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ADENOVIRUS AND CELL CYCLE CONTROL

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TABLE OF CONTENTS

1. ABSTRACT

Adenovirus infection of quiescent cells induces transition from G0 or G1 into the S phase of the cell cycle and allows cellular proliferation. This is beneficial for the virus since cells in S phase provide optimal conditions for viral replication. Adenovirus E1A, E1B and E4 gene products contribute to cell cycle deregulation. E1A proteins inactivate the pRb checkpoint, allowing the E2F transcription factor to activate genes involved in nucleotide metabolism and DNA replication, which are required in S phase. E1A also interacts with transcriptional modulators, including histone acetyltransferases, histone deacetylases, and other chromatin remodeling factors. These interactions

affect transcription of several cellular and viral genes, some of which are involved in cell cycle regulation. Cell cycle deregulation by E1A results in stabilization and accumulation of p53. To prevent cell cycle arrest and apoptosis that would be triggered by p53, the adenovirus E1B and E4orf6 gene products employ various mechanisms to inactivate the tumor suppressor. Additional E4 gene products also interact with and modulate cell cycle regulators. Cell cycle checkpoints targeted by adenovirus proteins are often compromised in human tumors as well. Thus, understanding the interactions between adenovirus and the cell cycle has facilitated the generation of adenovirus mutants, which can replicate only in cells with inactivated checkpoints. Such "oncolytic" viruses are being tested for their ability to specifically replicate in and lyse cancer cells.

2. INTRODUCTION

During infection of host cells, viruses rely heavily on cellular processes for various stages of their replication. Small DNA tumor viruses such as adenovirus (Ad), simian virus 40 (SV40), and human papillomavirus (HPV) depend on host cell mechanisms to replicate the viral genome. These viruses infect primarily quiescent cells, which do not provide an optimal environment for viral DNA synthesis due to rate-limiting levels of deoxynucleotides (1) and low levels of proteins involved in DNA synthesis. Various viruses have evolved different means to overcome this obstacle. The pRb and p53 regulatory pathways are two crucial pathways that control transitions from one cell cycle phase to the next. Not surprisingly, adenoviruses, as well as other DNA tumor viruses, target these pathways to induce quiescent cells to enter the S phase of the cell cycle.

Adenoviruses belong to the Adenoviridae family of viruses, which includes at least 50 human adenovirus serotypes, allocated to six subgroups (A through F). The adenovirus chromosome carries five early transcription units (E1A, E1B, E2, E3, and E4) (2). Modulation of the cell cycle is carried out by several adenovirus proteins, including E1A, E1B, and E4 gene products. The growth deregulatory functions of Ad are also essential for transformation of primary rodent cells. However, although all Ad serotypes are able to induce tumors in immunodeficient nude mice, only cells transformed by oncogenic Ad serotypes of groups A (Ad12, Ad18 and Ad31) and B produce tumors in immunocompetent rodents (3). In addition, the group D member, Ad9, is known to specifically induce mammary tumors in rats (4). Most of the work regarding adenovirus and cell cycle control was carried out using Ad2/5 or Ad12.

E1A and E1B are the only adenovirus genes consistently found to be integrated and expressed in adenovirus transformed cultured rodent cells. In general, E1A by itself is sufficient to activate the cell cycle, inducing expression of genes required for DNA synthesis and repeated rounds of cell division. However, proliferation of cells in response to E1A is often quite limited in the absence of E1B expression. With E1B coexpression, the number of primary cell colonies able to proliferate indefinitely in response to E1A expression is greatly elevated (5). E4 gene products were recently shown to enhance cellular transformation by E1A and E1B (6). In this review we will discuss the interaction of E1A, E1B and E4 from Ad2/5 and Ad12 with the cell cycle regulatory machinery.

3. THE E1A PROTEINS

E1A is the first viral transcription unit to be expressed after the adenoviral chromosome reaches the nucleus. Two mRNAs are transcribed from the Ad2/5 E1A unit during the early phase of infection: a 13S mRNA that encodes a 289R protein and a 12S mRNA that encodes a 243R protein. Experimentally, all the biological functions attributed to E1A can be carried out by one or both of these products (7). Three additional E1A mRNA species, with no definitive function, accumulate later in the infectious cycle. The two early mRNAs contain identical 5` and 3` ends, but differ internally due to differential splicing. They encode proteins that are identical except for an additional 46 amino acid segment (CR3) that is present in the larger polypeptide and encodes a transcriptional activation domain (8). When comparing E1A sequences from several human adenovirus serotypes it appears that the E1A products include three conserved regions (CR1, CR2 and CR3), separated by less highly conserved domains. These conserved regions along with arginine at position 2, a PXDLS motif and a short run of basic residues at the C-terminus are regions common to all Ad E1As. In the absence of any known enzymatic activity attributable to Ad E1A, and since E1A proteins are transcriptional activators that do not exhibit sequence-specific DNA binding, it is now considered that these proteins function through a series of protein-protein interactions with important cellular components. The three conserved regions mark domains that play major roles in mediating such interactions (Reviewed in (2, 9)). The oncogenic serotypes have an additional alanine-rich region between CR2 and CR3 that has been shown to be important for tumor induction (10-11). The CR3 region that is present in the 289R, but not in the 243R protein, is required for the activation of cellular genes and early viral transcription units (7). The CR3 domain binds several transcription factors and components of the general transcriptional machinery (7, 9, 12). Since both 243R and 289R proteins can promote cell cycle progression and cellular transformation, it is believed that CR3 is not absolutely required for these activities of E1A (13). Thus the role of CR3 in stimulating transcription is beyond the scope of this review. The 243R product can both activate and repress transcription, and the consequences of its effects on gene expression include cell cycle deregulation and virus replication.

E1A accomplishes cell cycle deregulation by binding to and perturbing the normal function of key negative regulators of cell growth. Ironically, the same activities of E1A that are required for productive virus infection and oncogenic transformation also stimulate programmed cell death, which causes abortive transformation unless E1A is coexpressed with anti-

apoptotic genes, such as adenovirus E1B proteins (Reviewed in (14-16)). E1A overrides normal regulatory constraints at the G1/S border by acting on at least three levels: (i) by inactivating pRb-family proteins and releasing active E2F transcription factor (section 3.2); (ii) by modulating the function of chromatin remodeling factors, such as the histone acetyltransferases (HAT) p300/CBP and PCAF, the SWI/SNF family member p400, and the complex containing CtBP and a histone deacetylase (HDAC) (section 3.4); and (iii) by targeting additional cellular proteins, including downstream targets of cdk2, and transcription factors involved in regulation of genes that participate in cell cycle control (section 3.3).

3.1 Early findings

In early studies of adenovirus, it was noticed that adenovirus infection influences the host cell cycle. Experiments with ts BHK cells that were arrested in G1 at the nonpermissive temperature and made quiescent by serum restriction revealed that Ad2 infection stimulated DNA synthesis at both the permissive and restrictive temperatures (17-19). A mutation analysis of Ad5 revealed that mutants in the E1A region were defective for the induction of cell cycle abnormalities, whereas an E1B mutant produced a wild type response. It was concluded that alteration of cell cycle progression was a direct effect of E1A (20-22). In several rat cell lines expressing the Ad12 E1A gene from a dexamethasone (DM)-regulatable promoter, DNA synthesis increased in density-arrested cells following DM induction (23). It was later shown that the 13S- or 12S-mRNA product alone had the ability to cause progression of the cell cycle at a similar rate (24). 293 is a cell line that has been immortalized and transformed by E1A and E1B (25). Transfection of 293 cells with an antisense E1A-expressing vector resulted in a transient inhibition of cellular DNA synthesis. Thus, even after extended periods of time in culture, cells immortalized by E1A still require E1A expression to activate the cell cycle and prevent them from senescing (26).

The origins of the understanding how E1A affects cell cycle regulation can be found in several reports describing a set of cellular proteins, which can be co-immunoprecipitated with E1A (27-30). The main co-precipitating proteins have molecular weights of about 33, 60, 80, 90, 105, 107, 130, 300 and 400 kDa, and several of these proteins have been shown to associate directly with E1A. The 105 kDa polypeptide was the first to be identified. It is the retinoblastoma tumor suppressor protein (pRb) (31), and the consequences of its interaction with E1A are discussed below (section 3.2). The other proteins include pRb family members (p107, p130); Cyclin A (p60) and Cdk2 (p33), which may associate with E1A by interacting with p107 and p130 (32-34); p300, which is closely related in its amino acid sequence to the CREB-binding protein (CBP), a histone acetyltransferase and a scaffold for the assembly of transcription complexes (35-36); and p400, which is a SWI2/SNF2-related protein (37). The interactions of E1A with these proteins are discussed below.

There are three regions in the 12S E1A protein that are required for transformation: the nonconserved amino terminus (aa 2 to 24), CR1 (aa 40 to 80) and CR2 (aa 120 to 140) (reviewed in (2)). These regions of E1A interact with two sets of cellular proteins: p300/CBP binds to the N terminus and CR1, and the pRb family members bind to CR1 and CR2 (figure 1). The interactions with pRb and p300/CBP influence functionally distinct growth-regulatory pathways (5). Both the pRb- and p300/CBP-interacting domains of E1A are required for cellular transformation, whereas either domain is sufficient for induction of DNA synthesis. Each domain alone is not enough to allow cells to pass the G2/M checkpoint and progress to mitosis (29, 38-40). E1B may contribute to induction of DNA synthesis by E1A, since it has been reported that E1A mutants, which fail to bind pRb, induce DNA synthesis at a significantly lower level in Ad5 lacking E1B than in Ad5 containing E1B (41). The C-terminus of E1A was shown to modulate transformation through binding to a protein called C-terminal binding protein (CtBP) (42-43). It has also been shown that a region near the C-terminus of the 12S protein is required for growth factor induction, which stimulates epithelial cell proliferation. Furthermore, this growth factor production is necessary for epithelial cells to survive past their normal life span in culture and become immortalized (44).

3.2 Targeting the pRb checkpoint by E1A

3.2.1 pRb

The pRb tumor suppressor has been shown by a number of assays to inhibit cell cycle progression and arrest cells at the G1 phase (45). pRb contains several functional domains, including domains A and B, which are highly conserved and which form a central “pocket” that is critical for the tumor suppressor function of pRb (46-47). The A and B domains are separated by a linker. The structural integrity of the “pocket” is required for the interaction of pRb with most of its associated proteins, including the E2F transcription factor (47).

The E2F family is a group of sequence-specific DNA-binding transcription factors that have been shown to regulate expression of genes required for entry into S phase and for DNA synthesis (47). “Free E2F”, the smallest E2F complex, is composed of heterodimers containing a subunit encoded by the E2F gene family and a subunit encoded by the DP family of genes. Six E2F genes and two DP genes are known in mammalian cells. Some of the E2F heterodimers are transcription activators and some act as repressors (48). Binding of pRb to E2F leads to repression of E2F transactivation, and at least two mechanisms are suggested to account for the repression. First, the pRb binding domain of E2F-1 through E2F-5 is embedded within their transactivation domains, and thus pRb binding can directly inhibit E2F transactivation (49). Second, pRb recruits repressors such as histone deacetylases and chromosomal remodeling SWI/SNF complexes to E2F-responsive promoters on DNA (47, 50).

pRb activity is regulated by phosphorylation. Hypophosphorylated forms of pRb predominate in early G1 phase and reappear during M phase, while hyperphosphorylated forms of pRb are present from late G1

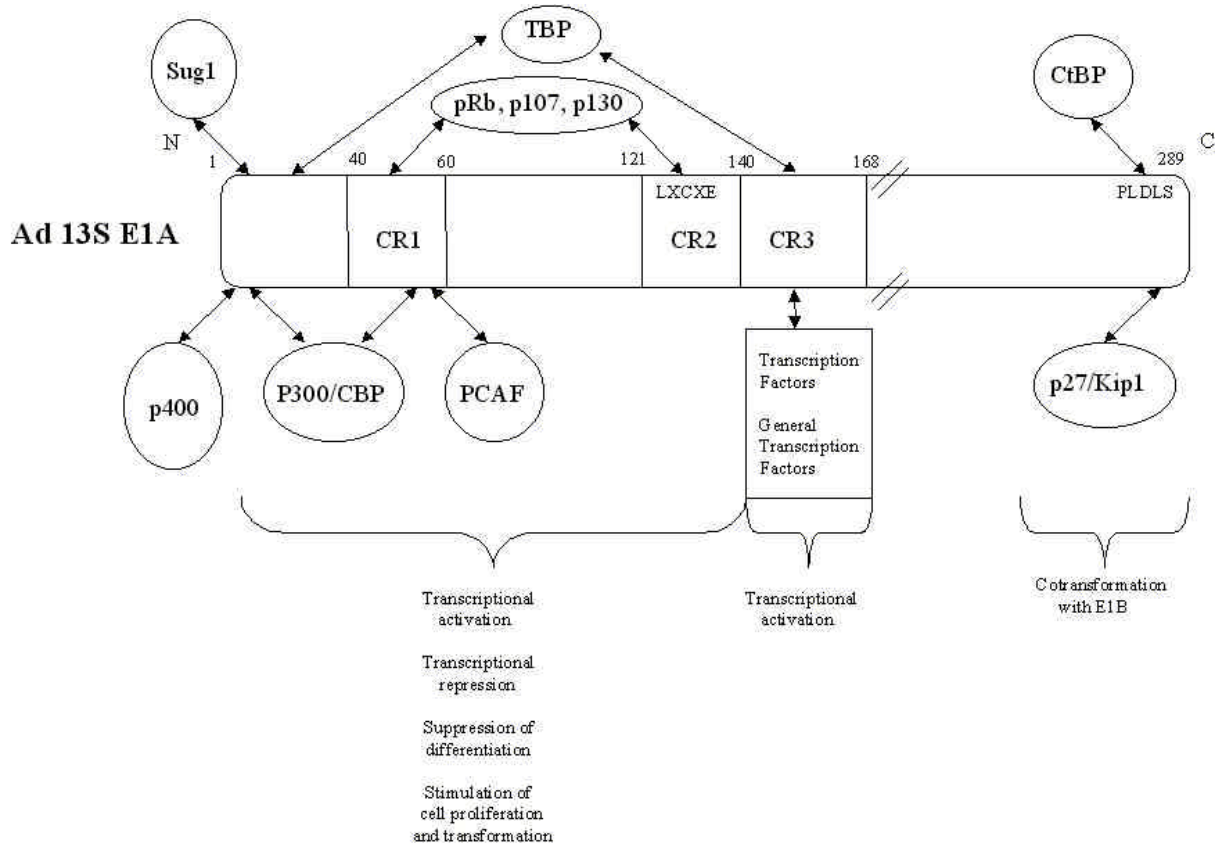

Figure 1. E1A domains involved in protein-protein interactions. Conserved E1A domains and sites for protein-protein interactions are shown. The functions attributed to the various domains are also represented. For details, see the text.

phase throughout S, G2 and M phases (51-52). Upon mitogenic stimulation of quiescent cells, accumulation of cyclin D-dependent kinases (Cdk4 and Cdk6) triggers the phosphorylation of pRb, leading to dissociation of E2F, which associates with the hypophosphorylated forms of pRb (47, 50). The activity of the cyclin D-Cdk complexes is inhibited in quiescent cells by a class of Cdk inhibitors called INK4 (named for their ability to inhibit Cdk4), which includes four members: p16INK4a, p15INK4b, p18INK4c and p19INK4d (50). Once freed from its association with pRb, E2F functions as a transcriptional activator and induces a battery of genes that regulate DNA metabolism (such as dihydrofolate reductase (DHFR), thymidine kinase, thymidylate synthase, DNA polymerase-alpha, and E2F-1 itself), as well as the cyclin E and A genes and Cdk2. The phosphorylation process is then accelerated by the cyclin E-cdk2 complex (53-54). Cyclin E-Cdk2 also phosphorylates additional substrates involved in DNA replication (50).

The interaction of E1A with pRb is mediated primarily by CR2, with sequences from the N-terminal portion of CR1 stabilizing the complex. The CR2 domain of E1A, containing the LXCXE motif required for E1A-pRb interaction, associates with pRb within domain B of the pocket, a region that is distinct from the site required for E2F interaction. As the E1A protein is bound to the pRb-E2F complex, the CR1 domain, which plays an auxiliary role, can compete for pRb binding to E2F. As a consequence of this interaction, E1A is left firmly bound to pRb and E2F is released, free to activate its target genes (Reviewed in (55)). In addition to releasing E2F from repression complexes, E1A has also been reported to cause stabilization of E2F, achieving a further increase in active E2F transcription factors (56). pRb acetylation is also modulated by E1A, as discussed in section 3.4.1.

3.2.2 pRb family members

Two additional pRb family members also associate with E2F: p107 and p130 (57-59). These proteins are structurally very similar to pRb, with the greatest sequence homology found in the pocket domain. p107 and p130 were found to share many biochemical similarities with pRb, as well as extensive functional properties. Like pRb, p107 and p130 have been found to block transcriptional activation by free E2F and to be capable of actively repressing E2F-regulated promoters (reviewed in (60)). Proteins such as the histone deacetylase HDAC1, which have been implicated in some aspects of pRb-mediated transcriptional repression, have also been reported to interact with p107 and p130. Furthermore, overexpression of p107 and p130 can arrest cells in G1 (61-62), similarly to pRb.

Despite the many similarities, several significant differences distinguish p107 and p130 from pRb. pRb, p107 and p130 associate with E2F during different portions of the cell cycle. p130-E2F complexes are found primarily in quiescent or differentiated cells and p107-E2F complexes are most prevalent in S phase, but can also be found in G1. pRb-E2F complexes are found in quiescent or differentiated cells, but are most evident as cells progress from G1 into S phase (reviewed in (48)). There are also differences in the identity of E2F family members, which associate with the various pocket proteins (60). The spacer region separating the A and B domains of the pocket is larger in p107 and p130 than in pRb, and the larger spacer serves as a site for direct binding of cyclin A/E-dependent kinases (63-65). The biological significance of these interactions is not clear. The p107 and p130-cyclin-Cdk complexes may represent enzyme-substrate complexes, although the stable nature of the interaction and the finding that these complexes include a large portion of the nuclear pool of p107 and p130 molecules may not be compatible with this interpretation. Alternatively, the interaction may target the kinase to other substrates. Interestingly, the substrate specificities of p107- and p130-associated kinases seem to differ from those of the free kinases (66). A third possibility that has been suggested is that p107 and p130 may serve as Cdk inhibitors by sequestering Cdks away from other substrates or by using their N-terminal domains to reduce the activity of the associated kinases (67-69). Currently, p107 and p130 are thought to be regulators of E2F and Cdks, although they may have additional, as yet unidentified, functions.

Similarly to pRb, E1A can bind p107 and p130, dissociating E2F from the complex. As for pRb, the interaction is mediated by the E1A CR2 region, and CR1 sequences were shown to contribute to stabilization of the E1A-p107 complex (38). However, the interactions of E1A with the various pocket proteins are not identical, and can be differentially affected by certain E1A mutants (70). Using such mutants, it was possible to show that interaction of E1A with the various pocket proteins could influence the expression of different genes (71). E1A does not disrupt the interaction of p107 and p130 with the cyclin-Cdk complexes (29, 65), and is thus found to be associated with kinase activities (30, 34, 72). p107 and p130 present in these complexes were reported by some investigators to be hyperphosphorylated (73), whereas others have shown that E1A blocks hyperphosphorylation of p107 and p130 (74). However, different phosphorylation sites may have been affected in the two cases. Although E1A was reported to modulate phosphorylation of p130 and p107, it is unclear whether a direct interaction with the pocket proteins is required (74). E1A was also shown to induce phosphorylation of pRB in normal quiescent cells, independently of binding to pRb (75). pRb phosphorylation may complement pRb inactivation by interaction with E1A. However, the functional significance of the E1A-pocket proteins-cyclin-Cdk complexes is uncertain. They could represent inactive complexes dissociated from E2F, or they may have additional, unknown functions. Interestingly, E1A itself was reported to be phosphorylated by various Cdks and phosphorylated E1A was found to be more efficient in binding pRb and disrupting pRb-E2F complexes (76).

In summary, E1A proteins disrupt a series of complexes consisting of different pocket proteins, several E2F subunits and cyclin-Cdks. These complexes normally regulate cell cycle progression and their disruption by E1A deregulates normal cell cycle control, leading to progression of quiescent cells into S phase.

3.3 Targeting cyclin-Cdks by E1A

In addition to interacting with cyclin-Cdk complexes (section 3.2.2), E1A was also shown to modulate the expression of genes encoding cyclins and Cdks. Cyclin A was shown to be transcriptionally activated by E1A, through a mechanism involving binding to p107 and relieving repression of the cyclin A promoter (71, 77). Cyclin D levels were reduced in Ad-transformed and infected cells, in a manner dependent on E1A, whereas Cyclin E was increased in response to Ad5 E1A (77-79). The E1A 243R protein has also been shown to induce elevated levels of Cdc2 in infected cells (80). Ad12 E1A similarly increased the levels of cyclins A, B1, E, Cdc2, and Cdk2, and decreased the levels of cyclins D1 and D3. These changes were suggested to depend on p107 and p130 (81). In addition, expression of E1A proteins in quiescent fibroblasts was reported to lead to an increase in Cdc25A tyrosine phosphatase activity and levels. This phosphatase activates Cdks by dephosphorylating an inhibitory phospho-tyrosine site in these molecules. Inhibition of Cdc25A function by antibody injection prevented virus-induced entry into S phase, presumably by inactivating the Cdc25A target Cdks (82). Thus, alterations in the levels of various cyclins and Cdk molecules and their modulators may also contribute to deregulation of the cell cycle by E1A.

In addition, E1A has been shown to directly affect Cdk inhibitors. E1A bound the cyclin-dependent kinase inhibitor p27/Kip1 in TGF-beta-treated cells and blocked its inhibitory effect. As a result, the activity of the cyclins E/A-cdk2 kinase complex was restored, and the TGF-beta growth-inhibitory effect was reversed (83). Mutation of two conserved motifs in CR2, GFP and SDDEDEE, was shown to impair the ability of E1A to overcome G1 arrest by the Cdk inhibitors p16 and p27, but did not prevent pRb binding by E1A (84). E1A can also interact with p21 and inactivate it (85-86). Thus, targeting Cdk activity appears to contribute to cell cycle deregulation by E1A. In addition, using a different experimental system, it was reported that E1A could prevent growth arrest by p27 without interacting with this Cdk inhibitor (87). These results suggest that E1A can also target downstream effectors of Cdk2 to modulate G1/S control.

3.4 E1A, HATs, HDACs, and chromatin remodeling

3.4.1 p300/CBP

A cellular protein of 300kDa has been found among the E1A-associating proteins. The p300 binding site on E1A includes the N-terminus (amino acids 1-25) and the C-terminal half of CR1 (5, 38). As described above, E1A mutants that lack CR2 and are unable to bind pRb are still

capable of inducing cellular DNA synthesis. Only mutants that lack both pRb- and p300-binding sites lose their ability to drive cells into S phase. Thus, E1A proteins contain two independent domains that can induce cell cycle progression from G1 to S phase (39-40, 88). In addition to S phase induction, the E1A p300-binding domain has also been shown to contribute to E1A's ability to repress enhancer function (38, 89-90). E1A can repress the activity of several enhancer elements, including some that control genes associated with cellular differentiation (91-93). The p300 sequence is highly homologous to the CREB-binding protein (CBP) (35) and both proteins are transcriptional co-activators involved in many physiological processes, including proliferation, differentiation and apoptosis (reviewed in (94)). Although differences in p300 and CBP functions have been reported, these proteins are generally believed to have highly overlapping activities. p300/CBP act as protein scaffolds, on which multicomponent transcriptional regulatory complexes are assembled. In addition, p300/CBP proteins possess a histone acetyltransferase (HAT) activity (95-96), and they are found in association with another HAT, PCAF. Histone acetylation plays an important role in the modulation of chromatin structure associated with transcriptional activation (97-99). It has been proposed that histone acetylation promotes destabilization of histone-DNA interactions in the nucleosome, resulting in increased accessibility of the chromatin to the transcription machinery. Although it has been shown that p300/CBP HAT activity is directly involved in chromatin remodeling, a growing body of evidence suggests that transcription factors and components of the transcription apparatus are also regulated by acetylation (94, 100). In the context of cell cycle regulation, it has been shown that the p300/CBP-PCAF protein complex can arrest cell cycle progression (101) and might regulate target genes that are involved in control of the G1/S transition, such as p21/WAF1 (102). p300 is also known to control E2F activity: as cells approach S phase, cyclin E/cdk2 phosphorylates E2F-5. This phosphorylation augments the physical interaction with p300, thereby enhancing the transcription of E2F target genes. The Cdk-stimulated interaction of p300 and E2F may be involved in irreversibly committing cells to cell cycle progression (103). Furthermore, it has been proposed that p300 has a dual role in controlling p53 activity. Current studies show that MDM2 mutants that can bind p53 but not p300 fail to promote efficient p53 degradation. Therefore it has been suggested that a ternary complex containing p300, p53 and MDM2 controls p53 stability. It has also been shown that a p300-interacting protein, JMY, augments p53-dependent apoptosis. Thus, p300 both augments p53 transactivation function and stimulates its turnover (Reviewed in (94)). P300/CBP proteins are also important for differentiation (section 3.5).

The binding of E1A to p300/CBP inactivates a number of cellular and viral promoters and enhancers (104-106). Various mechanisms have been proposed to explain the functional consequences of the interaction between E1A and p300/CBP. First, binding of E1A to p300/CBP disrupts coactivation complexes of p300/CBP containing PCAF or p/CIP (101, 107), and overexpression of PCAF in cells could counteract the mitogenic activity of E1A (101). Disruption of the complex containing PCAF is the consequence of the fact that E1A and PCAF bind to the same or overlapping region within p300/CBP. E1A can also bind PCAF independently of CBP, through CR1 (108). Second, E1A was reported to act as a potent inhibitor of the acetyltransferase activity of p300/CBP and PCAF *in vitro*. This inhibitory activity was necessary for E1A function *in vivo*: E1A inhibited p300-dependent transcription as well as nucleosomal modifications and p53 acetylation (109-110). Whereas the N terminus of E1A is required for the association with p300/CBP, CR2 and CR3 appear to be required for the inhibition of the acetyltransferase activity (109). Thus, inhibition of the HAT activity of p300/CBP may be dispensable for induction of G1 to S phase progression by E1A, but not for control of cell division. In contrast with the reports showing that E1A inhibited HAT activity of p300/CBP, one report demonstrated that E1A activated HAT activity (111). The authors of this report suggested that the different effects of E1A on CBP activity may depend on the system analyzed and the CBP transactivation domain involved. This may very well be the case, since p300/CBP proteins were shown to mediate E1A-induced transcriptional activation, rather than repression, through some effectors, such as YY1 (112) or CREB (113). In the latter case, CBP mediated transactivation of the proliferating cell nuclear antigen (PCNA), a crucial component of DNA replication complexes and an effector of E1A, which can contribute to induction of DNA synthesis.

Interestingly, the presence of pRb and p300/CBP in a multimeric protein complex with E1A has been reported to stimulate acetylation of pRb by p300/CBP (114). Acetylation of pRb hindered its phosphorylation by cyclin E-Cdk2 and increased its binding to MDM2. It is unclear, however, how acetylation of pRb could assist in E1A-induced cell cycle progression.

Contrasting reports also described the effect of E1A on p300/CBP phosphorylation. Eckner *et al*. showed that E1A stimulated p300/CBP phosphorylation, probably through cyclin-Cdk complexes (115). Banerjee *et al*., however, reported that E1A blocked p300/CBP phosphorylation by cyclin-Cdk complexes *in vitro*, (116). The C-terminal region of p300/CBP interacts with cyclin E-Cdk2, as well as with E1A. The 12S E1A protein, which is an inhibitor of p300-dependent transcription, does not affect the association between p300 and cyclin E-Cdk2. However, the 13S E1A product, which contains the transcriptional activation domain, enhances the association between p300 and cyclin E-Cdk2 (117). Furthermore, HAT activity was shown to be elevated at the G1/S border, and phosphorylation of the C-terminal region of p300/CBP by cyclin E-Cdk2 appeared to stimulate its HAT activity (111). Thus, if E1A stimulates HAT activity under some circumstances, it may mimic events occuring at the G1/S border. Another group, however, reported that cyclin E-Cdk2 negatively regulated p300-mediated coactivation of NF-kB (118), implying that phosphorylation may inhibit some p300 activities. Since the exact sites of phosphorylation on p300/CBP have not been mapped, it is

unclear to date how diverse phosphorylation events may influence the various activities of p300/CBP, and what input E1A has on these different events.

In summary, E1A associates with the HATs p300/CBP and PCAF and modifies their activities. Since these coactivators affect transcription of several genes, including cell-cycle related genes, the modulation of their activity contributes to E1A-induced G1 to S progression of the cell cycle. In contrast with E1A's interaction with pRb family members, which results in activation of E2F-regulated genes, the interaction of E1A with p300/CBP results in many cases in repression of genes that are normally up-regulated by p300/CBP.

3.4.2 p400

In addition to its interaction with the HATs p300/CBP and PCAF, E1A also associates with p400, a SWI2/SNF2-related protein. p400 has been found by peptide mapping to be related to p300 and it binds to the same domain on E1A as does p300/CBP (29, 73). The E1A-p400 complex contains additional components, including TRRAP, the DNA helicases TAP54 alpha/beta, actin-like proteins, and the human homologue of the *Drosophila* Enhancer of Polycomb (EPc) protein (37). EPc participates in epigenetic silencing mechanisms, such as position effect variegation (119). An E1A mutant, defective in p400 binding, is also defective in transformation. E1A has been shown to alter the subunit composition of p400 complexes, thus probably modulating their activities. TRRAP is a subunit of at least two other types of complexes, which include the HATs GCN5/SAGA and Tip60/NuA4. The recruitment of TRRAP appears to be required for oncogenic transformation by E1A and expression of dominant negative mutants of TRRAP block E1A-mediated oncogenic transformation (120-121).

3.4.3 CtBP

CtBP (C-terminal binding protein) is a 48 kDa cellular phosphoprotein that binds to the C-terminal region of adenovirus E1A proteins (43, 122). The interaction requires a PLDLS motif, with adjoining sequences influencing the affinity of the binding (123). A PXDLS motif is also found in many other proteins that bind CtBP and mediates their interaction with it. CtBP family proteins share a high degree of amino acid homology with NAD-dependent 2-hydroxy acid dehydrogenases, however, no significant acid dehydrogenase activity has thus far been detected. CtBP family proteins associate with a large number of DNA-binding proteins and function as transcriptional corepressors (124). CtBP1, the family member that associates with E1A, has been reported to associate with endogenous histone deacetylases such as HDAC2 and Sin3. CtBP-mediated repression of certain promoters has been shown to be sensitive to the HDAC inhibitor trichostatin (TSA), whereas repression of other promoters was insensitive to TSA. Thus, transcriptional repression by CtBP is either HDAC-dependent or -independent, depending on the promoter context. HDAC independent repression could involve interaction with the human Polycomb protein PcG (reviewed in (125)), or could be the result of direct interference with transcription initiation complexes (126). The binding of CtBP to the C-terminal region of E1A has been shown to be regulated by the nuclear acetylases p300/CBP and PCAF that interact with the amino terminus of E1A (127). A Lys residue found close to the PLDLS motif was acetylated by these HATs. E1A mutants that mimic the effect of the acetylation (Lys to Gln or Ala) were defective in CtBP binding, whereas a substitution of Lys to Arg enhanced CtBP binding. It could be suggested, therefore, that the HATs bound to the E1A N-terminus may decrease the interaction of the C-terminus with CtBP.

Deletions within the C-terminal region of E1A, which abolish CtBP binding, confer a hypertransforming phenotype to E1A in cooperative transformation assays with the activated Ras oncogene (42-43), and tumors expressing these E1A mutants are highly metastatic, while tumors expressing wild-type E1A are not. Furthermore, when E1A was artificially targeted to a reporter gene, it was shown that deletion of the CtBP-binding motif (PXDLS) in E1A increased E1A's transcriptional activity (128). The mechanisms by which CtBP binding may inhibit the oncogenic potential of E1A are not known, however, several possibilities have been suggested (125). First, CtBP binding to E1A may antagonize the activity of the HATs bound at the N-terminus by recruiting HDACs to the complex. This activity could then inhibit cell growth stimulation associated with the E1A-bound HATs. Second, interaction of CtBP with E1A may contribute to repression of certain cellular genes and deletion of the CtBP-binding region or acetylation of the Lys residue would relieve this repression. For example, the E1A mutants that mimic the effect of acetylation are defective in repressing CREB-stimulated transcriptional activation (127). Third, wild type E1A may activate some genes by removing CtBP from repression complexes. In support of this possibility, the E1A C-terminal region is involved in activation of some cellular genes that modulate oncogenesis (129-131). In addition to the repressor activity of CtBP, a context-dependent weak transcriptional activation function has been reported in 293 cells that express Ad E1A (132). Furthermore, coexpression with E1A turned CtBP from a potent repressor into a weak activator in NIH 3T3 cells, and mutations in E1A which delete residues 2-36 or abolish pRb binding greatly reduce the activation function. These results suggest that the interaction with E1A may expose a CtBP activation function, or that it changes the nature of multiprotein complexes involving CtBP.

The activities of several oncogenes and tumor suppressor genes appear to be modulated by interaction with CtBP. Thus, antagonizing CtBP action by E1A may contribute to the effect of the viral protein on cellular proliferation.

3.5 E1A and differentiation

Terminal differentiation involves two tightly linked phenomena: permanent withdrawal from the cell cycle and biochemical differentiation (133).

E1A can induce terminally differentiated cells to synthesize DNA and divide. This was shown for skeletal

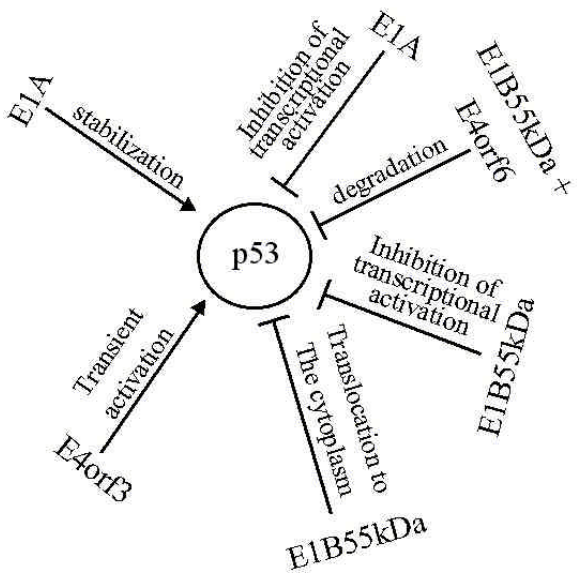

Figure 2. Adenovirus proteins affecting p53. Adenovirus proteins, which modulate p53 at multiple levels, are shown together with their effects on p53. The details are described in sections 4 and 5.

muscle cells (134-136), adipocytes and myocardiocytes (134, 137). In addition, E1A blocks the differentiation of a number of cell lines, including rat PC12 pheochromocytoma cells and a variety of myoblast cell lines (Reviewed in (7)). The regions of E1A involved in repression of differentiation closely correlate with regions required for transformation (7-8, 138-140). In particular, the extreme N-terminal region of E1A was consistently found to be critical, either alone or in conjunction with the CR2 region. Thus the interactions of E1A with p300/CBP and pRb family members are most likely involved in antagonizing differentiation processes.

Terminal differentiation is accompanied by pRb dephosphorylation. The cell cycle machinery, which propels cells through the G1/S transition, is frozen in this state and as a consequence pRb remains dephosphorylated (45). In addition, the amount of pRb protein, as well as that of p130, is greatly increased during terminal differentiation (Reviewed in (133)). The ability of mutant E1A proteins to bind pRb and/or its relatives p107 and p130 strongly correlates with their capacity to trigger DNA synthesis in postmitotic cells (136). E1A activates DNA synthesis by binding pRb family members, thus releasing transcription factors of the E2F family (45). This, in turn, would result in the transcriptional upregulation of host genes that are regulators of DNA synthesis. However, release of E2F is not sufficient for the reversal of differentiation. Although E2F is needed to trigger DNA synthesis in myotubes, as dominant negative DP-1 mutants inhibit E1A-mediated cell cycle reentry, E2F cannot replace E1A in inducing S phase, since overexpression of E2F could not reactivate those cells (141).

p300/CBP plays a critical role in mediating cell cycle arrest during terminal differentiation. Mutants of E1A, which were able to bind p300 but not pRb, were still able to block cellular differentiation in different cellular systems (133). Furthermore, it has been shown in several experimental systems that the expression of p21 (a Cdk inhibitor required for exit from the cell cycle), as well as terminal differentiation, are blocked if cells are transfected with an E1A mutant that retains the ability to bind to p300, but is not blocked by mutants that bind exclusively to pRb and not p300 (102, 142-144). MyoD is an example of an inhibitor of the cell cycle that binds p300/CBP and has been shown to be targeted by E1A. The interaction of MyoD with p300/CBP has been shown to be essential for cell cycle arrest and muscle-specific gene expression (105). By binding to p300/CBP, E1A inhibits these processes (145-146).

It has also been shown that a region within E1A (amino acid 40-61), which binds neither p300/CBP nor pRb, is necessary and sufficient for the repression of muscle-specific enhancers (147). In particular, a six amino acid motif (amino acids 55-60) is necessary for this effect. Apparently, mutagenesis of E1A residues 55-60 partially disrupts the binding to PCAF (a second PCAF binding site exists in E1A CR1) and thus partially disrupts the myogenesis blockade by E1A (108).

It appears, therefore, that E1A functions required for inducing non-proliferating cells to enter the cell cycle are also responsible for reversal of the differentiation state.

4. TARGETING THE p53 PATHWAY BY SEVERAL ADENOVIRUS PROTEINS

As discussed above, expression of E1A in quiescent cells can efficiently induce cellular DNA synthesis and transient cell proliferation (5). However, E1A expression is not sufficient to induce long-term growth of primary cells. This is because deregulation of the cell cycle activates p53, which triggers a cellular defense mechanism to implement growth inhibition and apoptosis. Thus, whereas E1A expression leads mostly to p53 accumulation (section 4.2), E1B-55kDa and E4orf6 employ various mechanisms to inactivate p53 (section 4.3.1), and E1B-19kDa antagonizes apoptotic processes initiated by p53. The multitude of mechanisms utilized by adenovirus proteins (as well as by several other viruses) to inactivate the p53 pathway (figure 2) underscores the importance of inactivating this pathway for virus replication in the host organism. Although the importance of p53 inactivation for adenovirus replication has lately become the subject of a heated debate (section 6), it is difficult to accept the notion that adenovirus invests so much effort to neutralize p53 without it having important consequences to virus replication. Possible explanations will be discussed in section 6.

4.1 The p53 tumor suppressor

The p53 protein was first identified more than twenty years ago as a binding partner of the SV40 T antigen (148-149). It was later found to associate with other viral proteins, such as the adenovirus E1B-55kDa protein (150), the adenovirus E4orf6 protein (151), the

human papillomavirus E6 protein (152), and others. The targeting of p53 by several tumor viruses drew attention to this protein, and its role in cell growth and tumorigenesis has been studied extensively.

The p53 gene encodes a tumor suppressor that is the center of cellular stress response pathways functioning to prevent the growth and survival of potentially malignant cells. The p53 protein is defective in most human cancers. In about half of these tumors, p53 is inactivated directly as a result of mutations in the p53 gene (153). In many others it is inactivated indirectly as a result of alterations in genes that belong to the p53 pathway, or through binding to viral proteins (154). Activation of p53 by cellular stress can induce several responses in the cells, including cell cycle arrest, apoptosis, DNA repair, inhibition of angiogenesis, differentiation, or senescence (reviewed in (155)).

4.1.1 Transcription modulation by p53

p53 is a sequence-specific transcription factor that can mediate many of its downstream effects by the activation or repression of a large number of target genes. Virtually all naturally occurring mutations in the p53 gene reduce the ability of the p53 protein to activate transcription (153). Furthermore, substitution of a gene encoding a transcriptionally inactive mutant p53 for the wild-type gene leads to loss of growth inhibitory activity in mice (156-157). These findings indicate the importance of the transcriptional activity of p53 to its function as a tumor suppressor. However, some activities of p53 have been reported that are entirely independent of transcriptional regulation (158-160). Whereas p53 activates transcription of several genes by binding to DNA in a sequence-specific manner, p53 mediates transcriptional repression of other genes independently of p53 DNA response elements. To mediate repression, p53 utilizes at least two mechanisms, association with histone deacetylases (161) or binding to the basal transcriptional machinery and interfering with transcriptional initiation (162-163). Genes that are upregulated by p53 and control cell cycle progression include p21/WAF1, 14-3-3 sigma, Gadd45, B99 and MCG10. Cell cycle regulatory genes whose expression is repressed by p53 include Wee1 (164), topoisomerase II alpha (165), cyclin B1 and Cdc2 (166-167).

4.1.2 p53 activation

The p53 pathway is usually inactive in normal cells, and is activated when cells are stressed. At least three independent pathways lead to activation of the p53 network. These pathways are triggered by DNA damage, aberrant growth signals (such as those resulting from expression of oncogenes), and chemotherapeutic drugs (154). All three pathways inhibit the degradation of the p53 protein, and cause its stabilization at high levels. The MDM2 protein, itself a transcriptional target of p53, is an E3 ubiquitin ligase that targets p53 for degradation by the proteasome (168-169), thus creating a negative feedback loop in which activation of p53 results in degradation of its protein product. MDM2 function is inhibited through various means, depending on the stress-induced pathway (170). One inhibitor of MDM2 is p14/ARF, which binds MDM2 (171) and neutralizes its activity by various means (171-173). Increased levels of the p53 protein are not sufficient for its activation. Other mechanisms include subcellular localization and protein modifications. p53 function depends on nuclear localization, and both nuclear import and export are tightly regulated (174). The ability of p53 to function as a transcription factor can be modulated by various post-translational modifications, including phosphorylation, acetylation, glycosylation, ribosylation, and sumoylation (154).

4.1.3 p53 regulation of the cell cycle

One of the first effects of p53 expression, in almost all cell types, is to block the cell cycle. p53 transcriptionally upregulates p21/WAF1, an inhibitor of the cyclin-dependent kinases (Cdk) (175). p21 binds and inactivates cyclin-Cdk complexes that mediate G1 progression and timely entry of cells into S phase. As a result, pRb is hypophosphorylated, E2F is sequestered by pRb, and the cell cycle is arrested at the G1/S border. Through its negative effect on other members of the Cdk family, p21 also contributes to inhibition of the transition from G2 to mitosis (175-176). p21 also associates with the proliferating cell nuclear antigen (PCNA) and prevents PCNA from mediating recognition of the DNA primer-template complex, leading to inhibition of the elongation step in DNA replication (177-178). Thus, p21 mediates interference of p53 in S phase progression as well.

Whereas p21 is the principal mediator of p53-induced cell cycle arrest at the G1/S border, several different gene targets of p53 are involved in regulation of the G2 to M transition (reviewed in (179)). In addition to p53-enhanced expression of p21, and the resulting inhibition of cyclin B1-Cdc2 activity (175), p53 also represses the transcription of cyclin B1 and Cdc2 (166, 167). Constitutive activation of the cyclin B1-Cdc2 complex overcomes p53-mediated G2 arrest. Moreover, in epithelial cells, p53 stimulates the expression of protein 14-3-3 sigma, which sequesters cyclin B1-Cdk1 complexes outside the nucleus, thus helping to maintain a G2 block (180). Another transcriptional target of p53 implicated in regulating the G2/M transition is Gadd45. Enhanced expression of Gadd45 in primary fibroblasts leads to G2 arrest. This effect is p53-dependent since overexpression of Gadd45 in p53-deficient fibroblasts fails to mediate G2 arrest (181). The effect of Gadd45 on G2/M transition may be due to its ability to dissociate Cyclin B1-Cdc2 complexes (179). Reprimo, a glycosylated, cytoplasmic protein, is also induced by p53, and overexpression of this protein leads to arrest of the cells in G2 (182). Two other genes induced by p53, and which may contribute to p53-mediated G2/M arrest are B99 and MCG10 (179). Cyclin G, which is induced by p53, may play a role in G2/M arrest or in induction of apoptosis (183-185).

4.2. E1A and p53

The p53 protein appears to be a target for several adenovirus proteins (figure 2), and some of these proteins affect p53 in more than one way. Ad E1A proteins have been shown to induce elevated p53 levels and to enhance p53-dependent apoptosis (186-187). p53-deficient primary fibroblasts expressing E1A are resistant to apoptosis and

become oncogenically transformed (188). Both the pRb- and p300/CBP-binding domains of E1A are required to promote p53 accumulation and apoptosis in primary cells (189-190). However, inactivation of pRb and not p107 or p130 is involved in these events (190). Other oncogenes, such as Myc or Ras, can also activate p53. The ability of E1A to induce p53 and its transcriptional targets is severely reduced in p14/ARF-null cells, which remain resistant to apoptosis following serum depletion or adriamycin signals. Thus p14/ARF mediates p53 activation induced by E1A proteins (191). As discussed above, E1A releases free E2F by binding pRb, thus activating E2F-dependent gene expression (section 3.2). E2F-1 transactivates p14/ARF (192), and p14/ARF protein binds MDM2 (171) and neutralizes its activity (171-173). Disruption of MDM2 function by p14/ARF allows p53 levels to rise, leading to induction of cell cycle arrest and apoptosis. In addition to E1A-pRb, the E1A-p300 interaction also affects p53 stability. Inhibition of p300 by E1A can interfere with either direct effects of p300-MDM2 complexes on p53 stability (193), or p53-dependent transactivation of MDM2 (194). Furthermore, it has been shown that p53 acetylation *in vivo* is mediated by p300/CBP. MDM2 actively suppresses p53 acetylation and this activity is abrogated by p14/ARF. Functionally, inhibition of deacetylation has been reported to promote p53 stability, suggesting that acetylation contributes to p53 accumulation (195). Since E1A was shown to modulate p300/CBP HAT activity both positively and negatively, depending on circumstances (see section 3.4.1), it is possible that E1A also modulates p53 acetylation by p300/CBP, and this could have an impact on p53 stabilization by E1A. In addition, it has recently been reported that E1A can bind Sug1, a regulatory component of the proteasome, (196) via E1A's N-terminus. This interaction may also play some role in stabilization of E1A target proteins, including p53 (197).

E1A may affect not only p53 stability but also its transcription. Recent studies in mice have shown that E1A can activate transcription of p53 by inducing the binding of the cellular transcription factors ETF and E2F to the p53 promoter. ETF binding is essential for activation of the p53 promoter while E2F binding is not (198).

In contrast with the positive effect E1A has on the accumulation of p53, E1A has been reported to have a negative effect on p53 function. E1A represses stimulation of transcription by p53 through a mechanism that does not affect p53 binding to DNA or phosphorylation of the p53 transactivation domain (199). Furthermore, some E1A-expressing cells have lost the ability to arrest in G1 after DNA damage, suggesting that E1A can inhibit the biological functions of p53. The inhibition of p53-mediated transactivation has been mapped to the p300/CBP binding domain of E1A (200). Mutant analysis revealed that p53 stabilization and inhibition of p53 transactivation were two independent functions of the p300/CBP binding region of E1A (200). Inhibition of p53-dependent p21 expression by E1A correlated with suppression of cell cycle arrest following DNA damage. The 13S E1A product can also antagonize the repression function of p53 by dissociating the complex formed between the carboxy-terminal domain of p53 and the TATA-binding protein (201). Thus, E1A counteracts, at least in part, its own activity as inducer of p53 accumulation, by inhibiting p53 function.

4.3 E1B proteins

The E1B transcription unit encodes two proteins, E1B-55kDa and E1B-19kDa, both of which can block p53-induced growth inhibition and apoptosis. The E1B-19kDa protein is homologous in sequence and function to the Bcl-2 family of apoptosis regulators, and the mechanisms by which it blocks both p53-dependent and p53-independent apoptosis are reviewed elsewhere (15-16). E1B-19kDa may also affect growth inhibition by p53, by alleviating p53-mediated repression of genes, while not blocking p53-mediated transactivation (202-203).

The E1B proteins cooperate with E1A to oncogenically transform cultured cells. The contribution of E1B proteins to cellular transformation is due, at least in part, to antagonizing apoptosis and growth arrest, which result from the stabilization of the cellular tumor suppressor protein p53 by E1A (186-187). Oncogenic transformation reflects the ability of these proteins to interfere with the normal function of cell cycle regulators and tumor suppressor genes.

4.3.1 E1B-55kDa protein

The E1B-55kDa protein plays an important role during human Ad5 productive infection in certain cell types, although it has been reported to be dispensable for virus replication in others (204-205). In the early phase of viral infection, E1B-55kDa counteracts E1A functions that would otherwise lead to the accumulation of p53 and the induction of cell cycle arrest and apoptosis, thus allowing efficient replication of the virus. During the late phase of infection, E1B-55kDa is required for efficient nucleocytoplasmic transport and translation of late viral mRNAs, as well as for the shutoff of host mRNA nuclear export and of host protein synthesis (reviewed in (2, 206)). Here we will focus on E1B functions involved in p53-dependent and -independent modulation of the cell cycle.

4.3.1.1 Transcriptional repression of p53-regulated genes by E1B-55kDa

The Ad5 E1B-55kDa protein binds to p53 in infected cells (150) and blocks its transcriptional activity. Analysis of mutant E1B-55kDa proteins has revealed a strong correlation between their ability to inhibit p53-mediated transcriptional activation and their ability to cooperate with the adenovirus E1A protein in the oncogenic transformation of primary cells (207). Since transcriptional activation by p53 accounts for most of its functions (section 4.1.1), including induction of cell cycle arrest, blocking p53 function by E1B contributes to the ability of the cell to transit from G1 to S phase, despite p53 stabilization. Expression of E1B-55kDa protein alone is not sufficient to stimulate quiescent cells to enter S phase, consistent with the finding that deletion of both p53 alleles does not directly lead to loss of regulated cell division (208). However, the finding that p53 is targeted by several viral oncoproteins suggests that the neutralization of p53 is important for the viral life cycle. Presumably, the E1B-

55kDa protein collaborates with E1A to more effectively activate quiescent cells. Since E1A expression leads to stabilization of p53, the E1B proteins must allow cell cycle progression of the activated cells by preventing a cell cycle block induced by p53, as well as preventing p53-induced apoptosis.

The E1B-55kDa protein binds to the amino-terminal transactivation domain of p53 (209). Mutation of p53 at either the proline at position 24, or the tryptophane at position 27, reduces the affinity of p53 for E1B-55kDa, although its transactivation function remains wild type (210). Thus, it is possible that the viral protein simply masks the p53 activation domain. However, interaction between E1B-55kDa and p53 was found to be necessary but not sufficient for the repression and transformation functions of E1B-55kDa, since an E1B-55kDa insertion mutant at position 443 bound to p53 with wild-type affinity but was defective for repression of p53 transcriptional activation and for transformation (207, 211). These studies have suggested that E1B-55kDa actively represses p53 transcription, and does not simply prevent p53 from binding DNA or sterically blocking its activation function. The mutation analysis of E1B-55kDa indicates that, whereas the p53-binding domain of E1B-55kDa lies in the center of the viral protein between residues 216 and 354 (209, 212), the carboxyl terminus of this protein is important for its repression functions (207, 211). This is supported by the report that phosphorylation of serine residues 490, 491 and threonine 495 within the carboxyl terminus of E1B-55kDa is required for repression of transcription, since substitution of these residues to alanines abolished repression activity of the viral protein (213-214).

The suggestion that E1B-55kDa has a general transcriptional repression activity gained support from transient transfection experiments in which Gal4-E1B-55kDa fusion protein was shown to repress expression from several target promoters containing Gal4 binding sites. These promoters did not contain common activator binding sites, suggesting that E1B-55kDa does not inhibit a specific activation mechanism, but rather inhibits a general process required for transcription. The inhibition was observed in cells lacking p53, suggesting that the repression function was independent of p53 (211). It appears, therefore, that E1B-55kDa acts as a general repressor of transcription, and that the repression is targeted to p53-activated promoters by binding of the viral protein to p53. Indeed, *in vitro* experiments demonstrated that E1B-55kDa bound directly to purified p53, causing a 10-fold increase in p53 affinity for tandem p53 binding sites, and specifically repressed p53 activation (215). Thus, E1B-55kDa protein converts p53 from a stress-regulated transcriptional activator into a strong constitutive repressor of p53-targeted genes. Using a highly purified *in vitro* transcription system, Berk and coworkers further analyzed p53 repression by E1B-55kDa (216). Since repression was observed in reactions containing purified general transcription factors, it was concluded that E1B-55kDa could operate through a mechanism that did not involve histone deacetylation. They could show that E1B-55kDa repression required tethering of the viral protein to the promoter, through its interaction with p53, since a point mutation in p53 that reduced the affinity to E1B-55kDa caused a markedly reduced repression. Furthermore, E1B-55kDa repressed transcription under conditions that did not allow p53 activation due to lack of TFIIA or substitution of TBP for TFIID. Thus, E1B-55kDa appeared to inhibit basal transcription, rather than block p53-specific activation. However, transcription could not be inhibited in reactions containing only purified general transcription factors, but required a corepressor that copurified with RNA polymerase II. The identity of the corepressor has not yet been published.

Although the large E1B proteins of Ad2/5 and the highly oncogenic Ad12 share the ability to neutralize p53, there are a number of differences between them. The Ad2/5 proteins bind p53 strongly, and the complex is localized to cytoplasmic dense bodies in E1-transformed cells (150, 217-219). The Ad12 large E1B protein interacts weakly with p53, and p53 and much of the E1B protein are found in the nucleus of Ad12-transformed cells. This is probably due in part to a nuclear localization signal found in Ad12, but not in Ad2/5 large E1B protein (212, 219). Furthermore, the large E1B protein from Ad5 but not Ad12 contains a nuclear export signal (NES) of the HIV-1 Rev-type, and continuously shuttles between the nucleus and the cytoplasm (220). The presence of a NES in Ad5 E1B-55kDa might further explain the differences observed in the subcellular localization of Ad5 and Ad12. Despite these differences, the binding sites for p53 on both types of large E1B proteins have been mapped to homologous sites in the center of the molecules (212). It has been reported that the large E1B protein of Ad12 is not only an inhibitor of transcription activation by p53, but can also interfere with p53-mediated repression of transcription through CAAT-TATA sequences (221). Expression of the Ad12 large E1B protein has been shown to result in the loss of G1 cell-cycle arrest after X-ray irradiation, presumably due to p53 neutralization (221). Disruption of the E1B-p53 complexes, using peptides identical to the p53 binding sites on both Ad2/5 and Ad12 large E1B proteins, resulted in release of transcriptionally active p53, as well as a reduction in cell growth and DNA synthesis due to cell cycle arrest, induced by p53 (222).

4.3.1.2 Acetylation of p53 as a target of E1B-55kDa

p53 can be acetylated by the p300 histone acetyltransferase on several lysine residues at its extreme carboxyl terminus (223), as well as by another HAT, PCAF, which acetylates p53 at a different location (224-225). Several groups showed that p53 can be acetylated *in vivo* in response to a variety of cellular stress signals (reviewed in (226)). The exact functional consequences of p53 acetylation are still controversial (226), and may include effects on DNA binding, coactivator recruitment, influencing transcriptional activity, stability and cellular localization. It is also possible that p53 acetylation might be a mechanism to recruit deacetylases to downregulate transcriptional activation by this protein, once p53 is no longer needed and the cell should overcome p53-mediated cell cycle arrest. It has been reported that p53 can associate with mSin3A, a corepressor reported to be involved in

transcriptional repression of several genes. This interaction is required for p53 to recruit HDAC1 and to repress at least two of its targets (161). It has later been shown that p53 can associate with additional histone deacetylases (226). It is not yet fully understood how the interactions with HDACs silence p53-activated expression, although it is likely to result, at least in part, from deacetylation of nucleosomes near the target promoter.

Akusjärvi and his colleagues have reported that the Ad2 E1B-55kDa protein forms an enzymatically-active complex with HDAC1 and the transcriptional corepressor protein mSin3A, both in Ad-infected and -transformed cells (227). However, although it is tempting to speculate that this complex contributes to repression of p53 as a transcriptional activator, they have not been able to demonstrate that overexpression of HDAC1 had an effect on E1B-55kDa repression of p53, or that E1B repression of basal transcription was affected by the HDAC inhibitor trichostatin. Liao and his coworkers found that E1B-55kDa protein specifically inhibited p53 acetylation by PCAF *in vivo* and *in vitro*, while acetylation of histones and PCAF autoacetylation were not affected (228). Moreover, the DNA binding activity of p53 was reduced in cells expressing E1B-55kDa. PCAF bound to E1B-55kDa and to a region near the COOH terminus of p53, surrounding Lys-320, the specific PCAF acetylation site. E1B-55kDa was shown to interfere with the physical interaction between PCAF and p53, suggesting that E1B-55kDa inhibits PCAF acetylation of p53 by preventing enzyme-substrate interaction. Liao and his colleagues propose that inhibition of p53 acetylation by E1B-55kDa complements the mechanism of direct targeting of DNA-bound p53 by the viral protein to achieve a more complete inhibition of p53. However, they did not directly show that interfering with the ability of E1B-55kDa to interact with PCAF (but not with p53) affects the ability of the viral protein to repress p53. Thus, the contribution of the interaction of E1B-55kDa with HATs and HDACs to repression of p53 transcriptional activation should be further investigated.

4.3.1.3 Regulation of p53 protein stability by the adenovirus E1B-55kDa and E4orf6 proteins

As described above, expression of the adenovirus E1A protein alone induces stabilization and accumulation of p53, however, accumulation of p53 is not usually observed upon infection of cells with wild-type adenovirus. Several studies have shown that the E1B-55kDa and E4orf6 proteins cooperate to counteract E1A-induced stabilization of p53 through accelerated p53 degradation (229-240). It appears that p53 degradation requires interactions of E1B-55kDa with both p53 and E4orf6, but occurs independently of MDM2 and p14/ARF, regulators of p53 stability in mammalian cells (235-236, 241). A protein fragment containing the amino-terminal 58 residues of the E4orf6 protein binds both E1B-55kDa and p53 (151, 242). A region toward the carboxyl terminus of p53 is involved in E4orf6 binding (151), whereas the E1B-55kDa-interacting domain is located at the amino-terminus of the tumor suppressor (209). The p53-binding domain of E1B-55kDa lies in the center of the viral protein (209, 212) and various domains in the E1B-55kDa protein contribute to stable association with E4orf6 (242). The sum of these interactions generates a ternary complex containing adenovirus E1B-55kDa and E4orf6 proteins and p53.

Several amino- and carboxyl-terminal domains on the E4orf6 protein are involved in p53 degradation *in vivo* (232, 235, 238-239, 241, 243), including the putative zinc-coordinating cysteine and histidine residues located at the amino-terminal p53-binding region. This sequence may share similarities with RING finger motifs, mediating protein interactions between ubiquitin conjugating enzymes (E2) and ubiquitin ligase complexes (E3), which target proteins for degradation by the proteasome (244). Another E4orf6 region involved in p53 degradation is the arginine-faced amphipathic alpha-helix located at the carboxyl terminus of the E4 protein (241, 245-246). This region appears to be required for many of the functions of the E4orf6 protein that contribute to efficient virus replication and is believed to be responsible for targeting E4orf6 and E4orf6-E1B-55kDa complexes to the nucleus (245-247). Identification of E4orf6-associating cellular proteins participating in a multiprotein complex has suggested a mechanism underlying adenovirus-mediated p53 degradation. The E4orf6-containing complex includes a novel Cullin-containing E3 ubiquitin ligase composed of Cullin family member Cul5, Elongins B and C, and the RING-H2 finger protein Rbx1(ROC1) (248). This complex is similar to the von Hippel-Lindau (VHL) tumor suppressor and SCF (Skp1-Cul1/Cdc53-F-box) E3 ubiquitin ligase complexes, and is capable of stimulating ubiquitination of p53 *in vitro* in the presence of E1/E2 ubiquitin-activating and -conjugating enzymes. Further studies are required to elucidate other levels of control that may regulate this complex.

4.3.1.4 Adenovirus proteins affect cellular localization of p53

When expressed alone, or in Ad5-transformed cells, E1B-55kDa can be detected in perinuclear, cytoplasmic bodies where it is associated with p53 (218-219). The presence of E1B-55kDa in the cytoplasm most likely reflects its continuous shuttling (220), and its lack of nuclear retention signal (212, 219). Physical sequestration of p53 outside the nucleus in Ad5-transformed cells could contribute to its inactivation. The tumor suppressor WT1 is also found in these cytoplasmic complexes, and Ad5 E1B was shown to abrogate WT1-mediated apoptosis (249). However, upon coexpression of the adenovirus E4orf6 gene product, an E1B-E4orf6 complex is formed (242). Subsequently, the equilibrium between nuclear export and import of the E1B-55kDa protein is changed and the E1B-55kDa-E4orf6 complex accumulates in the nucleus, where it is distributed evenly (247). During lytic infection, both proteins localize to the periphery of virus replication centers in the nucleus (250). E4orf6-mediated nuclear accumulation of E1B-55kDa requires an additional primate cell-specific factor (247). In addition, sumoylation of the E1B-55kDa protein has been reported to drive its nuclear accumulation, and a mutant that cannot be sumoylated is defective in nuclear transport and in inhibition of p53-mediated transcriptional activation (251). It is currently unknown whether E4orf6 modulates the sumoylation of E1B-55kDa.

The adenovirus protein E4orf3 also physically associates with E1B-55kDa (252, 253), and redirects it to nuclear tracks (254). p53 is then released from its association with E1B-55kDa, resulting in p53-mediated transcriptional activity. However, as described above (section 4.3.1.3), E4orf6 cooperates with E1B-55kDa to inactivate and degrade p53, even in the presence of E4orf3. During adenovirus infection, E4orf3 is expressed considerably earlier than E4orf6, suggesting that E4orf3 acts transiently on E1B-55kDa, allowing a passing activation of p53, before it is inactivated by E4orf6 and E1B-55kDa (254-255). Why a transient activation of p53 may be beneficial during adenovirus infection remains an open question.

4.3.1.5 E1B-55kDa protein does not target the p53 family members p63 and p73

p73 is a cellular protein that shows a significant homology to p53 (256). The homology between p53 and p73 is particularly extensive in the DNA binding domain and includes all residues known to form contact sites between p53 and DNA. When overexpressed, p73 can activate p53-responsive promoters, and can block cell proliferation and induce apoptosis in a manner analogous to p53 (256-257). The similarities between p53 and p73 raised the question whether viral oncoproteins were capable of inactivating p73, as they did p53. However, four different groups could not detect a physical interaction between Ad E1B-55kDa protein and p73 (236, 258-260). The difference in binding between p53 and p73 was traced to five amino acids near the amino termini of these proteins. When these residues, originating from the p53 protein, were transferred to p73, they conferred on this protein the ability to bind E1B-55kDa (236). The large E1B proteins of both Ad2/5 and Ad12 also have no effect on transcriptional activation by p73, and several studies have shown that E1B-55kDa and E4orf6 selectively target p53 for degradation, but not the related p63 and p73 proteins (236, 240, 260). Other viral oncoproteins, such as SV40 T antigen and the human papillomavirus E6 protein also target p53 but not p73 (259, 261), suggesting that inactivation of p53 is crucial for viral infection, whereas p73 can be tolerated. Similarly, p63/p51/KET, another p53 family member, is also not targeted by the viral oncoproteins tested (240, 262).

4.3.1.6 p53-independent interactions of E1B-55kDa with the cell cycle

By antagonizing p53-mediated cell cycle arrest and apoptosis, E1B-55kDa provides suitable conditions for virus replication and facilitates cellular transformation. However, recent reports by Goodrum and Ornelles (263-264) have demonstrated that E1B-55kDa relieves growth constraints imposed on viral replication by the cell cycle using additional mechanisms that are independent of p53. They showed that, in randomly cycling cells, an E1B-55kDa mutant virus was restricted for growth and produced progeny virus in only a fraction of infected cells. By contrast, the wild-type virus produced progeny in almost every infected cell. They further analyzed virus growth in synchronized populations of cells and showed that growth of the mutant virus was severely restricted in cells infected during G1, whereas this restriction was partially relieved in cells infected early in S phase. The E4orf6 and E4orf3 gene products of adenovirus also contributed to cell-cycle-independent adenovirus replication. However, replication of the E1B-55kDa mutant viruses was not related to the status of p53 in the cells (263, 265), suggesting that a p53-independent mechanism contributes to cell cycle-independent adenovirus replication. Thus, E1B-55kDa appears to deregulate the cell cycle by different mechanisms, all of which contribute to the lytic infection. In contrast to these findings, Gallimore and coworkers reported that viral replication occurred more efficiently in cycling cells, compared to quiescent cells, irrespective of whether they express E1B-55kDa (266). Thus, additional mechanisms to deregulate the cell cycle may be employed by adenovirus, depending on the cellular microenvironment. This by no means reduces the importance of p53 inactivation for adenovirus replication, at least in some cellular systems, as demonstrated by Harada and Berk (267). They have shown that in H1299, a p53-deficient cell line that contained a temperature-sensitive allele of wild-type p53, there was only a modest difference between the abilities of wild-type and E1B-55kDa mutant (*dl*1520) viruses to replicate at the non-permissive temperature. However, when a high level of p53 function was restored for 24 hrs prior to infection by shifting the cells to the permissive temperature, a substantial additional defect in the *dl*1520 yield was observed. Under these conditions, virtually no *dl*1520 replication occurred, whereas the presence of E1B-55kDa allowed replication to an extent similar to that for wild-type Ad5 in the parental H1299 cells. In contrast, Goodrum and Ornelles reported only a mild effect of p53 on replication of the same mutant virus (265). These results suggest that, under some cellular circumstances, E1B-55kDa is required to eliminate p53, whereas in other situations p53's presence may be tolerated.

5. INTERACTIONS OF ADENOVIRUS E4 PROTEINS WITH CELL CYCLE REGULATORY PATHWAYS

The E4 region includes seven open reading frames (orfs), some of which encode proteins that interact with cell cycle regulators, and contribute to cellular transformation by the virus. The role of adenovirus E4 proteins in virus replication and oncogenesis has recently been thoroughly reviewed (6, 268), and the interactions of E4 proteins with cell cycle regulators will be briefly mentioned here.

5.1 E4orf6

As described above (section 4.3.1.3), E4orf6 together with E1B-55kDa causes degradation of p53. However, a controversy exists in the literature whether E4orf6 also blocks p53- and p73-mediated transcriptional activation (reviewed in (268)). In addition, one group reports that E4orf6 moderately inhibits transcriptional activation by p63/p51 (240). E4orf6 also alleviates transrepression mediated by the carboxyl-terminus of p53 (151, 232), which correlates with the ability of the viral protein to enhance focus formation in primary cells in cooperation with the E1 genes. Besides its effect on p53,

overexpression of E4orf6 was also reported to result in accumulation of 293 cells in S phase. This S phase arrest was accompanied by degradation of cyclin A and inhibition of Cdc2 activity, caused by enhanced inhibitory phosphorylation on tyrosine (243). This effect of E4orf6 presents an additional mechanism to ensure that the infected cell provides the virus with the best conditions, i.e. S phase conditions, for its replication.

5.2 E4orf3

E4orf3 also cooperates with E1 in cellular transformation, however this effect is probably not linked to modulation of p53 function. The oncogenic properties of E4orf3 may involve its binding to E1B-55kDa and the reorganization of nuclear bodies, also called PODs (PML oncogenic domains) (253, 268). Reorganization of PODs may be involved in the cell cycle independent adenovirus replication mentioned in section 4.3.1.6 (264).

5.3 E4orf4

When expressed alone, the adenovirus E4orf4 protein induces p53-independent apoptosis in transformed cells, and this effect requires an interaction between E4orf4 and an active protein phosphatase 2A (PP2A) complex containing a B alpha/B55 subunit (269-272). E4orf4 also induces irreversible growth arrest in S. cerevisiae, by a mechanism requiring the yeast homologue of B alpha/B55 (CDC55) (273-275). The analysis in yeast suggests that E4orf4 targets PP2A to the anaphase-promoting complex/cyclosome (APC/C), leading to APC/C inactivation and arrest in mitosis (274). At the same time, E4orf4 partially counteracts its own inhibition of APC/C by enhancing Cdc28 (the Cdc2 kinase homologue in yeast) activity through a mechanism that requires MIH1, the yeast Cdc25 phosphatase homologue (274-275). However, the net effect of E4orf4 action in wild-type yeast cells is G2/M arrest. Similarly, E4orf4 can induce G2/M arrest in mammalian cells prior to induction of apoptosis (274). Other studies further indicate that unidentified E4 gene products (excluding E4orf6) cause a delay in cell cycle progression, associated with elevated levels of cyclin B (a substrate of APC/C) and partial G2 growth arrest. However, this effect is seen only in cells infected with adenovirus mutants lacking the E1 region (276-278). Thus, it is unclear to date how this interaction with the cell cycle machinery contributes to normal virus replication.

5.4 E4orf6/7

The E4orf6/7 protein is a viral transactivator that acts through an interaction with the E2F family of transcription factors, stabilizing their binding to DNA, to activate viral and cellular genes, including E2F-1 itself (reviewed in (6)). In addition, a recent study suggests that E4orf6/7 alone is sufficient to displace pRb and p107 from E2F complexes, hence activating genes controlled by E2F, even in the absence of E1A (279). Thus, E1A and E4orf6/7 complement each other in releasing E2F from repression by pRb family proteins, and enhance expression of E2F-driven genes, including those involved in cell cycle progression.

6. EXPLOITING THE INTERACTIONS BETWEEN ADENOVIRUS AND CELL CYCLE REGULATORY PATHWAYS TO GENERATE ONCOLYTIC VIRUSES FOR CANCER GENE THERAPY

As described in this review and summarized in figure 3, adenovirus E1A proteins eliminate the pRb checkpoint through direct binding to pRb and the related proteins p107 and p130. Subsequently, an active E2F transcription factor is released from a pRB-E2F complex, and is free to activate genes required for S phase entry and DNA replication. However, forced E2F activity results in p53 activation, and induction of both p53-dependent and -independent apoptosis. The adenovirus E1B and E4orf6 proteins are then required to antagonize p53 activity that may lead to induction of cell cycle arrest and apoptosis. The pRb-E2F and p53 pathways are the most frequently altered regulatory pathways in human cancers. Deregulation of E2F occurs in almost all cancers as a result of mutations or deletions of RB1 (encoding pRb) or CDKN2A (encoding the Cdk inhibitor p16 and p14/ARF), or overexpression of the CCND 9 (encoding D-type cyclins)-CDK4 complex (50). Thus viral oncogenes inactivate regulatory checkpoints that are also defective in cancer cells. Presumably, inactivation of these targets is beneficial for virus replication, and it has been suggested that mutant viruses unable to inactivate such targets are incapable of replicating in normal cells, where the checkpoints can be activated. However, they will be able to replicate in cancer cells with inactive checkpoints. Based on this principle, such mutant viruses, known as oncolytic viruses, will selectively replicate in and kill cancer cells, but will not harm normal cells, where the checkpoints are intact.

The most extensively studied oncolytic adenovirus to date is *dl*1520/ONYX-015. This virus, created by Berk and coworkers, contains a complete deletion of E1B-55kDa (280). Originally, based on results obtained from a few cell lines, it was suggested that neutralization of p53 by E1B-55kDa was required for virus replication and thus the E1B-55kDa mutant could replicate in and destroy tumor cells lacking p53, but not normal cells (281). After preliminary work in cell culture and in animals, the mutant virus was injected into tumors, and preliminary trials in patients revealed moderate success, greatly enhanced by the application of the mutant virus together with chemotherapy (282). However, the theoretical basis for this approach has been challenged by several groups who discovered that replication of the mutant virus does not always correlate with p53 status of infected cells (reviewed in (283)). In some cases, the mutant virus could not replicate well in p53-deficient tumors. This could be due to the fact that E1B-55kDa has functions other than p53 neutralization, such as its functions in late phase RNA transport and protein translation (206, 267). To solve this, more specific E1B-55kDa mutants should be investigated, in which only the p53 neutralization function is inactivated, but not the other E1B-55kDa functions (229). In other cases, *dl*1520/ONYX-015 could replicate well in cells containing wild-type p53. Further experiments showed that in many of these cells, upstream modulators of the p53

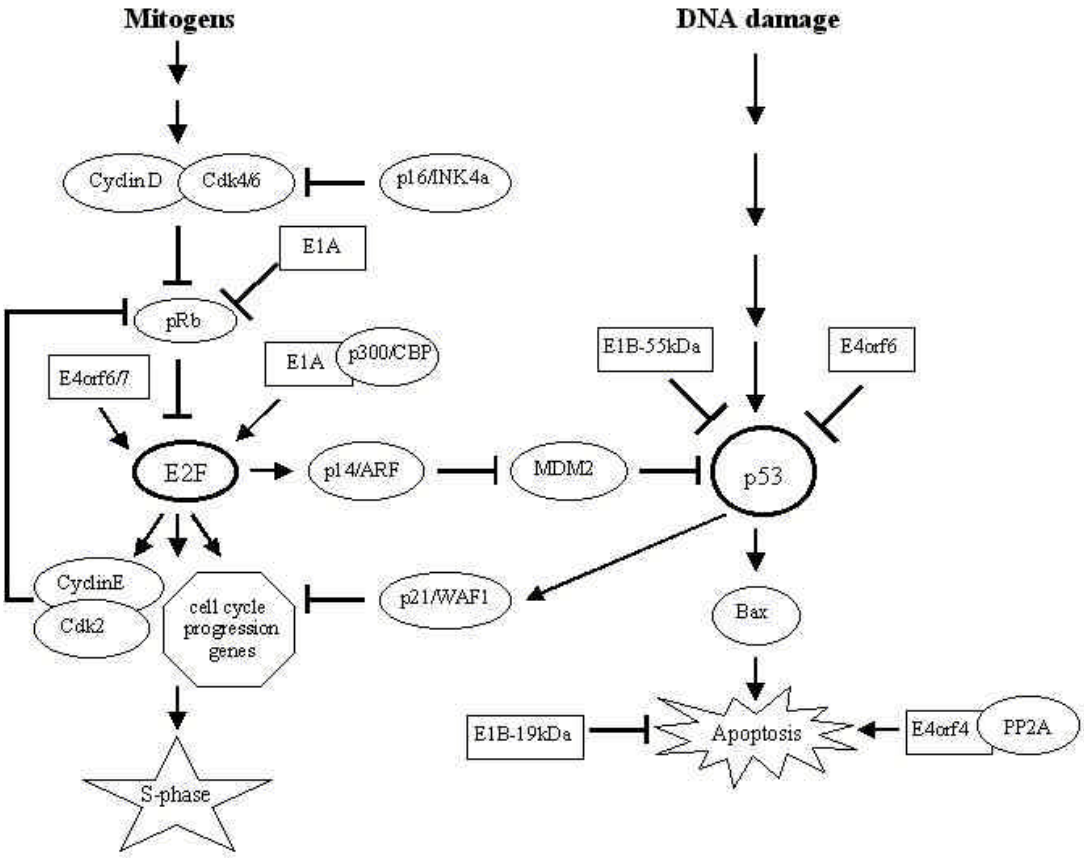

Figure 3. Interaction points between adenovirus proteins and major cell cycle regulatory pathways. Adenovirus proteins modulate the pRb and p53-regulated pathways. The E1A and E4orf6/7 proteins interact with the pRb pathway, whereas E1B-55kDa and E4orf6 affect the p53 pathway. Details are in the text.

pathway were missing. Specifically, loss of p14/ARF was shown to facilitate replication of the mutant virus in some tumor cells containing wild-type p53 (284). Yet another work utilized a p53 mutant, which was altered only in five residues that conferred on the protein resistance to inhibition and degradation by adenoviral proteins, but did not affect its ability to act as a transcriptional transactivator (285). This mutant did not inhibit viral replication in tumor cells and in primary endothelial cells. The authors concluded that active p53 did not inhibit adenovirus replication and could not serve as a basis for preferential replication of adenovirus in p53-deficient tumor cells. However, the findings that many DNA tumor viruses evolved numerous mechanisms to inactivate p53 indicate that, at least under some circumstances encountered in the host organism although not necessarily in all cultured cells, inactivation of p53 may be highly beneficial for the virus. It is also possible that p53 inactivation is redundant with other activities of adenovirus and is thus not required under some conditions. For example, p53-induced p21 activation leads to inhibition of pRb phosphorylation and cell cycle arrest. Thus p53 inactivation by E1B-55kDa and direct inactivation of pRb by E1A proteins provide redundant mechanisms converging on a checkpoint that must be inactivated by the virus to facilitate its replication. It is quite common for viruses to utilize several different mechanisms to ensure the performance of a vital function.

The interaction of the adenovirus E1A proteins with the pRb cell cycle checkpoint has been recently exploited for creating additional oncolytic adenoviruses. *dl*922-947 is a mutant E1A protein carrying a deletion of a short amino acid sequence in the CR2 region of E1A, required for the interaction between E1A and pRb family proteins. This virus was shown to replicate in and lyse a broad range of cancer cells with abnormalities in cell cycle checkpoints (286), but it demonstrated reduced S phase induction and replication in non-proliferating normal cells. In animals, this virus possessed a superior potency against tumors compared with other mutant adenoviruses.

A combination of both pRb- and p53-regulated pathways was exploited in a most recent example of oncolytic adenovirus (287). A protein containing the DNA-binding domain of E2F and the transrepression domain of pRb was constructed for use as an E2F antagonist. This protein is expressed under control of a p53-responsive promoter. Thus, the virus will express an E2F antagonist in normal cells containing wild-type p53, resulting in attenuation of virus replication. However, this virus will not

express the antagonist in tumor cells lacking p53, and will be able to replicate. These, and additional modifications, resulted in a vector that was attenuated in normal cells, but exhibited potent antitumor activity.

It is highly likely that several more variations on oncolytic adenoviruses, exploiting cell cycle checkpoints, will be described and utilized for cancer therapy in the future.

7. SUMMARY AND PERSPECTIVE

Adenovirus infection has long been known to induce quiescent cells to enter the S phase of the cell cycle. This phase is permissive for virus replication, presumably because host nucleotide pools and proteins involved in DNA synthesis are necessary for viral DNA replication. The E1A proteins are primarily responsible for transition of the host cell into S phase, by inactivating the pRb checkpoint and by activating or repressing transcription of several genes required for cell cycle progression. However, deregulation of the cell cycle by E1A also induces accumulation of p53. This inhibitor of cell proliferation has to be neutralized to facilitate continued proliferation of the cells. The adenovirus E1B and E4orf6 proteins employ several mechanisms to inactivate p53, thus preventing cell cycle arrest and apoptosis. Additional E4 gene products also contribute to cell cycle deregulation by the virus. Adenovirus mutants that cannot inactivate the pRb and p53 checkpoints are defective in their replication in cells with the intact checkpoints. The pRb and p53 pathways are compromised in most cancer cells. Thus, these cells could support replication of mutant adenoviruses that cannot normally replicate in cells with intact checkpoints. Understanding the interaction between adenovirus and the cell cycle allows us, therefore, to better understand the mechanisms of virus replication, and to devise viruses that can replicate only in cancer cells. Such viruses, called oncolytic viruses, are already being tested as new cancer therapeutic drugs. However, we obviously do not yet have a complete picture of the interactions between adenovirus and the cell cycle, giving rise to controversies such as the one involving the role of p53 inactivation in virus replication. Further analysis must be carried out in animals as well as in tissue culture, to solve such issues and allow the development of better oncolytic viruses.

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