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pRB, p107 and the regulation of the E2F transcription factor

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SUMMARY

Small DNA tumor viruses, such as adenovirus, encode proteins that deregulate the cell cycle. These proteins are potent transforming agents when tested in standard oncogenic assays. For adenovirus the best characterized viral oncoproteins are the early region 1A (E1A) products. Mutational studies have shown that E1A's oncogenic ability is determined primarily by its ability to bind to certain cellular proteins and interfere with their function. One of these cellular targets for E1A is the product of the retinoblastoma tumor suppressor gene, pRB. pRB is a negative regulator of cell proliferation, and its inactivation has been shown to be an important oncogenic step in the development of many human cancers. In adenovirus-mediated transformation, E1A binds to pRB and inactivates it, thus functionally mimicking the loss of pRB often seen in human tumors.

There is now compelling evidence to suggest that pRB regulates transcription at specific phases of the cell cycle by physically associating with key transcription factors. The best characterized target of pRB is the transcription factor E2F. The interaction of pRB and E2F leads to the inhibition of E2F-mediated transactivation. Most of the genes that are known to be controlled by E2F have key roles in the regulation of cell proliferation. During cell cycle progression, phosphorylation of pRB appears to change its conformation and E2F is released. In pathogenic settings E2F transactivation is not regulated by pRB binding. In human tumors with mutations in the retinoblastoma gene, functional pRB is absent and hence can no longer inhibit E2F activity. During adenovirus transformation, E1A binds to pRB and displaces E2F. In both these cases, E2F is released from pRB-mediated regulation at inappropriate times. The activation of these E2F-responsive genes may lead to the stimulation of cell proliferation. While we do not know whether E2F is the only target for pRB action, this work has formed a general picture of how tumor suppressor gene products such as pRB can control specific transcriptional events and act as negative regulators of cell growth.

Recent experiments have shown that E2F represents the combined activity of an extensive series of protein complexes. There are at least five genes that encode E2F polypeptides, and probably several more have yet to be identified. The E2F transcription factor is a heterodimer composed of two related polypeptides, one encoded by a member of the E2F gene family and the other by a member of the DP family. Intriguingly DP and E2F genes are also found in *Drosophila* and these may provide alternative approaches to the investigation of E2F function. In mammalian cells E2F/DP heterodimers are regulated, at least in part, by the formation of many larger complexes. E2F is found in separate complexes with pRB, p107/cyclin A/cdk2 or p107/cyclin E/cdk2, and additional complexes exist that have yet to be fully characterized. These E2F complexes are detected at specific points of the cell cycle and appear to provide different elements of E2F regulation.

Key words: pRB, p107, E2F transcription factor, regulation

pRB AND p107 ARE CELLULAR TARGETS FOR E1A-MEDIATED TRANSFORMATION

The E1A proteins are the first viral proteins to be synthesized during adenovirus infection (Lewis and Mathews, 1980; Nevins, 1981). Although these proteins are multifunctional, their overall function is to alter infected cells, changing their metabolism for a state more conducive for viral propagation. When E1A is studied by itself in the absence of other viral functions, it proves to be a potential nuclear oncoprotein. These activities are thought to drive cell cycle progression into S phase, where viral DNA synthesis can begin.

The regions of E1A that enable it to drive cell cycle progression correspond closely to the regions that are essential for E1A's oncogenic properties (Howe et al., 1990; Howe and Bayley, 1992). These regions include several domains that allow the interaction with cellular proteins (Whyte et al., 1989). There are two major classes of interaction, one with a cellular protein known as p300 and one with a group of related proteins that include pRB, p107, and p130. These two classes of interaction can be distinguished by the regions on E1A that make the contacts with the cellular proteins. Binding to p300 requires the amino-terminal regions of E1A between residues 1 and 76, while binding to pRB, p107, and p130 requires two independent interaction domains encompassing residues 35-60 and 120 to 127 (Whyte et al., 1989; Giordano et al., 1991a,b). Mutations in either region, which destroy the ability of E1A to bind to these cellular proteins, destroy E1A's ability to act as an oncogene.

Analysis of E1A's binding to pRB, p107, and p130 has

shown that these proteins are found in separate complexes, with E1A using similar sequences to bind to each protein independently. Adenovirus is not the only DNA tumor virus that encodes proteins that can bind to these cellular proteins. Both the polyomaviruses, best represented by SV40, and the human papillomaviruses encode proteins that bind to pRB and p107 (DeCaprio et al., 1988; Dyson et al., 1989a,b, 1992; Ewen et al., 1989). In vitro binding experiments suggest that these viral proteins will also bind to p130. Large T antigens of the polyomaviruses and the E7 proteins of human papillomaviruses show structural homologies to E1A in the regions that are needed to bind to pRB and p107 (Stabel, 1985; Figge et al., 1988; Phelps et al., 1988). In these viruses too, the pRB- and p107-binding regions are important for the viral proteins to act as oncogenes (Ewen et al., 1989; Munger et al., 1989; Larose et al., 1990). Thus, it appears that many small DNA viruses target cellular proteins such as p107 and pRB and that these interactions are an integral part of the oncogenic properties of their early products.

THE RETINOBLASTOMA TUMOR SUPPRESSOR GENE

The *RB-1* gene was the first tumor suppressor gene to be isolated and recognized for its involvement with human cancer. Inactivation of both copies of the *RB-1* gene has been found in 100% of retinoblastomas, and the inheritance of a mutated *RB-1* allele has been shown to be the genetic lesion in familial retinoblastoma (reviewed by Weinberg, 1988, 1992). Following the isolation of the *RB-1* gene considerable effort was focused into determining the biochemical function of its protein product (pRB). Although initial progress was slow, discoveries made during the last two to three years have allowed the development of models for the function of pRB. Many lines of evidence indicate that pRB serves as a regulator of transcription factors. Several factors have been shown to bind to pRB, including c-myc, N-myc, E2F (also called DRTF1), elf1, PU1, myoD, myogenin, ATF2 and abl (Bandara and La Thangue, 1991; Chellappan et al., 1991; Rustgi et al., 1991; Kim et al., 1992; Gu et al., 1993; Hagemeier et al., 1993; Wang et al., 1993; Welch and Wang, 1993). Of these, the best studied is the transcription factor E2F, and the pRB/E2F interaction is discussed in more detail below. E2F-mediated transcription is inhibited by its association with pRB (Hiebert et al., 1992; Zamanian and La Thangue, 1992; Helin and Harlow, 1993). pRB binding has also been proposed to repress several other factors, including c-myc, N-myc, Elf-1, PU1 (Rustgi et al., 1991; Hagemeier et al., 1993; Wang et al., 1993). Although these complexes are less well studied than E2F, the available data suggests that these interactions have many similarities to the E2F/pRB complex. In other cases (ATF-2, MyoD and myogenin) the pRB interaction is less well understood but may be fundamentally different since the association is proposed to cause transcriptional activation (Kim et al., 1992; Gu et al., 1993; Wang et al., 1993).

The *RB-1* gene is the prototype for all tumor suppressor genes. Loss or mutation of both *RB-1* alleles is a key step in the tumorigenic progression of many human cancers (see, for example, Weinberg, 1990). This loss or inactivation of functional pRB removes an inhibitor to cell proliferation and thereby promotes inappropriate division. Since E1A is a positively acting oncoprotein, the demonstration that pRB was one of the proteins bound to E1A led to a suggestion that E1A overcame pRB's negative regulatory role, mimicking the loss of pRB seen in many tumors (Whyte et al., 1988). Further work has shown that model to be correct.

A FAMILY OF POCKET PROTEINS - POTENTIAL CELL CYCLE REGULATORS

p107 was first identified through its interaction with the adenovirus early protein E1A (Yee and Branton, 1985; Harlow et al., 1986). The study of p107 has drawn further attention following the cloning of a partial cDNA by Ewen et al. (1991). This original clone represents the carboxy-terminal 90% of the full-length coding region for p107 recently isolated by Zhu et al. (1993). Many of the properties of p107, including how it interacted with E1A, suggested that it would be related to pRB. The sequence of the first p107 clone proved these suggestions correct (Ewen et al., 1991). p107 and pRB have 7 regions of recognizable sequence homology, the best homology showing 62% identity. Not surprisingly, a great deal of the homology was found in a region that corresponds to the binding site for E1A. This region, which has become known as the 'pocket', is composed of two essential segments separated by a 'spacer' region that is required for binding but whose sequence is irrelevant for interaction. In addition there is good homology in the carboxy-terminal region of pRB and p107.

The recent cloning of p130 has revealed that it is closely related to pRB and p107 (Hannon et al., 1993; Li et al., 1993; Mayol et al., 1993). The p130 protein also contains a pocket-domain structure that mediates its interaction with E1A. Sequence comparisons show that p130 and p107 are more closely related to one another than to pRB. This is most apparent in the comparisons of the spacer domains. Studies of p107 have shown that the spacer region contains a high-affinity binding site for the cyclin A/cdk2 and cyclin E/cdk2 kinases (Ewen et al., 1992; Faha et al., 1992, 1993) and analogous complexes may also exist for p130 (Hannon et al., 1993; Li et al., 1993). Although the spacer sequences of p107 and p130 are 44% identical neither sequence has any homology with the spacer domain of pRB and it appears that the stable association with cyclin-dependent kinases is a feature that distinguishes p107 and p130 from pRB. The function of these complexes are unknown; however, the stoichiometry is high. Up to 50% of the p107 found in the cell is associated with these cyclin/cdk complexes. This high level of interaction suggests that they are likely to play an important role in the action of p107 and p130.

Several approaches have been used to demonstrate that the reintroduction of pRB into tumor cells that lack a normal *RB-1* can cause cell cycle arrest (Goodrich et al., 1991; Hinds et al., 1992). Analogous experiments have shown that p107, when transiently expressed at high levels after transfection, can also cause cell cycle arrest (Zhu et al., 1993). In these experiments p107 was cotransfected with a second plasmid that expresses a cell surface marker that enables transfected cells to be identified after flow cytometry. Comparison between the growth suppression properties of pRB and p107 has enabled some initial conclusions to be drawn (Zhu et al., 1993). Many

cell lines are unaffected by the overexpression of pRB and p107, even cell lines such as J82 that lack a functional *RB-1* gene. A few cell lines, however, appear to be sensitive to these proteins and expression causes cell cycle arrest. Saos-2 cells, an osteosarcoma cell line, respond to pRB and p107 in an similar manner and arrest in G_1. However, the responses are not identical in other cells. In C33A cells, the overexpression of p107 caused cell cycle arrest in G_1, but the expression of pRB did not. This was one of the first indications that pRB and p107 have distinguishable functional characteristics. A second feature of these experiments reinforced this suggestion. These differences are apparent in the ability of various other genes to overcome growth arrest in G_1 induced by pRB or p107. Hinds et al. (1992) have demonstrated that co-transfecting G_1 cyclins overcomes the pRB arrest. However, in similar conditions these cyclins had little effect on the p107 block (Zhu et al., 1993). Similarly the over-expression of the E2F-1 gene overcame the pRB block but had no effect on the p107 arrested cells. Conversely the adenovirus E1A protein, which binds to both pRB and p107, overcame the p107 arrest with considerably greater efficiency than the pRB arrest. These different rescue properties provided a further indication that pRB and p107 have related but distinct potential for growth arrest.

THE E2F TRANSCRIPTION FACTOR

The first clues to the biochemical role of pRB came from other viral studies looking at the activation of the E2 promoter of adenovirus. Following infection, the E2 promoter is transactivated by E1A. One of the regions of the promoter that allowed E1A transactivation was called E2F for E2 factor (Yee et al., 1987). Adenovirus E1A activates transcription from the E2 promoter by releasing active E2F from inhibitory complexes (Bagchi et al., 1993). When the regions of E1A that were needed to cause this transactivation were mapped they corresponded with the regions that were needed to bind to pRB, p107, and p130 (Raychaudhuri et al., 1991). Purified E2F was examined and shown to be physically associated with pRB (Bagchi et al., 1991; Chellappan et al., 1991). More recent work has shown that by binding to E2F, pRB is able to inhibit E2F-mediated transcription (Hiebert et al., 1992; Zamanian and La Thangue, 1992; Helin and Harlow, 1993). Thus, one role of pRB is to bind to E2F and inhibit transcription. E1A breaks apart this interaction and leads to the activation of E2F-mediated transcription.

Purification of E2F on DNA-affinity columns suggested that it was a heterogeneous factor and that DNA binding required at least two different components (Huber et al., 1993). This impression has been confirmed by the cloning of at least 5 genes encoding components of E2F (Helin et al., 1992; Kaelin et al., 1992; Shan et al., 1992; Girling et al., 1993; Ivey-Hoyle et al., 1993; Lees et al., 1993). These genes fall into two classes, termed E2F or DP, whose products heterodimerize to produce a DNA-binding complex capable of activating transcription from promoters containing E2F sites (Bandara et al., 1993; Helin et al., 1993). In transient transfections the products of any one of the E2F-1, E2F-2, and E2F-3 genes heterodimerize with either of the DP-1 or DP-2 encoded proteins producing a transcriptional activator (J. Lees and C.-L. Wu, personal communication). Northern analyses suggest that some of these genes may be differentially expressed, but no functional differences between these complexes are known (Lees et al., 1993).

The cloning of E2F genes has allowed a detailed examination of the pRB/E2F interaction. pRB binds directly to the transactivation domain of E2F and represses its activity (Helin et al., 1994). This interaction appears to be regulated in several ways. One of the mechanisms of regulation appears to be at the level of protein phosphorylation. pRB contains several sites that are substrates for many of the cyclin-dependent kinases (cdks) that are active at discrete points of the cell cycle, and pRB becomes heavily phosphorylated as cells progress from G_1 into S phase (Buchkovich et al., 1989; Chen et al., 1989; DeCaprio et al., 1989; Mihara et al., 1989). Despite this, only the un- or underphosphorylated forms of pRB are found associated with E2F (Chellappan et al., 1991; Helin et al., 1992; Kaelin et al., 1992), thus the release of E2F from pRB may provide a temporal control to E2F activity.

POTENTIAL TARGETS FOR E2F

One of the outcomes of this regulation is that adenovirus infections lead to the activation of E2F-mediated transcription. While adenovirus has an early promoter that is activated by E2F, there are no E2F-responsive promoters in the polyomaviruses or the papillomaviruses. Therefore, the target promoters that are affected by viral proteins binding to pRB must be different for adenovirus. A potential answer to this problem comes from the demonstration that many cellular promoters that control genes involved in growth control also have E2F sites. These genes include those encoding c-myc, N-myc, b-myb, DNA polymerase-α, DHFR, thymidine kinase, thymidylate synthase, cdc2, and cyclin A (for a review see (Nevins, 1992; Helin and Harlow, 1993). In the c-myc, DHFR, b-myb, thymidine kinase, and cdc2 promoters the E2F sites have been shown to be essential for the transcriptional activation of these genes that occurs as serum-starved cells are stimulated to progress through the cell cycle (Blake and Azizkhan, 1989; Hiebert et al., 1989; Thalmeier et al., 1989; Dalton, 1992; Means et al., 1992; Lam and Watson, 1993). In this experimental system, the presence of a short element carrying 2 overlapping E2F sites has been shown to be sufficient for the temporal expression of the DHFR gene at the G_1 to S transition (Slansky et al., 1993). The view that emerges from these studies is that the transcription of a broad range of important growth-regulating genes appears to be regulated by E2F or E2F-related proteins.

Based on this information, the loss of pRB function is predicted to deregulate the transcription of E2F-responsive genes. The notion that this may contribute to the deregulation of cell cycle control is supported by several observations. First, the microinjection of E2F-1 protein stimulates DNA synthesis in quiescent cells (Johnson et al., 1993). Second, transfection of E2F-1 into WI38 fibroblasts inhibits cell cycle arrest upon serum withdrawal (Johnson et al., 1993). Third, the recent finding that Chinese hamster ovary cells are fully transformed by the cotransfection of E2F-1 and DP-1 (N. Heintz, personal communication). Fourth, the E2F-1 gene appears to undergo genetic rearrangements in some leukemias (Saito et al., 1994). All of these data point to an important role for E2F in the control of cell proliferation.

THERE ARE MANY E2F COMPLEXES

pRB is only one of several regulators of E2F. When extracts from various cell lines were tested for protein complexes that can bind specifically to E2F oligonucleotides, at least two major complexes were detected. One of these was identified as the pRB/E2F complex (Bandara and La Thangue, 1991; Chellappan et al., 1991). The other larger complex was originally demonstrated to contain cyclin A (Mudryj et al., 1991). The appearance of cyclin A in the complex prompted several labs to investigate whether other proteins might be found in this complex. Work from several groups showed that this E2F-binding complex contains p107, cyclin A, and cdk2 (Cao et al., 1992; Devoto et al., 1992; Shirodkar et al., 1992). Further analysis of p107/E2F showed that it actually contained two complexes, one with E2F/p107/cyclin A/cdk2 and one with E2F/p107/cyclin E/cdk2 (Lees et al., 1992). Surprisingly, these complexes exist in different stages of the cell cycle. The cyclin E/cdk2 version is found in G_1, while the cyclin A/cdk2 E2F/p107 complex is found in S phase.

Bandshift experiments using extracts from synchronized cells showed that serum-starved cells and cells that were early in G_1 phase contained E2F complexes that did not appear to contain either pRB or p107 (Mudryj et al., 1991; Shirodkar et al., 1992). Recently Cobrinik et al. (1993) have demonstrated that these complexes contain the p130 protein, and like p107, the p130/E2F complexes can also be formed with cyclin dependent kinases. By analogy with pRB, p107 and p130 may also be repressors of E2F activity. During adenovirus infection E1A dissociates all of the pRB-, p107-, and p130-E2F complexes and this correlates with the activation of E2F. Furthermore the transient overexpression of p107 has been shown to repress the activity of both endogenous and exogenous E2F (Schwarz et al., 1993; Zamanian and Thangue, 1993; Zhu et al., 1993).

The E2F complexes with p107 and p130 provide an added level of intrigue. Most of the E2F/p107 and E2F/p130 DNA-bound complexes contain stoichiometric levels of the cdks. These complexes provide a direct connection between the kinases that regulate cell cycle progression and the temporal expression of genes that are required for cell proliferation. It is widely speculated that this connection is important for the functions of both the kinases and E2F, but the consequence of the interaction is unclear. Many models have been proposed; examples are: that the complex targets the kinase to a DNA-bound substrate; that the complex targets the kinase to E2F/DP (conceivably it switches the factor on or off); that the kinase phosphorylates p107 or p130 and regulates the p107/E2F or p130/E2F interaction; that E2F/DP sequesters the kinase away from other substrates.

pRB AND p107 ASSOCIATE WITH DIFFERENT E2FS

Work in the last several months has emphasized the differences between pRB and p107. Analysis of the polypeptides that co-precipitate with p107 and pRB has revealed that DP-1 and DP-2 are associated with both pRB and p107 (Girling et al., 1993; C.-L. Wu and J. Lees, personal communication) but that the E2F components of the heterodimers differ (Dyson et al., 1993; Lees et al., 1993). The E2F polypeptides migrate differently on SDS-PAGE and yield different patterns after partial proteolytic digestion. Furthermore, antibodies raised against the products of cloned E2F genes differ in their ability to recognize the pRB-associated and p107-associated E2F proteins. Polyclonal antisera specific for E2F-1, E2F-2 and E2F-3 all immunoprecipitate pRB-associated E2F but fail to recognize p107-associated E2F (Dyson et al., 1993; Lees et al., 1993). To date only one E2F-1 antibody, a pan-reactive anti-peptide antibody that was raised against the pRB-binding domain of E2F-1, has been found to immunoprecipitate both pRB- and p107-associated E2F polypeptides. These findings suggest that pRB and p107 regulate different forms of E2F. Thus, although p107 and pRB both associate with E2F they appear to provide different elements of the regulation. It is unclear whether pRB-E2F and p107-E2F regulate a single set of genes in different ways or whether they act on different E2F-responsive promoters

ADDITIONAL TIERS OF E2F REGULATION

Several lines of evidence suggest that there may be tiers of regulation in addition to the interactions mentioned above. Phosphorylation of E2F is likely to be one mechanism of control. Early studies of partially purified E2F indicated that DNA-binding activity was influenced by phosphorylation state and many of the E2F polypeptides undergo extensive modifications (Bagchi et al., 1989; Yee et al., 1989). It is also clear that the levels of E2F proteins are an additional point of regulation. Initial studies of E2F-1 have revealed that the level of E2F-1 mRNAs and proteins are under strong regulation during the cell cycle, as E2F-1 mRNA is not found in G_0 cells (Kaelin et al., 1992). Raychaudhuri et al. (1991) have shown that dissociation of E2F complexes by detergent treatment greatly increases the total amount of E2F-DNA binding activity, suggesting that the DNA-binding activity of E2F was regulated by protein association. These workers purified a subset of E2F/pRB complexes that were unable to bind to DNA (Bagchi et al., 1991) and identified an associated inhibitory factor that modulated the DNA-binding properties of E2F/pRB complexes (Ray et al., 1992). Although the E2F/pRB complexes are not predicted to have transactivating properties, they may play an important role in regulation of gene expression. Such a role was suggested by Weintraub et al. (1992) who observed that E2F sites can act as silencing elements in the presence of high levels of pRB.

There are also some indications that the phosphorylation of pRB may not be the only regulator of the pRB/E2F interaction. Experiments using synchronized cells suggest that some of the pRB/E2F complexes persist late into S-phase (Shirodkar et al., 1992; Schwarz et al., 1993) by which time the majority of pRB is heavily phosphorylated and the transcription of many of the proposed E2F-regulated genes has already been elevated. This may indicate that different complexes are regulated at different times or that E2F transactivation has occurred prior to the disappearance of the pRB/E2F complex, presumably at a time when the pRB/E2F complex is not fully dissociated by pRB phosphorylation. It has been known for several years that some cell lines (the F9 embryonal carcinoma cell line is probably the best studied example) have normal levels of E2F and pRB but lack any of the larger E2F complexes (La Thangue et al., 1990). It has been suggested that these cells contain an E1A-

like activity that inhibits the pRB/E2F interaction, although the nature of this activity is unknown.

E2F GENES IN *DROSOPHILA*

All this supports the notion that the normal regulation of E2F in mammalian cells is a highly complex process, and it is clear that the E2F/pRB complex is only one aspect of an intricate network of controls. Currently, many groups are studying the biochemistry of E2F complexes. However, many of the fundamental questions about E2F function have yet to be addressed and a full investigation of E2F regulation will require both genetic and biochemical approaches.

To complement ongoing biochemical studies of E2F we have isolated E2F and DP genes from *Drosophila* and demonstrated that they are functional homologs of the human E2F genes in transactivation (Dynlacht et al., 1994). Both of the genes have been localized to regions of the *Drosophila* genome that have been extensively studied. If mutation of these genes leads to a clear phenotype then it may be possible to use genetic approaches to study the E2F pathway. Such a system may provide a unique opportunity to investigate the composition of the E2F pathway and its function. In particular it would be interesting to determine whether the E2F pathway is essential for viability and to investigate the role of E2F in normal development.

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