Journal of Cellular Biochemistry 75:698–709 (1999)

Interaction Between the pRb2/p130 C-Terminal Domain and the N-Terminal Portion of Cyclin D3

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Abstract An association between cyclin D3 and the C-terminal domain of pRb2/p130 was demonstrated using the yeast two-hybrid system. Further analysis restricted the epitope responsible for the binding within the 74 N-terminal amino acids of cyclin D3, independent of the LXCXE amino acid motif present in the D-type cyclin N-terminal region. In a coprecipitation assay in T98G cells, a human glioblastoma cell line, the C-terminal domain of pRb2/p130 was able to interact solely with cyclin D3, while the corresponding portion of pRb interacted with either cyclin D3 or cyclin D1. In T98G cells, endogenous cyclin D3-associated kinase activity showed a clear predisposition to phosphorylate preferentially the C-terminal domain of pRb2/p130, rather than that of pRb. This propensity was also confirmed in LAN-5 human neuroblastoma cells, where phosphorylation of the pRb2/p130 C-terminal domain and expression of cyclin D3 also decreased remarkably in the late neural differentiation stages. J. Cell. Biochem. 75:698–709, 1999.

Key words: Rb2/p130; cyclin D3; phosphorylation; protein-protein interaction; cell cycle; differentiation

The retinoblastoma susceptibility gene (*RB*) is the prototype of the class of tumor suppressor genes, whose lack of function relates entirely to cancer onset and progression [Klein, 1987; Weinberg, 1995]. The product of this gene, pRb, plays a key role in controlling the cell cycle at the G1/S transition. pRb functions are attributed mainly to its ability to bind to a number of cellular proteins, thus assembling complexes with peculiar regulatory properties [Weinberg, 1995]. A primary pRb function is to connect the cell cycle to the transcription machinery by binding to and modulating the activity of transcription factors, such as E2F, Myo-D, ATF-2, and Elf-1 [Riley et al., 1994]. Molecular dissection of the pRb protein has permitted its functional partition into distinct domains, which are usually referred to as (1) the N-terminal domain, (2) the A pocket domain, (3) the spacer, (4) the B pocket domain, and (5) the C-terminal domain, or C pocket [Weinberg, 1995; Paggi et al., 1996]. Specific protein-protein interactions have been described for most of these domains and can be summarized as follows: (1) the A/B pocket is involved in the binding of proteins displaying the LXCXE motif [Ewen et al., 1993]; (2) the C-terminal domain is involved in binding both the c-Abl tyrosine kinase [Welch and Wang, 1993] and the oncoprotein Mdm-2 [Xiao et al., 1995]; and (3) the large A/B pocket, which also embraces the spacer and spans within the C-terminal region up to amino acid 869 [Welch and Wang, 1995], modulates the binding to the E2F family of transcription factors [Qin et al., 1992; Hiebert, 1993].

Abbreviations used: GST, glutathione-S-transferase; SDS-PAGE, polyacrylamide gel electrophoresis-sodium dodecyl sulfate; PVDF, polyvinyldifluorene.

Grant sponsor: Associazone Italiana per la Ricerca sul Cancro (AIRC); Grant sponsor: Fondazione Italiana per la Ricerca sul Cancro (FIRC); Grant sponsor: Ministero della Sanità; Grant sponsor: National Institutes of Health; Grant number: R01-CA60999-01A1; Grant sponsor: Council for Tobacco Research.

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Received 4 June 1999; Accepted 7 June 1999

Binding of transcription factors to pRb is strongly dependent on its phosphorylation status [Weinberg, 1995; Paggi et al., 1996]. Specific kinase and phosphatase activities regulate pRb phosphorylation throughout the cell cycle. It is recognized that in order to act as a key negative cell cycle regulator, pRb, must be in its hypophosphorylated form. Progressive phosphorylation of pRb, performed by different cyclin/cdk heterodimers according to the cell cycle phases, leads to progressive release of sequestered transcription factors that become effective in promoting the G1-S transition and, ultimately, the cell cycle [Riley et al., 1994; Weinberg, 1995].

Two genes, namely *p107* [Ewen et al., 1991; Zhu et al., 1993] and *Rb2/p130* [Mayol et al., 1993; Hannon et al., 1993; Li et al., 1993] share structural homologies with pRb, mainly in the A/B pocket region. *RB, p107,* and *Rb2/p130* are members of the *retinoblastoma family* of genes and proteins, also referred to as the *pocket family* because of the common functional domain shared by these proteins [Ewen, 1994; Paggi et al., 1996]. Both *p107* and *Rb2/p130* possess marked growth suppressor properties, similar to, but with cell specificity distinct from, those of *RB* [Zhu et al., 1993; Claudio et al., 1994]. Among these genes, only *RB* is firmly established to act as a tumor suppressor. It has been found to be deleted or mutated in most retinoblastomas and sarcomas and in other types of human cancers as well [Weinberg, 1995; Riley et al., 1994]. Reinsertion of the wild-type *RB* gene in cancer cell lines with proven *RB* abnormalities reverts some important characteristics of the neoplastic phenotype [Huang et al., 1988; Bookstein et al., 1990; Takahashi et al., 1991]. Like pRb, p107 and pRb2/p130 also are nuclear phosphoproteins, whose phosphorylation is cell cycle regulated [Peeper et al., 1993; Baldi et al., 1995; Xiao et al., 1996]. In addition, the three pocket family members interact with common sets of proteins, including active cyclin/kinase complexes, transcription factors, and viral oncoproteins, although some interactions, such as that involving the C-terminal region of pRb and c-Abl, have not been reported for the other members of the pocket family [Welch and Wang, 1995; Whitaker et al., 1998].

The three pocket proteins also are concurrently involved in complex cellular processes such as embryonic development [Bernards et al., 1989; Szekely et al., 1992; Kim et al., 1995; Pertile et al., 1995; Jiang et al., 1997] or differentiation [Sidle et al., 1996; Richon et al., 1997; Raschellà et al., 1997, 1998; Kiess et al., 1995]. Despite these similarities, they often behave differently and do not exert redundant functions [Claudio et al., 1994; Jiang et al., 1997]. This is suggested mainly by the different phenotype associated with targeted disruption of *RB,* as compared with those of *p107, Rb2/p130,* and *p107-Rb2/p130* double knockout mice [Lee et al., 1992; Jacks et al., 1992; Cobrinik et al., 1996]. Additional data suggest a collaborative effect of *p107* and *Rb2/p130* in regulating several gene targets [Hurford et al., 1997]. Particular interest has been evoked recently by *Rb2/p130,* because it was found mutated in a human small cell lung carcinoma [Helin et al., 1997], suggesting that, besides *RB,* the lack of function of this gene also could contribute to tumorigenesis. In addition, the expression of pRb2/p130, evaluated by immunohistochemistry in biopsies of selected human cancers, appears correlated positively with tumor differentiation and with patient survival [Baldi et al., 1996; Susini et al., 1998].

With the purpose of detecting functionally relevant protein-protein interactions involving pRb2/p130 and, possibly, of enriching understanding of its specific functional role, we engineered a cDNA expression vector corresponding to the C-terminal domain of pRb2/p130. This region was chosen because it diverges significantly from the pRb C-terminal domain. We used the construct as bait to screen an expression library in the yeast two-hybrid system [Chien et al., 1991]. Our attention was focused on a consistently strong interaction we found with cyclin D3. This finding became the starting point for additional structural and functional characterization of this interaction.

METHODS

Yeast Two-Hybrid Selection

The C-terminal region (nt 3142–3489, corresponding to amino acids 1025–1139) of the *Rb2/p130* gene product (GenBank accession number X76061), was cloned into the *Sma*I-*Bam*HI restriction sites of vector pGBT9 (Clontech, Palo Alto, CA) inframe with the *GAL4* binding domain. Yeast strain HF7c [Feilotter et al., 1994], bearing UASg-His3 and UASg-LacZ as reporter genes, was co-transformed with the bait pGBT9-Rb2/p130-C-term and with a human brain cDNA library (Clontech) fused to the *GAL4*

activation domain, in the vector pACT2 (Clontech). Transformation was done using the lithium acetate method [Gietz et al., 1992]. Cells were plated directly on minimal synthetic defined (SD) medium: 2% glucose, 0.67% Bacto yeast nitrogen base (Difco Laboratories, Detroit, MI), supplemented with the required bases and amino acids, but lacking tryptophan (Trp), leucine (Leu), and histidine (His). Plates were incubated for 7 days at 30°C, then His^+ transformants were isolated. The His^+ colonies, replica-plated on SD -Leu-Trp-His medium and $LacZ^+$, were identified by a filter-lifting assay for β-galactosidase activity. Plasmid DNA was prepared from candidate clones and electroporated into *Escherichia coli* XLI-blue competent cells (Stratagene, La Jolla, CA). The recovered library-derived plasmids were analyzed further as positive candidates.

To investigate the cyclin D3 domains involved in the selected interactions, eight new prey carrying different cyclin D3 fragments (Fig. 1C) were constructed in the pGAD424 vector (Clontech).

Cell Lines

Human T98G glioblastoma multiforme cell line [Olopade et al., 1992] was cultured as described [Claudio et al., 1996]. The human LAN-5 neuroblastoma cell line [Seeger et al., 1982] was maintained in culture and then induced to differentiate with RA (all-trans-retinoic acid) (Sigma-Aldrich, Milan, Italy) as described by Raschellà et al. [1992, 1995].

Peptides

The 18-mer peptide EVIDLTCHEAGFPPSDDE, called CR2 wt, derived from the CR2 region of the E1A oncoprotein [Dowdy et al., 1993], and the E9K mutated form EVIDLTCHKAGFPPSDDE, called CR2 mut, were synthesized by Biogen (Rome, Italy) and dissolved in 10 mM NH_4OH immediately before use.

Co-precipitations and Co-immunoprecipitations Involving In Vitro-Translated Proteins

Plasmids encoding for mouse cyclins D1, D2, and D3 (provided by Dr. C.J. Sherr, Howard Hughes Medical Institute, Memphis, TN) were used individually to program a TnT rabbit reticulocyte lysate (Promega, Madison, WI) under control of the T7 polymerase. Aliquots of the reaction mixture were added to glutathione-Sepharose beads carrying protein portions chimerized with the N-terminal region of GST[3], i.e., GST-pRb2/p130 (1027–1139) or GST-pRb (792–928). Peptides (CR2 wt and CR2 mut)

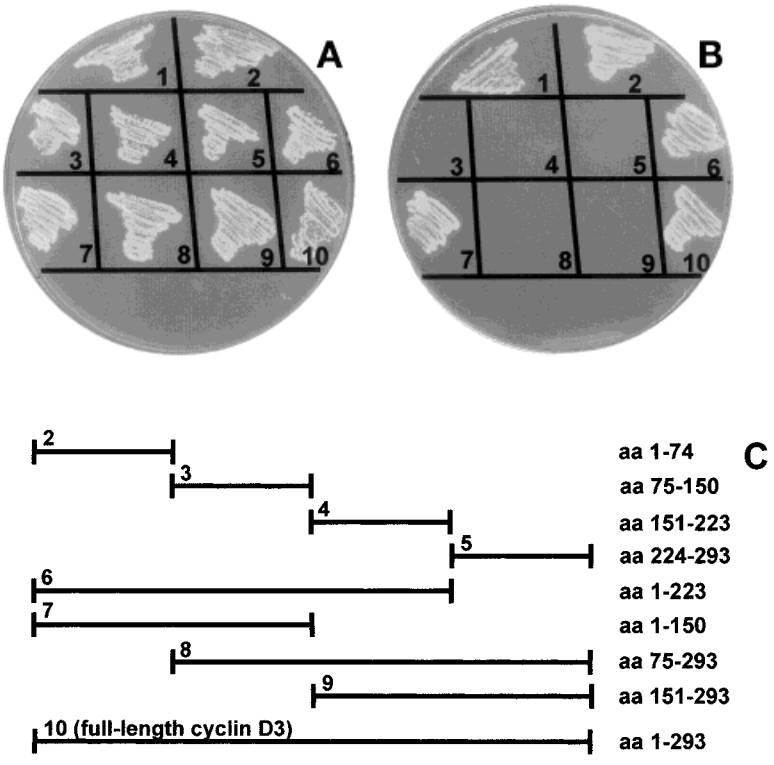


Fig. 1. Yeast two-hybrid system. Interaction between *pRb2/p130* C-terminal domain and cyclin D3. **A:** HF7c yeast cells plated in medium-Trp-Leu, after cotransfection with the bait pRb2/p130 C-terminal domain and with one of the different cyclin D3-related prey (2–10), indicated in **C** with the correspondent number, in order to verify the expression of both inserts. Numbers correspond to the transfected constructs represented in **C.** Clone 1 corresponds to the original cyclin D3 fragment isolated from the human brain cDNA library. **B:** The same yeast cells as in **A** plated in medium-Trp-Leu-His, to evaluate the interaction between bait and prey proteins, where only clones 1, 2, 6, 7, and 10 were able to grow. Panel 3: schematic representation of truncated and full-length cyclin D3 constructs cloned inframe with the activation domain of pGAD424. The relative amino acid stretches are indicated.

used for competition were added at a concentration of 50 μM before the programmed ^{35}S-labeled reticulocyte lysates were added. The same volume of solvent used for peptide solubilization (10 mM NH_4OH) was added to the control. Beads were washed three times in lysis buffer and dried; electrophoresis sample buffer was then added and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) performed. Gels were stained with Coomassie blue, treated for fluorography, dried, and then exposed at −70°C, using Kodak Biomax MS film with Du Pont Cronex intensifying screens.

In vitro-translated E1A synthesis and its co-immunoprecipitation with endogenous pRb were done as previously described [Paggi et al., 1994]. Peptides used for competition were added at a concentration of 50 or 150 μM before addition of the programmed ^{35}S-labeled reticulocyte lysates. The same volume of the solvent used for peptide solubilization (10 mM NH_4OH) was added to the control.

Co-precipitation with GST Chimeric Proteins, SDS-PAGE, and Western Blotting Analysis

The C-terminal region (nt 3142–3489, corresponding to amino acids 1027–1139) of *Rb2/p130* gene product (GenBank accession number X76061), was cloned into the *Bam*HI-*Sma*I restriction sites of vector pGEX-4T1 (Pharmacia-Biotech) inframe with the *GST* coding sequence. The C-terminal region (nt 2376–2790, corresponding to amino acids 792–928) of *RB* full-length cDNA (GenBank accession number M33647), was cloned into the *Bam*HI-*Eco*RI restriction sites of vector pGEX-2T (Pharmacia-Biotech) inframe with the *GST* coding sequence. T98G cells were lysed in 50 mM Tris-HCl pH 7.4, 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 0.1 mM Na_3VO_4, 0.1% Triton X-100, 1 mM PMSF, and 10 μg/ml leupeptin (lysis buffer) for 30 min at 4°C. After centrifugation, supernatants were transferred to a fresh tube and chimeric proteins GST-pRb2/p130 (1027–1139), GST-pRb2/p130 (1027–1104) (gift from Dr. L. Bagella, Thomas Jefferson University, Philadelphia, PA), and GST-pRb (792–928) conjugated to glutathione-Sepharose beads (Pharmacia-Biotech, Milan, Italy) were added and incubated for 90 min at 4°C. Beads were washed three times in lysis buffer and dried; electrophoresis sample buffer was then added. SDS-PAGE and protein transfer to a PVDF membrane was done as previously described [Baldi et al., 1995]. Cyclin D1 was detected using a rabbit polyclonal antibody (sc-92, Santa Cruz Biotechnology, Santa Cruz, CA). Cyclin D3 was detected using a rabbit polyclonal antibody (sc-182, Santa Cruz Biotechnology). Cdk4 was detected using a rabbit polyclonal antibody (sc-260, Santa Cruz Biotechnology). p21 was detected using a mouse monoclonal antibody (15091A, Pharmingen, San Diego, CA). p16 was detected using a rabbit polyclonal antibody (sc-467, Santa Cruz Biotechnology). HSP70 was detected using a mouse monoclonal antibody (HSP01, Oncogene Science, Manhasset, NY). Detection was carried out using the enhanced chemiluminescence (ECL) kit (Amersham, Milan, Italy), using Kodak Biomax MR film.

Cyclin-Associated Kinase Activity Assay

T98G cells were lysed as described above. Immunoprecipitation then was accomplished using antibodies anti-cyclin D1 (mouse monoclonal, sc-246; Santa Cruz Biotechnology) or anti-cyclin D3 (rabbit polyclonal, sc-182, Santa Cruz Biotechnology) conjugated to protein G- or protein A-agarose, respectively (Pierce, Prodotti Gianni, Milan, Italy). Beads were washed three times in lysis buffer. Associated kinase activity was assayed as previously described [Giordano et al., 1991; De Luca et al., 1997], using as substrates 0.5 μg/assay of GST-mouse pRb (769–921) (sc-4112, Santa Cruz Biotechnology), 5 μg/assay of GST-pRb2/p130 (1027–1139) and/or 5 μg/assay of GST-pRb (792–928), as indicated.

RESULTS

Physical Interaction Between pRb2 C-Terminal Domain and Cyclin D3

The yeast two-hybrid system was used to search for proteins capable of physically interacting with the C-terminal domain of pRb2/p130 (amino acids 1025–1139). The screening was done using a human brain complementary DNA (cDNA) library. Among an estimated 1×10^6 transformants screened, 49 clones able to proliferate on media lacking histidine were isolated. Seven were also positive in the β-galactosidase assay. Direct DNA sequencing of the rescued plasmids showed that all these clones encoded for cyclin D3, strongly suggesting its interaction with the C-terminal domain of pRb2/p130. In order to confirm this finding and to define more accurately the region of cyclin D3 involved in the binding, the full-length cyclin D3 and eight distinct constructs carrying differ-

ent regions (Fig. 1C) were analyzed. As shown in Figure 1A,B, the full-length cyclin D3 (amino acids 1–293) (construct 10) and the constructs coding for the amino acid stretch 1–74 (construct 2) or extending over this sequence (constructs 6 and 7) were the only ones allowing yeast growth in minimal SD medium lacking tryptophan, leucine, and histidine, as described under Methods. This let us to restrict the region of the cyclin D3 molecule involved in the binding to the C-terminal region of pRb2/p130 within the 74 N-terminal amino acids.

The LXCXE Motif of the D-Type Cyclins Was Dispensable for Binding to the C-Terminal Domains of pRb2/p130 and pRb

D-type cyclins, at the beginning of their N-terminal sequence, harbor the amino acid motif LXCXE, responsible for their binding to the pRb A/B pocket region [Dowdy et al., 1993; Ewen et al., 1993]. The binding investigated in the present study, however, involved the pRb2/p130 C-terminal domain interaction with cyclin D3. We restrained the region of cyclin D3 involved in the binding to the pRb2/p130 C-terminal domain to the 74 N-terminal amino acid sequence (Fig. 1). Therefore, we decided to assess the role of the N-terminal LXCXE motif of the D-type cyclins in the binding to the C-terminal domain of either pRb or pRb2/p130. To this end, the peptide EVIDLTCHEAGFPPSDDE, referred to as CR2 wt, a 18-mer derived from the CR2 (conserved region 2) of the adenovirus 5 E1A oncoprotein [Barbeau et al., 1992; Dowdy et al., 1993] was used to observe if it competed for the binding of cyclin D3 to the pRb2-p130 C-terminal domain. As a control, the peptide EVIDLTCHKAGFPPSDDE, referred to as CR2 mut, was used, bearing the substitution E9K (underlined in the amino acid sequence), according to a described nonfunctional mutation [Dowdy et al., 1993]. CR2 wt, as well as CR2 mut, had no effect on the binding of either GST-pRb2/p130 (1027–1139) or GST-pRb (792–928) to mouse D-type cyclins translated in vitro (Fig. 2A). These data can be also extended to human cyclins, because of the remarkable similarity between human and murine cognate D-type cyclins [Ewen et al., 1993]. In order to verify how these peptides were effective in competing for the binding of LXCXE proteins, we synthesized in vitro the whole 12S E1A oncoprotein and assayed its ability to coimmunoprecipitate with endogenous pRb [Paggi et al., 1994] in the T98G cell lysate. In this case, 50 or 150 μM CR2 wt peptide was able to reduce the binding of E1A to pRb consistently, while the same concentrations of CR2 mut were less efficient by far in competing with E1A for the pRb pocket (Fig. 2B). The concentrations of the peptides used for each competition assay were in the range of those reported by other investigators [Helin et al., 1992; Dowdy et al., 1993].

To ascertain whether the LXCXE motif of cyclin D3 was required for interaction with the C-terminal domain of pRb2/p130, using another technique we used the two-hybrid system, to analyze the ability to interact with pRb2/p130 C-terminal domain of two mutant cyclin D3 constructs derived from the one coding for the amino acid stretch 1–74. The first carried the E7K substitution, thus mutating the LXCXE motif to LXCXK, and the second carried a deletion of the first 7 amino acids, thus completely removing the LXCXE binding motif. As shown in Figure 3, when co-transformed in yeast with pRb2/p130 C-terminal domain, both constructs (3 and 4) allowed the growth of colonies in medium lacking tryptophan, leucine, and histidine, similar to the wild-type 1–74 cyclin D3 construct (2) and the full-length cyclin D3 (1). These results confirmed the dispensability of the LXCXE motif for the cyclin D3-pRb2/p130 C-terminal domain interaction.

Identification of the pRb2/p130 Portion Responsible for the Binding to Cyclin D3

We engineered a plasmid to produce a GST chimeric construct containing the C-terminal domain of pRb2/p130 [GST-pRb2/p130 (1027–1139)]. As a reference, an analogous construct, coding for the pRb C-terminal domain [GST-pRb (792–928)], was used as well. The correspondent proteins were employed in a coprecipitation assay. The glutathione-Sepharose-conjugated (immobilized) chimeric proteins GST-pRb2/p130 (1027–1139) and GST-pRb (792–928) were added to a T98G cell lysate, a $RB^{+/+}$ $p16^{-/-}$ human glioblastoma multiforme cell line [Olopade et al., 1992; Lukas et al., 1995]. Proteins coprecipitated with the immobilized GST fusion proteins were analyzed by SDS-PAGE and subsequent Western blotting for cyclins D1, D3, and cdk4. The results showed a strong interaction between GST-pRb2/p130 (1027–1139) and cyclin D3, clearly evident in Figure 4, as well as very weak interactions with

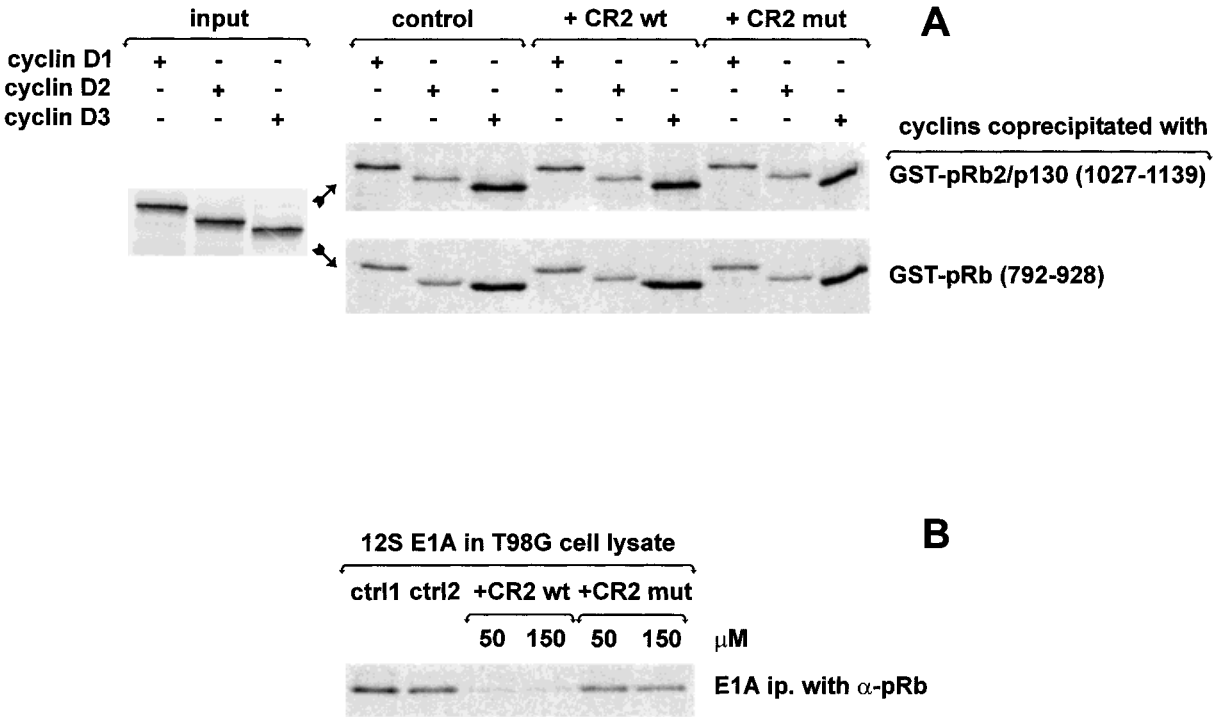


Fig. 2. CR2-mimicking peptide does not disrupt the interaction between in vitro-translated murine D-type cyclins and *GST-pRb2/p130* or *-pRb* C-terminal domains. **A:** ^{35}S-labeled in vitro-translated proteins were analyzed by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (left) or diluted in lysis buffer and incubated with the indicated immobilized GST chimeric constructs in the presence of 50 μM CR2 wt or CR2 mut peptides. Control was incubated with the same volume of 10 mM NH_4OH used to add diluted peptides (25 μl). In vitro-translated proteins used as input and proteins precipitated with the GST constructs were analyzed by 10% SDS-PAGE. Gel was treated for fluorography, dried, and then exposed to an autoradiography film. Bands represent co-precipitated D cyclins. **B:** ^{35}S-labeled in vitro-translated E1A was incubated in a T98G cell lysate in the presence of 50 or 150 μM CR2 wt or CR2 mut peptides. Control was incubated with the same volumes of 10 mM NH_4OH used to add diluted peptides (25 μl for ctrl1 and 75 μl for ctrl2). Immunoprecipitation was then performed using C36, an anti-pRb monoclonal antibody [Paggi et al., 1994]. E1A immunoprecipitated with pRb was analyzed by 10% SDS-PAGE. Gel was treated for fluorography, dried, and then exposed to an autoradiography film. Bands represent co-immunoprecipitated in vitro-translated E1A.

cyclin D1 and cdk4, both barely detectable. We also observed that GST-pRb (792–928) was clearly able to interact not only with cyclin D3, but also, in a comparable manner, with cyclin D1 (Fig. 4A). Another construct was used that contained a truncated C-terminal domain of pRb2/p130 [GST-pRb2/p130 (1027–1104)]. This last construct did not interact with cyclin D3 (Fig. 4B), thus suggesting that an epitope responsible for cyclin D3 binding should lie in the very C-terminal region of the pRb2/p130 molecule, precisely between amino acids 1105 and 1139.

The C-Terminal Domains of pRb2/p130 and of pRb as Substrates for Cyclin D1- and Cyclin D3-Associated Kinase Activity

It has been shown that specific domains of p107 and of pRb2/p130 can inhibit cdk2 kinase

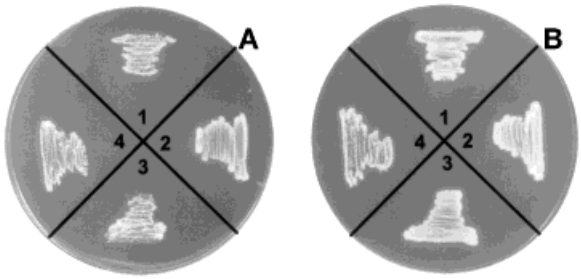


Fig. 3. Yeast two-hybrid system. Interaction between *pRb2/p130* C-terminal domain and cyclin D3 does not require the LXCXE motif. **A:** HF7c yeast cells plated in medium-Trp-Leu, after cotransfection with the bait pRb2/p130 C-terminal domain and with the following different cyclin D3-related prey: 1, full-length cyclin D3; 2, amino acids 1–74 of cyclin D3; 3, amino acids 1–74 of cyclin D3 bearing the mutation E7K; 4, amino acids 8–74 of cyclin D3, lacking the LXCXE motif. **B:** Same yeast cells as in **A** plated in medium-Trp-Leu-His, to evaluate the interaction between bait and prey proteins, where all the clones were able to grow.

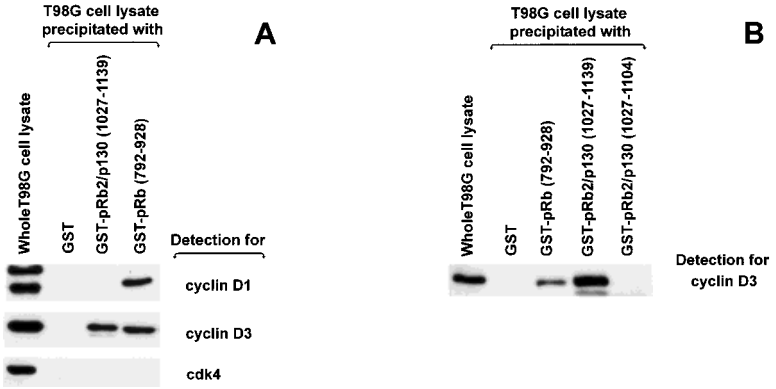
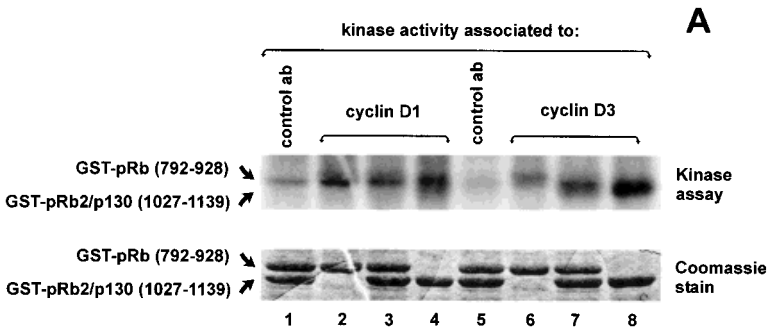
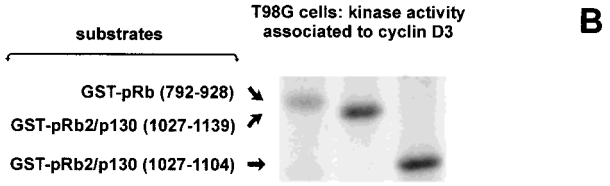


Fig. 4. Interaction of cyclins D1, D3, and cdk4 with GST chimeric constructs corresponding to *pRb2/p130* or *pRb* C-terminal domain domains. **A:** T98G cell lysates were incubated in the presence of immobilized GST or GST chimeric proteins, as indicated. Recovered interacting proteins were fractionated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto a polyvinyldifluorene (PVDF) membrane, and analyzed by means of Western blotting for cyclin D1, D3, or cdk4. Leftmost lanes show cyclins D1, D3, and cdk4 detection in crude T98G cell lysate. **B:** Same as **A,** but including the use of the truncated construct GST-pRb2/p130 (1027–1104). Detection was done for cyclin D3 only.

activity [Lacy and Whyte, 1997; Woo et al., 1997; De Luca et al., 1997; Castaño et al., 1998]. In order to elucidate the significance of the interactions between D-type cyclins and pRb or pRb2/p130 C-terminal portions, we assayed their effect on cyclin D1- and cyclin D3-associated kinase activity. We immunoprecipitated T98G cell lysate using antibodies anti-cyclin D1 or anti-cyclin D3. Immunoprecipitates were then assayed for associated kinase activity, using as a substrate GST-mouse pRb (769–921). Samples preincubated with either GST-pRb2/p130 (1027–1139) or GST-pRb (792–928) exhibited no change in activity, as evaluated by phosphorylation of the mouse pRb substrate, as compared with controls incubated with GST alone (not shown). In order to determine whether GST-pRb2/p130 (1027–1139) and GST-pRb (792–928) could be per se efficient substrates, the two chimeric proteins were incubated, individually or together, in the presence of cyclin D1- or D3-associated kinase activity (Fig. 5A). The two GST fusion proteins were suitable substrates for both associated kinase activity, but, when incubated simultaneously, the propensity of GST-pRb2/p130 (1027–1139) to become the preferential substrate for cyclin D3-associated kinase activity was evident (Fig. 5A, lanes 7, 8), while GST-pRb (792–928) was phosphorylated mainly by cyclin D1-associated kinase activity (Fig. 5A, lanes 3, 4). The amount of GST chimeric proteins that underwent phosphorylation slightly modified their SDS-PAGE migration pattern. Figure 5B shows that also the truncated GST-pRb2/p130 (1027–1104) molecule, despite its inability to interact with cyclin D3, was phosphorylated by the cyclin D3-associated kinase activity. It is worth mentioning that GST alone is not a substrate for cyclin/cdk heterodimers [Lin et al., 1991].

Our next step was to evaluate cyclin D3-associated kinase activity in the human LAN-5 neuroblastoma cell line. In this cell line, as well as in the murine neuroblastoma N1E-115 cell line, we demonstrated that expression pattern and phosphorylation status of pRb2/p130 differs from that of either pRb or p107, when these cells are induced to neural differentiation [Raschellà et al., 1997, 1998]. We then evaluated cyclin D3-associated kinase activity during LAN-5 differentiation on both substrates GST-pRb (792–928) and GST-pRb2/p130 (1027–1139) (Fig. 5C). In this case as well, GST-pRb2/p130 (1027–1139) was by far the preferred substrate throughout the differentiation process. However, the D3-associated kinase activity dropped down at the end of the differentiation process (10 d), in a way remarkably consistent with the amounts of cyclin D3, as evaluated by Western blotting at the same time intervals. Cyclin D1 and cdk4 levels also were determined, the former being slightly decreased and the latter substantially unchanged at the end of the process (10 d). p21 peaked at an intermediate differentiation time (3 d), whereas p16 was undetectable. HSP70 was used for normalization of the protein amounts loaded.





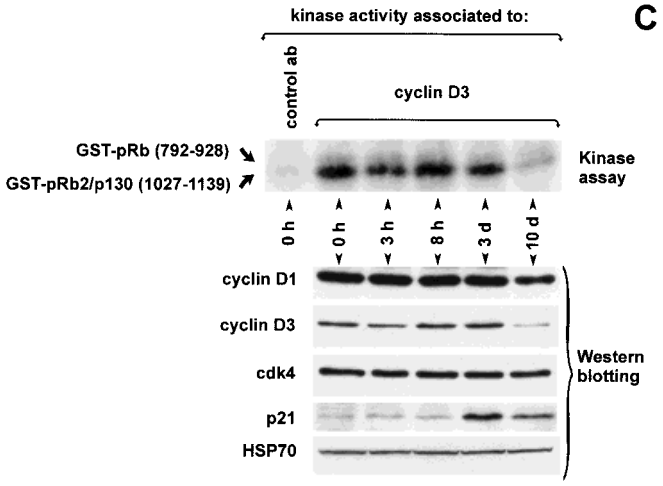


Fig. 5. Preferential phosphorylation of GST-Rb2/p130 C-terminal domain by cyclin D3-associated kinase activity. **A: Top,** after kinase assay in T98G cell lysate, proteins were separated by means of 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Gel was dried and then exposed to an autoradiography film. Phosphorylation of the indicated GST fusion proteins by cyclin D1- or D3-associated kinase activity is shown. **Bottom,** Coomassie stain of the dried and exposed gel in order to recognize presence and amount of the indicated GST fusion proteins utilized as substrates. **B:** Same kinase assay as in **A,** using the single substrate indicated, including the truncated construct GST-pRb2/p130 (1027–1104). **C:** After kinase assay in differentiating LAN-5 cell lysates, proteins were separated by means of 12% SDS-PAGE. Gel was dried and then exposed to an autoradiography film. Times after RA induction of differentiation are indicated. The uppermost part of the panel shows preferential GST-pRb2/p130 C-terminal domain construct phosphorylation and its modulation during the differentiation process. The lower parts of the panel show Western blotting determination (after 10% SDS-PAGE) of the indicated proteins at the same time points after induction of differentiation. HSP70 determination was used to visualize that a comparable amount of protein was loaded.

DISCUSSION

Phosphorylation of the pRb molecule causes neutralization of its growth-suppressive properties [Chen et al., 1989; Buchkovich et al., 1989; Goodrich et al., 1991], but maximal pRb functional inactivation is achieved by sequential phosphorylation of multiple sites of the molecule [Knudsen and Wang, 1996; Zarkowska and Mittnacht, 1997]. D-type cyclins are known to interact, via their LXCXE motif, with the A/B pocket of the *RB* gene product [Dowdy et al., 1993; Ewen et al., 1993]. These cyclins, coupled to cdk4/6, are responsible for the early pRb phosphorylation in G1. Although this step is crucial to allow further pRb inactivation by cyclin E/cdk2 [Connell-Crowley et al., 1997; Lundberg and Weinberg, 1998], the functional significance of the interaction between D-type cyclins and pRb remains controversial. In this context, a number of hypotheses have been proposed. Dowdy et al. [1993] suggest that D cyclins may interact with pRb to link this protein to cdk4/6, thus facilitating the interaction between the kinase and its substrate. Another

assumption states that the purpose of the binding via the LXCXE motif of the D cyclins to the A/B pocket of pRb will result into hiding the cyclin, thus sequestering it and neutralizing its growth-stimulating potential. Thus, a parallelism between the transcription factor E2F and D cyclins can be envisaged. Further pRb phosphorylation, also driven by additional cyclin/cdk molecules, can interfere with this interaction and can provide free D cyclins, which are functionally capable in promoting G1 progression. In addition, Ewen et al. [1993] hypothesize that E2F binding to pRb can be prevented by cyclin D1 engaged into the pRb pocket.

Despite the fact that both p107 and pRb2/p130 are phosphorylated and inactivated by D-type cyclins [Beijersbergen et al., 1995; Claudio et al., 1996; Johnson, 1995], and that pRb2/p130 has been shown to interact with in vitro-translated D-type cyclins [Hannon et al., 1993], precise mapping of the epitopes involved in these interactions is not currently available.

The LXCXE motif present in cyclin D1, although responsible for the binding to the A/B pocket of pRb, is not implicated in the ability of the heterodimer cyclin D1/cdk4 to phosphorylate pRb [Horton et al., 1995; Connell-Crowley et al., 1997]. This proves that binding to the A/B pocket is not responsible directly for kinase activation toward pRb. We describe here a binding of the D cyclins to the C-terminal domain of either pRb or pRb2/p130. In this scenario, it is reasonable to suggest that the interaction between D cyclins and pRb2/p130, as already postulated for pRb [Ewen et al., 1993], could account for more than one protein-protein interaction. This multiple interplay also could help in resolving the controversy about the functional role of the interaction of D cyclins with pRb and, possibly, with pRb2/p130. p107 was recently shown to inhibit cyclin A/cdk2 and cyclin E/cdk2 activities via dual cyclin-binding domains. Moreover, a cdk-binding region, reminiscent of $p21^{WAF1\text{-}CIP1}$, was mapped on the N-terminal portions of both p107 and pRb2/p130 [Castaño et al., 1998].

Our data permit improved analysis of the binding between pocket proteins and D-type cyclins on both sides of the interaction. It should be pointed out that, in the experiments involving in vitro-translated D-type cyclins, all the D-type cyclins seemed to interact in the same way, either with the pRb or with the pRb2/p130 C-terminal domain GST chimeric constructs. This result, in accordance with the data of Hannon et al. [1993], could be a partial artifact related to an incomplete reconstitution of the cellular environment, due, for example, to the absence of virtually any endogenous competitor (e.g., nuclear factors) in the rabbit reticulocyte lysate. However, the data obtained using either the CR2-mimicking peptide in co-precipitation, or the mutant cyclin D3 N-terminal constructs in the yeast two-hybrid system, excluded the possibility that the binding of cyclin D3 to pRb2/p130 could be mediated by the LXCXE motif, the one responsible for the interaction with the A/B pocket of pRb. Nevertheless, the epitope of cyclin D3 involved in binding to the C-terminal domain should lie within the 74 N-terminal amino acids or, more precisely, between amino acids 8 and 74.

In addition, our results permit precise definition of the pRb2/p130 epitope responsible for cyclin D3 binding. Basically, because of the lack of interaction between cyclin D3 and the truncated construct GST-pRb2/p130 (1027–1104), the amino acid stretch responsible for binding between aa 1105 and aa 1139 of the pRb2/p130 sequence was restrained. Despite its inability to bind to cyclin D3, the construct GST-pRb2/p130 (1027–1104) was nevertheless a good substrate for cyclin D3-associated kinase activity. Thus, the ability to interact with cyclin D3 and the propensity to become a substrate for cyclin D3-associated kinase activity (Fig. 5) appeared to be located in distinct regions of GST-pRb2/p130 (1027–1139). This has been also reported for the C-terminal portion of pRb, where distinct domains harboring phosphorylation sites or putative cyclins (mainly A and E)/cdk binding sites are described [Adams et al., 1999].

From our results, there was the experimental evidence that cyclin D3 coprecipitated using GST-pRb2/p130 (1027–1139) was not significantly associated with cdk4 (Fig. 4A). These data give credit to the hypotheses that cyclin D3 bound to the very C-terminal region of pRb2/p130 could be substantially ineffective in heterodimer formation with cdk4, thus giving to pRb2/p130 a peculiar role in modulating cyclin D3/cdk4 activity. Moreover, a recent paper by Stiegler et al. [1998] shows that the same C-terminal region of pRb2/p130 we found to interact with cyclin D3 results involved in binding to HDAC1.

A recent paper by Dong et al. [1998] presents for the first time direct proof that cyclin D3-

associated kinase activity is specifically involved in the phosphorylation of pRb2/p130 in mouse BALB/c 3T3 fibroblasts. A specific protein-protein interaction also is demonstrated between GST-p130 (amino acids 322–1139, spanning the A/B pocket and the C-terminal domain) and cyclin D3. Our data support these results and present more information about the portions of both proteins involved in the binding.

Specific roles have been depicted for pRb2/p130 in differentiation [Raschellà et al., 1997, 1998; Richon et al., 1997; Paramio et al., 1998; Kiess et al., 1995] and recently for cyclin D3 in either proliferation or differentiation [Bartkova et al., 1998]. In the LAN-5 human neuroblastoma differentiation model, cyclin D3 and its associated kinase activity were downmodulated at late differentiation stages (10 days after RA treatment), where the three pocket proteins appear primarily in their underphosphoryladed form [Raschellà et al., 1997].

We believe that an improvement of our knowledge on the interplay between the pocket family of proteins and the D cyclins could be helpful in interpreting their role in multifactorial regulatory phenomena, such as cell cycle, differentiation, and ultimately loss of growth control in cancer cells.

ACKNOWLEDGMENTS

F.B. and M.F. equally contributed to this work. F.B. is the recipient of an FIRC fellowship. We thank Drs. C. Passananti and M. Caruso (CNR, Rome, Italy), D. Lombardi (University of L'Aquila, Italy), P.G. Natali, R. Tecce, and A. Severino (Regina Elena Cancer Institute, Rome, Italy) for useful suggestions and criticism, Dr. G. Raschellà (ENEA, Rome, Italy) for the LAN-5 cell line, Dr. L. Bagella (Thomas Jefferson University, Philadelphia, PA) for the GST-pRb2/p130 (1027–1104) construct, and Dr. C.J. Sherr (Howard Hughes Medical Institute, Memphis, TN) for the murine D cyclins constructs.

This work was supported in part by grants from AIRC (to M.G.P. and A.F.) from FIRC (to A.D.L.) from Ministero della Sanità (to M.G.P.), and from the NIH (grant RO1-CA60999-01A1), and from the Council for Tobacco Research (to A.G.).

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