2 Viral Oncoproteins as Probes for Tumor Suppressor Function

Srikumar P. Chellappan, PhD

Contents

1. INTRODUCTION

Transformation by small DNA tumor viruses requires multiple events that induce normally quiescent cells into a state of proliferation *(1)*. This is necessary because the genomes of such viruses do not carry the machinery required for the replication of its own genome nor the components necessary for the transcription of its genes *(2)*. Hence, the common strategy utilized by this group of viruses is to make use of the transcription and replication machinery used by the host cells for their own purposes. The small DNA tumor viruses such as adenovirus (Ad), simian virus 40 (SV40), and human papillomavirus (HPV), are all capable of inducing a proliferative state in quiescent host cells, by the judicious use of their transforming oncoproteins *(2–5)*.

Apparently, the viral genome is designed so that the products of their early genes are capable of inactivating the major negative regulators of mammalian cell proliferation, namely, Rb and its family members and the p53 tumor suppressor protein *(3)*. Each of the three small DNA tumor viruses have proteins that can interact with these growth-suppressive proteins, and in every situation this interaction results in an inactivation of the tumor suppressor proteins *(6–9)*. The specific interaction between such viral oncoproteins (V-ONC) and the cellular tumor suppressor proteins have been studied in great detail, and this has led to a greater understanding of the biochemical processes involved in oncogenic transformation *(10)*. An important fallout of these detailed studies is that now it is possible to utilize these interactions for diagnostic purposes, because the V-ONC act as specific probes for the functional integrity of tumor suppressor protein.

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Though the small DNA tumor viruses are not closely related evolutionarily, they all share the ability to inactivate the Rb family proteins, as well as p53 *(2,11).* In the case of Ad, the *E1A* gene product can physically interact with the functional form of Rb and its family members *(12);* at the same time, products of the *E1B* gene can bind to and inactivate the p53 protein *(13–15).* Similarly, in the case of HPVs, the E7 protein interacts with the Rb family proteins *(16),* and a separate protein, the product of the *E6* gene, binds to and inactivates p53 *(5).* The situation is slightly different in the case of SV40: Here, the large T-antigen (T-Ag) is capable of interacting with both Rb family members and the p53 protein *(4,14).* But, in all cases, the viruses carry genes that can neutralize the Rb- and p53-mediated suppression of cell proliferation.

This review is organized so that first the V-ONCs that interact with the Rb family of tumor suppressors are discussed, followed by those that bind to p53. In the last subheading, the potential use of these interactions in assessing the presence and functional status of the cellular antioncogenes is discussed.

2. V-ONCS INTERACTING WITH RB AND RB FAMILY PROTEINS

2.1. Adenovirus E1A

2.1.1. Historical Perspective

A considerable amount of work was done in the early 1980s to understand the mechanism of oncogenic transformation by the early gene products of DNA tumor viruses, especially the *E1A* gene of Ad. Two major lines of investigation were undertaken by different groups: mutational analysis of the different regions of the *E1A* gene required for transformation, and analysis of different cellular proteins associating with Ad E1A proteins. Results of these studies converged on the potential mechanisms involved in the E1A-mediated cellular transformation, and laid a solid foundation for what is known about viral oncogenesis today.

Ad E1A is a phosphoprotein expressed at the early stage of infection, and the phosphorylation of E1A has been shown to affect its function. Differential splicing gives rise predominantly to two different polypeptides of different sizes, one 243 residues long (12S E1A) and the other 289 residues long (13S E1A) *(17).* There are additional smaller forms of E1A proteins also generated by differential splicing, but the complete transformation capacity of E1A requires the regions present in the 12S E1A protein. Analysis of the E1A structure revealed three regions highly conserved among different serotypes of Ads. These conserved regions (CR) have been named CR1, CR2, and CR3 (Fig. 1). Of these, CR1 and CR2 are present on both the 12S and 13S E1A, but CR3 is present only on the 13S E1A. As can be seen from the figure, the CR3 region almost perfectly overlaps the domain spliced out in the 12S E1A.

The CR1 and CR2 regions are derived from the exon 1 of the *E1A* gene, and the CR3 region is at the junction of exon 1 and exon 2. exon 2 of the *E1A* gene constitutes the multifunctional carboxy-terminal region, and is present in both the 12S and 13S forms of the protein *(18).* This C-terminal region is involved in effecting functions like transcriptional repression, suppression of cellular transformation, and mediated susceptibility to the host cytotoxic T-lymphocyte response *(17).*

It was discovered early that the Ad *E1A* gene could modulate the gene expression of both viral and cellular genes *(2).* This was based on studies using mutant viruses, which had inactive forms of the *E1A* gene. Further studies revealed that E1A regulates

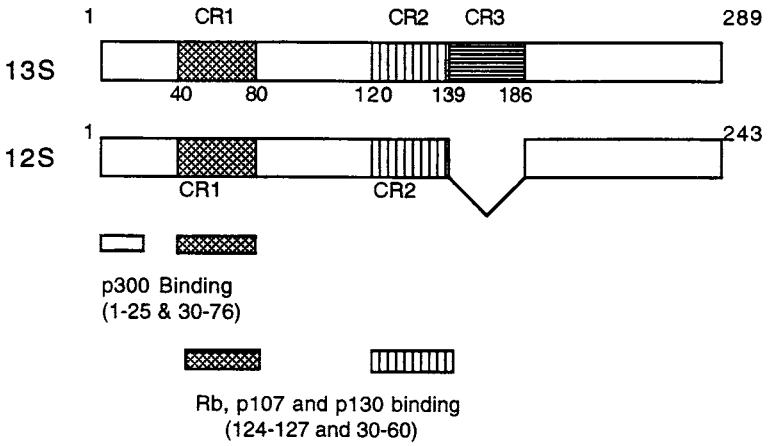

Fig. 1. Schematic of the Ad 5 E1A protein. Note that the CR3 domain present in the 13S protein is absent in the shorter 12S E1A. The conserved regions involved in binding to cellular proteins are also indicated.

gene expression, mostly at the level of transcription, and this is achieved through the mediation of many cellular transcription factors (TFs). E1A can induce, as well as repress, transcription from many cellular promoters, and these functions reside on different regions of the E1A protein. E1A does not bind to DNA directly, and all its cellular effects are mediated through targeting the cellular proteins of the host.

2.1.2. Immortalization and Transformation by E1A

It is well established that *E1A* is an oncogene, and is capable of immortalizing primary rodent cells on its own, and can effectively transform cells in cooperation with a second oncogene, such as *ras (1,2).* The transformation function of *E1A* has been studied in detail, especially the requirements of different regions necessary for functionally cooperating with other oncogenes. Such studies have revealed that sequences contained in the exon 1 of E1A are capable of cooperating with oncogenic ras to transform primary cells *(17).* But additional regions are necessary for transformation in collaboration with *E1B* gene or polyoma middle-T-Ag. For example, residues 140–193, overlapping with the CR3 region of the E1A protein, are required for cooperation with polyoma middle-T-Ag, and residues 266–276 in the C-terminal domain are required for cooperating with E1B in transformation.

Such detailed analysis of the regions of the E1A required for transformation helped identify three distinct domains within exon 1 sequences to be essential for transformation function. The CR1 region, as well as the CR2 regions, were absolutely necessary for transformation function, but were not sufficient by themselves. It soon became clear that the amino (N)-terminal residues, 2–25, which fell outside the span of CR1 sequences, were indispensable for transformation. Mutations within CR1 and CR2 significantly reduced the capacity of E1A to transform cells. Although the 12S E1A can bring about complete transformation in most primary cell lines, the CR3 region is essential for transformation of certain specific cell lines *(17).*

In addition to transforming cells, E1A was found to have certain growth-suppressive properties also *(17,19,20).* The first indication of this was obtained when it was found that

transformation of baby rat kidney cells by the 12S E1A was 100× more efficient than by the 13S E1A molecule. This raised the possibility that the CR3 domain, or other regions encoded by the exon 2, can function to suppress transformation. Further studies *(21–23)* showed that the repressive function resided within residues 237 and 283, especially in the region spanning 256–283. The molecular mechanisms by which this region mediates such suppressive properties are not yet clear, even though certain cellular proteins, such as CtBP, have been found to bind to this region. Repression of transformation by E1A exon 2 region may involve more indirect mechanisms also. For example, it is clear that E1A protein can induce the accumulation of the p53 protein, which has strong tumor-suppressive properties. E1A is thought to enhance p53 levels both by increasing the transcription of its genes, as well as by increasing the stability of the protein *(24,25).* The detailed molecular mechanisms involved in these processes are emerging now.

2.1.3. E1A Binding Proteins

The identification of different regions of E1A involved in cellular transformation facilitated detailed studies on the underlying molecular mechanisms. As mentioned earlier, one fruitful strategy was to identify cellular proteins that interact with E1A, and assess the potential effects of the interaction *(1).*

Early studies *(26)* identified a set of six cellular proteins that co-immunoprecipitated with Ad E1A from cellular extracts. They ranged in size from 300 to 33 kDa. The first protein to be positively identified as an E1A-binding protein was the Rb protein, the product of the retinoblastoma tumor suppressor gene. This finding was a milestone in the study of oncogenic mechanisms, because it provided an example of a V-ONC binding to and conceivably neutralizing a cellular tumor-suppressor protein. It further became evident *(12)* that both of the remaining Rb family members, p107 and p130 proteins, also bound to E1A equally well *(27).* E1A was found to bind to these proteins through the peptide sequence, LXCXE, which is present in the CR2 region. It was further found to be present in HPV E7 protein, as well as SV40 large T-Ag *(18,28).* As described in subheading 2.1.4., these oncoproteins, although derived from unrelated viruses, all appear to bind to the members of the Rb family. In the case of E1A, there appeared to be two distinct regions that made contacts with the Rb protein: the LXCXE motif in the CR2 region, and residues 30–60 of the CR1 region.

The 300 kDa protein that was found to associate with E1A has now been identified to be the transcriptional co-activator, p300/CBP *(29).* Many members of this co-activator family have been found to bind to E1A, and E1A can effectively block their transcriptional activity. It has also been reported that E1A can prevent the cyclin cyclin-dependent kinase (CDK)-mediated phosphorylation of p300. The p300/CBP family of transcriptional co-activators has indigenous histone acetylene transferase (HAT) activity, and they are associated with other HAT proteins, such as PCAF-1, in vivo. E1A is believed to affect these interactions, thus modulating the transcriptional activity of p300. Mutational studies of E1A protein had made it clear that the extreme N-terminal residues spanning 1–25 are essential for the transformation function of E1A *(30).* It is apparent that this region of E1A is involved in binding to p300, and that the subsequent inactivation is indispensable for E1A to transform cells.

The two proteins of 60 and 33 kDa have been identified to be cyclin A and CDK2. The CR2 region is chiefly involved in the binding to these proteins, and the CR1 region plays a secondary role in the interaction. Although it is established that E1A binds to

Table 1
Cellular Proteins Binding to E1A

Protein	*Region of E1A required for binding*
p400	**1–48**
p300	**1–25,** 30–76
Rb	**121–127,** 30–60
p107	**124–127,** 30–60
p130	**124–139,** 30–60
Cyclin A, p60	**124–127,** 30–60
P33CDK2	**124–127,** 30–60
BS69	**140–185,** 76–120
CtBP	**271–284**

cyclin A directly, it is not yet clear whether the interaction with CDK2 is direct or through cyclin A. Nevertheless, the pattern that is emerging suggests that E1A can bind to and affect the function of critical cellular proteins involved in cell cycle regulation. A list of the proteins known to bind directly to E1A, and the regions involved in the binding, is shown in Table 1.

2.1.4. Inactivation of Rb Function by E1A

Studies on the mechanisms involved in E1A-mediated transformation have highlighted the inactivation of Rb and its family proteins as an essential step in this process *(12).* Because Rb is a well-characterized tumor suppressor protein, the interaction between Rb and E1A has garnered maximum attention.

Rb is a nuclear phosphoprotein that plays a critical role in the progression of the mammalian cell cycle *(31).* Inactivation of Rb by phosphorylation is a necessary step for the transition of proliferating cells from G1 to S phase. It is well-established that it is the hypophosphorylated form of Rb that is functional in arresting cell proliferation, and inactivation of Rb in the mid-to-late G1 phase, by cyclins D and E and their associated kinases, facilitates the G1-to-S transition *(31–33).* E1A, as well as other V-ONCs, such as HPV E7 and SV40 large T-Ag, all preferentially binds to the functional hypophosphorylated form of Rb *(13).* It has now been shown that interaction with E1A, E7, or SV40 large T-Ag all bring about an inactivation of Rb that is equivalent to its phosphorylation by CDKs.

All three Rb family members have a central conserved domain, named "pocket domain" *(34).* The pocket domain imparts the growth-regulatory functions of the Rb protein *(35),* and almost all point mutations or deletions of the *Rb* gene found in human cancers map to this region *(36–38).* E1A binds to the functional pocket domain of the Rb protein. Studies in the past few years have established that interaction of E1A with Rb, or the phosphorylation of Rb, leads to its inactivation, which is equivalent to a deletion or mutation of the pocket domain of the gene.

As shown in Fig. 2, the pocket domain comprises two subdomains, named the A and B pockets. In the case of p107 and p130, these subdomains are separated by a spacer region *(39).* p107 and p130 are known to bind to cyclins A and E through the spacer region, through a sequence that is similar to the cyclin-A-binding domain of the

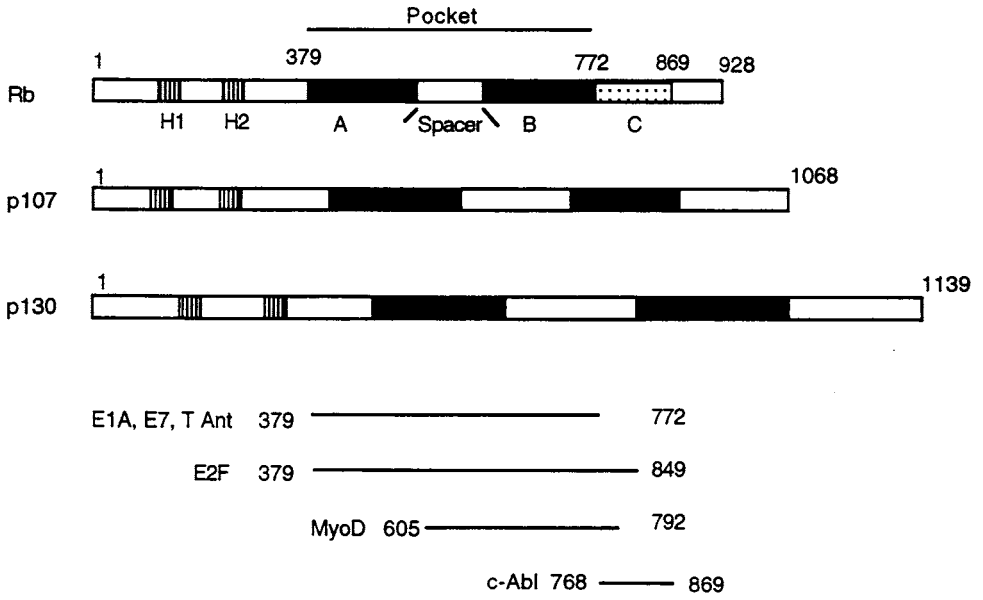

Fig. 2. Structure of the Rb family proteins. The conserved pocket domain is shown by the filled box; the H1 and H2 domains also share significant homology. The region 768–869 of Rb is involved in binding to c-Abl protein, and is referred to as the C box. The spacer region between A and B boxes are considerably larger and functionally distinct in p107 and p130, compared to Rb.

Waf1/cip1/p21 CDK-inhibitory protein *(40).* The spacer region is almost absent in Rb protein, although Rb can bind to the cyclin D proteins *(41).* E1A and other V-ONCs associate with Rb and Rb family members through the mediation of the A and B pockets.

The functional consequence of E1A interacting with the Rb family proteins has been elucidated. Studies in the early 1990s showed that Rb is associated with a cellular TF, E2F *(42–44),* which was originally identified as a factor necessary for the E1A-mediated induction of the Ad *E2* gene, and was found to bind to a sequence element, TTTCGCGC *(18).* There were two such elements present in the E2 promoter, and it was found that E1A induces cooperative binding of E2F to the sites, in association with the Ad E4 protein. It was also found that E1A can dissociate multiprotein complexes that contain the E2F TF *(45),* and it was already known that E1A bound to the Rb protein. Attempts were being made to identify the cellular proteins that bind to Rb as well, and such combined efforts revealed that E2F is a target for the Rb protein, and that E1A binding to Rb disrupts the interaction between Rb and E2F. The term E2F now refers to a family of six proteins, E2Fs 1–6. Of these, E2Fs 1–5 are transcriptionally active *(46),* and E2F6 is repressive in nature *(47).* Although the transcriptionally active E2Fs have similar DNA-binding abilities, they show a preference in their ability to bind to different Rb family members: E2Fs 1, 2, and 3 bind to the Rb protein *(39);* E2Fs 4 and 5 preferentially interact with p107 and p130 *(39,48,49).*

Further analysis of the functional consequences of the interactions involving Rb, E1A, and E2F showed that Rb binds to E2F, and represses its transcriptional activity *(50).* The binding of E1A could effectively reverse this Rb-mediated repression of E2F

to Rb *(50)*. Cloning of the E2F family members revealed that Rb binds to the transcriptional activation domain of E2F1, thus preventing its ability to function as a TF *(51,52)*. Recent studies have shown that Rb can block transcription from promoters containing E2F sites, by recruiting the cellular histone deacetylase enzyme, *HDAC1 (53–55)*.

In the case of E1A, it appeared that induction of E2F activity facilitated the expression of the *E2* gene. As described in later subheadings, HPV E7, as well as SV40 large T-Ag, were also capable of dissociating E2F from Rb, and thus inducing its transcription *(56)*. This was surprising, since none of the genes present in these viruses had E2F sites in their promoters. This raised the possibility that activation of E2F may be contributing to the expression of the *E2* gene in Ad, but it plays a more important role in the regulation of the cell cycle. It is now evident that the V-ONCs activate E2F to achieve a proliferative state, which is conducive for their DNA replication *(13,14)*.

It became apparent that many cellular promoters that were known to be induced by Ad E1A had E2F sites in their promoter *(57)*. Further, an analysis of the genes that are regulated by E2F showed a variety of cell-cycle regulatory genes as downstream targets of E2F. For example, genes for proteins such as DHFR, DNA polymerase α, ribonucleotide reductase, thymidylate kinase, thymidylate synthase, and so on, which are all necessary for DNA synthesis, are regulated by E2F. Further, many additional cell-cycle proteins, such as cyclins A and E, p107 CDK2, and soon were found to be regulated by E2F as well. The current model suggests that inactivation of Rb by phosphorylation, during the G1 phase of the cell cycle, releases free E2F activity, which transcribes this set of genes, required for cell cycle progression. In the case of viral infection, the products of the immediate early genes achieve the same end result, but by inactivating Rb by a direct interaction. Similarly, it may be imagined that, when the *Rb* gene is inactivated by mutation or deletion, there would be an abundance of active free E2F, which would contribute to uncontrolled cell cycle progression, and hence oncogenesis *(57)*.

The regions of E1A necessary for dissociation of E2F–Rb complexes have been worked out in detail. As discussed earlier, it is the CR2 region that chiefly mediates the interaction of 1A with Rb, but the CR1 region, especially residues 30–60 *(58),* contributes to the binding. Within this region, a tyrosine residue at position 47 is important for stable binding of the human Rb protein. There is no region in E7 or SV40 large T-Ag similar to this E1A CR1 motif. It appears that the same motifs are essential for the disruption of E2F-containing complexes by E1A. One model that has been proposed, based on extensive mutational analysis, as well as on competition experiments, is that the CR2 region would tether E1A to the Rb protein *(58)*. Once E1A is bound to Rb, the CR1 region blocks the region of Rb involved in binding to E2F, thus preventing the formation or existence of a Rb–E2F complex *(59)*. It has been proposed that cyclin D would be functioning in a similar fashion to disrupt Rb–E2F complex. Because cyclin D has an LXCXE motif, it may tether to the Rb protein and bring to its proximity the CDK4/6 kinases, which can phosphorylate Rb and disrupt an E2F–Rb interaction.

2.1.5. Interaction of E1A with p107 and p130

The interaction of E1A with Rb is the best-characterized interaction among all E1A-binding proteins, but all members of the Rb family interact with E1A in essentially the same fashion. It has been shown that interaction of E1A with p107, as well as with p130, can dissociate the E2F proteins associated with them *(60)*. The regions of E1A

involved in binding to p130 spans a few additional residues in the CR2 region; residues 121–127 are required for binding to Rb, as well as to p107, and the residues 121–139 are necessary for binding to p130 *(17)*.

The functional consequences of E1A binding to p107 and p130 may be the same, but it is not yet clear whether E1A must bind to and inactivate these proteins to induce cell proliferation. This is a question especially in the case of p107, in which it complexes with E2F in the S phase of the cell cycle *(61),* and hence the importance of inactivating a protein that functions past the G1–S transition point is questionable. The interaction of E1A with p130 may be more important, since Ad infects mostly quiescent cells, and p130–E2F complexes are prevalent in resting cells *(62)*. In a broader view, the interaction of E1A with either p107 or p130 appears to be less important than its interaction with Rb, simply because the role of these proteins in normal cell cycle regulation is not as significant as that of Rb. This is borne out by the fact that no mutations of the p107 gene has been reported in human cancers, and p130 has been reported to be mutated in a small subset of lung carcinomas. It may be assumed that, for the purposes of this review, the interactions of these proteins with E1A is not relevant.

2.2. HPV E7 Protein

Papilloma viruses are small DNA tumor viruses that have been linked to cancers of the genital tract *(63)*. About 70 types of papilloma viruses have been isolated, and are broadly classified into low-risk and high-risk HPVs, based on the correlation between their presence in benign genital warts or malignant cervical carcinoma *(64)*. HPVs are unique in that, although they share the same pathways of transformation as Ads and SV40, the latter two are not correlated with human cancer.

The genomes of HPV types are different, but the general organization is highly conserved *(3)*. There are three distinct regions within the genome: a region containing the regulatory elements for the transcription of viral genes, a region encoding six early genes, and one encoding two late genes. The most common HPV types found in cervical carcinoma are HPV16 and HPV18; for all practical purposes, their transforming proteins are identical. As in the case of Ads, there are two early genes that are crucial for transformation: the *E7* gene, which is functionally equivalent to Ad E1A; and the *E6* gene, which is comparable to the *E1B* gene *(9)*.

It has been found that integration of the viral genome into mammalian cells causes a loss of many viral genes, but maintains the *E7* and *E6* genes. Transformation experiments have suggested that *E7* gene from the high-risk HPV types can transform cells very efficiently; the *E6* gene has a lesser capacity to do so *(65)*. Studies using the soft-agar colony formation assay have shown that E7 protein, in association with a second oncogene, can transform a variety of primary rodent and mammalian cell lines, and the continued presence of the E7 protein is required for maintenance of the transformed phenotype. The *E6* gene has a lower capacity to transform cells, but it can efficiently cooperate with the *E7* gene to transform primary human keratinocytes, which are the natural hosts for HPV. Both genes, of low-risk HPV type, have weak transformation potential, thus correlating their activity with the tumorigenicity of the HPV type.

As mentioned earlier, the E6 protein is similar to Ad E1B protein functionally, and it can interact with the p53 tumor suppressor protein like E1A. Similarly, the E7 protein, which is functionally analogous to the Ad E1A, can bind to the Rb family of tumor suppressor proteins. This interaction is dealt with here first.

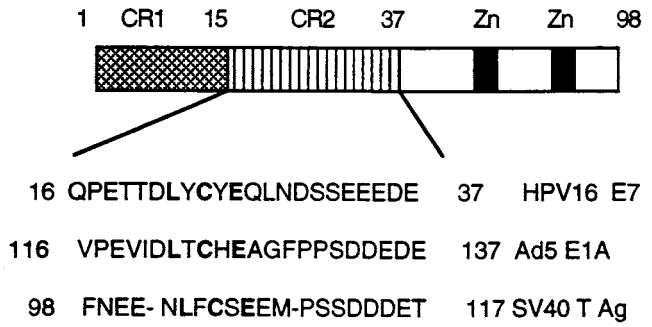

Fig. 3. Schematic of the HPV16 E7 protein. The conserved CR1 and CR2 domains are at the N-terminal half of the protein. The region of high-sequence homology between E7, E1A, and T-Ag is shown below, with the LXCXE motif in bold.

2.2.1. E7 Protein and its Interaction with Rb

The HPV E7 is a small acidic protein that localizes to the nucleus and nuclear matrix. This 98-amino-acid (aa) protein can be divided into three domains: CR1, CR2, and CR3, based on the structural similarity to the Ad *E1A* gene *(66).* CR1 and CR2 are at the N-terminal region of the protein, and the CR3 at the C-terminal end (Fig. 3). CR2 is essential for the binding to the Rb protein, and both CR1 and CR3 are required for the transformation function of E7. The CR3 region of the protein has two zinc-binding motifs, which are involved in dimerization of E7. Mutations in the zinc-binding region of E7 abolished its ability to transform cells, although it was able to bind to Rb protein very efficiently. There are two potential casein kinase sites at the C-terminus of the protein, and phosphorylation on these sites is believed to be important for the full transformation function of E7 *(3).*

The interaction of E7 with Rb family of tumor suppressor proteins has been studied in detail *(16,67,68).* In fact, the crystal structure of the CR2 region of E7, bound to the pocket domain of Rb, has been elucidated. As in the case of E1A, E7 protein binds to the pocket domain of Rb through a conserved LXCXE motif, and the binding to Rb is required for E7 to transform cells *(16).* The first indication of the correlation between Rb inactivation and the transformation by HPV E7 was obtained in 1991 *(69),* when an analysis of human cervical carcinoma cell lines revealed that those having an intact *Rb* gene had HPV E7 incorporated in the genome. In contrast, those that had a mutant *Rb* gene contained no E7. This suggested a direct correlation between the inactivation of Rb, either by mutation, or through the binding or HPV E7 protein.

Mutations in the Rb-binding motif of E7 totally abolished the ability of E7 to promote growth of primary cells in soft agar in cooperation with oncogenic *ras*. As already mentioned, low-risk HPV types have low capacity to transform cells, and, supporting this observation, HPV6 and HPV11 E7 can bind to Rb only weakly. Although the Rb-binding moieties of the high- and low-risk HPV types are similar, a comparison showed that HPV 16 E7 has an aspartic acid at position 22; HPV 6 E7 had a glycine *(3,70).* Substitution of the glycine with aspartic acid, in the HPV 6 E7, enhanced its ability to bind to Rb, and increased its ability to transform cells. This correlation does not appear universal, because certain low-risk types of HPV have an aspartic acid at

this position, and can bind to Rb efficiently. The correlation between the ability of E7 to transform cells and to bind Rb is still not clear, especially in the case of low-risk types. It has been proposed that, although the low-risk E7 proteins bind to Rb with comparable affinity, the functional consequences of the interactions may be different in the various types of HPV.

As in the case of Ad E1A, HPV E7 preferentially binds to the hypophosphorylated form of Rb. Similarly, binding of E7 could effectively dissociate Rb–E2F complexes in mammalian cell extracts. The crystal structure of HPV16 E7, bound to the pocket domain of Rb, has been solved recently, and the structure supports the earlier functional observations made on the interaction *(71)*. It was found that a 9-aa E7 peptide, carrying the LXCXE motif, binds to a highly conserved region within the B box of the Rb pocket. Both the A and B boxes seem to have structural similarities to cyclins and TFIIB, in that they all possess a five-helix cyclin fold. The LXCXE sequence was found to bind to a shallow groove in the B pocket, which was formed by three cyclin fold helices. Alternating Leu, Cys, Glu, and Leu side chains of the E7 peptide make intermolecular contacts with the B-box groove. In addition, there is a high density of van der Waals forces and hydrogen bond contacts distributed uniformly between the E7 peptide and the B box, contributing to the strength of the binding. The actual contact of the E7 peptide was to the groove of the B box, but the interphase of the A–B boxes appeared to contribute significantly to the binding. The structure also revealed a high degree of conservation of the B-box site, which binds to LXCXE motifs. Four residues that contact the backbone of the E7 peptide, Tyr709, Tyr756, Asn757, and Lys713 are identical in diverse species of Rb proteins, as well as in p107 and p130. The high level of conservation of this B-box site suggests that it plays a major role in the functioning of the Rb family proteins *(71)*.

It has been shown recently *(72)* that the half-life of the Rb protein is considerably reduced in cells stably transformed with the E7 protein *(72)*. An overexpression system showed that high levels of E7 protein can lead to an increased decay of the Rb protein, and this could be blocked by proteasome inhibitors. The degradation was limited to Rb, since there was no change in the stability of p107 or p130 proteins in response to E7. Further, this function appeared to be a specific feature of the HPV E7 protein, because neither Ad E1A nor SV40 T-Ag could affect the half-life of Rb *(72)*.

2.2.2. Binding of E7 to p107 and p130 Proteins

The binding of E7 to p107 and p130 has been studied in detail. Unlike in the case of E1A, there are apparent differences in the consequences of binding to Rb vs p107 or p130. First, it became apparent that E7 protein cannot dissociate p107–cyclin A–E2F complexes, unlike E1A; instead, it remains associated with the complex *(73)*. The association with the p107–cyclin A–E2F complex was also dependent on the LXCXE motif, and E7 from HPV 6 had a reduced capacity for association. It has been suggested that HPV E7 can target cellular genes like c-*myb* by targeting the p107–cyclin A–E2F complex in NIH 3T3 cells. Apparently, the expression of B-*myb* promoter in these cells correlates with the binding of distinct p107–E2F complexes at the E2F binding site, and Rb–E2F complexes do not appear to play a major role in this regulation *(74)*. It has been found that, although the inactivation of Rb family proteins and the induction of E2F activity correlates with the transformation function of E7, this alone is not sufficient. Despite the suggestion that E7 interacts differently with p107 and

p130, it is not yet clear whether the interactions of E7 with these proteins are important for its transformation function.

It may be concluded that the interaction of HPV E7 with Rb has been elucidated more clearly at the structural level, and the conclusions drawn from this can be extended to the other Rb-binding proteins, such as like E1A and SV40 T-Ag. Further, cellular proteins, such as cyclin D, may be targeting Rb through similar interactions *(71).*

3. V-ONCS INTERACTING IWTH p53

3.1. HPV E6 Protein

The HPV E6 is an 18 kDa 151-aa basic protein that also localizes to the nuclear matrix and cell membranes *(10).* Its most notable structural feature is the presence of four Cys motifs, which can form two well-defined Zn fingers. These motifs can bind Zn in vitro, and are highly conserved between all serotypes of HPV. The E6 protein has no homology to Ad E1B or SV40 T-Ag, but can function in a similar fashion. The major common feature of these three proteins is their ability to bind and inactivate p53 tumor suppressor protein. In certain high-risk HPV-infected cells, polycistronic E6 messages have been detected, which can give raise to full-length, or to a shorter, protein, E6* *(10).*

E6 protein from high-risk HPV types associate with p53 with higher efficiency than E6 from low-risk HPV types *(5).* The binding of E6 to p53 is enhanced by a cellular protein, E6AP (E6-associated protein). It has now been established that E6 protein binding leads to the degradation of p53, thus reducing its half-life. The proteolytic degradation of p53 through the ubiquitin–proteasome pathway has been studied in detail. Consistent with these observations, cell lines that carry a high level of E6 have very low amounts of p53 protein, mimicking situations in which *p53* gene is mutated. In vitro analyses have identified two domains of E6 that are involved in the binding and degradation of p53: the C-terminal end of E6 is required for binding; the N-terminal end is required for effecting degradation. E6 protein can inhibit the transcriptional activity of p53, and this does not require the activation of the proteasome pathway. In addition to p53, a variety of cellular proteins, ranging in mol wt from 33 to 212 kDa, have been found to associate with HPV E6, but their identities are not yet known.

3.2. Ad E1B Protein

The Ad 5 *E1B* gene has been studied with respect to its interaction with the p53 protein *(75).* Unlike the Rb-binding V-ONCs, there are no structural similarities between the p53-binding proteins *(8).* Thus, although Ad, HPV, and SV40 all encode proteins that can bind to p53, there are no conserved or shared domains between them. Further, the functional consequence of binding of these proteins to p53 are also different: the Ad E1B binding represses the transcriptional activity of p53; the HPV E6 protein leads to the degradation of p53 *(10).*

Ad *E1B* gene codes for two distinct protein products, one 55 kDa and the other 19 kDa in size. Only the 55 kDa E1b protein has been found to physically interact with p53. The p53-binding domain of E1B is not conserved, even in different serotypes of Ad, and E1B protein from certain strains, such as Ad12, cannot bind to p53 *(28).* Because p53 plays a major role in arresting cells in G1, in response to DNA damaging agents, or induces apoptosis if the DNA damage cannot be successfully repaired, it is believed that the V-ONCs that target p53 lead to a suppression of the cell death pro-

gram *(16)*. E1B proteins are believed to suppress cell death programs initiated by DNA damage, as well as by other viral proteins like E1A *(15,77)*. Thus, the V-ONCs that bind to p53 cause distinct functional effects than those binding to Rb and facilitating G1–S transition.

The functional characterization of p53 has shown that it is a TF, possessing distinct DNA-binding and activation domains. Further, it is very well established that p53 induces a wide variety of cellular genes, while repressing certain other genes. Studies on the functional consequences of E1b binding to p53 revealed that the 55 kDa protein targets the activation domain of p53, and thus inhibits p53-mediated transcriptional induction. This could influence the expression of vital cell cycle genes, such as the $p21^{Waf1/Cip1}$ proteins and Mdm2, which is a regulator of p53 itself. Detailed mutational analysis has shown that the ability of E1B 55 kDa protein to repress p53-mediated transcriptional activation strongly correlated with its ability to transform cells. Ad12 E1B 55 kDa protein, which was unable to bind to p53, was effective in blocking p53-mediated transcriptional activation. Conversely, the interaction between p53 and E1B was necessary, but not sufficient, for transcriptional repression, as well as transformation functions of E1B *(78,79)*. This conclusion is based on the finding that a single aa insertion at residue 443 abolishes the ability of E1B to bind p53, but it was effective in transcriptional repression and transformation. The C-terminal end of E1B outside the p53-binding region was required for its transformation function; phosphorylation at three sites within this region was also essential for the transformation function *(78,80)*.

The region of E1B 55 kDa that binds to p53 also is involved in binding to the Ad E4 protein Orf6 *(78)*. This led to the suggestion that the repressive properties of E1B are facilitated by the presence of E4Orf6. In addition, one study has shown that E1B, in cooperation with E4Orf6, modulates not only the transcriptional activity of p53, but also the levels of p53 protein. Because E4Orf6 has also been shown to bind to p53, it is thought that collective interactions among E1B55kd, E4Orf6, and p53 lead to modulation of the levels, as well as the activity, of p53.

It has been reported that the E1B 19 kDa protein can also affect p53 function, but that this does not require a direct interaction. Unlike E1B 55 kDa protein, the 19 kDa protein was unable to block the transcriptional activity of p53; since p53 is also known to repress the transcription of certain cellular genes, it is believed that E1B19 kDa protein affects the transcriptional repressive properties of p53.

3.3. SV40 T-Antigen

SV40 is an oncogenic DNA tumor virus that was originally discovered in rhesus monkey kidney cells. The oncogenic property of this virus resides in two early gene products, the large (T) and the small (t) tumor Ag *(4)*. The large T-Ag can transform cells on its own, but the small t-Ag cannot; the latter can enhance the transformation potential for the large T-Ag. The transformation function of the T-Ag was found to require its interaction with the Rb family tumor suppressor proteins, as well as the inactivation of p53. SV40 thus differs from Ad and HPV in having one protein that can inactivate Rb, as well as p53 pathways; these functions reside in separate proteins in the latter two. Further, SV40 T-Ag is also capable of binding to DNA, unlike E1A or E7 proteins *(81)*.

The SV40 T-Ag is a polypeptide of 708 aa, and is considerably larger than the other oncoproteins discussed thus far (Fig. 4). There are several distinct functional domains

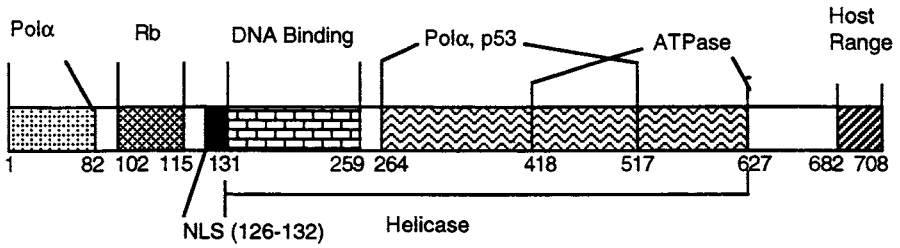

Fig. 4. Structure of the SV40 large T-Ag. The various functional and protein-binding domains are indicated.

that have been extensively characterized. A schematic of the T-Ag domain structure is shown in Fig. 4; as can be seen, the N-terminal end (1–82) and an internal domain is involved in binding to DNA polymerase α-primase. The Rb-binding domain carrying the LXCXE motif spans residues 102–115. Other well-defined domains include, progressively toward the C-terminal end, a NLS (126–132); a DNA-binding domain (131–259); a finger motif (302–320); a second DNA polymerase α-primase-binding domain (259–517), which partly overlaps with the p53-binding domain (275–517); and an adenosine triphosphatase (ATPase)/ATP-binding domain (418–627). A helicase domain extends from residue 131 to 627. There is a cluster of phosphorylation sites on the C-terminal region, which has been reported to be critical for T-Ag function. A small domain that defines the host range of (55)SV40 resides at the extreme C-terminal end, spanning residues 682–708. The ability of T-Ag to perform such a diverse array of functions enables it to facilitate all stages of viral replication and propagation *(4)*.

3.3.1. Interaction with Rb Family Members

The interaction of SV40 large T-Ag with Rb protein has been well-characterized, and the inactivation of Rb is essential for T-Ag to transform cells *(82)*. As mentioned earlier, the Rb-binding domain of large T-Ag has the conserved canonical LXCXE motif. The integrity of this motif is essential, but not sufficient, for the oncogenic activity of T-Ag. It is clear that interaction of T-Ag with Rb leads to an inactivation of Rb function, as in the case of E1A or HPV E7, leading to a dissociation and activation of E2F TF. Such studies have established that these three DNA tumor viruses all utilize common mechanisms for inducing the cells to enter S phase, creating an environment conducive to the replication of viral DNA. One difference in the case of T-Ag, however, is that it can perform considerably more functions than E1A alone, or even E1A and E1B combined *(7,83)*.

Consistent with the finding that T-Ag can efficiently dissociate Rb–E2F complexes, it was found that a human cell line, WI38-VA13, which stably expresses T-Ag, had no Rb–E2F complexes at all *(84)*. There was a reduced level of the cyclin A–E2F complex, and the loss of these complexes corresponded to an increase in the levels of free, transcriptionally active E2F. Like E1A and HPV E7, T-Ag also targets the pocket domain of hypophosphorylated Rb. Hence, biochemically, as well as functionally, the interaction of T-Ag with Rb is similar to that of E1A or E7.

It has been shown recently *(85)* that a region of T-Ag N-terminal to the conserved Rb-binding region is required for the functional inactivation of the Rb protein. This

region has been named the J-domain, because it has sequence homology to the J-domain of the DnaJ family of molecular chaperons of *Escherichia coli.* The J-domains are characterized by conserved histidine–proline–aspartate (HPD) residues, and this tripeptide is present at the N-terminus of the SV40 large T-Ag. Further, the J-domain of SV40 T-Ag, as well as certain other polyomaviruses, can functionally substitute for the J-domain of *E. coli* DnaJ chaperon.

Studies on the J-domain of T-Ag showed that it is required to overcome the G1–S arrest induced by all Rb-family members *(85,86).* In addition, it was required to reverse the repression of E2F activity brought about by Rb, as well as p130. From these experiments, it appears that, in the case of T-Ag also, the LXCXE motif tethers the protein to Rb, while a N-terminal region functionally inactivates it *(86).* This general pattern is similar to the one seen in the E1A-mediated inactivation of Rb *(58,59).*

It has also been reported that the J-domain of T-Ag can affect the phosphorylation status of p107 and p130 proteins. In cells stably expressing T-Ag, there is a reduced amount of phosphorylated forms of p107 and p130. There was also a faster turnover of p130 protein, which could be a result of its aberrant phosphorylation status. These effects of T-Ag appear to be J-domain-dependent, because point mutations in the HPD motif abolished these changes. Further, replacement of the N-terminal J-domain of T-Ag with J-domain motifs from cellular proteins restored this ability. Similarly, the transformation function of T-Ag also appeared to require a functional J-domain. The biochemical basis for the functioning of the J-domain is not yet clear, but it appears to be as important as the LXCXE motif in the Rb-binding domain for full T-Ag function.

3.3.2. Interaction of T-Ag with p53

As in the case of other p53 binding vONCs, the region of T-Ag that is involved in binding to p53 is large and not conserved. The biochemical effect of the binding of T-Ag to p53 is also different from the binding of E6 or E1B. One interesting facet of this interaction is that phosphorylation of both T-Ag and p53 appear to be necessary for binding in murine cells. In addition, the p53-binding region of T-Ag overlaps with the binding sites for DNA polymerase α and ATP, raising the possibility that T-Ag–p53 complexes may affect the viral functions regulated by T-Ag *(4).* This notion is supported by the finding that a single point mutation (Pro to Leu), at position 584 of T-Ag, resulted in a loss of p53 binding, along with changes in the ATPase activity of T-Ag, as well as its ability to oligomerize. This mutation also resulted in reduced stability of T-Ag, and resulted in defective replication and reduced transformation functions.

It has been found that wild-type murine p53 can block the binding of T-Ag to DNA polymerase α; in addition, wild-type p53 could effectively reduce the replication of viral origins, but mutant p53 molecules were unable to do this *(6).* This could possibly result from p53 competing for the binding to T-Ag to DNA polymerase α or another cellular protein involved in replication. One other interesting aspect of the interaction between T-Ag and p53 is that the phosphorylation, as well as the stability, of the latter increase, upon T-Ag-mediated cellular transformation. The relative importance of p53 binding in T-Ag-mediated transformation of primary murine cells was highlighted in one study, in which it was found that the N-terminal region of T-Ag, up to residue 250, was not necessary for this function. This ruled out a role for Rb-binding, nuclear translocation, and DNA binding abilities of T-Ag. In that study, residues 251–626 were

found to be vital for immortalization, suggesting that p53 binding and inactivation is indispensable for the transformation function of T-Ag *(87).*

The modulation of p53 by T-Ag extends beyond enhancing the stability of the former. It has been shown that T-Ag can block the DNA-binding activity of p53, which correlated with T-Ag inhibiting the transcriptional activation functions of p53. One recent study showed that the N-terminal domain of T-Ag, which is not involved in DNA binding nor binding to p53, could effectively repress p53-mediated transcription. This suggests that the N-terminal region of T-Ag may be affecting p53-mediated transcription indirectly, through other cellular factors involved *(6).*

Overall, it may be summarized that, although the interaction of V-ONCs with Rb family members has been characterized in great detail, ambiguity still exists as to the nature of their interactions with p53, and its functional consequences, partly because the p53-binding domains of the V-ONCs are large and not very well defined, and they do not share extensive homology. Despite these drawbacks, it appears to be a fruitful endeavor to study the interactions of these proteins with p53 in greater detail.

4. V-ONCS AS PROBES FOR RB FUNCTION

The observations described above make it clear that V-ONCs interact with Rb family members specifically and with high affinity. The most notable aspect of the interaction is that the V-ONCs specifically target the active form of Rb and Rb family members, which leads to an alteration of their normal function. These features make the V-ONCs efficient probes for assessing the functional status of the Rb protein in a given cell.

The functional effects of V-ONCs on Rb have been studied more extensively in Ad E1A, but the recent structural studies on HPV E7 peptide would make it a more adaptable probe for Rb function. The 9-aa peptide derived from E7 appears to bind to Rb efficiently, and may provide a good model for designing custom probes for Rb function *(88,89).* Since V-ONCs bind specifically to the functional, wild-type, hypophosphorylated form of Rb, the binding itself may be considered as a measure of the functional status of Rb. In addition, because the binding of such V-ONCs leads to a perturbation of Rb activity, methods could be designed to evaluate such changes in Rb function. Many novel methods have been developed recently to measure protein–protein interactions in vivo in living cells *(90),* mostly using fluorescent probes *(91,92).* Such methods generally measure changes in the fluorescence properties of the tagged protein or peptide when it interacts with another component. It may be imagined that a tagged E7 peptide, or one derived from Ad E1A, would be able to detect the functional status of the Rb protein, using such methods.

The tagged V-ONCs may be used to detect functional Rb proteins in biopsy samples of human tumors, or to evaluate whether the Rb protein is expressed in its functional form after being introduced into cells for gene therapy purposes *(93).* Although constitutively active, phosphorylation site mutants of Rb are expected to be used for this purpose, the assay using V-ONCs would be of immense help in assessing the amounts, as well as the functional status, of the protein. The advantage of such an assay system is that it would be able to detect functional Rb protein, even in single cells, and the assay may be modified easily to an in vitro diagnostic system very efficiently.

The V-ONCs would be especially useful for detecting functional Rb protein, because, unlike the p53 protein, there are no good antibodies that can distinguish

between a functional form of the Rb protein and a mutated protein. Further, because even small peptides derived from the oncoproteins can specifically interact with functional Rb makes this approach feasible and attractive. It may be imagined that such specific biological probes would be of value in assessing the functional status of the vital growth regulatory proteins.

5. V-ONCS AS PROBES FOR P53 FUNCTION

The use of V-ONCs to assess the levels or the functional status of p53 tumor suppressor protein is not as attractive or feasible as in the case of the Rb family proteins. The chief reasons for this are that the regions of V-ONCs interacting with p53 are large, and they do not specifically interact with the functional form of p53 alone. In contrast, there are excellent immunological reagents available that can distinguish between the functional and inactive forms of p53. But it still remains an option, which could be utilized in circumstances in which antibodies may not be effective or accessible. Again, one of the many recent techniques for detecting protein–protein interactions in vivo and in vitro may be modified for this purpose. It would appear that polypeptides derived from the Ad E1B 55 kDa protein or the HPV E6 protein would be more suitable for this purpose.

It appears an exciting possibility that the oncoproteins of DNA tumor viruses may be harnessed to detect and quantitate cellular proteins that can prevent cell proliferation and oncogenesis. This would be a valuable addition to the repertoire of modern techniques to combat cancer.

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