The Retinoblastoma Gene Product (Rb) Induces Binding of a Conformationally Inactive Nuclear Factor-κB*

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Nuclear factor-κB (NF-κB) regulates expression of several viral and cellular genes including the human immunodeficiency virus long terminal repeat, major histocompatibility complex class I, and interleukin 2Rα cytokine genes. Here we report that the retinoblastoma gene product (Rb) stimulates binding of the NF-κB p50 homodimer. The addition of Rb protein to an in vitro gel shift binding assay stimulated p50 binding greater than 10-fold. Interestingly, by analyzing NF-κB-dependent transcription activity in vitro, we demonstrate that Rb suppresses transcriptional activity of p50. Chymotrypsin analysis suggests that Rb induces a conformational change in the NF-κB-DNA complex, resulting in binding of a transcriptionally inactive complex. Finally, we demonstrate by coimmunoprecipitation analysis that the Rb-p50 complex is present in Jurkat cell extracts. Our results suggest that Rb may play an important role in regulation of NF-κB transcriptional activity.

NF-κB was initially identified as a DNA binding factor in the immunoglobulin (Ig) κ light chain enhancer in B lymphocytes (1–3). Members of the NF-κB/Rel family include p65 (Rel A), Rel B, p52/100 (NF-κB1), p50/p105 (NF-κB2), v-rel, and the product of the c-rel proto-oncogene (4–8). All members of the NF-κB/Rel family of transcription factors share a 300-amino acid region of homology in their N termini, referred to as the Rel homology domain. The activity of NF-κB/Rel family of transcription factors is controlled at multiple levels due, in part, to the complexity of the individual NF-κB molecules. One level of regulation is through the association of 1κB with NF-κB/Rel proteins (9, 10). Another level of regulation is provided by the different combination of NF-κB/Rel dimers formed and the κB sites recognized or activated by them (5, 11–13). For example, the p50 homodimer binds different κB sites such as Ig-κB, H2-κB, and IFN-β-κB with equally high affinity but only activates expression from H2-κB and Ig/HIV κB sites (12). Subsequent to DNA binding, NF-κB may activate transcription through interactions with the basal transcription factors (14–16). Studies have shown that c-rel and v-rel interact with TBP and TFIIB in vivo and in vitro.

The retinoblastoma susceptibility gene (Rb) is a member of a class of cellular genes referred to as tumor suppressor genes, anti-oncogenes, or recessive oncogenes (17). These proteins are associated with a subset of human cancers, including retinoblastoma, small cell lung cancer, osteosarcoma, and carcinoma of the bladder and breast, which are due to the frequent loss or mutational inactivation of the Rb gene (18, 19). Rb is a ubiquitously expressed nuclear protein, the phosphorylation state of which changes during the cell cycle, with a role in regulating cellular proliferation and gene expression (20–23). Phosphorylation of Rb at the G1-S transition allows the progression of cells into S phase and through the cell cycle. Sequences in the Rb protein, commonly referred to as the pocket (approximately 400 amino acids), are important for protein-protein interactions (24–26) and contain homology with basal transcription factors TBP and TFIIB (27).

Rb interacts with, and negatively regulates, the cellular transcription factor E2F (28, 29). Transient cotransfection experiments have demonstrated that an Rb expression plasmid inhibits E2F-dependent transcription, which is correlated with the ability of Rb to interact with E2F (30, 31). Rb is physically associated with the E2F DNA-protein complex. It has been proposed that Rb binds to the E2F activation domain and blocks its function as a transcription factor. Independent studies have further suggested that Rb-E2F is an active repressive complex, which inhibits activation of other promoter elements when bound to the E2F sites (28). Constructs containing E2F sites cloned into a promoter with a TATA box and an ATF transcription factor site were tested for activation by ATF in the presence of E2F-Rb. The presence of E2F sites inhibited activation of this promoter regardless of the presence of ATF. This attributes a critical function to Rb since it is described as a regulator that affects promoter activity by switching a E2F site from positive to negative elements. The interaction of E2F and Rb is restricted to the G1-S phase of the cell cycle since only underphosphorylated Rb forms complex with E2F. Subsequent phosphorylation of Rb disrupts the Rb-E2F interactions, allowing progression into S phase. Activation of E2F stimulates transcription of S-phase progression genes such as the E2F family, dihydrofolate reductase, thymidine kinase, thymidine synthase, ribonucleotide reductase, and c-myc.

The interaction of Rb with other transcription factors such as elf-1 (32), PU.1 (27), c-myc (33), and ATF (34) have been reported. Interestingly, interaction of Rb with elf-1 suppresses transcription similar to that seen with Rb and E2F. The functional consequence of the interaction of Rb with c-myc or PU.1 remains to be determined but appears to be distinct from that seen with E2F since Rb does not directly inhibit PU.1 or c-myc transcriptional activity in the absence of a binding site for E2F (32). In this report, we present experiments that demonstrate that Rb regulates NF-κB p50 transcriptional activity.
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EXPERIMENTAL PROCEDURES

Cell Lines and Plasmids—Jurkat E6.1 cells were obtained from the NIH AIDS repository. Cells were grown in RPMI 1640, supplemented with 10% fetal calf serum (Life Technologies, Inc.), 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in 5% CO2 incubator. Extracts were prepared using the Manley protocol (35). The p50 expression vector, pETp50, was provided by P. F. Lindholm, pGEX-2T, pGEX-2T RB (379–792), and pGEX-2T RB (379–928) were provided by B. Kaelin. The LTR-chloramphenicol acetyltransferase and AdML plasmids used for the in vitro transcription assays have been described previously (36,37).

Protein Expression and Purification—GST RB fusion or GST proteins were generated in Escherichia coli by growth of the transformed HB101 strain in Luria broth with 100 μg/ml ampicillin. The cultures were grown to an absorbance of 0.6, followed by induction with 0.5 mM isopropyl-β-D-galactopyranoside for 3 h. The GST fusion proteins were obtained by sonication of the bacterial pellet in buffer containing 50 mM Tris, pH 7.5, 250 mM NaCl, 0.1% Nonidet P-40, and 1 mM phenylmethylsulfonyl fluoride. The purified preparations were analyzed by SDS-polyacrylamide gel electrophoresis and then stained with Coomasie Brilliant Blue. The concentrations of the 27-kDa GST, 71-kDa GST, and 86-kDa GST RB (379–928) were estimated by comparison with a bovine serum albumin standard. One unit is defined as 100 ng of protein. [35S]Methionine-labeled NF-κB proteins were generated in vitro transcription reactions using the TNT system (Promega Corp.). Protein production and yield was confirmed by SDS-polyacrylamide gel electrophoresis and autoradiography.

In Vitro Transcription in Whole-Cell Extracts—Templates were prepared by digestion of 100 μg of plasmid DNA with 5–10-fold units excess of restriction enzymes for up to 2 h under buffer conditions recommended by New England BioLabs. After the termination of the digest, the DNA was subjected to two phenol/chloroform/isomyl alcohol (50:50:1) extractions and a subsequent ethanol precipitation. All incubations were done at 30°C. The in vitro transcription buffer contained 10 mM HEPES, pH 7.9, 50 mM KCl, 0.5 mM EDTA, 1.5 mM dithiothreitol, 6.25 mM MgCl2, and 8.5% glycerol. Two to ten μg/ml of the HIV-1 LTR chloramphenicol acetyltransferase and AdML DNAs were linearized by digestion with EcoRI and BamHI, respectively. One hundred twenty-five to 500 ng of template were used per reaction. HeLa whole-cell extracts were prepared as described previously (35) and added to a final concentration of approximately 3.75 mg/ml (~55 ng/reaction). The reactions also contained nucleiide triphosphates in water (500 μM), [α-32P]UTP (400 Ci/mmol) (15 μCi), purified GST fusion protein (1.0 unit), and purified p50 (Promega) at various concentrations. Transcription reactions were terminated by the addition of 20 μM Tris-HCl, pH 7.8, 150 mM NaCl, and 0.2% SDS. Labeled RNA was purified and analyzed on a 4% polyacrylamide-urea gel under denaturing conditions as described previously.

Coimmunoprecipitation Assay—Jurkat cells were stimulated with 50 ng/ml 12-O-tetradecanoylphorbol-13-acetate for 1 h. Whole-cell extracts were prepared, and immunoprecipitations were performed as follows. Cells were washed twice with phosphate-buffered saline and resuspended in 400–600 μl of lysis buffer containing 100 mM Tris-HCl, pH 7.5, 10 mM EDTA, 300 mM NaCl, and 0.1% Nonidet P-40. Aprotinin and leupeptin were added fresh at a final concentration of 1 μg/ml. After 40 min of incubation, the cells were vortexed briefly, centrifuged at 14,000 rpm for 5 min, and the supernatant was retained for immunoprecipitation. Three hundred μg of whole-cell extract was used in each assay. The immunoprecipitation was performed using equivalent amounts of GST-coupled, GST-RB, or rabbit IgG beads. After 3 h of incubation at 4°C, the antibodies were precipitated using protein A-protein G beads (previously blocked with 4% bovine serum albumin) for 1 h at 4°C. Beads and the complexes bound to them were washed two times with the lysis buffer. The immunoprecipitates were separated on a 4–20% Tris-glycine gel, transferred onto a nitrocellulose filter, and immunoblotted with anti-Rb antibodies. After 30 min in blocking buffer, as described above, the blot was incubated with the Rb antibody (1:10,000; Upstate Biotechnology). The blot was washed three times in blocking buffer. Peroxidase-labeled anti-mouse antibodies (1:10,000; Amersham Corp.) was added to the primary antibodies. The signal was developed using Amersham ECL Western blotting reagents. The image was developed using Kodak XAR autoradiography film.

RESULTS

The DNA Binding Activity of the NF-κB p50 Homodimer Is Induced by the Rb Pocket—E. coli-purified GST Rb pocket (amino acids 379–792) fusion protein was tested for its ability to regulate the DNA binding activity of NF-κB p50. Approximately 100 ng of purified p50 was incubated with the Igκ NF-κB oligonucleotide probe. The specificity of the NF-κB gel shift complex was also demonstrated by adding 3 μl of anti-p50 polyclonal antibodies (Santa Cruz Biotechnol- ogy, Inc.) to the p50 gel shift reactions, producing a supershift complex. NF-κB Protein and GST RB Fusion-Protein Binding Assays—For gel shift assays, the [35S]methionine-labeled NF-κB proteins were incubated with approximately 0.5 μg of either GST or GST Rb proteins in buffer D with 0.5% Nonidet P-40 and 0.5 mM dithiothreitol for 2 h at 4°C. Glutathione-Sepharose beads (100 μl of 50% suspension) were added for an additional 2 h at 4°C with gentle mixing. The reactions were washed three times with the same buffer, followed by SDS-polyacrylamide gel electrophoresis and autoradiography.

For gel shift assays, the [35S]methionine-labeled NF-κB proteins were incubated with approximately 0.5 μg of either GST or GST Rb proteins in buffer D with 0.5% Nonidet P-40 and 0.5 mM dithiothreitol for 2 h at 4°C. Glutathione-Sepharose beads (100 μl of 50% suspension) were added for an additional 2 h at 4°C with gentle mixing. The reactions were washed three times with the same buffer, followed by SDS-polyacrylamide gel electrophoresis and autoradiography.
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The effect of Rb (379–792) on the p50-induced transcription was analyzed (Fig. 2D). Control reactions with HIV-1 LTR and GST or GST Rb were included as controls (Fig. 2D, lanes 1 and 2). The addition of p50 to the control reactions with GST produced a bell-shaped induction curve (Fig. 2D, lanes 3–7). The difference in p50 titration curves seen in Fig. 2, A and D, are likely due to a difference in specific activity of the p50 used in the two experiments. When Rb (379–792) was included in the p50-induced transcription assay, a suppression of the p50-induced transcription activity was detected (Fig. 2D, lanes 8–12). These results are quantitatively demonstrated in Fig. 2E. To demonstrate that Rb does not nonspecifically repress transcription, GST Rb was added to transcription reactions with a control AdML template (Fig. 2F). Transcription from the AdML promoter was compared with reactions containing GST or GST Rb. No differences were observed in the transcription reactions of AdML alone or with GST or GST Rb (379–792) (Fig. 2F, lanes 1–3).

One interpretation of the transcription experiments might be that since Rb induces p50 binding, the bell-shaped p50 induction curve is simply offset in the presence of Rb. We, therefore, compared the transcriptional activity of equivalent p50 DNA binding activities in the absence or presence of Rb (Fig. 2G). Gel shift assays were performed with in vitro transcription reaction buffer (Fig. 2G). Under those conditions, equivalent p50-DNA binding activity was observed at 14 ng of p50 in the presence of GST and 3 ng of p50 in the presence of GST Rb. At 56 ng of p50, equivalent p50 binding was observed in the presence of GST or GST Rb (Fig. 2G). By comparing the percentage of p50 bound to the κB site and the fold induction of transcriptional activity, we conclude that p50 is not able to activate transcription in the presence of Rb (Fig. 2G).

Rb Induces Conformational Change of NF-κB p50—The transcription analysis suggested that the Rb-induced NF-κB complex was transcriptionally inactive. Previous studies by Fujita et al. (12) have demonstrated that NF-κB p50 provides strong transcriptional activation only when adopting a chymotrypsin-resistant conformation. Therefore, we analyzed the conformation of p50 bound to the κB motif in the presence of Rb using a chymotrypsin sensitivity assay. Two sets of gel shift reactions were studied and compared; the first contained p50 (Fig. 3A, lanes 1–5), and the second contained the Rb-induced p50 gel shift complex (Fig. 3A, lanes 8–12). The p50 gel shift complexes were subsequently treated with chymotrypsin for various times. The reactions were stopped upon the addition of chymostatin, and the complex containing the labeled κB motif was analyzed by electrophoretic mobility shift assay. Quantitative analysis of chymotrypsin digestion demonstrated that the control NF-κB and Rb-induced NF-κB complex were digested at different rates (Fig. 3A, lanes 1–5 and 7–11; Fig. 3D). For example, at the 10-min time point, the Rb-induced NF-κB gel shift complex was reduced by approximately 55%. In contrast, no significant reduction in the amount of control NF-κB complex was observed. The difference in chymotrypsin sensitivity is further evident if one compares the NF-κB gel shift complex intensity at 0 and 15 min. Although there is 5–10-fold more complex in the Rb-induced control incubation, the signal at 15 min of digestion is equivalent between the two samples (Fig. 3A, lanes 1 and 5, 7 and 11). The chymotrypsin-sensitive nature of the Rb-induced NF-κB complex correlates with decreased transcriptional activity.
FIG. 2. A, in vitro transcriptional activation of wild-type HIV-1 LTR by NF-κB p50 subunit. In vitro transcription reactions were carried out as described previously (23). Lane 1, 125 ng of HIV LTR template DNA; lane 2, 125 ng of HIV LTR template DNA plus 1 μg/ml α-amanitin. Lanes
Coimmunoprecipitation of Rb Protein with NF-κB p50 in Vivo—Rb induction of NF-κB binding activity might be mediated through direct protein-protein interaction. Therefore, coimmunoprecipitation assays were performed with Jurkat whole-cell extracts. The whole-cell extract was incubated with anti-p50, anti-Rb, or rabbit IgG. The proteins from these immunoprecipitations were separated on a 4–20% SDS-polyacrylamide gel, transferred to nitrocellulose, and immunoblotted with anti-Rb antibody. Rb protein was detected in immunoprecipitation reactions that contained anti-Rb or anti-p50 antibodies (Fig. 4A, lanes 1 and 2). In the control reaction, Rb failed to coprecipitate with preimmune rabbit antiserum (Fig. 4A, lane 3). Because the IgG heavy chain migrates at ~50 kDa, we were unable to perform the reciprocal coimmunoprecipitation of p50 with Rb, i.e. precipitate the Rb protein and immunoblot with anti-p50.

The interaction between Rb and p50 was also demonstrated with in vitro GST binding assays. Glutathione-Sepharose beads containing equal quantities of GST or GST Rb (379–792) proteins were incubated with in vitro translated p50. NF-κB p50 was specifically precipitated in the presence of GST Rb (Fig. 4B, lane 2) but not in the presence of control GST protein. Similar binding assays with other members of the NF-κB family, including p65 and c-rel, suggests that the Rb pocket interacts with the Rel homology domain (data not shown).

**DISCUSSION**

The activity of the NF-κB/Rel family of transcription factors is regulated at multiple levels. In this study, we discovered a novel interaction between the cellular transcription factor NF-κB p50 and the product of the retinoblastoma susceptibility
addition to its DNA binding activity. Studies have shown that Rb binds to several transcription factors in vivo and in vitro and, as a result, regulates their function. Given the physical interaction between Rb and p50 and the function of Rb in repression of E2F activity, one interpretation of these results might be that Rb is a part of the NF-κB/DNA complex, blocking the interaction of NF-κB with the basal transcription machinery. Using antibody supershift and biotinylated DNA pull-down assays, we have been unable to demonstrate that Rb is a stable component of the DNA-protein complex.

It has been demonstrated previously that the p50 homodimer provides transcriptional activation only when adopting a chymotrypsin-resistant conformation (12). Our present results suggest that the conformation of the p50 homodimer is altered in the presence of Rb, reducing its transcriptional activity and functioning as a transcriptional repressor. This repression, therefore, would be functionally different from that observed with E2F and explains why NF-κB was not detected in screening assays to identify promoter elements that are normally targeted by Rb (36). Along these lines, it will be of interest to determine if Rb phosphorylation regulates the interaction of Rb and p50 in a cell cycle-dependent manner.

The results presented in this report suggest that Rb is one of several cellular proteins that function to regulate p50 binding and activity. It has been demonstrated that the cellular protooncogene, Bcl-3, specifically inhibits binding of homodimeric p50 to DNA (37, 38). Interestingly, when coexpressed, Bcl-3 and p50 both localize to the nucleus and form a protein-protein complex that is detected in nuclear extracts. In contrast, IκB-α and IκB-γ inhibit p50 homodimer activity by inhibiting nuclear translocation. In this regard, Rb and Bcl-3 both function as nuclear inhibitors of p50. There are, however, distinct differences in the activities of Bcl-3 and Rb. Although Bcl-3 inhibits binding of the p50 homodimer, it does not inhibit binding of the p50-p65 heterodimer that can bind to the NF-κB site and activate transcription. Rb, in contrast, stimulates the binding of an inactive p50 homodimer to the NF-κB site, resulting in a decrease in transcription. NF-κB plays an important role in the response of lymphocytes to antigens and cytokines. In addition to the cytoplasmic regulation of NF-κB transport, Rb induction of transcriptionally inactive p50 homodimer binding may represent another important pathway for the regulation of nuclear NF-κB activity.

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