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Functional Analysis of pRb2/p130 Interaction with Cyclins

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Abstract

The retinoblastoma (Rb) family consists of the tumor suppressor pRb and related proteins p107 and pRb2/p130. Ectopic expression of pRb and p107 results in a growth arrest of sensitive cells in the G₁ phase of the cell cycle. We demonstrated here that the growth-suppressive properties of pRb2/p130 were also specific for the G₁ phase. The A-, E-, and D-type cyclins as well as transcription factor E2F1 and the E1A viral oncoprotein were able to rescue the pRb2/p130-mediated G₁ growth arrest in SAOS-2 cells. The rescue with cyclins A and E correlated with their physical interaction with pRb2/p130, which surprisingly has been found to occur over all phases of the cell cycle. The phosphorylation status as well as the kinase activity associated with pRb2/p130 dramatically increased near the G₁-S-phase transition. This suggests that, like the other Rb family members, pRb and p107, the phosphorylation of pRb2/p130 is controlled by the cell cycle machinery and that pRb2/p130 may indeed be another key G₁-S-phase regulator.

Introduction

Since the discovery of the first tumor suppressor gene, much energy has been directed toward the identification and characterization of such genes which serve as negative regulators of the cell cycle machinery and the elucidation of their involvement in human malignancies. According to Knudson’s “two-hit” hypothesis (1), the development of several human cancers is thought to involve loss of heterozygosity of putative tumor suppressor genes, several of which are yet to be identified (2). One of the most extensively studied tumor suppressor genes is the retinoblastoma susceptibility gene (Rb), whose gene product (pRb) is a nuclear phosphoprotein that has been shown to serve as a negative regulator of cell cycle progression through G₁-S-phase transition, at least in some specific cell types (3). G₁ growth arrest by pRb is dependent on the sequences necessary for its physical interaction with the E2F family of transcription factors as well as with a number of oncoproteins from human DNA viruses (4–7). Binding to these proteins occurs at a specific “pocket region” in pRb, shared by two additional E1A-associated proteins, which has led to the identification of two members of the Rb family, p107 and pRb2/p130 (8–11). pRb2/p130 maps to human chromosome 16q12, an area in which deletions have been found in several human neoplasias including breast, ovarian, hepatic, and prostatic cancers (12). Moreover, the gene responsible for familial cylindromatosis, a rare autosomal dominant disease characterized by multiple neoplasias originating from the skin appendages, has been recently localized to chromosome 16q12-q13, thereby further lending credence to the hypothesis that the Rb2/p130 gene is a tumor suppressor gene (13). The human Rb2/p130 gene, recently characterized by us, consists of 22 exons, spanning over 50 kb of genomic DNA (14). Recent functional studies of p107 and pRb2/p130 indicated that although the Rb family members may be able to complement each other, the proteins are not fully functionally redundant (15, 16). Like pRb, ectopic expression of either p107 or pRb2/p130 is able to suppress the growth of the osteosarcoma cell line SAOS-2 which lacks a functional pRb (7, 15, 16). The T98G human glioblastoma cell line is refractory to the effects of p107 and pRb2/p130 but undergoes growth arrest from pRb2/p130, indicating that pRb2/p130 is not merely a surrogate for either pRb or p107 and that there are differences in the specific mechanisms of growth inhibition employed by the three family members (15).

The interplay between the Rb⁶ family and the E2F family is hypothesized to regulate transcription and progression of the cell cycle and offers unique models for the growth suppression by the Rb family members. Several sources of data suggest that pRb, p107, and pRb2/p130 associate with distinct E2F species that modulate their activity in a sequential manner (17–21). The Rb-E2F complexes may in turn be modulated by the cell cycle machinery through the phosphorylation of Rb family members by cdkks, resulting in the release of active E2F species to stimulate the transcription of important genes involved in DNA synthesis and progression of the cell cycle. The ability of pRb and p107 to negatively regulate E2F correlates strongly with their ability to arrest cell growth (6, 7, 22, 23). The phosphorylation of pRb and p107 has been shown to be cell cycle regulated (24–26). At this point, it is uncertain as to how and whether the cell cycle machinery regulates the growth suppressive action of pRb2/p130. pRb2/p130 binds to cyclins A and E in vivo and to cyclins D₁, D₂, and D₃ in vitro (9, 10). We present data herein that support a cell cycle regulation for the phosphorylation of pRb2/p130. We have found pRb2/p130 to be highly phosphorylated at the G₁-S transition. We have also investigated the effects of known components of the cell cycle machinery on pRb2/p130-mediated G₁ growth arrest. We have compared and contrasted our rescue analysis of pRb2/p130 with that previously obtained by others for pRb and p107 in SAOS-2 cells (16, 27) in an attempt to decipher the actual mechanisms of inhibition by the family members. Our studies have been furthered by delving into the regulatory processes involving pRb2/p130 throughout the various stages of the cell cycle.

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5 The abbreviations used are: cdk, cyclin-dependent kinase; FBS, fetal bovine serum; FACS, fluorescence-activated cell sorting; Rb, retinoblastoma; PVDF, polyvinylidene difluoride.

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Materials and Methods

Plasmids. The plasmids pCDNA3 pRb2/p130 sense and antisense, pCMV CD20 and pCMV 107 have been described elsewhere (15, 16).

Cell Culture and Transfection. The human cell lines SAOS-2 (osteosarcoma), MG63 (osteosarcoma), MCF7 (breast adenocarcinoma), and T98G (glioblastoma) were used. The cell lines were purchased from the American Type Culture Collection. SAOS-2 and MCF7 cells were cultured into DMEM with the support of 10% FBS, l-glutamine, and penicillin-streptomycin. MG63 and T98G were cultured into Eagle’s MEM with the support of 10% FBS, l-glutamine, and penicillin-streptomycin.

All of the cell lines were transfected using the standard suspension calcium-phosphate precipitation method. (16, 28).

Colony Formation Assay. The colony formation assay was performed as described previously (15). MG63 and MCF7 cells were selected with 600 µg/ml G418, whereas SAOS-2 cells were selected with 800 µg/ml G418 for 3 weeks.

Flow Cytometry Analysis. Flow cytometry analysis was carried out according to the procedure described previously (16), with few modifications. Ten µg of either DNAs pCMV cyclins A, B1, D1, D2, D3, E, and F, pCMV E1A wild type, and pCMV E2F1 were cointransfected with 10 µg ΔcDNA3 pRb2/p130 sense and 2 µg pCMV-CD20.

Eighteen h after transfection, the cells were washed twice with PBS, once with culture medium, and incubated with fresh medium at 37°C. At 48 h, the cells were collected in PBS containing 3 mM EDTA, pelleted by centrifugation, and incubated with 20 µl FITC-conjugated anti-CD20 monoclonal antibody (Becton Dickinson) for 20 min on ice. The cells were washed with PBS supplemented with 1% serum, pelleted by centrifugation, and fixed in 70% ethanol at 4°C. Before FACS analysis, the cells were washed with PBS supplemented with 1% serum and incubated at 37°C with 20 µg/ml propidium iodide and 200 µg/ml RNase A for 30 min. FACS analysis was performed on a Coulter Elite apparatus, and data from 1 × 10⁶ CD20-positive cells were used to determine the cell cycle distribution of the selected cells.

Cell Cycle Synchronization. Synchronized cells were prepared as described previously (29, 30, 31). Briefly, confluent plates of SAOS-2 cells were serum starved with medium containing 0.1% FBS for 4 days. The cells were then released from the G0 block by splitting them at a 1:3 dilution into medium containing 10% FBS for a period of 12 h to achieve G1, synchronization. To reach a G2 and M synchronization, exponentially growing cells were treated with quercetin (70 mM final concentration) and nocodazole (2.6 mM final concentration), respectively, and were serum starved with medium containing 0.1% FBS for 4 days. The cells were then released from the G2/M block by splitting them at a 1:3 dilution into medium containing 10% serum and 20 µl/ml propidium iodide (100 µg/ml final concentration) for 24 h and then released in hydroxyurea-free medium for 4 h. Phases of the cell cycle were confirmed by FACS analysis.

Antibodies. The polyclonal α-Rb2/p130, α-cyclin A, and α-cdk2 have been described elsewhere (32–34).

Western Blot, Kinase Assay, and Immunoprecipitation. These techniques were performed as described elsewhere (33, 35, 36).

Results and Discussion

Cell Cycle-dependent Phosphorylation of pRb2/p130 and Associated Kinase Activity. It has been demonstrated that pRb2/p130 as well as pRb and p107 display shifts in electrophoretic mobility according to their degree of phosphorylation (30, 37, 38). A rabbit polyclonal serum was raised against the pRb2/p130 C-terminal 20-amino acid peptide. The immune serum recognized all phosphorylated isoforms of pRb2/p130 in both immunoprecipitation and in Western blot, showing no cross-reaction with pRb or p107 (data not shown). Western blot for pRb2/p130 was performed on SAOS-2 cells, synchronized in each cell cycle phase as described in “Materials and Methods.” Fig. 1a shows pRb2/p130 changes in expression and in phosphorylation during the different phases of the cell cycle. Essentially, two major forms were evident in G0 and G1, while a third slower migrating band appeared during the S-phase, remained through G2, and decreased during M. The overall amount of protein peaked at G2.

The pRb2/p130-associated kinase activity was then determined in the same synchronized cells. An equal amount of total protein for each fraction was immunoprecipitated with antisera to pRb2/p130 and assayed for histone H1 kinase activity. Columns, cpm values of a representative experiment. The kinase activity associated with the pRb2/p130 immunocomplex was found to peak in the enriched S-phase fraction. The Western blot was normalized with the monoclonal antibody heat shock protein 72/73 (HSP 72/73 purchased from Oncogene Science). c, pRb2/p130-associated kinase activity of the same synchronized SAOS-2-enriched fractions. The fractions were lysed and an equal amount of total protein from each fraction was immunoprecipitated with antisera to pRb2/p130 and assayed for histone H1 kinase activity. Columns, cpm values of a representative experiment. The kinase activity associated with the pRb2/p130 immunocomplex was found to peak in the enriched S-phase fraction. The experiment was repeated four times, and the result varied <5% each time. Columns, pRb2/p130 kinase activity.

Fig. 1. a, Western blot analysis with pRb2/p130 antiserum on total lysates of the human osteosarcoma cell line SAOS-2 in different phases of the cell cycle. Total lysates were run on a 7% SDS-PAGE gel and transferred onto a PVDF membrane. First lane on the left, cell lysate from an asynchronous population. Second lane to the sixth lane contain run lysates from enriched fractions of cells synchronized in the G0, G1, S-phase, G2, and M, respectively. b, the Western blot was normalized with the monoclonal antibody heat shock protein 72/73. c, pRb2/p130-associated kinase activity of the same synchronized SAOS-2-enriched fractions. The cells were lysed and an equal amount of total protein from each fraction was immunoprecipitated with antisera to pRb2/p130 and assayed for histone H1 kinase activity. Columns, cpm values of a representative experiment. The kinase activity associated with the pRb2/p130 immunocomplex was found to peak in the enriched S-phase fraction. The Western blot was normalized with the monoclonal antibody heat shock protein 72/73 (HSP 72/73 purchased from Oncogene Science). c, pRb2/p130-associated kinase activity of the same synchronized SAOS-2-enriched fractions. The cells were lysed and an equal amount of total protein from each fraction was immunoprecipitated with antisera to pRb2/p130 and assayed for histone H1 kinase activity. Columns, cpm values of a representative experiment. The kinase activity associated with the pRb2/p130 immunocomplex was found to peak in the enriched S-phase fraction. The Western blot was normalized with the monoclonal antibody heat shock protein 72/73 (HSP 72/73 purchased from Oncogene Science).
state of pRb2/p130 changes dramatically at the G1-S transition in SAOS-2 human osteosarcoma cells. The functional significance of this posttranslational modification is yet to be determined. It may regulate the functional activity of the protein by affecting its interactions with the transcription factors E2F4 and E2F5, and in essence controlling the temporal expression of early response genes, as is thought to occur with the Rb family members pRb and p107.

**pRb2/p130-Cyclin/cdk Complex Formation.** To decipher the effectors of pRb2/p130 phosphorylation in this system, combined immunoprecipitation/Western blot analysis was performed on asynchronous SAOS-2 cells. Physical interactions between pRb2/p130 and cdk2 as well as with cyclins A and E were found (Fig. 2). The associations of pRb2/p130 with cdk2, cyclin A, and cyclin E are consistent with data previously obtained by others (9, 10).

pRb2/p130 associations with cyclins A and E were then analyzed in cell cycle-enriched fractions of SAOS-2 cells (Fig. 3, a and b, respectively). Only two major forms of pRb2/p130 were evident in Western blotting analysis after immunoprecipitation with an anti-cyclin A antibody. pRb2/p130 association with cyclin A was lowest in the G0 and M phases. For the pRb2/p130-cyclin E association, almost no detectable cyclin E was found in G0 cells, while it increased in G1, peaked in the S-phase and G2, and remained through M. In this respect, pRb2/p130 may be serving to modulate the kinase activity of cyclin A-cdk2 and/or cyclin E-cdk2.

Since basal levels of cyclin A or cyclin E may possibly be present throughout the cell cycle, as has been found in certain tumor cell lines, pRb2/p130 may serve to sequester the early active forms of cyclin A-cdk2 and/or cyclin E-cdk2 to keep them from pushing cells prematurely into the S-phase by phosphorylating their desired cellular targets too soon. The fact that pRb2/p130 was found complexed with cyclin A-cdk2 and cyclin E-cdk2 over all phases of the cell cycle, except G0 for cyclin E, lends credence to this hypothesis. Such a scenario has recently been proposed for p107 and cyclin A-cdk2 and cyclin E-cdk2 complexes (39).

**G0/G1 Growth Arrest by pRb2/p130.** A common feature of p53 and pRb, two well-characterized tumor suppressor gene products, is their ability to suppress cellular proliferation when overexpressed in sensitive cell lines (40). By colony formation assay, we have previously demonstrated that pRb2/p130 exhibits growth suppressive properties (15) which has held true in all cell lines tested thus far. The cell lines MCF7, human breast adenocarcinoma, and MG63, human osteosarcoma, which are refractory to the growth suppressive effects of both pRb and p107 (16), undergo growth arrest when transfected with pRb2/p130 as confirmed by colony assay (data not shown).

We then investigated whether the growth suppression by pRb2/p130 was specific to a certain stage of the cell cycle, using flow cytometry assays. The pRb2/p130, p107, or pCDNA3 vector alone was cotransfected with a plasmid expressing a cell surface protein (CD20) that normally is only expressed in B lymphocytes, and that can be identified by immunofluorescent cell staining. After transfection, positive cells were identified by using an anti-CD20 monoclonal antibody, and the DNA content of the sorted cells was determined by propidium iodide staining. SAOS-2 cells were selected for FACS analysis, since they have been used previously for the rescue analysis of both pRb and p107, which allows for comparison of the effects on Rb family members (16, 27). Additionally, the SAOS-2 cells are pRb and p53 deficient (27, 41).

In SAOS-2 cells, transfection with pRb2/p130 resulted in a significant decrease in the proportion of the S-phase cells and a corresponding increase in the fraction of G0-G1 cells. FACS analysis demonstrated that pRb2/p130 elicited a rise in the fraction of cells in G0-G1 from 31.8% ± 4.9 to 58.4% ± 2.4. This resulted in an 84% increase in the number of cells in G0-G1 compared to that of the vector alone (Fig. 4). As expected, p107 was also able to cause G0-G1 growth arrest.

7. A. De Luca, unpublished data.
of the SAOS-2 cells, as previously demonstrated by Zhu et al. (16). Therefore, all three family members are able to elicit a G1 growth arrest of certain cell lines.

Rescue of pRB2/p130-mediated Growth Arrest. To examine the abilities of different proteins that have been involved in the functional roles of the Rb family members to influence the growth suppression activity of pRB2/p130, a number of rescue assays have been performed.

For each of the rescue assays, 10 µg pRB2/p130 and 10 µg of each of the plasmid DNAs were cotransfected along with 2 µg CMV-CD20 into SAOS-2 cells. Cotransfection of either cyclin A or cyclin E with pRB2/p130 was able to rescue the cells from G0-G1 growth arrest and to cause an increase in the fraction of the cells in S-phase with respect to vector alone. This was consistent with their association with pRB2/p130 as mentioned previously (Fig. 2, b and c). However, growth suppression by wild-type p107 is not rescued efficiently by cyclin A or cyclin E (16). Therefore, an alternative explanation for the interaction of cyclin A-cdk2 and/or cyclin E-cdk2 with pRB2/p130 may be that these kinases instead modulate the activity of pRB2/p130 by altering the phosphorylation status of the protein itself and/or other proteins such as E2F4/DP1 and E2F5/DP1 found in association with pRB2/p130. Interestingly, cyclins D1 and D2 were also able to rescue the growth suppression of pRB2/p130 and to drive the cells into S-phase. Cyclin D3 also resulted in rescue of the cells from growth arrest but had its largest increase in the G2-M fraction. This phenomenon seen with the D-type cyclins, which have only been found to complex with pRB2/p130 in vitro, may be explained in two ways. Since various phosphorylated forms of pRB2/p130 are found throughout the cell cycle, the results of the rescue analysis may suggest that different cyclin-cdk complexes are important in modulating the functional activity of pRB2/p130. Of course, the interaction between a kinase and a substrate may be too fleeting to allow detection by immunoprecipitation. On the other hand, the rescue by the D-type cyclins may simply be due to their intrinsic ability to drive the cell cycle into a certain phase and not due to any specific interaction with pRB2/p130. This however is unlikely to be the case, since cotransfection of either cyclin B1 or F, which are known to push cells through the G2-M transition, resulted in no significant change from the cell cycle profile produced by the transfection of pRB2/p130 alone.

The overall results of the rescue analysis showed that pRB2/p130, and p107 as a positive control, transfected individually elicited a G0-G1 growth arrest. Cotransfection of pRB2/p130 with E1A, cyclins A, E, D1, and D2, or transcription factor E2F1 resulted in a marked increase in the fraction of cells in the S-phase. The rescue by E2F1 is...
consistent with the published observation previously (20). Cotransfection of E2F1 is able to rescue SAOS-2 cells arrested by either pRb or pRb2/p130 overexpression (16) or pRb2/p130 overexpression, as demonstrated in Fig. 4. E2F1 cotransfection has no significant effect on p107-mediated growth suppression (16). Since E2F1 is found in association with pRb in vivo, the rescue of pRb-mediated growth arrest may be explained by enhanced levels of functional E2F1 due to a quantitative inability of pRb to sequester and to abrogate E2F1 transcriptional activity. pRb/p2130, however, has not been shown to form a complex with E2F1 in vivo. Recent findings that pRb2/p130 is a negative regulator of E2F1 gene expression and that D-type cyclin kinase activity relieves E2F-mediated repression of E2F1 transcription (42) offer a possible explanation of E2F1 and the D-type cyclins’ rescue of pRb2/p130 suppression. In this scenario, pRb would be involved in the posttranslational modification of E2F1 function and pRb2/p130 would regulate transcriptional activity of the E2F1 gene. In essence, the family members pRb and pRb2/p130 via two different mechanisms would be regulating E2F1 activity that is necessary for maintaining normal cellular proliferation.

Cyclin A-cdk2, cyclin E-cdk2, and cyclin D-cdk4 have all been shown to be capable of regulating the function of pRb (27, 43, 44); however, stable in vivo associations have only been demonstrated with cyclin D-cdk4. Overexpression of cyclin A, cyclin E, and cyclin D2 or D3 results in the rescue of pRb-mediated cell cycle block (27, 44). p107, on the other hand, is not effectively rescued by either cyclin A or cyclin E overexpression (16). However, expression of cyclin D1-cdk4 overcomes p107-mediated growth arrest (38). Considering the timing of induction and activation of the cyclins along with the phosphorylation status of pRb, cyclin D-cdk4 seems the most likely candidate to be involved in the G1-S phosphorylation of pRb (40). The D-type cyclins have also been implicated in negatively regulating the function of p107 by phosphorylation (38). This may explain the lack of growth suppression by both pRb and p107 in T98G and MCF7 cells. T98G and MCF7 cells lack the cdk inhibitor p16 (45), which inhibits the function and activation of cdk4 and cdk6 that associate with the D-type cyclins; therefore, there is possibly an enhanced activity level of these kinases which can compensate for the increased levels of pRb and p107 by phosphorylating and thus rendering the proteins nonfunctional (40). Since we have shown previously that T98G cells are instead growth arrested by pRb2/p130 (15), this may suggest that pRb2/p130 is able to bypass the D-type cyclins-cdk regulation and is possibly modulated through a different transduction pathway. However, our results from the rescue analysis demonstrate that the D-type cyclins are able to effectively rescue pRb2/p130-mediated growth arrest in SAOS-2 cells. This effect may be dependent on the absolute levels of the D-type cyclins or other pathways may be altered or enhanced in the T98G cells, rendering the cells refractory to pRb and p107 but not to pRb2/p130.

FACS analysis results of the rescue by cyclins A, E, D1, D2, and D3 as well as for E1A and E2F1 were confirmed by colony assay, using the same conditions as mentioned above (Fig. 4).

Our results suggest that cyclin A-cdk2 and cyclin E-cdk2 are promising candidates for the modulation of pRb2/p130 activity, since both were shown to form in vivo complexes with pRb2/p130. More studies however are underway to confirm the functional consequences and significance of these interactions. Here, we show that the cell cycle block mediated by pRb2/p130 is effectively rescued by cyclin A, cyclin E, cyclin D1, cyclin D2, and cyclin D3, with cyclin A eliciting the most dramatic effects. Cyclin D3 advanced the cells into G2-M when cotransfected with pRb2/p130 (Fig. 4). Cyclin B1 and cyclin F fail to rescue pRb2/p130-mediated G1-S-phase block as expected, since both cyclins are involved in the G2-M transition (40). Of importance, our rescue data determined using FACS analysis have been substantiated by colony assay, thereby lending credibility to these results.

The data presented herein point to different members of the cell cycle machinery being capable of influencing the function and in effect the regulation of the different Rb family members. This suggests that, while individual Rb family members may have overlapping effects on certain signal transduction pathways, they also demonstrate unique characteristics in being able to influence a specific pathway more than another member. For example, cyclin E-cdk2 and cyclin A-cdk2 may be able to overcome the cell cycle block of pRb and pRb2/p130 but would fail to do so for p107. Cyclin D-cdk4 should be able to overcome the block elicited by pRb, p107, and pRb2/p130. The growth arrest by pRb2/p130 of the T98G cell line, which lacks p16, is an interesting paradox at this point. An emphasis in the future needs to be placed on further delineation of the various pathways involved in the functioning and regulation of the Rb family members and identifying areas of overlap and uniqueness in function.

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