A novel form of pRb expressed during normal myelopoiesis and in tumour-associated macrophages

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Received 15 September 2004; revision accepted 29 September 2004

Abstract. The retinoblastoma (Rb) tumour suppressor promotes cell cycle exit, terminal differentiation and survival during normal development and is functionally inactivated in most human cancers. We have identified a novel myeloid-specific form of retinoblastoma protein (pRb), termed Δ Rb-p70, that exists *in vivo* as an N-terminally truncated form of full-length pRb. Δ Rb-p70 appears to be the product of alternative translation and is expressed in primary myeloid cells in fetal liver, bone marrow and spleen. It is also expressed in the human myelomonocytic cell line U937 and is down-regulated as U937s are induced to differentiate. We have also detected Δ Rb-p70 expression in primary human breast tumours and we have determined that Δ Rb-p70 is specifically expressed in tumour-associated macrophages. These data identify a novel mechanism for regulating pRb expression that is unique to the myeloid system.

INTRODUCTION

The retinoblastoma (Rb) tumour suppressor gene is required for coordinating cell cycle exit, with tissue-specific gene expression occurring during terminal differentiation (Classon & Harlow 2002; Liu *et al.* 2004). The Rb tumour suppressor is also critical for cellular survival under conditions of stress, including DNA damage and cytokine deprivation (Chau & Wang 2003).

Control of retinoblastoma protein (pRb) activity is regulated by the phosphorylation of key residues mediated by cyclin-dependent kinases largely during the G₁ and S phases of the cell cycle (Mitnacht 1998). Expression of pRb is also controlled by proteolysis. This may be mediated by caspase cleavage at the C-terminal end of the protein during apoptosis, induced by treatment of cultured cells with Fas, tumour necrosis factor- α , or staurosporine, and in B cells deprived of interleukin-3 (Janicke *et al.* 1996; Chen *et al.* 1997; Tan *et al.* 1997; Gottlieb & Oren 1998; Chau *et al.* 2002).

The Rb tumour suppressor plays critical roles in the haematopoietic system, including intrinsic cell functions in erythropoiesis (Clark, Doyle & Humbert 2004; Spike *et al.* 2004) and other less well-defined roles in myelopoiesis (Klappacher *et al.* 2002). Given the importance of the Rb

tumour suppressor to haematopoietic homeostasis, we have investigated how its expression is regulated during haematopoietic development and in different haematopoietic lineages. Our data have identified and characterized a novel form of pRb that is uniquely expressed in myeloid cells.

MATERIALS AND METHODS

Mice, histological procedures and immunohistochemistry

Timed matings were set up as described previously to generate E11.5, 12.5, E13.5, E14.5, E15.5, or E16.5 mouse embryos (Macleod K. *et al.* 1996). Fetal liver was dissected from embryos for use in various protocols and was disaggregated in phosphate buffered saline minus Ca^{2+} and Mg^{2+} (PBSA) to a single cell suspension by serial passage through a 23G syringe needle followed by a 27G needle.

Tissues were fixed in 10% neutral-buffered formalin and processed for sectioning. Immunohistochemistry was carried out using heat denaturation to expose epitopes. Primary antibodies [anti-Ki-67 (A-0047, DAKO, Glostrup, Denmark), anti- $\Delta\text{Rb-p70}$ (IMG-395, Imgenex, San Diego, CA, USA), or antimyeloperoxidase (A-0398, DAKO)] were diluted to 1 : 500 in phosphate-buffered saline (PBS)/serum and developed using Vectastain reagents, as described previously (Macleod *et al.* 1996).

Western blot, immunoprecipitation and gel shift procedures

All whole cell protein lysates were prepared by high salt extraction in the presence of 0.5 mM phenylmethylsulphonyl fluoride, 1 $\mu\text{g/mL}$ aprotinin, leupeptin, pepstatin A, and 0.1 mM sodium orthovanadate, as described previously (Macleod *et al.* 1996). Equal quantities [determined by Bradford assay (50 μg)] were loaded onto gels for sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). Western blotting for pRb was carried out on 7% SDS–PAGE, with 1-h blocking in PBS/milk, overnight incubation at 4 °C in 0.03% PBS-Tween (PBS-T) with a 1 : 250 dilution of primary antibody (G3-245, Pharmingen, San Diego, CA, USA, cat. no. 14001 A or $\Delta\text{Rb-p70}$ -specific IMG-395, Imgenex) followed by 2 washes in 0.03% PBS-T and 1 h in anti-mouse secondary antibody and enhanced chemiluminescence. Similar conditions were used to detect p107 and p130 by Western blot except that 0.05% PBS-T was used for incubation with rabbit anti-p107 (Santa Cruz sc-318, Santa Cruz, CA, USA) or rabbit anti-p103 (Santa Cruz sc-317). Immunoprecipitations were carried out as described previously (Macleod *et al.* 1995) using the following anti-pRb antibodies: G99 (I-240), XZ104, XZ-133 (all BD-Pharmingen, San Diego, CA, USA), C-15 (sc-50 from Santa Cruz) and the following anti-E2f antibodies: E2f-1 (sc-2280), E2f-2 (sc-633), E2f-3 (sc-878), E2f-4 (sc-866) from Santa Cruz.

The gel shifts for the E2f proteins were carried out as described previously (Macleod *et al.* 1996) except that nuclear extracts were used instead of whole cell lysates (Schreiber *et al.* 1989). Immunoprecipitation-gel shifts were carried out as described previously (Lee *et al.* 2002). The supershifting antibody for E2f-1 was KH95 (Pharmingen, cat. no. 14971A) while the E2f-2 and E2f-3 supershifting antibodies were the same as those used for Western blotting (as above). Both pRb and p107 were supershifted using the 21C9 and SD15 antibodies described previously (Macleod *et al.* 1996).

Polymerase chain reaction (PCR) and *in vitro* translation

PCR was carried out using Pfx polymerase (Invitrogen, Grand Island, NY, USA) using primers specific to different regions of mouse Rb, as indicated in the figure legends. Mutant forms of pRb

were generated by PCR-mediated mutagenesis and confirmed by DNA sequencing. Different mutant forms of pRb were expressed *in vitro* in rabbit reticulocyte lysates according to the manufacturer's guidelines (T7 coupled reticulocyte lysate system, Promega, Madison, WI, USA). Specific caspases and/or their cognate inhibitors (Calbiochem, San Diego, CA, USA) were added to the TNT lysate after 90 min of synthesis and incubated for a further 15 min at 30 °C.

Flow cytometry and cell sorting

The following antibodies were obtained from Pharmingen/Clontech (San Diego, CA, USA) and used for surface labelling of cells: fluorescein isothiocyanate-conjugated rat anti-mouse CD117 (c-Kit) (cat. no. 01905B/553354), R-phycoerythrin-conjugated rat anti-mouse TER119 (cat. no. 09085B), biotin-conjugated rat anti-mouse TER119 (cat. no. 553672), streptavidin-conjugated cychrome (cat. no. 13038A), allo-phycoerythrin-conjugated rat anti-mouse Mac-1 (cat. no. 553312) and phycoerythrin-conjugated rat anti-mouse Gr1 (cat. no. 553128). R-Phycoerythrin-conjugated rat anti-mouse F4-80 (cat. no. RM2904) was obtained from CalTag laboratories (Burlingame, CA, USA). Surface-labelled cells were analysed by FACSscan (Becton-Dickinson, San Diego, CA, USA) and cells were sorted using the MoFlo (DAKO Cytomation).

RESULTS

Identification of novel forms of pRb in haematopoietic tissues

Gene targeting in the mouse has revealed critical functions for the Rb tumour suppressor in fetal liver (FL) haematopoiesis (Clarke *et al.* 1992; Jacks *et al.* 1992; Lee *et al.* 1992). Our group performed immunoblots to examine the expression of pRb during wild-type FL development. We observed high-level expression of both hypo- and hyper-phosphorylated forms of pRb (p110/p105) throughout FL development, from E11.5 to E16.5 (Fig. 1a). However, we also observed two lower molecular weight forms of pRb, with apparent molecular weights of approximately 90 kDa and 70 kDa, that were first detected at E13.5 of FL development (Fig. 1a, lane 3) and continued to be expressed throughout FL haematopoiesis (Fig. 1a, lanes 3–6). The timing of expression of these lower molecular weight forms of pRb, which we have collectively termed Δ Rb, coincided with the timing of a haematopoietic defect in the FL and death of Rb-null embryos (Jacks *et al.* 1992).

As shown in Fig. 1(a) (lower panels), the expressions of p107 and p130 were not similarly affected during FL development, suggesting that the mechanism acting to produce Δ Rb was specific to this member of the pocket protein family. Δ Rb was not expressed in Rb-null FL demonstrating that it was encoded by the Rb locus (Fig. 1b, lane 6). Δ Rb expression was also restricted to the haematopoietic system during embryogenesis (Fig. 1b, lane 5).

Δ Rb expression was also restricted to the haematopoietic system in adult mice (Fig. 1c). Δ Rb was detected in adult mouse bone marrow (BM) and spleen (Fig. 1c, lanes 9 and 12) but not in adult mouse brain, kidney, gut, heart, skeletal muscle, lung, or testes (Fig. 1c, lanes 1–7). Although Δ Rb is expressed in FL, BM and spleen, we did not detect Δ Rb in thymus (Fig. 1c, lane 10) suggesting that Δ Rb is restricted to the myeloid system.

We detected an additional form of pRb in the small intestine, migrating with a molecular weight of approximately 100 kDa (Fig. 1c, lane 3). The molecular weight of the gut-specific form of pRb suggests that it may be the product of caspase cleavage (Janicke *et al.* 1996; Chen *et al.* 1997; Tan *et al.* 1997; Gottlieb & Oren 1998) and indeed mice engineered to express a caspase-resistant form of pRb (Rb-MI) show resistance to endotoxin-induced apoptosis in the gut (Chau *et al.* 2002).

Figure 1. Identification of myeloid specific forms of pRb. (a) Immunoblot analysis of pRb, p107 and p130 expression in primary mouse fetal liver. (b) Immunoblot analysis of pRb expression in whole cell lysates from E13.5 mouse embryos. (c) Immunoblot analysis of pRb expression in adult tissues. (d) Immunoblot analysis of pRb expression in flow cytometric sorted populations of fetal liver cells, including differentiating erythroblasts (TER119⁺), haematopoietic progenitors (cKit⁺) and myeloid cells (Mac-1⁺ Gr1⁺ Mac-1⁺ F4-80⁺).

To further delineate the cell type expressing Δ Rb within the haematopoietic system, we sorted cells from E15.5 FL based on the expression of surface markers, including TER119 (erythroid), c-Kit (stem cells and progenitors) and Mac-1 (stem cells, myeloid progenitors and granulocytes/monocytes). We were unable to detect Δ Rb in TER119⁺ erythroblasts (Fig. 1d, lane 1) and the majority of Δ Rb was expressed in Mac-1⁺ TER119⁻ cells (Fig. 1d, lane 3) suggesting that it is restricted to myeloid progenitors or to granulocytes/monocytes. To clarify this, we examined sorted populations from BM, and detected Δ Rb in Mac-1⁺ F4-80⁺ macrophages (Fig. 1e, lane 9) and Mac-1⁺ Gr1⁺ granulocytes (Fig. 1e, lane 11). The onset of Δ Rb expression in E13.5 FL is consistent with the timing of macrophage migration into FL hepatic cords where they compose the central macrophages around which erythroblastic islands develop. These islands of erythroblasts depend upon the macrophages for proliferation and maturation signals (Sasaki *et al.* 1993).

Δ Rb-p70 is generated by alternative translation

Δ Rb could be generated by a number of different mechanisms including alternative splicing of the Rb message, alternate promoter usage, alternative translation and proteolysis. We examined each of these possibilities in turn and excluded both alternative splicing and alternate promoter usage as the mechanism for generation of the Δ Rb. As shown in Fig. 2(a), reverse transcriptase-PCR using total RNA from E15.5 FL, generated PCR products of exactly the predicted sizes based on the intron–exon structure of mouse Rb (Bernards *et al.* 1989).

Δ Rb-p90 was not present in lysates prepared by direct lysis of E15.5 FL in 2 $\times$ Laemmli buffer (Fig. 2b). This suggested that Δ Rb-p90 was the product of proteolysis during extraction. However, Δ Rb-p70 was still detectable following direct lysis of E15.5 FL, E16.5 FL and of BM (Fig. 2b, lanes 1–3) suggesting that it was expressed *in vivo*.

Figure 2. Mechanism of synthesis of Δ Rb. (a) Primers spanning the indicated exons of mouse Rb give rise to the predicted fragment sizes when used in RT-PCR on RNA derived from E15.5 fetal liver. (b) Immunoblot of lysates prepared by direct lysis of fetal liver, bone marrow and spleen in 2 $\times$ Laemmli buffer. (c) *In vitro* translation of pRb in TNT rabbit reticulocyte lysates in the presence of exogenously added caspase 1 (lane 2) or caspase 2 (lane 5), caspase inhibitors (YVAD, lanes 3, 4; XVDAD, lanes 6, 7). (d) PCR-mediated point mutagenesis carried out to mutate specific methionine residues (M1, M107, M202 and M213) to alanine and to introduce a 2-base-pair frameshift mutation (FSM) between ATG1 and ATG2 at the *KpnI* site. Sequence analysis (lower panel) reveals that ATG5 lies within a particularly good Kozak consensus sequence for translational initiation. (e) pRb point mutants were transiently expressed in Rb-deficient Saos2 osteosarcoma tumour cells and lysates were harvested for immunoblotting to examine pRb expression. (f) Diagrammatic representation of pRb, point mutations introduced and the antibodies used in immunoprecipitations. (g) Immunoprecipitation of endogenous pRb and Δ Rb-p70 with antibodies to E2fs (lanes 2–5) or with domain-specific anti-pRb antibodies (lanes 1, 6–9) followed by Western blotting with G3-245 to pRb.

pRb has potent anti-apoptotic functions and is eliminated by caspase cleavage during programmed cell death induced by various stresses, including DNA damage, cytokine deprivation and Fas treatment (Janicke *et al.* 1996; Chen *et al.* 1997; Tan *et al.* 1997; Gottlieb & Oren 1998; Fattman *et al.* 2001). The caspase cleavage site in pRb (amino acids 883–887) maps to the C terminus of the protein (Fig. 2f) and cleavage at this site results in the release of a 5 kDa fragment and a 100 kDa cleaved product that is no longer able to bind to E2fs to inhibit their activity (Janicke *et al.* 1996; Chen *et al.* 1997; Tan *et al.* 1997; Gottlieb & Oren 1998). However, the molecular weight of Δ Rb-p70 was not consistent with cleavage at the caspase site in pRb. When pRb was expressed *in vitro* in rabbit reticulocyte lysates, we observed expression not only of full-length pRb (p105) but also of the p70 form of Δ Rb (Fig. 2c, lane 1). We also observed a higher molecular weight form of pRb that we have determined to be phosphorylated pRb (p110). These observations suggested that the molecular machinery required to express Δ Rb-p70 was present in reticulocyte lysates. We added exogenous caspases to reticulocyte lysates following Rb synthesis and looked for cleavage of full-length pRb to the size of Δ Rb-p70. We observed cleavage of pRb *in vitro* with caspases 1 and 2 (Fig. 2c, lanes 2 and 5). Both forms of pRb (full-length p105 and Δ Rb-p70) were shortened by approximately 5 kDa following caspase

cleavage (Fig. 2c, lanes 2 and 5) suggesting that both forms had an intact caspase cleavage site (amino acids 883–887). Specific inhibitors YVAD and XVDAD blocked the cleavage of pRb and Δ Rb *in vitro* by caspases 1 and 2 (Fig. 2c, lanes 4 and 7). These results suggest that although pRb can be cleaved by caspases, the generation of Δ Rb is not the result of caspase cleavage.

Cathepsins are highly expressed in haematopoietic tissues but we were unable to detect cleavage of pRb by exogenously added cathepsin B or L (data not shown). Similarly, calpain treatment of *in-vitro*-synthesized pRb failed to reduce full-length pRb to the molecular weight of Δ Rb-p70. Overall, our results suggest that Δ Rb-p70 is not the product of proteolysis.

To determine whether Δ Rb-70 could be generated by alternative translation, we generated mutant forms of pRb and expressed them *in vitro* in reticulocyte lysates (Fig. 2d). Mutation of the first in-frame ATG (Δ A1) or introduction of a frameshift mutation between ATG1 and ATG2 (FSM) resulted in loss of expression of full-length pRb but Δ Rb-p70 continued to be expressed (Fig. 2d, lanes 2 and 3), suggesting that Δ Rb-p70 was generated by alternative translation in reticulocyte lysates. Δ Rb-p70 is most likely translated from the fifth in-frame ATG because its expression is specifically abolished by mutation of this codon (Fig. 2d, lane 5). The presence of a pyrimidine at position +4 in the Kozak sequence surrounding the first in-frame ATG (Fig. 2d, lower panel) may cause it to be by-passed by the ribosomal initiation complex and leaky scanning may then permit initiation downstream from ATG5 that has a consensus guanosine residue at +4 (Kozak 1986, 1987; Hellen & Sarnow 2001).

These Rb mutants were then expressed in Saos2 cells (Fig. 2e). The Δ A1 mutant drove the expression of four forms of pRb that appear to be translated from the second (A2), third (A3), fourth (A4) and fifth (A5) in-frame ATGs, with the A2 and A5 forms being predominant (lane 2). The FSM mutant drove expression of the A2 form only in Saos2 cells (lane 3) whereas it directed expression of multiple forms in reticulocyte lysates (Fig. 2d, lane 2). These differences in Δ Rb expression suggest that the mechanisms determining selection of the initiating ATG are different *in vivo* and *in vitro*. The Rb5 mutant (an N-terminal deletion mutant) drove expression of the A5 form only in Saos2 cells (lane 4). These results demonstrate that pRb can be translated *in vivo* from downstream ATGs.

To confirm the identity of Δ Rb-p70, we have tried to perform N-terminal protein sequencing but on all occasions, the N-terminal end of the purified protein was blocked. Nevertheless, we believe that our mutagenesis data *in vitro* and *in vivo* provide compelling evidence that Δ Rb-p70 may be generated by alternative translation from the fifth in-frame ATG.

Δ Rb-p70 may also be subject to the same proteolysis as full-length pRb (that gave rise to Δ Rb-p90 during extraction) because Δ Rb-p70 detected in BM and FL (Fig. 1c, lane 8 and Fig. 2e, lane 1) is noticeably shorter than Δ Rb-p70 in spleen (Fig. 1c, lane 12) or expressed exogenously in Saos2 (Fig. 2e, lane 4).

Here, we have used antibodies specific to different regions of pRb (Fig. 2f) to characterize the regions missing in Δ Rb *in vivo* (Fig. 2g). pRb-105, Δ Rb-p90 and Δ Rb-p70 were all immunoprecipitated with the 21C9, XZ104 and XZ133 antibodies, indicating that all three forms had an intact central portion of the protein including the A domain and spacer region (Fig. 2g, lanes 1, 7 and 8). The C15 antibody failed to immunoprecipitate Δ Rb-p90 (lane 9), suggesting that Δ Rb-p90 was missing the C-terminal 15 amino acids of pRb-p105. However, the C15 antibody did recognize Δ Rb-p70 indicating that its C terminus was intact (lane 9). Unfortunately, the G99 antibody raised against the N terminus of human pRb does not cross-react with mouse pRb in immunoprecipitation experiments. Given that Δ Rb-p70 can be immunoprecipitated with antibodies to all other regions of the protein, it seems likely that its reduced molecular weight is the result of the loss of N-terminal sequences, consistent with it being generated by alternative translation. This is supported by work with the G99 antibody on human pRb (Fig. 3d).

Figure 3. Δ Rb is down-regulated during myeloid differentiation. (a) Immunoblot of pRb expression in human tumour cell lines: U937 myelomonocytic leukaemia (lane 1), Jurkat T cells (lane 2), MCF7 breast tumour (lane 3), Rb-deficient MDA-468 breast tumour (lane 4), HL60 monocytic leukaemia (lane 5), p53-deficient MDA-231 breast tumour (lane 6), U2OS osteosarcoma (lane 7), NB7 neuroblastoma (lane 8) and L8057 megakaryoblast (lane 9). (b) Immunoblot analysis of pRb expression in U937s induced to differentiate with 10 nM phorbol 12-myristate 13-acetate (PMA). (c) Flow cytometric analysis of Mac-1 (myeloid-specific) and F4-80 (macrophage-specific) expression in differentiating U937s. (d) Immunoprecipitation with the indicated antibodies from undifferentiated U937s and immunoblotting with G3-245 antibody for pRb. (e) Electrophoretic mobility shift assays were used to examine E2f and pRb binding in nuclear lysates from undifferentiated U937s (lanes 1–7) or from U937s, induced to differentiate for 24 h with PMA (lanes 8–14).

pRb-105 could be immunoprecipitated with antibodies to E2f-1, E2f-2 and E2f-3 (Fig. 2g, lanes 2–4) but not with antibodies to E2f-4 (lane 5). Δ Rb-p90 could not be immunoprecipitated with any of the E2f antibodies, which is consistent with the loss of its C terminus (lane 9). By contrast, Δ Rb-p70 could be immunoprecipitated with antibodies to E2f-1, -E2f-2 and E2f-3 (lanes 2–4) as expected if Δ Rb-p70 expressed an intact C terminus and pocket domain (lanes 7–9). Both pRb-105 and Δ Rb-p70 were more readily immunoprecipitated with antibodies to E2f-2 (lane 3) than with antibodies to E2f-1 (lane 2) or E2f-3 (lane 4), most likely reflecting the high level of E2f-2 expression in FL. In summary, Δ Rb-p70 appears to lack an intact N terminus

consistent with generation by alternative translational initiation from the fifth in-frame ATG and retains a functional E2f-binding domain.

Δ Rb expression in leukaemic cell lines is attenuated during differentiation

Δ Rb was uniquely expressed in the human U937 myelomonocytic cell line (Fig. 3, lane 1) and not in any other lines tested (Fig. 3, lanes 2–9). Δ Rb expression disappeared in U937 cells within 24 h of induction to differentiate with phorbol esters (Fig. 3b). Δ Rb was the predominant form of pRb in cycling U937s but by 8 h following treatment with PMA, full-length pRb-110 had increased while Δ Rb-p70 had decreased (Fig. 3b, lane 3). By 24 h post-induction, Δ Rb was undetectable and hypophosphorylated pRb was highly expressed (lane 5). Loss of Δ Rb expression was coincident with the differentiation of U937s along the monocyte/macrophage pathway, as measured by flow cytometry for expression of macrophage marker F4-80 and up-regulation of myeloid marker, Mac-1 (Fig. 3c).

Δ Rb-p90 in U937s did not immunoprecipitate with the C15 antibody (Fig. 3d, lane 4) while Δ Rb-p70 did not immunoprecipitate with the G99 antibody (lane 2), suggesting that Δ Rb observed in U937 cells is structurally similar to mouse Δ Rb. When we examined the interaction of Δ Rb with E2fs in cycling U937s, we confirmed that Δ Rb-p90 lacked E2f-binding activity (Fig. 3d, lanes 5–8) while Δ Rb-p70 possessed strong binding activity for both E2f-1 and E2f-3 (Fig. 3d, lanes 5 and 7). E2f-2 was not expressed in U937s (Fig. 3d, lane 6). We were unable to detect pRb in E2f-4 immunoprecipitates from cycling U937 cells (Fig. 3d, lane 8), consistent with mouse FL (Fig. 2g).

When we examined the DNA-binding activity of pRb–E2f complexes in cycling or differentiating U937s, we observed very little pRb supershift in extracts from cycling cells (Fig. 3e, lane 2). In these cells, free E2f-3 (lane 6) and E2f-4 (lane 7) formed the major DNA-bound complexes. However, antibodies to pRb shifted complexes containing E2f-3 and E2f-4 in lysates from differentiating U937s (lane 9). Combined with the results of immunoprecipitation–Western blotting experiments, these observations suggest that Δ Rb-p70–E2f complexes have reduced DNA-binding activity compared to pRb–E2f complexes.

Δ Rb is expressed in primary tumours in tumour-associated macrophages

We examined the expression of pRb in primary human tumours (Fig. 4a,b). We observed expression of Δ Rb in all gastric tumours (T) examined (Fig. 4a, lanes 2, 4, 6, 8 and 10) but only at very low levels in normal control gastric tissue (N) from the same patient (Fig. 4a, lanes 1, 3, 5, 7 and 9). When we examined primary breast tumour tissue by Western blot analysis, we observed Δ Rb expression in approximately half of all tumours examined (Fig. 4b, lanes 2, 5 and 6). Δ Rb-p90 was observed in most of the gastric tumour samples (Fig. 4a, lanes 2, 4, 6 and 8) but in only one of the breast tumour samples examined (Fig. 4b, lane 5). Consistent with our hypothesis that Δ Rb-p90 is the product of proteolytic cleavage and is missing its C terminus, we failed to detect Δ Rb-p90 with the C15 antibody (Fig. 4b, lower panel, lane 5). We detected Δ Rb-p70 expression with both the 245 and C15 antibodies, suggesting that the Δ Rb-p70 we observed in primary breast tumours is the same or similar to that which we described in mouse FL (Fig. 2) and in U937 cells (Fig. 3).

We made use of an antibody (Imgenex, #IMG-395) that specifically recognizes Δ Rb-p70 expressed in U937s or in primary human breast tumour material, which does not cross-react with full-length pRb (Fig. 4c). Using immunohistochemical approaches, we used this antibody to examine Δ Rb-p70 expression *in situ* and observed that breast tumour cells were negative for Δ Rb-p70 expression (Fig. 4f,h). Δ Rb-p70 expression was restricted to non-tumour cells in the tumour microenvironment and to cells lining the ducts of the breast (Fig. 4f,h). In contrast,

Figure 4. Δ Rb is expressed in tumour-associated macrophages. (a) Immunoblotting of whole cell lysates from primary human gastric tumours (T) and normal tissue controls (N). (b) Immunoblotting of whole cell lysates from six representative primary human breast tumours with the G3-245 antibody or the C15 antibody. (c) Immunoblotting of primary tumour and U937 whole cell lysates with the G3-245 antibody and with an antibody specific to Δ Rb-p70 (Imgenex, IMG-395). Immunohistochemistry on sections of primary human breast tumours using antibodies specific to: (d) pRb (G3-245), (e) the proliferation antigen Ki-67, (f) Δ Rb-p70 (IMG-395), and (g) myeloperoxidase. All tumours used in this study were provided by the Medical Research Council UK/Cancer Research UK Tissue Bank and their use was approved by the Regional Ethics Committee, Ninewells Hospital, Tayside.

tumour cells expressed Ki-67 (Fig. 4e), which is indicative of a high proliferative index and they also expressed full-length pRb (Fig. 4g).

Given the haematopoietic specificity of Δ Rb-p70 expression in the mouse and in U937 cells, and the unexpected staining pattern in primary tumours (in non-tumour cells), we considered whether the cells staining positive for Δ Rb-p70 expression in tumour sections were tumour-associated macrophages (TAMs). TAMs are involved in tumour-promoting activities such as expression of epidermal growth factor, matrix metalloproteinases, vascular endothelial growth factor and tumour necrosis factor, and are an important target of cancer therapy (Pollard 2004). To test whether Δ Rb-p70 was specifically expressed in TAMs, we stained adjacent tumour sections with an antibody to the macrophage-specific marker, myeloperoxidase (MPO) and observed that expression of Δ Rb-p70 (Fig. 4f) colocalized with cells expressing MPO (Fig. 4g), suggesting that Δ Rb-p70 was indeed expressed in TAMs. These data suggest that Δ Rb-p70 is a marker of TAMs and may be used to identify tumours with macrophage infiltration.

DISCUSSION

We have identified a novel form of the Rb tumour suppressor, termed Δ Rb-p70, that is expressed exclusively in the myeloid system. Our data suggest that Δ Rb-p70 is the product of alternative translation from the fifth in-frame ATG and thereby lacks the conventional N-terminal sequences of pRb, but nevertheless retains a functional E2f-binding domain.

Although several previous studies have suggested that the N terminus of pRb is dispensable, including functional assays reporting that the 'large pocket' works as well as full-length pRb in growth arrest (G_1) assays (Qin *et al.* 1992) and/or in pituitary tumour suppression assays *in vivo*

(Yang *et al.* 2002), other studies suggest that the N terminus may have key functions. For example, the N terminus of pRb is highly conserved between different species, including *Xenopus laevis* (Destree *et al.* 1992), chicken (Boehmelt *et al.* 1994), mouse (Bernards *et al.* 1989), and human (Hong *et al.* 1989) but not between the pocket protein homologues, p107 and p130 (Mulligan & Jacks 1998). Furthermore, tumour mutations have been identified in this region of pRb in humans, for example, deletion of exon 4 in bilateral retinoblastoma (Hogg *et al.* 1993), frameshifts in exon 4 in unilateral retinoblastoma, and deletion of exon 4 in low penetrance retinoblastoma (Dryja *et al.* 1993). Functional assays have shown that the N terminus is required for 'flat cell' formation in Saos-2 cultures (Qian *et al.* 1992), phosphorylation of pRb (Hamel *et al.* 1990) and G₂ arrest (independent of the 'pocket') (Karantzsa *et al.* 1993). Further evidence supporting a functional role for the N terminus comes from *in vivo* mouse studies. These show that in contrast to wild-type *Rb* transgenes, mutation/deletion of the N terminus of pRb-generated dominant negative transgenes, which were unable to rescue the developmental defects in Rb-null mice, also had reduced tumour suppressor activity (Riley *et al.* 1997).

At the molecular level, it has been suggested that the N terminus of pRb is required for interaction with a variety of key cellular regulators, including a G₂/M kinase (Sterner *et al.* 1995), MCM7 (Sterner *et al.* 1998), hsp 70 (Inoue *et al.* 1995), a nuclear death receptor (Doostzadeh-Cizeron *et al.* 1999) and the glucocorticoid receptor (Singh *et al.* 2001). Furthermore, the N terminus of pRb is required for proper phosphorylation of pRb by cyclin-dependent kinases and deletion of the N terminus creates a constitutively active form of the protein (Hamel *et al.* 1990; Hinds *et al.* 1992). Decoupling of E2f-binding activity, located in the C terminus of pRb, from other activities dependent on a functional N terminus may thus have unique significance within the myeloid system.

The use of alternative translational initiation codons is regulated as a function of cell cycle phase (Pyronnet *et al.* 2000) and can be induced, for example under conditions of hypoxia (Stein *et al.* 1998). The selection of alternate initiation codons is determined by recruitment of ribosomes through either cap-dependent or cap-independent/internal ribosome entry site-dependent mechanisms (Sachs 2000). The absence of a good IRES sequence in the Rb message and a weak Kozak consensus around ATG1 leads us to suspect that alternative translation of pRb from ATG5 occurs as a consequence of leaky scanning. The presence of a pyrimidine at position +4 in the Kozak sequence surrounding the first in-frame ATG may cause it to be by-passed by the ribosomal initiation complex, and leaky scanning may then permit initiation downstream from ATG5 that has a consensus guanosine residue at +4 (Kozak 1987, 1986; Hellen & Sarnow 2001).

Alternative translation as a mechanism to regulate gene expression is not unusual in the haematopoietic system. The GATA-1, C/EBP α , C/EBP β , AML1 and Scl transcription factors are all subject to alternative translational initiation (Calligaris *et al.* 1995; Pozner *et al.* 2000; Calkhoven *et al.* 2000, 2003) and the alternative forms of some of these proteins have been shown to regulate cell fate in a distinct manner. We have shown that pRb can be alternatively translated *in vitro* and *in vivo* from ATG5 to render a protein lacking its N terminus. This may have significance in myeloid differentiation because we have shown that Δ Rb is down-regulated as U937 myelomonocytic leukaemia cells differentiate along the macrophage or granulocyte pathways.

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