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*J Immunol* 1998; 161:268-276;
http://www.jimmunol.org/content/161/1/268
Both PU.1 and Nuclear Factor-κB Mediate Lipopolysaccharide-Induced HIV-1 Long Terminal Repeat Transcription in Macrophages

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We recently reported that LPS stimulation of monocyteic cells leads to the activation of PU.1, a member of the Ets family of transcription factors. Phosphorylation of PU.1 by protein kinase CK2 was found to up-regulate its trans-activation function, but not its DNA binding activity. Previous studies suggested that Ets proteins could bind to NF-κB motifs at the tetrameric core sequence TTCC. In macrophages, LPS-inducible HIV-1 gene expression is mediated in part by binding of NF-κB to identical tandem binding sites located within the long terminal repeat (LTR). Thus, we performed additional studies to determine whether PU.1 also played a role in regulating HIV-1 gene expression in macrophages. Our functional studies revealed that activation of the HIV-1 LTR in LPS-stimulated cells requires both NF-κB and PU.1. Extensive mutagenesis of the HIV-1 LTR revealed that PU.1-dependent activation requires the Ets motif within the upstream NF-κB site, whereas NF-κB itself binds to the downstream site. We also found that insertion of five additional nucleotides between the NF-κB sites abolished LPS inducibility, suggesting a direct interaction between factors that bind these sites. Lastly, we found that mutation of PU.1 at serine 148, which prevents its phosphorylation by CK2, blocked its ability to activate the HIV-1 LTR in response to LPS. These effects were promoter specific because PU.1 did not affect LPS-inducible activation of a distinct NF-κB-dependent promoter. While these data do not demonstrate direct binding of PU.1 to the HIV-1 LTR, they illustrate a novel role for PU.1 in activation of the HIV-1 LTR by LPS. The Journal of Immunology, 1998, 161: 268–276.

A ctivation of HIV-1 gene expression can be induced in human monocytes by both LPS and cytokines (1, 2). Inducible viral gene expression is mediated by transcription factors that bind to the long terminal repeat (LTR).3 Inducible activation of HIV-1 gene expression in macrophages has been reported to be predominantly mediated by the transcription factors NF-κB (1, 3, 4), SPI (5, 6), and C/EBP (7, 8). NF-κB is a dimer comprised of several related family members, including p50, p52, p65 (RelA), c-Rel, and RelB (reviewed in Ref. 9). NF-κB binds to the decameric DNA consensus sequence GGGGNNYYYCC and thereby activates transcription. The LPS-inducible element within the HIV-1 LTR contains two tandem copies of the NF-κB binding sequence GGGACCTTCC (3). Deletion or mutation of both NF-κB binding sites within the HIV-1 LTR abolished LPS-inducible viral gene expression (1, 4, 10), although recent studies have suggested that NF-κB alone is not sufficient for transcriptional activation of the LTR (11, 12).

Several lines of evidence prompted us to examine whether the tandem NF-κB motifs within the HIV-1 LTR bind additional factors that mediate LPS-stimulated HIV-1 transcription in monocytes. Seth et al. noted that the pyrimidine-rich sequence TTCC, located within the 3’ half of the NF-κB consensus motif and base-paired with GGAAG on the complementary strand, is the core binding site recognized by members of the Ets family of transcription factors (13). These authors also presented indirect evidence that Ets proteins could regulate HIV-1 LTR function. More recently, Bassuk et al. reported that Ets proteins could physically interact with the p50 NF-κB subunit in a DNA-dependent manner and that this interaction was required for synergistic transcriptional activation of the HIV-1 LTR (14). Furthermore, Flory et al. suggested that Raf-1-dependent activation of the HIV-1 LTR in T cells was mediated by the binding of GABP, and not NF-κB, to the tandem NF-κB motifs (15). GABP is a heterodimeric transcription factor consisting of an Ets-like α-chain and a β-chain that contains ankyrin-like repeats. These findings led us to address the possibility that PU.1, a major Ets protein found in monocyteic cells, could mediate LPS-inducible HIV-1 LTR function.

PU.1 has been identified as a transcription factor that regulates several myeloid-specific genes, including IL-1β, the macrophage CSF receptor, the macrophage scavenger receptor, the human high affinity IgG receptor (FcγR1b), CD11b, and the common β subunit of the IL-3, granulocyte-macrophage CSF, and IL-5 receptors (reviewed in Ref. 16). PU.1 has also been shown to control gene expression via interaction with other transcription factors. In B cells, PU.1 promotes binding of the nuclear factor, PIP (also termed NF-EM5 and IRF-4), to an adjacent site on the Ig κ light chain 3’ enhancer (17). This interaction between PU.1 and PIP required phosphorylation of serine residue 148 on PU.1, a known site for phosphorylation by protein kinase CK2 both in vitro and in

1 This work was supported in part by National Institutes of Health Grant RO1GM57053 (to M.J.F.).

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3 Abbreviations used in this paper: LTR, long terminal repeat; CAT, chloramphenicol acetyl transferase; GABP, GA-binding protein; EL, Ets-less; 3’EL, mutations at the downstream NF-κB site that abolished Ets protein binding; 5’EL, mutations at the upstream NF-κB site that abolished NF-κB binding; dbl-EL, mutations at both Ets and NF-κB sites; IL-2R, IL-2R, α-chain.
vivo (18). We recently demonstrated that the trans-activation function of PU.1 was up-regulated in LPS-stimulated murine macrophages (19, 20). This finding was notable because PU.1 is constitutively expressed in macrophages, and its DNA-binding activity is unchanged following LPS stimulation. We also found that LPS stimulation resulted in the phosphorylation of PU.1 at serine 148, an event that was required for enhancement of its trans-activation function. In this study we tested the hypotheses that PU.1 could regulate LPS-inducible activation of the HIV-1 LTR and that phosphorylation of PU.1 at serine 148 by CK2 was required for this regulation. In LPS-stimulated macrophages, the phosphorylation of PU.1 at serine 148 by CK2 was required for enhancement of its trans-activation function. We also found that LPS stimulation resulted in the phosphorylation of PU.1 at serine 148, which is unchanged following LPS stimulation. We also found that LPS stimulation resulted in the phosphorylation of PU.1 at serine 148, which is unchanged following LPS stimulation.

Materials and Methods

Cell lines
MonoMac 6-transformed human monocytic cells were maintained in AIM-V serum-free medium (Life Technologies, Gaithersburg, MD). Cells were cultured at 37°C in the presence of 5% CO2 in a humidified incubator. The HT1080/CD14 human fibroblast cell line was previously described (21). HT1080/CD14 cells were maintained in high glucose DMEM culture medium (BioWhittaker, Walkersville, MD) supplemented with 10% heat-inactivated FBS (HyClone Laboratories, Logan, UT), 10 mM HEPES (BioWhittaker), 2 mM L-glutamine (BioWhittaker), 100 U/ml penicillin (BioWhittaker), and 100 µg/ml streptomycin (BioWhittaker). These cells were discarded after 1 mo in culture, and fresh cultures were prepared from frozen stocks. The murine macrophage RAW264.7 cell line was obtained from the American Type Culture Collection (Rockville, MD). RAW264.7 cells were maintained in DMEM culture medium with supplements as described above. LPS levels in all medium components were <10 pg/ml (final concentration). Cells were stimulated by adding LPS (Escherichia coli serotype 055:B5, Sigma, St. Louis, MO) to a final concentration of 1 µg/ml for the periods indicated in the text.

Plasmids
The IκB-2N dominant-negative mutant IκB expression plasmid, generated by site-directed mutagenesis to replace the serine codons at positions 32 and 36 with alanine codons, was provided by Dr. John Hiscott (McGill University, Montreal, Quebec, Canada) and was previously described (22). The Tat expression plasmid pCMV Tat, which contains a cDNA including both coding exons of HIV-1 Tat under the transcriptional control of the CMV immediate early promoter, was provided by Dr. Michael Green (University of Massachusetts Medical Center, Worcester, MA) and was previously described (23). Full-length native and mutant murine PU.1 cDNAs cloned into the eukaryotic expression plasmid PECE were provided by Dr. Charles Van Beveren (Sidney Kimmel Cancer Center, San Diego, CA) and were previously described (18). Expression of these cDNAs was under the control of the SV40 promoter and enhancer. The mutant PU.1 cDNA (termed S148A) was generated by site-directed mutagenesis to replace the serine codon at position 148 with an alanine codon.

The reporter plasmids pILIC-CAT and pdI-NF-CAT were described previously (19, 20). pILIC-CAT contains a portion of the LTR from HIV-1 LAV, including the entire U3 region and extending to position 81 relative to the start site of transcription. This primer also contained a 23-bp tag sequence as 5′-TACTTCGATCGATCGGTCAAG-3′ corresponding to the HIV-1 NF-κB binding site. The pCMV5 contains the CMV immediate early promoter and human growth hormone transcriptional termination and polyadenylation sequences (26). A Rous sarcoma virus-luciferase reporter plasmid was provided by Dr. Stefan Doerre (Boston University School of Medicine) and was previously described (28).

Oligonucleotide-directed mutagenesis
Two rounds of PCR were used to generate four reporter plasmids containing 6-bp (TTCGAA) substitution mutations sequentially positioned between nucleotide positions −110 and −86 of the HIV-1 LTR. The first round of PCR used a 5′ primer (5′-TACTTCGATCGATCGGTCAAGCGACCTTCAAGAAGCTCAGTACATCG-3′) corresponding to the HIV-1 NF-κB motifs and the LTR mutant reporter plasmids. Shown are the tandem decameric linker-scanning mutants (m87, m93, m99, and m105), the EL mutants (5′ EL, 3′ EL, and dbl-EL), the PZ mutants (3′ PZ and dbl-PZ), and the 5-bp spacer mutants. Mutated nucleotides are indicated in bold.

Transient transfection and reporter assays
The HT1080/CD14 line was engineered by stably transfecting HT1080 human fibosarcoma cells with an expression plasmid containing the human CD14 cDNA and was previously described (21). The HT1080/CD14 cells were transfected by lipofection as previously described (20). The RAW264.7 cells were transfected by the same method with the following modifications. Briefly, RAW264.7 cells were cultured at a density of 2 × 105 cells 2 days before transfection in 35-mm tissue culture dishes (Costar, Cambridge, MA). Before transfection, cells were washed and cultured in serum-free DMEM supplemented with 10 mM HEPES (1 ml/35-mm dish). Plasmid DNA (3 µg of total DNA) was ethanol precipitated and resuspended in serum-free medium at a final concentration of 1 µg/ml. For each transfection, 3 µg of total DNA was added to 100 µl of serum-free medium and mixed with 6 µl of Lipofectamine reagent (Life Technologies) and an additional 100 µl of serum-free medium. The Lipofectamine/DNA mixture was incubated for 15 min at room temperature. Following incubation, 800 µl of serum-free medium was slowly overlaid onto the Lipofectamine/DNA complexes and allowed to incubate for an additional 15 min at room temperature. The entire Lipofectamine/DNA mixture (1.1 ml) was then
incubated with the cells for 5 h. After 5 h, the cells were diluted with 2 ml of medium containing 20% FBS and then incubated overnight. The next day, the culture medium was replaced with fresh DMEM. LPS was then added to some dishes at a final concentration of 1 μg/ml as indicated in the text. Cells were cultured for an additional 24 to 48 h as indicated in the text. Cells were then harvested and lysed, and the protein concentrations were determined. Lysates (40 μg each of lysates prepared from each transfection) were assayed in duplicate for CAT activity using the two-phase fluor diffusion assay exactly as previously described (29). CAT activity is measured as the average slope (time vs accumulation of acetylated chloramphenicol) ± SEM. All transfection experiments were repeated at least three times using different plasmid preparations, and a single representative experiment is shown.

MonoMac 6 cells were seeded at a density of 2 – 3 × 10⁵ cells/ml 3 days before transfection. These cells (5 × 10⁶ cells) were transfected by electroporation in 1 ml of serum-free medium. A Life Technologies Cell-Porator was used with settings of 800 μF and 300 V. All electroporations were performed at room temperature. Fifteen micrograms of reporter plasmid was used for each transfection. When included, 2.5 μg of the Tat expression plasmid or 15 μg of the dominant negative mutant IκB plasmid were used. Additional pSV2d-CAT DNA was added to bring the total amount of DNA used in each transfection to 30 μg. Transfections were performed in duplicate and then split into two aliquots, one of which was treated with LPS (1 μg/ml) as indicated in the text. Cells were harvested 24 h after treatment and lysed, and intracellular CAT activity was measured as described above.

Results

**PU.1 is required for LPS-inducible activation of the HIV-1 LTR**

Based on evidence that the Ets-like protein GABP α could regulate HIV-1 LTR activation in T cells (15), we asked whether PU.1 could serve a similar function in LPS-stimulated cells. HT1080/CD14 cells (which naturally lack PU.1) were cotransfected with a PU.1 expression plasmid and either a CAT reporter under the control of the HIV-1 LTR or an LTR in which the tandem NF-κB motifs were deleted (positions −117 to −70). Following transfection, the cells were stimulated with LPS and were harvested 48 h later. Duplicate transfected cell cultures were harvested without LPS stimulation. As shown in Figure 2A, both reporter constructs did not express CAT activity in the absence of either PU.1 or LPS stimulation. In contrast, cotransfection with the PU.1 expression plasmid was able to induce a small amount of CAT activity in an NF-κB-independent manner. Most strikingly, the LTR-CAT reporter plasmid could not be activated by LPS alone, whereas cells that were cotransfected with both the reporter plasmid and the PU.1 expression plasmid expressed high levels of CAT activity following LPS stimulation. The mutant LTR reporter plasmid lacking the tandem NF-κB sites did not show any increase in CAT activity following LPS stimulation in the presence or the absence of PU.1. These data suggest that PU.1 can activate the HIV-1 LTR in LPS-stimulated cells, and that the tandem NF-κB motifs are required for this effect. The inability of LPS to induce CAT expression in the absence of PU.1 was not due to a lack of NF-κB, because our previous studies demonstrated the presence of nuclear NF-κB in LPS-stimulated HT1080/CD14 cells (21). Western blot analysis showed that the NF-κB subunits p50 and p65 (RelA) were present in nuclear extracts from these cells (data not shown). Experiments reported below will show that this nuclear NF-κB is capable of activating transcription of a PU.1-independent promoter.

The inducibility of the wild-type HIV-1 LTR in unstimulated HT1080/CD14 cells was further confirmed by cotransfection of the reporter plasmid with an expression plasmid encoding the viral *trans*-activator protein Tat. We found that Tat could induce substantial HIV-1 LTR expression in unstimulated HT1080/CD14 cells (Fig. 2B), thereby demonstrating that the HIV-1 LTR is functional in these cells. Similar results were observed in the MonoMac 6 cells (data not shown). We also observed that a heterologous promoter containing a single copy of the HIV-LTR NF-κB site could be activated in HT1080/CD14 cells by LPS (data not shown).

**Mutation of PU.1 binding sites within the NF-κB motifs abolishes LPS inducibility of the HIV-1 LTR**

The 3′ half of each HIV-1 LTR NF-κB motif contains a core Ets binding site (GGAAT in the reverse orientation). Several studies have suggested that Ets proteins can bind to the LTR NF-κB motifs at these core sequences (13–15). To identify sequences within the NF-κB motifs that were required for PU.1-dependent expression of the LTR in LPS-stimulated cells, we tested a series of reporter plasmids containing specific mutations within the 5′ and 3′ halves of each NF-κB motif. These linker-scanning mutations were generated as described above and are shown in Figure 1. These mutant reporter plasmids were transfected into MonoMac 6 cells, stimulated with LPS, and then harvested 48 h later. These cells constitutively express high levels of endogenous PU.1 (data
FIGURE 3. Mutation of putative PU.1 binding sites within the NF-κB tandem repeats abolishes LPS inducibility of the HIV-1 LTR. A, Wild-type (wt hiv) and mutant (m87, m93, m99, and m105) HIV-1 LTR reporter plasmids were transiently transfected into the human monocytic cell line MonoMac 6. See Figure 1 for a description of the individual mutant plasmids. Following transfection, cells were stimulated with 1 μg/ml LPS (dark-striped bars) for 24 h. Unstimulated (light-striped bars) duplicate transfected cell cultures were also harvested. Cells were then harvested and lysed, and intracellular CAT activity was measured as described above. CAT activity is expressed as the slope ± SEM. B, Wild-type and mutant reporter plasmids were transiently transfected into the murine RAW264.7 macrophage cell line, stimulated with 1 μg/ml LPS (dark-striped bars), and then harvested 24 h later. Unstimulated (light-striped bars) duplicate transfected cell cultures were also harvested. Cells were then lysed, and intranuclear CAT activity was measured. CAT activity is expressed as the slope ± SEM. C, A PU.1 expression plasmid and either wild-type or mutant HIV-1 LTR reporter plasmids were transiently cotransfected into HT1080/CD14 cells. Following transfection, cells were stimulated with 1 μg/ml LPS (dark-striped bars) and were harvested 24 h later. Unstimulated (light-striped bars) duplicate transfected cell cultures were also harvested. Cells were then lysed, and intracellular CAT activity was measured. CAT activity is expressed as the slope ± SEM.

not shown), As shown in Figure 3A, we found that mutations at either NF-κB 5′ half-site (m105 and m93) could still be activated by LPS stimulation. In contrast, mutations at either NF-κB 3′ half-site (m99 and m87, which destroy the putative PU.1 binding site) could not be activated by LPS. The same results were obtained using RAW264.7 macrophages (Fig. 3B). These cells also constitutively express high levels of endogenous PU.1 (20). We subsequently cotransfected the mutant reporter plasmids along with a PU.1 expression plasmid into HT1080/CD14 cells, stimulated the cells with LPS, and then harvested the cells 24 h later. We again found that mutation of either NF-κB 3′ half-site (m99 and m87) abolished LPS inducibility (Fig. 3C), thus confirming the results obtained using the MonoMac 6 and RAW264.7 cells. Together, these data demonstrate that at least one functional Ets motif contained within the NF-κB 3′ half-site is necessary for LPS-inducible expression of the HIV-1 LTR. In contrast to data obtained using the monocytic cells, the LTR-CAT reporter construct alone was not responsive to LPS stimulation in HT1080/CD14 cells (Fig. 3C), presumably due to the absence of endogenous PU.1 in these cells. Because LPS is known to activate NF-κB in these cells (30), this finding demonstrates that NF-κB alone is insufficient to direct activation of the viral LTR. These data do not argue against a role for NF-κB in LPS-inducible expression of the HIV-1 LTR, but suggest that PU.1 is also required for LPS responsiveness. Data directly demonstrating a role for NF-κB in LPS responsiveness of the HIV-LTR will be presented later in this section.

Orientation of PU.1/Ets and NF-κB binding within the HIV-1 LTR

The results described above showed that both NF-κB 3′ half-sites within the tandem repeat are required, but did not indicate which factor requires which site. Previous studies demonstrated that only one of the tandem NF-κB binding sites is likely to be occupied at any one time (31), suggesting that the adjacent site could be bound by either PU.1 or a PU.1-inducible Ets protein. To determine which site was bound by such an Ets protein, we used a panel of Ets-less (EL) mutations in which the TTCC core motif was mutated to CTCC. This generated an NF-κB binding site that did not have the capacity to bind PU.1 or other Ets proteins. HIV-1 LTR reporter plasmids were generated in which the upstream, downstream, or both Ets sites were mutated. These reporter plasmids were transfected into RAW264 cells, stimulated with LPS, and harvested 24 h later. As shown in Figure 4A, we found that mutations at the downstream NF-κB site that abolished Ets protein binding (3′EL) could still be activated by LPS stimulation. In contrast, mutations at the upstream NF-κB site that abolished Ets protein binding (5′EL) could not be activated by LPS. Similarly, mutations at both NF-κB sites (dbl-EL) could not be activated by LPS. These data demonstrate that NF-κB binds to the downstream NF-κB site, whereas an Ets protein binds to the upstream site. The same results were obtained when these mutant reporter plasmids were cotransfected with a PU.1 expression plasmid into HT1080/CD14 cells (Fig. 4B). Although our data are consistent with the findings of Sweet et al., who used gel mobility shift assays to show that PU.1 bound to the upstream, but not to the downstream,
The conclusion that NF-κB bound to the downstream site was inconsistent with our result obtained using the m93 mutant, which showed that mutations designed to block NF-κB binding at this downstream site did not substantially block LPS induction of the LTR (Fig. 4, A and B). One explanation for this inconsistency is that m93 could still bind NF-κB. To test this possibility, we constructed an additional mutant reporter plasmid in which the downstream NF-κB site (GGGACTTTCC) was mutated to CTCACTTCCC (designated 3'PZ). These substitutions were previously shown to block NF-κB binding and function (1). The 3'PZ and m93 reporter plasmids were transfected into RAW264 cells, stimulated with LPS, and then harvested 24 h later. As shown in Figure 4C, we found that the m93 mutant could be activated by LPS stimulation, whereas the 3'PZ mutant could not be activated. Similarly, mutations at both NF-κB sites (dbl-PZ) could not be activated by LPS, consistent with results obtained using the dbl-EL mutant (Fig. 4, A and B). The same results were obtained when these mutant reporter plasmids were cotransfected with a PU.1 expression plasmid into HT1080/CD14 cells (data not shown). Together, our data suggest that LPS responsiveness of the m93 mutant results from the inability of this mutant to completely prevent NF-κB binding to the downstream site in the living cell. Subsequent experiments revealed that unlabeled competitor DNA containing a single copy of the same mutant NF-κB site used in the m93 mutant (CGAACTTCCC) could effectively compete for NF-κB binding in a gel-shift assay (data not shown), thus further supporting our contention that the m93 mutant could bind NF-κB, albeit with lower affinity.

A dominant-negative IκB mutant protein blocks LPS-inducible activation of the HIV-1 LTR

Our data demonstrate that PU.1 is required for LPS-inducible expression of the HIV-1 LTR in LPS-stimulated cells, but do not exclude the possibility that NF-κB is also required (i.e., that each factor is required, but only both are sufficient) for LPS-inducible expression. To determine whether NF-κB is also necessary to activate the HIV-1 LTR, we used a dominant-negative IκBα mutant protein to prevent dissociation of NF-κB from IκBα following LPS stimulation. RAW264.7 cells were cotransfected with the HIV-LTR reporter plasmid and a dominant-negative IκBα mutant expression plasmid. After transfection, cells were stimulated with LPS and were harvested 24 h later. Duplicate transfected cell cultures were harvested without LPS stimulation. As shown in Figure 5A, CAT expression was observed in cells transfected with the HIV-1 LTR reporter plasmid and stimulated with LPS. This LPS-inducible activation of the HIV-LTR was completely blocked by

\[ \text{PF.1 and NF-κB mediate HIV-LTR transcription} \]

\[ \text{FIGURE 4. PU.1 binds upstream of NF-κB within the HIV-1 LTR. A, Wild-type (wt hiv) and EL mutant (5'EL, 3'EL, and dbl-EL) HIV-1 LTR reporter plasmids were transiently transfected into RAW 264.7 cells. See Figure 1 for a description of the individual mutant plasmids. Following transfection, a portion of the cells was stimulated with 1 μg/ml LPS (dark-striped bars), and cells were harvested 24 h later. Unstimulated (light-striped bars) duplicate transfected cell cultures were also harvested and lysed, and intracellular CAT activity was measured. CAT activity is expressed as the slope ± SEM. B, A PU.1 expression plasmid (PU.1) and either wild-type (wt hiv) or EL mutant (5'EL, 3'EL, and dbl-EL) HIV-1 LTR reporter plasmids were transiently cotransfected with into HT1080/CD14 cells. See Figure 1 for a description of the individual mutant plasmids. Following transfection, a portion of the cells was stimulated with 1 μg/ml LPS (dark-striped bars), and cells were harvested 24 h later. Unstimulated (light-striped bars) duplicate transfected cell cultures were also harvested and lysed, and intracellular CAT activity was measured. CAT activity is expressed as the slope ± SEM. C, Wild-type (wt hiv) and mutant (m93, 3'PZ, and dbl-PZ) HIV-1 LTR reporter plasmids were transiently transfected into RAW 264.7 cells. See Figure 1 for a description of the individual mutant plasmids. Following transfection, a portion of the cells was stimulated with 1 μg/ml LPS (dark-striped bars), and cells were harvested 24 h later. Unstimulated (light-striped bars) duplicate transfected cell cultures were also harvested and lysed, and intracellular CAT activity was measured. CAT activity is expressed as the slope ± SEM. Similar results were obtained using HT1080/CD14 cells cotransfected with a PU.1 expression plasmid (data not shown).} \]
the dominant-negative IκBα mutant. Virtually identical results were obtained using LPS-stimulated HT1080/CD14 cells cotransfected with the PU.1 expression plasmid (Fig. 5B). The specificity of these results was confirmed by the demonstration that expression of neither a reporter gene under the control of the NF-κB-independent Rous sarcoma virus LTR nor a reporter gene under the control of the PU.1-dependent CD11b promoter was affected by the dominant-negative IκBα mutant (data not shown). Together, these results strongly support the conclusion that both PU.1 and NF-κB are required for LPS-induced activation of the HIV-1 LTR.

**Genetic evidence for PU.1/Ets interaction with NF-κB**

The tandem NF-κB binding sites within the LTR are located approximately 10 bp apart, and therefore one helical turn apart, on the DNA. This raised the possibility that PU.1 or a PU.1-inducible Ets protein could physically interact with NF-κB when bound to the LTR. To test this possibility, we constructed an additional mutant reporter plasmid in which the upstream and downstream NF-κB sites were separated by a 5-bp spacer. The spacer mutant reporter plasmid was transfected into RAW264 cells, stimulated with LPS, and then harvested 24 h later. As shown in Figure 6, we found that the spacer mutant could not be activated by LPS stimulation. The same results were obtained when this mutant reporter plasmid was cotransfected with a PU.1 expression plasmid into HT1080/CD14 cells (data not shown). This finding suggests that physical interaction between an Ets protein and NF-κB is required for activation of the LTR by LPS. Such an interaction is consistent with the findings of Bassuk et al., who showed that the p50 NF-κB subunit could physically bind to Ets proteins (14). These associations were mediated by interaction between the Rel homology domain and the Ets homology domain of the two proteins.

Additional control experiments were performed to demonstrate that the various mutations introduced into the HIV-1 LTR solely affected PU.1- and NF-κB-dependent regulation and did not affect the ability of this promoter to be regulated by the basal transcriptional machinery. The mutant reporter plasmids were cotransfected with the Tat expression plasmid into both RAW264 and HT1080/CD14 cells. The cells were then stimulated with LPS and were harvested 24 h later. In both cell lines, Tat was capable of activating reporter gene expression (data not shown), consistent with the PU.1- and NF-κB-independent nature of Tat action (33).

**Serine 148 on PU.1 is necessary for LPS-inducible activation of the HIV-1 LTR**

We previously showed that serine 148 was necessary for LPS-inducible expression of a PU.1-dependent reporter plasmid (20). Therefore, we hypothesized that the same residue would be important for PU.1-dependent activation of the HIV-1 LTR in LPS-stimulated cells. HT1080/CD14 cells were cotransfected with the HIV-LTR reporter plasmid and either a wild-type PU.1 expression plasmid or a mutant PU.1 expression plasmid in which the serine 148 codon was mutated to an alanine codon (S148A). After transfection, cells were stimulated with LPS and harvested 48 h later. Duplicate transfected cell cultures were harvested without LPS stimulation. As shown in Figure 7, LPS-inducible CAT activity was observed only when the HIV-1 LTR reporter plasmid was

**FIGURE 5.** A dominant-negative IκBα protein blocks LPS-inducible activation of the HIV-1-LTR. A, An expression plasmid encoding a dominant-negative IκBα mutant was used to prevent the dissociation of IκBα from NF-κB following LPS stimulation. RAW264.7 cells were transiently cotransfected with the HIV-1 LTR CAT reporter plasmid (wt hiv) and a dominant-negative IκBα mutant expression plasmid (dn IκB). Following transfection, cells were stimulated with 1 μg/ml LPS (dark-striped bars) and were harvested 24 h later. Unstimulated (light-striped bars) duplicate transfected cultures were also harvested. Cells were then lysed, and intracellular CAT activity was measured. CAT activity is expressed as the slope ± SEM. B, The tandem NF-κB sites are separated by a 5-bp spacer (5 bp; see Fig. 1) were transiently transfected into the murine RAW 264.7 macrophage cell line. Following transfection, cells were stimulated with 1 μg/ml LPS (dark-striped bars) and were harvested 24 h later. Unstimulated (light-striped bars) duplicate transfected cultures were also harvested. Cells were then lysed, and intracellular CAT activity was measured. CAT activity is expressed as the slope ± SEM.

**FIGURE 6.** Evidence for interaction between PU.1 and NF-κB. Reporter plasmids containing the wild-type HIV-1 LTR (wt hiv) and a mutant LTR in which the upstream and downstream NF-κB sites are separated by a 5-bp spacer (5 bp; see Fig. 1) were transiently transfected into the murine RAW264.7 macrophage cell line. Following transfection, cells were stimulated with 1 μg/ml LPS (dark-striped bars) and were harvested 24 h later. Unstimulated (light-striped bars) duplicate transfected cultures were also harvested. Cells were then lysed, and intracellular CAT activity was measured as described above. CAT activity is expressed as the slope ± SEM.
cotransfected with the wild-type PU.1 expression plasmid. The S148A mutant was unable to mediate LPS-induced LTR activation. These results demonstrate that serine 148 is required for the ability of PU.1 to activate the LTR. These data are consistent with the functional importance of serine 148 previously demonstrated in B cells (18) and macrophages (20).

**LPS-inducible IL-2 receptor promoter function is PU.1 independent**

Our data suggest that PU.1 can regulate HIV-1 LTR expression based on its ability to use NF-κB 3’ half-sites that contain the TTCC Ets core motif. Many inducible cytokine promoters contain functional NF-κB sites that possess potential Ets binding sites (e.g., IFN-β, TNF-α, IL-6, IP-10, and lymphotoxin). We subsequently evaluated the ability of PU.1 to regulate the expression of a distinct NF-κB-dependent promoter. For these studies we used a reporter plasmid containing the human IL-2R promoter. The NF-κB site within this promoter (GGGAATCTCC) has been shown to be critical for IL-2R expression (25). While this NF-κB site lacks a TTCC Ets core motif within the 3’ half-site, it does possess the complementary sequence (GGAA) within the 5’ half-site. We cotransfected HT1080/CD14 cells with this IL-2R-CAT reporter plasmid and a PU.1 expression plasmid to determine whether PU.1 had any effect on LPS activation of the IL-2R promoter. As shown in Figure 8, cells transfected with the IL-2R reporter construct expressed CAT activity following LPS stimulation. Furthermore, cotransfection with the PU.1 expression plasmid had no effect on LPS-inducible CAT expression. These findings demonstrate that PU.1 does not mediate LPS-inducible activation of the NF-κB-dependent IL-2R promoter and suggest that only a subset of NF-κB-dependent promoters is also PU.1 dependent. We hypothesize that some NF-κB motifs that contain 3’ PU.1 core binding sites might confer PU.1-dependent LPS-inducible expression, whereas NF-κB motifs that lack a 3’ PU.1 core binding site would probably be PU.1 independent. Potential explanations for the inability of the Ets core motif within the 5’ half-site to confer PU.1-dependent expression will be discussed below.

**Discussion**

The goal of these studies was to determine whether PU.1, the predominant Ets-like transcription factor found in macrophages and monocytes, was required for LPS-inducible activation of the HIV-1 LTR. We used genetic approaches to test the hypothesis that PU.1 could use NF-κB sites within the viral LTR at conserved TTCC motifs. An additional aim of these studies was to determine whether LPS-induced phosphorylation could modulate the ability of PU.1 to regulate HIV-1 gene expression in macrophages following LPS stimulation. We have shown here that activation of the HIV-1 LTR in LPS-stimulated cells requires both PU.1 and NF-κB. PU.1 appears to use the upstream NF-κB site within the LTR at the TTCC motif, whereas NF-κB binds to the downstream site. Activation of NF-κB in an LPS-responsive cell line lacking PU.1 was not sufficient to induce HIV-1 LTR transcription. Furthermore, transfection of this line with a PU.1 expression plasmid was sufficient to restore LPS-inducible transcription of the HIV-LTR. We also found that mutation of PU.1 at serine 148, which prevents its phosphorylation by CK2, blocked its ability to activate the HIV-1 LTR in response to LPS. These effects were promoter specific, because PU.1 was not required for LPS-inducible activation of the NF-κB-dependent IL-2R promoter. Thus, there appears to be a subset of NF-κB motifs that can interact with PU.1. These studies demonstrate a novel role for PU.1 in activation of the HIV-1 LTR by LPS.

Our data revealed that mutation of either NF-κB 3’ half-site within the HIV-1 LTR (i.e., the putative Ets binding sites) abolished LPS-inducible expression, and expression of a dominant-negative IκBα mutant protein also abolished LPS-inducible expression of the viral LTR. Furthermore, we confirmed an earlier finding that mutation at both 5’ half-sites abolished LPS-inducible expression (1). Together, these findings demonstrate that both NF-κB and PU.1 are required for activation of the HIV-1 LTR by LPS. Two previous studies add to our understanding of the molecular basis for activation of the viral LTR by LPS. First, Gaynor et al. reported that each of the tandem NF-κB motifs was capable of binding factors independently, and that the HIV-1 LTR was occupied by only one NF-κB molecule at any one time (31). Second, Baldwin and Sharp used methylation interference footprinting.
to show that NF-κB binding to DNA involves contact with guanidine residues within the GGAA core PU.1 binding motif (34). Furthermore, we have previously shown that PU.1 also contacts the DNA at these guanidine residues (35). Taken together, these two observations suggest that a single NF-κB motif could not bind PU.1 and NF-κB simultaneously. These data are consistent with a model in which a single LTR binds a single molecule of PU.1 and a single molecule of NF-κB. Because PU.1 and NF-κB potentially bind to sequences located 10 bp apart, a distance of one helical turn of DNA, we considered the possibility that PU.1 and NF-κB could lie in close physical proximity and could directly interact. We observed that a 5-bp insertion mutation that displaced the two factors by one-half of a helical turn on the DNA abrogated LPS inducibility, suggesting that PU.1 and NF-κB directly interact.

Our demonstration that PU.1 is capable of regulating the function of the HIV-1 LTR via interaction with the NF-κB motifs is consistent with previous reports by other laboratories. For example, Bassuk et al. recently showed that the p50 NF-κB subunit could physically bind to Ets proteins (14). Flory et al. reported that the Ets-like protein GABP α could bind to and regulate the function of HIV-1 LTR NF-κB motifs in a human T cell line (15). These investigators proposed that both HIV-LTR NF-κB motifs were bound by the GABP heterodimer in T cells. This contrasts with our data, which support a model in which both PU.1, or a PU.1-inducible Ets protein, and NF-κB bind to the HIV-1 LTR. It is unlikely that PU.1/Ets binds to both NF-κB motifs based on our finding that a dominant-negative IkBα mutant blocked LPS-inducible expression of the LTR. Furthermore, other investigators have used gel mobility shift assays to show that PU.1 binds only to the upstream NF-κB site (32). It should be emphasized that our data do not directly demonstrate binding of PU.1 to the HIV-1 LTR and do not exclude the possibility that additional Ets proteins may also regulate activation of the HIV-1 LTR. PU.1 may also be required for expression of an additional Ets protein that directly binds to the HIV-1 LTR.

Our finding that the PU.1 mutant S148A could not mediate LPS-inducible expression of the HIV-1 LTR in transfected HT1080/CD14 cells suggests that CK2 is part of the LPS signal transduction pathway that leads to activation of HIV-1 gene expression by LPS. A similar role for CK2 was previously reported to be required for activation of the Ig κ-chain 3’ enhancer by PU.1 (17, 18). We recently showed that S148A failed to activate a PU.1-dependent promoter in LPS-stimulated HT1080/CD14 cells (20). The studies reported here used the PU.1-deficient LPS-responsive HT1080/CD14 cell line to demonstrate a requirement for both PU.1 and serine 148, a site for phosphorylation by CK2. While it would have been desirable to show that S148A could block LPS-inducible HIV-1 LTR expression in monocytic cells, the high levels of wild-type PU.1 that are constitutively present in these cells prevent effective use of S148A as a dominant-negative mutant. Interestingly, CK2 was recently reported to be a selective target of flavonoids that inhibit HIV-1 replication (36). Thus, flavonoids may inhibit HIV-1 gene expression by blocking the phosphorylation and activation of PU.1 by CK2. Experiments are currently underway to test this hypothesis.

A survey of NF-κB binding sites within the promoters of several LPS-inducible genes reveals that many contain internal PU.1/Ets binding motifs consisting of the consensus sequence TTCC. These genes include TNF, IL-1R antagonist, inducible nitric oxide synthase, IP-10, IL-6, and IL-8. The ability to directly observe binding of PU.1 to these NF-κB motifs in vitro may depend on flanking sequences surrounding the TTCC core motif. For example, PU.1 does not bind measurably to the HIV-1 LTR NF-κB sites under standard gel-shift assay conditions, but it does bind well to the NF-κB site within the IL-1R antagonist promoter (M. J. Fenton and M. J. Smith, Jr., unpublished observations). The IL-1R antagonist promoter NF-κB motif contains the extended ANTTCCNT PU.1 consensus sequence, whereas the HIV-1 LTR NF-κB motifs lack the outermost flanking nucleotides. The importance of flanking sequences in determining the affinity of PU.1 for a specific site containing the TTCC core motif has been previously reported (37). The lack of specific flanking sequences required for PU.1 binding may explain why the IL-2R promoter, which contains a GGAA core motif and lacks consensus flanking nucleotides, is not PU.1-dependent. Experiments are currently underway to determine the role of PU.1 in the regulation of other NF-κB-dependent cytokine promoters.

The effects of LPS on HIV-1 replication are complex and may reflect action at more than one point in the virus replication cycle. Kornbluth et al. initially reported that treatment of HIV-1-infected, monocyte-derived macrophages with LPS repressed virus replication (38). Repression was observed after LPS pretreatment and was associated with a decrease in both integrated provirus and viral RNA accumulation, suggesting an effect on reverse transcription and/or integration. In a more recent study it was shown that LPS could either stimulate or repress HIV-1 LTR-directed expression depending on the differentiation status of the cell (39). Specifically, LTR-directed expression was repressed in terminally differentiated macrophages, but was activated in both a promonocytic cell line and in freshly isolated peripheral monocytes. Whether this differential effect of LPS on HIV-1 transcription results from distinct interactions between NF-κB and Ets-like proteins at the core enhancer remains to be determined.

Acknowledgments

We thank Drs. Stefan Doerre and David Hume for plasmids, advice, and helpful discussions. Drs. Hume and Matthew Sweet are also acknowledged for sharing data with the authors before publication. Shuyan Wang is acknowledged for providing technical assistance.

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