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Evidence for the Involvement of a Nuclear NF-κB Inhibitor in Global Down-Regulation of the Major Histocompatibility Complex Class I Enhancer in Adenovirus Type 12-Transformed Cells

XIAOHONG LIU,† RUOWEN GE,‡ AND ROBERT P. RICCIARDI*

Department of Microbiology, School of Dental Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104

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Diminished expression of major histocompatibility complex class I antigens on the surface of adenovirus type 12 (Ad12)-transformed cells contributes to their high tumorigenic potential by enabling them to escape immune recognition by cytotoxic T lymphocytes. This low class I antigen expression is due to a block in class I transcription, which is mediated by Ad12 E1A. Genetic analysis has shown that the class I enhancer is the target for transcriptional down-regulation. In this study, we show that the ability of the R1 element of the class I enhancer to stimulate transcription is greatly reduced in Ad12-transformed cells. The loss of functional activity by the R1 element was attributed to loss of binding by the NF-κB p50-p65 heterodimer. NF-κB binding appears to be blocked within the nucleus rather than at the level of nuclear translocation. Significantly, NF-κB binding activity could be recovered from the nuclear extracts of Ad12-transformed cells following detergent treatment, suggesting that the block is mediated through a nuclear inhibitor present in the Ad12-transformed cells. These results, taken together with the fact that the R2 element of the class I enhancer exhibits strong binding to the transcriptional repressor COUP-TF, suggest that the class I enhancer is globally down-regulated in Ad12-transformed cells.

While all serotypes of human adenovirus are able to transform nonpermissive rodent cells, only a certain subset can generate tumors in immunocompetent animals. The high tumorigenic potential of adenovirus type 12 (Ad12) correlates with diminished expression of the major histocompatibility complex (MHC) class I antigens on the surface of Ad12-transformed cells (8, 16, 48). By contrast, Ad5-transformed cells, which are nontumorigenic, are not reduced in class I expression. The low level of class I antigens on Ad12-transformed cells apparently contributes to their ability to form tumors, since these transformed cells have a greater opportunity to escape recognition by immunosurveillant cytotoxic T lymphocytes (8, 52, 56).

In Ad12-transformed cells, class I expression is blocked at the level of transcription (1, 18), and Ad12 E1A is the only viral gene required for this down-regulation to occur (55). Genetic analysis has revealed that the MHC class I enhancer is the target of Ad12 E1A-mediated transcriptional down-regulation (20). Strong binding activity to the R2 element of the class I enhancer (Fig. 1) by the transcriptional repressor COUP-TF suggested that this region of the enhancer acts negatively in Ad12-transformed cells (20, 26, 30). Indeed, in the context of synthetic promoters, only the wild-type R2, and not a mutated R2 element defective in COUP-TF binding, was able to repress transcription in Ad12-transformed cells (26).

In this study, we showed that the R1 element of the MHC class I enhancer, which normally is responsible for 70% of the enhancer activity (11), is also down-regulated in Ad12-transformed cells. The R1 element contains a κB consensus binding sequence for the NF-κB/Rel protein family, members of which have been implicated in the regulation of MHC class I expression (5, 22, 24, 45, 49). Specifically, we show here that lack of transcriptional stimulation by the R1 element in Ad12-transformed cells is due to loss of binding by the NF-κB p50-p65 heterodimer. Only the transcriptionally inactive p50-p50 homodimer binds to the R1 element in Ad12-transformed cells. Most interestingly, our results indicate that the inhibition of NF-κB binding in Ad12-transformed cells occurs within the nucleus, rather than at the well-characterized regulatory step of nuclear translocation. The fact that detergent treatment of Ad12 nuclear extract could completely recover NF-κB binding activity suggests that the inhibition is mediated through a nuclear inhibitor present in the Ad12-transformed cells. A model for global down-regulation of the MHC class I enhancer through the R1 and R2 elements in Ad12-transformed cells is discussed.

MATERIALS AND METHODS

Cell lines and antibodies. Ad12-transformed (F10-12 and 12A1) and Ad5-transformed (KAd5-3 and Wt5a) cells were derived from primary mouse BALB/c ($H\text{-}2^d$) cells (16). Peptide antibodies against different NF-κB family members (kindly provided by Nancy Rice) include anti-p65 N-terminal (antibody 1207) and anti-p65 C-terminal (1226), anti-p50 N-terminal (1263) and anti-p50 C-terminal (1157), and anti-Rel C-terminal (1266) antibodies (41, 42). Anti-p52 (1495) and anti-RelB (1318) antibodies are directed against the N termini of murine p100 and RelB, respectively (40). IκBα and IκBβ antibodies were raised against recombinant proteins (kindly provided by Rodrigo Bravo and Sankar Ghosh) (15, 54).

Plasmid constructions, transfection, and CAT assay. pH2-37, pH2-H2E, and pH2-(R1)2 are reporter constructs containing the chloramphenicol acetyltransferase (CAT) gene under the control of the $H\text{-}2K^b$ basal promoter (pH2-37), the $H\text{-}2K^b$ enhancer juxtaposed to the $H\text{-}2K^b$ basal promoter (pH2-H2E), and two copies of the R1 element linked to the $H\text{-}2K^b$ basal promoter [pH2-(R1)2],

* Corresponding author. Mailing address: Department of Microbiology, School of Dental Medicine, University of Pennsylvania, 4010 Locust St., Philadelphia, PA 19104. Phone: (215) 898-3905. Fax: (215) 898-8385. Electronic mail address: ricciardi@biochem.dental.upenn.edu.

† Present address: Department of Pharmacology, School of Medicine, University of California at San Diego, La Jolla, CA 92093-0636.

‡ Present address: Department of Zoology, Faculty of Science, National University of Singapore, Singapore 0511, Singapore.

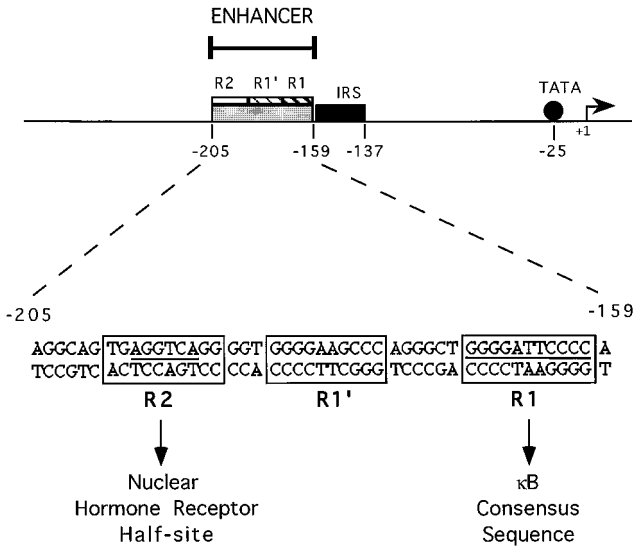

FIG. 1. Regulatory elements of the *H-2K*^{b} MHC class I promoter. Indicated are the transcription initiation site (arrow), TATA box, interferon response sequence (IRS), and class I enhancer. The class I enhancer is composed of R2, R1′, and R1 elements (boxed). The R2 element can be recognized by RARβ/RXRβ and COUP-TF nuclear hormone receptors. The R1 element contains a κB consensus sequence, which can be recognized by members of the NF-κB/Rel protein family.

respectively (20, 25). pH2-H2EM1 is the same as the wild-type pH2-H2E except that there is a consensus site mutation within the R2 element in which AGGTCA is changed to ATTTCA. Transfection and CAT assays were performed as described elsewhere (20). For each transfection, 5 μg of each reporter plasmid was cotransfected with 4 μg of pRSV-β-gal and 11 μg of pBluescript. β-Galactosidase activity was used to normalize transfection efficiencies.

Cell extract preparation. Nuclear extract was prepared as described previously (30) with modifications. All the steps were performed on ice or in a 4°C cold room to prevent proteolysis. Cells were lysed on ice for 5 to 8 min in 2 packed cell volumes of lysis buffer (9 mM Tris-HCl [pH 7.5], 135 mM NaCl, 0.9 mM $MgCl_2$, 0.5 mM dithiothreitol [DTT], 0.5 mM phenylmethylsulfonyl fluoride [PMSF], and 0.3% Triton X-100, with DTT and PMSF added fresh). The extent of lysis (>95%) and the integrity of nuclei were checked microscopically. The nuclei were then pelleted at 3,500 rpm for 5 min in an Eppendorf microcentrifuge, washed once in lysis buffer without Triton X-100, and then resuspended in 2 packed cell volumes of Dignam buffer C (25% glycerol, 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.9], 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT) (14). The nuclear proteins were extracted by rocking at 4°C for 45 min, and insoluble material was pelleted at 14,000 rpm for 15 min at 4°C in an Eppendorf microcentrifuge. The supernatant was designated the nuclear extract. The supernatant from the first spin after cell lysis was saved as the postnuclear fraction, mixed with 0.11 volume of Dignam buffer B (0.3 M HEPES [pH 7.9], 1.4 M KCl, 0.03 M $MgCl_2$) (14), rocked in the cold room for 15 min, and spun at 14,000 rpm in an Eppendorf microcentrifuge for 45 min. The supernatant after this spin was designated the cytoplasmic extract. Both the nuclear and cytoplasmic extracts were then dialyzed at 4°C for 1 h against Shapiro's buffer D [20 mM HEPES (pH 7.9), 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.2 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 2 mM DTT, 0.5 mM PMSF, 0.5 mg of leupeptin per liter, 0.7 mg of pepstatin A per liter] (50), and precipitated proteins were removed by centrifugation at 12,000 rpm for 15 min at 4°C in an Eppendorf microcentrifuge. Extracts were quick-frozen in a dry ice-ethanol bath and stored at −80°C. The protein concentrations of nuclear and cytoplasmic extracts were measured by the Bradford assay (10) (Bio-Rad). The ratio of the total nuclear protein recovered versus the total cytoplasmic protein was around 1:3.5. Where mentioned, a sucrose pad was used to purify the nuclei. Briefly, after cell lysis, nuclei were washed once and layered onto 30% sucrose in 9 mM Tris-HCl (pH 7.5)–135 mM NaCl–0.9 mM $MgCl_2$–0.5 mM DTT–0.5 mM PMSF. After a 10-min spin at 2,200 rpm (950 × *g*) at 4°C in a benchtop Beckman centrifuge, nuclear pellet was resuspended in Dignam buffer C and extraction of nuclear proteins was continued as described above.

For preparation of whole-cell extracts, cells were lysed directly in 2× sodium dodecyl sulfate (SDS) sample buffer (100 mM Tris-HCl [pH 6.8], 4% SDS, 200 mM DTT, and 20% glycerol). The protein concentrations of whole-cell extracts were measured with a Micro BCA Protein Assay Reagent Kit (Pierce).

Western blot (immunoblot). Adjusted amounts of nuclear extract (9 μg) and cytoplasmic extract (31.5 μg) were used to derive equal cell equivalents of these subcellular fractions based on the 1:3.5 ratio of the total nuclear versus the total cytoplasmic protein (3). Whole-cell extract (50 μg) was used where indicated. All extracts were fractionated on SDS–10% polyacrylamide gels and transferred to an Immobilon-P membrane (Millipore). After blocking with TBST (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.05% Tween 20) containing 5% Carnation nonfat dry milk, the membrane was incubated for 1 h at room temperature (RT) with primary antibody at the following dilutions: 1:1,000 for anti-p65 (antibody 1226), anti-p52 (1495), and anti-IκBα; 1:1,200 for anti-IκBβ; and 1:3,000 for anti-p50 (1157). Following 1 h of incubation with 1:1,000-diluted goat anti-rabbit immunoglobulin G-horseradish peroxidase conjugate, the membrane was developed with the Amersham enhanced chemiluminescence detection reagent.

Band shift and supershift. The sequence of double-stranded R1 oligonucleotide has been described elsewhere (26). For band shift, 4.5 μg of nuclear extract was incubated at 30°C for 30 min with 3×10^4 cpm of ^{32}P-labeled R1 probe and 4 μg of poly(dI-dC) · poly(dI-dC) in R1 binding buffer (10 mM Tris-HCl [pH 7.5], 100 mM NaCl, 1 mM EDTA, 1 mM DTT, and 5% glycerol). The DNA-protein complexes were then fractionated at RT on a 5% nondenaturing polyacrylamide gel in 0.5× TBE buffer (45 mM Tris [pH 8.3], 45 mM boric acid, 0.5 mM EDTA). For supershift, antibody was preincubated with nuclear extract and 4 μg of poly(dI-dC) · poly(dI-dC) in R1 binding buffer at RT for 15 min. The labeled probe was then added to the binding reactions and incubated at 30°C for 30 min. Treatment of nuclear extract with the ionic detergent sodium deoxycholate (DOC) was performed as described by Baeuerle and Baltimore (3, 4). Briefly, DNA band shift and supershift reactions were performed as described above, and then 0.2% Nonidet P-40 (final concentration) and 0.8% DOC (final concentration) were added and incubated at RT for 5 min. Nonidet P-40 was then made up to a 1.2% final concentration, and after another 5-min incubation at RT, the DNA binding reaction mixtures were loaded onto a 5% nondenaturing polyacrylamide gel in 0.5× TBE buffer.

RESULTS

Transcriptional stimulation by the R1 element of the class I enhancer is reduced in Ad12-transformed compared with Ad5-transformed cells. We previously demonstrated that when examined in the context of synthetic promoters, the R2 element of the class I enhancer was able to down-regulate transcription in Ad12- but not Ad5-transformed cells (26). This negative regulation by the R2 element in Ad12-transformed cells correlated with strong binding of a transcriptional repressor, COUP-TF (30). In surprising contrast, class I repression in Ad12-transformed cells could not be relieved when a mutated R2 element, which was defective in COUP-TF binding, was examined in the context of the natural class I enhancer (Fig. 2, compare wild-type pH2-H2E with mutant pH2-H2EM1). It is noted that in Ad5-transformed cells R2 functions as a positive element, which explains the partial drop in transcriptional activity observed with pH2-H2EM1 in these cells. This inability of the mutated R2 element to derepress class I transcription raised the possibility that at least one other element of the class I enhancer, in addition to the R2 element, is also down-regulated in Ad12-transformed cells. Because the R1 element of the class I enhancer is known to be essential for stimulating class I transcription (11, 13), we investigated whether its functional activity is altered in Ad12-transformed cells. As shown in Fig. 2, a R1 dimer construct [pH2-(R1)2] was much less transcriptionally active in Ad12-transformed compared to Ad5-transformed cells. These results indicate that the R1 element of the MHC class I enhancer is another target of Ad12 E1A-mediated repression. The fact that the R1 element is functionally inactive in Ad12-transformed cells serves to explain why mutating the negative R2 element failed to relieve repression.

Loss of NF-κB binding to the R1 element in Ad12-transformed cells. The R1 element has a κB consensus binding sequence for NF-κB/Rel family members, which largely function in regulating transcription of genes involved in immune and inflammatory responses (32, 51, 53). Members of the mammalian NF-κB/Rel family include p50, p52, p65, Rel, and RelB. All the members contain at their N termini the so-called Rel homology domain, which is required for dimerization, DNA binding, and nuclear translocation. The NF-κB/Rel fam-

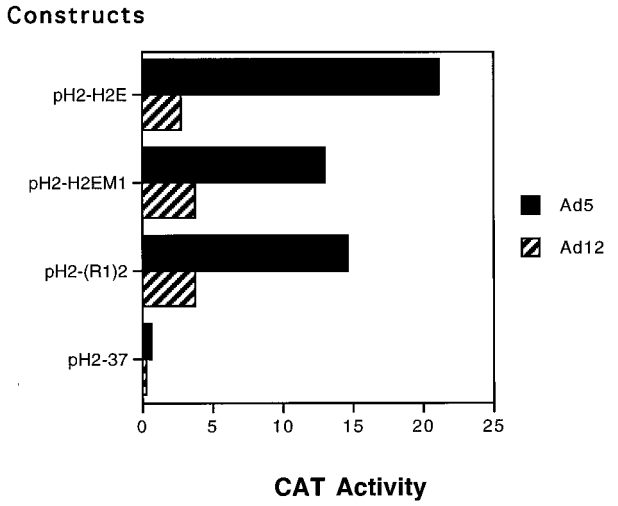

FIG. 2. Mutation of the R2 element in the context of the natural class I enhancer does not relieve repression in Ad12-transformed cells. The reporter CAT constructs were driven by the *H-2* basal promoter alone (pH2-37) or the *H-2* basal promoter linked to the wild-type *H-2* enhancer (pH2-H2E), the M1 mutant *H-2* enhancer in which AGGTCA was converted to ATTTCA within the R2 element (pH2-H2EM1), or an R1 dimer [pH2-(R1)2]. The constructs were transfected into Ad12 (12A1)- and Ad5 (KAd5-3)-transformed cells and analyzed for CAT activity. Transfection efficiency was normalized against β-galactosidase activity. Similar results were observed with other Ad12- and Ad5-transformed cells (data not shown).

ily members can either homo- or heterodimerize through their N-terminal Rel homology domains, and the nature of the homo- or heterodimer that is formed will influence its ability to recognize a specific κB binding sequence (9, 19, 27, 28, 36, 44).

To determine whether the reduced transcriptional activity of the R1 element in Ad12- versus Ad5-transformed cells is due to differences in their R1 binding activities, band shift experiments using a labeled R1 oligonucleotide and nuclear extracts from Ad12- and Ad5-transformed cells were performed. As shown in Fig. 3, nuclear extract from Ad12-transformed cells generated a single DNA-protein complex (lane 1), whereas nuclear extract from Ad5-transformed cells showed an additional, more slowly migrating complex (lane 7). To identify which members of the NF-κB/Rel family are present in these R1 complexes, supershift experiments using a panel of peptide antibodies against different NF-κB/Rel family members were conducted. Only the more slowly migrating complex unique to Ad5-transformed cells was supershifted by an anti-p65 antibody (lane 9). However, this more slowly migrating R1 complex, as well as the faster-migrating R1 complex common to both Ad12- and Ad5-transformed cells, was supershifted by an anti-p50 antibody (Fig. 3, lanes 2 and 8). Neither the more slowly migrating nor the faster-migrating R1 complex was recognized by anti-p52, anti-Rel, or anti-RelB antibodies (lanes 4 to 6 and 10 to 12). These results indicated that the more slowly migrating R1 complex from Ad5-transformed cells consists of p50-p65 NF-κB heterodimer and that the faster-migrating R1 complex common to both Ad12- and Ad5-transformed cells represents the p50-p50 homodimer. Thus, nuclear NF-κB binding activity to the R1 element is greatly diminished in Ad12-transformed cells, whereas the binding activity of the p50-p50 homodimer is not perturbed. Our results, which through the use of specific antibodies have definitively characterized the components of the respective R1 complexes in Ad12- and Ad5-transformed mouse cells, are consistent with the previous suggestion that NF-κB binding activity is reduced in Ad12-transformed rat cells (31, 34).

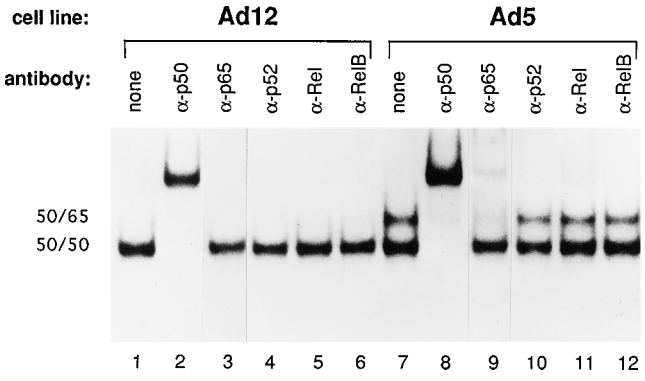

FIG. 3. There is a lack of NF-κB p50-p65 binding to the R1 element in Ad12-transformed cells. ^{32}P-labeled R1 oligonucleotide was incubated with nuclear extracts from Ad12-transformed (12A1) or Ad5-transformed (KAd5-3) cells, without antibody (lanes 1 and 7) or with peptide antibodies against different NF-κB/Rel family members (lanes 2 to 6 and lanes 8 to 12). The specific antibodies used are anti-p50 (1263), anti-p65 (1207), anti-p52 (1495), anti-Rel (1266), and anti-RelB (1318). The positions of p50-p65 heterodimer (NF-κB) and p50-p50 homodimer are indicated on the left. Similar results were obtained with other Ad12- and Ad5-transformed cells (data not shown).

The loss of NF-κB (p50-p65 heterodimer) binding to the R1 element in Ad12-transformed cells (Fig. 3) accounts for the low transcriptional activity of the R1 element in these cells (Fig. 2). This is because only the p50-p65 heterodimer activates transcription, because of a transactivation domain present in p65. By contrast, the p50-p50 homodimer does not activate transcription since p50 lacks a transactivation domain (6, 17, 23, 37, 43, 44, 46).

The block of NF-κB binding to the R1 element in Ad12-transformed cells is not controlled at the level of nuclear translocation. Regulation of NF-κB by nuclear translocation has been widely studied. The transport of NF-κB into the nucleus is regulated by cytoplasmic inhibitors (IκBs), which physically retain NF-κB in the cytoplasm in an inactive form (7, 32, 51). To determine in Ad12-transformed cells whether the block of NF-κB binding to the R1 element is controlled at the level of nuclear translocation, a Western blot was performed using anti-p65 antibody on both the nuclear and cytoplasmic fractions. As shown in Fig. 4A, similar levels of p65 were present in nuclear extracts from Ad12- and Ad5-transformed cells. In addition, no major differences were observed in the cytoplasmic levels of p65. Furthermore, no significant differences were observed for the p50 subunit in either the nuclear or the cytoplasmic fractions (Fig. 4B). As expected from these findings, whole-cell extracts from Ad12- and Ad5-transformed cells were found to contain similar levels of p50 and its precursor p105, respectively (Fig. 4B, lanes 9 and 10). These results indicated that the block of NF-κB binding to the R1 element in Ad12-transformed cells is not regulated by nuclear translocation, since similar levels of p65 and p50, respectively, are present in the nuclei of both cell types.

The block of NF-κB binding to the R1 element in Ad12-transformed cells is attributed to a nuclear inhibitor. Because the p65 and p50 subunits of NF-κB are already present in the nuclei of Ad12-transformed cells, the lack of NF-κB DNA binding activity in the nuclear extracts of Ad12-transformed cells could be due to a nuclear inhibitor. To test whether we could disrupt such a putative nuclear inhibitor from its association with NF-κB (or the p65 subunit), nuclear extract from Ad12-transformed cells was treated with the detergent DOC. DOC is known to recover NF-κB DNA binding activity from

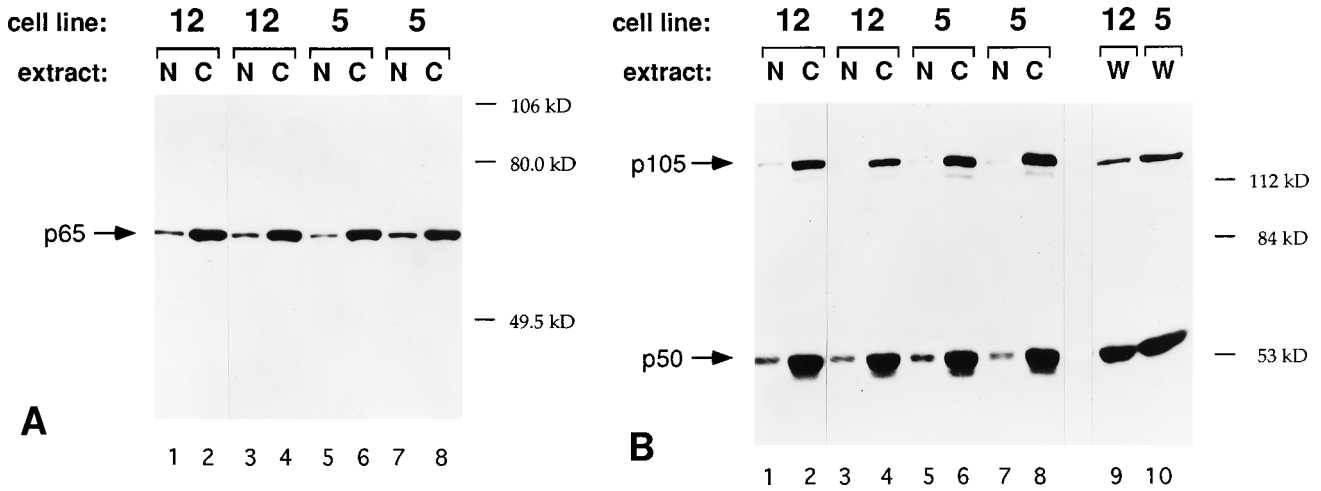

FIG. 4. NF-κB subunits are present in the nuclei of Ad12- and Ad5-transformed cells. Nuclear (N), cytoplasmic (C), or whole-cell (W) extracts from Ad12- and Ad5-transformed cells were analyzed on Western blots probed with either anti-p65 antibody (1226) (A) or anti-p50 antibody (1157) (B). The amounts of nuclear and cytoplasmic extracts used were adjusted to give equal cell equivalents of subcellular fractions. The actual cell lines are F10-12 (lanes 1 and 2), 12A1 (lanes 3, 4, and 9), KAd5-3 (lanes 5, 6, and 10) and Wt5a (lanes 7 and 8). The positions of p65, p50, and p105 (the p50 precursor) are indicated by arrows on the left, and molecular mass markers are denoted on the right.

the non-DNA-binding cytoplasmic fraction, presumably by dissociating the cytoplasmic inhibitor IκBα or Iκβ from NF-κB (3, 4). Indeed, treatment of Ad12 nuclear extract with DOC fully recovered the NF-κB DNA binding activity (Fig. 5, lanes 3 to 6). While the nuclear extract from Ad5-transformed cells also showed a modest increase in NF-κB binding upon DOC treatment (lanes 1 and 2), the increase in Ad12-transformed cells was much more dramatic. These results strongly argue that a nuclear inhibitor that prevents NF-κB from binding to the R1 element of the class I enhancer is present in Ad12-transformed cells. Interestingly, the similar NF-κB binding activity after DOC treatment of both cell types (lanes 2 and 4) suggests that Ad12-transformed cells have much more of the nuclear inhibitor than Ad5-transformed cells and is consistent with there being similar amounts of p65 and p50, respectively, in the nuclei of Ad12- and Ad5-transformed cells (Fig. 4).

Since IκBα is a major cytoplasmic inhibitor for the nuclear translocation of NF-κB (7) and it has been reported that in certain circumstances IκBα can translocate to the nucleus and inhibit the DNA binding and transcriptional activities of NF-κB (2, 12, 57), it was important to learn whether the nuclear inhibition of NF-κB binding to the R1 element in Ad12-transformed cells involves IκBα. To address this question, the subcellular distribution of IκBα was analyzed by Western blot on nuclear and cytoplasmic fractions from both Ad12- and Ad5-transformed cells. Figure 6 clearly shows that IκBα resides only in the cytoplasm in both cell types, indicating that the putative nuclear inhibitor in Ad12-transformed cells is distinct from IκBα. The fact that IκBα was not detected in the nuclear extracts was also useful in ruling out cytoplasmic contamination. In addition, no differences were observed between Ad12- and Ad5-transformed cell extracts when the Western

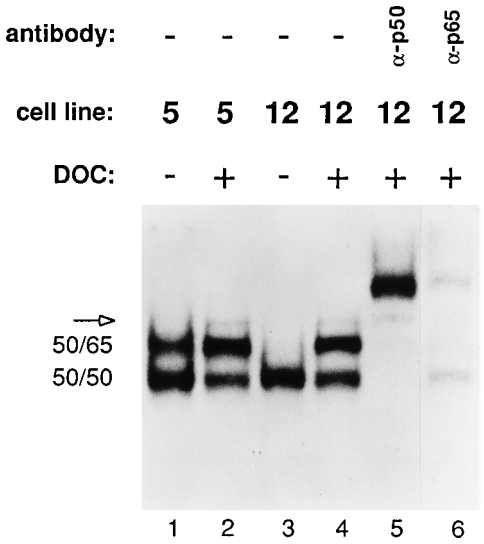

FIG. 5. DOC treatment recovers NF-κB DNA binding activity in Ad12-transformed cells. Nuclear extracts were subjected to band shift or supershift reactions using labeled R1 oligonucleotide as the probe. Reaction mixtures were either untreated or treated with DOC before being loaded onto the gel. The positions of the p50-p65 heterodimer and the p50-p50 homodimer are shown on the left. The faint band indicated by the arrow likely represents a p65-p65 homodimer. Similar results were obtained with other Ad12- and Ad5-transformed cells (data not shown).

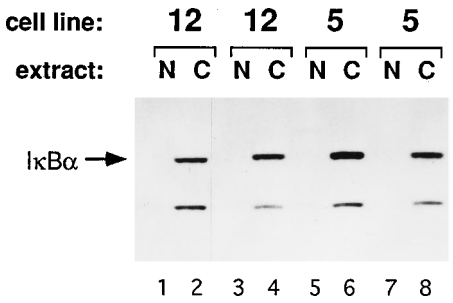

FIG. 6. IκBα resides in the cytoplasm. Nuclear (N) and cytoplasmic (C) extracts from Ad12- and Ad5-transformed cells were analyzed by Western blot as described in the legend to Fig. 4 except that the blot was probed with anti-IκBα antibody. The actual cell lines are F10-12 (lanes 1 and 2), 12A1 (lanes 3 and 4), KAd5-3 (lanes 5 and 6), and Wt5a (lanes 7 and 8). The position of IκBα is indicated by an arrow on the left. The faster-migrating band detected is likely a breakdown product of IκBα.

blots were probed with antibodies against other NF-κB cytoplasmic inhibitors, IκBβ, p100, and p105.

It is noted that trace amounts of p105, which is the precursor of p50 and also a cytoplasmic inhibitor of NF-κB, were detected in the nuclear extracts of both Ad12- and Ad5-transformed cells (Fig. 4B), even when the nuclei were purified through a sucrose cushion (data not shown). While generally regarded as a cytoplasmic component because of masking of its nuclear localization signal, small amounts of p105 in nuclear extracts may result from a minor translocation of this precursor protein into the nucleus (33). Most importantly, the facts that trace amounts of p105 were detected in the nuclear extracts of both Ad12- and Ad5-transformed cells and that inhibition of NF-κB by p105 is insensitive to DOC treatment (42) indicate that p105 is not contributing to the differential NF-κB binding activity observed between these cells.

Taken together, our results indicate that reduced NF-κB binding is responsible for the diminished transcriptional activity of the R1 element of the class I enhancer in Ad12-transformed cells. The block in NF-κB binding occurs within the nucleus rather than at the level of nuclear translocation. Recovery of NF-κB binding activity by detergent suggests the involvement of a nuclear inhibitor. While the identity of this inhibitor remains to be determined, it does not appear to be one of the known cytoplasmic inhibitors of NF-κB.

DISCUSSION

Diminished class I transcription in tumorigenic Ad12-transformed cells enables them to evade immune detection by cytotoxic T lymphocytes (8, 52, 56). We have previously demonstrated that the class I enhancer is the target of this transcriptional down-regulation (20), which is mediated by Ad12 E1A (55). In this study, we have shown that the R1 element of the class I enhancer, which is critical for class I transcription, is functionally inactive in Ad12-transformed cells compared to the nontumorigenic Ad5-transformed cells. The inability of the R1 element to stimulate transcription in Ad12-transformed cells is due to a selective inhibition of binding by the NF-κB p50-p65 heterodimer. In contrast, binding to the R1 element by the p50-p50 homodimer is not altered. The fact that the p50-p65 heterodimer is transcriptionally active, whereas the p50-p50 homodimer is transcriptionally inactive (6, 17, 23, 37, 43, 44, 46), provides an explanation as to why the R1 element fails to stimulate transcription in Ad12-transformed cells.

Nuclear translocation serves as a key step in the natural regulation of NF-κB binding activity (7, 32, 51). The block of NF-κB binding to the R1 element in Ad12-transformed cells does not occur at the step of nuclear translocation, since Ad12- and Ad5-transformed cells have similar levels of p65 and p50, respectively, present in their nuclei. These findings support the notion that the block of NF-κB binding to the R1 element occurs after NF-κB enters the nucleus. That this is indeed the case was demonstrated by the fact that NF-κB binding activity was recovered following treatment of Ad12 nuclear extract by the detergent DOC. Most importantly, the DOC treatment experiment suggests that there is a nuclear inhibitor complexed with NF-κB (or the p65 subunit) which can be dissociated by the detergent. In further support of this conclusion, preliminary findings suggest that an active inhibitor can be separated from endogenous NF-κB upon DOC treatment of Ad12 nuclear extract and used to inhibit the NF-κB binding activity in Ad5 nuclear extract. In addition, NF-κB binding activity is diminished in Ad5/Ad12 somatic hybrid transformed cells (unpublished data), which are also low in class I expression (21), further arguing that Ad12-transformed cells contain an inhibitor that actively down-regulates NF-κB and class I transcription.

A recent report suggests that newly synthesized IκBα, induced by tumor necrosis factor alpha and interleukin-1 treatment, could enter the nucleus and negatively regulate the DNA binding and transcriptional activities of NF-κB (2). However, our Western blot analysis with anti-IκBα antibody on nuclear and cytoplasmic fractions from Ad12- and Ad5-transformed cells clearly indicated that the cytoplasmic NF-κB inhibitor IκBα remains in the cytoplasm in both Ad12- and Ad5-transformed cells. Thus, the nuclear inhibitor in Ad12-transformed cells is distinct from IκBα. Furthermore, the nuclear inhibitor does not appear to be any of the other known cytoplasmic NF-κB inhibitors, e.g., IκBβ, p100, and p105.

Ad12 E1A per se could be the nuclear inhibitor in Ad12-transformed cells. However, preliminary results suggest that Ad12 E1A does not directly prevent NF-κB from binding to DNA, since recombinant Ad12 E1A protein failed to inhibit NF-κB binding activity. Alternatively, Ad12 E1A may indirectly mediate the nuclear block of NF-κB binding through inducing or stabilizing a cellular nuclear inhibitor. In particular, sequences in the first exon of Ad12 E1A, which include a nonconserved 20-amino-acid stretch that is not present in Ad5 E1A, were shown to be responsible for mediating the loss of NF-κB binding (29). The fact that nuclear extract from Ad5-transformed cells also showed some increase in NF-κB binding activity upon DOC treatment supports the notion that the nuclear inhibitor is of cellular origin. It is interesting to speculate that such a nuclear inhibitor could normally function as a fine-tuning mechanism for the regulation of NF-κB target genes by modulating the amounts of active p50-p65 heterodimer to inactive p50-p50 homodimer and that Ad12 E1A could usurp this mechanism to mediate down-regulation of class I transcription.

A nuclear inhibitor for NF-κB has also been implicated in down-regulating NF-κB binding activity to the human immunodeficiency virus type 1 (HIV-1) long terminal repeat in chronically HIV-1-infected monocytes, which may be responsible for the restricted HIV-1 expression in these cells (38). The inhibition was specific for NF-κB, since p50-p50 homodimer binding was not changed and DOC treatment was able to restore the NF-κB DNA binding activity (38). Whether the nuclear inhibitor present in the chronically HIV-1-infected monocytes is the same as the nuclear inhibitor in Ad12-transformed cells remains to be learned. Regardless, different viruses may utilize similar mechanisms to deregulate the DNA binding and transcriptional activities of cellular transcription factors in becoming tumorigenic or persistent.

In contrast to our findings, which suggest the existence of a nuclear inhibitor, a very recent study proposed that down-regulation of NF-κB in Ad12-transformed rat cells is attributed to reduced levels of p50, due to interference of p105 processing (47). There are several possible explanations for this discrepancy. First, our analysis is based on the use of nuclear extracts, whereas Schouten et al. employed whole-cell extracts. However, when we did examine whole-cell extracts from Ad12- and Ad5-transformed cells (Fig. 4B), we observed no major differences in the levels of p50 or p105, respectively. The second possibility is that this study used adenovirus-transformed mouse, as opposed to rat, cells. However, in a separate study using Ad12- and Ad5-transformed rat cells, we did not detect any major difference in the levels of p50 or p105 (29). Finally, the sources of the p50 antibodies could account for the different interpretations. For example, the SC-114 anti-p50 antibody

was recently shown to be nonspecific in Western blot analysis (35).

Our overall studies imply that Ad12 E1A mediates global down-regulation of MHC class I enhancer in transformed cells by inactivating each of the enhancer elements. As shown here, there is lack of NF-κB binding to the R1 element in Ad12-transformed cells, which explains why we failed to observe transcriptional stimulation by the R1 element in these cells. Moreover, there is high binding activity by the transcriptional repressor COUP-TF to the R2 element (30). Preliminary evidence further suggests that the R1′ element also contributes to class I repression in Ad12-transformed cells. The significance of global down-regulation of the MHC class I enhancer could be to ensure complete shutdown of class I transcription in Ad12-transformed cells. Through global down-regulation of the class I enhancer, the cell surface class I antigens would remain at low levels, enabling Ad12-transformed cells to escape immune surveillance and to form tumors. It is interesting to consider that this mechanism of global down-regulation may be used by Ad12 to establish persistent infection in the human host (39).

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