A retinoblastoma ortholog controls stalk/spore preference in Dictyostelium

Harry MacWilliams1,*, Kimchi Doquang1,2, Roberto Pedrola3, Gytha Dollman1, Daniela Grassi3, Thomas Peis1, Adrian Tsang2 and Adriano Ceccarelli3

We describe rblA, the Dictyostelium ortholog of the retinoblastoma susceptibility gene Rb. In the growth phase, rblA expression is correlated with several factors that lead to ‘preference’ for the spore pathway. During multicellular development, expression increases 200-fold in differentiating spores. rblA-null strains differentiate stalk cells and spores normally, but in chimeras with wild type, the mutant shows a strong preference for the stalk pathway. rblA-null cells are hypersensitive to the stalk morphogen DIF, suggesting that rblA normally suppresses the DIF response in cells destined for the spore pathway. rblA overexpression during growth leads to G1 arrest, but as growing Dictyostelium are overwhelmingly in G2 phase, rblA does not seem to be important in the normal cell cycle. rblA-null cells show reduced cell size and a premature growth-development transition; the latter appears anomalous but may reflect selection pressures acting on social ameba.

KEY WORDS: Ameba, Cell cycle, Differentiation, Evolution

INTRODUCTION

Among the higher eukaryotes, multicellularity is thought to have arisen independently in the lines leading to animals and higher plants (Bonner, 1974; Kaiser, 2001), but some gene families are known to be important in the multicellular development of both of these groups. Such genes may have initially acquired their developmental functions in the unicellular ancestors of modern forms. The study of unicellular differentiation may thus provide insights about the original functions of such conserved developmental mechanisms.

The Amebozoa are a group of unicellular and facultative multicellular organisms which separated from the lines leading to animals and plants about when these split from one another (van der Peer et al., 2000; Eichinger et al., 2005). Like most microorganisms, Amebozoa proliferate indefinitely when provided with an adequate food supply. When they starve, however, the amebae transform to cysts, dehydrated and encapsulated cells that can survive for long periods under hostile conditions. If food again becomes available, the cysts germinate and re-enter the proliferative phase. The best studied Amebozoan, Dictyostelium discoideum (reviewed by Kessin, 2001), is unicellular in the proliferative phase, but shows a primitive form of multicellular development. Cells aggregate when the nutrition is exhausted and after a short multicellular migration phase, most of the amebae differentiate as cyst-like cells called spores. A minority of the cells enter a second differentiation pathway to form stalk cells; the stalk elevates the spore mass from the substrate and is thought to facilitate dispersal.

Dictyostelium is probably derived from a purely unicellular Amebozoan [S. Baldauf, personal communication; see also Alvarez-Curto et al. (Alvarez-Curto et al., 2005)], and this implies that its simple multicellularity evolved independently from that of animals and plants. Dictyostelium nonetheless has orthologs of important plant and animal differentiation regulators, and these may be involved in Dictyostelium development. One example is the homeodomain gene variai, a negative regulator of stalk differentiation (Han and Firtel, 1998); another is the Dictyostelium β-catenin ortholog aardvark, also a repressor of the stalk pathway (Coates et al., 2002; Coates, 2003).

The decision for stalk or spore differentiation depends on an interaction of cell-autonomous properties determined in the unicellular stage with intracellular signals in the aggregate. If two different Dictyostelium cultures are mixed just before development, one frequently observes that the cells of one culture preferentially form stalk, while the other amebae preferentially make spores; if unmixed, both cultures would make normally proportioned fruiting bodies. ‘Pathway preferences’ may result from differences in the composition of the growth medium or in stage of the growth curve when harvested. Preferences may also be found among the cells of a single culture, and here they depend on the cell cycle position of individual amebae at the moment when development begins (reviewed by MacWilliams et al., 2001). Preferences are not absolute but relative, so that cells can be placed in a linear hierarchy from most stalk-prefering to most spore-prefering (Leach et al., 1973; Fortunato et al., 2003). During the multicellular stage, the proportions of spores and stalk cells are regulated by negative feedback (Rafols et al., 2001) and theoretical models suggest that pathway preferences modulate the sensitivity of cells to negative-feedback signals (Blaschke et al., 1986). Recent studies suggest that nutritional state and cell cycle position modulate the sensitivity of cells to the negative feedback regulator DIF, a chlorinated hydroxyphenone made by cells of spore pathway that promotes stalk differentiation (Kay and Thompson, 2001).

In a database survey for Dictyostelium genes that might link pathway preferences with the cell cycle, we came across an ortholog of the ‘retinoblastoma-susceptibility gene’ Rb, a gene that regulates both the cell cycle and differentiation in animals and plants (reviewed by Classon and Harlow, 2002). Rb was discovered as a tumor suppressor that is inactivated, directly or indirectly, in most cancers (Sherr, 1996). In the cell cycle, the protein pRb blocks the G1/S transition through its interaction with transcription factors of the E2F family. E2Fs target many genes required in the S phase, and...
pRb both sequesters ‘activator E2Fs’ and is recruited to the promoter by ‘inhibitor E2Fs’ where it participates in inhibitory chromatin remodeling complexes (reviewed by Trimarchi and Lees, 2002). pRb is neutralized at the end of G1 when it is phosphorylated by cyclin-dependent kinases, which leads it to dissociate from E2Fs of both classes.

The role of Rb in cell differentiation is perhaps best illustrated by the phenotype of the Rb-null mouse; this is superficially normal in early development, but dies before birth with massive defects in many differentiated tissues, including muscle, blood and nerves (Zacksenhaus et al., 1996). During normal development, Rb is strongly expressed in these tissues (Szekely et al., 1992; Jiang et al., 1997) and pRb interacts with tissue-specific transcription factors to promote both cell cycle withdrawal and the expression of terminal genes (Gu et al., 1993; Novitch et al., 1999; Thomas et al., 2001; Chen et al., 1996; Cole et al., 2003; Iavarone et al., 2004; Toma et al., 2000; Batsché et al., 2005). Rb may also have additional roles in mammalian differentiation. There are intriguing reports of Rb acting as a ‘selector gene’, favoring white over brown adipose differentiation (Hansen et al., 2004) or specifically inhibiting neurendocrine cell fate in the lung (Wikenheiser-Brokamp, 2004); the Rb-related gene p130 favors neuronal rather than glial differentiation (Jori et al., 2001). In a particularly fascinating case, pRb may revere the interaction of activin and nodal signaling by competing with goosecoid for binding to the transcription factor PU.1 (Konishi et al., 1999). Rb-family genes also affect differentiation in Drosophila (Du and Dyson, 1999), Caenorhabditis (Lu and Horwitz, 1998; Myers and Greenwald, 2005) and Arabidopsis (Ebel et al., 2004).

The widespread role Rb-family genes in multicellular development encouraged us to investigate the role of the Dictyostelium retinoblastoma ortholog rblA in the development of this Amebozoan. A priori, any of a variety of functions were conceivable, ranging from cell-cycle control, regulation of cell cycle exit and selector gene function. Our results suggest that the major role of rblA may be to modulate pathway preference. rblA also affects the Dictyostelium growth-development transition, but its action here is unusual: whereas Rb in animals and plants promotes cell cycle exit and differentiation, rblA inhibits the onset of development. We interpret this as an adaptation specific to social ameaeae, and suggest that in ancestral Amebozoans, the role of Rb in development was similar to that in higher organisms.

MATERIALS AND METHODS

Dictyostelium strains and basic methods

The axenic line AX2 (Watts and Ashworth, 1970) and derivatives were used in all experiments. For disruption of rblA, exponentially growing cells were electroporated with 10-20 μg of transforming DNA in 0.1 cm cuvettes (Pang et al., 1999) and selected in HL5 containing 10 μg/ml blasticidin. For the reporter- and overexpression constructs, we used the transformation method previously described (Wetterauer et al., 1996) and modified (Deichsel et al., 1999). Cell cycle synchronization, BrdU staining and β-galactosidase measurements were performed as previously described (MacWilliams et al., 2001). Measurement of the DIF response was as described (Thompson and Kay, 2000).

Vector construction

The rblA disruption construct contains the first 903 bp of the rblA-coding region, followed by a blastcidin-resistance cassette (Adachi, 1994) and bp 1699-2949 of rblA. The reporter rblA::x-gal contains 1126 bp of genomic sequence upstream of the rblA ATG plus the first 21 rblA codons, with upstream XbaI and downstream BglII sites, fused to the reporter i-Scotland (Gaudet et al., 2001). The overexpression vector A15::rblA contains the actin 15 promoter followed by eight actin codons, a BglII/HindIII fusion, 10 codons specifying a myc epitope, a BglII site, followed by the entire rblA-coding region and ~1500 bp of downstream genomic sequence, culminating in an actin 8 terminator. The cassette was assembled in V18m5 turbo (Deichsel et al., 1999).

Measurement of nuclear DNA content

Measurements were performed essentially as described previously (Weijer et al., 1984). Axenically grown cells or spores harvested from bacterial plates were collected by centrifugation, resuspended in 70% ethanol and maintained at 4°C until needed. For staining, cells or spores were collected once again by centrifugation and suspended in 1 μg/ml DAPI. In most experiments, cells and spores were mixed and viewed in a chamber made of two coverslips separated by spacers. Images were captured using a Zeiss 63×/1.25 oil objective, a Till Photonics Polychrome IV illuminator, a Sensicam 370-KL cooled CCD camera and Tillvision software. A short through-focus film was obtained from each field and the best frame selected for each cell or spore. Polygon ROIs were drawn about the nucleus and the cell body as a whole, and average and integrated pixel values measured; this allowed correction of nuclear fluorescence for cytosolic background.

The alignment of the predicted protein with sequences from different organisms revealed two regions of high similarity. One, near the N terminus (Fig. 1A,B), encompasses the pRb interaction domains with the transcription factor SP1 and the pRb kinase cdk4/cyclin D (Connell-Crowley et al., 1997). Here, the sequence is 20% identical and 35% similar to human pRb. The remaining region (23% identical/38% similar) encompasses the pfam RbA and RbB domains (Marchler-Bauer et al., 2003) (Fig. 1A,C). Eighteen out of the 27 amino acids important for generation of the ‘pocket motif’ are conserved, as are 10 of the 12 residues that interact with the LXCGXE motif (Umen and Goodenough, 2001; Lee et al., 1998). In its overall size, rblA resembles the related pocket proteins p107 and p130 more closely than pRb (Fig. 1D), and the resemblance to the latter is also greater in pairwise blastP matches of the N-terminal domains (expected values of 2e-07 and 6e-05 versus 9e-1). The match is better to pRb in the spacing of the RbA and RbB boxes, and the closer resemblance in this region is also detected by the blastP algorithm (3e-18 versus 2e-10 and 2e-11). rblA lacks two...
Fig. 1. Structure of the Dictyostelium retinoblastoma ortholog rblA with Rb family members from other species. (A) Overview showing blocks of similarity. The gray rectangle N corresponds to the conserved N-terminal region, while blocks A and B are the RbA and RbB domains, respectively. The regions that interact with SP1 and Rb kinase in the human gene are indicated by thin lines. (B, C) Alignment of rblA with Rb proteins from the indicated organisms. Conserved residues are boxed, and identical shaded in gray. (B) The N-terminal region; (C) the RbA and RbB boxes, and the intervening spacer. Black circles indicate positions relevant to the folding of RbA and RbB domains; asterisks indicate amino acids interacting with the LXCXE peptides. (D) Comparison between rblA and human members of the pocket protein family. The scale is the same as in A. The sequences have been arbitrarily aligned at the N-terminal side of the B box.
conserved features of p107 and p130, a cdk inhibitor domain and a bipartite B box (Classon and Dyson, 2001) and in this aspect is also closer to pRb.

Role of \textit{rblA} in development

When Northern blots of polyA+ RNA prepared from \textit{Dictyostelium} at various stages of the life cycle were probed with a randomly labeled DNA fragment complementary to the middle region of \textit{rblA}, we obtained bands of the size expected for a complete transcript. In a representative experiment, the expression was maximal at T12 and T16 (Fig. 2A), when cell type-specific reporters are first expressed. An exposure of several days was necessary, suggesting that the mRNA is of very low abundance. In several further experiments the timing was similar or slightly later. The upregulation during development suggests the main role of the gene may be in cell differentiation.

To determine whether \textit{rblA} expression is cell-type specific or common to all developing cells, we constructed a reporter, \textit{rblA}:opgal, in which genomic sequences upstream from \textit{rblA} drive a short-lived Galactosidase reporter. Using a sensitive chemiluminescent assay, we could detect Galactosidase activity in vegetative cells; this increased about 200-fold during development (Fig. 2B). When allowance is made for differences in developmental timing and the developmental asynchrony that is common in transformants, the pattern is consistent with that seen in the \textit{rblA} northern blot. As the construct contains all but 300 bp of the intergenic region separating \textit{rblA} from the divergently transcribed upstream neighbor, \textit{talA}, it seemed possible that its behavior could be influenced by \textit{talA} promoter elements. The temporal regulation of \textit{talA} is very different from that of \textit{rblA}, however; it is expressed at moderate levels in vegetative cells, peaks at 6-9 hours of development and subsequently decreases (A. Mueller-Taubenberger, personal communication). We conclude that the reporter reflects \textit{rblA} promoter activity. Using X-gal as a substrate, reporter expression was not detectable during proliferation, but readily visible in multicellular stages (Fig. 2C) in positions where cells of the spore pathway are characteristically found (Fig. 3). In differentiation, \textit{rblA} is essentially specific for the spore pathway.

We then wished to know whether \textit{rblA} is necessary for normal spore formation. We inactivated the gene by homologous recombination. Candidate clones were screened by PCR and two independent clones were found by southern blotting to contain a unique insertion of the blasticidin resistance cassette into the \textit{rblA} locus (not shown). In northern blots, the \textit{rblA} mRNA was replaced by a ~1.2 kb truncated message fragment (not shown). If this is translated, the product would be a fragment of the N-terminal domain, which in pRb inhibits substrate binding (Goodrich, 2003), and is not predicted to lead to a partial-activity phenotype. Nonetheless, there is a formal possibility that some features of the null phenotype result from this message.

---

**Fig. 2. Expression of \textit{rblA}.** (A) Northern blot showing expression beginning at 12 hours. (B) β-Galactosidase activity in extracts of developing cells carrying the \textit{rblA}:opgal reporter, detected using chemoluminescence. Two independent clones were measured. The gray curves (left scale) show vegetative activity and an increase beginning at 8-10 hours; the black curves (right scale) show an ultimate 200-fold upregulation. (C) Colony lift of growing clone of \textit{rblA}:opgal cells, stained with X-gal and counterstained with Ponceau Red. The heavy red band (left) is the clone edge, made up of proliferating cells. Multicellular stages (slugs and fruiting bodies) are on the right. Expression is visible only at multicellular stages.

**Fig. 3. Expression pattern of the \textit{rblA}:opgal during development.** (A) In a tight mound (14 hours), the central core, which is occupied by stalk precursors, remains unstained. (B) In the slug stage (17 hours), staining is confined to the rear two-thirds; the anterior prestalk region is negative. (C) In a fruiting body (22 hours), staining is almost exclusively found in the future spore mass. For clarity, the prestalk region and the stalk have been outlined in B and C.
Cells of both rblA-null clones developed normally and formed fruiting bodies containing both stalk cells and spores; the spores were morphologically normal and resisted both desiccation and freezing. We transformed both clones with the stalk-cell specific reporter ST-gal (Ceccarelli et al., 1991) and the spore-specific reporter spiA-gal (Richardson and Loomis, 1992), as well as the prestalk-specific construct ecmA-gal (Early et al., 1993). The staining patterns were all normal (not shown); rblA is not necessary in either the stalk or spore differentiation pathways.

Proliferating Dictyostelium cells have a long G2 phase, while G1 is not normally detectable (Weijer et al., 1984). A recent study (Chen et al., 2004) suggests that spores arrest in G1, and we wondered rblA expression in the spore pathway might be responsible. In this case, one would expect the G1/S block to be abolished in rblA-null cells, so that rblA-null spores would have twice as much DNA as the wild type. We compared the nuclear DNA contents of rblA-null and wild-type spores using the nuclei of wild-type cells as an internal standard. The relative fluorescence values (mean±s.d.) were 0.98±0.04 for wild-type spores, 1.07±0.04 for rblA-null spores and 1 for the standard. The data thus do not support the idea that rblA brings about G1 arrest during spore differentiation. It appears, moreover, that, under our conditions, wild-type spores have the same nuclear DNA content as proliferating cells (Fig. 4A), i.e. they are predominantly/ exclusively G2. As we used a different Dictyostelium strain from Chen and co-workers (Ax2 versus Ax4), estimated DNA content using a different dye (DAPI versus propidium iodide) and employed a different measurement principle (nuclear fluorescence via imaging, versus whole-cell fluorescence via flow cytometry), it is not immediately apparent why our results differ from theirs.

To determine whether rblA might play a role in the more subtle phenomenon of pathway preference, we marked both clones of rblA-null cells with a vector that confers constitutive β-galactosidase expression and mixed these with unmarked wild-type cells. In an independent set of experiments, GFP was used as a strain marker. In both cases, the rblA-null cells were found mainly in the anterior prestalk region and the posterior rear-guard zone (Fig. 5D; GFP results not shown). In a third series of experiments, we marked the mutants with green CMFDA Cell Tracker (Invitrogen) (Dormann and Weijer, 1997), and mixed them with wild type. In fruiting bodies (Fig. 5A-C) the rblA-null cells were found mainly in the papilla and stalk, with a minor population in the spores. In two experiments, the fraction of stained cells was 0.15 and 0.20 in the mix before development, and the fraction of stained spores 0.01 and 0.05. rblA-null cells thus show a strong bias for the stalk fate.

Pathway preference is normally determined by the composition of the growth medium, the stage of the growth curve and the cell cycle phase, and we wished to determine whether these factors influence rblA expression. In cells carrying the rblA::oppGal gal reporter, which had been synchronized by cold release, reporter expression (measured by chemoluminescence) was maximal 3 hours before S phase, defined as the peak nuclear of BrdU incorporation (Fig. 6). Given a doubling time of 10-12 hours, a 30-minute M phase and a negligible G1 (Weijer et al., 1984), rblA is maximally expressed in the late G2 phase, when cells have a strong prespore-differentiation preference (Thompson and Kay, 2000; MacWilliams et al., 2001). We then diluted stationary-phase cells into normal or...
glucose-free media. The reporter expression increased sharply after dilution in both media and decreased again as the cells approached stationary phase (Fig. 7); the maximal activity was about threefold higher in glucose-containing than in glucose-free media. \( rblA \) expression is thus highest in cells growing in normal medium, intermediate during growth without glucose, and lowest in stationary phase cells; this is exactly the order of spore-formation preferences found in classical studies (Leach et al., 1973).

This correlation led us to ask whether pathway preference might depend on \( rblA \). We thus grew \( rblA \)-null cells in normal and glucose-free media, mixed them and followed their fates using a gfp marker that was present only in the cells grown without glucose. In the same experiment with the wild type, the glucose-starved cells are recovered preferentially in the prestalk zone of the slug (Kay and Thompson, 2000), but we could detect no such preference when exclusively \( rblA \)-null cells were used (Fig. 8). It did not appear that \( rblA \)-null cells have a problem in using glucose, as they reached a higher saturation density in glucose-containing medium (not shown) than we found in the wild type (Fig. 7). This suggests that \( rblA \)-deficient cells specifically lack the ability to translate internal metabolic signals into pathway preference, and is consistent with the idea that \( rblA \) is intimately involved in the control of this phenomenon.

Pathway preferences are thought to act by modulating the sensitivity of cells to a negative-feedback regulator of stalk-spore proportioning (Blaschke et al., 1986) and recent work suggests that both cell cycle position and composition of the growth medium modulate the sensitivity of cells to the negative-feedback regulator DIF (Kay and Thompson, 2001). We accordingly wished to determine whether \( rblA \)-null cells show altered DIF responsiveness. Wild-type and \( rblA \)-null cells were transformed with the stalk-cell specific reporter ecmB-gal (Early et al., 1993), and the DIF response was measured in monolayers (Thompson and Kay, 2000) using β-galactosidase activity as a measure of stalk induction (Fig. 9). \( rblA \)-null cells appeared to be about threefold more sensitive than wild type; the difference was significant at \( P<0.05 \).

**Role of \( rblA \) during proliferation and at the growth-development transition**

We used a Coulter-type cell counter (HC-333, Boehringer-Mannheim, USA) to measure the vegetative growth rate of \( rblA \)-null and wild-type cells over 150 hours in shaking suspension cultures (see Fig. S1A in the supplementary material). In a second experiment, cells growing attached in 12-well plastic tissue culture plates were labeled continuously with BrdU over 12 hours; cells...
were fixed hourly, then stained and counted to follow the labeling curve (see Fig. S1B in the supplementary material). As neither experiment showed a difference between mutant and wild type, we conclude that \textit{rblA} does not affect the cell cycle length. There does appear to be an effect on cell size. \textit{rblA}-null and wild-type cells, grown surface-attached and harvested at about 10\% confluence, were measured by flow cytometry. The null cells showed lower forward scattering and were thus smaller than the controls (Fig. 10). Interpreted with calibrated marker beads (not shown), the data suggest that the average null cell is about 20\% smaller in diameter than the average wild-type cell and thus has about 50\% of the average wild-type volume.

In vertebrates, developmental upregulation of \textit{Rb} is thought to promote cell cycle exit at the beginning of terminal differentiation and to stabilize the differentiated state. In \textit{Dictyostelium}, to our surprise, \textit{rblA}-null cells showed accelerated, rather than retarded, development. When cultured in bacteriological dishes, \textit{rblA}-null cells ceased growing before confluence and formed patterns suggestive of aggregation streams (Fig. 11A); at higher magnification, many cells had an elongate form typical of aggregating cells (Fig. 11B). Wild-type cells, by contrast, grow well past confluence and never initiate development in growth medium. When plated for development on non-nutrient agar, \textit{rblA}-null strains developed more rapidly than normal, completing the program in 13-15 hours, compared with 20-24 hours in the wild-type cells (Fig. 11C). About half of this difference appeared to be due to acceleration of aggregation; thus, pre-aggregative stages are completed in 5-6 hours, compared with the 10-12 hours necessary in the AX2. Post-aggregative development, however, also appeared to be faster in \textit{rblA}-null strains.

\textit{Rb}-deficient vertebrate myoblasts can sometimes be induced to differentiate by growth factor withdrawal, but the myofibrils are unstable and the nuclei re-enter S-phase if replaced in normal growth medium (Gu et al., 1993). Wild-type \textit{Dictyostelium} cells that have begun development will resume vegetative growth if nutrients are re-supplied, but only after a period of several hours (Soll and Wadell, 1975). We wondered if this delay might reflect the stabilization of cell cycle withdrawal by \textit{rblA}. We therefore allowed wild-type and \textit{rblA}-null cells to develop on filters for 10 hours, after which they were washed free and resuspended in growth medium. Both strains...
resumed growth after 5 hours (see Fig. S2 in the supplementary material), at which point BrdU labeling also resumed (not shown). No difference between the strains was apparent.

In view of these rather unexpected results, we wished to confirm that rblA is a functional analog, as well as an ortholog of vertebrate Rb. As the G1 phase is normally undetectable in proliferating Dictyostelium and because a basic function of Rb is to block the cell cycle at the G1/S boundary (Qin et al., 1992; Huang et al., 1988; Goodrich et al., 1991), we reasoned that overexpressing rblA might create a stable G1 phase, and that this would provide strong evidence that rblA is functionally a retinoblastoma-family gene. We constructed a vector (A15::rblA) in which rblA-coding sequences are under the control of the Dictyostelium actin15 promoter, which is widely used as a strong and unspecific promoter in Dictyostelium studies. Transformed clones grew exceptionally slowly, though the cells appeared healthy under phase-contrast optics. Clones were stained in situ with DAPI and the nuclear DNA contents measured, using wild-type spores as an internal standard. The results suggested that the cells were predominately G1, though the distribution also included some G2 cells (Fig. 4B). Thus, rblA can produce a long-lasting G1 phase in proliferating Dictyostelium cells when expressed at non-physiological levels.

**DISCUSSION**

**Retinoblastoma and its classic partners in Dictyostelium**

In this paper, we present a first characterization of rblA, the Dictyostelium representative of the ‘pocket protein’ family known in plants, animals and some unicellular organisms. The gene, which was initially identified in a database search for proteins containing similarities to the vertebrate retinoblastoma RbA and RbB domains, is the only member of this family in Dictyostelium. An alignment of rblA with pRb from phyllogenetic distant organisms shows conservation of several features important for binding to target proteins in vertebrates. The RbA and RbB domains, which in the vertebrate protein are known to form a ‘binding pocket’ are well conserved, as is the LxCxE interaction motif. Less well conserved but still clearly recognizable is a region near the N terminus that interacts in mammals with the transcription factor SP1 and the Rb-kinase CDK4/cyclin D.

Very little has been published on potential rblA-interacting molecules in Dictyostelium, but a survey of genomic data (www.dictybase.org) suggests some conservation of proteins whose animal orthologs participate in Rb-containing complexes. There is a fairly clear E2F homolog (DDB0216397) and a related gene (DDB0220101) with closer affinities to the E2F-binding partner DP. Two particularly notable genes are RbbD (DDB0232003) and lin9 (DDB0232078). The human orthologs are RbpAp48 and LIN9; the former is found in several complexes involved in pRB-mediated repression (Lai et al., 2001; Nicolas et al., 2001; Vaute et al., 2002) while the human ortholog LIN9 interacts with pRb in osteoblast differentiation (Gagrica et al., 2004). The Caenorhabditis orthologs lin-53 and lin-9 interact with Rb in the synMuvB group to regulated vulva differentiation (Lu and Horvitz, 1998; Beitel et al., 2000). The Drosophila orthologs CAF1p55 and Mip130 are found with pRb in the dREAM complex, which localizes to transcriptionally silent chromatin and represses differentiation- and sex-specific genes (Korenjak et al., 2004; Taylor-Harding et al., 2004). Orthologs of RbbD and lin9 have also been described in plants (Ach et al., 1997; Bhatt et al., 2004). A fourth potential pRb-interactor in Dictyostelium is RbbB (DDB0220639), the vertebrate ortholog of which, Rbhp2, recruits HDACs to promoters repressed by Rb/E2F (Gray et al., 2005). Dictyostelium has a histone methyltransferase (Chubb et al., 2006), two putative histone deacetylases (DDB0189724; DDB0190980) and genes for four chromatin-remodeling ATPases of the SNF2 group, including one apparent SWI/SNF homolog (DDB022695) the vertebrate relatives of which mediate retinoblastoma-repression of cyclin A and the polo-like kinase (Siddiqui et al., 2003; Gunawardena et al., 2004) and the plant homologs of which are extensively involved in the control of development (Sarnowski et al., 2005; Zhou et al., 2003).

Dictyostelium has a ‘minimal set’ of cell cycle genes, initiating comparison with Ostreococcus (Robbens et al., 2005). There are three cyclins of the cell-cycle group, cycA (DDB0231774), cycB (DDB0185035) and cycD (DDB0231773), and one cyclin-dependent kinase, cdk1 (DDB0185028), in the superfamily defined by animal Cdns 1/2 and plant Cdns A/B. There is no cdk4/6 homolog, and no E-type cyclin, as in plants.

**rblA in Dictyostelium development**

Dictyostelium expresses rblA primarily in cells of the spore differentiation pathway. During growth, when the promoter is very weakly expressed, its activity is correlated with the ‘spore differentiation preference’ of cells: it is highest in the late cell cycle (Fig. 5), in the exponential phase of the growth curve and in cells grown in rich medium (Fig. 6). During development, the promoter activity increases 200-fold and can be demonstrated by X-gal staining in the periphery of tight aggregates (where prespore gene expression begins), in the prespore zones of slugs and in the nascent spores (Fig. 3). The message is detectable in northern blots only during the period of cell-type-specific gene expression.

Although the expression pattern suggests a function in spore differentiation, rblA-null mutants make spores which are grossly indistinguishable from those of the wild type, and withstand dessication and freezing; rblA is thus dispensable for spore formation. Experiments with cell-type-specific reporters demonstrate, furthermore, that rblA-null slugs have normal or near-normal ratios of prestalk and prespore cell types. Although Dictyostelium spores have been reported to show G1 arrest, spores from rblA-null strains do not differ in nuclear DNA content from the wild type.

As rblA expression is correlated with spore differentiation preference, we finally considered the possibility that rblA might be involved in this elusive phenomenon. We found in fact that rblA-null cells are strongly enriched in the prestalk zones of chimeric slugs (Fig. 4), while they are depleted in the prespore zones and spores. When rblA-null cells are given a free choice of the stalk and spore pathways, they show a strong preference for stalk differentiation. Such preferences are well known in Dictyostelium, and have been explained as differences in sensitivity to the stalk-cell inducer DIF (Thompson and Kay, 2000). We therefore tested the DIF responsiveness of rblA-null cells and found them significantly more sensitive than the wild type (Fig. 7). Our data suggest that the factors controlling pathway preference in Dictyostelium influence rblA promoter activity, and that rblA in turn controls the responsiveness of cells to DIF.

**rblA in the Dictyostelium cell cycle and at the growth-development transition**

rblA has only relatively subtle effects during cell growth and at the growth-development transition. In contrast to mammalian cells, where a triple knockout of all three Rb-family genes reduces the cell cycle length by about 35% (Sage et al., 2000), deletion of the single Rb ortholog of Dictyostelium has no significant effect on the
generation time. In this respect, Dictyostelium resembles the unicellular alga Chlamydomonas, in which cells deficient in mat3 show unchanged mass doubling rate (Umen and Goodenough, 2001). This difference presumably reflects a fundamental difference in multicellular and unicellular life style; in microbes, the cells proliferate as fast as nutrition (or in the case of algae, light) allows, while in multicellular organisms, proliferation is regulated by growth factor signaling and is usually slower than metabolic resources alone would sustain. Removing Rb family genes in mammalian cells thus eliminates a regulatory level that is not functional in unicellular organisms. In Dictyostelium, we nonetheless found that rbla can block the cell cycle in the presence of nutrition if expressed at nonphysiological levels; raw material is thus present from which multicellular-type controls could be fashioned.

Proliferating rbla-null cells are significantly smaller than wild-type cells (Fig. 8). Similar phenomena are seen in triple-Rb family-knockout mammalian cells and Chlamydomonas; in all three cases, the volume reduction is ~50%. The mechanism underlying this effect is unknown.

Given the dramatic increase in rbla expression in development, we were surprised to find that Rbla-null cells initiate development earlier and complete development more rapidly than the wild type (Fig. 9). The growth-development transition in Dictyostelium is a complex process in which the yakA kinase plays a central role (Souza et al., 1998; Souza et al., 1999). Both the rbla-null and the yakA overexpressor cease growth prematurely, so that one could speculate that rbla null suppresses yakA. The relationship between these two signals is not straightforward, however, as yakA also affects cell size and, here, rbla-null resembles the yakA-null.

**Dictyostelium, retinoblastoma and evolution**

The retinoblastoma-susceptibility gene Rb was discovered as a vertebrate tumor suppressor, the protein product of which, pRb, inhibits or represses many genes required at the G1/S transition or in S phase. Vertebrate Rb is upregulated during the differentiation of multiple tissues; it is required for normal differentiation in many of these and, in several, pRb is known to synergize with tissue-specific transcription factors. These interactions often have a strong positive-feedback or feed-forward character. They are usually specific to the hypophosphorylated form of pRb, which occurs only in G1, and in addition to promoting the expression of terminal differentiation genes, the interacting factors often induce either pRb itself or other inhibitors of the G1/S transition, such as p27, which potentate the interaction by increasing the fraction of pRb in the hypophosphorylated state. In addition, the interacting partners may induce further transcription factors with which pRb also interacts (Novitch et al., 1999). In such systems, the effect of pRb upregulation is to make cell cycle exit irreversible (Thomas et al., 2004). Retinoblastoma family genes are also upregulated in the development of Drosophila (Keller et al., 2005) and maize (Huntley et al., 1998).

Consistent with this pattern, rbla in Dictyostelium is dramatically upregulated during the formation of spores. As in other organisms, this may well be under positive-feedback control, as spores are formed by cells that have higher initial rbla expression. In contrast to these conserved features, however, the relationship between rbla and cell cycle exit is not typical. Retinoblastoma-deficient mutants show supernumerary cell cycle activity in differentiating tissues in vertebrates (Lee et al., 1992), Drosophila (Du and Dyson, 1999), Caenorhabditis (Lu and Horvitz, 1998) and plants (Ebel et al., 2004). In Dictyostelium, however, rbla-null cells do not linger in the proliferative phase but enter development prematurely. Moreover, the signal that initiates in Dictyostelium, starvation, does not induce rbla but represses it.

These anomalous features can plausibly be regarded as adaptations to the way of life of the social ameba. Dictyostelium is almost certainly derived from ancestors that were solitary in both proliferative and developmental stages. Solitary Amoebozoans generally encyst when starved, and these cysts may resemble Dictyostelium spores in function (Mazur et al., 1995) and in aspects of morphology (Chavez-Munguia et al., 2005). It thus seems likely that encystment in the non-social ancestors of Dictyostelium involved an Rb-family gene. In solitary amebae, starvation induces 100% of the population to encyst; and the role of Rb in this process may have differed little from the one familiar in multicellular organisms today. In particular, starvation may have initiated Rb expression, and Rb expression may have activated mutually reinforcing processes of cell cycle withdrawal and differentiation.

In social amebae, there are two differentiated cell types, and a mechanism is needed to allocate two these pathways. Positive-feedback systems are predestined to work as switches, and a proportioning system could have been constructed simply by coupling the Rb-driven encystment programs of the individual amebae via a diffusible negative regulator (Gierer and Meinhardt, 1972; Lewis et al., 1977). Here, the details can have great selective consequences, as stalk cells die while spores remain in the gene pool. If starvation induces Rb in such a system, then the proportioning mechanism will preferentially shunt starving cells to the spore pathway, while ameba that, for whatever reason, have done particularly well in the competition for nutritional resources will be diverted to the ‘ altruistic’ stalk fate. Here, it seems likely that fitness could be improved by reversing the relationship between starvation and Rb expression. Starvation must of course continue to control cell cycle exit, but alternatives to Rb could be found for this role.

This scenario thus suggests that in the evolution of the social ameba, the link of Rb to cell cycle exit was lost while the relationship with differentiation was retained. This is consistent with the idea that the connections between retinoblastoma-family genes and specific differentiation pathways are particularly stable in evolution. Control of differentiation may thus be an ancient function of retinoblastoma signaling.

We thank the Dictyostelium Genome Consortium and the Dictybase team, without whose generous assistance this project would not have been possible. We are indebted to Charles David for material and moral support and for his astute comments on the manuscript. This work was partly supported by grants FINE60-2002- and PRIN2004-#133COF04CE to A.C.

**Supplementary material**

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/133/7/1287/DC1

**References**


which acts in an Rb-related pathway, is required for gonadal sheath cell development and encodes a novel protein. Gene 22, 253-263.


Rb and differentiation in Dictyostelium RESEARCH ARTICLE


