

Activation of HIV-1 LTR by Rad51 in microglial cells

Inna Rom, Armine Darbinyan, Martyn K. White, Jay Rappaport, Bassel E. Sawaya, Shohreh Amini and Kamel Khalili*

Department of Neuroscience; Center for Neurovirology; Temple University School of Medicine; Philadelphia, PA USA

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Infection with HIV-1 induces a variety of biological alterations to the host that are beneficial to the life cycle of the virus but may have adverse effects on the host cell. Here we demonstrate that expression of Rad51, a major component of the homologous recombination-directed DNA repair (HRR) pathway, is induced upon HIV-1 infection of microglial cells. Activation of Rad51 expression positively impacts on HIV-1 LTR transcription through a region of the viral promoter known for binding the inducible transcription factor NFκB. Rad51 showed the ability to form a complex with the p65 subunit of NFκB and regulate the level of p65 interaction with LTR DNA encompassing the κB motif. This study provides evidence for reciprocal interaction of HIV-1 and a host DNA repair protein that impacts on expression of the viral genome. These results also point to the ability of HIV-1 to recruit proteins involved in DNA repair that are necessary for retroviral DNA integration, efficient replication and prevention of viral-induced cell death.

Introduction

Even after the advent of the highly active antiretroviral therapy (HAART), clinical disease of the CNS accounts for a significant degree of morbidity of HIV-1-infected subjects.¹ In the brain, HIV-1 can replicate in microglia, which can release neurotoxic molecules leading to CNS damage.^{2,3} The early phase of HIV-1 infection begins with viral entry and involves all steps leading to integration of viral DNA into the chromosome of the host cell. During the integration process, a staggered cut is introduced into host DNA, generating an overhanging 5' phosphorylated end, followed by joining of the 3' end of viral DNA to the 5' end of the host genome.⁴ It has been suggested that both the unintegrated double-stranded viral DNA as well as gaps formed during integration of viral DNA may trigger DNA damage signaling and induce the DNA damage response (DDR).⁴ There is evidence that non-homologous end-joining (NHEJ), which is involved in repair of DNA double-strand breaks (DSBs), is required for completion of retroviral DNA integration, efficient replication and prevention of viral-induced cell death.⁵⁻⁷ Components of nucleotide excision repair (NER) and base excision repair pathway (BER) also have been implicated in the early steps of HIV-1 infection.^{8,9} A possible role for the other major DNA DSB repair pathway, homologous recombination-directed DNA repair (HRR), in progression of HIV-1 infection remains largely unknown.

Earlier studies also described a role for HIV-1 Tat in the induction of the HRR protein Rad51 via stimulation of its promoter in astrocytic cells and cultured neuronal cells.¹⁰ In astrocytes, increased levels of Rad51 led to stimulation of HIV-1 LTR

activity through collaboration with HIV-1 Tat and the transcription factors C/EBPβ and CHOP.¹¹ Similarly, we also demonstrated that Tat stimulates HRR DNA repair of I-SceI-induced DSB and the nuclear appearance of Rad51 foci in mouse embryo fibroblasts.¹²

In the present study, we asked whether infection of human microglial cells affects the level of Rad51 expression in these cells and if so, what is the impact of Rad51 on the activity of the HIV-1 LTR. Our results show a positive feedback interaction involving the inducible transcription factor NFκB, which is a powerful positive regulator of the LTR.¹³ This interaction between Rad51 and NFκB leads to a synergistic stimulation of the HIV-1 LTR in microglial cells.

Results

Expression of Rad51 is enhanced in HIV-infected primary human microglia. The ability of HIV-1 Tat protein to upregulate the expression of Rad51 in glioblastoma cell line (U-87 MG)¹¹ gave us a rationale to validate our findings in primary human cultures of microglia. Primary human microglia were infected with the JR-FL strain of HIV-1 as described in Materials and Methods. Cells were harvested every second day following infection until day 10 post-infection (p.i.), total microglia lysates were prepared and subjected to Western blot analysis for Rad51. Grb2 was the loading control. Densitometric analysis shows a quantitation of the differences in Rad51 levels and revealed that the induction of Rad51 at day 10 p.i. was 12-fold when normalized to the level of Grb2 protein. Rad51 was gradually induced

*Correspondence to: Kamel Khalili; Email: kamel.khalili@temple.edu

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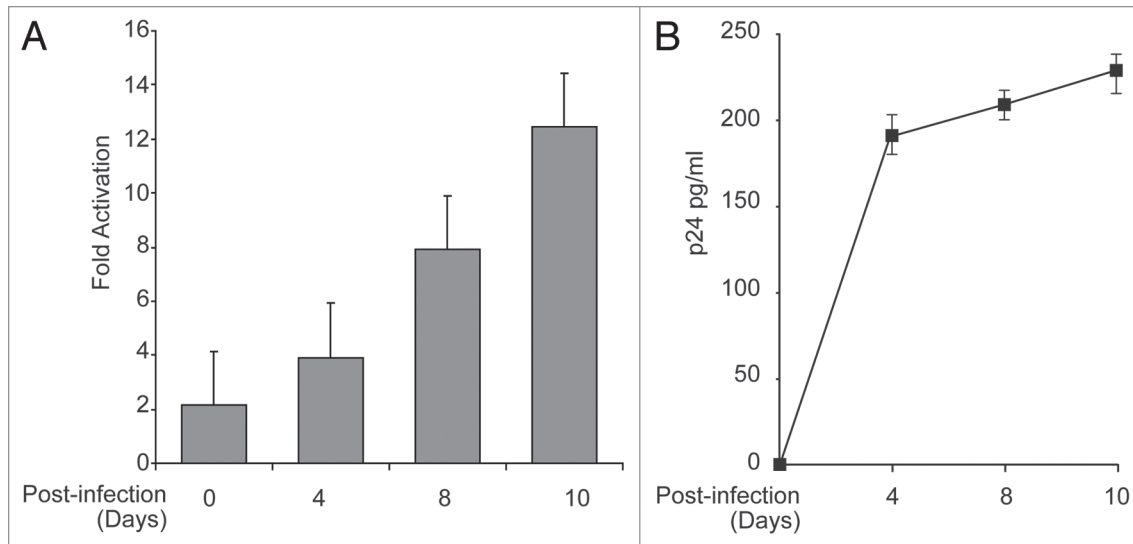


Figure 1. Expression of Rad51 in HIV-1 infected primary human microglial cells. (A) Quantitation of the changes in Rad51 levels was performed by densitometric analysis of the scanned X-ray films. The results were normalized relative to expression of Grb2 and are presented as a histogram. (B) p24 levels in the supernatant of the infected microglial cells during the course of HIV-1 infection as measured by ELISA and is shown over time in days post-infection.

following HIV-1 infection and reached maximal levels at day 10 p.i. (Fig. 1A). Supernatants collected from the microglia cultures were analyzed by ELISA for the presence of HIV-1 p24 protein, which is an indicator of viral replication (Fig. 1B). In parallel, p24 was not detected in uninfected microglia that were used as a control.

Rad51 stimulates transcriptional activity of the HIV-1 LTR in primary human microglia. Using a glioblastoma cell line (U-87 MG), we previously showed that Rad51 activates the HIV-1 promoter through its physical interplay with the transcription factor C/EBP β .¹¹ Note that C/EBP β binds to the core promoter region of the LTR¹⁴ as shown schematically in Figure 2A. Therefore, we investigated the impact of Rad51 induction on HIV-1 LTR that encompasses regions of LTR starting from the position -167 to +66. Primary cultures of human fetal microglia were transfected with luciferase reporter plasmids either alone or together with plasmids expressing Rad51. As shown in Figure 2B, expression of Rad51 resulted in 50, 42 and 10-fold, enhancement in transcription activity driven by HIV-1 LTR promoter -167/+66 and deletion mutants, respectively. These results led to the conclusion that the region, which contains binding site for NF κ B is necessary for Rad51-mediated LTR activation.

Since Rad51 failed to activate the LTR in the absence of NF κ B-binding site, we next examined whether a functional interplay exists between Rad51 and NF κ B. Primary human microglia were co-transfected with HIV-1 LTR deletion mutants along with plasmids expressing Rad51 and/or NF κ B subunit, p65. Interestingly, an additive activation of the LTR transcription by Rad51 and NF κ B was observed (Fig. 2B). These results suggest Rad51 activates the LTR mainly through the NF κ B and not C/EBP β , binding region.

Specific small interfering RNAs inhibits the transcriptional activity of the HIV-1 LTR in primary human cultures

of microglia. The requirement of NF κ B DNA motifs for Rad51-mediated activation of HIV-1 LTR and additive activation of LTR transcription by Rad51 and NF κ B in co-transfection studies prompted us to investigate the necessity of NF κ B protein for Rad51 stimulation of HIV-1 LTR. Using primary human microglia, we found that depletion of endogenous p65/NF κ B by siRNA caused a significant decline (80%) in the level of HIV-1 LTR activity compared to basal level of LTR activity in cells transfected with non-targeting siRNA (Fig. 2C). Expression of Rad51 in cells stimulated the LTR (50-fold) but transfection with p65 siRNA partially reduced luciferase expression (25-fold). In a reciprocal experiment, downregulation of the expression of endogenous Rad51 (Fig. 2E) resulted in a 70% decrease in the level of HIV-1 LTR transcriptional activity, compared to activity in control cells transfected with non-targeting siRNA. Silencing of Rad51 mRNA in cells where p65/NF κ B was expressed also significantly inhibited LTR-driven gene expression: more than 70% compared to level of LTR activity in microglia transfected with p65/NF κ B along with non-targeting siRNA. These data suggest that presence of both proteins Rad51 and NF κ B is important for efficient stimulation of HIV-1 LTR in microglial cells. The expression levels of Rad51 and p65/NF κ B proteins and the efficiency of their silencing in these two experiments were monitored by Western blot analysis (Fig. 2D and F).

Expression and subcellular localization of Rad51 and NF κ B in primary human microglia. NF κ B is sequestered in the cytoplasm but when activated, it translocates into the nucleus. Rad51 has a diffuse nuclear and cytoplasmic pattern of expression, but rapidly appears in nuclei in response to DNA damage to form distinct, nuclear foci, which are sites of HRR DNA repair.¹⁵ The expression and subcellular localization of p65/NF κ B and Rad51 were examined in primary human fetal microglial cells following transient transfection with plasmids expressing Rad51

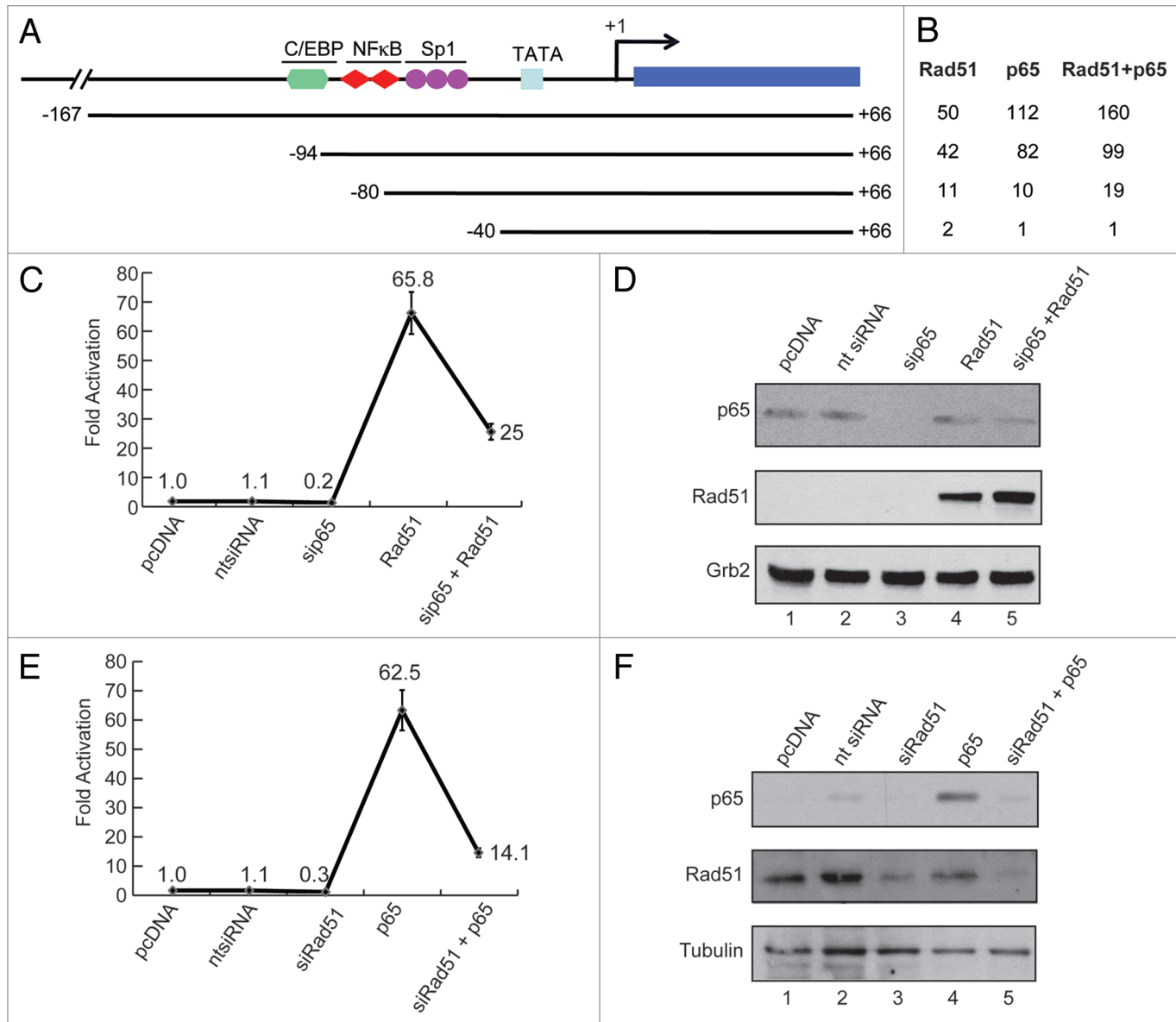


Figure 2. Effect of Rad51 or siRNA-Rad51 or p65 on HIV-1 LTR activity. (A) Schematic representation of the structural organization of the HIV-1 LTR enhancer region and basal/core promoter and the positions of the deletion mutants that were used in this study. (B) Primary human microglial cells were transfected with 100 ng of the HIV-1 LTR deletion mutants (-167/+66, -94/+66, -80/+66, -40/+66) fused to the luciferase reporter gene either alone or in combination with 300 ng of plasmids expressing Rad51 and/or p65 subunit of NF κ B. The numbers represent the fold change in the HIV-1 promoter activity mediated by Rad51 and/or p65/NF κ B. (C) The effect of silencing of p65 subunit of NF κ B gene expression on HIV-1 LTR activity was assayed when by transfection the HIV-1 LTR plasmid (-167/+66) alone or in combination with 100 nM of p65 siRNA (sip65), non-targeting siRNA (ntsRNA) or plasmid-expressing Rad51 with or without p65 siRNA. (E) Effect of silencing of Rad51 expression was also assayed by transfection of the HIV-1 LTR with 100 nM of Rad51 siRNA (siRad51), non-targeting siRNA (ntsRNA) or plasmid-expressing p65/NF κ B with or without Rad51 siRNA. The amounts of DNA in each transfection mixture were normalized with pcDNA. Luciferase activity was determined 48 hours after transfection. (D and E) The levels of expression of p65/NF κ B and Rad51 were analyzed by Western blots for the lysates from the experiments shown in (C and E), respectively. Grb2 served as a loading control.

in fusion with green fluorescent protein and p65/NF κ B. The Rad51-GFP fusion protein was marked by the nuclear appearance of speckled green fluorescence in response to treatment of cells with the DNA damage-inducing agent cisplatin (data not shown). Cells transfected with GFP-Rad51 showed bright green staining that was cytoplasmic (predominantly perinuclear) and nuclear (punctate) (Fig. 3D). p65/NF κ B protein was detected by

immunofluorescence in both nuclear and cytoplasmic compartments of transfected cells (Fig. 3B and E). Superimposition of both fluorochromes shows colocalization of both proteins in the perinuclear region and in the nuclei of cells (Fig. 3G). DAPI stain was used for staining nuclear DNA (Fig. 3C and F).

Interaction of Rad51 with NF κ B and identification of the region within Rad51, which is important for its interaction

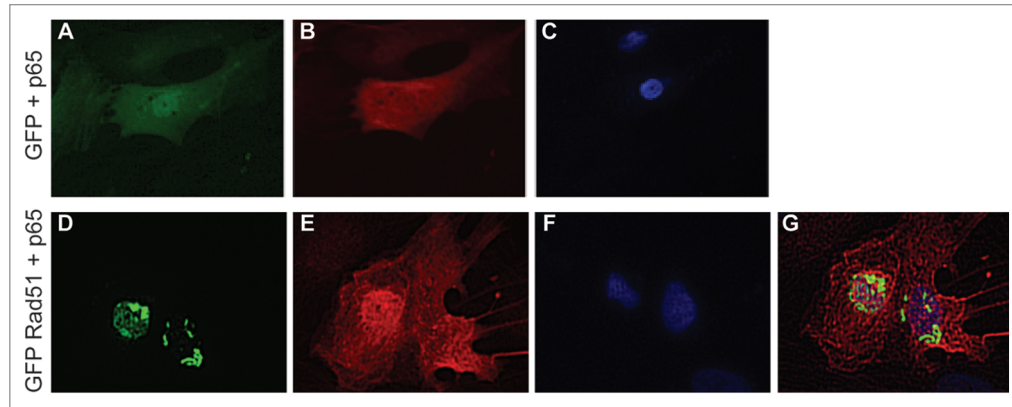


Figure 3. Colocalization of Rad51 and p65/NFκB. Microglia were transfected with plasmid expressing p65/NFκB together with plasmids expressing either GFP-Rad51 or GFP. Cells transfected with GFP-Rad51 show bright green staining that is cytoplasmic, predominantly perinuclear and punctuate staining in the nucleus (D). Green staining in GFP-transfected control cells was uniformly distributed in nuclei and cytoplasm (A). By immunofluorescence we detected p65/NFκB protein in both nuclei and cytoplasm of infected cells (B and E rhodamine). Superimposition of both fluorochromes shows colocalization of both proteins in the perinuclear region and in the nuclei of cells (G). DAPI was used for labeling nuclear DNA (C and F).

with NFκB. Our observations provided a rationale to investigate if there was a physical interaction of Rad51 with NFκB. The association of Rad51 with NFκB was detected by immunoprecipitation/western blot analysis (Fig. 4A) in U-87MG cell extracts expressing endogenous levels of both proteins. Results from GST-based protein-binding assays using extracts prepared from U-87MG cells revealed the ability of p65/NFκB to interact with full-length Rad51 protein (Fig. 4B). Analysis of a series of Rad51 deletion mutants highlighted the importance of the region of Rad51 that spans amino acids 40–80, in this interaction. Interestingly, the region in Rad51 (40–80 aa), which is necessary for interaction with NFκB, is one of the most highly conserved segments among Rad51 paralogs across all major kingdoms of living organisms.¹⁶ Figure 4B illustrates p65/NFκB western blots following the GST pull-downs and Figure 4C shows the integrity of GST-Rad51 and its mutant variants, which were verified by SDS-PAGE. Figure 4D illustrates the linear organization of full-length Rad51 and its deletion mutants and their relative binding to p65/NFκB.

Rad51 enhances the p65/NFκB binding to NFκB response element within HIV-1 LTR. In HIV-1-infected cells, NFκB binds to the two NFκB sites present in the HIV-1 LTR and stimulates transcription. To determine if the association of Rad51 with NFκB affects binding of p65/NFκB with κB motif, we performed electrophoretic mobility shift assays (gel shift) using a [γ^{32} P]-labeled 28 base pair double-stranded oligonucleotide containing the κB motif (Fig. 5A). Incubation of control nuclear extracts prepared from U87 MG cells transfected with pcDNA empty vector plasmid resulted in the formation of a faint band of DNA-protein complexes with slower electrophoretic mobility (lane 2), which we attributed to the ability of the endogenous NFκB to bind to its recognition motif. The same result was observed when probe was incubated with nuclear extracts prepared from cells transfected with non-targeting siRNA (NTsiRNA, lane 5). Silencing of Rad51 mRNA resulted in a significant decrease of DNA-protein complex formation (lanes 3 and 4). Expression of Rad51 caused an increase in the binding of endogenous proteins

to the probe (lane 6). The specificity of *in vitro* DNA binding was tested in competition assay with excess unlabeled probe (compare lane 7 to lane 8). Co-incubation of the reaction with anti-p65 antibody (lane 9), but not non-immune mouse serum (NMS, lane 10) resulted in the formation of a higher molecular weight complex (supershift) of lower mobility indicating the presence of p65/NFκB in the observed DNA-protein complex. As a control, extracts prepared from cells transfected with p65 were used (lane 11) and the presence of p65 in the complex (lane 13) as well as the specificity of the p65-DNA interaction (lanes 12 and 14) were determined. The expression of Rad51 was assessed by Western blot assay (Fig. 5B). These data suggest that Rad51 enhances the ability of NFκB to interact with the κB motif.

Rad51 and NFκB functional interaction. To investigate the relevance of Rad51:NFκB interaction to the functional cooperativity of these two proteins, we examined the effect of Rad51 on the LTR (-176/+66)-luciferase reporter using Rad51, full-length and deletion mutants in primary human microglial cells. As shown in Figure 5C, the activity of LTR was enhanced 50 fold in the presence of full-length Rad51 (1–339). The LTR activity was less with the expression of the Rad51 mutants with expanding deletion of C-terminus (39 and 13 fold) and was completely abrogated in the presence of Rad51 1–40 and Rad51 80–339 (5- and 3-fold respectively). Thus mutants of Rad51 (1–40 and 80–339), which exhibited no binding activity toward NFκB, showed reduced ability to stimulate transcription from HIV-1 LTR. These observations provide evidence that physical association between Rad51 and NFκB may be important for their coordinated effect on the expression of the viral genome. Expression of Rad51 and deletion mutants was verified by western blot analysis of cell lysates prepared from transfected cells using antibody against MYC-tagged Rad51 (Fig. 5D).

Discussion

Microglial cells together with CNS macrophages and astrocytes are the main targets for HIV-1 in the brain and serve as a reservoir

for the virus. While astrocytes display only a restricted infection, activated microglia and macrophages support productive infection and release proinflammatory cytokines and neurotoxins that contribute to HIV-1 neuropathogenesis. Here, we report a novel function of the DNA repair protein Rad51 in the regulation of transcription of the HIV-1 LTR in primary cultures of human microglial cells. Rad51 was found to enhance the action of the transcription factor NFκB, which is a key regulator of the basal activity of the HIV-1 LTR. Depletion of endogenous Rad51 by siRNA diminished NFκB-mediated stimulation of HIV-1 transcription. Similarly, silencing of NFκB by siRNA, caused a significant suppression of viral transcription, which was partially rescued by ectopic expression of Rad51, revealing a role for Rad51 in HIV-1 gene expression. A direct physical interaction between Rad51 and NFκB p65/NFκB was indicated *in vivo* by immunoprecipitation experiments and *in vitro* by GST-pull down assays while gel shift experiments indicated that Rad51 enhances the binding of p65/NFκB to its target DNA sequence within the HIV-1 LTR.

With regard to the mechanism whereby NFκB stimulates the HIV-1 LTR, Barboric et al.¹⁷ (2001) previously found that the ability of the NFκB to stimulate elongation of transcription depends on the P-TEFb kinase activity which phosphorylates the carboxyl-terminal domain (CTD) of the RPB1 subunit of RNAPII and thereby increases transcriptional processivity. It is also possible that a similar mechanism of activation of LTR transcription is involved for Rad51:NFκB. This may be important early after HIV-1 infection prior to the expression of Tat, which is a powerful stimulator of transcriptional elongation via its ability to stimulate RNAPII phosphorylation.¹⁸ In this scenario, Rad51 upregulation occurs soon after HIV-1 infection, perhaps as a consequence of activation of the DNA damage response pathway by proviral DNA⁴ and cooperates with NFκB to stimulate the HIV LTR while Tat begins to accumulate.

The importance of HIV-1 replication in the brain is suggested by numerous studies which demonstrate that in subjects without neurocognitive disease, most of the cerebrospinal fluid (CSF)

virus originates from the systemic compartment in contrast to subjects with HIV-1-associated dementia who have a genetically compartmentalized virus indicative of replication within the brain.^{19,20} Since HIV-1 does not productively infect neurons, indirect mechanisms of neuronal injury are thought to be involved in the progression of dementia. HIV-1-associated cognitive impairment correlates closely with neuroinflammation, monocyte

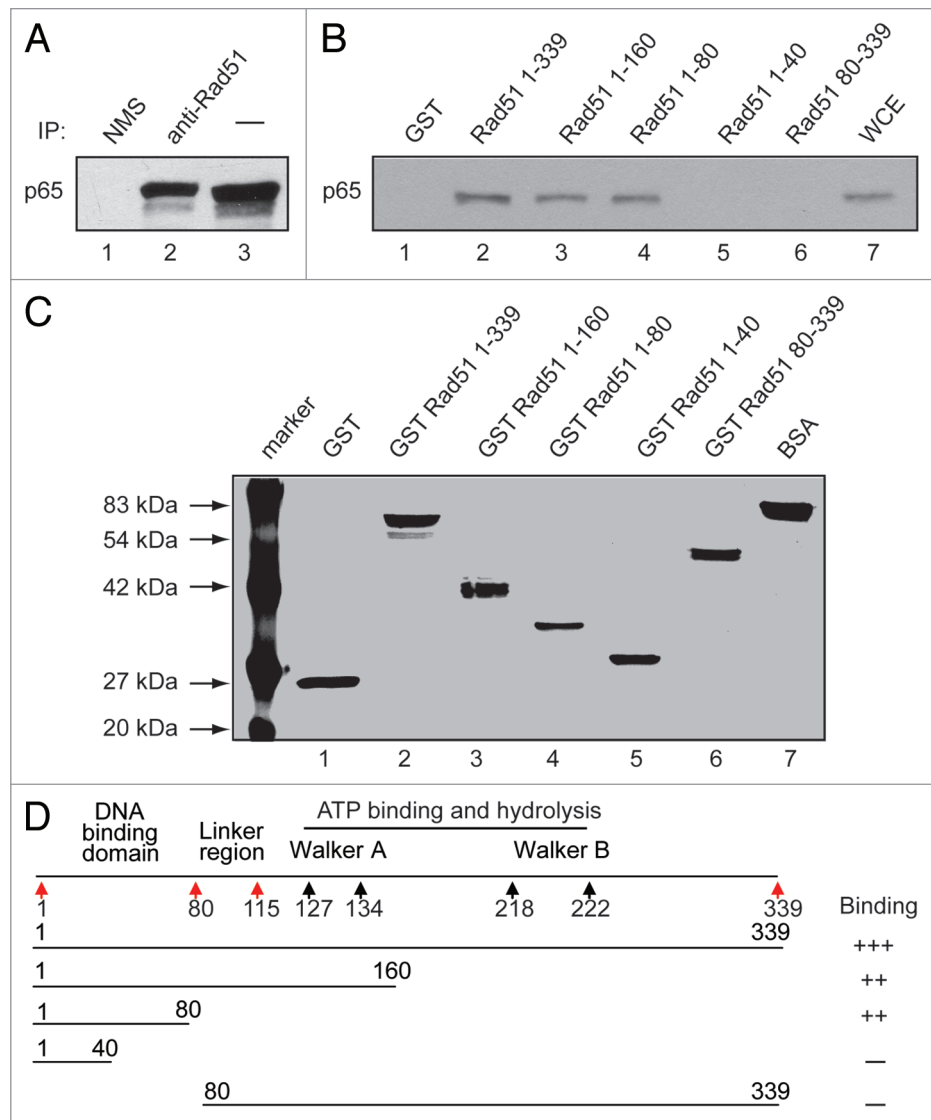


Figure 4. Physical interaction between Rad51 and p65/NFκB. (A) Interaction between p65/NFκB and Rad51 was evaluated by immunoprecipitation (IP)/western blot analysis. Total cell lysate prepared from U-87MG cells was immunoprecipitated using anti-Rad51 antibody (lane 2). Nonimmune mouse serum (NMS) was used for the negative control for the IP reaction (lane 1). After IP, protein complexes were washed, separated by SDS-PAGE followed by Western blot analysis using anti-p65 antibody. Fifty micrograms of the total cell lysate protein extract was included on the gel as a positive control (lane 3). (B) Protein extracts from U-87MG cells were incubated with GST-Rad51 or its deletion mutants (1 μM) in a GST pull-down assay for identification of the region of Rad51 that binds to p65/NFκB. GST was used as a negative control for the binding reaction (lane 1). NFκB/p65 was assessed by Western blot. Fifty micrograms of total cell protein extract was used as a positive control for protein expression (lane 7). (C) The integrity of GST and the GST-Rad51 fusion proteins used in the assay was demonstrated directly by SDS-PAGE followed by Coomassie blue staining. (D) Schematic representation of the organization of Rad51, the deletion mutants and their relative binding to p65/NFκB.

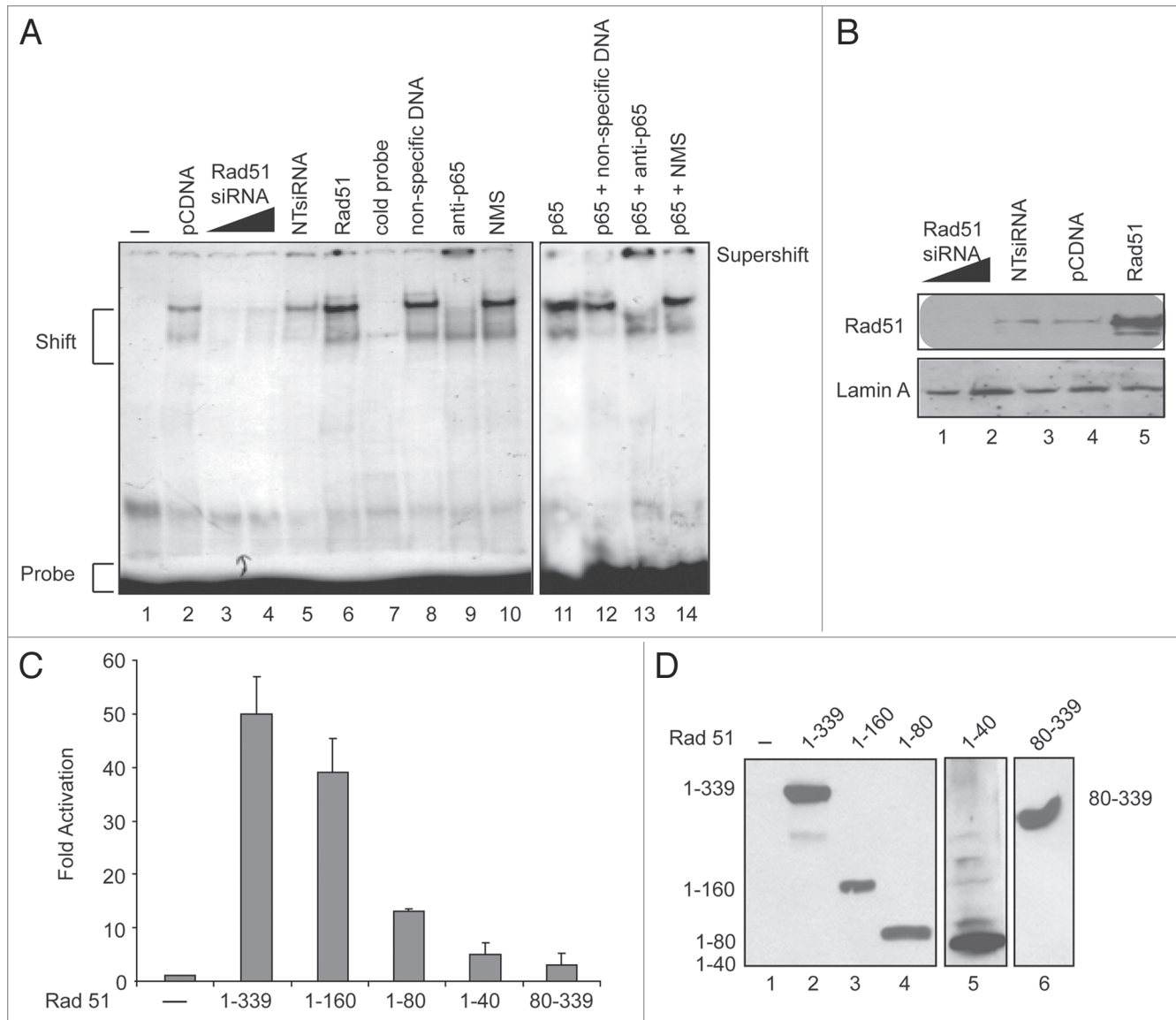


Figure 5. Effect of Rad51 on the interaction of p65/NFκB with κB DNA sequence element of HIV-1 LTR measured by gel shift in nuclear extracts from U87 MG cells. (A) Approximately 100,000 cpm of synthetic [γ - 32 P]-labeled double-stranded DNA oligonucleotide probe corresponding to the HIV-1 LTR κB site was incubated with 10 μg of nuclear extracts prepared from U87 MG cells transfected with 100 nmol siRNA for Rad51 (lane 3) or 200 nmol siRad51 (lane 4), 200 nmol non-target siRNA (lane 5), pcDNA6A-RAD51 (1–339) (lane 6), with p65/NFκB (lanes 11–14) and control pcDNA (lane 2). Labeled probe was also incubated with nuclear extracts prepared from pcDNA-transfected U87 MG cells in the presence of a specific DNA competitor (cold probe, lane 7), non-specific competitor (an unrelated dsDNA) (lanes 8 and 12), anti-p65 antibody (lanes 9 and 13) and normal mouse serum (NMS) (lanes 10 and 14). (B) Western blots were performed to determine the levels of expression of Rad51 with Lamin A as a loading control (lower part). (C) To identify the region of Rad51 involved in activation of the HIV-1 LTR, primary human microglial cells were transfected with 100 ng of LTR-luciferase plasmid (-167/+66) either alone or in combination with 300 ng of plasmids expressing Rad51: full length (1–339), Rad51 (1–160), Rad51 (1–80), Rad51 (1–40) and Rad51 (80–339). The amount of DNA in each transfection mixture was normalized with pcDNA6A. Luciferase activity was determined 48 hours after transfection. (D) Expression of Rad51 and deletion mutants was verified by western blot analysis of cell lysates prepared from the transfected primary human microglial cells using antibody against MYC-tagged Rad51.

infiltration and the presence of activated, though not necessarily infected, microglia and macrophages in the brain.^{21,22} Activated microglia release inflammatory mediators, cytokines and oxidative radicals at neurotoxic levels leading to stimulation of other bystander microglia and macrophages, astrocyte dysfunction and neuronal injury.^{23,24} In this regard, the activation of the NFκB signaling system of microglia, as described here, regulates not

only HIV-1 gene expression but also the expression of cellular genes associated with various important functions. These include genes involved in cell proliferation, oncogenesis, inflammation and immune response.²⁵ For example, it is known that NFκB modulates the expression of cytokines such as IL-6, IL-1β, TNFα and GM-CSF, chemokines such as IL-8, RANTES, MIP-1α and other molecules that may be important in the inflammatory

response such as iNOS, COX-2, VCAM and ICAM-1.²⁶ Thus it is possible that the stimulation of NFκB signaling by Rad51 that we have observed may also contribute to the aberrant expression of chemokines in the HIV-1-infected brain, thus amplifying the cascade of pathologic events. Given the importance of the role of the activation of microglia and inflammatory processes in the neuropathogenesis of HIV-1 encephalitis it would be of great interest to study the role of NFκB/Rad51 cross-communication in the inflammatory response.

Materials and Methods

Cell culture and transfection. Primary human fetal microglia were prepared from 8- to 12-week-old human fetal brain tissue (Advanced Bioscience Resources Inc., Alameda, CA USA) by a modified procedure based on the methods by Cole and de Vellis,²⁷ and Yong and Antel.²⁸ The human monocytic cell line U-937 was purchased from the ATCC. The human glioblastoma cell line, U-87MG, was grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum. All the transfection assays including transfection of RNAi and siRNA transfections were performed using Oligofectamine kit (Life Technologies, Inc., Carlsbad, CA). Rad51 siRNA was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Plasmids. The HIV-1 LTR-luciferase reporter plasmid and its deletion mutants were created by PCR and cloned into KpnI/BglII of pGL3 as described previously.¹¹ CMV-NFκB(p65) expression plasmid has been described previously.²⁹ pLEGFP-C1 Rad51 was created using a DNA fragment containing the human Rad51 gene from pMX-flag-Rad51 (gift from T. Skorski, Temple University) using primers: forward -5', GCT GCG GAC GGA TCC ATG GCA ATG CAG ATG CAG-3' and reverse -5', GAA AAA CCC GTC GAC TCA GTC TTT GGC ATC TCC-3'. The BamHI/SalI DNA fragment was cloned in the pLEGFP-C1 plasmid into BglII/SalI sites (Clontech, Palo Alto, CA). Rad51 full length and/or deletion mutant fragments were generated using standard PCR technique with the following primers (below) and cloning into pLEGFP-C1 (1-6), pGEX-5X-1 and pcDNA6-Myc/His-A (7-12):

1- coding:-5', GGG ATC CAA AAT GGC AAT GCA GAT GCA GCT T, -3' (fragments 1-339, 1-160, 1-80, 1-40);

2- coding:-5', GGG ATC CAA AAT GGC TTA GTT CCA ATG GGT TTC ACC A, -3' (fragment 81-339);

3- non-coding:-5', GAG CGG CCG CCA GTC TTT GGC ATC TCC CAC, -3' (fragments 1-339 and 81-339);

4- non-coding:-5', GAG CGG CCG CTT AAT GTA CAT GGC CTT TCC TTC A, -3' (fragment 1-160);

5- non-coding:-5', GAG CGG CCG CTT TTT AGC TGC CTC AGC CAG AAT T, -3' (fragment 1-80);

6- non-coding:-5', GAG CGG CCG CTT TTT CTT CAC ATC GTT GGC AT, -3' (fragment 1-40).

7- coding:-5', GGG ATC CCC ATG GCA ATG CAG ATG CAG CTT, -3' (fragments 1-339, 1-160, 1-80, 1-40);

8- coding:-5', GGG ATC CCC TTA GTT CCA ATG GGT TTC ACC A, -3' (fragment 81-339);

9- non-coding:-5', GAG CGG CCG CTC AGT CTT TGG CAT CTC CCA C, -3' (fragments 1-339 and 81-339);

10- non-coding:-5', GAG CGG CCG CTC AAT GTA CAT GGC CTT TCC TTC A, -3' (fragment 1-160);

11- non-coding:-5', GAG CGG CCG CTC ATT AGC TGC CTC AGC CAG AAT T, -3' (fragment 1-80);

12- non-coding:-5', GAG CGG CCG CTC ATT TCT TCA CAT CGT TGG CAT, -3' (fragment 1-40).

Luciferase assay. Cells were harvested at designated time points and protein extracts (20 μg) were used to examine the level of luciferase activity with the dual-luciferase reporter assay system (Promega, Madison, WI). The pRLTK plasmid was used as an internal control for transfection efficiency.

Protein extracts and western blot analysis. For whole cell extract preparation, cells were lysed for 30 min on ice in LB1 (50 mM HEPES, pH 7.5/150 mM NaCl/1.5 mM MgCl₂/1 mM EGTA/10% glycerol/1% Triton X-100) buffer containing protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) and 0.2 mM sodium orthovanadate. Cell debris was removed by centrifugation at 14,000 rpm for 15 min at 4°C. The supernatant was assayed for protein content by Bradford analysis (Bio-Rad, Hercules, CA). Nuclear and cytoplasmic fractions were prepared using NE-PER nuclear and cytoplasmic extraction reagents (Pierce Biotechnology, Rockford, IL). Western blot analysis was performed as described.³⁰ Fifty micrograms of whole cell extract, 10 μg of nuclear extract or 30 μg of cytoplasmic extract were used. Bound antibody was detected using the ECL enhanced chemiluminescence detection kit (Amersham, Arlington Heights, IL).

Expression and purification of recombinant GST-Rad51 fusion protein and its deletion mutants. The expression and purification of the GST fusion proteins have been described previously.³¹ The integrity of proteins was verified by SDS-polyacrylamide gel electrophoresis followed by Coomassie blue staining.

In vitro protein-protein interactions (GST pull-down assay). GST pull-down assays were performed as we have previously described.³⁰ For in vitro binding assays, 350 μg of whole-cell protein lysate prepared from U-87 MG cells was incubated with 1 μM of GST or GST-Rad51 fusion proteins (full length and/or deletion mutants). The bound proteins were eluted and separated by SDS-PAGE. GST-fusion proteins were detected by western blot analysis with anti-p65 antibodies.

Co-immunoprecipitation. Three hundred and fifty micrograms of total protein extract prepared from U-87 MG cells were incubated with either an anti-Rad51 antibody or normal mouse serum (NMS) overnight at 4°C. Immunocomplexes were precipitated by the addition of protein A-Sepharose beads and resolved by SDS-PAGE followed by western blotting using an anti-p65 antibody.

Electrophoretic mobility shift assay (EMSA). This assay was performed as previously described.³² Briefly, [³²P]-labeled 28-bp double-stranded primer containing the NFκB motif of the HIV-1 LTR (coding: 5'-TCG ACA GAG GGG ACT TTC CGA GAG GC-3'; non-coding: 5'-GCC TCT CGG AAA GTC CCC TCT GTC GA-3') was incubated with 10 μg of nuclear extracts

prepared from U87 MG cells transfected with plasmids expressing p65/NFκB, Rad51 full length (1–339) and deletion mutants (Rad51 1–160 and Rad51 1–80). The presence of p65 in the complexes was determined using anti-p65 antibodies or normal mouse serum (NMS). Cold non-labeled specific competitor was also used. The binding mixture was resolved by electrophoresis in a 6% native polyacrylamide gel and analyzed by autoradiography. The integrity and equal loading of proteins used in these assays were verified by SDS-PAGE.

HIV-1 virus preparation and infection. Primary human microglial cells (1×10^6) were infected with JR-FL strain of HIV-1 as follows. Fifty nanograms of p24-containing virus stock (prepared from HIV-infected U937 cells) were added to the cells followed by incubation in a small volume of serum-free media for 2 h at 37°C. The cells were then washed twice with PBS and fresh media containing 2% of FBS was added. Supernatants from the infected cells were collected at 2, 4, 6, 8 and 10 days after infection. HIV-1 p24 was measured by ELISA kit (PerkinElmer).

Detection of fluorescent proteins. Primary human microglial cells (5×10^4) were transfected with 1 μg of GFP-C1 or GFP-Rad51 expression plasmids and seeded in poly-L-lysine-coated glass chamber slides. Cells were fixed in 4% paraformaldehyde, blocked with 5% BSA and then incubated with anti-p65 rabbit

polyclonal primary antibodies for 1 h. Control cells were left without primary antibody. Cells were then washed and incubated with rhodamine-conjugated anti-rabbit secondary antibody for 45 min. Expression of Rad51 and p65 was visualized by fluorescence microscopy.

Antibodies. Anti-α-tubulin, clone B512 was from Sigma-Aldrich and anti-*myc* antibody from Invitrogen. Rabbit anti-Grb2 (cat# 3972), anti-lamin A (cat# 2032) and phospho-Rpb1 CTD (Ser2/5, cat# 4735) were from Cell Signaling Technology, Inc., (Danvers, MA). Rabbit anti-Rad51 and mouse anti-Rad51 (Ab-2) were from Calbiochem (EMD Chemicals, Gibbstown, NJ). Mouse anti-p65/NFκB (F6, sc-8008), anti-IκB-α (C-21, sc-371) and anti-IκKγ (FL-419, sc-8330) were obtained from Santa Cruz Biotechnology, Inc., (Santa Cruz, CA). Rhodamine-conjugated, goat anti-rabbit IgG (H + L) was from Pierce (Rockford, IL).

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