A Unique Domain of pRb2/p130 Acts as an Inhibitor of Cdk2 Kinase Activity*

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The Cdk2 kinase has long been known to be involved in the progression of mammalian cells past the G1 phase restriction point and through DNA replication in the cell cycle. The Rb family of proteins, consisting of pRb, p107, and pRb2/p130, has also been shown to monitor progression of G1 phase, mostly through their interaction with E2F family members. p107 is able to inhibit Cdk2 kinase activity through this interaction via a p21-related domain present in the C terminus of the protein. We show here that pRb2/p130 also possesses this activity, but through a separate domain. Moreover, we correlate the increased expression of pRb2/p130 during various cellular processes with the decreased kinase activity of Cdk2. We hypothesize that pRb2/p130 may act not only to bind and modify E2F activity, but also to inhibit Cdk2 kinase activity in concert with p21 in a manner different from p107.

The mammalian cell cycle is presently thought to be driven by the sequential activation and deactivation of various cyclin/cyclin-dependent kinase (Cdk) pairs (1). The prototypic cell cycle kinase, Cdc2, was originally discovered in yeast and found to be involved in regulating both DNA replication and mitosis. The prototypic cell cycle. The Rb family of proteins, consisting of pRb, p107, and pRb2/p130, has also been shown to monitor progression of G1 phase, mostly through their interaction with E2F family members. p107 is able to inhibit Cdk2 kinase activity through this interaction via a p21-related domain present in the C terminus of the protein. We show here that pRb2/p130 also possesses this activity, but through a separate domain. Moreover, we correlate the increased expression of pRb2/p130 during various cellular processes with the decreased kinase activity of Cdk2. We hypothesize that pRb2/p130 may act not only to bind and modify E2F activity, but also to inhibit Cdk2 kinase activity in concert with p21 in a manner different from p107.

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growth of a cell line that is resistant to Rb growth suppression. We show here that pRb2/p130 is also able to inhibit Cdk2 kinase activity in a manner similar yet distinct from p107. Although both proteins are known to stably bind these complexes, pRb2/p130 does so from a separate domain, the spacer region. We also find that pRb2/p130 protein levels are increased upon differentiation of myocytes, coincident with Cdk2 kinase inhibition, where p107 protein levels have been shown previously to decrease. These results suggest that pRb2/p130 may act not only to bind and modify E2F activity, but also to inhibit Cdk2 kinase activity in concert with p21.

**EXPERIMENTAL PROCEDURES**

**Construct Preparation**—We used a prokaryotic expression vector pGEX-2T (Stratagene) and the polymerase chain reaction to generate chimeric glutathione S-transferase. The primers used to amplify the polymerase chain reaction fragments that were subcloned in the pGEX-2T were derived from the 5'- and 3'-ends of the NH$_2$, A, Spacer, B, and COOH domain of pRb2/p130 and the Spacer domain of pRb and p107. The pGEX-2T fusion proteins generated are shown in Fig. 2.

**GST\(^{-}\)Fusion Protein Preparation**—XL1-Blue bacteria carrying pGEX-2T vectors were grown to midlog phase then induced to express protein by the addition of isopropyl-1-thio-\(\beta\)-d-galactopyranoside to the media to 0.25 mM. The cultures were then shaken for 4 h. Bacteria were then pelleted and resuspended in NENT buffer (20 mM Tris, pH 8, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40). Cell suspensions were sonicated and pelleted, and the supernatant was collected. The remaining bacteria were then resuspended in NENT + 2% N-lauroyl sarcosine and pelleted, and the supernatant was again collected. The combined supernatants were incubated with glutathione-agarose (Pharmacia) overnight at 4°C. The agarose was then pelleted and washed three times in NENT buffer.

**Immunoblotting**—Cell lysates were prepared by resuspending pelleted cells in 200 μl of lysis buffer (50 mM Tris, 5 mM EDTA, 250 mM NaCl, 50 mM NaF, 0.1% Triton, 0.1 mM Na$_3$VO$_4$, plus protease inhibitors). 50 μg of protein were run on a 7% polyacrylamide gel. Proteins within the polyacrylamide gel were transferred to a polyvinylidene difluoride membrane (Millipore) in CAPS buffer (10 mM CAPS, 20% methanol, pH 11). The membrane was blocked with 5% milk in TBS-T buffer (2 mM Tris, 13.7 mM NaCl, 0.1% Tween 20, pH 7.6) and then washed in TBS-T. Primary antibody was incubated with the membrane in 3% milk and then washed in TBS-T. The membrane was then incubated with anti-rabbit Ig coupled with horseradish peroxidase (Amersham) and washed in TBS-T. The presence of secondary antibody bound to the membrane was detected using the ECL system (NEN Life Sciences).

**Kinase Assays**—Cell lysates were prepared by resuspending pelleted cells in 200 μl of lysis buffer (50 mM Tris, 5 mM EDTA, 250 mM NaCl, 50 mM NaF, 0.1% Triton, 0.1 mM Na$_3$VO$_4$, plus protease inhibitors). An equal amount of protein for each fraction was immunoprecipitated with a specific antibody. Prior to detecting the presence of histone H1 kinase activity, each sample was incubated with an equal amount of the pGEX-2T constructs previously described for 30 min at 4°C. Protein kinase assays were performed as described (18). Kinase assays were repeated at least three times giving an interassay standard deviation within 10% after normalization for protein amount.

**RESULTS**

pRb2/p130 has been shown to be a pocket protein that may be functionally specific for G1 phase of the cell cycle. In part by interacting with E2F5 and E2F4, pRb2/p130 holds cells in a quiescent or differentiated state. Since pRb2/p130 has also been shown to bind cyclins, we sought to find whether an increase in pRb2/p130 protein coincides with an alteration of associated Cdk activity. Certain models have suggested that there is an inverse relationship between these two phenomena. ML1 myeloma cells when undergoing differentiation in vitro show a marked increase in pRb2/p130 protein by terminal differentiation, while associated Cdk2 kinase activity decreases to basal levels. The murine hematopoietic progenitor cell line FDC-P1 displays a high amount of pRb2/p130 in early G1 phase which is coupled to a decrease in associated Cdk2 histone H1 kinase activity. As protein levels decline in late G1, kinase activity is restored to Cdk2 (19).

In this study we have focused on the myoblast cell line, C2C12. When cultured in medium containing 2% horse serum, C2C12 cells undergo cell cycle arrest, fuse with neighboring cells, and elongate into fully differentiated, multinucleated muscle fibers. After 5 days in this differentiation medium, cells had obtained a complete myotubular morphology. Immunoblotting of protein extracts from 24-h time points of these cells shows an increase of more than twice the amount of pRb2/p130 protein (Fig. 1A). pRb2/p130 complexes were also immunoprecipitated from these extracts and subjected to a kinase assay using histone H1 as substrate to assess associated Cdk2 activity. Cdk2 kinase activity decreased more than half by the end of the differentiation pathway (Fig. 1B). To confirm that the protein levels of Cdk2 were equal in all samples which underwent kinase analysis, half of the immunoprecipitated samples were assessed for Cdk2 levels by immunoblotting (Fig. 1A).

Previous studies have shown that p107 protein decreases during muscle differentiation (20). pRb2/p130 therefore may differ in its functional similarities to p107 with respect to binding cell cycle machinery. These results taken together show concomitantly that as pRb2/p130 protein levels increase, associated Cdk2 activity decreases, suggesting that pRb2/p130 may play a role in inhibiting the activity of this kinase.

Since there is a correlation of pRb2/p130 protein levels and Cdk2 kinase activity, we sought to determine whether pRb2/p130 can directly inhibit Cdk2. A panel of mutants, representing different regions of pRb2/p130, p107, and pRb as control, were developed and expressed as GST-fusion proteins (Fig. 2). Cdk2 complexes were immunoprecipitated from lysates of exponentially growing ML1 myeloma cells, and to the precipi-

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1 The abbreviations used are: GST, glutathione S-transferase; CAPS, 3-(cyclohexylamino)propanesulfonic acid.

2 D. Leibermann, personal communication.
tates was added 20 μg of the indicated GST-fusion protein. The mixtures were then subjected to a kinase assay using histone H1 as substrate. The A and B domains of the pocket and C-terminal regions of pRb2/p130 had little or no effect on the kinase activity of Cdk2 compared with precipitates treated with GST alone, and the N terminus had a moderate inhibitory activity. However, the spacer region, whose amino acid sequence is specific to pRb2/p130, decreased Cdk2-dependent histone phosphorylation dramatically (Fig. 3A). The spacer region’s effect on Cdk2 activity was found to be dose-dependent (Fig. 3B). This was unexpected as although pRb2/p130 and p107 share many structural and functional qualities such as common binding partners E2F4, Cdk2, and cyclins A and E, pRb2/p130 differs in its inhibitory affect on Cdk2. As opposed to pRb2/p130, the N-terminal region of p107, containing a p21CIP1-like motif, is responsible for this activity. Nucleotide homology analysis of the N terminus of pRb2/p130 reveals a similar region of identity with p21CIP1 (data not shown); however, as is shown in Fig. 3A, this is not able to inhibit Cdk2 kinase activity as strongly as the spacer region.

To determine if this inhibitory region is conserved in only pRb2/p130, spacer regions from all Rb protein family members were expressed as GST-fusion proteins and added in large excess to kinase assays of Cdk2. Only pRb2/p130 was able to inhibit Cdk2 kinase activity significantly through this region; therefore pRb2/p130 is unique in this ability (Fig. 4). Finally, to determine if this inhibition is a generality found with all Cdc2-family members, Cdc2, Cdk2, Cdk4, and Cdk5 were immunoprecipitated from ML1 cells and were subjected to a kinase assay using either histone H1 or Rb as substrate (Fig. 5). The spacer region of pRb2/p130 was only able to decrease Cdk2 kinase activity, demonstrating that the inhibitory activity associated with pRb2/p130 is specific for the kinase that it is able to bind stably in cells, Cdk2.

**DISCUSSION**

The possibility of pRb2/p130 acting as a Cdk2 kinase inhibitor raises the notion that Cdk2s may be negatively controlled by a variety of mechanisms, including phosphorylation and known Cdk inhibitor binding. It has been demonstrated that the pRb2/p130 gene product is a nuclear protein whose phosphorylation is cell cycle-regulated (21). Previous reports suggest that pRb2/p130 may primarily act in early G1 phase, shown by its association with the G0-specific transcription factor E2F5 and high expression in differentiated or quiescent cells. In addition to potential modification of E2F protein functions, pRb2/p130 may also contribute to G0/G1 arrest by decreasing the activity of kinases that may allow the cell to enter S phase. The highly homologous p107 protein has also been shown to have this effect on Cdk2. However, we feel that the effect we see of
pRb2/p130 on Cdk2 differs in three respects. One, as p107 is added in increasing amounts to Cdk2 kinase assays, pRb and histone H1 substrates decrease in phosphorylation, while p107 itself becomes increasingly phosphorylated. This suggests that p107 acts more like a preferential substrate, not an inhibitor. pRb2/p130 does not become phosphorylated in our in vitro assays (data not shown), therefore not acting as an alternative substrate, but as a binding protein with inhibitory properties.

Second, the region of pRb2/p130 that displays the most dramatic effect is one that is poorly conserved between the two proteins. The Rb family of proteins may functionally diverge through this region of low homology, and this might be one of those functions. Finally, it has been stated that p107 may not act so much as a true Cdk inhibitor, but simply masks Cdk2/cyclin A from its substrate, E2F4. We propose that pRb2/p130 acts differently since the spacer region is very close to the predicted E2F binding site, the C terminus, and that pRb2/p130 seems to increase at times of Cdk2 inhibition in vivo, whereas p107 does not.

In conclusion, we have shown that a unique domain of the pRb2/p130 protein is able to inhibit Cdk2 kinase activity in vitro, and that in model in vivo systems, pRb2/p130 protein increases are coincident with Cdk2 inhibition, suggesting that pRb2/p130 may not only act to regulate E2F activity, but also kinase activity as well.

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