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Interaction of the pRB-family proteins with factors containing paired-like homeodomains

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The specific loss of pRB or p107 together with p130 disrupts the normal development of only a very limited spectrum of tissues. These developmental defects have been attributed primarily to deregulation of E2F activity and consequent uncontrolled proliferation. We hypothesized, however, that the tissue-specific nature of these defects may also reflect deregulation of pRB-family associated factors that are specifically involved in determining cell fate. We report here that the pRB-family members interact with transcription factors which contain paired-like homeodomains such as MHox, Chx10 and Pax-3. The interaction between the pRB-family and the paired-like homeodomain proteins was initially identified in a yeast two-hybrid screen where the N-terminal portion of p130 was used to isolate interacting factors from an embryonic mouse library. This interaction was confirmed by *in vitro* binding and co-immunoprecipitation assays. We show further that co-expression of Pax-3 dependent pRB, p107 or p130 with Pax-3 causes repression of activated transcription from the c-met promoter. These data demonstrate that the pRB-family proteins can modulate the activity of factors which specifically control cell fate and/or differentiation as well as controlling cell cycle regulators.

Keywords: mhox; paired-like homeodomain; PGX-3; pRB

Introduction

Progression through or exit from the eukaryotic cell division cycle requires the expression and activity of both positive and negative cell cycle regulatory factors (Cross *et al*., 1989; Hartwell and Weinert, 1989; Hunt, 1989; Laskey *et al*., 1989; Lewin, 1990; Murray and Kirschner, 1989; Sherr, 1996). One important class of negative regulatory factors is the pRB-family proteins, which includes pRB, p107 and p130. Their apparent role as negative regulators of cell cycle progression was established when it was determined that they were important targets of the virally encoded transforming proteins of the DNA tumor viruses, SV40, adenovirus and human papilloma virus. Specifically, Large T antigen (T_{Ag}) (DeCaprio *et al*., 1988), Ela (Dyson *et al*., 1989) and E7 (Dyson *et al*., 1989; Münger *et al*., 1989), respectively, all contain sequences in their transformation domains which specifically bind the pRB-family proteins (Dyson *et al*., 1992). Mutation of these closely related sequences in T_{Ag}, E1a or E7 and the resultant failure to bind pRB, p107 and p130 prevent cellular transformation by these transforming viral proteins (Ewen *et al*., 1989; Heck *et al*., 1992; Howley *et al*., 1991; Jewers *et al*., 1992; Larose *et al*., 1991; Munger *et al*., 1991; Whyte *et al*., 1988).

Binding of pRB to E1a led to the critical observation that one role of the pRB-family proteins was complex formation with and resultant repression of activated transcription by the E2F-family of transcription factors (Bandara and La Thangue, 1991; Beijersbergen *et al*., 1994; Buck *et al*., 1995; Hamel *et al*., 1992; Helin *et al*., 1992, 1993; Hiebert *et al*., 1992; Hijmans *et al*., 1995). Data from a number of labs have demonstrated that pRB, p107 and p130 have different binding affinities for specific E2F-family members (Beijersbergen *et al*., 1994; Buck *et al*., 1995; Hijmans *et al*., 1995; Lees *et al*., 1993; Sardet *et al*., 1995). It is also clear that distinct complexes predominate at different points in the cell cycle or in cells that have exited the cell cycle during terminal differentiation. For example, p130 is predominantly bound to E2F4 in terminally differentiated and/or quiescent cells (Cobrinik *et al*., 1993; Corbeil *et al*., 1995; Kiess *et al*., 1995; Shin *et al*., 1995; Smith *et al*., 1996). As cells enter the cell cycle, p130 appears to be replaced by p107 in these complexes (Moberg *et al*., 1996).

While the pRB-family proteins have been described primarily as cell cycle regulatory factors, a number of observations indicate that these proteins may also play an important role in normal embryonic development. pRB, for example, was originally identified as the product of the retinoblastoma susceptibility gene, *RB1* (Friend *et al*., 1986). Loss of pRB in the developing human retina results in the formation of retinoblastoma tumors (Dunn *et al*., 1988; Horowitz *et al*., 1990). Additionally, mice homozygously deleted for pRB died *in utero* by E14.5 (Clarke *et al*., 1992; Jacks *et al*., 1992; Lee *et al*., 1992). Rather than showing uniform disruption of all developing tissues, however, these mice exhibited defects that were restricted to particular cells of the central nervous system and developing erythropoietic cells. Furthermore, while mice homozygously deleted for either p107 (Lee *et al*., 1996) or p130 (Cobrinik *et al*., 1996) developed normally, the double nullizygous mice ($p107^{-/-}/p130^{-/-}$) died at birth due to skeletal defects (Cobrinik *et al*., 1996). The chondrocyte-specific defects in the $p107^{-/-}/p130^{-/-}$ mice were attributed to a delay in terminal differentiation due to deregulation of the cell cycle.

While the interaction between the pRB-family proteins and the E2F-family of cell cycle regulatory

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transcription factors has been well characterized, it is clear that they also complex several factors which are involved in determining cell fate. For example, pRB forms specific complexes with the myogenic determinants, MyoD and myogenin (Gu *et al*., 1993; Schneider *et al*., 1994). Interaction of pRB with these tissue specific transcription factors is apparently required for potent transcriptional activation of genes regulated by MyoD or myogenin (Skapek *et al*., 1996). Interestingly, a recent report demonstrated that expression of a weak pRB allele on the pRB nullizygous background resulted in profound disruption of muscle structures during development, supporting a role for pRB during myogenesis (Zacksenhaus *et al*., 1996). Other tissue-specific or developmental targets for pRB that have been reported include the ets-related protein, Elf-1 (Wang *et al*., 1993), members of the C/EBP-family protein, NF-IL6 (Chen *et al*., 1996b), and C/EBPα, β and δ (Chen *et al*., 1996a) as well as a new member of the HMG-box group of transcription factors, HBP1 (Tevosian *et al*., 1997).

The developmental defects observed in mice and humans following loss of pRB or p107 together with p130 have been attributed primarily to deregulation of E2F activity and consequent failure to exit the cell cycle as cells differentiate. We hypothesized, however, that the tissue specific effects of losing specific pRB-family proteins may also be a consequence of deregulation of the activity of other novel pRB-associated factors which are involved in determining cell fate. We report here that the pRB-family proteins form complexes with a class of important developmental factors which all contain paired-like homeodomains and includes MHox, Pax-3, Chx10, and Alx3. We report further that the consequence of these interactions is repression of activated transcription mediated by these paired-like homeodomain proteins.

Results

Isolation of novel pRB-family-associated proteins

A yeast two-hybrid screen was performed on an E11.5 mouse embryonic library to search for novel developmental factors that interacted with the pRB-family proteins. The N-terminal portion of p130 (amino acids 1–415; see also Figure 3a) was used as the 'bait' in this screen since we had determined previously that a number of unknown nuclear factors complexed this region of p130 in an *in vitro* binding assay (OW; unpublished observation). Eight cDNAs encoding proteins that specifically interacted with the N-terminus of p130 were isolated in the two-hybrid assay. Six of these cDNAs had no significant sequence similarity in their first 200 bp to known genes. However, one cDNA encoded the mouse paired-like homeodomain protein, MHox (Cserjesi *et al*., 1992). Another cDNA encoded a novel protein, B4, which is greater than 80% identical in a 150 bp region to the paired-like homeodomain in MHox and to the paired-like homeodomain regions of Chx10 (Liu *et al*., 1994), Cart-1 (Zhao *et al*., 1993), A1x3 (Rudnick *et al*., 1994), Pax-3 (Goulding *et al*., 1991) and Drosophila *aristaless* (Schneitz *et al*., 1993). The high degree of similarity between B4 and MHox exclusively in their paired-like homeodomain regions and the fact that the B4 clone encoded only 40 amino acids outside of the paired-like homeodomain indicate the paired-like homeodomains in MHox and B4 are the binding sites for the N-terminus of p130.

We next confirmed complex formation between the pRB-family proteins and the paired-like homeodomain proteins in an *in vitro* binding assay (Figure 1). GST- or His-tagged versions of Pax-3, Chx10 and the novel paired-like homeodomain protein, B4, (see Figure 1a) were isolated from bacteria. Nuclear lysates prepared

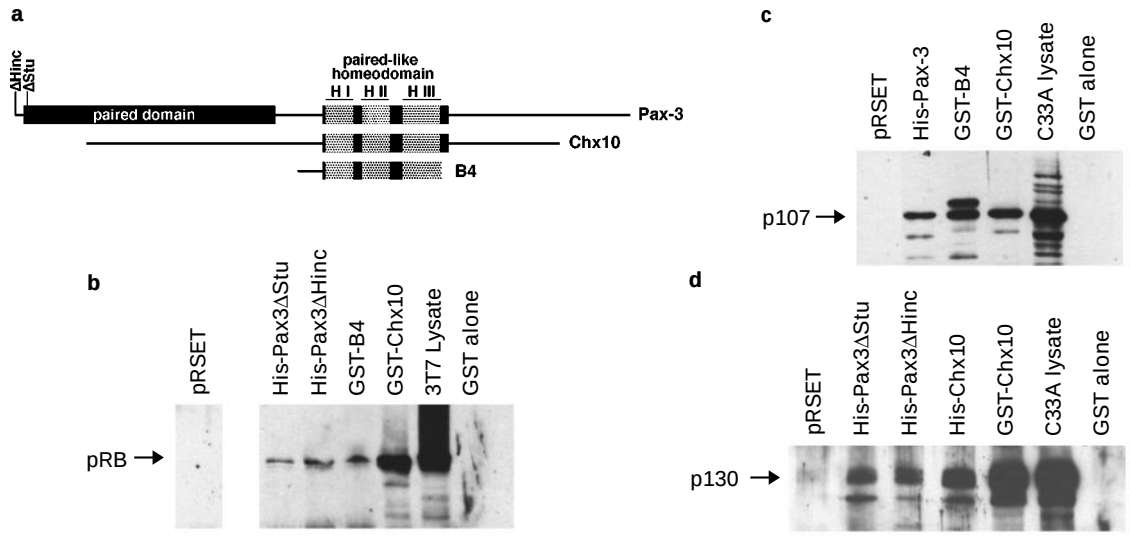

Figure 1 Binding of pRB-family proteins to transcription factors that contain paired-like homeodomains. GST- or His-tagged versions (**a**) of Pax-3, Chx10 or the novel homeodomain protein, B4, were purified from bacteria and used to assess binding of the pRB-family proteins. Nuclear lysates containing native pRB (**b**), p107 (**c**) and p130 (**d**) were prepared from the mouse T cell lymphoma, 3T7 (pRB), or the human cervical carcinoma cell line, C33A (p107 and p130), and were passed over affinity columns containing the various homeodomain proteins. Binding was assessed by Western analysis using antibodies specific for each of the pRB-family proteins. Note that the extra band in the B4 lane for p107 binding is due to cross reaction of the α-p107 antibody with a bacterial protein which co-purifies with GST-B4 (data not shown)

from the human cervical carcinoma pRB-deficient cell line, C33A, or the murine T cell lymphoma, 3T7, were then passed over affinity columns containing the fusion proteins. pRB (Figure 1b), p107 (Figure 1c) and p130 (Figure 1d) specifically bound to Pax-3, B4 and Chx10 but did not bind to beads containing lysates from bacteria expressing GST alone or the empty His-tagged vector, pRSET. These binding assays were repeated in the presence of ethidium bromide (Lai and Herr, 1992) or following addition of DNase 1 (Lai and Herr, 1992) in order to eliminate the possibility that complex formation was mediated through non-specific binding of the pRB-family proteins to DNA (data not shown).

Binding of pRB-family proteins to the homeobox proteins was also confirmed in GST-binding experiments using *in vitro* translated Pax-3 or Chx10 (Figure 2). Rabbit reticulolysates, programmed to express Pax-3 or Chx10 protein, were passed over glutathione beads containing GST alone, GST-pRB and/or GST-p130 and binding was determined following SDS–PAGE. A very weak background signal for both Pax-3 and Chx10 was seen for columns containing GST-alone. In contrast, Pax-3 bound strongly to GST-pRB. Likewise, Chx10 bound strongly to GST-p130 although weaker binding to GST-pRB was seen for this paired-like homeodomain protein in this assay.

Complex formation between the pRB-family proteins and one of the paired-like homeodomain proteins, Pax-3, was further confirmed in co-immunoprecipitation assays from whole cell lysates (Figure 3). Endogenous p107 or p130 was immunoprecipitated from C33A cells programmed to stably express an HA-tagged version of Pax-3. As Figure 3 clearly demonstrates, Pax-3 forms stable complexes with both of these pRB-family proteins *in vivo*.

We next determined that the paired-like homeodomain proteins preferentially associate with the active (unphosphorylated) form of pRB (Figure 4). Similar to the binding experiments described above, untreated COS cell lysates (L) or lysates treated with λ-phosphatase (P) were passed over columns containing GST-Chx10 or GST-PHox, and pRB binding assessed by western analysis. The first lane demonstrates that pRB migrates as two distinct bands: the slower migrating, inactive, hyperphosphorylated form and the faster migrating, unphosphorylated form. Treatment with λ-phosphatase (lane P) converts all of the pRB to the faster migrating, unphosphorylated form. When lysates were passed over columns containing GST-Chx10, this fusion protein clearly bound to the unphosphorylated pRb present in the λ-phosphatase treated (P) lysates. However, GST-Chx10 also bound exclusively to the unphosphorylated form of pRB in untreated lysates (L) and does not pull down the hyperphosphorylated species. Likewise, GST-PHox preferentially bound the unphosphorylated form of pRB present in the untreated COS cell lysates. Thus, analogous to E2F-binding to pRB, Chx10 and PHox preferentially associate with the active, unphosphorylated form of the pRB protein.

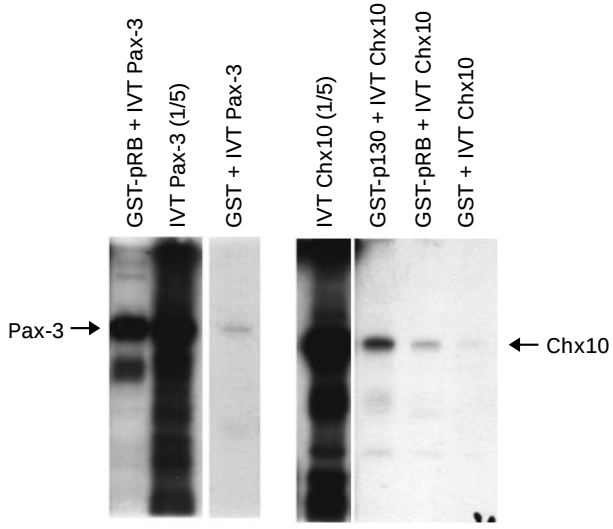

Figure 2 Binding of *in vitro* translated paired-like homeodomain proteins to GST-pRB-family proteins. Rabbit reticulolysates were programmed to express ^{35}S-methionine labelled Pax-3 (left panel) or Chx10 (right panel) and passed over glutathione columns containing GST alone, GST-pRB and/or GST-p130. Specific binding was determined following autoradiography of SDS–PAGE gels and compared to the signal from 20% of the Pax-3 (IVT Pax-3 1/5) or Chx-10 (IVT Chx10 1/5) input

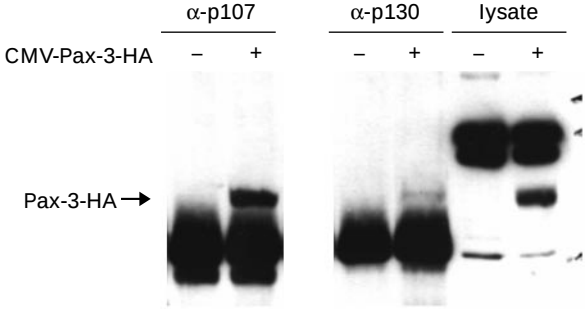

Figure 3 Co-immunoprecipitation of Pax-3 with p107 and p130. HA-tagged Pax-3, under the control of the CMV promoter, was stably expressed in C33A cells. Cell lysates were prepared from these cells (+) or control cells (−) and endogenous p107 (left panel) or p130 (middle panel) was immunoprecipitated from these cells. Co-immunoprecipitated Pax-3-HA was detected using the anti-HA antibody, 12CA5. The Pax-3-HA signal was compared to the Western analysis of the lysate (right panel)

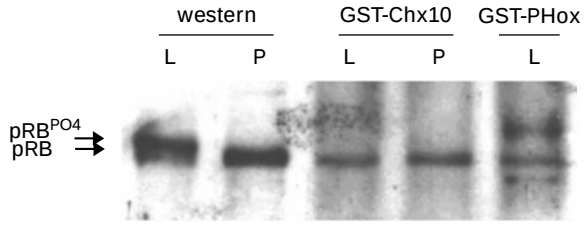

Figure 4 The paired-like homeodomain proteins bind the unphosphorylated form of pRB. Untreated (L) COS lysates or lysates treated with λ-phosphatase (P) were passed over glutathione columns containing GST-Chx10 or GST-PHox. pRB bound to the fusion proteins was then compared to the pRB signal seen in the lysates (western lanes). These data reveal that the unphosphorylated form of pRB (pRB) exclusively binds to the paired-like homeodomain proteins while the hyperphosphorylated form of pRB (pRB^{PO4}) shows little affinity for Chx10 or PHox. No binding to GST alone was observed (data not shown; see also Figures 1 and 6)

The N-terminus and small-pocket regions of pRB bind to the homeodomains

We next wished to determine the regions in the pRB-family proteins which are responsible for complex formation with the paired-like homeodomain proteins (Figure 5). GST-fusion proteins of full-length pRB or various fragments of pRB (see Figure 5a) were isolated from bacteria. Nuclear lysates prepared from C33A cells stably expressing HA-tagged Pax-3 or PHox were then passed over beads containing the GST-fusion proteins or a column containing GST alone and bound Pax-3-HA (Figure 5b) or PHox-HA (Figure 5c) were detected by western analysis. Binding activities of Pax-3-HA and PHox-HA were similar when tested against regions of pRB. Specifically, little binding to the C-terminal portion of pRB could be demonstrated while binding to full length (pRB^{FL}), large pocket (pRB^{LP}), small pocket (pRB^{SP}) and N-terminal (pRB^{N}) regions was easily detected. Binding to the N-terminal portion of pRB indicates that the association between Pax-3/PHox and pRB involves multiple contacts. This binding is distinct from pRB-binding to E2F-1 where the large pocket region and the C-terminus are required (data not shown and Helin *et al*., 1993; Huang *et al*., 1992).

While Pax-3 and PHox exhibit similar binding activities towards the domains in pRB, binding of Pax-3 or PHox to the N-terminal domains of p130 or p107 reveal preferential binding activities. Specifically, PHox, the mouse homologue (MHox) of which was isolated in the original $p130^{N}$ two-hybrid screen, has significant binding activity towards the N-terminal domain of p130 and p107. In contrast, Pax-3, which was not isolated in our original two-hybrid screen, has only very weak binding activity towards the N-terminal regions of p107 and p130 in this assay.

Helices I and II in the homeodomain are required for pRB-family binding

The three-dimensional structure of a prototype paired-like homeodomain has been solved (Wilson *et al*., 1995). This protein structural motif is comprised of three alpha helical regions which together form the paired-like homeodomain. Helix III is responsible for recognition of and interaction with specific sequences on DNA, while Helices I and II are exposed and could interact with proteins which associate with homeodomain proteins. For PHox, all three helices must be intact for DNA-binding activity and for interactions with other nuclear proteins (Grueneberg *et al*., 1995).

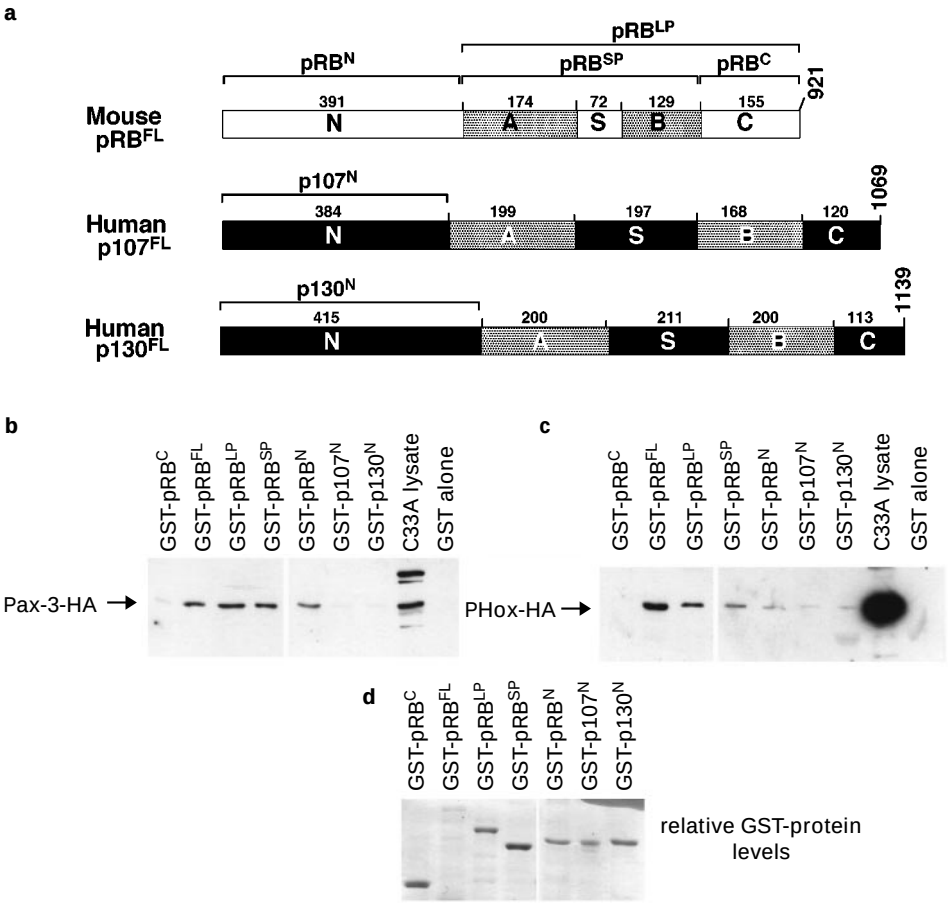

Figure 5 The N-terminal and pocket domains of pRB interact with the paired-like homeodomain proteins. The N-terminal region (pRB^{N}), small pocket (pRB^{SP}), large pocket (pRB^{LP}), C-terminal region (pRB^{C}) or full length pRB (pRB^{FL}) as well as the N-terminal region of p107 ($p107^{N}$) or p130 ($p130^{N}$, **a**) were expressed as GST-fusion proteins in bacteria and isolated on glutathione beads. Whole cell lysates prepared from C33A cells programmed to stably express an HA-tagged version of Pax-3 or PHox were then passed over columns containing the various GST-fusion proteins. Binding of Pax-3-HA to these proteins was detected by Western analysis using an α-HA antibody (**b**) or PHox (**c**). The coomassie stained gel in (**d**) demonstrates that the each GST-fusion protein is present on these beads at relatively similar levels with the exception of the full length pRB ($GST\text{-}pRB^{FL}$) which is present at relatively low levels

In contrast to PHox, which contains a single paired-like homeodomain, Pax-3 contains a paired domain in addition to its paired-like homeodomain (see Figures 1a and 6b). These two structurally distinct domains clearly influence each other's functional activities (Chalepakis *et al*., 1994; Underhill *et al*., 1995). We tested whether deletion of specific helices from the paired-like homeodomain in Pax-3 would abrogate its binding to p107 (Figure 6). Deletion of the helix III (Pax-3ΔH3) did not affect the ability of Pax-3 to bind to p107 (Figure 6a, lanes 1–3). However, removal of all three helices prevented the formation of Pax-3-p107 co-complexes. These data confirm that the pRB-family proteins specifically interact with the paired-like homeodomain of Pax-3. Additionally, binding of p107 to the Pax-3ΔH3 mutant suggests that the important sites of interaction in the paired-like homeodomain for binding the pRB-family proteins are present in Helix I and Helix II.

pRB-family members repress Pax 3-dependent transcriptional activation

Complex formation between the E2F and pRB-family proteins results in repression of activated transcription by E2F (Helin *et al*., 1992). We, therefore, tested whether the pRB-family proteins would also repress activated transcription mediated by the paired-like homeodomain proteins in transient co-transfection assays (Figure 7). These assays were performed in exponentially growing, undifferentiated mouse P19 embryonal carcinoma cells since these cells express low or undetectable levels of Pax-3 (Pruitt, 1992), p130 (Corbeil *et al*., 1995) and pRB (Slack *et al*., 1993). Two model promoters, $(PRS9)_6$-TK-CAT, which contains both paired domain and homeodomain binding sites (Chalepakis *et al*., 1991; Figure 7a) and $(P3)_3$-CAT, which contains homeodomain binding sites (Wilson *et al*., 1993; Figure 7b) were initially tested. Pax-3 strongly activated transcription from $(PRS9)_6$-TK-CAT while co-expression of pRB, p107 or p130 consistently repressed Pax-3-dependent activated transcription 2–3-fold. Similarly, Pax-3 activated transcription from the reporter construct, $(P3)_3$-CAT (Figure 7b). Pax-3-dependent transcription from $(P3)_3$-CAT was also repressed by all three pRB-family proteins 2–3-fold. This extent of repression of Pax-3 by pRB is similar to the levels we have observed previously for pRB-mediated repression of E2F-dependent transcription in these undifferentiated P19 cells (Hamel *et al*., 1992).

A number of studies have demonstrated that Pax-3 regulates the expression of several important developmental genes. One such regulatory target of Pax-3 is c-*met* (Daston *et al*., 1996; Epstein *et al*., 1996; Yang *et al*., 1996). The c-*met* promoter contains a paired domain binding site, and expression of c-*met* in lateral somites and in the dermamyotome is dependent on Pax-3 activity. We next tested whether the pRB-family proteins affected Pax-3-dependent transcription from the c-*met* promoter (Figure 7c). Expression of Pax-3 strongly activated transcription from the c-*met*-CAT construct. Co-transfection of Pax-3 with the pRB or p107 expression vector repressed Pax-3-dependent c-*met* promoter activity 3–4-fold.

Other potential targets of homeodomain proteins are the promoters of myogenic factors. The myosin light chain (MLC) promoter, for example, contains both a homeodomain binding site and an A-T rich muscle response element (MRE) upstream of two E-boxes (Ernst *et al*., 1991; Rao *et al*., 1996). In addition, Pax-3 expression can inhibit differentiation of C_2C_{12} myoblasts in culture (Epstein *et al*., 1995). We tested the effect of Pax-3 and pRB on the activity of the myosin light chain (MLC) promoter (Figure 7d). MLC promoter activity was minimal in undifferentiated P19

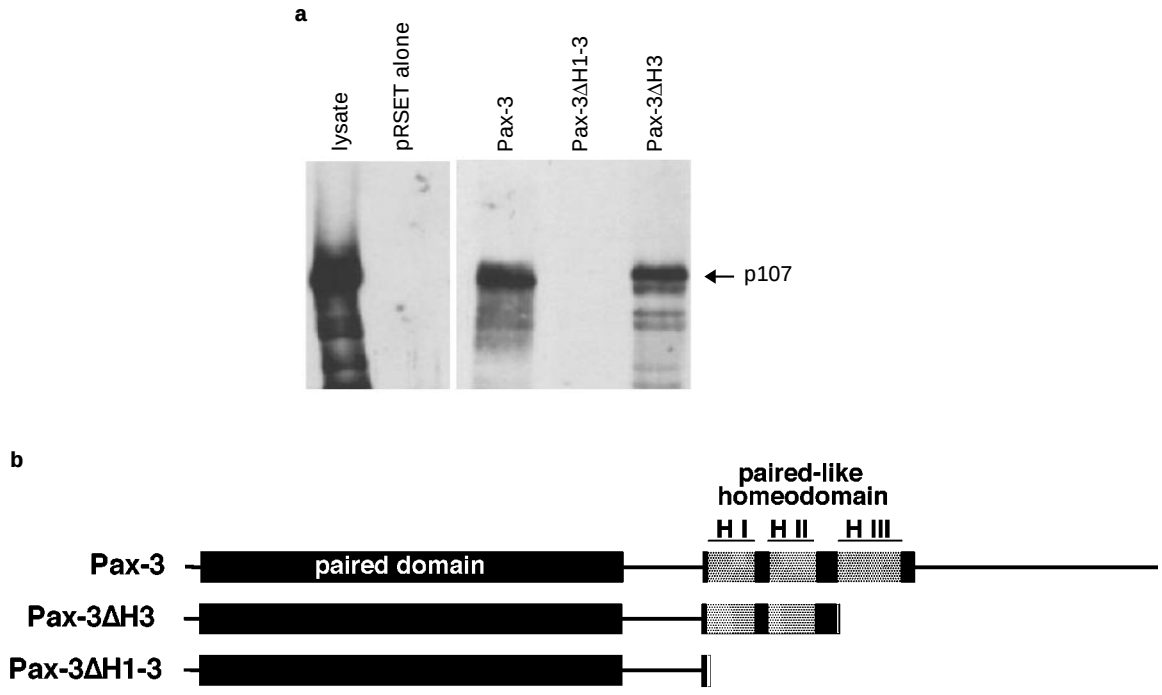

Figure 6 The homeodomain is required for pRB-family binding. Similar amounts of His-tagged Pax-3 homeodomain proteins, from which either helix III or all three helices had been deleted (**b**), were purified from bacteria and tested for binding of endogenous p107 from C33A cell lysates (**a**)

cells. Individually, both MyoD and Pax-3 activated transcription from the MLC promoter. In the presence of pRB, however, MyoD-dependent transcriptional activity was augmented, as has been previously described (Skapek *et al*., 1996), whereas Pax-3-dependent transcriptional activity was repressed. Thus, in contrast to increasing activated transcription by myogenic factors such as MyoD, pRB represses Pax-3-dependent activated transcription of promoters containing either paired-domain or homeodomain binding sites.

Discussion

We have determined that pRB-family members are able to interact with and repress the transcriptional activity of proteins that contain paired-like homeodomains. This class of transcription factors, which are involved in determination of cell fate, represents a novel class of targets for regulation by the pRB-family and provides a potential mechanism for integrating the processes of cellular proliferation and differentiation.

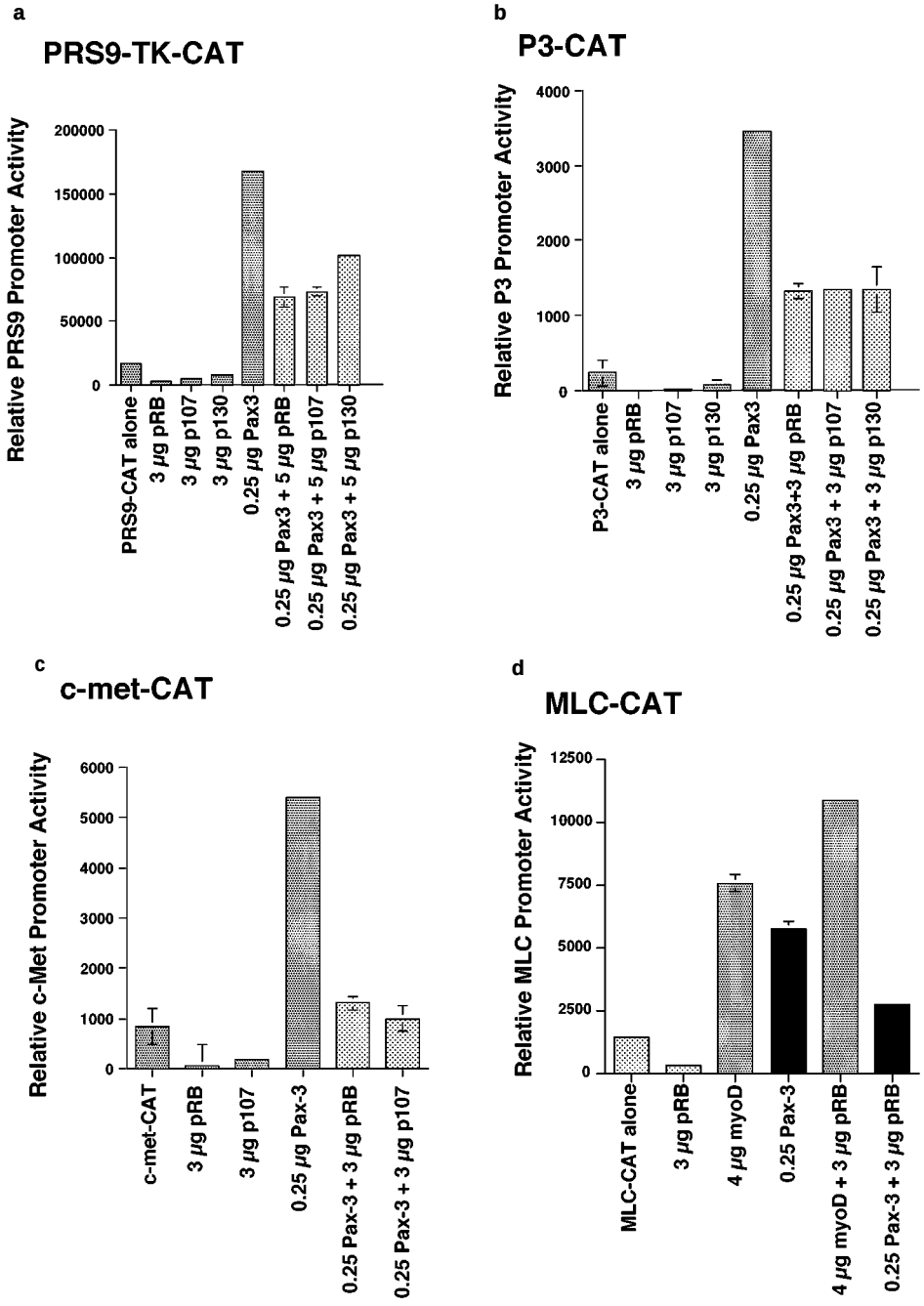

Figure 7 pRB represses Pax-3-dependent activated transcription. CAT reporter constructs with either PRS9 motifs, which have binding sites for both a paired domain and homeodomain (**a**), the homeodomain binding site, P3 (**b**), the c-Met (**c**) or the myosin light chain (**d**) promoters were transfected into undifferentiated P19 cells in the presence or absence of Pax-3 and/or the pRB-family proteins, pRB, p107 or p130. Addition of Pax-3 strongly increased transcription from the respective promoters while co-transfection of any of the pRB-family proteins repressed Pax-3 dependent activated transcription 2–3-fold

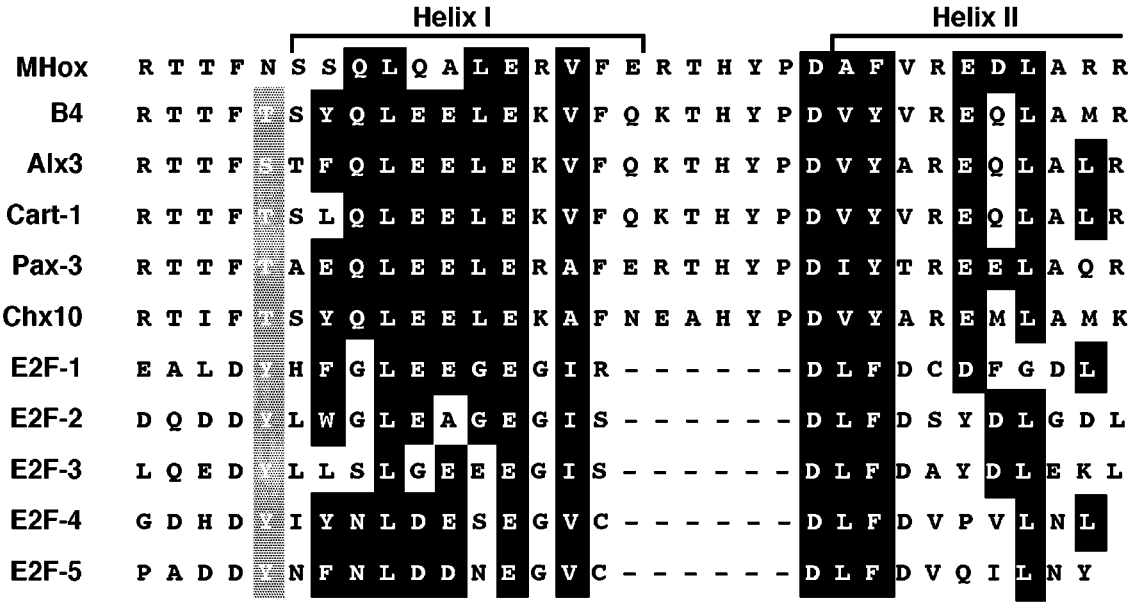

Figure 8 Comparison of amino acid sequences of paired-like homeodomain proteins with the pRB-family binding site of the E2F-family members. Helix I and part of Helix II of the homeodomain-containing proteins, Alx3, Cart-1, Pax-3, Chx10 and B4 are aligned with the pRB-family binding site at the C-terminus of the E2F-family proteins (Helin *et al*., 1993; Shan *et al*., 1996). Blocked out regions represent identical or highly conserved amino acids amongst these proteins. An amino acid containing a free hydroxyl group is also present at position -1 relative to Helix 1 in all of these proteins

Sites of interaction between the pRB-family proteins and paired-like homeodomains

After identifying paired-like homeodomain proteins as a novel target of pRB-family binding, we compared the sequences of these proteins to the well characterized pRB-family binding site in E2F (Figure 8; Helin and Harlow, 1994; Helin *et al*., 1993; Shan *et al*., 1996). A striking similarity can be seen between the amino acid sequence of Helix I and II of the paired-like homeodomains (Kissinger *et al*., 1990) and the pRB-family pocket-binding motif that is located in the transactivation domain of the E2F-family proteins (Helin *et al*., 1992). That these two helices are accessible for binding other nuclear factors can be appreciated from the crystal structure of the paired-like homeodomain of the *Paired* transcription factor from *D. melanogaster* (Wilson *et al*., 1995). Helix III of the paired-like homeodomain is the DNA recognition helix and sits in the DNA major groove. In contrast, while making contacts with the backbone of DNA, Helix I and II lie outside the DNA binding site and would be accessible for binding by other proteins. Given the sequence similarity of Helix I and II to the pRB-binding site on the E2F-family proteins, we propose that these two helices represent a possible binding site for pRB-family members on the paired-like homeodomains. This hypothesis is supported further by our data demonstrating that *in vitro*, p107 can bind to His-tagged Pax-3 that lacks Helix III (Pax-3ΔH3) of the paired-like homeodomain but not to a mutant of Pax-3 that lacks all three helices (Pax-3ΔH1-3) of this region (see Figure 6). The fact that p107 was able to bind to Pax-3 from which helix III of the homeodomain had been deleted (Pax-3ΔH3) suggests that the paired domain of Pax-3 may stabilize the structure of the remaining two helices of the paired-like homeodomain and facilitate binding of pRB-family proteins. This hypothesis is consistent with the previously described interdependence between the paired domain and paired-like homeodomain of Pax-3 (Underhill *et al*., 1995).

In the well-characterized pRB-E2F interaction, pRB requires both an intact pocket domain and C-terminal domain for strong binding to E2F (Cress *et al*., 1993; Hiebert *et al*., 1992; Welch and Wang, 1995b). In contrast, the C-terminal portion of pRB is sufficient and necessary for c-*abl* binding to pRB (Welch and Wang, 1993, 1995a,b). We isolated the paired-like homeodomain proteins, MHox and B4, in a yeast two-hybrid screen using only the N-terminus of p130. The binding experiments with these proteins indicate that the N-termini in addition to the pocket region of the pRB-family proteins make important contacts with the paired-like homeodomains. The C-terminus of pRB, however, does not specifically bind to the paired-like homeodomains. Thus, we have defined a paired-like homeodomain-binding motif in the pRB-family proteins which is distinct from the E2F-binding region. This motif is similar to that previously described for pRB-binding of the interferon-inducible protein, p202 (Choubey and Lengyel, 1995).

It appears that at least one paired-like homeodomain protein, Pax-3, has preferential binding activity amongst the homeodomain-binding motifs in the N-termini of the pRB-family proteins. Specifically, Pax-3 binds strongly to the N-termini and small pocket regions of pRB but only weak association with the N-terminal portion of p130 or p107 was observed. This result is consistent with our failure to isolate Pax-3 in the original two-hybrid screen of the mouse embryonic library using the N-terminus of p130 as bait. In contrast, B4, MHox and its human homologue, PHox, bind the N-termini of all three pRB-family proteins, consistent with their isolation in the two hybrid screen. We note, however, that our experiments do not address whether there are significant differences

in the binding affinity of different homeodomain proteins toward individual full length pRB-family proteins.

Biological consequences of the paired-like homeodomain/pRB-family interaction

We have demonstrated a novel interaction between the pRB-family proteins and a class of factors which are fundamental to determining cell fate. These data imply an important role for the pRB-family proteins in the regulation of differentiation during embryonic development. For example, during muscle development, myogenic precursors must migrate from the lateral dermamyotome to the limb bud (Cossu *et al*., 1996). This migration is dependent on expression of the c-*met* receptor (Bladt *et al*., 1995), whose transcription is induced by Pax-3 (Daston *et al*., 1996; Epstein *et al*., 1996; Yang *et al*., 1996). After the myoblasts have colonized the limb buds, they exit the cell cycle and being to express muscle-specific markers. Thus, the role of pRB-family members in myogenesis may be threefold: (1) pRB-family proteins can repress E2F-mediated transcription and facilitate cell cycle exit; (2) pRB-family proteins can augment transcriptional activation by myogenic bHLH transcription factors; and (3) pRB-family proteins can repress Pax-3 activity.

It was suggested that the bone defects observed in $p107^{-/-}/p130^{-/-}$ mice may reflect the fact that p107 and p130 are required for control of E2F activity and cellular proliferation at a critical point during chondrocyte differentiation (Cobrinik *et al*., 1996). However, in addition to deregulation of normal E2F-dependent control of chondrocyte proliferation, our data suggest that the chondrocyte defects may also be due to deregulated activity of paired-like homeodomain proteins, such as Cart-1, which are involved in chondrocyte development (Zhao *et al*., 1993). Similarly, in the human retina, loss of pRB may result in both constitutive activity of cell cycle regulatory factors such as E2F, as well as deregulated activity of paired-like homeodomain factors such as Chx10 and Pax-6 (Burmeister *et al*., 1996; Hill *et al*., 1991; Liu *et al*., 1994).

A clearer understanding of the possible biological consequences of the interaction between proteins that contain a paired-like homeodomain and members of the pRB-family will require a more precise characterization of their patterns of expression during each stage of embryogenesis. However, our data demonstrate that the pRB-family proteins may directly influence cell fate by interacting with and modifying the activity of paired-like homeodomain proteins in addition to regulating the activity of cell cycle control factors. pRB-family proteins may, therefore, provide one mechanism of coordinating cellular proliferation and differentiation programs.

Materials and methods

Plasmids and antibodies

The CMV-Pax-3-HA expression vector and the $(P3)_3$-CAT reporter construct were generously provided by J Epstein (University of Pennsylvania, Philadelphia, PA). c-met-CAT was constructed from the c-met-luc previously described (Epstein *et al*., 1996). The 320 bp c-*met* promoter region was cut out with *Bam*HI and *Hind*III, blunt ended and ligated between the blunt ended *Hind*III and *Sal*I sites in pCAT-basic (Stratagene, Richmond, CA). $(PRS9)_6$-TK-CAT (Epstein *et al*., 1996) and myosin light chain-CAT reporter (Ernst *et al*., 1991) have been described previously. The monoclonal α-pRB antibody, 14001A, was purchased from Pharmingen (Richmond, CA) and the α-p107 (SC-318) and α-p130 (SC-317) polyclonal rabbit antibodies were purchased from Santa Cruz Biotechnology (Richmond, CA).

Cell culture

Murine P19 embryonal carcinoma cells (McBurney, 1993; Rudnicki and McBurney, 1988) were grown in α-MEM supplemented with 7.5% bovine serum (Cansera) and 2.5% fetal clone II (Hyclone). Human C33A cervical carcinoma cells were grown in DMEM supplemented with 10% fetal calf serum. Murine 3T7 lymphoma cells were grown in RPMI supplemented with 10% fetal calf serum.

Yeast two-hybrid screen

The N-terminus of human p130 (amino acids 1–415) was subcloned C-terminal to the GAL4 DNA binding domain (amino acids 1–147) in the yeast two-hybrid 'bait' vector pGBT9 (Clonetech). *Saccharomyces cerevisiae* HF7c, which expresses *HIS3* under the control of the *GAL1* promoter, was first transformed with pGBT9-$p130^N$ and then with a pGAD10 library (Clonetech) of murine embryonic E11.5 cDNAs fused to the GAL4 transcriptional activation domain (amino acids 768–881). 7.5×10^6 transformants were plated on minimal media lacking histidine and containing 5 mM 3-AT.

Binding assays

For the *in vitro* binding assays, GST- and His-tagged homeodomain proteins were expressed in bacteria and purified as has previously been described (Kiess *et al*., 1995). Nuclear lysates were prepared from the mouse 3T7 lymphoma (pRB binding) or human C33A cervical carcinoma cells (p107 and p130 binding) as previously described (Kiess *et al*., 1995). 100 μg of cell lysate was then added to glutathione-sepharose beads or nickel-coated beads bearing GST- or His-tagged fusion proteins, respectively, and the beads were washed five times with lysis buffer (120 mM NaCl, 50 mM Tris-HCl pH 8; 0.5% NP40). The beads were then boiled in Laemmli sample buffer, separated on 6% SDS–PAGE gels and electrophoretically transferred to nitrocellulose. Western blots were then probed with antibodies specific for pRB, p107 or p130, and developed using a chemiluminescent reaction (Kierkegaard and Perry) according to the manufacturer's instructions. For the co-immunoprecipitation experiments, p107 or p130 was immunoprecipitated from total cell lysates prepared from C33A cells that transiently expressed HA-tagged Pax-3. The samples were then boiled in Laemmli sample buffer, separated on 8% SDS–PAGE and transferred to nitrocellulose, and the blots were probed with the anti-HA monoclonal antibody, 12CA5.

λ-phosphatase treatment

COS cell lysates were prepared as described above for 3T7 and C33A lysates. Prior to performing the binding experiments, a portion of the COS lysates was treated with λ-phosphatase (New England Biolabs) as we have previously described (Kiess *et al*., 1995).

Promoter assays

Undifferentiated murine P19 embryonal carcinoma cells were co-transfected with either the $(PRS9)_6$-TK-CAT, $(P3)_3$-CAT, c-met-CAT or myosin light chain-CAT reporter constructs, a CMV-βgalactosidase control plasmid and plasmids expressing murine Pax-3 and/or murine pRB, human p107 or human p130 as described previously (Hamel *et al*., 1992). Cells were harvested, lysates were prepared, and CAT and βgal activities were determined as previously described (Hamel *et al*., 1992). Relative CAT activity was then determined by normalization to βgal activity.

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