Two Domains within the Adenovirus Type 12 E1A Unique Spacer Have Disparate Effects on the Interaction of E1A with P105-Rb and the Transformation of Primary Mouse Cells

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Transformation of primary rodent cells by functions of the adenovirus type 12 (Ad12) early region 1 (E1) is reduced severalfold compared with transformation by E1 of Ad2. We analyzed whether the unique spacer region of Ad12 E1A that borders the conserved region (CR) 2 and represents an oncogenic determinant of Ad12 E1A is involved in this impaired transformation property, putatively by modulating transformation-relevant biological E1A functions. We show that a mutant (E1ASpm1) that lacks 12 amino-terminal residues of the spacer binds p105-Rb and p130 as Ad12 E1A wild type (E1Awt), whereas a second spacer mutant (E1ASpm2) that lacks an adjacent stretch of six alanines exhibits highly reduced binding to p105-Rb. The binding of this mutant to the p130 pocket protein is, however, little impaired. E1ASpm1 diminishes the formation of the p105-Rb-E2F complex more efficiently than E1Awt or, least efficient, E1ASpm2. These properties of the spacer mutants to target and to disintegrate the p105-Rb-E2F complex correspond with their ability to transform primary mouse cells in combination with E1B. E1ASpm1 (plus Ad12 E1B)-transfected cells could be easily established as cell lines, comparable to Ad12 E1Awt- or Ad2 E1Awt-transformed cells. In contrast, cells transfected with E1ASpm2 or Ad12 E1AΔCR2 (lacking the entire CR2) died within 6–10 weeks after replating, although foci were formed in all cases. Of note, the E1ASpm1-transformed cells grow as fast as the Ad2 E1Awt-transformed cells, with a doubling rate of 15 h, whereas the doubling of the Ad12 E1Awt-transformed cells takes ~120 h. Moreover, in the established cell lines, the affinity of E1ASpm1 to p105-Rb was higher than with that of E1Awt. Our data suggest the presence of a transformation-suppressing domain within the carboxyl-terminal 12 residues of the Ad12 E1A-unique spacer; whereas the hydrophobic stretch of six alanines in the spacer is required for stable transformation.

INTRODUCTION

E1A oncoproteins from human adenoviruses (Ad) reprogram quiescent cells to progress from G0/G1 to S phase, thereby overriding the growth suppressive signals of the untransformed cell (Jansen-Dürr, 1996). This inappropriate up-regulation of the cell cycle can lead to mitosis and, especially in rodent cells, the transformation of the cells. Functions of E1A alone are able to immortalize primary rodent cells (Gallimore et al., 1985; Lynch and Trainer, 1989), but in most cases the fully transformed phenotype can be achieved only by coexpression of a second oncogene, such as the activated cellular Ha-ras (Ruley 1983) or the adenoviral E1B (Williams et al., 1995), that prevents E1A-induced apoptosis (White, 1996).

Alternative splicing of the E1A transcription unit gives rise to five (Ad2/5) or six transcripts (Ad12; Brockmann and Esche, 1995). From these, the functions of the 13S and 12S mRNA products are most frequently examined. E1A proteins do not bind DNA in a sequence-specific manner (Ferguson et al., 1985). They mediate all of their known functions by physical interactions with cellular proteins, such as transcription factors/cofactors like activator protein-1, necrosis factor-κB, ATF-2, p300, or TBP (Whyte et al., 1989; Lee et al., 1991; Wang et al., 1993; Geisberg et al., 1994; Zantema and van der Erb, 1995; Parker et al., 1997) or tumor suppressor proteins like p105-Rb (Nevins, 1995; Jones, 1995). Most of these functions involve the nonconserved amino-terminus and/or the three conserved regions (CR) 1–3 of E1A that are highly homologous among different Ad serotypes. As a result of these interactions, E1A trans-regulates transcription of its own (Kirch et al., 1993; Kawamura et al., 1993; Pützer et al., 1997) and of other viral and cellular genes (Berk et al., 1979; Nevins, 1981; Shenk and Flint, 1991). The interaction of E1A with the p105-Rb-E2F transrepressor complex (Weintraub et al., 1992) has drawn much attention and involves the CR1 and the CR2 (Dyson et al., 1992). The CR2 domain was found to make the first contact between E1A and p105-Rb, allowing the CR1 to compete with E2F for the pocket protein binding (Ikeda and Nevins, 1993; Fattaey, 1993). The released E2F transcription factor trans-activates several cellular genes (Bagchi et al., 1990) that are required for cell cycle progression, like DHFR (Slansky et al., 1993), c-myc, and DNA-polymerase (Nevins, 1995).

In contrast to the nononcogenic serotypes (e.g., Ad2), functions of the E1A plus E1B proteins of the oncogenic...
serotypes (e.g., Ad12) can induce tumors in immunocompetent rodents. The oncogenic determinants are located within the Ad12 E1A proteins (Williams et al., 1995). Although functionally and structurally related, a considerable diversity evolved among the E1A genes of oncogenic and nononcogenic subgroups, especially when the nonconserved regions are considered (Brockmann and Esche, 1995). Thus in contrast to the E1A proteins of Ad2, a p300-independent transcriptional trans-activation domain is located within the 29 amino acids of the amino-terminus (Lipinski et al., 1997). Moreover, Ad12 E1A lacks two transcriptional activation domains (AR1 and AR2) that were found in Ad5 E1A to be involved in CR3-mediated transcriptional activation (Bondesson et al., 1992; Strom et al., 1998).

Another important difference is the presence of a unique spacer region in Ad12 E1A that is located between CR2 and CR3. It consists of a block of 20 amino acids that are not present in Ad2 E1A (Fig. 1A) and exhibit a considerable homology to transcriptional suppressor proteins found in the embryonic development of Drosophila (Licht et al., 1990). Ad5/Ad12 hybrid virus experiments have shown that the integrity of the spacer is important for the oncogenicity of the virus (Jelinek et al., 1994; Telling and Williams, 1994). Despite its additional oncogenic properties, the transformation rate of primary rat cells induced by transfection with Ad12 E1A/E1B was ~80% lower as for Ad2 E1A/Ad12 E1B (Leclère et al., 1993). CR1 of Ad12 was found to be one determinant of the poor Ad12 E1A transformation properties. Interestingly, an Ad2/Ad12 E1A hybrid protein that contains the CR1 of Ad2 and the CR2 plus the spacer of Ad12 E1A was still 40% less efficient in transformation (Leclère et al., 1993). The poor transformation property of Ad12 E1A corresponds with very recent results, where in contrast to Ad2 E1A, the expression of Ad12 E1A in human cells resulted in the prevention of mitosis by S-phase arrest (Grand et al., 1998). Thus because CR2 is nearly identical between Ad12 and Ad2/5 E1A, a region carboxyl-terminal from the CR2, possibly the spacer, might be involved in the reduced transformation by Ad12 E1A.

Here we examined whether parts of the Ad12 E1A unique spacer modulate the CR2-dependent binding to p105-Rb and p130, the disruption of p105-Rb- and/or p130-E2F complexes, and a putative participation in the transformation of primary mouse cells. We provide evidence for two domains within the spacer that have different effects on the pocket protein interaction and transformation properties of Ad12 E1A.

**FIG. 1.** Role of Ad12 E1A-specific spacer regions for the physical interaction of E1A with p105-Rb and p130. (A) Schematic presentation of the amino acids 106–159 of Ad12 E1A<sub>wt</sub> in comparison with the Ad12 E1A mutants and Ad2/5 E1A. The Ad12 unique spacer and the binding domains of the pocket proteins with respect to previous findings with Ad2/5 E1A (Barbeau et al., 1994) are indicated. (B and C) Binding of the pocket proteins p105-Rb and p130 from 1 mg of nuclear extracts to 50 μg of different GST-Ad12 E1A fusion proteins were examined by combined protein interaction/Western blot experiments; one fourth of the nuclear proteins that bound to E1A (from 1 mg originally applied nuclear extract) was loaded onto the Western blot gel. The lanes A549 NE and U937V NE contain 20 μg of nuclear extracts; GST<sub>escher</sub> indicates GST-tag without fused E1A proteins. The appearance of p105-Rb and p130 proteins on the blots is indicated. Rb, p130, and E1A were subsequently detected on the same blot. The intensive bands in case of the E1A antibody are due to the high levels of E1A, released from the GT-Sepharose.
RESULTS AND DISCUSSION

The E1A proteins from the oncogenic Ad12 and the nononcogenic Ad2/5 exhibit differences in their interaction with cellular proteins, resulting in altered oncogenicity (Williams, 1995), different transformation capacity in primary rodent cells (Leclère et al., 1993), and opposite ability to induce mitosis in quiescent human cells (Grand et al., 1998). In the present study, we examined whether the Ad12 E1A unique spacer influences the targeting of the pocket proteins, the disruption of pocket protein-E2F complexes, or the transformation properties of Ad12 E1A.

The hydrophobic hexa-alanine stretch in the carboxyl-terminal half of the Ad12 E1A unique spacer region participates in the efficient binding of E1A to p105-Rb but not to p130.

We analyzed the binding of GST-13S-E1A (E1Awt) or spacer mutant fusion proteins (Fig. 1A), immobilized on GT-Sepharose, to p105-Rb and p130 from nuclear extracts by in vitro protein interaction analysis and subsequent detection of E1A and the E1A-bound pocket proteins by Western blotting. Nuclear extracts were prepared from nearly confluent A549 cells or from U937V cells at a cell density of 2 × 10⁶ cells/ml medium. Under these conditions, p105-Rb and p130 were easily detectable in 20 μg of nuclear extracts from U937V (Fig. 1b) and A549 (Fig. 1c) cells. For both cell lines, Ad12 E1Awt bound 7–15% of the input (with respect to the amount in nuclear extracts, as calculated from the cell control Western blot signals; Figs. 1, b and c). As shown for Ad5 E1A (Barbeau et al., 1994), the Ad12 E1AΔCR2 mutant that lacks the entire CR2 was unable to bind p105-Rb or p130 (Figs. 1, a and c), underscoring the prominent role of CR2 as pocket protein targeting domain. The E1AΔsm1 mutant that lacks the 12 amino-terminal residues of the spacer (Fig. 1a) binds both p130 and p105-Rb as well as E1Awt, indicating that this region is dispensable for efficient binding of Ad12 E1A to p105-Rb and p130. Binding of E1A to p130 also is only little affected when the hydrophobic stretch of six alanines in the carboxyl-terminal half of the spacer are deleted (E1AΔsm2 mutant; Fig. 1a). In contrast, the binding of the E1AΔsm2 mutant to p105-Rb was very weak (Figs. 1, b and c) compared with E1Awt or E1AΔsm1. Thus the hexa-alanine stretch in the spacer seems to be required for an efficient binding of Ad12 E1A to p105-Rb.

Influence of the Ad12 E1A spacer mutants on the formation of the p105-Rb-E2F and p130-E2F complexes

To determine whether the binding properties of the spacer mutants to the pocket proteins correspond with the disruption of p105-Rb/p130-E2F complexes, we examined the formation of these complexes in gel shift experiments with the E2F-binding motif from the DHFR promoter in the presence and absence of the E1A proteins (Fig. 2). For these experiments, 2 μg of nuclear extracts from U937V and H596 cells (that do not express p105-Rb; Harbour et al., 1988) were incubated with the [α-32P]dCTP-labeled DHFR-E2F site-containing oligonucleotide.

Four complexes (Fig. 2A, a–d) are formed in gel shift experiments with the U937V nuclear extracts. Complex b contains p105-Rb and complex a contains p130 as judged from antibody perturbation analysis (Fig. 2A). The complex c was previously found to contain free E2F-1 (Kirch et al., 1997). In the case of the p105-Rb-deficient H596 cell line, the most shifted complex contained p130 (Fig. 2B). The addition of 6 μg of the E1Awt protein disrupted both the p105-Rb-containing (Fig. 2A) and the p130-containing (Figs. 2, A and B) complexes, whereas the E1AΔCR2 did not affect any of the complexes (Figs. 2, A and B), supporting the results of the protein interaction experiments (Figs. 1, b and c). As E1AΔsm1, 6 μg of both the E1AΔsm1 and the E1AΔsm2 mutant, respectively, are able to disrupt the p105-Rb-E2F and p130-E2F complex. Because these high amounts of E1A proteins might mask a fine tuning of pocket protein E2F disruption, we examined whether the spacer modulates this E1A function using lower levels of E1Awt, E1AΔsm1, and E1AΔsm2 GST-fusion proteins (Fig. 3). At 0.5 μg of GST-E1A protein, disruption of the p105-Rb-containing complex b is incomplete for E1Awt and even less effective for E1AΔsm2. In contrast, the formation of complex b was significantly reduced in the presence of 0.5 μg of the E1AΔsm1 mutant protein, resulting in a ranking of the order of E1AΔsm1 > E1Awt > E1AΔsm2 (Fig. 3). This ranking is observed for as much as 2 μg of E1A proteins (data not shown), whereas no difference among the E1Awt, E1AΔsm1, and E1AΔsm2 proteins was found at 4 μg, even after long exposure of the gel shift (long exposure, Fig. 3, 4 μg). Efficient disruption of the p130-containing complex a requires >4 μg of E1A proteins (compare Figs. 2A and 3), possibly due to a preferred interaction of E1A with the p105-Rb-E2F complex. All complexes were efficiently competed by a 10- to 500-fold molar excess of the unlabeled DHFR-oligonucleotide, whereas an E2F-1-binding site mutant oligonucleotide [DHFRm], prepared according to the 62/60 mutant described by LaThangue et al. (1990)] had no effect on the complex formation, except for complex d, which was unaffected by a 10-fold but diminished by a 100- and 500-fold excess of DHFRm (Fig. 2C), indicating a lower affinity of the binding partners compared with the other complexes.

In summary, two regions within the Ad12 E1A spacer have disparate effects on the interaction of E1A with the p105-Rb pocket protein. The hexa-alanine stretch contributes to the p105-Rb targeting by E1A (Fig. 1b) and efficiently supports the disruption of p105-Rb-E2F complexes. In contrast, the 12 amino-terminal residues of the spacer do not seem to contribute to the targeting of
FIG. 2. Disintegration of p105-Rb/p130-E2F complexes from U937V and H596 cells by E1A proteins. (A) Gel shift experiments using an α-32P-dCTP-labeled oligonucleotide containing the E2F-binding motif of the DHFR promoter and 2 μg of U937V nuclear extracts in the presence or absence of either anti-p105-Rb (αRb) or anti-p130 (αp130) antibodies or 6 μg of GST-E1A fusion proteins (as indicated) in comparison with GST alone; complexes were labeled a-c, as indicated (see text for details). (B) Gel shift experiments as in A with nuclear extracts from H596 cells that do not express p105-Rb (Harbour et al., 1988); the position of the p130-containing complex is indicated. (C) Gel shift competition experiments using an unlabeled DHFR oligonucleotide and an E2F-site mutant (DHFRm) oligonucleotide according to the 62/60 mutant described by LaThangue et al. (1990); s indicates supershifted band. *Top of the gel.
p105-Rb by E1A (Figs. 1, a and b) and the presence of this region in E1A proteins reduces their ability to disrupt the p105-Rb-E2F complex (Fig. 3).

The hexa-alanine stretch and the amino-terminal part of the spacer express disparate functions in the transformation of primary mouse cells

To analyze whether the hexa-alanine stretch and the amino-terminal part of the spacer have an effect on E1A functions in vivo, we examined the transformation properties of E1A in cooperation with Ad12 E1B by focus formation analysis (Table 1). We cotransfected mouse kidney cells prepared from neonatal NMRI mice (days 5–7 after birth) with equal amounts (0.5 and 4 μg) of GST-E1A (E1Awt, E1ASpm1, and E1ASpm2) fusion proteins. 4 μg" indicate long exposure of the gel part, in which 4 μg of E1A proteins were used. Numbering of the complexes is as described in Fig. 2A.

FIG. 3. Effects of low E1A protein levels on the disintegration of p105-Rb/p130-E2F complexes. Gel shift experiments were performed as described in Fig. 2 with U937V nuclear extracts in the presence of different amounts (0.5 and 4 μg) of GST-E1A (E1Awt, E1ASpm1, and E1ASpm2) fusion proteins. 4 μg* indicate long exposure of the gel part, in which 4 μg of E1A proteins were used. Numbering of the complexes is as described in Fig. 2A.
A unique spacer with opposite abilities in cell transformation: the amino-terminal part of the spacer seems to mediate a less aggressive growth of Ad12 E1-transformed cells, whereas the hydrophobic stretch of six alanines is indispensable for efficient transformation, which corresponds with its importance for the targeting of p105-Rb by Ad12 E1A.

Oligoalanine stretches, as in Ad12 E1A spacer or in other oncogenic Ad like SA7 (Williams, 1995), are a feature of transcriptional suppressor proteins found in the embryonic development of Drosophila, like the "Kruppel" (Kr) zinc finger containing polypeptide (Licht et al., 1990). Interestingly, the transcriptional repressor functions of Kr map within the alanine-rich amino-terminus of the protein (Licht et al., 1990). If, however, the hexaalanine stretch in the Ad12 E1A 13S and/or 12S mRNA-encoded proteins mediates repression of cellular genes and if such a kind of transcriptional repressor function contributes to the transformation properties of Ad12 E1A remain to be established.

**MATERIALS AND METHODS**

**Cell culture**

The A549 small cell lung carcinoma cell line (American Type Culture Collection) was maintained in DMEM, 10% FCS, and 2 mM glutamine. The U937 variant (V) histiocytic lymphoma cell line (Kirch et al., 1997; Kloke et al., 1992) and the non-small lung cancer NCI-H596 cell line (kindly provided by Dr. F. Kaye, National Cancer Institute-Navy Oncology Branch, Bethesda, MD) that lacks...
p105-Rb expression (Harbour et al., 1988) were cultured in RPMI, 10% FCS, and 2 mM glutamine.

**E1A expression constructs**

We generated two spacer mutants of the large, 13S mRNA-encoded wild-type E1A protein (E1A<sub>wt</sub>), lacking either the amino-terminal (E1A<sub>Spm1</sub>) or the very carboxyl-terminal part of the spacer consisting of the hydrophobic Ala<sub>6</sub> stretch (E1A<sub>Spm2</sub>, Fig. 1A) by primer-introduced sequence modification PCR as described previously (Kirch et al., 1993). These mutants, as well as E1A wild type (E1A<sub>wt</sub>) and a mutant lacking the CR2 (E1A<sub>delCR2</sub>, Fig. 1A), were cloned into the pGEX-2T bacterial expression vector (kindly provided by Dr. M. A. Blanar, Department of Molecular Biology, Princeton University). After IPTG induction of protein synthesis, the amino-terminal glutathione-S-transferase (GST)-E1A fusion proteins were purified by glutathione (GT)-Sepharose chromatography as recently described (Kirch et al., 1998).

**Combined protein interaction Western blot experiments**

Purified GT-Sepharose-bound E1A proteins were used for protein interaction/Western blot experiments, in which 50 μg of E1A proteins was incubated with 1 mg of twice GT-Sepharose-precleared nuclear extracts that were prepared as described previously (Kirch et al., 1997) and washed three times with the incubation buffer (20 mM HEPES, pH 7.4, 0.1% Tween 20, 1 mM NaF, 1 mM Na<sub>2</sub>VO<sub>4</sub>, 150 mM NaCl). Subsequently, GST-E1A-bound nuclear proteins were eluted by incubation with 120 μl of Laemmli’s buffer at 95°C for 10 min, containing 5% of β-mercaptoethanol, and one fourth of the eluted proteins was analyzed by Western blotting (Kirch et al., 1997).

**Immunoprecipitation**

Immunoprecipitation experiments were performed using 100 μg of nuclear extracts from the Ad12 E1A<sub>wt</sub>, Ad12 E1A<sub>Spm1</sub>, and Ad2 E1A<sub>wt</sub> plus Ad12 E1B-transformed baby BMK cells. Immunoprecipitation was performed as described in Materials and Methods using the anti-105-Rb antibody. The amount of pocket protein-bound E1A proteins was analyzed by Western blotting using either an anti-Ad12 E1A antibody (kindly provided by P. Gallimore) or an anti-Ad5 E1A antibody (M73; Santa Cruz).

**Gel shift experiments**

For gel shift experiments, the binding of proteins from nuclear extracts to the E2F-binding motif from the DHFR promoter was examined in the presence and absence of the (GST)-E1A proteins. For these experiments, 2 μg of nuclear extract was incubated with the[α-<sup>32</sup>P]dCTP-labeled DHFR-E2F site-containing oligonucleotide (5'-GGGCTGCGATTTCGCGCCAAACTTGACGGCA-3') as recently described (Kirch et al., 1997). For competition experiments, the unlabeled DHFR-E2F site oligonucleotide and an E2F-binding site mutant oligonucleotide [GGGCTGCGATTTCGATAAAAACCTGGACGG, according to the E2F-binding deficient E2-promoter-mutant 62/60, described by La Thangue et al. (1990)] were used. For antibody perturbation experiments, nuclear proteins were preincubated with either 0.3 μg of the IF8-anti-p105-Rb antibody (Santa Cruz) or 2 μg of the C20-anti-p130 antibody (Santa Cruz) for 4 h at 4°C before the addition of the oligonucleotide. For E1A interference analysis, 0.5, 4, or 6 μg of recombinant E1A protein was incubated with the nuclear proteins for 4 h at 4°C.

**Foci formation analysis**

Mouse kidney cells were prepared from neonatal NMRI mice (days 5–7 after birth). The cells were transfected, using the FuGENE 6 transfection reagent (Boehringer-Mannheim) according to the manufacturer’s protocol, with 0.75 μg of pRc/RSV-Ad12-E1B plus 0.75 μg of pRc/RSV-Ad12-E1A<sub>wt</sub>, -E1A<sub>Spm1</sub>, -E1A<sub>Spm2</sub>, -E1A<sub>delCR2</sub> or -Ad2 E1A or with the pRc/RSV eukaryotic expression vector alone. Cells were cultured in DMEM, 10% FCS, and 2 mM glutamine. Medium was changed twice a week, and foci formation was determined weekly. All experiments were performed in triplicate with 2 × 10<sup>5</sup> cells each.
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