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Human Cytomegalovirus pp71: A New Viral Tool to Probe the Mechanisms of Cell Cycle Progression and Oncogenesis Controlled by the Retinoblastoma Family of Tumor Suppressors

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Abstract The DNA tumor virus oncogenes (adenovirus E1A, simian virus 40 (SV40) large T antigen, and papillomavirus E7) have been instrumental in illuminating the molecules and mechanisms of cell cycle progression and carcinogenesis. However, since these multifunctional proteins target so many important cellular regulators, it is sometimes difficult to establish the functional importance of any individual interaction. Perhaps a herpesvirus protein, newly defined as a cell cycle regulator, can help address these issues. Like the DNA tumor virus proteins, the human cytomegalovirus (HCMV) pp71 protein contains a retinoblastoma protein (Rb) binding motif (LxCxD), and stimulates DNA synthesis in quiescent cells. Unlike E1A, T antigen, and E7, pp71 expression does not induce apoptosis, nor does it cooperate to transform primary cells. Determining how pp71 induces cell cycle progression without invoking apoptosis or leading to cellular transformation may help in defining the signals that ultimately lead to these processes. J. Cell. Biochem. 93: 37–45, 2004.

Key words: retinoblastoma; Rb; p107; p130; E2F; cell cycle; cytomegalovirus; pp71

VIRAL PROTEINS AS TOOLS

The contributions that the study of viral proteins has made to our understanding of cell cycle progression, immortalization, and transformation of mammalian cells cannot be overstated, and the oncoproteins of the DNA tumor viruses are the leaders of the pack [Helt and Galloway, 2003]. These proteins (E1A, T antigen, and E7) utilize a common motif, denoted by the single amino acid code LxCxE (where x stands for any amino acid) to bind to the pocket domain of the retinoblastoma tumor suppressor (Rb) and its family members, p107 and p130. The Rb family members bind to well over 100 cellular proteins [Morris and Dyson, 2001], including many transcription factors, and thus control not only cell cycle progression, but differentiation and apoptosis as well. Many, but certainly not all of the activities of the Rb family are mediated by their major binding partners, the E2F family of transcription factors (E2F-1, -2, -3, -4, -5). E2F proteins dimerize with either DP-1 or DP-2 and bind to DNA sequences located in the promoters of numerous genes, modulating their transcription [Trimarchi and Lees, 2001].

Transcriptional activation by E2F is inhibited when it is bound by the Rb proteins, and when these Rb–E2F complexes are recruited to E2F-responsive promoters. Rb–E2F complexes modulate transcription in at least two ways. Rb sterically blocks the activation domain of E2F when bound to it, thus preventing E2F-mediated activation [Flemington et al., 1993; Helin et al., 1993]. Furthermore, Rb–E2F complexes actively repress transcription from genes that respond to E2F by recruiting histone deacetylases (HDACs) that utilize an L/IxCxE motif to bind to the Rb family members [Brehm

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et al., 1998; Luo et al., 1998; Magnaghi-Jaulin et al., 1998]. Since the protein products of many E2F responsive genes are required for cell cycle progression out of quiescence (G_0), through G_1 and into the S phase, Rb–E2F complexes arrest cell cycle progression in G_0/G_1.

During normal cell cycle progression, the cyclin dependent kinases phosphorylate the Rb family members, disrupting Rb–E2F complexes [Weinberg, 1995]. This results in both the de-repression and activation of E2F-responsive promoters, and thus cell cycle progression through G1 and into S. By utilizing their LxCxE motifs, the DNA tumor virus oncoproteins bind in the pocket domain of the hypophosphorylated forms of the Rb family members, and dissociate both HDAC-Rb–E2F, and Rb–E2F complexes. This leads not only to cell cycle progression, but also to the induction of apoptosis, a cellular response to unwarranted or overzealous growth signals. In cooperation with additional viral or cellular oncogenes, E1A, T antigen, and E7 lead to the immortalization and transformation of primary rodent cells [Helt and Galloway, 2003].

In addition to Rb and E2F, these viral proteins also interact with the p300/CBP tumor suppressor [Goodman and Smolik, 2000]. p300 is a transcriptional co-activator that acts as a bridge between numerous sequence-specific DNA-binding transcription factors and the general transcriptional machinery. p300 also acts as an acetyltransferase, and thus regulates gene expression in multiple ways. However, these two important classes of tumor suppressors do not nearly represent the full repertoire of proteins that interact with the DNA tumor virus oncoproteins. In fact, E1A itself binds to over 40 cellular proteins (http://www.geocities.com/jmymryk.geo/protein.html). The sheer number of cellular factors that E1A, T antigen, and E7 interact with makes it difficult to assign specific functions to each interaction. This becomes even more complicated by the fact that many of these binding regions overlap. Thus, showing that any individual interaction is either necessary or sufficient for the biological activities of these viral protein can be technically challenging.

VIRAL MODULATION OF THE CELL CYCLE

The DNA tumor viruses have small genomes with limited coding potential, thus they create multifunctional proteins that are extremely powerful and efficient, but could be described as lacking a certain sense of style. They are like the "bull in the china shop" that plows through everything that gets in its way. Larger viruses have the luxury of a higher coding capacity, giving them the capability of separating functions into different gene products. If the synthesis and/or degradation of these proteins were differentially regulated, it might allow these viruses to attack cells as efficiently, but in more subtle ways.

HCMV is the largest DNA virus, and it has been estimated to encode 192 gene products [Murphy et al., 2003]. By comparison, adenovirus has less than 40 genes [Mei et al., 2003], papillomavirus has no more than 10 [Duensing and Munger, 2003], and SV40 has only 7 genes [Saenz-Robles et al., 2001]. HCMV infection induces quiescent cells to renter the cell cycle and progress through the G1 phase, but then arrests them at the G1/S border [Kalejta and Shenk, 2002]. At least four HCMV proteins can modulate cell cycle progression [Castillo and Kowalik, 2004], UL69, IE1, IE2, and pp71. UL69 arrests cells in G1 through an unknown mechanism [Lu and Shenk, 1999]. IE1 drives quiescent cells into the S phase in the absence of p53 or p21 function [Castillo et al., 2000], and IE2 can both stimulate and arrest cell cycle progression [Wiebusch and Hagemeier, 1999; Castillo et al., 2000; Murphy et al., 2000]. Because the major immediate early region of HCMV (that produces IE1 and IE2) could compliment an E1A-deficient adenovirus mutant [Spector and Tevethia, 1986], the IE1 and/or IE2 proteins have long been thought of as E1A homologs. Their ability to stimulate the cell cycle and bind to the Rb family members supports this concept, but since these HCMV proteins lack a consensus Rb-binding LxCxE motif, they appear to be functional but not sequence homologs of the DNA tumor virus proteins. Therefore, it is likely that they stimulate cell cycle progression through a mechanism completely different from that of the DNA tumor virus proteins.

THE NEW KID IN TOWN: PP71

However, another HCMV protein called pp71 does contain a motif (LxCxD) that is closely related to the Rb-binding LxCxE motif. Moreover, pp71 stimulates G1 cell cycle progression [Kalejta and Shenk, 2003a], drives quiescent

cells out of G_0, through G_1 and into the S phase [Kalejta et al., 2003], and binds to the hypophosphorylated forms of all three Rb family members. A single point mutation in the LxCxD motif abrogates the ability of pp71 to induce DNA synthesis in quiescent cells. Thus, pp71 has both sequence and functional homology to the DNA tumor virus oncoproteins.

pp71 binding to the hypophosphorylated forms of Rb, p107, and p130 induces their degradation, and mutation of the LxCxD motif inhibits the ability of pp71 to degrade the Rb family members [Kalejta et al., 2003]. This degradation occurs through an interesting, albeit ill defined mechanism that requires proteasome function, but does not involve ubiquitin conjugation to the Rb family members. In fact, the pp71-mediated degradation of Rb, p107, and p130 continues under conditions that maintain proteasome function but inhibit ubiquitin-mediated proteolysis, indicating that the degradation, while proteasome-dependent, is ubiquitin-independent [Kalejta and Shenk, 2003b].

IS PP71 JUST ANOTHER E1A/T ANTIGEN/E7?

The DNA tumor virus oncogenes attack the Rb family through an LxCxE motif, leading to the induction of DNA synthesis in quiescent cells, and either eventual cell death by apoptosis, or in cooperation with other viral or cellular oncogenes, the immortalization and transformation of primary rodent cells [Helt and Galloway, 2003]. While pp71 also has an LxCxD motif, targets the Rb family, and stimulates cell cycle progression, it fails to induce apoptosis, and cannot cooperate to transform cells [Kalejta and Shenk, 2003a]. Thus, while pp71 shares a subset of the functions of E1A, T antigen, and E7, it also apparently lacks additional activities important for apoptosis induction and transformation. To begin to investigate how pp71 interacts with the Rb pathway to induce cell cycle progression but not apoptosis or transformation, we must first review how E1A, T antigen, and E7 induce each of these outcomes, and then determine if and how the activities of pp71 differ from these DNA tumor virus oncoproteins.

CELL CYCLE INDUCTION

When the DNA tumor virus oncogenes utilize their LxCxE motifs to bind to the pocket domains of Rb, p107, and p130, they disrupt the binding of the Rbs and HDACs [Brehm et al., 1998; Ferreira et al., 1998; Magnaghi-Jaulin et al., 1998], as well as the Rbs and the E2Fs [Chellappan et al., 1992]. This results in both the de-repression and activation of E2F responsive genes.

The E2Fs that bind to the Rb family members can be separated into two classes, those that mediate repression and those that mediate activation [Trimarchi and Lees, 2001]. The repressive E2Fs are E2F-4 and E2F-5. E2F-4 is responsible for the majority of E2F binding activity in cells, is found in both quiescent and cycling cells, and binds to Rb, p107, and p130 [Moberg et al., 1996]. The different Rb family members predominate during different periods of the cell cycle, with p130 levels high in quiescent cells, Rb being present in all cell cycle phases, and p107 found mostly during the S and G2 phases [Grana et al., 1998]. Overexpression of E2F-4 weakly drives quiescent cells into the S phase [DeGregori et al., 1997]. Attaching a nuclear localization signal to E2F-4 dramatically increased its ability to stimulate S phase entry [Muller et al., 1997].

E2F-1, -2, and -3 are the activating E2Fs and their overexpression leads to efficient S phase entry [DeGregori et al., 1997]. They associate mainly with Rb, but can also bind to p130. Not only are they strong transcriptional activators of E2F-responsive genes [Lees et al., 1993], but their own transcription is controlled by E2F activity. Thus, the model for cell cycle progression from quiescence to S phase invokes a cascade of E2F activity (Fig. 1A). First, the repressive p130–E2F-4 complexes are disrupted through p130 phosphorylation by the cyclin dependent kinases. This allows a de-repression of the *E2F-1*, *-2*, *-3* genes, which are synthesized, but these newly made E2F proteins are immediately sequestered by Rb. When they are released from Rb (by phosphorylation), these activating E2Fs stimulate the transcription of genes that are rate limiting for progression into the S phase [Nevins, 1998]. Viral oncoproteins such as E1A bind, through their LxCxE motifs, to the pocket domains of the hypophosphorylated forms of the Rb family members and disrupt Rb–E2F complexes, leading to cell cycle progression (Fig. 1B).

pp71 contains an LxCxD motif, and thus presumably binds in the Rb pocket domain, although that has not been directly addressed. As mentioned above, both HDACs and E2Fs

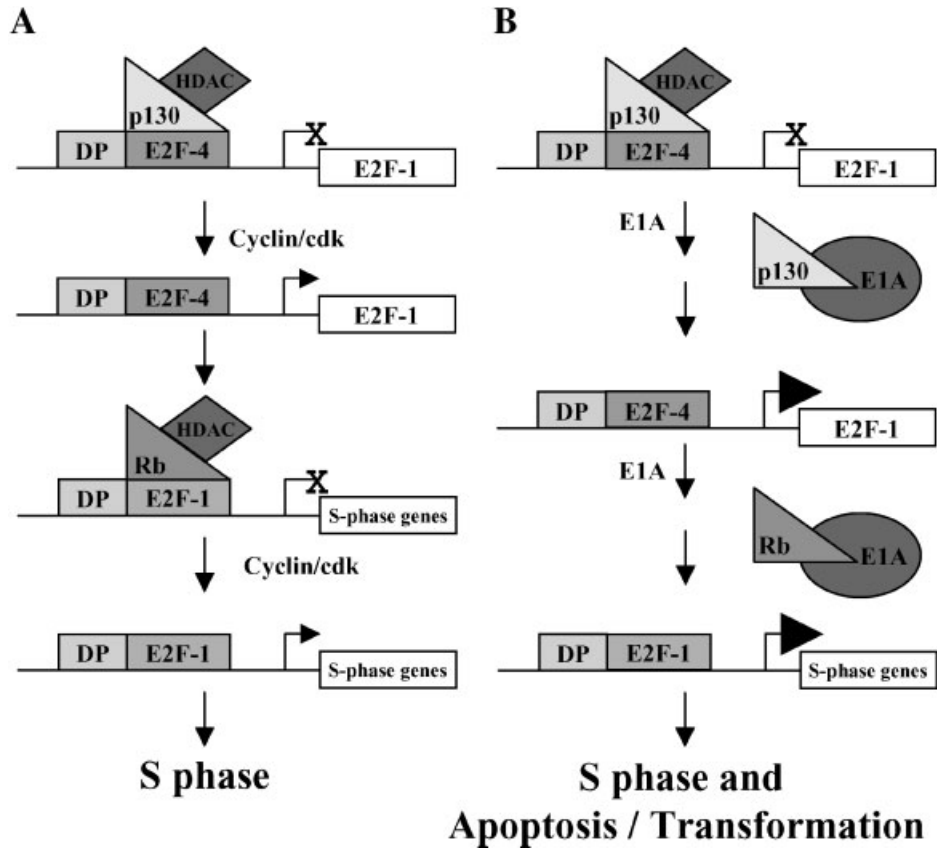

Fig. 1. A cascade of E2F activity drives cell cycle progression. **A**: E2F-responsive genes are repressed (X) in quiescent (G_0) cells by HDAC–p130–E2F4 complexes. Serum stimulation induces cyclin-dependent kinase activity that phosphorylates p130, releasing it from E2F4, and resulting in the activation (arrow) of the gene for E2F1. Newly made E2F1 in complex with Rb occupies the promoters of genes that are rate limiting for entry into the S phase. As Rb becomes phosphorylated by cyclin-dependent kinases, it is released from E2F1, which now can activate the expression of its target genes, leading to progression into the S phase. **B**: Expression of the adenovirus E1A oncoprotein disrupts p130–E2F4 complexes, activating the expression of E2F1. E1A also sequesters Rb, so the newly made E2F1 is unregulated and is immediately free to transactivate its target genes. This unregulated activation leads to cell cycle progression as well as the induction of either apoptosis or transformation. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

bind within the pocket domain, HDACs through and L/IxCxE motif [Magnaghi-Jaulin et al., 1998], and E2Fs through other sequences [Xiao et al., 2003]. Therefore, one would predict that the LxCxD motif of pp71 might compete with HDACs for binding in the pocket. However, this binding competition does not necessarily have to extend to the E2Fs. E1A, T antigen, and E7 each require sequences in addition to the LxCxE motif to disrupt Rb–E2F complexes [Helt and Galloway, 2003], and such a sequence has not yet been identified in pp71. Furthermore, while the DNA tumor virus oncoproteins have all been shown to disrupt Rb-HDAC [Brehm et al., 1998; Ferreira et al., 1998; Magnaghi-Jaulin et al., 1998] and RB–E2F complexes [Chellappan et al., 1992], the ability of pp71 to accomplish these tasks has yet to be addressed. These are crucial experiments to determine the mechanism through which pp71 stimulates the cell cycle.

APOPTOSIS

E2F-1 is a strong inducer of apoptosis [Qin et al., 1994; Shan and Lee, 1994; Wu and Levine, 1994], E2F-2 and E2F-3 may induce apoptosis under certain experimental conditions [Vigo et al., 1999], and the repressive E2Fs (E2F-4 and -5) lack the ability to induce apoptosis [DeGregori et al., 1997]. E2F-1 induces apoptosis through multiple pathways by transcriptionally activating pro-apoptotic genes such as *p19ARF* [Ginsberg, 2002], caspaces [Nahle et al., 2002], *APAF-1* [Moroni et al., 2001], and the checkpoint kinase Chk2 [Rogoff et al., 2004].

As E1A, T antigen, and E7 each liberate E2F-1, this is the most likely scenario for how they induce apoptosis. Since pp71 does not induce apoptosis, it will be interesting to determine if pp71 can liberate E2F-1 from its Rb complexes, and if this results in the transcriptional activation of the *p19ARF*, caspace, *APAF-1*, or *Chk2* genes. It should be noted that E1A and E7 have viral co-conspirators that inhibit the apoptosis that they induce (E1B and E6, respectively) by inhibiting or degrading p53 [Scheffner et al., 1990; Debbas and White, 1993]. Furthermore, T antigen itself inhibits p53 activity, thus it both induces and subsequently inhibits apoptosis [Mietz et al., 1992]. It does not appear that pp71 inhibits apoptosis, since it could not prevent apoptosis induced by E2F-1 overexpression [Kalejta et al., 2003]. Thus, pp71 does not appear to first induce and then inhibit apoptosis, but likely never induces it.

TRANSFORMATION

A major focus of research on the DNA tumor virus oncogenes has been trying to determine how they induce transformation (carcinogenesis) in primary rodent cells [Helt and Galloway, 2003]. Transfection of expression plasmids for E1A, or E7 alone rarely leads to transformation, because of the induction of apoptosis. Thus, co-transfection of cooperating oncogenes to inhibit the p53 pathway to apoptosis (such as adenovirus E1B or papillomavirus E6) is required for efficient transformation. As T antigen also targets p53, it can transform cells alone.

The approach to study transformation by these proteins has been a simple one. Mutant alleles of these proteins are created and co-transfected into rodent cells, and the number of transformed foci produced are counted. This approach has identified the regions of these proteins that are required to induce transformation [Helt and Galloway, 2003]. For all three proteins, they are comprised of the LxCxE motif and other sequences that target Rb–E2F complexes, as well as sequences that mediate binding to the p300/CBP tumor suppressors. The p53-binding region of T antigen is also required. The caveat to these experiments is that E1A, T antigen, and E7 are multifunctional proteins that bind to numerous cellular factors through overlapping sites. Thus, it is difficult to determine if the Rb family members, p300/CBP, and p53 are the only important targets for cellular transformation attacked by these proteins.

pp71 does not transform cells [Kalejta et al., 2003], either alone, or in cooperation with E1B or other inhibitors of apoptosis such as Bcl2 or dominant-negative p53. It also fails to cooperate with cellular oncogenes such as Myc or activated Ras. Co-transfection of pp71 and E1A/E1B did not reduce the number of foci compared to E1A/E1B alone (Kalejta and Shenk, unpublished observations), so pp71 does not appear to inhibit transformation. The big question is how does pp71 stimulate the cell cycle without transforming cells?

One possible explanation is that pp71 interacts inefficiently with the Rb family because its LxCxD sequence deviates from the canonical LxCxE. Interestingly, a mutant E1A protein in which the LxCxE motif was changed to LxCxD still bound to Rb and transformed cells, but did so less efficiently than the wild type protein [Corbeil and Branton, 1994]. Furthermore, E7 proteins from some low-risk papillomaviruses contain an LxCxE motif but fail to transform cells [Heck et al., 1992]. These proteins bind weakly to the Rb family members because they lack an upstream acidic residue present in E7 proteins from the high-risk papillomaviruses, as well as in E1A and T antigen. However, pp71 does have an upstream acidic residue, and binds strongly enough to the Rb family to induce cell cycle progression [Kalejta et al., 2003]. Recently, the inability to degrade Rb, not binding efficiency, has been suggested as an explanation for why E7 proteins from the cutaneous low-risk papillomaviruses fail to transform cells [Gonzalez et al., 2001]. However, pp71 does degrade the Rb family members but still fails to transform cells, indicating that degradation of these proteins does not lead directly to transformation.

Ineffective targeting of the Rb family by pp71 is not the only possible reason why pp71 fails to transform cells. Sequence elements important in binding to p300, although somewhat poorly defined in E1A, T antigen, and E7, have not yet been found in pp71, indicating that pp71 may not interact with p300. E1A helps mediate the acteylation of Rb by p300 [Chan et al., 2001]. This may indicate that, not only is binding to these two tumor suppressors important for E1A-induced transformation, but having a single molecule bind to them simultaneously is important as well. Furthermore, E1A interacts with the CtBP protein, and this binding attenuates the transformation efficiency of E1A

[Chinnadurai, 2004]. Although it is possible that pp71 might also have an activity that attenuates transformation, it does not inhibit the ability of E1A to transform cells (Kalejta and Shenk, unpublished observations), making this possible explanation for the lack of transforming capability of pp71 appear less likely.

Thus, the question remains, is pp71 unable to transform cells because it modulates the Rb pathway in a unique manner that leads to cell cycle stimulation but not transformation, or does it lack a function retained in E1A, T antigen, and E7, such as the ability to modulate the activity of the p300/CBP tumor suppressors? Experiments designed to test these models will be important in determining how pp71 stimulates cell cycle progression without inducing transformation. Does pp71 bind to p300/CBP? Can the pp71 LxCxD motif, if substituted for the LxCxE in E1A bind to Rb with enough efficiency to lead to transformation? Can pp71 cooperate with LxCxE-mutant, transformation-deficient alleles of E1A, T antigen, or E7 to lead to transformation? These straightforward experiments should be very telling in determining why pp71 fails to transform cells.

UNIQUE FEATURES OF PP71

An interesting question regarding pp71 is how its ability to degrade the Rb family members, and its unique method for doing so contributes to its biologically properties? While E7 also degrades all of the Rb family members [Gonzalez et al., 2001], degradation of these proteins does not appear to be necessary for transformation since T antigen only degrades p130 [Stubdal et al., 1997], and E1A does not alter the stability of any of the Rb family members [Berezutskaya et al., 1997]. Furthermore, why has pp71 chosen to degrade the Rb family members in a proteasome-dependent, ubiquitin-independent fashion? While this degradation mechanism is not unheard of, it is certainly uncommon [Orlowski and Wilk, 2003]. Does this play an important role in pp71's function as a cell cycle regulator? Experiments to uncover a more detailed mechanism for the degradation reaction should begin to answer this question.

MODELS TO TEST AND BUILD ON

Could pp71 act in a way that is fundamentally different from E1A, T antigen, and E7? pp71 has a long stretch of acidic residues, and since it is a transcription factor [Liu and Stinski, 1992], this sequence may constitute an acidic activation domain. Since binding to the Rb family members could tether pp71 to E2F-responsive promoters, in theory a transcriptional activation domain of pp71 could then activate transcription from these promoters (Fig. 2A). Some acidic transcriptional activation domains are also signals for rapid proteolysis [Salghetti et al., 2000], which could explain the degradation of the Rb family members by pp71. This model would require either that pp71 gets degraded along with the Rb family member, or that the acidic domain of pp71 functions in trans to signal the degradation of Rb. However, a caveat to this model is the potential requirement for ubiquitin-conjugation in situations where acidic activation domains contribute to the turnover of the transcription factor [Muratani and Tansey, 2003], and the ubiquitin-independence of the pp71-mediated degradation reaction [Kalejta and Shenk, 2003b].

Alternatively, pp71 may degrade both the Rb and E2F component of the complex it targets, which might send an attenuated growth signal. However, fibroblasts lacking the three activating E2Fs (E2F-1, -2, -3) cannot progress through the G_1 phase [Wu et al., 2001], making this model less likely. If it targeted only certain Rb–E2F complexes (e.g., those containing E2F-4), pp71 expression could result in the de-repression of E2F-responsive promoters, but not their activation, perhaps leading to cell cycle stimulation, but not transformation. This model (Fig. 2B) could also explain why pp71 fails to induce apoptosis, because complexes containing the activating E2Fs would not be targeted. With the finding that E2F-1 makes unique complexes with Rb that can be distinguished from other Rb–E2F complexes [Dick and Dyson, 2003], this becomes a distinct possibility. Each of these models although speculative, is testable, and can act as a starting point to explore the relationship between pp71, the Rb and E2F families, cell cycle progression, and carcinogenesis.

CATCHING UP WITH E1A, T ANTIGEN, AND E7 (AND THEN PASSING THEM!)

pp71 represents an opportunity to expand our vision of the cell cycle, apoptosis and transformation, since it shares some, but not all of the properties of the DNA tumor virus proteins.

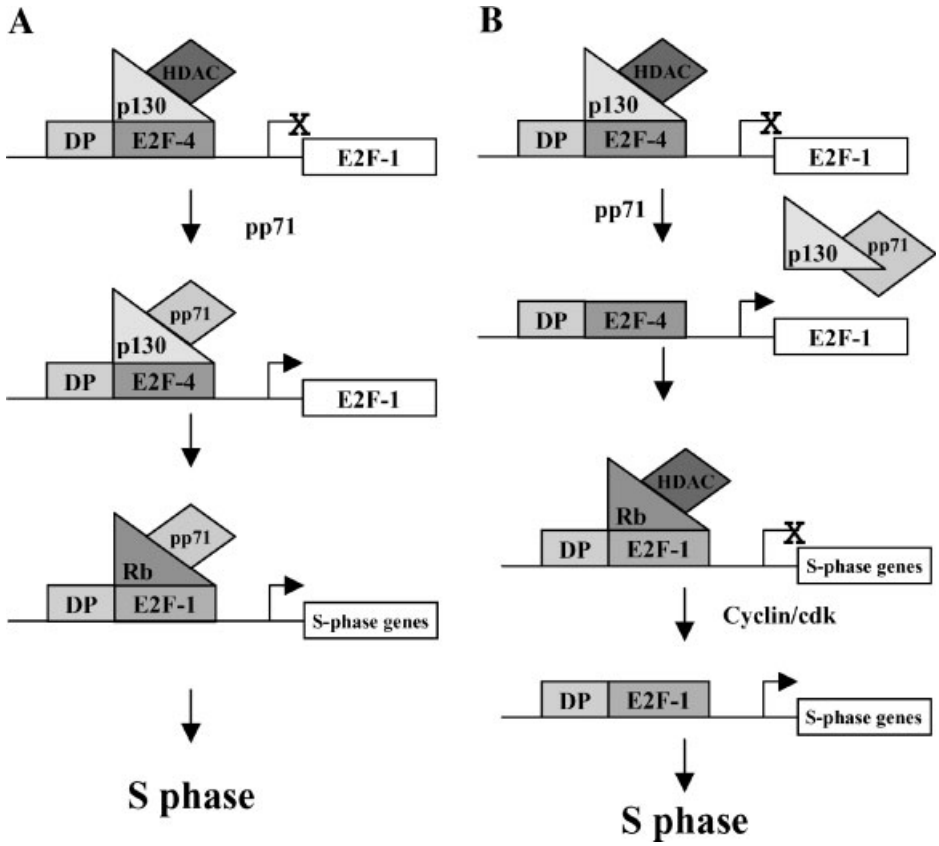

Fig. 2. Potential models for pp71 activity. **A**: pp71 dissociates HDAC from the p130–E2F4 complex. This new complex remains bound to the promoter and the activation domain of pp71 induces the expression of the *E2F-1* gene. pp71 also interacts in a similar fashion with Rb–E2F1 complexes, resulting in the stimulation of cell cycle progression, but neither transformation nor apoptosis. **B**: Expression of pp71 disrupts p130–E2F4 complexes, activating the expression of E2F1, but pp71 cannot disrupt Rb–E2F1 complexes. However, cell cycle progression has been initiated, and thus cyclin dependent kinase activity disrupts these complexes. This leads to cell cycle progression into the S phase, but neither transformation nor apoptosis. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Thus, it serves as a good foil for these valuable viral tools, and has the potential to help us focus in on the truly critical molecules that these viral proteins target to alter the cell cycle, apoptosis, differentiation, and carcinogenesis.

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