Interaction between Cell Cycle Regulator, E2F-1, and NF-κB Mediates Repression of HIV-1 Gene Transcription*

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The NF-κB/Rel family of transcription factors is one of the main targets of cytokines and other agents that induce HIV-1 gene expression. Some of these extracellular stimuli arrest cells in the G1 phase of the mitotic division cycle and modulate the activity of the tumor suppressor protein Rb and its partner E2F-1. Earlier studies indicated that E2F-1, a transcription factor that stimulates expression of S-phase-specific genes, is able to repress transcription directed by the human immunodeficiency virus (HIV-1) type-1 promoter in a variety of cells, including those of glial and lymphocytic origin. Here, we demonstrate that E2F-1 may regulate the activity of the HIV-1 long terminal repeat through its ability to bind sequences in the NF-κB enhancer region and to interact with the NF-κB subunit, p50. Gel retardation and methylation interference assays show that E2F-1 is able to bind specifically to a site embedded within the two NF-κB elements. Gel retardation/immunoblot analysis using purified E2F-1 and p50 homodimers reveals the presence of complexes containing both proteins. Affinity chromatography and co-immunoprecipitation assays provide evidence for direct interaction of E2F-1 and p50 in the absence of their DNA target sequences. In vitro transcription assay demonstrates that E2F-1 represses NF-κB mediated transcription in a cell-free system. Functional studies in Jurkat T lymphocytic cells point to the importance of both the E2F and NF-κB binding sites in E2F-1 mediated repression of HIV-1 promoter, in vivo. The results of this study suggest that NF-κB activity may be regulated by its interaction with the cell cycle regulatory protein, E2F-1.

Infection with the human immunodeficiency virus type-1 (HIV-1)1 gives rise to a progressive disease which can be separated into three clinical phases. The acute phase of infection occurs within 1–3 weeks after exposure and is characterized by mononucleosis-like symptoms and plasma viremia (1–3). This initial phase is followed by an extended asymptomatic phase during which there is often a slow, but progressive decline in immune function that leads to the final stage, acquired immunodeficiency syndrome (AIDS). During the stage of clinical latency, the viral burden in the peripheral bloodstream is markedly diminished, but viral replication persists in the lymph nodes (4–7). Although there is no direct correlation between clinical and viral latency, the discrepancy between the number of cells harboring viral DNA and the small number of cells expressing viral RNA during this phase, suggests that factors which regulate HIV-1 gene expression may play a role in delaying the onset of AIDS (4, 5).

Cellular proliferation and differentiation are among the many parameters involved in determining the extent of HIV-1 replication in different host cells. For example, mitogenic activation of peripheral blood lymphocytes and stimulation of peripheral blood monocytes and promonocytic cell lines to differentiate are required for productive replication of HIV-1 (8–10). Quiescent (G0) T lymphocytes and monocytes and γ-irradiated (G1 arrested) monocyte-derived macrophages are not permissive to HIV replication because of their inability to complete the reverse transcription process (11–13). However, many agents that promote the arrest of proliferating T lymphocytes and monocytes in G1 (UV irradiation, transforming growth factor-β, and tumor necrosis factor-α, sodium butyrate, etc.) stimulate viral replication by facilitating later stages in the viral life cycle including transcription (14–18). These observations suggest that although the macromolecular environment provided by cells during the G0 and early G1 phase is not suitable for early steps in the viral life cycle, the repertoire of cellular transcription factors in the G1 phase can increase levels of HIV-1 transcription.

Normal progression of cells from the G1 to S phase is regulated in part by E2F-1. E2F-1 belongs to family of proteins including E2F-1 to -5 which are thought to play an important role in both promoting and restricting cell cycle progression (19, 20). Overexpression of E2F-1 in tissue culture cells stimulates them to enter S-phase by activating transcription of a subset of S-phase genes. These genes contain the consensus E2F-binding site (5′-TTTTRRCG-3′) in their promoters (20). During early G1, the retinoblastoma protein, Rb p105, binds to E2F-1. This interaction not only prevents the latter from activating transcription, but also promotes active repression of some promoters by the complex (21–24). Phosphorylation of Rb later in G1 abolishes the interaction between Rb and E2F-1 (25). Subsequent completion of S-phase requires inactivation of E2F-1 DNA binding activity (26–28). Recently, E2F-1 knockout mice showed a high incidence of unusual tumors and abnormalities in T cell maturation suggesting that in vivo, E2F-1...
Two-dimensional Gel Retardation Assay (GR-Western)—Gel retardation assay was performed as described previously (36). Bands were excised, soaked in SDS sample buffer for 1 h, and loaded onto a 10% denaturing (SDS) polyacrylamide (29:1) gel. Samples were resolved by SDS-PAGE and blotted to Western blot (37).

Immunoprecipitation-Western—Nuclear extracts were prepared as described previously (37). Subsequent steps were also performed at 4 °C. 300 µg of precleared nuclear extract were incubated with primary antibodies overnight. Immunocomplexes were precipitated with Sepharose A. The pellets were washed three times with TNN buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Nonidet P-40, 0.2 mM phenylmethylsulfonyl fluoride) and resuspended in SDS sample buffer. Proteins were resolved by SDS-PAGE and analyzed by Western blot (37).

GST Pull-down—GST pull-down experiments were performed in GST binding buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Nonidet P-40, 5 mM EDTA, 50 mM sodium fluoride). GST fusion proteins were prepared as described above, and kept on the glutathione beads. 200 µg of bacterial lysate were included in reactions using recombinant NF-xB subunits to eliminate nonspecific interactions. Pellets were washed 3 times in GST binding buffer and resuspended in SDS sample buffer.

Antibodies—KH95 (Santa Cruz) is a mouse monoclonal specific for human and mouse E2F-1. PC10 (Santa Cruz) is a mouse monoclonal antibody that recognizes mouse and human PCNA. BF683 (Santa Cruz) is a mouse monoclonal antibody specific for human cyclin A. Sc-114 (Santa Cruz) is a rabbit polyclonal antibody raised against a nuclear localization signal of p50. NR1157 is a rabbit polyclonal antibody which recognizes p50 and was kindly provided by Dr. Nancy Rice (National Cancer Institute, Frederick, MD).

Transient Transfection and Chloramphenicol Acetyltransferase Assay—Jurkat cells were transfected by electroporation using the conditions specified by the manufacturer (Bio-Rad). 1 × 10^6 cells were co-transfected with a total of 20 µg of DNA. Whole cell extracts were prepared by freeze-thawing cells harvested 48 h after transfection. Chloramphenicol acetyltransferase assay was performed as described previously (38).

Plasmids—The following chloramphenicol acetyltransferase reporter plasmids were used in transient transfection assay: -117/+3, mE2F, mtE2B, mtE2Bmut. The plasmid -117/+3 has been described previously (38). The name delineates the portion of the HIV-1 LTR included in each construct; nucleotide positions are described with respect to the +1 transcription start site. Polymerase chain reaction-mediated site-directed mutagenesis was used to introduce point mutations in the -117/+3 CAT reporter construct (39). The mtE2F construct contains CC to AT substitution in the putative E2F-binding site (5'-TTTCCGC-3') and the mtE2B construct contains a GGG to TCT substitution in the first -3 (GGGACTTTC). The plasmid, mtE2Bmut, was constructed by isolating the TaqI-BglII fragment from the HIV-CATΔB construct which contains a GGG to TCT substitution in both NF-xB-binding sites. The TaqI-BglII fragment spans from nucleotides -117 to +24 of the HIV-1 LTR. The CMV-neo-E2F-1 expression plasmid has been described previously (36).

In Vitro Transcription Assay—In vitro transcription using a highly purified reconstituted system was described previously (40, 41). The reactions contained purified general transcription factors, the co-activator fraction (USA) and tag-flagged TFIIID. The HIV-1 LTR (pMHIV wt) and the adenovirus major late promoter (pMLA53)-driven constructs served as templates (42).

RESULTS

Recombinant E2F-1 Interacts Specifically with the HIV-1 LTR in Vitro—We previously demonstrated that a functional E2F-1 responsive element lies within the enhancer region of the HIV-1 LTR (31). The ability of E2F-1 to bind to this element in vitro was demonstrated with gel retardation (GR) and methylation interference assays. GR analysis using recombinant GST-E2F-1 and a 37-base pair oligonucleotide probe containing the minimal responsive region (nucleotides -117 to +24 with respect to the transcription start site, +1) showed that E2F-1 is able to interact with the LTR sequence (Fig. 1A, lanes 1–3). The E2F element from the DHFR promoter served as a positive control demonstrating the fidelity of the recombinant GST-E2F-1 (Fig. 1A, lanes 4–6). The GST component of the fusion protein had no binding activity for either the HIV-LTR or DHFR E2F elements (data not shown). Specificity of E2F-1 binding to the HIV-LTR was verified by competition analysis
FIG. 1. Binding of recombinant E2F-1 to the HIV-1 kB motif. A, gel retardation assay was performed using 25, 50, and 100 ng of purified bacterially produced GST-E2F-1 (lanes 1–3). Radiolabeled double-stranded oligonucleotides containing two copies of HIV-1 kB (2kB) (Panel D) and DHFR E2F (5′-ATCTTG-TCGCCTCTTCCTCCGCTTCTCTC-3′) were used as probes. B, 50 ng of GST-E2F-1 were incubated with radiolabeled 2kB probe in the absence (lane 1) or presence (lanes 2–10) of 25- and 125-fold molar excess of unlabeled competitor DNA: 2kB (lanes 2 and 3), 1kB (5′-ACAAAGGACTTTCCTATGGG-3′) (lanes 4 and 5), 1kBm (5′-ACAAAGGACTTTCCTATGGG-3′) (lanes 6 and 7), and DHFR E2F (lanes 8 and 9). In lane 10, 100 ng of monoclonal antibody KH95 (Santa Cruz) was included in the binding reaction. Position of supershifted complex is marked by an arrow. C, methylation interference was performed as described previously (37). Individual strands of the 2kB probe were end-labeled using [γ-32P]ATP and poly nucleotide kinase and hybridized to the unlabeled complementary strand. The probe was methylated using DMS and subsequently incubated with 250 ng of GST-E2F-1. Peridinin cleavage and analysis of the DNA is detailed under “Experimental Procedures.” Asterisks mark the protected sites on the upper strand (lanes 1–3) and lower strand (lanes 4–6). Lanes 1, 3, 4, and 6 contain free 2kB probe (F); while lanes 2 and 5 contain the bound probe (B). D, diagram of NF-κB enhancer region including the two well characterized NF-κB-binding sites and the putative E2F-binding site. Bold faced letters depict the nucleotides protected in the methylation interference assay. E, 25 and 50 ng of bacterially produced GST-E2F-1 were incubated with 2kB (WT), two copies of 1kB as described above, and 2kBmutant (Mut) oligonucleotides identical to 2kB except for a 2-base pair substitution in the E2F-1 binding site (as shown above for 1kBm) probes, and binding was analyzed by gel retardation assay.

utilizing a variety of unlabeled competitor oligonucleotides. The wild type HIV-1 sequence containing two copies of the kB motif (2kB) and the DHFR E2F sequences competed for E2F-binding (Fig. 1B, lanes 1–3, 8, and 9), while the oligonucleotide containing one copy of kB sequence (1kB) and its mutant variant (1kBm) were less effective (Fig. 1B, lanes 4–5 and 6–7). The addition of an E2F-1 specific monoclonal antibody (KH95, Santa Cruz) to the binding reaction decreased the intensity of the major complex and induced the formation of a novel band with slower electrophoretic mobility, demonstrating the presence of E2F-1 in the original complex (Fig. 1B, lane 10). Methylation interference assay using purified components revealed that GST-E2F-1 interacts with the guanine residues on both strands of the sequence (Fig. 1, C and D). Mutation of two critical guanine and cytosine residues of the coding strand completely abrogated the association of E2F-1 with the 2kB probe (Fig. 1E, compare lanes 1 and 2 to 3 and 4). Gel retardation analysis using various E2F-1 deletion constructs demonstrated that the basic helix-loop-helix domain of E2F-1 which is responsible for binding to the consensus E2F-1 site also mediates its interaction with the site in the HIV-1 LTR (data not shown).

E2F-1 Alters Binding of p50 Homodimers to DNA—Since the binding site for E2F-1 partially overlaps the cis-elements recognized by the NF-κB subunits, we speculated that E2F-1 may prevent association of NF-κB with their targets in the LTR. Results from the gel retardation assay indicated that the addition of increasing amounts of E2F-1 to binding reactions containing p50 homodimers alters the electrophoretic mobility of the p50 complex and leads to the formation of novel bands (Fig. 2A). Two-dimensional gel electrophoresis where the individual bands from the GR assay were analyzed for their protein content by Western blot analysis indicated that the intermediate complexes contain both E2F-1 and p50 (Fig. 2B). Novel complexes containing E2F-1, p50, and p65 were formed when E2F-1 and baculovirus-produced p50/p65 heterodimers were used in an identical assay (data not shown). The E2F-1 deletions containing amino acids 88–241 or amino acids 241–437 were used to further analyze the binding of E2F-1 and p50 in GR assay (Fig. 2A). The appearance of distinct novel complexes containing both the DNA-binding domain of E2F-1 (88–241) and p50 (Fig. 3, A, lanes 1–5; B, lanes a-d) demonstrated that both proteins can bind simultaneously to the HIV-1 LTR. This suggests that the novel complexes seen in Fig. 2A may represent displacement of p50 homodimers by E2F-1. However, the E2F-1 mutant (241–437) that does not interact with DNA participates in a DNA-binding complex when incubated with p50 (Fig. 3, A, lanes 6–10; B, lanes e–g). This indicated that in
addition to displacing p50, E2F-1 may interact directly with p50.

E2F-1 Interacts with the 50-kDa Subunit of NF-kB—Immunoprecipitation assay was performed to detect the presence of endogenous p50/E2F-1 heterodimers. Since deletion of the first 88 amino acids of E2F-1 did not alter its ability to repress the HIV-1 LTR when used in transient transfection assays (data not shown), nuclear extract was prepared from NIH3T3 cells that constitutively expressed detectable levels of a truncated version of human E2F-1 containing amino acids 88–437. Immunoprecipitation with an antibody specific for E2F-1 resulted in the precipitation of p50 (Fig. 4A, lane 1) and E2F-1 (88–437) (data not shown). The control antibodies against PCNA and human cyclin A were unable to precipitate significant amounts of p50, demonstrating the specificity of the reaction (Fig. 4A, lanes 2 and 3). The small amount of p50 in lane 2 is likely to result from an indirect interaction, as the truncated ectopically expressed E2F-1 was immunoprecipitated with the antibody against PCNA (data not shown). However, we cannot exclude the possibility of a weak interaction between PCNA and p50. GST pull-down assays demonstrated that GST-E2F-1 binds directly to the baculovirus-produced p50 (Fig. 4B, lanes 1 and 2), but interacts to a lesser extent when p50 is in the form of a complex with its cellular partner, p65 (Fig. 4B, lanes 3 and 4). This suggests that the ratio between E2F-1, p50, and p65 may dictate the extent of their interactions and consequent modulation of transcription. GST pull-down assay using various E2F-1 deletions indicated that the C-terminal region of E2F-1 (amino acids 241–437) contains the p50-binding domain (Fig. 4C). Furthermore, since deletion of amino acids 1–88 reduced the amount of p50 pulled down (Fig. 4C, compare lanes 2 and 3), a region within the first 88 amino acids may be an important contributor to the interaction between p50 and E2F-1 in vitro. However, it is important to note that this region does not appear to have a critical functional role with respect to HIV-1 transcription (as discussed above).

Mutation of E2F and NF-kB-binding Sites Attenuates Ability of E2F-1 to Repress Transcription—To assess the relative contributions of NF-kB and E2F-1 to the observed regulatory event in vivo, transient transfection studies were performed. Since treatment of cells with phorbol myristate acetate induces nuclear translocation of p50 and p65 and increases NF-kB-mediated transcription of HIV-1 promoter (32), it was of interest to examine the effect of E2F-1 overexpression on HIV-1 transcription in phorbol myristate acetate-treated T-lymphocytic cells. As shown in Fig. 5A, E2F-1 was able to repress phorbol myristate acetate-stimulated levels of HIV-1 LTR-directed transcription. This observation confirms that E2F-1 suppresses b-mediated activation. In the next series of experiments, several mutant reporter constructs containing nucleotide substitutions...
in the κB and E2F-binding sites were created. The mutation designed to inhibit E2F-1 binding is also likely to disrupt binding of AP-2 to its target sequence and abolish its positive contribution to the activity of the HIV-1 LTR (43). Therefore, the reduced basal activity exhibited by the mtE2F construct may represent a combination of the loss of a positive regulator and a negative regulator (Fig. 5B). Mutation of the E2F-1 binding site (mtE2F) decreased the level of responsiveness to E2F-1 from 91 to 60% (Fig. 5B), suggesting that the E2F-binding site serves to facilitate repression of the HIV-1 LTR, perhaps by stabilizing a complex containing E2F-1 and p50. The E2F-1 mutant (88–241) that contains the DNA-binding domain, but not the p50-binding domain, did not repress HIV-1 transcription in the absence of NF-κB. This provides additional evidence that binding of E2F-1 to its target site is not sufficient for mediating repression of transcription directed by the HIV-1 LTR. Disruption of the 5’ NF-κB-binding site (mtxκB1) in the HIV-1 LTR reduced repression caused by the wild type E2F-1 to 50% (Fig. 5B). Mutation of both NF-κB-binding sites (mtxκB2) showed a more drastic effect on the response of the promoter to E2F-1 (Fig. 5B). These data demonstrate that the κB motifs, which upon binding to NF-κB subunits bring these activators into close proximity of the basal transcription complex, are essential for E2F-1-mediated transcriptional repression. Thus it is likely that E2F-1 exerts its effect on the HIV-1 promoter by interacting with the p50 subunit, and disrupting the ability of NF-κB to activate transcription.

**Discussion**

Earlier studies indicated that E2F-1 inhibits both basal levels of transcription and Tat-induced activation of the HIV-1 LTR in a variety of cell types (31). The responsive site was mapped to the region of the promoter encompassing the two NF-κB-binding sites. In this study we demonstrate that E2F-1 binds to an element embedded within these two NF-κB-binding sites in the HIV-1 LTR and also interacts with the 50-kDa subunit of NF-κB. Although the E2F-1-binding site is required...
for maximal repression, its presence in the HIV-1 LTR is not essential for repression by E2F-1. By contrast, intact NF-κB-binding sites are essential for E2F-1-mediated down-regulation of the HIV-1 promoter in vivo. In addition, E2F-1 specifically represses NF-κB (p50/p65) mediated activation in vitro. Together, these data suggest that E2F-1 down-modulates transcription by virtue of its ability to associate with p50. Furthermore, binding of E2F-1 to a site in close proximity to the NF-κB-binding site may facilitate this interaction. Although, previous reports have suggested that the role of NF-κB may be dispensable in both basal as well as Tat-mediated activation of the HIV-1 LTR, it is evident that the presence of NF-κB results in the enhancement of viral growth and replication (44–46). In fact, the presence of NF-κB sites in most HIV-1 genomes invariably makes the virus susceptible to regulation by NF-κB subunits and factors, including E2F-1, that influence NF-κB mediated transactivation. In support of this notion, our earlier studies demonstrated that E2F-1 reduces HIV-1 replication in a human cell line (31).

Activation of the HIV-1 LTR by purified NF-κB subunits requires the presence of co-factors in addition to general transcription initiation machinery (42, 43, 47). Similarly, it has been postulated that transactivation by Tat may require co-factors to facilitate the interaction between basal transcription factors, including TFID, and the NF-κB proteins (38, 48, 49). Thus, it is possible that binding of E2F-1 to p50 disrupts the association of NF-κB with another cellular transcription factor(s), resulting in suppression of both basal and Tat-induced activation of the HIV-1 LTR.

E2F-1 interacts with a number of proteins, including Sp-1, cyclin-A, Dp-1, Rb, TBP, and p53 (26, 50–57). The binding of Rb to the C-terminal region of E2F-1 during the G1 phase is thought to prevent E2F-1 from functioning as a transcriptional activator by inhibiting its association with the basal transcription complex via TBP (50, 51). In this study we demonstrate that the C terminus of E2F-1 spanning amino acids 241 to 437 also contains a binding site for p50. Thus, it is possible that Rb, TBP, and p50 may have to compete for binding to E2F-1. In support of this notion, recent studies showed that the underphosphorylated form of Rb, which interacts with E2F-1, can activate HIV-1 transcription through the NF-κB enhancer region. It is possible that Rb activates transcription by competing with p50 for binding to E2F-1 and inhibiting the repressive activity of E2F-1.

Because the activity of E2F-1 is regulated in a cell-cycle dependent manner through changes in its expression level and its interactions with Rb and other proteins (20), E2F-1 may provide a link between host cell cycle and HIV-1 transcription and replication. Early in the G1 phase, underphosphorylated Rb may compete with p50 for binding to the C-terminal region of E2F-1 and prevent E2F-1 mediated repression of HIV-1 LTR activity. Later in G1, when Rb is phosphorylated and E2F-1 is released, E2F-1 may not only activate transcription of S-phase genes, but also modulate the transcriptional activity of the NF-κB complex. Our data suggest that the E2F-1/NF-κB is less active in induction of transcription than NF-κB alone. Although the inhibitory effect of E2F-1 on HIV-1 transcription may seem small (2–3-fold), the link provided by E2F-1 and other cell cycle proteins which regulate HIV-1 gene expression may have implications for viral latency, since many of the extracellular stimuli which affect the activation and proliferation state of infected cells also modulate HIV-1 replication. Tumor necrosis factor-α, transforming growth factor-β, and UV irradiation, for example, have been shown to inhibit proliferation of T-lymphocytes and stimulate HIV-1 replication in these cells (14–18). The model which we propose suggests that the molecular signaling pathways which mediate the effects of certain cytokines and other agents on cellular proliferation may coincide with those involved in modulating HIV-1 replication.

In addition to its relevance for HIV-1 replication, the data presented in this paper may have more global significance. Since E2F-1 is involved in down-regulating the prototype NF-κB responsive promoter, the HIV-1 LTR, by interacting directly with p50, it is likely that it modulates other genes encompassing the NF-κB motif. One consequence of constitutive NF-κB activation is overexpression of NF-κB responsive genes including tumor necrosis factor-α, interleukin-2, granulocyte-macrophage colony-stimulating factor, major histocompatibility complex class I, V-CAM, and E-selectin, which are involved in regulating the growth rate and activation state of cells (58). The association of constitutive activation of NF-κB family members with the incidence of human tumors (59–61), the decreased growth rate observed in tumor cells treated with p50 and p65 antisense oligonucleotides (62, 63), and the nature of the genes subject to regulation by NF-κB (58, 64) provide compelling evidence that dysregulated NF-κB activity may be involved in tumor formation and/or progression. Despite the fact that E2F-1 is involved in promoting S-phase in tissue culture cells, E2F-1 knockout mice exhibit abnormalities in T lymphocyte development and develop a broad spectrum of unusual tumors, which suggests that E2F-1 may also be involved in restraining cell growth. Given the association between NF-κB overexpression and tumor progression, and the high incidence of tumors in E2F-1 knockout mice, it is reasonable to speculate that one of the normal functions of E2F-1 that is associated with its role as a tumor suppressor may be to down-regulate NF-κB responsive genes in a timely manner.

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