The Rb/E2F pathway and cancer

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Over the past decade, studies focusing on the mechanisms controlling cellular proliferation have converged with equally intensive efforts directed at the analysis of oncogenic pathways associated with human cancer. These convergent studies have revealed the central role played by the pathway that controls the activity of the retinoblastoma tumor suppressor protein (Rb), which in turn regulates the E2F transcription factor. In particular, it is now clear that the Rb/E2F pathway is critical in regulating the initiation of DNA replication. It is also clear that the control of the pathway is disrupted in virtually all human cancers. Questions remain, however, as to the specific role played by individual activities within the pathway in the control of cell growth and their participation in the development of cancer.

THE Rb/E2F PATHWAY IN CELL PROLIFERATION

The retinoblastoma gene was initially identified as a genetic locus associated with the development of an inherited eye tumor (1,2). The realization that it was a loss of function of Rb that was associated with disease established the tumor suppressor paradigm (3). Subsequent work identified the E2F transcription factor activity as a key target for the growth suppressing action of the Rb protein (4–6). Additional work demonstrated that Rb function, including the ability to interact with E2F, was regulated by phosphorylation and that the primary kinase responsible was the D-type cyclin-dependent kinases (7–9). D cyclin/cdk4 activity is induced by growth stimulation, thus initiating the cascade of events that leads to E2F accumulation and S-phase entry (10) (Fig. 1).

The role of E2F transcription factor activity in controlling the transition from G1 to S phase has become clear from a large number of studies that have identified the E2F regulated genes (11,12). These include genes that encode DNA replication activities and cell cycle regulatory activities. Indeed, it is now clear that in addition to the various replication proteins such as DNA polymerase α and proliferating cell nuclear antigen, nucleotide biosynthetic activities including thymidine kinase, thymidylate synthase and ribonucleotide reductase, and various DNA repair activities such as RAD51, virtually the entire apparatus of initiation factors that assemble a pre-replication complex at origins of replication is under the control of the Rb/E2F pathway.

First, as discussed above, the transcriptional activation targets of the pathway, the genes subject to control by the E2F family of transcription factors, include the majority of genes encoding DNA replication proteins. Second, the deregulation of the pathway is the primary function of each of the DNA tumor virus oncoproteins that promote cellular proliferation (11). This includes the adenovirus E1A protein, SV40 T antigen and human papillomavirus E7 protein. In each case the viral protein targets Rb and inactivates its function. Indeed, it is interesting to note the studies of Howley and colleagues (16) examining the nature of mutations found in human cervical carcinoma cells, which revealed a relationship between the status of Rb and the expression of the HPV E7 protein which functions to inactivate Rb. Cells that were E7 positive were Rb wild-type and those tumors that showed no evidence of papillomavirus involvement, and were thus E7 negative, showed evidence of Rb mutation. Third, as discussed in detail below, the pathway is disrupted in virtually all human tumors; not a surprising observation given the critical role of the pathway in regulating the transition of cells from a quiescent state into S phase, but nevertheless representing important additional evidence regarding the critical role of the pathway.

THE Rb/E2F PATHWAY IN CELL FATE DETERMINATION

In addition to the role of the Rb/E2F pathway in the control of cell proliferation, it is also clear that this pathway is linked to events that determine cell fate through an induction of apoptosis. Two observations were key in providing this link. First, studies of adenovirus mutants revealed a role for the viral E1A protein in the induction of p53-dependent cell death, dependent on E1A domains known to be involved in binding to Rb (17,18). Second, initial studies by Wu and Levine (19) demonstrated that the deregulated expression of E2F1 in quiescent cells led to an induction of p53-dependent apoptosis. Further studies...
have shown that this is an activity largely unique to E2F1 and involves an ability of E2F1 to trigger p53 protein accumulation (20).

Additional evidence for a role of E2F1 as a signal for apoptosis can be found in the analysis of E2F1 knockout mice. The original description of the E2F1 null phenotype included both an increased rate of tumor development (21) and a decreased rate of thymic apoptosis (22). More recently, loss of E2F1 function has been shown to severely impair thymocyte negative selection (23) and also to reduce apoptosis seen in the lens of Rb-deficient embryos (21). These results thus suggest a role for E2F1 in the increased apoptosis seen as a result of loss of Rb function as well as a physiological role for E2F1 in maintaining the homeostatic balance of T lymphocytes.

Recent work that has detailed a pathway controlling the accumulation of p53 has provided a link between the Rb/E2F pathway and the p53 response (Fig. 2). This follows from the identification of the p19ARF gene (24) and subsequent work that established a role for ARF in the control of Mdm2 function (25–27). Mdm2 had previously been shown to function as a p53 ubiquitin ligase, targeting p53 for destruction (28,29). Thus, the accumulation of ARF blocks Mdm2 and hence allows p53 to accumulate. The link to the Rb/E2F pathway was established by the observation that the p19ARF gene is an E2F target (30,31). Thus, the induction of E2F1 accumulation that results from loss of Rb function then activates the ARF/Mdm2/p53 pathway, leading to induction of apoptosis.

It is also true that E2F1 can induce apoptosis independent of p53 and one mechanism appears to involve the p73 homologue of p53. Recent work shows that E2F1 induces p73 transcription and that p53-independent apoptosis in response to E2F1 is reduced in cells that are deficient for p73 (32,33).

### Rb/E2F Pathway Mutations in Cancer

Given the clearly important role played by the Rb/E2F pathway in controlling cell growth, representing the critical series of events leading to induction of DNA replication and S phase, it is not surprising that oncogenic mutations are seen to disrupt the normal function of the pathway. In principle, one need only to inactivate one step in the regulatory pathway outlined in Figure 1 to achieve a deregulation of E2F activity and thus a deregulation of cellular growth control. Indeed, the analysis of human cancers has shown that mutations within the Rb/E2F pathway are usually not duplicative, i.e. a tumor carrying an Rb mutation does not generally exhibit a mutation in a second gene within the pathway.

The analysis of human tumors has revealed a wide spectrum of mutations that alter the Rb/E2F pathway. Although mutation of the Rb gene was first observed in inherited retinoblastoma (2), it is clear that loss of Rb function contributes to a wide array of human cancers (34–36). Indeed, the role of Rb in the development of sporadic tumors of somatic origin is considerably greater, in terms of numbers of cases, than the contribution of Rb to inherited eye tumors. Rb mutations, consistent with loss of Rb function, have been identified in a wide spectrum of tumors including osteosarcomas, small cell lung carcinomas, breast carcinomas and others.

After Rb, the most frequent mutation in human cancers disrupting the regulation of the Rb/E2F pathway involves the p16INK4a cyclin kinase inhibitor. The p16 protein is responsible for the control of D cyclin/cdk4 kinase activity. Thus, in the absence of p16, D/cdk4 activity is elevated, leading to Rb phosphorylation and E2F accumulation. As such, the absence of p16 activity is functionally equivalent to loss of Rb. Inherited mutation of p16INK4a and subsequent loss of the wild-type allele in tumors is observed in melanoma but like Rb, loss of p16 function is much more prevalent in sporadic cancers of a variety of types.

Finally, deregulated expression of the D-type cyclins, as well as the cdk4 gene, leads to an increased level of D/cdk4 activity and thus deregulation of the pathway. Both amplification as well as translocation of the D1 cyclin gene has been observed in a variety of human cancers and amplification of the cdk4 gene has been seen in sarcomas and gliomas (35).

Strikingly absent from the list of genetic alterations involving the Rb/E2F pathway are the E2F genes themselves, the activity of which is the ultimate event in the activation of the pathway. Based on the analysis of E2F function in cell culture as well as mouse models, one might expect to find deregulation of E2F2 or E2F3, genes that appear to play a positive role in cell proliferation, or loss of E2F4 or E2F5, the products of which function together with Rb family members to repress transcription in quiescent cells (11,12). In addition, one might...
also expect to observe loss of E2F1 in human tumors, given the role of the E2F1 protein as a signal for apoptosis. Nevertheless, although loss of E2F1 in the mouse does lead to tumor formation (37), no such examples have been found in human cancers. Moreover, although there have been a few isolated descriptions of mutations in the trinucleotide repeat element found in the E2F4 gene (38–41), these have not been widespread, nor proven to be causative of the cancer phenotype.

**QUESTIONS**

Although the role of the Rb/E2F pathway in normal cell proliferation is firmly established, as is the fact that deregulation of the pathway is a common occurrence in human cancer, a number of critical questions still remain. These include issues of the relationship of particular gene mutations with particular cancer types, the apparent special role for Rb in the development of retinoblastoma and the absence of mutations in other members of the family of proteins that function in the pathway.

In light of the central role played by Rb/E2F in the control of cellular proliferation, one might expect that alterations in the pathway would be central to all tumors. Yet the analysis of colon carcinoma has so far shown little evidence for mutations within the genes encoding proteins in the Rb/E2F pathway (35,42–45). Of course, it is possible that yet to be identified activities that directly participate in the control of Rb/E2F function will be shown to be mutated within these cancers. Alternatively, it is possible that, for whatever reason, mutations that are outside the pathway but nevertheless impact on the regulation of the pathway are primary in these cancers. One such case of relevance to colon cancer is the recent demonstration of a role for β-catenin/Tcf4 in the activation of cyclin D1 expression (46). Since β-catenin accumulation is regulated by adenomatous polyposis coli (APC), and mutations in APC are found in most human colon cancers, activation of the Rb/E2F pathway would be one consequence of loss of APC function. An additional possibility relates to the role of the Myc protein in affecting the Rb/E2F pathway. Recent studies have implicated Myc in the control of Cdk4 expression (47). Although the Myc gene is not frequently amplified in colon cancers, there is frequently overexpression of the Myc gene. One possible cause could again relate to APC control, since other work has provided evidence for a role for β-catenin/Tcf4 in the control of Myc transcription (48).

Finally, additional work has demonstrated a role for Myc in controlling the activation of the E2F1, E2F2 and E2F3 genes (49,50) and recent evidence demonstrates a requirement for these E2F genes for full Myc function (G. Leone, R. Sears, E. Huang, R. Rempel, F. Nuckolls, C.H. Park, S.J. Field, M.A. Thompson, H. Yang, Y. Fujiwara et al., unpublished data). Thus, it is possible that deregulation of Myc as a result of loss of APC control as well as gene amplification, contributes in multiple ways to the activation of the E2F/Rb pathway (Fig. 3).

Although individuals that inherit one mutant form of the retinoblastoma gene develop retinoblastoma with nearly 100% probability, they exhibit only a moderately increased risk for other forms of cancer, most notably osteosarcoma. One must assume that an individual carrying only one functional copy of the Rb gene is likely to have many instances during their lifetime of loss of the second allele within a dividing cell. A possible explanation for the fact that these events do not lead to tumor formation, and the preponderance of tumors within particular tissues, could relate to the role of the Rb/E2F pathway linking to the p53 response. Most likely, a cell that suffers a complete loss of Rb function due to loss of a chromosome during mitosis will activate the p53 response as well as p73, undergo apoptosis and thus not survive to generate a tumor mass. Only when such a cell also harbors a mutation within the p53 response pathway would the deregulation of Rb have the potential to further develop. Thus, at least some of the tissue specificity might be attributed to differences in the potential for p53 mutagenic events.

This logic, which couples a p53 response to the deregulation of the Rb/E2F pathway, falls in contrast to the situation seen in retinoblastoma. Unlike most other cases, these tumors appear to arise primarily as a result of loss of function of one gene—the Rb gene. Although other loci are implicated in the development of retinoblastoma, such as a locus at chromosome 6p (51), the involvement of multiple genes other than Rb does appear to be limited and is in contrast to the development of most other tumor types. Most striking is the absence of p53 mutations in retinoblastoma, in contrast to the situation in the vast majority of other tumors. Thus, retinoblastoma appears to be, in fact, a ‘special case’, one that develops without the usual multiple genetic alterations characteristic of other somatic tumors. Why this is, and why there is an absence of a p53 response seen in other tissue types, is unclear.

It is also true that the mutations seen in human cancers do not extend to all members of the families of proteins operating in the pathway. For instance, although mutation of the Rb gene is frequent, mutations in other members of the Rb family, which includes the p130 and p107 genes, are not common events in human cancers. Why this might be the case is not clear, although it does appear that Rb plays a more widespread role in the control of E2F activity than the other Rb family members (52). Nevertheless, although mutations involving p107 have not been described, one study has identified loss of function mutations within the p130 gene in small cell lung carcinoma cell lines (53). More recently, a screen for p130 mutations in a series of lung carcinoma samples revealed that 79% of the samples exhibited p130 mutations (54), suggesting that the...
loss of p130 function could be an important component in the development of these tumors.

Perhaps most striking is the general lack of mutations or chromosomal alterations in members of the E2F family. Although homozgyous deletion of E2F1 in the mouse does lead to increased tumor formation in several tissues (37), possibly linked to a role for E2F1 as a signal for apoptosis, a link between E2F1 and human cancer has not been established. Likewise, mutations or alteration that would lead to loss of expression or deregulated expression of other E2F proteins have not been clearly established in human tumors. Thus, even though E2F3 appears to play a particularly important role in the control of cellular proliferation (13,55) and E2F4 appears to play a widespread role in determining cellular differentiation (56), direct alteration of these genes does not appear to contribute significantly to the development of human cancer. Alterations in the E2F4 gene involving changes in the trinucleotide repeat element have been observed, but there is no evidence as yet that these alterations have an impact on E2F4 function or that they contribute to the cancer phenotype. The potential explanations for the apparent lack of direct alterations of E2F genes in cancer are numerous but perhaps the most likely reason is the overlap in function that is evident within the family, as well as the potential disruptive role of deregulated expression, which might not allow a productive event.

IMPLICATIONS

Clearly, understanding the role of the Rb/E2F pathway in the development of human cancer has had a profound impact on the understanding of the process of normal cell growth and cell determination. As this understanding continues to grow, one hopes that insights will offer new clues to therapeutic approaches to treat cancer. Targeting the Rb/E2F pathway is particularly attractive given the fact that virtually all human cancers exhibit alterations in this pathway. In this regard, strategies focused on the control of E2F proteins hold particular promise, since activation of E2F activity is the ultimate consequence of deregulation of the Rb pathway, irrespective of the nature of the mutation. In this sense, targeting E2Fs could be a more generic approach. The problem, of course, is that the activation of E2Fs is part of the normal process of cell growth and thus any attempt to control E2F activity will have a negative impact on normal tissue that has a proliferating component. In that sense, therapeutics focused on E2F activities would not be particularly different from drugs that inhibit DNA replication activities. Moreover, as discussed above, most cancers do not involve direct alterations of the E2F genes and thus, the elevated levels of E2F activities present in tumors represent normal proteins. The more attractive possibility is to take advantage of small alterations in the normal balance of events—those changes that are not typical of the normal cell.

A variety of gene therapy-based approaches using gene replacement or gene addition as the general strategy have been described, either to block the proliferative capacity of the Rb/E2F pathway or to enhance the apoptotic role of the pathway. Although logical, these approaches are nevertheless unlikely to be effective as efficient mechanisms to stop or kill tumors. Rather, the development of small molecules that have specificity for key components of the pathway, which may play particularly important roles in deregulated events of cancer, is more likely to be successful. More specifically, Hartwell et al. (57) have proposed a therapeutic development strategy that takes advantage of a synthetic lethality concept, a process well documented in yeast whereby the combined loss of function of two genes becomes lethal. In either case, it is the development of a detailed understanding of the critical pathways and gene interaction relationships, as has now begun to develop with the Rb/E2F pathway, that will be critical for engineering the new generation of therapeutic approaches to cancer.

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