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The Rb2/p130 Gene Product Is a Nuclear Protein Whose Phosphorylation Is Cell Cycle Regulated

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Abstract The Rb2/p130 protein has been shown to have a high sequence homology with the retinoblastoma gene product (pRb), one of the most well-characterized tumor suppressor genes, and with pRb-related p107, especially in their conserved pocket domains, which display a primary role in the function of these proteins. In this study, we report on the biochemical and immunocytochemical characterization of the Rb2/p130 protein, using a polyclonal antibody developed against its "spacer" region included in the pocket domain of the whole protein. We show that pRb2/p130 is a phosphoprotein located at the nuclear level and that its phosphorylation pathway can be dramatically reduced by phosphatase treatment. Moreover pRb2/p130, with p107, is one of the major targets of the E1A viral oncoprotein-associated kinase activity, showing a phosphorylation pattern which is modulated during the cell cycle, reaching a peak of activation at the onset of S-phase.

Key words: tumor suppressor gene, retinoblastoma gene, Rb2/p130, pocket protein, nuclear phosphoprotein, E1A oncoprotein, cell cycle

According to Knudson's "two-hit" hypothesis, many types of human cancers are thought to be caused by a loss of heterozygosity of putative tumor suppressor genes [Knudson, 1971, 1984]. One of the most well-characterized members of this gene category is the retinoblastoma susceptibility gene (RB), whose protein product (pRb/p105) has been shown to behave as a negative regulator of cell cycle progression [Huang et al., 1988; Takahashi et al., 1991]. The ability of pRb/p105 to exert its growth-suppressive activity is due to its interaction with transcription factors, such as the family of the E2F molecules, whose function is to promote the transcription of genes required for cell-cycle progression [Sang et al., 1995]. A number of DNA tumor viral oncoproteins, such as adenovirus E1A, SV40 T antigen, and papillomavirus E7, have been found to complex to pRb/p105 at the pocket region, thus impairing its ability to bind to transcription factors and thus achieving cell proliferation [Chellapan et al., 1991; Hiebert et al., 1992; Qian et al., 1992; Qin et al., 1992; De Caprio et al., 1988; Dyson et al., 1989; Whyte et al., 1988]. Two other proteins, p107 and pRb2/p130, previously identified by their interaction with adenovirus E1A oncoproteins [Harlow et al., 1986], have been cloned and have been shown to be structurally and, in many aspects, functionally related to pRb/p105 [Ewen et al., 1991; Mayol et al., 1993]. pRb2/p130 and p107 include in their structure a pocket domain, which contains the highest identity with pRb/p105, and both dis-

Abbreviations used: ABC, avidin-biotin complex; cdk, cyclin dependent kinase; DMEM, Dulbecco's modified Eagle medium; DTT, dithiothreitol; FCS, fetal calf serum; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulphate; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of SDS.

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play growth suppressive properties [Claudio et al., 1994; Zhu et al., 1993].

pRb2/p130 confers the same cell-growth inhibitory effects on SAOS-2 cells, an Rb −/− human osteosarcoma cell line, as pRb/p105 and p107, and it is also able to elicit growth arrest of the T98G human glioblastoma cell line, which is refractory to pRb- and p107-mediated growth arrest [Claudio et al., 1994]. Additionally, pRb2/p130 and p107 interact and form complexes with different members of the E2F family of transcription factors, as pRb does, with varying temporal order of complex formation [Schwarz et al., 1993; Cobrink et al., 1993; Cao et al., 1992; Shirodkar et al., 1992; Sardet et al., 1995; Vairo et al., 1995]. In addition, p107 maps to human chromosome 20q11.2, which is not commonly found altered in human neoplasias [Ewen et al., 1991], whereas pRb2/p130 resides on human chromosome 16q12.2, which has been frequently found altered in several human neoplasias, such as breast, ovarian, hepatic, and prostatic carcinomas [Yeung et al., 1993]. These findings suggest that the biological functions of pRb2/p130 and p107 are related to, more than overlapping, those of pRb/p105 yet may occur through different signal transduction pathways.

In this study, we report on the biochemical and immunocytochemical characterization of the pRb2/p130 protein. Our data are relevant to better understand the functional properties of this protein and its role in cell-cycle regulation and in cancer development.

MATERIALS AND METHODS

Biological Reagents

The antigen used to develop the polyclonal anti Rb2/p130 serum was obtained as follows: briefly, the "spacer" region of Rb2/p130 cDNA, described by Mayol et al. [1993], was PCR-amplified and subcloned in pGEX-2T (Pharmacia). Expression of the fusion protein was performed as previously reported [Smith et al., 1988; Frangioni et al., 1993]. Bacterially expressed glutathione S-transferase-Rb2/p130 spacer fusion protein was used to immunize rabbits. C36 and M73 monoclonal antibodies, respectively, against pRb/p105 and the E1A oncoprotein [Whyte et al., 1988; Harlow et al., 1985] were kindly provided by the Cold Spring Harbor Facility (Cold Spring Harbor, NY).

In Vitro Transcription-Translation

In vitro transcription of the Rb/p105, Rb2/p130, and p107 cDNA clones was performed by a T7 RNA polymerase capping reaction. Briefly, after phenol/chloroform extraction and ethanol precipitation, the transcription products were used as substrates for in vitro translation using a rabbit reticulocyte lysate (Promega) and ^{35}S-methionine as a radioactive label.

Cell Culture and Biological Assays

The human tumor cell lines SAOS-2 (osteosarcoma), ML-1 (lymphocytic leukemia), and 293 (adenovirus 5–transformed kidney embryonic cells) were all obtained from the American Type Culture Collection and maintained in culture in Dulbecco's modified Eagle medium (DMEM) with 10% fetal calf serum (FCS) at 37°C in a 10% CO_2–containing atmosphere. For cell-cycle analysis, cell culture, cell labeling, and centrifugal elutriation were performed as described [Giordano et al., 1989, 1991a]. Immunoprecipitation and kinase assay were carried out as previously described [Giordano et al., 1991]. Potato acid phosphatase treatment was done as described [Buchkovich et al., 1989].

Immunocytochemistry

Immunocytochemical studies were performed following standard avidin-biotin-peroxidase technique, using SAOS-2 as target cells. The cells were grown on slides and fixed in 50% acetone/methanol at 4°C for 5 min. After sequential washings with PBS, they were quenched in hydrogen peroxide and blocked with goat normal serum (Vector Laboratories) at a 1:100 dilution in PBS for 30 min at room temperature. Slides were then incubated for 1 h at room temperature with rabbit polyclonal immune serum raised against the spacer region of pRb2/p130 at a 1:500 dilution and then incubated with goat antirabbit biotinylated antibody (Vector Laboratories) for 60 min at room temperature. After washings in PBS, the slides were processed by the ABC method for 30 min at room temperature. Diaminobenzidine was used as the final chromogen. A negative control was included by replacing the primary immune serum with preimmune serum.

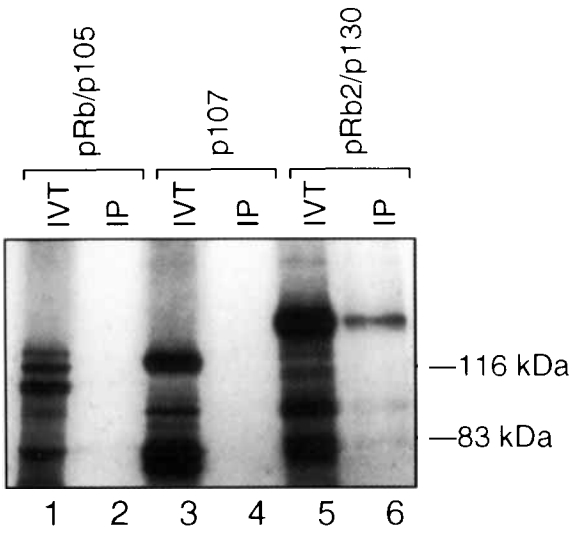

Fig. 1. In vitro translated (IVT) forms of pRb/p105, p107, and pRb2/p130 (*lanes 1, 3, 5*) were immunoprecipitated with the immune serum against the spacer region of pRb2/p130 (*lanes 2, 4, 6*). The polyclonal serum was able to immunoprecipitate only the in vitro form of pRb2/p130.

RESULTS

As indicated in Figure 1, the anti-pRb2/p130 serum was found to not cross-react with the other two pocket proteins, pRb/p105 and p107. The cDNAs for Rb/p105, p107, and Rb2/p130 were in vitro translated (Fig. 1, lanes 1, 3, 5) and immunoprecipitated using the rabbit polyclonal serum against the glutathione S-transferase-Rb2/p130 spacer (Fig. 1, lanes 2, 4, 6). The polyclonal serum was able to immunoprecipitate only the in vitro translated form of pRb2/p130 molecule, whereas no reactivity was found with the other two proteins.

The retinoblastoma gene product Rb/p105 and p107 have been reported to be constitutively expressed in all human tissues and to have nuclear localization [Hollingsworth et al., 1993; Ewen, 1994]. In order to investigate the cellular localization of pRb2/p130, an immunocytochemical study was performed on fixed SAOS-2 cells, a human osteosarcoma cell line bearing a defective pRb molecule that lacks exons 21–27 and is unable to have a specific nuclear localization [Shew et al., 1990]. As shown in Figure 2, pRb2/p130 was localized in the nucleus in a high percentage of target cells. However, a certain degree of heterogeneity in the intensity of pRb2/p130 staining was observed within the global cell population. In fact, actively dividing cells (i.e., mitotic cells) showed a stronger nuclear staining mostly concentrated around the nuclear membrane and sometimes also present in the cytoplasm. This pattern of immunoreactivity might reflect differences in pRb2/p130 levels during the different phases of the cell cycle.

It has been reported by immunoprecipitation and SDS-PAGE analysis that pRb/p105 migrates with an heterogeneous pattern due to various degrees of phosphorylation [Chen et al., 1989; De Caprio et al., 1992; Durfee et al., 1993]. As shown in Figure 3A, by using a cell lysate from ^{35}S-labeled ML-1 cells, we observed that pRb2/p130 migrates with an apparent molecular mass ranging between 138 and 130 kDa (Fig. 3A, lane 3). This suggests the presence of different levels of phosphorylation. This phenomenon was drastically reduced after acid phosphatase treatment (Fig. 3A, lane 4), where only a major band, with an apparent molecular mass of 130 kDa, was evident. In Figure 3A (lanes 1, 2) the phosphatase effect on the pRb molecule using the C36 monoclonal antibody is represented as a control.

To validate the phosphorylated nature of the slow migrating bands, protein extracts from ^{32}P-labeled ML-1 cells were immunoprecipitated using both C36 and anti-pRb2/p130 antibodies. In Figure 3B, lanes 1 and 2 show the phosphorylated forms of pRb and pRb2/p130, respectively.

We next investigated the pattern of association of pRb2/p130 with the adenovirus E1A oncoprotein, since E1A coprecipitates a series of different cellular proteins, including those of the pocket family [Giordano et al., 1991b]. In particu-

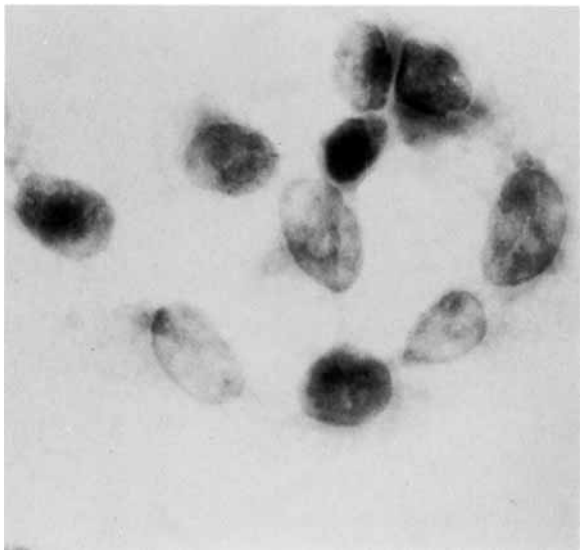

Fig. 2. Immunoreactivity of anti-pRb2/p130 immune serum on SAOS-2 cells.

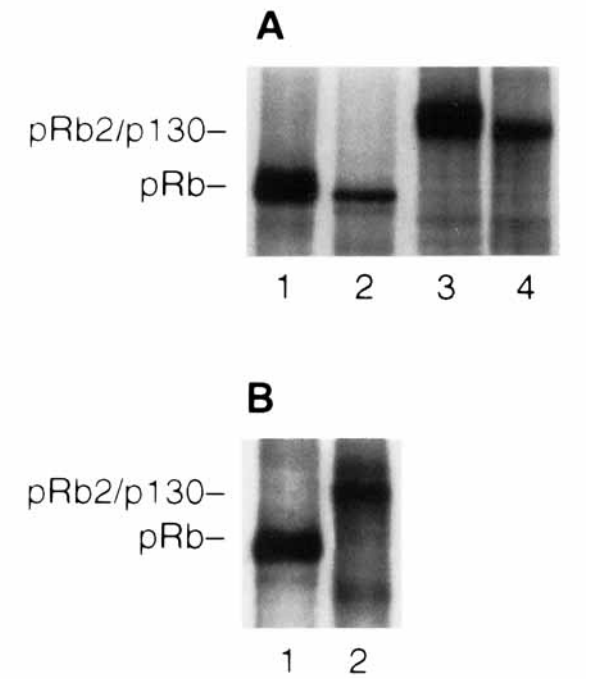

Fig. 3. **A:** SDS-PAGE analysis (8%) of cellular extracts from ^{35}S-labeled ML-1 cells after immunoprecipitation of pRb and pRb2/p130 by means of the C36 anti-pRb monoclonal antibody and of the anti-pRb2/p130 immune serum. *Lane 1:* Immunoprecipitated pRb. *Lane 2:* Immunoprecipitated pRb treated with acid phosphatase. *Lane 3:* Immunoprecipitated pRb2/p130. *Lane 4:* Immunoprecipitated pRb2/p130 treated with acid phosphatase. **B:** SDS-PAGE analysis (8%) of cellular extracts from ^{32}P-labeled ML-1 cells after immunoprecipitation of pRb and pRb2/p130 by means of the C36 anti-pRb monoclonal antibody and of the anti-pRb2/p130 immune serum. *Lane 1:* Immunoprecipitated pRb. *Lane 2:* Immunoprecipitated pRb2/p130.

lar, we tested the ability of pRb2/p130 to act as a substrate for the cyclin-cdk complexes coprecipitated with E1A [MacLachlan et al., 1995]. For this purpose, human 293 cells, which are immortalized by a fragment of the adenovirus 5 genome and constitutively express the E1A oncoprotein [Graham et al., 1977], were separated into populations from various portions of the division cycle by means of centrifugal elutriation. The diagram at the bottom of Figure 4 represents the propidium iodide flow cytometric analysis of 293 cells: an enrichment of cells was achieved. Immediately following elutriaton, cells were lysed and immunoprecipitation with the anti-E1A M73 monoclonal antibody was carried out. In vitro kinase assay of the endogenous substrates associated with E1A for all the elutriated fractions was performed (Fig. 4, upper panel). At the transition from a population containing predominantly cells in G1 to one containing more S-phase cells (between fractions 4 and 9) phosphorylation of both p107 and pRb2/p130 increased, reaching the maximum level at fraction 8 (Fig. 4, upper panel). pRb was not visible, since its phosphorylated form does not bind E1A [Paggi et al., 1994]. It seems that pRb2/p130, as well as the cognate protein p107, were the two major substrates of the E1A-associated kinase activity, and that their state of phosphorylation could change during the cell cycle.

DISCUSSION

In this paper we report on the biochemical and immunocytochemical characterization of pRb2/p130 by using a polyclonal antibody developed against its spacer region. We have demonstrated that the immune serum was highly specific for pRb2/p130, since it did not cross-react with the in vitro translated forms of the other two members of the pocket family.

The staining pattern of anti-pRb2/p130 antibody in SAOS-2 cells closely resembles that already described for pRb/p105 [Cordon-Cardo et al., 1994]. The presence of reactivity in the cytoplasm of some cells may be explained by disruption of the nuclear membrane during the mitotic phase of the cell cycle. It is then reasonable that in this situation pRb2/p130 diffuses out of the nucleus into other subcellular compartments. On the other hand, the discrepancies in the intensity of the nuclear staining observed among these cells may reflect differences in pRb2/p130 expression levels during the different phases of the cell cycle.

We show that pRb2/p130 has an electrophoretic mobility ranging between 138 and 130 kDa, thus implying the existence of different phosphorylated forms of the protein. Consistent with this hypothesis, we have found that only the fastest migrating band is detected following phosphatase treatment of pRb2/p130 immunoprecipitates.

Our findings on the pRb2/p130 cell-cycle-dependent phosphorylation pattern indicate that the highest activity of this protein is at the G1–S-phase of the cell cycle. This seems to be confirmed by several reported studies. In fact, the newly characterized E2F family members, E2F-4 and E2F-5, have been shown to interact in vivo with pRb2/p130 and p107 and to be expressed in the mid-G_1 phase of the cell cycle, suggesting a possible functional role of these two proteins in the regulation of the early phases of the cell cycle [Vairo et al., 1995; Sardet et al., 1995]. Our results are further strengthened by

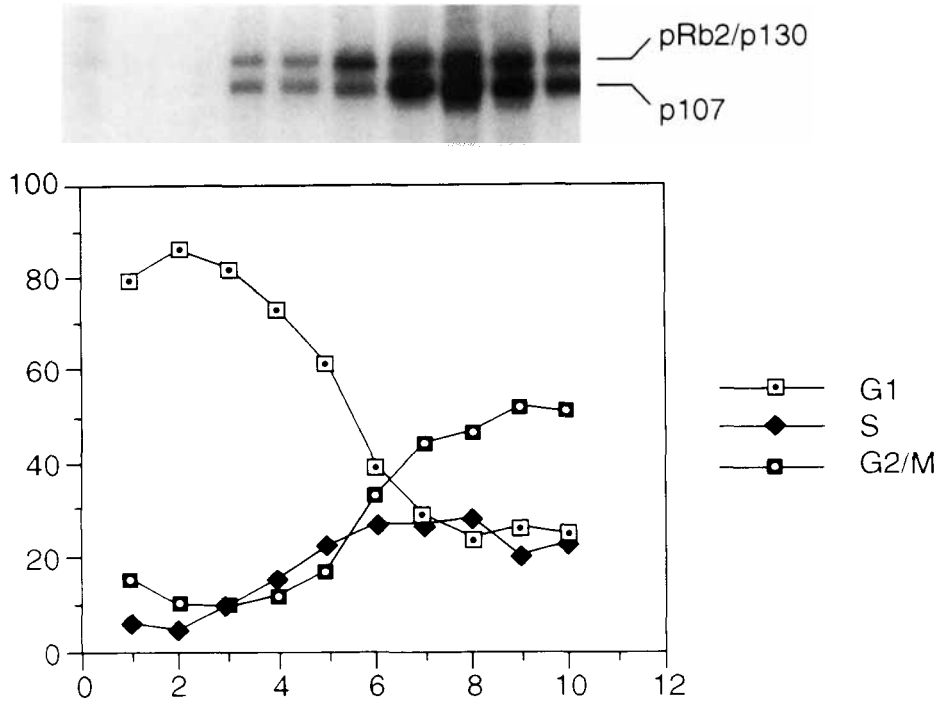

Fig. 4. Cell-cycle-dependent kinase activity on p107 and on pRb2/p130. **Upper panel:** SDS-PAGE (8%) after E1A immunoprecipitation by means of the M73 monoclonal antibody from unlabeled elutriated 293 cells and subsequent assay of kinase activity; bands represent phosphorylated p107 and pRb2/p130. **Lower panel:** Scheme representing the cell-cycle phase enrichment of 293 cells by elutriation; fractions were analyzed by flow cytometry. Values on the abscissa represent elutriation fraction numbers and refer also to the related bands on the upper panel. Values on the ordinate represent the percentage of cells in each phase of the cell cycle.

our observation that pRb2/p130 associates in vivo with cyclin A cyclin E, and CDK2 and that the ectopic expression of pRb2/p130 stops cells in G1 phase [Claudio et al., submitted].

However, many issues remain to be addressed: 1) the sequential order of interactions between the pocket proteins and the E2F family of transcription factors; 2) the mechanisms which regulate the E1A binding to pRb2/p130 and p107, since this may be independent from the status of their phosphorylation, as it is the opposite for pRb/p105; and 3) the mechanisms by which pRb/p105, p107, and pRb2/p130 exert their growth-suppressive activity in different tumor cell lines.

In summary, our findings suggest that the pRb2/p130 protein, which displays a high sequence homology with the tumor suppressor protein pRb/p105 and with the Rb-related p107, demonstrates some functional overlap with the other pocket family members. Moreover, this study will also help to better understand the role of pRb2/p130 in cell-cycle regulation and/or in the onset and progression of tumor formation.

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