPG (10–20 μM), core-PI (10 μM), PHA (3 μg/ml; PHA-P, Sigma), TNF-α (2 ng/ml; R&D Systems, Minneapolis, MN), and sodium butyrate (NaB; 1 mM; Sigma) for 24 h at 37°C. When indicated, cells were pretreated for 1 h at 37°C with specific inhibitors (herbimicin A, W7, BAPTA, HA1004, and MDL12330A) before incubation with the described stimuli. Next, luciferase activity in cellular extracts was monitored as described previously (43). Briefly, 100 μl of cell-free supernatant was withdrawn from each well, and 25 μl of cell culture lysis buffer (25 mM Tris phosphate (pH 7.8), 2 mM DTT, 1% Triton X-100, and 10% glycerol) was added before incubation at room temperature for 30 min. An aliquot of cell extract (20 μl) was mixed with 100 μl of luciferase assay buffer (20 mM tricine, 1.07 mM $(MgCO_3)_4{\cdot}Mg(OH)_2{\cdot}5H_2O$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 270 μM coenzyme A, 470 μM luciferin, 530 μM ATP, and 33.3 mM DTT), and the sample was introduced into the counting chamber of a standard liquid scintillation counter equipped with single-photon monitor software (Beckman Instruments, Fullerton, CA). The total number of photo-events were measured over a 30-s time lapse.

Binding of LPG on monocytoid and T lymphoid cells

Cells (1 × 10^6) were first incubated with 20 μM *L. donovani* LPG for 30 min at 37 or 4°C. Next, the cells were washed twice with PBS, pH 7.4, and resuspended in 100 μl of PBS containing 1 μg of CA7AE, a monoclonal anti-LPG IgM Ab (Cedarlane, Hornby, Ontario, Canada) (44, 45). Cells were incubated for 30 min on ice. Samples were washed twice in PBS and left for 30 min on ice with 1 μg of FITC-conjugated goat anti-mouse IgM (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). After two washes with PBS, samples were fixed with 1% (v/v) paraformaldehyde and analyzed by cytofluorometry (EPICS XL, Coulter Corp., Miami, FL).

Preparation of nuclear extracts

Nuclear extracts were prepared according to the microscale preparation protocol described by Andrews and Faller (46). Briefly, 1G5 cells (10^7) were treated for 1 h at 37°C with 10 μM *L. donovani* LPG. Cells were washed twice with PBS, and pelleted cells were resuspended in 400 μl of cold buffer A (10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, and 0.2 mM PMSF). After 10 min on ice, the lysate was vortexed for 10 s, and samples were centrifuged for 10 s at 12,000 × *g*. The supernatant fraction was discarded, and the cell pellet was resuspended in 100 μl of cold buffer B (20 mM HEPES-KOH (pH 7.9), 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, and 0.2 mM PMSF) and incubated on ice for 20 min. Cellular debris was removed by centrifugation at 12,000 × *g* for 2 min at 4°C, and the supernatant fraction was stored at −70°C until assayed.

Electrophoresis mobility shift assays

Electrophoresis mobility shift assays were conducted using 7 μg of nuclear extracts. Protein concentrations were determined by the bicinchoninic assay with a commercial protein assay reagent (Pierce, Rockford, IL). Nuclear extracts were incubated for 30 min at 23°C in 15 μl of buffer C (100 mM HEPES (pH 7.9), 40% glycerol, 10% Ficoll, 250 mM KCl, 10 mM DTT, 5 mM EDTA, 250 mM NaCl, 2 μg poly(dI-dC), and 10 μg nuclease-free BSA fraction V) containing 0.8 ng of 5′ end ^{32}P-labeled dsDNA oligonucleotide. dsDNA (100 ng) was labeled with [gamma-^{32}P]ATP and T4 polynucleotide kinase in a kinase buffer (New England Biolabs, Beverly, MA). This mixture was incubated for 30 min at 37°C, and the reaction was stopped with 5 μl of 0.2 M EDTA. The labeled oligonucleotide was extracted with phenol/chloroform and passed through a G-50 spin column. The dsDNA oligonucleotide, which was used as a probe or a competitor, contained the consensus NF-κB-binding site corresponding to the sequence 5′-ATGTGAGGGGACTTTCCCAGGC-3′. A dsDNA oligonucleotide containing a mutated NF-κB-binding site (bold print) was also used (5′-ATGTGAG**CTC**ACTTTCCCAGGC-3′). Oligonucleotides were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). DNA-NF-κB complexes were resolved from free labeled DNA by electrophoresis in

native 4% (w/v) polyacrylamide gels containing 50 mM Tris-HCl (pH 8.5), 200 mM glycine, and 1 mM EDTA. The gels were subsequently dried and autoradiographed. Cold competitor assays were conducted by adding 1-, 10-, and 100-fold molar excesses of homologous unlabeled dsDNA NF-κB oligonucleotide simultaneously with the labeled probe.

Statistical analysis

Statistically significant differences between groups were performed with the analysis of variance module of SAS software (version 6.07, SAS Institute, Cary, NC) using Fisher's least significant difference test. $p < 0.05$ was considered statistically significant (p values are given in the figure legends). All data are presented as the mean ± SD.

Results

We have previously shown that one of the major surface components of *L. donovani*, LPG, can activate virus replication in OM-10.1 and U1, two monocytoid cell lines latently infected with HIV-1 (31). Since the CD4-positive T cells are the primary target for HIV-1 in human peripheral blood (47), we wanted to determine whether LPG could lead to a similar HIV-1-activating effect in T cells. We first evaluated the capacity of LPG to bind to several lymphoid and monocytoid cell lines by flow cytometric analysis. The binding of LPG to monocytoid cells was monitored based on our previous results indicating that *L. donovani* LPG is capable of activating latent HIV-1 proviral DNA in cells of monocytoid origin (31). As expected, the great majority of U937, U1, and OM-10.1 monocytic cells was found to incorporate high amounts of LPG (>95% of cells were positive, with a mean fluorescence intensity between 102–183). More importantly, LPG was also found to strongly interact with a high percentage of T lymphoid MT-2 and 1G5 cells (data not shown). These results are in agreement with a previous study showing that LPG can efficiently bind to the human T lymphoid cell line MT-2 (44). In addition, an LPG binding assay was performed at 4°C and revealed that the majority of cells were positive for LPG (>95%). However, it should be noted that the efficiency of binding of LPG at 4°C was reduced compared with that of cells incubated at 37°C (mean fluorescence intensity of 8).

To study the putative effect of LPG on the regulatory elements of HIV-1 (LTR), we used the human T lymphoid 1G5 cell line. This cell line has stably integrated constructs made of the luciferase gene driven by the HIV-1_{SF2} LTR (34). It thus allows for a rapid and sensitive evaluation of external stimuli that can positively modulate transcription from the HIV-1 LTR. In this set of experiments, a basal level of luciferase activity was achieved using untreated 1G5 cells (negative control), while incubation of cells with the mitogenic agent PHA was used as a positive control, since 1G5 cells were expressing the TCR/CD3 complex (M. J. Tremblay and B. Barbeau unpublished observations). Incubation of 1G5 cells with increasing concentrations of LPG (1–10 μM) for 24 h resulted in a statistically significant increase in HIV-1 LTR-driven luciferase activity (3.5-fold, 10 μM) compared with the basal level of reporter gene activity in untreated cells (Fig. 1*A*). These studies were also conducted using core-PI, a fragment of LPG. Similarly, core-PI was found to potently activate HIV-1 transcription (Fig. 1*B*). Data from these experiments clearly indicate that LPG, one of the major surface constituents of the protozoan parasites of the genus *Leishmania*, can activate HIV-1 LTR-dependent gene expression in T cells.

Next, we investigated the intracellular second messengers participating in the *L. donovani* LPG-induced signaling pathway(s) leading to activation of HIV-1 LTR transcription in T cells. This goal was attained using several specific inhibitors that are known to be useful tools for studying the role of particular signaling pathways in cellular activation. It should be noted that subcytostatic and subcytotoxic concentrations of each inhibitor were used in our experiments, and none of them showed a capacity to activate HIV-1 LTR transcription per se (data not shown). First, the involvement of protein tyrosine kinases (PTK) in the LPG-mediated activation of the transcriptional state of HIV-1 LTR elements was monitored by pretreating 1G5 cells with increasing concentrations of herbimycin A (0.01, 0.1, and 1 μM), a potent PTK inhibitors. Herbimycin A at a concentration of 1 μM abrogated the LPG-dependent enhancement of HIV-1 LTR-driven reporter gene expression (Fig. 2*A*), implicating PTK in this activating effect. Implication of protein kinase A (PKA), PKC, and/or protein kinase G (PKG) was next evaluated despite the previous demonstration that LPG acts as a potent inhibitor of PKC activity in monocytes (48). 1G5 cells were pretreated with H7, a selective serine/threonine kinase inhibitor that can inhibit PKA (K_i = 3.0 μM), PKC (K_i = 6.0 μM), and PKG (K_i = 5.8 μM). The *L. donovani* LPG-mediated activation of HIV-1 transcription was totally abolished by

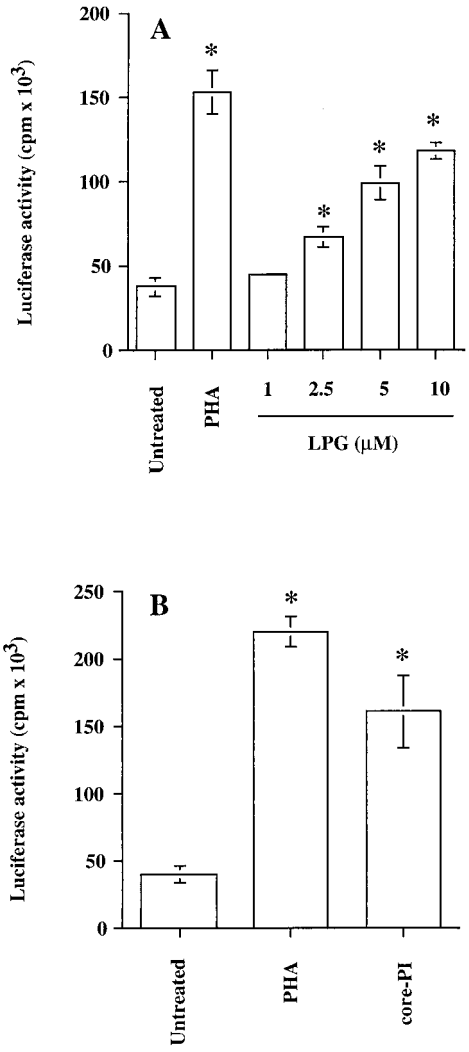

FIGURE 1. Activation of HIV-1 transcription in T cells by LPG and core-PI. 1G5 cells were incubated for 24 h in the absence or the presence of LPG (0–10 μM; *A*) and a fragment of LPG, the core-PI (10 μM; *B*). Untreated cells were used as a negative control, while incubation with PHA (3 μg/ml) served as a positive control. Cell lysates were evaluated for luciferase activity by scintillation count. Results shown are the mean ± SD of triplicate samples. Asterisks indicate significant differences from untreated 1G5 cells ($p < 0.01$).

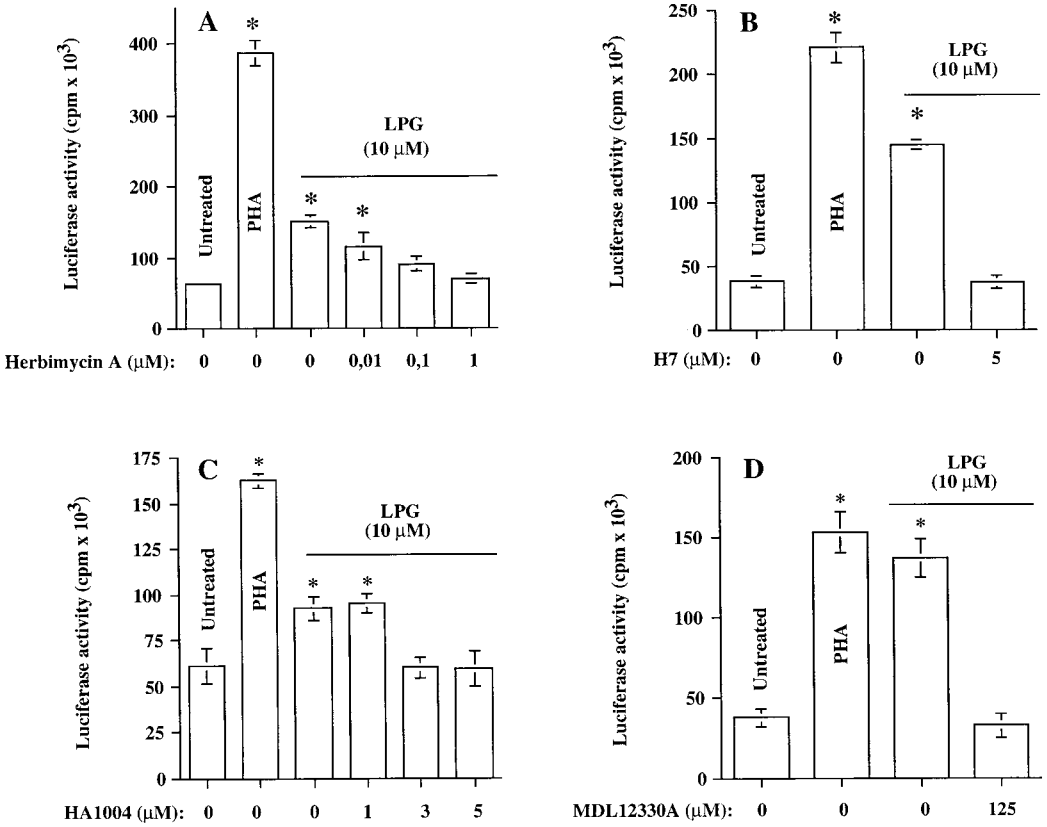

FIGURE 2. Involvement of PTK and PKA in LPG-mediated activation of HIV-1 LTR activity. 1G5 cells were pretreated with the indicated concentrations of herbimycin A (0.01, 0.1, and 1 μM; *A*), H7 (5 μM; *B*), HA10004 (1, 3, and 5 μM; *C*), or MDL-12,330A (125 μM; *D*) before incubation for 24 h with *L. donovani* LPG (10 μM). Cells lysates were evaluated for luciferase activity by scintillation counting. The results shown are the mean ± SD of three determinations. Asterisks indicate significant differences from untreated 1G5 cells ($p < 0.01$).

pretreating 1G5 cells with H7 at a concentration sufficient to inhibit PKA (5 μM) as well as partially inhibit PKC and PKG (Fig. 2*B*). To discriminate the exact role played by PKA, PKC, or PKG, 1G5 cells were next pretreated with HA1004, a serine/threonine kinase inhibitor that preferentially inhibits PKA (K_i = 2.3 μM) and PKG (K_i = 1.3 μM) over PKC (K_i = 40.0 μM). The *L. donovani* LPG-induced activation of HIV-1 transcription was totally abolished by pretreatment with HA1004 at concentrations sufficient to inhibit PKA and PKG (3 and 5 μM; Fig. 2*C*), but not PKC. Thus, these observations strongly support the implication of PKA and/or PKG in *L. donovani* LPG-mediated up-regulation of HIV-1 LTR-dependent gene activity. The pivotal role of the PKA-dependent signaling pathway in LPG-induced HIV-1 LTR activation has been clearly established with the use of specific cAMP inhibitors (MDL12330A) (49). At a concentration known to completely abolish cAMP activity (125 μM), HIV-1 transcription was completely antagonized in response to LPG stimulation (Fig. 2*D*).

It has been previously shown that *L. donovani* and LPG can alter Ca^{2+} homeostasis and induce Ca^{2+} influx in monocytic cells (50) (M. Olivier and S. J. Turco, unpublished observations). To evaluate whether Ca^{2+}-dependent signaling events could be involved in the effect mediated in T cells by *L. donovani* LPG, we tested the abilities of the Ca^{2+} chelator BAPTA (5 μM) and the Ca^{2+}/calmodulin inhibitor W7 (5, 10, and 20 μM) to modulate the action of *L. donovani* LPG on HIV-1 LTR activity. Treatment of 1G5 cells with BAPTA or W7 at a concentration of 5 μM before the addition of *L. donovani* LPG totally abrogated the activating effect on HIV-1 LTR transcription (Fig. 3, *A* and *B*). These results indicate that Ca^{2+} and calmodulin are important elements in the intracellular signaling events that are initiated in T cells by LPG.

The transcription factor NF-κB has been shown to be a major constituent in HIV-1 LTR regulation (51). Thus, it was of interest to determine whether the NF-κB binding site located within the HIV-1 LTR sequence was involved in the LPG-induced activation of HIV-1 LTR-driven gene expression. This possibility was tested using Jurkat E6.1 cells transiently transfected with a wild-type HIV-1 LTR-driven luciferase molecular construct and its NF-κB-mutated version before treatment with LPG. As depicted in Figure 4, PHA, TNF-α, and NaB were all potent activators of HIV-1 LTR transcription. On the other hand, PHA and TNF-α, which are known to activate HIV-1 LTR transcription via an NF-κB-dependent signaling pathway, were not able to do so in Jurkat E6.1 cells transfected with the NF-κB-mutated construct. It should be noted that NaB still led to a threefold increase in HIV-1 LTR activity in cells transfected with pmκBLTRLUC. This result was expected due to the reported ability of NaB to enhance HIV-1 LTR activity in an NF-κB-independent fashion. Indeed, it has been postulated that NaB potentially acts on relaxation of chromatin by histone acetylation (52). More importantly, LPG was no longer capable of inducing HIV-1 LTR-driven gene activity in cells carrying the NF-κB-mutated version of the molecular construct. Therefore, these results suggest that LPG-mediated activation of HIV-1 LTR-dependent gene expression in T cells involved the κB binding sites located within the HIV-1 LTR.

To further substantiate the putative role played by the NF-κB region in the LPG-mediated increase in HIV-1 LTR-driven activity, we next performed band-shift assays using a NF-κB consensus binding site in the presence of nuclear extracts from 1G5 cells untreated or treated with LPG. A noticeable shift was observed in

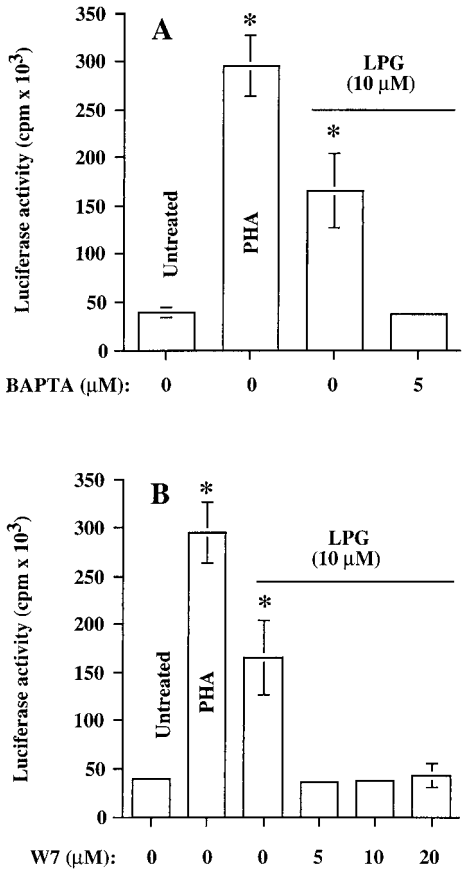

FIGURE 3. Effects of Ca^+ and calmodulin antagonists on LPG-induced activation of HIV-1 LTR-driven activity. 1G5 cells were pretreated with the calcium chelator BAPTA (5 μM; *A*) or the calmodulin antagonist W7 (5, 10, and 20 μM; *B*) before incubation for 24 h with LPG (10 μM). The cell lysates were evaluated for luciferase activity by scintillation counting. The results shown are the mean ± SD of three determinations. Asterisks indicate significant differences from untreated 1G5 cells ($p < 0.01$).

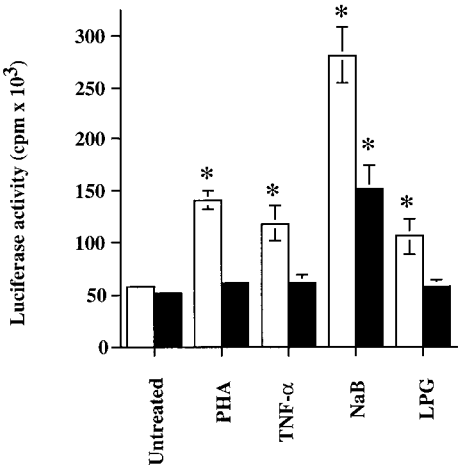

FIGURE 4. LPG induction of HIV-1 LTR activity requires intact NF-κB binding sites. Jurkat E6.1 were transiently transfected with either wild-type (empty bars) or NF-κB-mutated (filled bars) HIV-1 LTR-dependent luciferase constructs. Next, cells were either left untreated or were treated for 24 h with PHA (3 μg/ml), TNF-α (2 ng/ml), NaB (1 mM), or LPG (10 μM). The cell lysates were evaluated for luciferase activity by scintillation counting. The results shown are the mean ± SD of triplicate samples. Asterisks indicate significant differences from untreated transfected Jurkat E6.1 cells ($p < 0.01$).

nuclear extracts from LPG-treated cells (Fig. 5, *lane 3*). The unlabeled wild-type NF-κB oligomer completely inhibited LPG-induced binding, while no competition could be seen with unlabeled mutant NF-κB oligonucleotide, demonstrating that the binding was NF-κB specific (*lanes 4–7*).

Finally, further proof of the importance of the NF-κB sequence in LPG-mediated activation of HIV-1 transcription was provided by the use of the pκB-TATA-LUC vector, which consists of the luciferase reporter gene placed under the control of a minimal promoter as well as HIV-1 NF-κB elements (41). As depicted in Figure 6, LPG treatment of Jurkat cells transfected with this construct led to a nearly fourfold increase in luciferase activity. Moreover, treatment of these transfected cells with sodium salicylate, an inhibitor of NF-κB (53), almost completely abrogated LPG-dependent activation of transcription.

Discussion

Leishmania is highly prevalent in many areas of the world, and it has been found to be an opportunistic infection in immunosuppressed individuals, including patients infected with HIV-1 (54). Recent figures demonstrate that as many as 3 to 7% of individuals infected with HIV-1 in southern Europe develop leishmaniasis (55). The results from these epidemiologic studies coupled with our previous observation indicating that *L. donovani* and its major surface molecule LPG up-regulate virus replication in monocytoid cells (31) have led us to introduce the concept that *Leishmania* can act as a cofactor in the pathogenesis of HIV-1 infection (22). Our previous findings and the high incidence of serious complications associated with this parasitic infection in HIV-1-infected patients have been the compelling force for the present experiments, which were aimed at investigating the putative molecular interactions between these two micro-organisms. These studies were conducted in CD4-positive T cell lines because they represent the primary target for HIV-1, as indicated by the fact that such newly infected cells are thought to produce >99% of circulating virions in infected individuals (56).

In this study we demonstrate for the first time that the *L. donovani* major surface molecule LPG can stimulate the HIV-1 LTR in CD4-positive cells of T cell origin. This up-regulation of HIV-1 LTR activity was seen 24 h after the addition of *L. donovani* LPG, thereby suggesting a direct effect on HIV-1 LTR-dependent gene expression. We attempted to identify the various intracellular second messengers implicated in the LPG-mediated activating effect on HIV-1 LTR sequence using a variety of specific chemical inhibitors and appropriate plasmids. We have shown that both PTK- and PKA-dependent pathways play a pivotal role in the LPG-induced activation of HIV-1 LTR transcription. Indeed, their inhibition by specific inhibitors was found to totally abrogate the LPG-mediated up-regulating effect on the regulatory elements of HIV-1. The divalent cation Ca^{2+} and the Ca^{2+}-binding protein calmodulin were also found to serve as major physiologic effectors for the *L. donovani* LPG-induced effect, because the Ca^{2+} and calmodulin inhibitors BAPTA and W7 inhibited activation of HIV-1 LTR by LPG. This observation was expected considering that a vast array of molecules involved in signal transduction (e.g., kinases and phosphatases) are dependent upon Ca^{2+} regulation (57). Our previous studies have indicated that Ca^{2+} mobilization is rapidly induced in leukocytes by the protozoan parasite *Leishmania* and its surface molecule LPG as well as by its structural LPG components (50) (M. Olivier and S. J. Turco, unpublished observations). Therefore, it was not surprising to discover that Ca^{2+} and calmodulin are

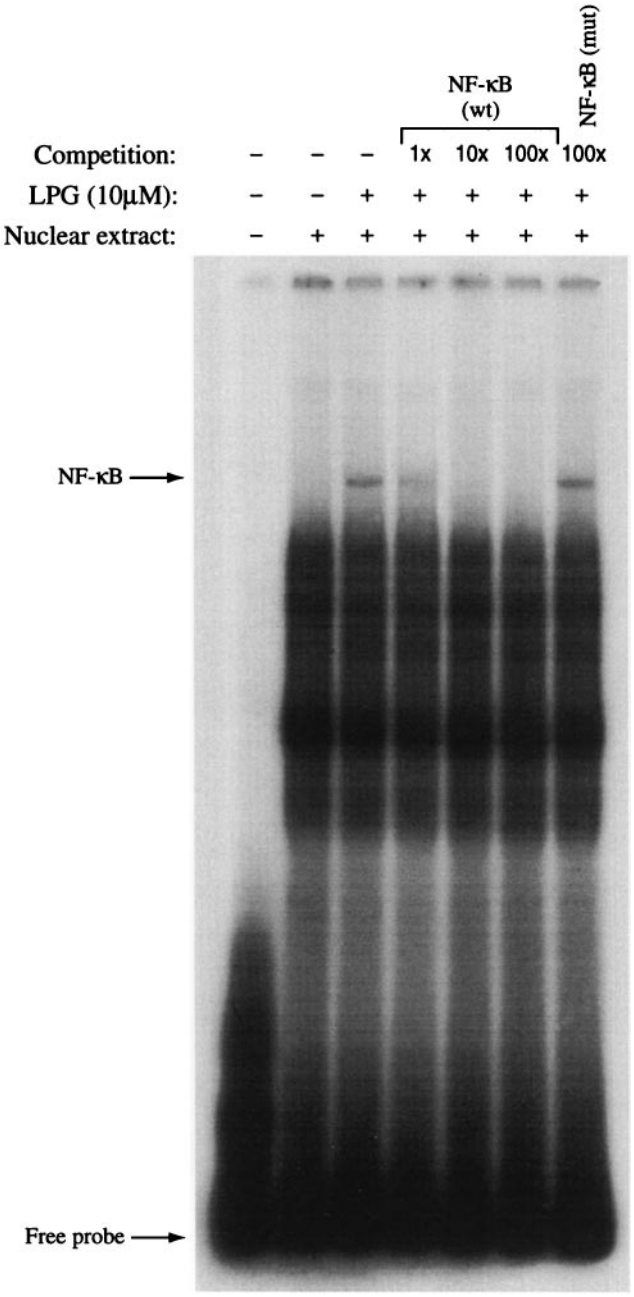

FIGURE 5. LPG-mediated nuclear translocation of NF-κB. 1G5 cells were incubated for 1 h in the absence or the presence of *L. donovani* LPG (10 μM). Seven micrograms of each nuclear extract were incubated with ^{32}P end-labeled synthetic double-stranded NF-κB probe. *Lane 1*, Free probe; *lane 2*, untreated 1G5; *lane 3*, 1G5 cells incubated with 10 μM of *L. donovani* LPG. *Lanes 4* to *6*, the κB-specific band is competed away by 1-, 10-, and 100-fold molar excesses of an unlabeled synthetic wild-type NF-κB cold competitor, while no similar competition could be observed with 100-fold molar excess of an unlabeled mutant NF-κB oligonucleotide (*lane 7*). The position of the specific complex bound by the κB site probe is indicated by an arrow on the *left side*.

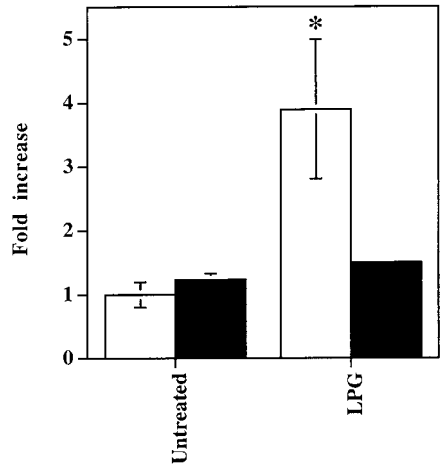

FIGURE 6. Induction by LPG is mediated via HIV-1 κB consensus sequence and is sensitive to sodium salicylate. Jurkat E6.1 cells were transiently transfected with the pκB-TATA-LUC vector and were either left untreated (empty bars) or were pretreated (filled bars) for 1 h with sodium salicylate (2.5 mM). Next, cells were either left untreated or were treated with LPG (20 μM). The cell lysates were evaluated for luciferase activity by scintillation counting. The results shown are the mean ± SD of triplicate samples and are presented as the fold increase over the value in the untreated sample. Asterisks indicate significant differences from untreated 1G5 cells ($p < 0.01$).

key elements in LPG-mediated activation of HIV-1 LTR-driven reporter gene activity.

The *L. donovani* LPG-induced signaling cascade was ultimately found to mediate its effect through the NF-κB binding region. This statement is based on transfection experiments using Jurkat E6.1 cells transfected with HIV-1 LTR-driven luciferase vector carrying either wild-type or mutated κB binding sites. In this set of experiments, LPG-mediated HIV-1 LTR activation was completely inhibited by mutations of the double NF-κB binding sites, thereby directly implicating the NF-κB transcription enhancer element on the HIV-1 LTR in the observed phenomenon. Additional evidence came from experiments with the pκB-TATA-LUC construct in which a similar induction of transcription was observed in the presence of LPG, an induction that was sensitive to sodium salicylate. The results of an electromobility shift assay further support the idea that *L. donovani* LPG leads to activation and nuclear translocation of NF-κB. Even though no direct proof has been provided to directly implicate NF-κB, our results suggest of the participation of this transcription factor complex in the signaling cascade mediated by LPG.

Several protein kinases, including PKA, PKC, and PTK, as well as the Ca^{2+}-dependent phosphatase calcineurin, have been reported to play an important role in the regulation of NF-κB (58, 59). Thus since most HIV-1 LTR inducers have been shown to act either completely or partially via the NF-κB transcription factor (39, 60–63), it is plausible that the inhibitory capacity of the diverse second messenger antagonists that we used to block LPG-induced HIV-1 LTR activation may have acted on signaling events necessary for NF-κB translocation. In addition, it is well established that cellular stimulation by component from bacteria such as LPS or lipoarabinomannan of *M. tuberculosis* are potent inducers of NF-κB activation (18, 64). These data are supportive of our findings indicating that *L. donovani* LPG is up-regulating HIV-1 LTR activity mainly via an NF-κB-dependent signaling pathway.

Previous studies have shown that the NF-κB complex is sequestered in the cytoplasm as an inactive precursor complexed with a repressor termed IκBα that masks the nuclear localization signal of the transcriptional complex (65, 66). Phosphorylation of IκBα on both serine 32 and 36 residues leads to the release and degradation of IκBα (67), thereby allowing the rapid translocation of NF-κB from the cytoplasm to the nucleus and binding on regulatory regions of genes bearing the NF-κB binding sites (65, 66, 68). It should be noted that purified PKA was shown to be sufficient to

phosphorylate and dissociate IκBα, allowing the NF-κB transcription factor to bind to its DNA sequence (68, 69). PKA-dependent NF-κB activation has been shown to be inducible by calcium ionophore (64, 70), thus providing a suggestive link for the involvement of LPG-inducible Ca^{2+}-dependent events in HIV-1 LTR activation. The inhibition of LPG-mediated activation of HIV-1 LTR transcription by the PTK antagonist herbimycin A is not unanticipated based on the postulate that NF-κB might be activated by PTK such as $p59^{fyn}$ (71).

Another important finding of our report is that one of the intramembrane structural components of LPG, named core-PI, could also act as a potent inducer of HIV-1 LTR activity. This observation is of prime importance, since core-PI is the only detectable LPG moiety present at the surface of the intracellular amastigote form of the parasite under which the infection progressed within the host. In addition, repeated units of this surface constituent of *Leishmania* could be encountered on the surface of infected mononuclear phagocytic cells (32, 72). Therefore, cell-to-cell interaction during antigenic presentation between *Leishmania*-infected macrophages and T cells may lead to the physical binding of *Leishmania* molecules to CD4-positive T lymphocytes infected with HIV-1. LPG can thus act as an exogenous stimulator of viral chromosomal DNA synthesis, since the majority of infected $CD4^+$ T cells are known to harbor a transcriptionally latent HIV-1 provirus (73).

The exact mechanism by which LPG could induce HIV-1 LTR activation remains unclear. It is uncertain whether receptor-dependent or -independent LPG/cell interaction is the basis for this transcription factor induction. In fact, it might be argued that membrane intrusion of LPG (receptor-independent) is the inducing mode based on FACS results showing that most LPG/cell interaction occurs at 37°C. However, although membrane insertion of LPG is a well-documented phenomenon, receptor binding of LPG has been equally well described (reviewed in Ref. 74). Since equal concentrations of LPS were not shown to activate HIV-1 LTR activity in 1G5 cells (data not shown), mere nonspecific membrane insertion of lipidic molecules does not seem in these circumstances to account for LTR activation and thus suggests a certain specificity in the mode of action of LPG, which might lend credence to a receptor-dependent mechanism. Hence, the cell binding of LPG at 4°C might be the relevant interaction for activation through the NF-κB binding site, although being much weaker than the 37°C counterpart. Further analyses will be required of these two interesting possibilities.

Easterbrook et al. have recently demonstrated that rapid association of LPG to the surface membrane of human T lymphoid cells could inhibit HIV-1-induced syncytia formation (44). Since virus-mediated multinucleated giant cell formation has been proposed as an important event in the pathogenesis of HIV-1 infection (75–77), they postulated that *Leishmania* LPG represents an agent that could inhibit progression of the disease. However, our past (31) and present observations indicate that caution must be taken in designing therapeutic strategies that would be based on the use of LPG to modulate the fate of HIV-1 infection.

In summary, *L. donovani* LPG and its core-PI moiety activate HIV-1 LTR transcription in T cells via the NF-κB motif. This suggests that exposition of HIV-1-carrying T cells to parasite-infected mononuclear phagocytes might be sufficient to trigger activation of latent provirus DNA. Our observations further reinforce the fact that members of the *Leishmania* genus might be envisaged as cofactors responsible for the switch from the clinical latency state to HIV-1-related diseases. A better understanding of the cellular, biochemical, and molecular events that occur following coinfection with *Leishmania* and HIV-1 may permit the development of more appropriate and efficient ways to control the diseases associated with the dual infection.

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