The N Terminus of Adenovirus Type 12 E1A Inhibits Major Histocompatibility Complex Class I Expression by Preventing Phosphorylation of NF- κ B p65 Ser²⁷⁶ through Direct Binding[∇]

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The immune-escape strategy employed by human oncogenic adenovirus type 12 (Ad12) involves downregulation of major histocompatibility complex class I (MHC-I) transcription by disabling the transactivator NF- κ B (p50/p65). This is accomplished by the Ad12 E1A protein (E1A-12), which prevents NF- κ B from becoming phosphorylated by the protein kinase A catalytic subunit (PKAc). In this study, we examined the interactions between E1A-12 and NF- κ B. Our data show that an E1A-12 mutant retaining the N-terminal 66 amino acids was as effective as the wild-type E1A-12 protein (266 amino acids) in binding p65, preventing phosphorylation of p65-Ser²⁷⁶, and inhibiting transactivation. In contrast, the nontumorigenic adenovirus type 5 E1A protein (E1A-5) and other E1A-12 mutants lacking the N-terminal regions were severely defective in these activities. Further studies revealed that an N-terminal peptide consisting of residues 1 to 40 of E1A-12 was able to associate directly with p65 *in vitro* and prevent PKAc from phosphorylating p65-Ser²⁷⁶. In the absence of the N terminus, there is an almost complete loss of E1A-12 binding to p65. These findings provide solid evidence for the role of the E1A-12 N terminus as an NF- κ B binding domain. Significantly, this study indicates that the E1A-12 N terminus prevents PKAc from gaining access to p65 to account for Ser²⁷⁶ hypophosphorylation. The E1A-12 N terminus interaction with p65 serves as a key explanation of how Ad12 downregulates MHC-I transcription and contributes to oncogenesis by escaping cytotoxic T lymphocytes.

Adenovirus type 12 (Ad12), the most studied tumorigenic strain of adenovirus, is capable of generating tumors in adult rodents with intact immune systems (34). The E1A and E1B genes of both Ad12 tumorigenic and Ad5 nontumorigenic strains are sufficient to transform and immortalize cells in culture. Viral transformation is largely due to the association of E1A with the retinoblastoma protein (pRB), which results in stimulation of the cell cycle, and to the association of E1B with p53, which prevents apoptosis (27). However, these viral transforming proteins differ in that only Ad12 E1A (E1A-12) has the additional property of being tumorigenic (34).

One major feature of Ad12 tumorigenic cells is the loss of major histocompatibility complex class I (MHC-I) antigens on the cell surface (1, 6, 9, 28, 31), which provides a means of escaping immune-surveillance by cytotoxic T lymphocytes (CTLs) (4, 35). E1A-12 is solely responsible for MHC-I diminution as this is observed in cells that express E1A-12 as the only gene of Ad12 (31). This reduction of MHC-I expression occurs at the level of transcription (1), with the class I enhancer serving as the targeted DNA element (10). Detailed analysis of the class I enhancer revealed strong binding of a nuclear hormone receptor, COUP-TFII, to the enhancer R2 site (22) and loss of binding of the activator NF- κ B (p50/p65) to the en-

hancer R1 site (21). These dual actions by E1A-12, which enable repressor binding and disable activator binding, likely provide a fail-safe mechanism to ensure that MHC-I surface antigens remain unexpressed under different physiological conditions, e.g., fluctuations in cytokine levels (13).

In Ad12 tumorigenic cells, NF- κ B is synthesized and constitutively translocated to the nucleus but fails to bind to its DNA recognition site (R1) of the class I enhancer (21). This inability of NF- κ B to bind DNA is largely attributed to hypophosphorylation of its p50 subunit (17). It was further revealed that in order for the p50 subunit to bind DNA, it must be phosphorylated on serine residue 337 (Ser³³⁷) and that the protein kinase A catalytic subunit (PKAc) performs this enzymatic function (11, 14).

In addition, we recently observed that in Ad12 tumorigenic cells, E1A-12 prevents PKAc from phosphorylating the corresponding residue on the p65 subunit of NF- κ B, i.e., serine 276 (p65-Ser²⁷⁶) (12), whose phosphorylation is required for transactivation. It has been suggested that phosphorylation of Ser²⁷⁶ promotes transcriptional transactivation by releasing the p65 C-terminal transactivation domain from an intramolecular masking by the p65 N-terminal region (37, 38). This unmasking of the p65 transactivation domain would enable a bivalent interaction with the coactivator CBP/p300 (38). Thus, by preventing phosphorylation of the PKAc sites on both the p50 and p65 subunits, E1A-12 could disable NF- κ B from both binding to DNA and activating transcription. Importantly, E1A-12 does not prevent phosphorylation of NF- κ B by directly inhibiting PKAc as enzymatic activity of this kinase in Ad12 tumor-

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igenic cells remains the same as in Ad5 nontumorigenic cells (12). This is consistent with the fact that prevention of global PKAc activity will eventually lead to cell death, as we have shown by treating cells with the PKAc inhibitor H89 (11, 12). Thus, E1A-12 appears to target NF- κ B specifically.

In this study, we reveal the mechanism by which the E1A-12 oncoprotein prevents NF- κ B from activating transcription. We show that the N-terminal 40-amino-acid region of E1A-12 directly binds p65, which acts to impede phosphorylation of p65-Ser²⁷⁶. This unique property of E1A-12 disables NF- κ B from activating transcription of the MHC-I promoters. This mechanism, which results in downregulation of MHC-I cell surface antigen expression, serves as a means by which Ad12 tumorigenic cells escape lysis by CTLs.

MATERIALS AND METHODS

Cell culture. The COS-7 cell line was grown in Dulbecco's modified Eagle's (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μ g/ml streptomycin.

DNA constructs. To construct N-terminally FLAG-tagged E1A-12 and E1A-5, pCMV-Ad12E1A and pCMV-Ad5E1A (where CMV is cytomegalovirus) were used as templates to amplify 13S cDNAs of E1A-12 and E1A-5, respectively. FLAG-containing cDNA fragments of full-length or mutant E1A-12 were generated by using primers harboring a HindIII restriction site immediately upstream of a sequence coding for the FLAG epitope (Sigma) at the 5' end and an XbaI site downstream of the E1A-12 coding sequence at the 3' end. The amplified cDNA fragments were digested with HindIII and XbaI, gel purified, and subcloned into pRC/CMV (Invitrogen) individually to construct pRC/CMV-E1A12wt (where wt is wild type), an E1A-12 mutant consisting of amino acids 1 to 66 (aa1-66), aa1-232, aa1-240, aa31-266, aa95-266, and aa202-266. pRC/CMV-E1A5wt encoding full-length E1A-5 with an N-terminal FLAG epitope was produced in a similar fashion. To generate a glutathione S-transferase (GST) and p65 fusion protein, the human p65 cDNA was subcloned into pGEX4T-1 (Amersham Pharmacia Biotech). Construction of pCMV-hp65, a His₆-tagged p65 plasmid, as well as the pSG424-p65 plasmid expressing the Gal4-p65 fusion protein was previously described (12). The Gal4-Luc reporter plasmid and pSG424 were kind gifts from T. D. Gilmore (Boston University, Boston, MA). To create N-terminally His₆-tagged wild-type E1A-12 and its truncated forms (aa1-40 and aa41-266), each amplified cDNA fragment with an upstream His₆ coding sequence was treated with NdeI and SapI and inserted into the same sites of pTXB1 (New England Biolabs).

Cell transfection, Western blotting, and immunoprecipitation. Transfection of COS-7 cells was carried out by using FuGENE 6 (Roche) according to the manufacturer's instructions. For the coexpression of p50 and p65 with FLAG-tagged E1A-5 or E1A-12 in COS-7 cells, 300 ng of pCMV-hp65 and 500 ng of pRC/CMV-E1A5 or pRC/CMV-E1A12 expressing wild-type or truncation E1A-12 mutants were cotransfected into COS-7 cells on six-well plates. Cells were harvested at 40 h posttransfection. Extraction of cellular proteins was performed as previously described (11, 17). The precleared whole-cell lysates were incubated at 4°C overnight with rabbit anti-FLAG antibody (Cell Signaling) or normal rabbit serum (Santa Cruz) in NETN buffer (150 mM NaCl, 1 mM EDTA, 20 mM Tris-Cl, pH 8.0, 0.2% Nonidet P-40, 1 mM Na₃VO₄, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, and 2 μ g/ml aprotinin). Protein A agarose beads (Invitrogen) were then added to the mixture and incubated at 4°C for 2 h. Immunocomplexes were harvested following intensive washes with NETN buffer. Western blotting was conducted as previously reported (17). Rabbit monoclonal anti-p65 antibody (Epitomics) and rabbit anti-phospho-p65 (Ser²⁷⁶) antibody (Cell Signaling) were used to detect the presence of pan-p65 and phospho-p65 (Ser²⁷⁶), respectively. Alternatively, cell lysates were incubated with goat anti-p65 agarose conjugate (sc109G-AC; Santa Cruz) or normal goat serum (Santa Cruz) in NETN buffer, followed by washing, elution, and Western blotting using rabbit anti-FLAG antibody (Cell Signaling).

In vitro kinase assay. The *in vitro* kinase assay was conducted as described previously (12, 14). Briefly, 0.4 μ g of His₆-p65 was first incubated with various concentrations (0 to 50 μ M) of E1A-12 peptide (Invitrogen) or Kaposi's sarcoma-associated herpesvirus (KSHV) processivity factor 8 (PF8) peptide consisting of residues 1 to 22 (Biosynthesis) at 30°C for 1 h. Both peptides were synthesized and purified by high-performance liquid chromatography (HPLC). Two units of PKAc (Sigma) was added, and the incubation was continued for another 30 min

in kinase buffer (25 mM HEPES, pH 7.5, 10 mM MgCl₂, 10 mM NaF, 1 mM Na₃VO₄, 1 mM CaCl₂, 5 mM dithiothreitol, 20 μ M ATP). Total p65 and p65 phosphorylated at Ser²⁷⁶ were visualized by Western blotting using the pan-p65 antibody (Epitomics) and the anti-phospho-p65 (Ser²⁷⁶) antibody (Cell Signaling), respectively. To assess the effect of the synthesized E1A-12 peptide consisting of residues 1 to 40 (pep1-40) on phosphorylation of CREB by PKAc, increasing concentrations (0 to 5 μ M) of biotinylated CREB-Ser¹³³ peptides (Cell Signaling) were incubated with the E1A-12 pep1-40 at 30°C for 30 min in the kinase buffer containing 2 units of PKAc. The reactions were stopped with 50 mM EDTA (pH 8.0), and mixtures were then allowed to bind to streptavidin-coated microplates (Roche Applied Science) at room temperature for 60 min. Following four washes with phosphate-buffered saline containing 0.1% Tween-20 (PBS-T), rabbit anti-phospho-PKA substrate [RRX(S/T)] antibody diluted in 1% bovine serum albumin (BSA) in PBS-T was added to each well and incubated at room temperature for 2 h with gentle rocking. All wells were then rinsed four times with PBS-T prior to a standard enzyme-linked immunosorbent assay (ELISA) using horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Bio-Rad) and ABTS [2,2'-azinobis(3-ethylbenzthiazolinesulfonic acid; Roche)]. The absorbance at 405 nm was recorded using a Genios Pro plate reader (Tecan).

Luciferase assay. p65 transactivation under the influence of E1A-12 was investigated with a Dual-Glo Luciferase Assay System (Promega) as described previously (12). Briefly, subconfluent COS-7 cells were grown on six-well plates. Plasmids that were used to transfect each well include *Renilla* luciferase reporter pRL-TK (30 ng), Gal4-Luc reporter (200 ng), empty vector pSG424 (250 ng), or pSG424-p65 (250 ng), as well as 500 ng of either pRC/CMV-E1A12wt or one of the mutants (pRC/CMV-E1A12-aa1-66, -aa1-232, -aa1-240, -aa31-266, or -aa95-266). At 36 h posttransfection, cells were lysed, and luminescent signals were measured using a Genios Pro plate reader. Each transfection was performed in duplicate, and each experiment was repeated three times.

In vitro protein-binding assays. Ni-nitrilotriacetic acid (NTA) Superflow agarose beads (Qiagen) were washed with PBS supplemented with 0.2% NP-40 (PBS-NP). Pulldown assays were performed by incubating 1 μ g of recombinant His₆-E1A12wt or its truncated forms (His₆-E1A12aa1-40 and His₆-E1A12aa41-266) with 100 μ l of slurry (50%, vol/vol) of prewashed Ni-NTA agarose at 4°C for 2 h. The beads were then washed extensively with cold PBS-NP, followed by blocking at 4°C overnight in the presence of 5% BSA. Next, 2 μ g of GST-p65, GST-p50, or GST control was added and further incubated with the beads at 4°C for 3 h. Following five washes with PBS-NP, bound proteins were eluted with 300 mM imidazole and subjected to Western blotting with the monoclonal p65 antibody (Epitomics) or GST antibody (GE). As a control, recombinant GST-p65 or GST-p50 also was incubated with the BSA-blocked Ni-NTA agarose in the absence of His₆-tagged E1A-12 proteins, followed by the same procedures of elution and Western blotting.

FACS analysis. Cells were treated with 5 mM EDTA in PBS and resuspended in PBS containing 3% bovine serum albumin (PBSB). Cells were then incubated with two fluorescein isothiocyanate (FITC)-conjugated monoclonal anti-mouse MHC-I antibodies (34-1-2S from eBioscience and 34-1-2S from Santa Cruz Biotechnology) in PBSB for 45 min on ice. After cells were washed with PBS three times, they were fixed in 0.5 ml of 4% paraformaldehyde in PBS and then subjected to fluorescence-activated cell sorting (FACS) analysis.

RESULTS

The N terminus of E1A-12 prevents Ser²⁷⁶ of p65 from becoming phosphorylated. We previously demonstrated that E1A-12 hinders PKAc from phosphorylating NF- κ B both *in vivo* and *in vitro* (12). In order to define the region of E1A-12 responsible for preventing phosphorylation of p65, a set of N- and C-terminal truncation mutants of E1A-12 were generated (Fig. 1A). The E1A-12 mutants, wt E1A-12, and wt E1A-5, each containing a FLAG tag appended to the N terminus, were cotransfected with a p65 plasmid into COS-7 cells. The phosphorylation of p65-Ser²⁷⁶ was examined by Western blotting using a phospho-Ser²⁷⁶ antibody (Fig. 1B). As expected from our earlier studies (12), phosphorylation of p65-Ser²⁷⁶ was greatly reduced in the presence of wt E1A-12 (Fig. 1B, lane 2) compared to wt E1A-5 (lane 9). As with wt E1A-12, all of the three C-terminal deletion mutants aa1-240, aa1-232, and aa1-66 (Fig. 1A) were able to mediate remarkable reduction of

FIG. 1. The N terminus of E1A-12 inhibits phosphorylation of p65-Ser²⁷⁶. (A) Schematic diagram showing wild-type E1A-12 and truncated E1A-12 mutants. The three conserved regions (CR1, CR2, and CR3) and a unique spacer region involved in tumorigenesis are indicated. (B) Constructs of N-terminally FLAG-tagged wt E1A-12, E1A-12 deletion mutants, or wt E1A-5 were cotransfected into COS-7 cells with a p65 plasmid. At 40 h posttransfection, whole-cell lysates were prepared and subjected to Western blot analysis using a phospho (P)-p65-Ser²⁷⁶ antibody (top panel). The same blot was stripped and reprobed with an antibody recognizing total (both phosphorylated and unphosphorylated) p65 (middle panel) and an antibody against the FLAG tag (lower panel). Nonspecific (NS) protein bands are boxed with dashed line.

p65-Ser²⁷⁶ phosphorylation (Fig. 1B, lanes 3 to 5), even when the deletion reached into conserved region 1 (CR1) (Fig. 1A) and left only 66 amino acids at the N terminus (mutant aa1-66) (Fig. 1B, lane 5). In contrast, deletion of the N-terminal 30 residues (aa31-266) (Fig. 1A) abolished the ability of E1A-12 to inhibit p65-Ser²⁷⁶ phosphorylation (Fig. 1B, lane 6). The same results were obtained when further deletions (aa95-266 and aa202-266) (Fig. 1A) were made toward the C terminus (Fig. 1B, lanes 7 to 8). The total protein levels of p65 expressed in COS-7 cells were comparable (Fig. 1B). The E1A protein levels in these cells were also comparable (Fig. 1B, lower panel). This rules out variation in expression as an explanation for the proteins' differential impact on Ser²⁷⁶ phosphorylation. These results indicate that suppression of p65 phosphorylation at Ser²⁷⁶ is attributable to an inhibitory activity contained within the N-terminal 66 residues of E1A-12.

The N-terminal region of E1A-12 inhibits p65 transactivation. It is well established that phosphorylation of p65-Ser²⁷⁶ by PKAc is critical for transcriptional activation by NF- κ B (23, 36, 38). This implied that the E1A-12 mutants that retain the N-terminal region and prevent p65-Ser²⁷⁶ phosphorylation

FIG. 2. Transcriptional stimulation by p65 is suppressed by the N-terminal region of E1A-12. Plasmids expressing a Gal4-p65 fusion protein and luciferase reporters that contain a Gal4 binding site were cotransfected into COS-7 cells in the absence (bar 2) or the presence of FLAG-tagged E1A-12 wt (bar 3) and mutant (bars 4 to 8) proteins. Transfection with the plasmid expressing Gal4 alone served as a negative control (bar 1). Luciferase activity driven by p65 was measured at 40 h posttransfection. For each transfection assay, a *Renilla* luciferase reporter was included for normalization of gene expression. Each transfection was performed in duplicate and repeated two to three times. Error bars represent standard deviations. Cell lysates from transfections were subjected to Western blotting using anti-p65 and anti-FLAG antibodies to probe the levels of Gal4-p65 and E1A-12 proteins, respectively. Nonspecific (NS) protein bands are boxed with dashed line.

(Fig. 1B) should also abolish the ability of p65 to stimulate transcription. To ascertain this, a Gal4-p65 fusion protein construct and a luciferase reporter containing a Gal4 DNA binding site upstream of the luciferase coding region (12) were cotransfected into COS-7 cells with either wt E1A-12 or one of the E1A-12 deletion mutants. As shown in Fig. 2, E1A-12 mutants aa1-66, aa1-232, and aa1-240, all of which contain the N-terminal region of E1A-12, impaired p65 transactivation to the same extent as wt E1A-12 (Fig. 2, compare bars 3 and 6 to 8). In contrast, the two E1A-12 N-terminal deletion mutants, aa31-266 and aa95-266, had a minimal effect on p65 transactivation (bars 4 and 5), which was comparable to the activity derived from Gal4-p65 in the absence of E1A-12 (bar 2). Taken together, these results (Fig. 1B and Fig. 2) are consistent in showing that the E1A-12 N terminus (residues 1 to 66) mediates impairment of both p65-Ser²⁷⁶ phosphorylation and transactivation.

The N terminus of E1A-12 interacts with p65. Previously, we proposed that E1A-12 might block phosphorylation of p65-Ser²⁷⁶ by physically associating with this subunit of NF- κ B (12, 27). Indeed, as shown in Fig. 3A, full-length wt E1A-12 transfected into COS-7 cells was able to immunoprecipitate p65 (lane 3), whereas E1A-5 was completely incapable of binding p65 (lane 18), consistent with the inability of E1A-5 to prevent

FIG. 3. The N-terminal region of E1A-12 is required to interact with p65 *in vivo*. (A) Coimmunoprecipitation of p65 by E1A-12 containing an intact N terminus. COS-7 cells were cotransfected with plasmids that express FLAG-tagged wt E1A-12, E1A-12 deletion mutants, or WT E1A-5. After 40 h, expressed E1A proteins were immunoprecipitated (IP) from cell lysates with a rabbit FLAG antibody, followed by Western blotting using a rabbit anti-p65 antibody. The interactions of p65 with wt E1A-12 (lane 3), aa1-240 (lane 6), aa1-66 (lane 9), aa31-266 (lane 12), aa95-266 (lane 15), and E1A-5 (lane 18) are shown. As a negative control, immunoprecipitation was performed with normal rabbit serum (lanes 2, 5, 8, 11, 14, and 17). p65 input (2% of whole-cell lysate) for each immunoprecipitation assay is also shown (lanes 1, 4, 7, 10, 13, and 16). The positions of p65 and IgG heavy chain are indicated. IgG ctrl, control IgG; IgG-h, IgG heavy chain. (B) Coimmunoprecipitation of E1A-12 that contains an intact N terminus by p65. Cell lysates in panel A were also subjected to reciprocal immunoprecipitation (goat α-p65) and Western blot (rabbit α-FLAG) analysis. The coprecipitated wt E1A-12 (lane 3), aa1-240 (lane 6), aa1-66 (lane 9), aa31-266 (lane 12), aa95-266 (lane 15), and E1A-5 (lane 18) are shown. Control immunoprecipitations were performed with normal goat serum (lanes 2, 5, 8, 11, 14, and 17). E1A input (2% of whole-cell lysate) for each immunoprecipitation assay is also shown (lanes 1, 4, 7, 10, 13, and 16).

p65 phosphorylation. We then inquired whether the E1A-12 deletion mutants aa1-240 and aa1-66, which retain the N terminus and interfere with p65-Ser²⁷⁶ phosphorylation (Fig. 1), also interact with p65. As expected, these two E1A-12 mutants were able to immunoprecipitate p65, albeit aa1-66 (Fig. 3A, lane 9) was slightly less capable than wt E1A-12 or aa1-240 (lanes 3 and 6). In contrast, the E1A-12 mutants lacking their N termini (aa31-266 and aa95-266) failed to immunoprecipitate p65 (lanes 12 and 15). The expression of these E1A wt and mutant proteins was comparable (data not shown). These results were further substantiated by reciprocal immunoprecipitation reactions, in which an antibody specific for p65 was able to coimmunoprecipitate wt E1A-12 and deletion mutants aa1-240 and aa1-66 (Fig. 3B, lanes 3, 6, and 9) but not mutant aa31-266 or aa95-266 or wt E1A-5 (lanes 12, 15, and 18). These data indicate that the N-terminal region within the first 66 amino acids of E1A-12 interacts with p65 in such a manner that p65 phosphorylation at Ser²⁷⁶ and transactivation are impeded.

An N-terminal 40-amino-acid peptide of E1A-12 blocks p65-Ser²⁷⁶ phosphorylation. The N-terminal sequence consisting of amino acids 1 to 66 of E1A-12, which is capable of preventing p65-Ser²⁷⁶ phosphorylation and *trans*-activation, is shown in Fig. 4. A bioinformatic interrogation of this segment of E1A-12 failed to identify any cellular or nonadenovirus genes with sequence similarity. The first 39 residues are unique and are contiguous with CR1 (Fig. 4, underlined sequence). The

shaded residues shown in Fig. 4 are particular to E1A-12, and boxed residues share homology with the tumorigenic strains Ad40 and Ad41 (3). Based on this sequence information, we investigated whether the N-terminal amino acids of E1A-12 that precede CR1 can functionally inhibit p65-Ser²⁷⁶ phosphorylation. We generated a synthetic peptide (pep1-40) containing amino acids 1 to 40 of E1A-12 and tested whether it is capable of blocking phosphorylation of p65-Ser²⁷⁶ in an *in vitro* kinase assay containing PKAc (PKA catalytic subunit), ATP, and recombinant p65. As shown in Fig. 5A, pep1-40 prevented PKAc from phosphorylating p65-Ser²⁷⁶ in a dose-dependent manner (right panel, lanes 3 to 6). It is noted that in the absence of PKAc, no other kinase activity was observed for p65-Ser²⁷⁶ phosphorylation (Fig. 5A, left panel, compare lanes 1 and 2). As a control, the *in vitro* kinase assay was conducted using an irrelevant peptide representing the N-terminal 22 residues of processivity factor 8 (PF8) of the Kaposi's sarcoma-associated herpes virus (KSHV). Consistent with our previous finding that nonspecific proteins including GST and E1A-5 did not inhibit p65-Ser²⁷⁶ phosphorylation (12), this KSHV peptide failed to prevent p65 phosphorylation at Ser²⁷⁶ (Fig. 5B). These results indicate that residues 1 to 40 of the E1A-12 N terminus specifically prevent p65-Ser²⁷⁶ phosphorylation.

Two possibilities account for the inhibition of p65-Ser²⁷⁶ by E1A-12. The first is that pep1-40 binds the PKAc catalytic site, thereby keeping p65 from becoming phosphorylated. In this situation, PKAc would be globally impaired from phosphorylating its numerous substrates, which, predictably, should be lethal to the cell. The second possibility is that pep1-40 selectively binds p65, such that it masks this PKAc substrate singularly without interfering with the general kinase activity of this cellular enzyme. In this situation, p65-Ser²⁷⁶ phosphorylation would be specifically blocked. In order to distinguish these two possibilities, we assessed whether pep1-40 could also prevent phosphorylation of a PKAc peptide substrate that contains

FIG. 4. Sequence of the N terminus (residues 1 to 66) of E1A-12 that interacts with p65 and disables p65 from stimulating transcription. The shaded residues are completely unique to E1A-12, and the boxed residues with asterisks are chemically conserved among tumorigenic strains Ad40 and Ad41. The underlined residues comprise a portion of CR1.

FIG. 5. The N-terminal 40 amino acid residues of E1A-12 prevent PKAc from phosphorylating p65-Ser²⁷⁶ *in vitro*. (A) E1A-12 N-terminal 40-amino-acid peptide blocks p65-Ser²⁷⁶ phosphorylation in the PKAc assay. Recombinant His₆-p65 (0.4 μ g) was either left untreated or incubated with increasing amounts (0, 10, 20, and 50 μ M) of the N-terminal 40-amino-acid peptide (pep1-40) of E1A12 in the presence of PKAc and ATP. Phosphorylation of p65 was examined by Western blotting with an antibody that specifically recognizes phosphorylated p65-Ser²⁷⁶. As a quantitative control, the same blot was probed with anti-p65 antibody that recognizes total p65. The pep1-40 peptide included in each assay was detected by silver staining. (B) Control KSHV peptide does not block p65-Ser²⁷⁶ phosphorylation. A similar *in vitro* kinase assay was performed as in panel A except that the E1A-12 peptide was replaced with a peptide corresponding to the N terminus (residues 1 to 22) of the KSHV processivity factor PF8. (C) E1A-12 pep1-40 peptide does not alter phosphorylation of CREB in the PKAc assay. Increasing amounts (0, 0.25, 0.5, 1.0, 2.0, 3.0, 4.0, and 5.0 μ M) of biotinylated CREB peptide that contains a PKA phosphorylation site were incubated with PKAc and ATP in the presence of different amounts (0, 20, or 50 μ M) of E1A-12 pep1-40 peptide. The CREB peptide was then bound to streptavidin-coated plates. ELISA was performed using a phospho-PKA substrate [RRX(S/T)] antibody to detect CREB phosphorylation, and the absorbance was measured at 405 nm. Data represent averages of the results from three independent experiments. Error bars indicate standard deviations.

Ser¹³³ of CREB. In this assay, the CREB peptide was incubated with PKAc and ATP in the presence of pep1-40, and the phosphorylation level of CREB-Ser¹³³ was analyzed by ELISA using an antibody against phospho-PKA substrate [RRX(S/T)]. As shown in Fig. 5C, pep1-40 had no inhibitory effect on

FIG. 6. The N-terminal 40 amino acid residues of E1A-12 bind to p65 directly. The interaction between p65 and E1A-12 was determined by His₆ pulldown assays. Recombinant His₆-tagged WT E1A-12, aa1-40, or aa41-266 (1 μ g each) was bound to Ni-NTA agarose beads, followed by incubation with 2 μ g of recombinant GST-p65 or GST. After extensive washing, protein complexes were eluted with 300 mM imidazole and analyzed by Western blotting using p65 antibody (right panel) or GST antibody (left panel). As a negative control, the same amounts of GST-p65 or GST were incubated with Ni-NTA agarose beads that were not pretreated with His₆-tagged E1A-12 proteins, followed by the same procedures as described above. Each input (lanes 1 and 6) contains approximately 20 ng of GST-p65 or GST.

the phosphorylation of CREB-Ser¹³³, even at a high concentration (50 μ M), indicating that E1A-12 does not directly target PKAc. Therefore, our data strongly argue that the diminished phosphorylation of p65-Ser²⁷⁶ results from the ability of E1A-12 to prevent PKAc from gaining access to its p65 substrate rather than directly targeting PKAc.

The N-terminal 40-amino-acid region of E1A-12 directly interacts with p65. We next pursued the notion that the N-terminal 40-amino-acid region of E1A-12 directly binds p65, thus causing a block in Ser²⁷⁶ phosphorylation and a subsequent loss of NF- κ B (p50/p65) transactivation. A pulldown experiment was performed to evaluate the physical interaction between wt or mutant E1A-12 proteins with p65. Purified wt and deletion mutant E1A-12 proteins containing N-terminal His₆ epitope tags were incubated with GST-p65. As shown in Fig. 6, full-length wt E1A-12 protein was able to bind p65 directly (lane 3). Remarkably, E1A-12 mutant aa1-40, which comprises only the N-terminal 40 amino acids, retained the ability to bind p65 (Fig. 6, lane 4). However, deletion mutant aa41-266, which lacks the first 40 amino acid residues of E1A-12, displayed a drastic reduction in p65 binding (Fig. 6, lane 5). Of note, the GST moiety of the p65 fusion protein did not contribute to E1A-12 binding activity as GST alone did not yield any binding activity (Fig. 6, right panel). In summary, these results demonstrate that the 40-amino-acid N terminus of E1A-12 is capable of directly targeting and blocking phosphorylation of p65-Ser²⁷⁶ to disable NF- κ B from stimulating transcription.

To examine if prevention of p65-Ser²⁷⁶ phosphorylation by the E1A-12 N-terminal region leads to inhibition of NF- κ B-transactivated MHC-I expression, we transiently transfected plasmids expressing aa1-66, aa31-266, or wt E1A-12 into NIH 3T3 cells. Following transfection, FACS analyses were conducted to quantitate the surface levels of MHC-I antigens on NIH 3T3 cells. As shown in Fig. 7, expression of the N-terminal aa1-66 mutant resulted in a modest, but consistent reduction of MHC-I antigens on the surface (bar 2) compared with empty vector transfection (bar 1). In agreement with this, MHC-I mRNA levels were reduced by aa1-66, as demonstrated by reverse transcription-PCR (RT-PCR) (data not shown). Im-

FIG. 7. E1A-12 N-terminal region downregulates MHC-I. NIH 3T3 cells were transiently transfected with same amounts (15 μ g) of empty vector (control) or plasmids expressing aa1-66, aa31-266, or wt E1A-12. At 72 h posttransfection, FACS analyses of MHC-I surface antigens were conducted. Data represent the means of fluorescence intensity of cell surface MHC-I staining from two independent FACS analyses.

portantly, the decreased MHC-I expression on cell surfaces induced by aa1-66 is comparable to that obtained from wt E1A-12 (bar 3). In contrast, the mutant aa31-266 failed to inhibit MHC-I expression (bar 4). These data clearly demonstrate that the E1A-12 N-terminal region plays a critical role in downregulating MHC-I expression by preventing NF- κ B phosphorylation and transactivation, thus providing Ad12 tumor cells with a mechanism of immune escape.

DISCUSSION

NF- κ B is the master transcriptional regulator of immune response gene products, including MHC-I proteins that present foreign antigens to CTLs. By downregulating MHC-I transcription, E1A-12 enables Ad12 tumorigenic cells to escape immune surveillance and lysis by CTLs. In this study, we have revealed that the 40-amino-acid N terminus of E1A-12 inhibits NF- κ B from stimulating transcription by blocking phosphorylation of subunit p65-Ser²⁷⁶. The C-terminal sequences of E1A-12 did not inhibit phosphorylation of p65-Ser²⁷⁶. The possibility that the E1A-12 N terminus directly targets PKA as a means of preventing phosphorylation of p65-Ser²⁷⁶ was ruled out by showing that CREB, a major kinase substrate of PKA, was not affected by the E1A-12 peptide. Rather, the inhibition was shown to be accomplished through the direct interaction of the E1A-12 N terminus with p65.

It is significant that the N terminus of E1A-12 alone was capable of (i) binding p65, (ii) blocking p65-Ser²⁷⁶ phosphorylation, and (iii) preventing p65-dependent transcriptional activation, as demonstrated by the assays employed in this study. Neither the C terminus of E1A-12 as exhibited by aa31-266 nor full-length E1A-5 was able to inhibit any of these activities. The N termini of E1A-5 and E1A-12, which share limited homology, are able to bind multiple cellular proteins (24). For example, The N-terminal 41 amino acids of E1A-5 have been shown to bind at least 15 cellular proteins, e.g., AP-2 (29), myogenin (30), pCAF (26), and p300 (2). Proteins that bind to the E1A-12 N terminus include p300 (32) and, as shown in this report, p65. Moreover, residues 1 to 29 of E1A-12 have been shown to exhibit a significant transactivation activity that drives the viral E2 promoter by potentially forming a complex with

general transcription factors TATA-binding protein (TBP)-TFIIF on the promoter (18, 20). In this respect, the E1As have been referred to as highly versatile hub proteins, which have the ability to interact with many of other proteins to form networks that can effectuate diverse cellular functions (24). The distinct features of the N-terminal regions might help interpret the striking difference of tumorigenicity levels between serotypes of Ad5 and Ad12 (27, 34). In this regard, it will be interesting to discern how both E1A-5 and E1A-12 can bind p300 (7, 32, 33), whereas only E1A-12 binds p65. Mutational analysis will discern whether the conserved residues Ile¹⁸ and Leu¹⁹ of E1A-12 that are involved in p300 binding (19) are not essential for p65 binding. Similarly, this approach will evaluate if the nonconserved E1A-12 residues (Thr³, Pro⁷, Tyr¹², Gln¹³, Asn²⁵, Asn²⁸, Glu²⁹, Ser³², Asp³³, Lys³⁶, Tyr³⁷, and Val³⁸) are required to mediate p65 binding.

While the E1A-12 N-terminal region is sufficient to prevent p65-Ser²⁷⁶ phosphorylation in cells, it is not known if this event occurs in the cytoplasm or nucleus. Considering that the nuclear localization signal in E1A-12 is located at the very C terminus and that all of the C-terminal deletion mutants including aa1-66 retained the ability to inhibit p65-Ser²⁷⁶ phosphorylation and transactivation, it is reasonable to speculate that the E1A-12 N terminus may exert its inhibitory effect in the cytoplasm. However, we cannot rule out the possibility that these E1A-12 mutants, especially aa1-66, can freely enter the nucleus due to their relatively small size. Alternatively, E1A-12, through its association with p65, may cotranslocate to the nucleus, where p65-Ser²⁷⁶ phosphorylation by PKAc is prevented. Nevertheless, our results suggest a model whereby the E1A-12 N terminus masks the p65-Ser²⁷⁶ kinase recognition site, making it inaccessible to PKAc. However, the precise manner of this inhibition has yet to be resolved. One possibility is that binding of E1A-12 to p65 directly bars PKAc from gaining access to Ser²⁷⁶. Alternatively, upon binding, E1A-12 could induce a conformational change in p65 that disables PKAc recognition of Ser²⁷⁶. Stable interactions between E1A-12 and PKAc are unlikely because the presence of the 40 residue N-terminal peptide of E1A-12 had no effect on phosphorylation of CREB but effectively blocked phosphorylation of p65 under similar experimental settings. Of note, the direct targeting of the p65 substrate by E1A-12 rather than its kinase concurs with the fact that active PKAc is essential for regulating a wide spectrum of cellular substrates necessary for cell survival. Previous studies demonstrated that phosphorylation of Ser²⁷⁶ causes major conformational changes in p65, which expose the C-terminal transactivation domain of p65 and thereby facilitate its transcriptional activity (36–38). It is likely that E1A-12, through its N-terminal binding domain, stably interacts with p65 in order to retain NF- κ B as an inactive transcription factor. In the larger context, because hypophosphorylated p65 is seriously defective in transactivation, NF- κ B will be unable to promote transcription of MHC-I genes, which provides a means by which Ad12 tumorigenic cells escape lysis by CTLs. Indeed, this study demonstrates that the N-terminal 66-amino-acid region of E1A-12 is capable of mediating MHC-I downregulation in cells.

Our previous study demonstrated that E1A-12 inhibits p50 from being phosphorylated at Ser³³⁷ by PKAc, analogous to inhibition of p65-Ser²⁷⁶ (12). Based on this finding and in light

of the three-dimensional space resemblance of the p65 and p50 subunits (5, 8), it is compelling to speculate that the E1A-12 N-terminal peptide also binds to p50 in a fashion similar to p65. Indeed, examination of E1A-12 mutants revealed that the E1A-12 N terminus including the N-terminal 40-residue peptide binds p50 as well (J. Jiao, H. Guan, and R. P. Ricciardi, unpublished data). Since p50 DNA binding activity requires phosphorylation of Ser³³⁷, as we recently reported (11, 14), the E1A-12 N terminus should disable both NF- κ B recognition of the MHC-I enhancer and transactivation. The requirement of the E1A-12 N-terminal region for inhibition of NF- κ B phosphorylation, activation, and MHC-I transcription is in accord with earlier studies of an Ad5/Ad12 hybrid E1A showing that the E1A-12 N terminus (residues 1 to 42) plays an important role in downregulating MHC-I expression (15, 16, 25), which is a prerequisite for E1A-12-induced tumorigenesis (27, 34).

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