Induction of the Cell Cycle in Baby Rat Kidney Cells by Adenovirus Type 5 E1A in the Absence of E1B and a Possible Influence of p53

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From previous studies on the induction of DNA synthesis in quiescent primary baby rat kidney cells by adenovirus type 5 (Ad5) E1A deletion mutants, we concluded that induction is prevented only when cellular proteins p300 and pRb are both uncomplexed with E1A (J. A. Howe, J. S. Mymryk, C. Egan, P. E. Branton, and S. T. Bayley, *Proc. Natl. Acad. Sci. USA* 87:5883–5887, 1990). We have now examined induction by these same mutants in virus lacking the E1B region, so that cellular p53 was no longer complexed to the E1B 55-kDa protein. E1A mutants that fail to bind pRb induced DNA synthesis at a significantly lower level in Ad5 lacking E1B than in Ad5 containing E1B. Apparently, therefore, uncomplexed p53 can partially replace p300 in cooperating with pRb to suppress DNA synthesis in baby rat kidney cells.

Infection of quiescent baby rat kidney (BRK) cells by human adenovirus type 5 (Ad5) causes them to enter the cell cycle and divide (2). These effects are brought about by E1A proteins (1, 3, 18, 24), and using a series of E1A deletion mutants, we have mapped the essential regions of the proteins to those shown in Fig. 1 (10, 11). These regions coincide with those necessary for E1A to transform BRK cells in cooperation with *ras* (12) and for E1A proteins to bind to pRb and to another cellular protein, p300 (7) (Fig. 1). Our experiments and those of most other authors on these effects were performed with viruses that expressed E1B. It is therefore legitimate to ask what effect removing E1B would have on the ability of E1A to induce quiescent BRK cells to progress through the cell cycle.

E1B must affect the growth pattern of cells, as it is essential for transformation by adenovirus. However, E1B is incapable of bringing about these changes in the absence of E1A. In lytic infection and in transformation by adenovirus, E1A is necessary for transactivating E1B expression, yet even when E1B is expressed efficiently without E1A, it fails to transform (4). Therefore, when adenovirus infection affects cell growth, the role of E1A is paramount, but E1B must participate as well.

The Ad5 E1B region produces two major, unrelated proteins of 19 and 55 kDa. The 19-kDa protein has been reported to associate with the nuclear lamina and intermediate filaments, disrupting their organization (22). It also appears to stabilize DNA. Thus, it prevents degradation of cellular DNA during apoptosis, or programmed cell death, induced by E1A (20). It also appears to stabilize the DNAs of infecting virus and transfected plasmids so as to enhance expression from their genes (9).

Obviously, therefore, in some fraction of cells infected with Ad5 that lacks E1B, the absence of the E1B 19-kDa protein will allow E1A to induce apoptosis, bringing the

growth of these cells to an abrupt end. In other infected cells, however, in which apoptosis does not occur, the growth induced by E1A may be affected in more subtle ways by the absence of the E1B 55-kDa protein, as this protein binds to the cellular protein p53. p53 is a growth suppressor that appears to prevent cells from leaving the G₁ phase of the cell cycle. The main evidence for this is that when wild-type p53 is overexpressed in transformed cells, these cells are arrested at G₁. This result has been obtained both with rodent cells transformed by viral oncogenes and with human tumor cells and cell lines, although other transformed cells were not prevented from proliferating (reviewed in references 15 and 21). Two mechanisms by which p53 could act have been suggested: one is that it blocks initiation of DNA replication, and the other is that it inhibits expression of genes required for progression from G₁ into the cell cycle either directly as a repressor or by activating the expression of repressors (15, 21, 23). In the normal cell, p53 may itself be regulated by changes in its level of phosphorylation (21). Many features of p53 are therefore reminiscent of pRb, the product of the retinoblastoma susceptibility gene, and it has been suggested that the functions of these two growth suppressors may be interconnected (14). The similarities between p53 and pRb extend further, as both are bound by the transforming proteins of DNA tumor viruses, and as with pRb, p53 is believed to be inactivated as a suppressor when complexed with viral protein (21).

To determine what effect E1B has on cell cycle progression produced in BRK cells by E1A, we have compared adenoviruses containing and lacking E1B. Experiments with adenovirus are particularly worthwhile for studying p53, as this virus causes chromosomal aberrations in cells (3, 5) and p53 may participate in the control of cell cycle progression following some types of DNA damage (13). In addition, experiments on primary BRK cells have the advantage of not being complicated by mutated forms of p53 or of other oncogene products that may be present in established cell lines.

In our previous experiments (10, 11), we reduced killing of BRK cells by the virus by using E1A mutations rescued into Ad5 *dl520*, which produces the smaller 243-residue (243R)

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FIG. 1. Map of the 243-residue E1A protein of Ad5. (A) Positions of deletions used in this study and the residues deleted. (B to D) Regions of the protein required for (B) binding to cellular proteins p300 and pRb (based on results in BRK cells with the deletions in the Ad5 *dl520* background [19]; deletions in the Ad5 *dl520E1B⁻* background gave similar results), (C) transformation of BRK cells in cooperation with *ras* (12) and induction of mitosis in BRK cells (10), and (D) induction of cellular DNA synthesis in BRK cells (11) (only E1A proteins with two deletions, one in the hatched region and one in the stippled region, are defective).

E1A protein but not the larger, transactivating 289R E1A protein. In the present study, we compared the effects of these viruses to those of viruses containing the same E1A mutations but in a background of E1B-deleted Ad5 *dl520* (*dl520E1B⁻*). Ad5 *dl520* viruses with the deletions shown in Fig. 1 have been described elsewhere (11). To produce Ad5 *dl520E1B⁻* and E1A deletion mutants of it, a plasmid containing the *Xho*I C fragment of Ad5 (up to nucleotide 5788) but lacking the E1B region, nucleotides 1682 to 3533, was constructed from pXC-1 (17). Into this we inserted *dl520* and the various E1A exon 1 deletions. The mutated E1A and E1B regions were then rescued into Ad5 *dl309* as described previously (16). Mutant viruses were plaque purified twice.

We tested the effect of deleting E1B on the ability of *dl520* to induce mitosis by infecting quiescent BRK cells with *dl520* or *dl520E1B⁻*. The procedure was the same as described previously (10) except that we used 2 PFU per cell rather than 10 to minimize apoptosis. At infection or 24 h later, we added nocodazole for 24 h and then fixed the cells, stained them with propidium iodide, and measured the mitotic index on batches of 500 to 1,000 cells. Averaged over three experiments, the mitotic indices for 0 to 24 and 24 to 48 h were as follows: with *dl520*, 28 and 12%, respectively; with *dl520E1B⁻*, 20 and 6%, respectively; and with mock infection, less than 1% for both intervals. Summed over the whole 48-h period, the mitotic index with *dl520E1B⁻* was therefore 65% of that with *dl520*. Clearly, the lack of E1B cannot prevent, and at most only reduces, induction of mitosis by E1A.

Even though the change in mitotic index was small, it was possible that more subtle effects of E1B in controlling entry into the cell cycle could be revealed by studies with E1A mutants. As we explain below, the E1A mutants of most interest were those that fail to bind to p300 (e.g., *dl1101/520*) or pRb (*dl1107/520* and *dl1108/520*) (Fig. 1). These mutants are all severely defective at inducing mitosis (10), so that we were unlikely to detect a further decrease in the induction of mitosis by these mutants in the absence of E1B. A more promising approach was to study the induction of cellular DNA synthesis by E1A in infected BRK cells. Earlier we found that this induction is not affected by single deletions in

TABLE 1. Cellular proteins not complexed in the presence of different Ad5 mutants and the hypothetical and actual induction of S phase in quiescent BRK cells

Mutant	Uncomplexed cellular protein			Entry into S phase	
	p300	pRb	p53	Hypothetical ^a	Actual ^b
<i>dl520</i>					Yes
<i>dl1101/520</i>	X				Yes
<i>dl1107/520</i>		X			Yes
<i>dl1108/520</i>		X			Yes
<i>dl01/07/520</i>	X	X			No
<i>dl02/06/520</i>					Yes
<i>dl520E1B⁻</i>			X	Yes	Yes
<i>dl1101/520E1B⁻</i>	X		X	No	Yes
<i>dl1107/520E1B⁻</i>		X	X	No	Reduced
<i>dl1108/520E1B⁻</i>		X	X	No	Reduced
<i>dl01/07/520E1B⁻</i>	X	X	X	No	No
<i>dl02/06/520E1B⁻</i>			X	Yes	Yes

^a Assuming that p53 together with either p300 or pRb can suppress entry into S phase from G₀/G₁ (see text).

^b Results for *dl520* viruses from reference 11; those for *dl520E1B⁻* from this work.

E1A, even when these deletions remove sequences necessary for the E1A protein to bind to either p300 or pRb (11). The only E1A mutants that are defective for induction are those in which deletions remove sequences necessary for binding both p300 and pRb, such as the double E1A deletion

FIG. 2. Incorporation of [³H]thymidine into DNA over a 72-h period by BRK cells infected with the Ad5 mutant viruses indicated. For each mutant, the average incorporation for the number of experiments indicated in parentheses is shown as a percentage of the incorporation by Ad5 *dl520* in the same experiments. Error bars show standard deviations.

FIG. 3. Incorporation of [³H]thymidine per cell into DNA over a 72-h period by BRK cells infected with the Ad5 mutant viruses indicated. For each mutant, the incorporations per cell for the three successive 24-h intervals were summed and the total expressed as a percentage of that for *dl520* and averaged over the number of experiments indicated in parentheses (A) and as the fold increase over that in mock-infected cells and averaged over all experiments (B).

dl01/07/520 (Fig. 1; Table 1). Thus, when E1A is present, cells appear to be prevented from entering the cell cycle only when both p300 and pRb are uncomplexed to E1A and so are free in the cell. There is strong suggestive evidence that p300, like pRb, is a growth suppressor preventing cells from entering the cell cycle by repressing gene expression (19). The results just described suggest that these two suppressors must cooperate to prevent entry into S phase, as either alone is insufficient. If p53 is also a suppressor, can it modify this requirement for both p300 and pRb? That is, when p53 is uncomplexed with the E1B 55-kDa protein in the cell, can it cooperate with p300 or pRb to prevent entry into S phase? If this were the case, then single deletions such as *dl1101*, *dl1107*, and *dl1108*, which induce DNA synthesis in the *dl520* background, should fail to do so in *dl520E1B*⁻ (Table 1).

To investigate this, we measured the incorporation of [³H]thymidine by quiescent BRK cells after infection with E1A mutants of Ad5 *dl520E1B*⁻ and Ad5 *dl520*. Primary BRK cells were infected at 2 PFU per cell and labeled with [³H]thymidine at 0 to 24, 24 to 48, and 48 to 72 h after infection. Floating cells and cells adhering to the plate were harvested, and incorporation of ³H into DNA was measured as described previously (11). For each mutant, incorporations for the three labeling periods were summed, expressed as a percentage of the total incorporation for *dl520*, and averaged over all experiments (Fig. 2). All of the E1A mutants in *dl520* except *dl01/07/520* induced incorporations significantly greater than those in mock-infected cells, and in this respect the effects were essentially the same as we observed before (11). The incorporation induced by

dl520E1B⁻ was very similar to that induced by *dl520*, but those induced by E1A mutants *dl1101*, *dl1107*, and *dl1108* in *dl520E1B*⁻ were consistently less than those induced by the corresponding mutants in *dl520*. We observed the same relative differences between the E1B⁻ and E1B⁺ mutants when we infected at 1 and 4 PFU per cell. At most, therefore, lack of E1B reduced but did not prevent the induction of DNA synthesis by E1A mutants that fail to bind to p300 or pRb.

Because of apoptosis induced by E1A in the absence of E1B, the *dl520E1B*⁻ viruses killed more cells in the experiments of Fig. 2 than did the *dl520* series. Therefore, at least part of the reduction in incorporation for E1A mutations in the E1B⁻ background seen in Fig. 2 could have been due to increased cell death. To correct for this, we next measured the incorporation per cell for cells adhering to the plate. We did this by preparing duplicate sets of dishes of infected cells; one set was labeled as before, but we measured only incorporation into cells still adhering to the plates; with the duplicate set, we counted the numbers of cells adhering to the plates at times corresponding to the ends of labeling periods. In these experiments, we included the double mutants *dl02/06/520* and *dl02/06/520E1B*⁻, in which the deletions *dl1102* and *dl1106* do not affect the binding of the E1A protein to either p300 or pRb (Fig. 1).

Figure 3 shows the average total incorporation per cell over 72 h for the various mutants, expressed in Fig. 3A as a percentage of that for *dl520* and in Fig. 3B as the fold increase over that for mock-infected cells. As a further comparison, in Table 2 we give the ratios of the incorpora-

FIG. 4. Fluorograph of an SDS-polyacrylamide gel of immuno-precipitates from ³⁵S-labeled extracts of BRK cells infected with Ad5 mutant viruses. The infecting virus is indicated for each lane. Ad5 *dl*312 lacks the E1A region. Bands representing E1A proteins and the cellular proteins p300 and pRb are marked.

tions per cell induced by each mutant in the two back-grounds for the individual experiments in Fig. 3. We present the results in these three ways because any single comparison puts undue emphasis on the accuracy of the result chosen as the standard, for example, that for *dl*520 in Fig. 3A and for mock infection in Fig. 3B.

All of the single mutants and the double mutants *dl*02/06/520 and *dl*02/06/520E1B⁻ induced more than twice as much DNA synthesis as occurred in mock-infected cells; only *dl*01/07/520 and *dl*01/07/520E1B⁻ induced poorly (Fig. 3). The absence of E1B did not reduce induction by *dl*520 or *dl*02/06/520, and if it caused a decrease with *dl*1101/520, this decrease was not statistically significant (Fig. 3; Table 2). However, the absence of E1B significantly decreased induction with *dl*1107/520 and *dl*1108/520. These reductions were not due to the failure of these mutants *dl*1107/520E1B⁻ and *dl*1108/520E1B⁻ to produce E1A proteins. Analyses on sodium dodecyl sulfate (SDS)-gels of E1A proteins immuno-precipitated with E1A-specific M73 antiserum from labeled BRK cells by a method described previously (19a) showed that *dl*520E1B⁻ and all of the E1B⁻ mutants produced similar and readily detectable levels of E1A that were comparable to the level produced by *dl*520 (Fig. 4). These analyses also confirmed that the binding of mutant E1A proteins to cellular proteins p300 and pRb (Fig. 1) was unaffected by the removal of the E1B region. Thus, deletion *dl*1101 prevented binding to p300 in mutants *dl*1101/520E1B⁻ and *dl*01/07/520E1B⁻ (Fig. 4, lanes 3 and 6), and deletions *dl*1107 and *dl*1108 prevented binding to pRb in mutants *dl*1107/520E1B⁻, *dl*1108/520E1B⁻, and *dl*01/07/520E1B⁻ (Fig. 4, lanes 4 to 6). Furthermore, binding was unaffected

TABLE 2. Ratios of incorporations of [³H]thymidine per cell by BRK cells infected with different Ad5 E1A mutants in E1B⁻ and E1B⁺ backgrounds

Expt	Ratio of incorporations/cell							
	<i>dl</i> 520E1B ⁻ / <i>dl</i> 520	<i>dl</i> 1101/520E1B ⁻ / <i>dl</i> 1101/520	<i>dl</i> 1107/520E1B ⁻ / <i>dl</i> 1107/520	<i>dl</i> 1108/520E1B ⁻ / <i>dl</i> 1108/520	<i>dl</i> 01/07/520E1B ⁻ / <i>dl</i> 01/07/520	<i>dl</i> 02/06/520E1B ⁻ / <i>dl</i> 02/06/520	<i>dl</i> 1101/520E1B ⁺ / <i>dl</i> 1101/520	<i>dl</i> 1107/520E1B ⁺ / <i>dl</i> 1107/520
1	1.3	1.4						
2	0.9	0.6			0.5		1.3	1.0
3	1.2	1.0			0.3		0.9	1.4
Mean	1.1	1.0			0.4		1.1	1.2

by deletions *dl1102* and *dl1106* in mutant *dl02/06/520E1B⁻* (Fig. 4, lane 7).

It seems likely that the poorer incorporations per cell induced by *dl1107/520E1B⁻* relative to *dl1107/520* and by *dl1108/520E1B⁻* relative to *dl1108/520* were due to the absence of binding of p53 by the E1B 55-kDa protein rather than to the lack of the E1B 19-kDa protein. The reason for this is that from the known properties of the E1B 19-kDa protein described earlier, it is not easy to see how the absence of this protein could have affected these two E1A mutants differently from all the others. However, the absence of the E1B 55-kDa protein appeared to affect the ability of E1A to induce quiescent BRK cells to enter S phase of the cell cycle only with E1A mutants that allowed uncomplexed p53 to cooperate with another growth suppressor as follows.

Deletions *dl1107/520* and *dl1108/520* lack sequences essential for E1A to bind to pRb (Fig. 1), so that with these mutants, pRb is uncomplexed in the cell. The reduced incorporations induced by these mutations in the E1B⁻ background suggest that when p53 and pRb were free together in the cell, they may have partially suppressed DNA synthesis induced by E1A binding to p300 (Table 1). With *dl1101/520*, which removes a sequence required for binding to p300, removal of E1B caused little if any change. This finding indicates that p53 and p300 free together in the cell had little effect in preventing DNA synthesis. Ad5 *dl520* and *dl02/06/520*, which were unaffected by deleting E1B, both produce E1A proteins that bind to p300 and pRb. Therefore, when p53 was the only uncomplexed protein of the three, it could not suppress induction of DNA synthesis by E1A.

Thus, in the suppression by uncomplexed proteins of DNA synthesis induced by E1A (Table 1), these results suggest that only p300 and pRb together suppressed completely; pRb and p53 together suppressed partially; p300 and p53 together and all three proteins separately had little effect. Conversely, in terms of the induction of DNA synthesis in quiescent BRK cells by E1A, the binding of p53 by E1B had no effect when E1A bound p300 and pRb together or pRb alone, but binding of p53 increased induction when E1A bound p300 alone. One explanation for these observations is that p300 and pRb control entry into the cell cycle in different ways, possibly by suppressing the expression of different genes (19), and that p53 can partially replace p300 in this control.

The transforming proteins of several other DNA tumor viruses, such as the large T antigen of simian virus 40, have properties resembling those of the E1A and E1B proteins of Ad5. In a study on the stimulation of DNA synthesis in quiescent CV-1P monkey cells by simian virus 40 large T antigen, Dobbstein et al. (6) concluded that three functions of T antigen contribute to its full potential for inducing growth; one is the binding of pRb, another is probably the binding of p53, and the third involves the DNA-binding domain of T antigen. With the E1A and E1B proteins of Ad5 in BRK cells, there are also three functions, namely, the binding of p300, pRb, and p53. However, there the similarity ends; with large T antigen in CV-1P cells, the three functions are additive, but with Ad5 proteins in BRK cells, they were not. More significantly, the three Ad5 functions were by no means equally important. The binding of p300 or pRb by E1A was essential for induction (as expected from what we described at the beginning of this note), whereas the binding of p53 by E1B only had a minor effect, possibly as a partial substitute for p300.

The difference between these two systems, the different responses of tumor cells to added p53 (21), and the different effects of inactivation of the *Rb* gene in different species of cells (8) all show what is almost certainly the case generally, namely, that the action of growth suppressors such as p53 and pRb varies between different types of cells. Evidently, mammalian genomes must contain genes for a variety of growth suppressors of overlapping functions, and cells must select from them appropriately, according to the situations that cells find themselves in.

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