Inducible pRb2/p130 Expression and Growth-suppressive Mechanisms: Evidence of a pRb2/p130, p27Kip1, and Cyclin E Negative Feedback Regulatory Loop

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ABSTRACT

The retinoblastoma family of proteins, pRb/p105, p107, and pRb2/p130, cooperate to regulate cell cycle progression through the G1 phase of the cell cycle. Each of the family members realize their common goal of G1-S checkpoint regulation through overlapping and unique growth regulatory pathways. We took advantage of a tetracycline-regulated gene expression system to control the expression of RB2/p130 in JCV virus-induced hamster brain tumor cells to study in vivo the molecular mechanisms used by pRb2/p130 to elicit its growth-suppressive function. We have previously used this system to demonstrate that induction of pRb/p130 expression suppresses tumors growth in vivo by overcoming neoplastic transformation mediated by the large T-antigen oncoprotein of JCV (JCV TAg). Here we found that induction of pRb2/p130 in vivo specifically inhibits cyclin A- and cyclin E-associated kinase activity and by doing so induces p27Kip1 levels presumably by inhibiting p27Kip1-targeted proteolysis by cyclin E-Cdk2 phosphorylation of p27Kip1. RB2/p130 induction also decreased cyclin A and the transcription factor E2F-1 while increasing cyclin E at both the transcriptional and protein levels of expression. The growth inhibitory activity of pRb2/p130 also correlated with its E2F-binding capacity. Furthermore, p27Kip1 and pRb2/p130 were found to be targets of the JCV TAg oncoprotein and to interact in vivo with each other independently from the presence of TAg. Interestingly, pRb2/p130 expression negatively modulated the binding of p27Kip1 to JCV TAg. These data suggest that pRb2/p130 and p27Kip1 may cooperate in regulating cellular proliferation, and both may be involved in a negative feedback regulatory loop with cyclin E.

INTRODUCTION

The Rb1 family of proteins, pRb/p105, p107, and pRb2/p130, are defined by a structural and functional domain known as the pocket region. They are highly homologous within the pocket region that is responsible for many of the important protein-protein interactions for their growth-suppressive functions. The pocket domain consists of A and B subdomains divided by a spacer region. Rb family members, p107 and pRb2/p130, form their own subfamily because of their similarity in the spacer region. Each of the Rb family members are nuclear phosphoproteins that regulate G1 progression, are implicated in various forms of differentiation, are regulated in a cell-cycle-dependent manner by phosphorylation, are growth-suppressive in a cell-type-dependent manner, and are critical targets for inactivation by transforming oncoproteins of DNA tumor viruses (1, 2).

Intriguingly, several lines of evidence indicate that the functional overlap between the proteins does not extend to complete redundancy. Each of the Rb family proteins binds to and modulates the activity of the E2F family of transcription factors that stimulate the transcription of genes needed to progress through the S phase. However, the timing of this regulation varies between Rb family members, and they each bind to distinct members of the E2F family (3). pRb2/p130 in association with E2F-4 is the most abundant E2F complex found in resting or quiescent cells in G0, and this complex is thought to help maintain a state of transcriptional silence (4, 5). The pRb2/p130-E2F-4 complex negatively modulates the expression of the E2F-1 promoter linked to a CAT reporter system by a repressive E2F site in the promoter (6). In this manner, pRb2/p130 also regulates the expression of the Rb/p105 and p107 genes because each contains E2F sites in their promoters (7, 8). As the cells start to re-enter the cell cycle, E2F-4 is still found in association with pRb2/p130 in early G1, pRb2/p130 is then replaced by p107 in mid to late G1, and then by pRb/p105 in late G1 and S phases (5, 9–11). In vivo, only pRb/p105 is found in association with E2F family members E2F-1, E2F-2, and E2F-3 (12). Both E2F4 and E2F-5 were cloned for their ability to bind to distinct members of the E2F family (3). pRb2/p130 in association with E2F family members E2F-1, E2F-2, and E2F-3 is found in association with pRb/p130 in late G1 and S phases (5, 9–11).

Each of the Rb family proteins are negatively regulated by phosphorylation, which frees the E2F family of transcription factors to induce the transcription of genes whose protein products are necessary for S-phase progression. The phosphorylation status of each of the Rb family members varies throughout the cell cycle. Several cyclin-dependent kinases are implicated in this process (1). The best candidates for phosphorylation of pRb/p105 and p107 are the cyclin D1-Cdk4/6 complexes (1, 13). In vitro studies indicate that cyclin D1-Cdk4/6 complexes (1, 13). In vitro studies indicate that cyclin D1-Cdk4 complexes are capable of using pRb/p105 and p107 as a substrate. The timing of the activation of the cyclin D3-Cdk4 complex coincides with the phosphorylation profile of pRb/p130. Furthermore, pRb2/p130 associates with cyclin D3 both in vitro and in vivo, making the cyclin D3-Cdk4 complex the best candidate for phosphorylation of pRb/p130 (14).

The question of whether the cyclins are targeting these Rb family members for phosphorylation and functional inactivation or whether p107 and pRb2/p130 are modulating the function of these kinases is a complex issue. Interestingly, p107 and pRb2/p130 are modulating the function of these kinases is a complex issue. Interestingly, p107 and pRb2/p130 contain a p21-like kinase inhibitory domain that has been shown to inhibit Cdk2 kinase activity in vitro, in vivo for p107 (21, 22), and in vitro for pRb/p130 (22, 23). Additionally, a distinct kinase inhibitory domain in pRb2/p130 is located in the spacer region that specifically inhibits Cdk2 kinase activity in vitro (23).

Studies examining the growth-suppressive mechanisms used by the proteins reveal distinct differences. The growth-suppressive activity for the Rb family members is cell type-specific. Saos-2 cells, human
osteosarcoma, are growth-arrested in the G_{0}/G_{1} phase of the cell cycle by each of the Rb family members (20, 24, 25). Certain cell lines, such as the C33A human cervical carcinoma cell line, are inhibited by overexpression of p107 (20) and pRb2/p130 (26) but not by pRb/p105. Furthermore, the T98G cell line, human glioblastoma, is sensitive to the growth-suppressive effects of pRb2/p130, yet refractory to that of family members pRb/p105 and p107 (24). This suggests that there are at least some fundamental differences in the molecular pathways that the Rb family proteins influence to elicit cell cycle control.

To explore the in vivo effects of induction of pRb2/p130 expression on the cell cycle machinery, we used a modified tetracycline inducible expression system in hamster glioblastoma cells transformed by the JC virus (27), the human polyoma virus that is the etiological agent of progressive multifocal leukoencephalopathy (28). In this system, pRb2/p130 expression is repressed in the presence of tetracycline and induced upon the withdrawal of tetracycline from the cellular medium (27). The JC virus contains a large TAg oncprotein that is 72% homologous to its SV40 counterpart (28, 29) and that effectively targets and functionally inactivates each of the Rb family members (27, 30–33). Therefore, in the uninduced state, this system is essentially functionally null for each of the Rb family members. We have previously demonstrated that induction of pRb2/p130 in this system is able to overcome JCV TAg-mediated cellular transformation. Induction of pRb2/p130 expression results in nearly 90% of the cells growth-arrested in the G_{0}/G_{1} phase of the cell cycle, and growth suppresses tumor formation both in vitro and in vivo (27). RB2/p130 has recently been shown to be mutated and/or functionally inactivated in patients with lung cancer, mesothelioma, nasopharyngeal carcinoma, and Burkitt’s lymphoma (35–39). Restoration of RB2/p130 by way of viral-mediated gene delivery led not only to inhibition but actual regression of tumor formation in vivo (35), thereby demonstrating that RB2/p130 behaves as a true tumor suppressor gene. In this paper, we examined in detail the molecular mechanisms involved in pRb2/p130-mediated growth suppression.
The kinase activity associated with cyclin D3 also increased somewhat (Fig. 1, row 6). Because coexpression of both cyclin D1 and cyclin D3 with pRb2/p130 rescues pRb2/p130-mediated growth suppression (19), this increase in activity may be attributable to pRb2/p130 serving as a substrate for the D-type-associated kinases. This is also supported by the finding that pRb2/p130 associates with cyclin D3, and the variation in the phosphorylation status of pRb2/p130 correlates with the maximal activation of cyclin D3-associated kinase activity (14). The background kinase activity was determined by assaying the kinase activity associated with immunoprecipitations using NRS, as shown in Fig. 1 (row 7). The effects of pRb2/p130 induction on the following proteins' associated kinase activity in the same cell extracts: row 2, pRb2/p130-associated kinase activity; row 3, cyclin A-associated kinase activity; row 4, cyclin E-associated kinase activity; row 5, cyclin bD1-associated kinase activity; row 6, D3-associated kinase activity; row 7 (background), NRS-associated kinase activity; row 8, WB analysis with anti-Cdk2, demonstrating that the amount of Cdk2 remains the same in the HJC12 cells in the induced and uninduced states. pRb/p105 was used as the substrate for determining the kinase activity associated with cyclins D1 and D3. Otherwise, histone H1 was used as the substrate.

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pRb2/p130 Down-Regulates Cyclin A and E2F1 but Induces p27Kip1 and Cyclin E Expression. We next decided to examine the effects of pRb2/p130 induction on the expression of a number of key members of the cell cycle machinery by WB analysis as shown in Fig. 2. The parental control cell line HJCΔ5 was included to demonstrate that the effects were specific to increased levels of pRb2/p130 and not caused by the Vp16 tet transactivator. Induction of pRb2/p130 expression caused p27Kip1 and cyclin E to be downregulated, while pRb2/p130 induction caused p27Kip1 and cyclin E to be downregulated, while p27Kip1 and cyclin E to be downregulated, while p27Kip1 and cyclin E to be downregulated, while p27Kip1 and cyclin E to be downregulated, while p27Kip1 and cyclin E to be downregulated.
pression led to dramatic decreases in the expression of both cyclin A and the transcription factor E2F-1.

E2F-1 expression was sensitive to even the small amount of leak of pRb2/p130 expression in this batch of cell lysates. E2F-1 protein levels were further reduced with higher pRb2/p130 expression levels. The inhibition of cyclin A expression may explain in part the vast reduction in cyclin A-associated kinase activity. Intriguingly, induction of pRb2/p130 expression also led to a substantial induction in p27Kip1 levels by decreasing or inhibiting targeted proteolysis of p27Kip1. In this manner, pRb2/p130-mediated inhibition of cyclin2 kinase activity leads to a positive feedback loop by inducing p27Kip1 levels that can go on to further inhibit Cdk activity, ensuring that pRb2/p130 does not become the substrate of other Cdns and inactivated by phosphorylation.

**JCV TAG BINDS p27KIP1.** Because the E1A oncoprotein has been previously shown to bind the p27Kip1 protein directly (53), we decided to examine whether or not JCV TAG is also able to associate with p27Kip1. We performed a series of immunoprecipitations of HJCA5 and HJC12 cells in both the uninduced (+ tetracycline) and induced states (− tetracycline) with NRS as a negative control, with an anti-pRb2/p130 polyclonal antibody and Western blotting with the PAB 416 anti-TAg monoclonal antibody.

To examine whether or not JCV TAg is also able to associate with the PAB 416 anti-TAg monoclonal antibody. Precipitates underwent electrophoresis on a 10% polyacrylamide SDS gel and were Western-blotted with the PAB 416 anti-TAg monoclonal antibody.
sequence labeled with $^{32}$P, we detected an E2F complex that was effectively competed with cold wild-type oligonucleotide but not with a point mutant oligonucleotide that abrogates E2F binding to DNA (Fig. 5, Lanes 1 and 2); therefore, these bands were specific. The band of the E2F complex was supershifted by incubation with an antibody that specifically recognizes pRb2/p130 (Fig. 5, Lanes 5 and 8) as well as by an antibody that specifically recognizes E2F4 (Fig. 5, Lanes 4 and 7) in HJC12 cells in the presence or absence of tetracycline. Almost the entire E2F complex was shifted in the HJC12 cells in the induced state (−tetracycline) by incubation of the pRb2/p130 antibody (Fig. 5, Lane 8), indicating that most of the E2F is bound by pRb2/p130 in these cells. However, in the HJC12 cells in the uninduced state (+tetracycline), only a small fraction of the E2F complex was supershifted by the pRb2/p130 antibody (Fig. 5, Lane 5), a reflection of the low endogenous expression level of pRb2/p130 in the proliferating HJC12 cells (Figs. 1, 2, and 5).

We have shown previously by flow cytometry analysis (fluorescence-activated cell-sorting analysis) that upon induction of pRb2/p130 expression (HJC 12−tetracycline), nearly 90% of the cells are found in the G$_S$/G$_1$ stage corresponding to a mean increase of 41.6% in the G$_S$/G$_1$ population (95% confidence intervals, 40.1–43.1%) in three separate experiments. This effect was specific for pRb2/p130 expression. Time-course WB analysis of cyclin E protein levels in HJC12 cells after induction of pRb2/p130 expression in the HJC12 cells provided an abundance of pRb2/p130 that could effectively sequester E2F activity, thereby leading to growth arrest. Therefore, the ability of induced levels of pRb2/p130 to block proliferation correlated with its E2F-binding capacity.

Transcriptional Repression by pRb2/p130 Induction. Because cyclin A and E2F-1 both contain E2F sites in their promoter regions, their down-regulation may have been caused by pRb2/p130 repression of E2F-mediated transcription (44, 54). We first demonstrated that the down-regulation of cyclin A by pRb2/p130 functioned at the transcriptional level because others have previously reported that the E2F-1 promoter linked to a CAT reporter gene is effectively down-regulated by transfection with a pRb2/p130 expression plasmid (6). HJC12 cells were transfected with the minimal region of the cyclin A promoter (−89/+11), which contains two E2F binding sites and displays the same cell cycle regulation profile as the 7.5-kb full promoter construct (54), linked to a luciferase reporter gene. Cells were then placed in the presence or the absence of tetracycline for 48 h. As shown in Fig. 6, induction of pRb2/p130 expression greatly inhibited luciferase activity by a >3.2-fold reduction; therefore, increased pRb2/p130 levels silenced expression from the cyclin A minimal promoter. This is in agreement with previous transfection studies demonstrating that histone deacetylase 1 enhances the ability of pRb2/p130 to inhibit E2F-dependent transcription from the cyclin A promoter (55).

Transcription from the minimal cyclin E promoter (−94/+263 promoter region) linked to a luciferase reporter gene was repressed by 4.2-fold upon pRb2/p130 induction (Fig. 6). This region contains an E2F-binding site that has previously been shown by mutational analysis to be required for G$_1$-specific activation of the cyclin E promoter.

![Graph showing luciferase activity](image)

**Fig. 6.** Luciferase assay of the cyclin A (−89/+11), cyclin E (−94/+263), and cyclin E (−543/+263) promoter HJC12 cells induced (−tetracycline, for 48 h) or not induced (+tetracycline). Induction of pRb2/p130 expression greatly inhibited the luciferase activity of the cyclin A minimal promoter (−89/+11 promoter region) by >3.2-fold (column 4) and by 4.2-fold that of the minimal cyclin E promoter (−94/+263 promoter region). Transcription from the −543/+263 cyclin E promoter region was enhanced by 1.8-fold upon pRb2/p130 expression (column 8). The graph represents the mean of three separate experiments each performed in triplicate with error bars included.

![Graph showing steady accumulation of cyclin E protein](image)

**Fig. 7.** Steady accumulation of cyclin E protein with increasing levels of pRb2/p130 expression. Time-course WB analysis of cyclin E protein levels in HJC12 cells after induction of pRb2/p130 expression using an anticyclin E polyclonal antibody (Santa Cruz). The cells were harvested at the indicated minutes after withdrawal from tetracycline. **Arrows,** the steady accumulation of the M$_r$ 42,000 and 50,000 isoforms of cyclin E. Total cell extracts were electrophoresed on a 10% SDS polyacrylamide gel.

![Graph showing cell cycle profiles](image)

**Fig. 5.** The effect of pRb2/p130 induction on E2F-binding capacity. EMSA of HJC12 cells in the induced (−tetracycline, for 48 h) or uninduced (+tetracycline) status using a $^{32}$P-end-labeled double-stranded oligonucleotide of the consensus E2F DNA-binding site as a probe, which was competed with cold wild-type and mutant double-stranded oligonucleotides as indicated to show the specificity of the bands. Incubation of the extracts with an anti-pRb2/p130 antibody as indicated demonstrated the presence of pRb2/p130 in the E2F complexes (Lanes 5 and 8). Incubation of the extracts with an anti-E2F4 antibody as indicated demonstrated the presence of E2F4 in the E2F complexes (Lanes 4 and 7).
Fig. 8. Hypothesized model of the cooperation between pRb2/p130 and p27Kip1 in regulating cellular proliferation and their proposed involvement in a negative feedback regulatory loop with cyclin E. Thickened arrows, the effects of pRb2/p130 induction on the normal progression (thin arrows) of the cell cycle. X, an unidentified molecule that may facilitate the pRb2/p130 interaction with p27Kip1. The enzymatic mediators and modulators are indicated for each step next to the arrow indicating their influence on the equilibrium of the reaction. A detailed explanation is provided in the “Results and Discussion” section.

(44). This fits the predicted model of pRb2/p130 repression of E2F-mediated transcription.

Unexpectedly, transcription from the −543/+263 cyclin E promoter region linked to a luciferase reporter gene was enhanced by 1.8-fold upon pRb2/p130 expression (Fig. 6). This 806-bp fragment has been shown to retain full promoter activity and the cell cycle regulation of the promoter. In our system, the −94/+263 promoter region exhibited ~50% of the promoter activity of the −543/+263 construct in the uninduced state, which is consistent with previously published data (44). Additionally, our work supports the notion that there are additional potential E2F-binding sites as well as other regulatory sites within this 806-bp region that are functionally different from the E2F site within the −94/+263 region (44). This was concordant with the protein levels of cyclin E in HJC12 cells in the induced and uninduced states (Fig. 2). Upon withdrawal of tetracycline from HJC12 cells, the M, 50,000 and 42,000 forms of cyclin E continued to increase as the levels of pRb2/p130 accumulated over time (Fig. 7). Pulse chase experiments using HJC12 cells (± tetracycline) revealed that the half-life of cyclin E was greatly extended to beyond 48 h upon pRb2/p130 induction.4 We presently cannot explain this occurrence. We can only hypothesize that protein levels of cyclin E must remain high in the presence of pRb2/p130 expression because cyclin E-associated kinase activity is hypothesized to be the rate-limiting factor involved in the G1-S transition (56, 57). In this manner, cyclin E is present to serve as an early immediate response gene when the cell is signaled to divide. Both pRb2/p130 and cyclin E may exhibit negative feedback regulation of each other. Cyclin E expression may be maintained by pRb2/p130-mediated induction at the transcriptional level (as shown here) and possibly by stabilizing the short half-life of the cyclin E protein thought to be mediated by its PEST sequences (58, 59). Cyclin E is maintained in an inactive form by pRb2/p130 inhibition of cyclin E-associated kinase activity both directly and by induction of the universal CDK inhibitor p27Kip1. Then as the cell prepares for DNA replication, pRb2/p130 is inactivated most likely by phosphorylation, thus releasing its inhibition on cyclin E-Cdk2 kinase activity, which may now enhance pRb2/p130 phosphorylation and inactivation as well as the phosphorylation and degradation of p27Kip1 allowing the cell to progress through the G1-S phase transition. Then cyclin E is rapidly degraded without the induction and/or protection of pRb2/p130, thus preventing endoreduplication, maintaining DNA fidelity, and allowing the cycle to renew (60). Fig. 8 is a graphic depiction of our hypothesized model of the negative feedback regulatory loop involving pRb2/p130, p27Kip1, and cyclin E.

Induction of the −543/+263 cyclin E promoter region demonstrates that the reduction in the expression of the cyclin A promoter was specific to pRb2/p130 expression and not merely attributable to the cells being in a growth-arrested state. These same assays were also performed in the HJCΔ5 cells, and no significant effects upon luciferase activity were seen with any of the promoter constructs in the presence or absence of tetracycline (data not shown). Therefore, the effects were specific to pRb2/p130 induction.

The down-regulation of the cyclin A promoter by pRb2/p130 may not only be attributable to pRb2/p130 sequestering E2F activity, but it may also be attributable to the induction of p27Kip1 expression by pRb2/p130. Others have previously demonstrated that p27Kip1 inhibits the expression of E2F-regulated genes, specifically the cyclin A and cyclin E genes, by inducing the accumulation of repressor complexes of E2F (61). The fact that pRb2/p130 induction down-regulates the cyclin A promoter but induces the cyclin E promoter may be a reflection of the presence of JCV TAg in the experimental system. Repression of the cyclin E promoter may be more dependent upon the presence of p27Kip1 Cdk-inhibitory activity that may be effectively sequestered by JCV TAg.

Our results indicate that pRb2/p130 elicits growth suppression by impacting upon multiple molecular pathways. Induction of pRb2/p130 does specifically inhibit cyclin A- and cyclin E-associated kinase activity in vivo. This evidence confirms previous in vitro data that demonstrated a p21-like kinase inhibitory domain within the amino terminus of p107 and pRb2/p130 as well as a second kinase inhibitory domain found only within the spacer region of pRb2/p130, which inhibited Cdk2 kinase activity (21–23). However, in vivo the scenario is not this straightforward. pRb2/p130 also increases the protein level of the Cdk inhibitor p27Kip1. Because p27Kip1 is a universal Cdk inhibitor, this may provide a positive feedback loop for enhancing the growth regulatory function of pRb2/p130. By inhibition of cyclin E-Cdk2 kinase activity, pRb2/p130 may prevent p27Kip1 from being targeted for ubiquitin-mediated proteasome degradation.
In turn, p27Kip1 can inhibit the function of several Cdk5s (62), thus blocking the phosphorylation and functional inactivation of the Rb family proteins. The down-regulation of cyclin A expression by induction of pRb2/p130 would further inhibit Cdk2 kinase activity. Additionally, the repression of E2F-regulated complexes by pRb2/p130 and p27Kip1 further links the growth regulatory functions of these two proteins.

The demonstration that JCV TAgs associate with p27Kip1 further testifies to the importance of this protein in regulating normal cell division. JCV TAgs much like E1A may sequester p27Kip1 function. Alternatively, TAgs may use its association with p27Kip1 to alter the phosphorylation state of pRb2/p130 and p107 because SV40 and BK virus TAgs have been shown to alter the phosphorylation states of p107 and pRb2/p130 and to decrease the half-life of pRb2/p130 (63–65). If pRb2/p130 and p27Kip1 were competing for the same binding site on JCV TAgs, that would decrease the likelihood of such a scenario. However, increased degradation of pRb2/p130 mediated by TAgs would free more TAgs to bind and sequester the activity of p27Kip1. The fact that two DNA tumor viruses simultaneously evolved a mechanism to target the Rb family proteins and p27Kip1 (27, 30–33, 53) further supports a functional link between the Rb family, specifically pRb2/p130, and p27Kip1.

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