RBF*, a novel RB-related gene that regulates E2F activity and interacts with *cyclin E* in *Drosophila

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Genetic studies have shown that *cyclin E* and *dE2F* are critical regulators of S-phase entry during *Drosophila* embryogenesis. Whereas the ectopic expression of *cyclin E* activates *dE2F*-dependent transcription, it has been proposed that cyclin E does not act directly on dE2F but targets a negative regulator of E2F activity. Such a regulator might be analogous to the family of RB-related proteins (pRB, p107, and p130) that associate with E2F in humans; however, extensive efforts have failed to find such homologs in *Drosophila*. We have developed a two-hybrid approach that allows transcription activators to be used as bait for interacting proteins. From a screen using *Drosophila* E2F (dE2F and dDP) as bait, we identified a novel gene, *RBF*. *RBF* combines several of the structural features of pRB, p107, and p130, suggesting that it may have evolved from a common ancestor to the three human genes. RBF associates with dE2F and dDP in vivo and is a stoichiometric component of E2F DNA-binding complexes. RBF specifically repressed E2F-dependent transcription and suppressed the phenotype generated by ectopic expression of dE2F and dDP in the developing *Drosophila* eye. RBF was phosphorylated by a cyclin E-associated kinase in vitro, and loss-of-function *cyclin E* mutations enhanced an RBF overexpression phenotype, consistent with the idea that the biological activity of RBF is negatively regulated by endogenous cyclin E. The properties of RBF suggest that it is the intermediary factor that was proposed to allow cyclin E induction of E2F activity. These findings indicate that RBF plays a critical role in the regulation of cell proliferation in *Drosophila* and show that analogous pathways regulate S-phase entry in a diverse range of species.

[Key Words: *Drosophila*; RB, E2F; S-phase; cyclin E; cell cycle]

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It has been a matter for conjecture whether the mechanism of G₁ control in *Drosophila* resembles the events regulating the proliferation of mammalian cells. In *Drosophila*, several different variations of the cell cycle occur and only a few cell types are known to progress through a cell cycle that contains a G₁ phase (for review, see O'Farrell 1992; Foe et al. 1993; Edgar 1995). However, recent studies suggest that S-phase entry in *Drosophila* is regulated by proteins homologous to the important regulators of the mammalian cell cycle. *Drosophila* homologs of cdk2 (*Dmcdc2c*; Lehner and O'Farrell 1990), cyclin E (Richardson et al. 1993), and E2F (*dE2F*, *dDP*; Dynlacht et al. 1994; Ohtani and Nevins 1994) have been described that may carry out functions similar to their mammalian counterparts.

In *Drosophila*, cyclin E is required for DNA synthesis during embryogenesis (Knoblich et al. 1994), and ectopic expression of cyclin E drives cells into S-phase (Duronio and O'Farrell 1994; Knoblich et al. 1994). Cyclin E associates with *Dmcdc2c* in vivo, generating a kinase that is

regulated during G₁ to S progression (Sauer et al. 1995). At least some of the functions of cyclin E in the *Drosophila* embryo appear to be mediated by dE2F. Embryos homozygous for mutant alleles of *dE2F* are unable to activate a program of gene expression that normally accompanies G₁ to S progression. Similar to *cyclin E* mutant embryos, *dE2F* mutant embryos show severe defects in DNA synthesis (Duronio et al. 1995). Because dE2F is expressed in a relatively uniform pattern in wild-type embryos, the temporal changes in dE2F-dependent transcription are likely to result from regulation of the activity of the gene product.

Duronio and O'Farrell (1995) have shown that the functional relationship between *cyclin E* and *dE2F* varies in different developmental situations. During G₁17, in the endocycling cells of the midgut, *cyclin E* appears to act downstream of *dE2F*. In contrast, in the CNS *cyclin E* is required for dE2F-dependent transcription and appears to act upstream of *dE2F*. Ectopic expression of cyclin E in wild-type embryos induces transcription of dE2F-regulated genes in many cell types (Duronio and O'Farrell 1994, 1995; Sauer et al. 1995). By analogy with

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mammalian cells it has been proposed that cyclin E does not act directly on dE2F but that this regulation is mediated by a homolog of the retinoblastoma protein (pRB), or related proteins (Duronio and O'Farrell 1994, 1995). However, despite extensive searches no such homologs have been found, and direct evidence for such a regulator has been elusive. The identification of such a regulator is essential for a detailed understanding the molecular mechanisms controlling the G₁ to S transition in *Drosophila*.

pRB provides a constraint to the proliferation of many types of mammalian cells (for review, see Riley et al. 1994; Weinberg 1995). The inactivation of both copies of the *Rb* gene appears to be the rate-limiting event in the genesis of retinoblastoma, and *Rb* mutations are seen in many different types of tumors. pRB is also a target for the transforming regions of several viral oncoproteins. These viral products inactivate pRB by competing with several cellular proteins that normally interact with the pRB "pocket" domain. The same regions of the viral proteins make similar contacts with two pRB-related proteins, p107 and p130, which suggests that the inactivation of the family of proteins is important (for review, see Dyson and Harlow 1992).

pRB is required for normal mouse development (Clarke et al. 1992; Jacks et al. 1992; Lee et al. 1992). *Rb*-nullizygous embryos die by day 13.5–14.5 of development. In these embryos, as in pRB-deficient tumor cells, the presence of p107 and p130 is unable to compensate for the absence of pRB, indicating that these proteins must have distinct functions. However, biochemically, pRB, p107, and p130 appear to have similar activities. All three proteins associate with E2F and, in several experimental systems, repress E2F-dependent transcription (for review, see Cobrinik 1996). In addition, pRB, p107, and p130 are each phosphorylated by cyclin-dependent kinases (cdks) during G₁ to S progression, and these modifications are thought to inhibit association with E2F and other potential partners (for review, see Cobrinik 1996).

Presently, it is unclear to what extent pRB, p107, and p130 share overlapping functions and to what extent their functions are unique. Although pRB, p107, and p130 are all regulators of E2F, they associate with specific subsets of E2F dimers and associate with E2F at different times during the cell cycle. In addition, p107 and p130 contain a binding site for cdks in their spacer region that lies between the two halves of the pocket domain (Ewen et al. 1992; Faha et al. 1992; Hannon et al. 1993; Li et al. 1993; Mayol et al. 1993). Overexpression of this cdk-binding domain arrests cells in G₁ (Zhu et al. 1993, 1995). The spacer region of pRB differs in sequence from the p107 and p130 sequences and lacks cdk-binding or cell cycle arrest activities.

It would be advantageous to study the function of the pRB gene family in an organism that is genetically tractable. Whereas RB homologs have been found in mammals (Bernards et al. 1989), frogs (Destree et al. 1992), and chicken (Boehmelt et al. 1994), no homologs have been reported in invertebrates, raising speculation that this

group of proteins may have appeared late in evolution. Here we report the isolation of a cDNA encoding the retinoblastoma family homolog (RBF), a *Drosophila* protein that is widely expressed and combines features of pRB, p107, and p130. We show that RBF physically associates with dE2F/dDP and regulates E2F activity in transient transfection experiments and during development. RBF is a substrate for cyclin E-dependent kinases in vitro, and genetic assays are consistent with the notion that the activity of RBF is negatively regulated by cyclin E. Taken together, these data suggest that RBF is the missing component that mediates induction of E2F activity by G₁ cdks. Thus, both the structure and function of RB-related proteins are conserved between mammals and flies.

Results

Drosophila E2F complexes contain dE2F, dDP, and an unidentified protein

The carboxyl terminus of dE2F shows a limited but significant homology to the pRB-binding domain of human E2F proteins (Helin et al. 1992, 1993a; Kaelin et al. 1992; Shan et al. 1992; Ivey-Hoyle et al. 1993; Lees et al. 1993; Beijersbergen et al. 1994; Dynlacht et al. 1994; Ginsberg et al. 1994; Ohtani and Nevins 1994; Sardet et al. 1995; Fig. 1A). To search for evidence of E2F-associated proteins, electrophoretic mobility shift assay (EMSAs) were carried out with *Drosophila* cell extracts. As shown in Figure 1B, the E2F DNA-binding activity from the untreated extracts (complex A) can be specifically competed by wild-type oligonucleotides but not by mutant sequences. Furthermore, addition of an antibody to either dE2F or dDP completely supershifts or abolishes complex A. Interestingly, when the extract was treated with deoxycholate (DOC), a faster migrating E2F-binding activity was observed (complex B). As shown in Figure 1B, complex B still binds specifically to the E2F site (lanes 8–10) and contains both dE2F and dDP subunits (lanes 11–13), indicating that the native E2F-binding activity contains dE2F, dDP, and an unidentified protein. Mammalian E2F complexes that contain pRB, p107, or p130 are known to be sensitive to DOC treatment and can be readily disrupted to liberate free E2F (Bagchi et al. 1990). The observation that E2F complexes in *Drosophila* are similarly affected by DOC suggested that dE2F/dDP might be regulated by a binding protein analogous to pRB-related proteins.

A two-hybrid screen to identify proteins that interact with dE2F and dDP

Attempts to isolate pRB-related cDNAs from *Drosophila* cDNA libraries by direct screening approaches were unsuccessful. Because the potential regulator of dE2F/dDP was found to be physically associated with E2F, we elected to screen for interacting proteins using a yeast two-hybrid system (Fields and Song 1989). However, the dE2F activation domain, in which the potential pRB-

Figure 1. Indications that a pRB-like protein might exist in *Drosophila*. (A) The carboxy-terminal region of dE2F has sequence homology to the pRB-binding motif of human E2F-1 and to similar sequences in other human E2F genes. Amino acids that are identical between dE2F and the five cloned human E2F genes are highlighted. The 18 amino acids of E2F-1 shown to be both necessary and sufficient for binding to pRB (Hein et al. 1993a) are underlined. (B) DOC treatment converts the major E2F-binding activity in *Drosophila* cell extracts to a faster migrating complex. An E2F EMSA was performed using *Drosophila* SL2 cell extracts (lanes 1–7) or extracts that were treated with DOC (lanes 8–13). Specific E2F-binding activities (complexes A and B) are competed by an excess of unlabeled wild-type competitor DNA (lanes 2,9) but not by a mutant competitor (lanes 3,10). Both the slow migrating E2F complex (A) and the faster migrating complex (B) that are generated by DOC treatment are eliminated or supershifted by anti-dE2F and anti-dDP sera (lanes 5,6,12,13) but not by a normal mouse serum (NMS) control (lanes 4,11).

binding domain is embedded (Fig. 1A), showed strong transcriptional activity in yeast when fused to the Gal4 DNA-binding domain (data not shown), which makes it unsuitable as a bait in a conventional yeast two-hybrid screen.

Therefore, we developed a method that allows transcription activators to be used as bait (Fig. 2). In our modified version of the two-hybrid screen, the full-length dE2F-encoding sequence was fused to the Gal4 activation domain (AD–dE2F) as the bait fusion; a *Droso-*

Figure 2. A modified yeast two-hybrid system used to identify potential dE2F/dDP interactors. (A) Strategy for the two-hybrid screen. The full-length dE2F was fused to the Gal4 activation domain (amino acids 768–881; AD) and coexpressed along with a fusion between full-length dDP and AD (AD–dDP) as bait. A *Drosophila* cDNA library was fused to the Gal4 DNA-binding domain (amino acids 1–147; DB) and transformed into yeast cells that expressed AD–dE2F/AD–dDP and contained the *GAL1::HIS3* reporter gene. Potential interactors were selected by replica-plating of the cDNA library-containing transformants onto plates containing 3AT. As the majority of 3AT^R colonies contained cDNAs that encode an activation domain (Self-activator false positives) rather than a dE2F-interacting protein (Potential interactors), a second screen was used. The 3AT^R colonies obtained were replica plated onto plates containing both 3AT and cyclohexamide (CHX). As the bait plasmid contained the wild-type *CYH2* gene conferring dominant sensitivity to CHX and the host yeast strain was mutated at the corresponding locus (*cyh2R*) [Durfee et al. 1993], CHX resistance selects for colonies that have lost the bait plasmid. Potential interactors were unable to grow in the absence of the bait plasmid (i.e., 3AT–CHX^S), but false positives are 3AT^R even in the absence of the bait (3AT–CHX^R). (B) Diagram representing the different dE2F alleles used to test specificity in C. AD–dE2F deletion alleles were used that either lacked or contained only the carboxy-terminal homology domain. Exact amino acid junctions are indicated. (C) Clone 16 encodes a protein that interacts with the carboxyl terminus of dE2F. (Top row) Patches of the *GAL1::HIS3 GAL1::LacZ SPAL10::URA3* MaV103 yeast strain [Vidal et al. 1996] cotransformed with plasmids expressing the Gal4 DB–clone 16 fusion (DB–Clone16) and three alternative fusions of dE2F to Gal4 AD. (Bottom row) Patches following cotransformation with five different pairs of control plasmids: (lane 1) the DB and AD domains expressed separately; (lane 2) DB–pRB/AD–E2F1; (lane 3) DB–Fos/AD–Jun; (lane 4) full-length Gal4 and AD; (lane 5) DB–dE2F/AD–dDP [Vidal et al. 1996]. Three individual transformants were tested for each allele, one of which is shown here. Patches of cells growing on plates selective for the presence of both plasmids [Sc–L–T–H] were replica plated onto plates lacking histidine and containing the indicated concentration of 3AT [Sc–L–T–H+3AT(10 mM)] or containing X-gal, a substrate for the *lacZ*-encoded enzyme.

phila cDNA library was fused with the Gal4 DNA-binding domain. In addition, as it was shown in mammalian

cells that pRB/E2F-1 interaction is stronger in the presence of DP-1 (Helin et al. 1993b), a single bait plasmid

was constructed to allow coexpression of AD-dE2F and AD-dDP fusion proteins. A two-step screening was used to identify interacting genes (see Fig. 2A for details). In an initial selection based on the expression of a *GAL1::HIS3* reporter gene, ~5000 colonies were selected on 3-aminotriazole (3AT)-containing plates from ~2 million transformants. As has been shown previously for random *Escherichia coli* sequences (Ma and Ptashne 1987), we expected the majority of these colonies to contain cDNAs encoding a transcriptional activation domain. Thus, in a second step, we screened these colonies to identify clones that activated *GAL1::HIS3* only in the presence of the AD-dE2F/AD-dDP-encoding bait plasmid. Convenient screening was achieved by use of a bait plasmid containing the counter-selectable marker *CYH2* (Durfee et al. 1993) as described in Figure 2A. Forty-six positives were found to require the AD-dE2F/AD-dDP bait plasmid for growth on 3AT plates. The corresponding DB-cDNA-encoding plasmids were isolated and retested in fresh yeast cells. Upon further analysis, these positives were found to represent 21 distinct cDNAs.

RBF encodes a protein with homology to pRB, p107, and p130

The positive DB-cDNA fusion proteins were next screened against a panel of dE2F deletion mutants to determine whether any interacted specifically with the carboxy-terminal homology region of dE2F, as would be expected for a pRB-like regulator. One clone (clone 16) was found to interact with full-length dE2F and with a small carboxy-terminal fragment of dE2F but not with a mutant in which the homology region was deleted (Fig. 2B,C). Clone 16 was used to rescreen a *Drosophila* cDNA library (Hafen et al. 1987) to obtain full-length cDNA clones. Sequencing of the longest clone obtained revealed that it contains an open reading frame of 797 amino acids, with an in-frame stop codon upstream of the first ATG codon (data not shown). The encoded protein has significant homology with pRB, p107, and p130 (Fig. 3). This homology extends throughout almost the full length of the open reading frame but is most striking in regions homologous to the pocket domains of pRB, p107, and p130 that are essential for binding to viral oncoproteins, to E2F, and to other partners (Hu et al. 1990; Huang et al. 1990; Kaelin et al. 1990; Ewen et al. 1991; Hannon et al. 1993; Li et al. 1993; Mayol et al. 1993). The *Drosophila* sequence shows slightly higher identity with p107 and p130 than with pRB; however, the organization of the protein resembles pRB more closely than p107 and p130. Most notably, the *Drosophila* protein lacks the spacer domain that is highly conserved between p107 and p130 and mediates their stable association with cdks (Ewen et al. 1992; Faha et al. 1992; Lees et al. 1992; Hannon et al. 1993; Li et al. 1993; Mayol et al. 1993; Zhu et al. 1995). In addition, the B-half of the pocket domain of the *Drosophila* cDNA resembles pRB rather than p130 and p107 where these sequences differ by a long insertion in otherwise highly conserved sequences. Interestingly, the *Drosophila* protein contains a

cluster of potential cdk phosphorylation sites immediately downstream of the pocket domain in a position similar to the sites that are thought to regulate the activity of pRB, p107, and p130 (Ewen et al. 1991; Lees et al. 1991; Lin et al. 1991; Hannon et al. 1993; Li et al. 1993; Mayol et al. 1993). Taken together, the sequence and organization of the *Drosophila* cDNA appears to be intermediate between pRB, p107, and p130 and we have therefore termed it an RB family homolog *RBF*.

RBF is associated with dE2F and dDP in vivo

Based on sequence similarities, it appeared likely that *RBF* might regulate E2F activity in *Drosophila*. To test this notion, anti-RBF antibodies were used to determine whether *RBF* was associated with E2F in vivo. The anti-RBF sera specifically recognized a 95-kD protein in *Drosophila* cell extracts that corresponded in size to the in vitro-translated full-length *RBF* protein (data not shown). As shown in Figure 4A, *RBF* was coimmunoprecipitated from *Drosophila* cell extracts with both dE2F- and dDP-specific antibodies. To determine whether *RBF* was a component of the native E2F/DNA complexes detected in Figure 1B, anti-RBF antibodies were added to an E2F EMSA. As shown in Figure 4B, anti-dDP and anti-dE2F antibodies completely eliminated or supershifted E2F complexes both in native cell extracts and in extracts that were treated with DOC. In contrast, anti-RBF antibodies only eliminated the slow migrating E2F/DNA complex found in native extracts but had no effect on E2F/DNA complexes found in DOC-treated extracts. This effect of anti-RBF antibodies on the E2F DNA-binding activity is not likely attributable to cross-reactivity to other proteins, as the same antibody only recognizes a single band from the dE2F and dDP immunoprecipitation (Fig. 4A). Thus, *RBF* is a stoichiometric component of endogenous E2F DNA-binding activity, which is released by DOC treatment.

RBF represses E2F-dependent transcription and suppresses the phenotype of dE2F/dDP transgenic flies

To assess whether *RBF* association regulates E2F activity, we investigated the effect of *RBF* expression on dE2F/dDP-dependent transcription. Cotransfection of *Drosophila* SL2 cells by dE2F and dDP activated the transcription of an E2F reporter construct by 50-fold. This activation was suppressed completely by the coexpression of *RBF* (Fig. 4C). In contrast, cotransfection of *RBF* had no effect on C/EBP-dependent transcription activation (Fig. 4C), indicating that the repression by *RBF* was specific to dE2F/dDP.

To test whether *RBF* has similar properties in vivo, we investigated whether raising the level of *RBF* could suppress the phenotype of transgenic flies carrying GMRdE2F and GMRdDP transgenes. The GMR promoter allows gene expression to be specifically targeted to the eye. Previously, we found that coexpression of dE2F and dDP in the *Drosophila* retina caused ectopic

Figure 3. RBF encodes a protein that is homologous to pRB, p107, and p130. Shown is sequence alignment of RBF with pRB, p107, and p130. The numbers refer to the RBF amino acid sequence. Amino acids that are identical between RBF and one or more of the human proteins are indicated. The A and B halves of the pocket domain of pRB are underlined. The arrow indicates the 5' end of RBF sequences found in the initial isolate. RBF has 26% amino acid identity with pRB, 35% with p130, and 37% with p107. Unlike p107, and p130, RBF lacks both the conserved spacer sequences that lie between the A and B segments and the insertions in the B segment of the pocket. Like pRB, p107, and p130, RBF contains a cluster of potential cdk phosphorylation sites immediately downstream of the pocket domain (T⁷¹⁵, S⁷²⁸, S⁷⁶⁰, S⁷⁷¹).

S-phases in regions of the eye disc that normally contain only postmitotic cells (Du et al. 1996). Under scanning electron microscopy (SEM), the adult eyes of the flies carrying two copies of GMRdE2F and two copies of GM-RdDP are rough (GMRdE2F²dDP²; Fig. 5C,D) and are characterized by abnormal patterns of photoreceptors and cone cells, and by additional bristles (Du et al. 1996).

Transgenic lines were established carrying GMRRBF, so that RBF could be expressed in the same cells as dE2F and dDP. A chromosome carrying two copies of the transgene (designated GMRRBF²) was used for these experiments, and the eyes of adult flies carrying GMRRBF² were normal (e.g., see Fig. 7A,B, below). When a GMRRBF² chromosome was introduced into the

Figure 4. RBF is associated with dE2F and dDP in vivo and represses the transcriptional activity of dE2F and dDP. (A) Western blots probed with RBF (lanes 1–5), dE2F (lanes 6–10), and dDP (lanes 11–15) antibodies. Immune complexes were prepared from SL2 cell extracts with the antibodies indicated. PAb419, XZ77, and XZ37 are monoclonal antibodies to SV40 large T antigen (Harlow et al. 1981), human pRB, and human pRB/p107 (Hu et al. 1991), respectively. They do not recognize RBF and serve as negative controls. RBF, dE2F, and dDP were each found in anti-dE2F and anti-dDP immunoprecipitations (lanes 3,4,8,9,13,14). (B) RBF is a component of the E2F/DNA complex and can be released from E2F complexes by treatment with DOC. EMSA with E2F site as probe was carried out using native SL2 cell extracts or extracts that were treated with DOC as described in Fig. 1B. The slow-mobility E2F/DNA complex found in native extracts (lanes 1–7) is specifically eliminated or supershifted by anti-RBF, anti-dE2F, and anti-dDP antisera (lanes 5–7). In contrast, the faster-mobility E2F/DNA complex found in DOC-treated extracts (lanes 8–14) was eliminated or supershifted by anti-dE2F and anti-dDP antisera but was unaffected by anti-RBF antibodies (lanes 12–14). An additional nonspecific band was generated by the addition of the anti-RBF, anti-dE2F, and anti-dDP antisera (lanes 5–7 and 12–14). (C) RBF is a specific repressor of E2F-dependent transcription. SL2 cells were transfected with the expression and reporter constructs as indicated. Cotransfection of RBF inhibited transcriptional activation by dE2F/dDP but had no effect on C/EBP activity. Duplicate samples are shown.

Figure 5. RBF suppresses the eye phenotype caused by ectopic expression dE2F and dDP. Shown are SEMs of *Drosophila* compound eyes. The genotypes are as follows: Oregon R (A,B); GMRdE2F²dDP²; +/TM6B (C,D); and GMRdE2F²dDP²; GMRRBF²/TM6B (E,F). Magnifications, 200 $\times$ in A, C, and E; and 1000 $\times$ in B, D, and F. Expression of dE2F and dDP disrupts normal eye development, resulting in disorganized ommatidia and additional bristles. This phenotype is suppressed by coexpression of RBF in the developing eye. The eyes of GMRRBF²/TM6B flies were completely normal (see Fig. 7A,B).

GMRdE2F²dDP² background, the expression of RBF suppressed the rough eye phenotype caused by dDP and dE2F, restoring the normal arrangement of ommatidia and bristles (Fig. 5A,B,E,F). We conclude that expression of RBF not only inhibits the transcriptional activity of dE2F and dDP in transient transfection but that RBF can suppress the biological consequences of dE2F/dDP overexpression during development.

Although a single copy of the GMRRBF² chromosome did not perturb normal eye development, two copies of this chromosome (GMRRBF²/GMRRBF²; designated GMRRBF⁴) resulted in eyes that contained fused ommatidia and that lacked bristles almost entirely (Fig. 6A,B). Immunostaining of GMRRBF⁴ eye imaginal discs indicated that RBF was expressed at a level $\sim$ 10-fold higher than normal. As expected, the GMRRBF⁴ phenotype was suppressed by the coexpression of dE2F and dDP (Fig. 6C,D), indicating the abnormal eye development was a result of RBF expression. Retinal sections of GMRRBF⁴ eyes revealed that most ommatidia contained the normal number of photoreceptors (Fig. 6F) and that occasional fusion of ommatidia occurred in regions where pigment cells were missing. In addition, phalloidin staining of pupal retinas revealed that a significant portion of ommatidia ($\sim$ 10%–20%) contained three cone cells rather than the normal complement of four cells (Fig. 6G,H). The appearance of fused ommatidia and the absence of bristles in GMRRBF⁴ flies are also seen in trans-

genic flies expressing the human p21 gene (de Nooij and Hariharan 1995). In GMRp21^B/+ flies, the expression of human p21 completely inhibits S-phases posterior to the morphogenetic furrow (second mitotic wave) and reduces the number of cells available for formation of the adult eye, resulting in missing pigment cells and bristle cells. The GMRRBF⁴ phenotype, although less severe than the GMRp21 phenotype, may result from a similar reduction in cell proliferation.

Deletion of cyclin E enhances a GMRRBF phenotype

Because the phenotype generated by GMRRBF is dosage sensitive, these lines provide an opportunity to identify genes or pathways that interact with RBF in vivo. We have introduced mutant alleles of genes that encode components of cell cycle machinery into the GMRRBF² background to determine whether halving the dosage of any of these endogenous components could enhance the effects of RBF expression. As shown in Figure 7, A and B, eyes carrying the GMRRBF² chromosome were completely normal. However, introduction of a null allele of *cyclin E* (*Cyc E*-AR95; Knoblich et al. 1994) into this background, resulted in eyes with fused ommatidia and missing bristles, similar to the GMRRBF⁴ flies (Fig. 7C,D). Adults heterozygous for *cyclin E* have normal eyes indicating that this phenotype results from an interaction between GMRRBF² and *Cyc E*-AR95. In contrast, mutant alleles of *cyclin A*, *cyclin B*, *Dmcdc2*, *Dmcdc2c*, and *string* did not enhance the GMRRBF² phenotype (data not shown), indicating that the phenotype was not sensitive to a twofold reduction in gene dosage for these components. As RBF contains several motifs that are consensus sites for phosphorylation by cdks (Fig. 3), the enhancement by *cyclin E* mutation suggests that RBF may be negatively regulated by cyclin E-associated kinases. In embryo extracts cyclin E has been shown to be associated primarily with Dmcdc2c (Sauer et al. 1995). To determine whether RBF serves as a substrate of this kinase, purified RBF protein was added to immune complexes prepared from embryo extracts with cyclin E- or Dmcdc2c-specific antibodies and incubated under the appropriate conditions. RBF was readily phosphorylated by cyclin E- and Dmcdc2c-associated kinase (Fig. 7E). These observations are consistent with the idea that RBF is negatively regulated by cyclin E/cdc2c kinase through phosphorylation.

Discussion

cyclin E and *dE2F* are essential genes that act as positive regulators of the *Drosophila* cell cycle. Recent studies have shown that the epistasis of *cyclin E* and *dE2F* varies in different developmental situations, indicating that the regulation of these functions is plastic and that their integration is critical for control of cell cycle progression (Duronio and O'Farrell 1995). A detailed understanding of these processes will not be possible until the important components of these pathways are identified. In cells where cyclin E is required to activate dE2F-depen-

dent transcription, it has been suggested that cyclin E does not act directly on dE2F, instead it is proposed that cyclin E may phosphorylate a negative regulator of E2F activity (Duronio and O'Farrell 1995). This model is appealing, as mammalian cells contain a family of proteins (pRB, p107, and p130) that regulate E2F activity and interact in multiple ways with cdks. However, despite extensive searches, no homologous genes or activities have been identified in *Drosophila*. Given that many *Drosophila* cells do not contain a G₁ phase, it was unclear whether such a homolog existed and how closely the networks that regulate G₁ to S progression in mammalian cells were likely to be conserved in flies. The isolation and properties of RBF reveal the existence of analogous pathways that connect the activation of cdks in G₁ to specific gene expression.

RBF has all the expected properties of an E2F regulator. RBF is found in stoichiometric amounts in dE2F/dDP complexes and represses the transcriptional activity of dE2F/dDP in transient transfection. Moreover it contains a pocket that is homologous to the E2F-binding domains of pRB, p107, and p130. Perhaps more importantly, RBF was found to antagonize dE2F/dDP activity in vivo. The ectopic expression of dE2F and dDP in the eye drives cell proliferation in postmitotic cells. The resulting eye phenotypes were suppressed by expression of RBF at levels that by themselves gave no discernible effect. Conversely the high level expression of RBF gave a phenotype that was, in turn, suppressed by dE2F and dDP. Because these proteins interact directly and have antagonistic activities in cell culture and in vivo, we conclude that RBF serves to regulate E2F activity in *Drosophila*.

RBF contains seven sites that resemble the consensus sites of phosphorylation of cdks. Four of these sites are clustered immediately downstream of the pocket domain in a position analogous to the clusters of phosphorylation sites found in human pRB. As RBF was phos-

phorylated by cyclin E-associated kinase in vitro, and an RBF-dependent eye phenotype was enhanced by the mutation of one copy of the *cyclin E* gene, it appears likely that the activity of RBF is directly regulated by a cyclin E-associated kinase. Given the properties of RBF, it is highly likely that the activation of E2F-dependent transcription that occurs following ectopic expression of cyclin E is mediated by phosphorylation of RBF and release of associated E2F complexes. Interestingly, cyclin E may not be the only kinase to phosphorylate RBF. Duronio and O'Farrell (1995) have shown that cyclin E appears to act downstream of dE2F during G₁/S phase progression in endocycling cells. In these cells an alternative kinase may be responsible for the activation of E2F-dependent transcription. It is tempting to speculate that in these cells RBF is phosphorylated by a cyclin D-dependent kinase. However, this hypothesis is not testable at present.

E2F-dependent transcription is down-regulated when G₁ control first appears during *Drosophila* development (G₁/S; Duronio et al. 1994). Exit from G₁/S requires dE2F (Duronio et al. 1995) and is accompanied by a transient increase in dE2F-dependent transcription. Because RBF is a negative regulator of E2F activity, it was possible that some of these changes in E2F activity might be attributable to changes in RBF expression. However, in situ hybridization and immunostaining with RBF antibodies revealed that RBF is broadly and uniformly expressed in stage 12 and stage 13 embryos (data not shown). Moreover, Western blots reveal that RBF is abundant in early embryos. Thus, changes in RBF expression do not appear to play an important role in regulating these early cycles. Because dE2F expression is also relatively uniform, it appears that changes in E2F activity are attributable to factors acting upstream of RBF.

Intriguingly, RBF is not a clear homolog of pRB, p107, or p130 but combines features of all three proteins, raising the possibility that RBF may have evolved from a

Figure 6. Characterization of the GMRRBF⁴ eye phenotype. (A,B) SEM of GMRRBF⁴ showing fused ommatidia and missing bristles. (C,D) SEM of GMRRBF⁴; GMRRBF⁴ showing suppression of the GMRRBF⁴ phenotype by coexpression of dE2F and dDP. (E) Section of a wild-type eye. (F) Section of a GMRRBF⁴ eye. The arrow indicates a region in which the absence of pigment cells causes fused ommatidia. (G) Phalloidin staining of wild-type pupal retina. (H) Phalloidin staining of GMRRBF⁴ pupal retina. RBF expression caused the absence of bristles and the appearance of ommatidia with three cone cells (arrow) rather than the normal four cells.

Figure 7. Cyclin E mutation enhances the *GMRRBF*² phenotype in vivo, and RBF is phosphorylated by a cyclin E-associated kinase in vitro. (A,B) SEM of *GMRRBF*²/*TM6B* (C,D) SEM of *cyclin E*^{AR95/+} and *GMRRBF*²/*TM6B*, illustrating reduced bristles and fused ommatidia. (E) in vitro kinase reactions using recombinant RBF as a substrate. Immune complexes were isolated from *Drosophila* embryo extracts using polyclonal antibodies to either cyclin E, Dmcdc2c, or normal rabbit serum. Complexes were incubated with recombinant RBF under standard kinase conditions and separated on 8% SDS-PAGE.

gene that was a common ancestor to the three human genes. This is consistent with the observation that all of the slow mobility E2F–DNA complex detected in *Drosophila* cell extracts was eliminated by RBF antibodies and raises the possibility that RBF may be functionally analogous to the family of mammalian proteins. Curiously, RBF contains sequences homologous to most regions of the pocket domains but lacks any sequences homologous to the spacer regions of p107 and p130. To date, we have been unable to find evidence for a stable RBF/cyclin E complex in cell extracts. The spacer regions of p107 and p130 contain p21 homology domains that bind with high affinity to cdks and can cause cell cycle arrest when overexpressed. However, the biological function of the spacer region is unclear. Among the hypotheses proposed for the spacer regions of p107 and p130 are the ideas that they may enable the associated kinase to be targeted either to substrates that bind into the pocket domain or to nearby DNA bound substrates. Because the spacer region is absent from RBF it appears that such a function is either dispensable or performed by other means.

Identification of a *Drosophila* homolog of RB-family proteins facilitates genetic studies based on the function of this group of proteins. Although a specific mutant of RBF has yet to be identified, the overexpression of RBF in the eye yielded a dosage-sensitive phenotype that has enabled some candidate genes to be tested. As RBF is expected to regulate cell cycle progression, we initially tested mutants in cell cycle genes for genetic interaction. Loss of one copy of *cyclin E* enhanced the *GMRRBF*² phenotype, suggesting that under these conditions, phosphorylation of RBF by cyclin E-dependent kinase is limiting. Enhancement of the *GMRRBF*² phenotype was relatively specific as twofold changes in other cell cycle genes had no effect. Although cyclin E and Dmcdc2c are known to form an active kinase, the failure of *Dmcdc2c*

mutation to modify the *GMRRBF* phenotype was not surprising as cyclins are generally considered to be the limiting subunit for most cdks. Interestingly, previous studies have shown that a twofold reduction in the gene dosage of *cyclin A* or *string* strongly suppresses the eye phenotype of *roughex* mutations (Thomas et al. 1994). This suggests that *roughex* and RBF, while both being negative regulators of the cell cycle, interact with the cell cycle machinery in different ways. These results indicate that more extensive screens for modifiers of RBF activity will be possible and may identify additional components of this key regulatory pathway.

Materials and methods

E2F EMSAs

EMSA and DOC treatment of cell extracts were carried out essentially as described in Cao et al. (1992) and Wu et al. (1995). A 100-fold molar excess of unlabeled oligonucleotide was used for the competition reactions. One microliter of mouse polyclonal sera was added where indicated. Anti-dE2F and anti-dDP sera were raised against the full-length histidine-tagged proteins (Harlow and Lane 1988).

Two-hybrid screening

A cDNA library was synthesized from *Drosophila* SL2 cell poly(A)⁺ RNA, as described in Sambrook et al. (1989), and was cloned into the pPC86DB vector. To construct the dE2F and dDP coexpression bait plasmid, dE2F and dDP were cloned into the pPC97AD vector, and the AD–dE2F expression cassette was isolated and inserted into the pPC97AD–dDP plasmid. The expression of both dE2F and dDP in yeast MaV103 (Vidal et al. 1996) was confirmed by mating with yeast MaV106 carrying the pPC86DB–dDP or the pPC86DB–dE2F plasmid, respectively. For the library screen, yeast MaV103 carrying the pPC97AD–dE2F/AD–dDP plasmid was transformed with the pPC86DB–cDNA library, transformants were replica plated

onto plates lacking histidine and with 3AT [Sc-L-T-H+3AT (10 mM)], resistant colonies were re-replica plated onto plates with Sc-T-H+3AT (10 mM)/CHX and potential interactors were picked from the initial 3AT-selective plates. pPC86DB-cDNA plasmids were recovered and reintroduced into yeast MaV103 cells with one of the following four plasmids: pPC97AD, pPC97AD-dE2F, pPC97AD-dDP, or pPC97AD-dE2F/AD-dDP. Ten cDNA clones were found to be able to interact with dE2F alone (class 1), whereas 11 cDNAs required both dE2F and dDP for interaction (class 2). To test the binding specificity, clones from class 1 were cotransformed with different mutants of dE2F into MaV103 cells in the absence of AD-dDP.

The GenBank accession number for the RBF cDNA is X96975. The position of the *RBF* gene was localized by in situ hybridization to polytene chromosomes and mapped to 1C-D.

Antibody preparation, immunoprecipitation, Western blot analysis, and kinase assay

Immune complexes were collected from SL2 cell extracts on antibodies covalently coupled to protein A-Sepharose beads. Proteins were separated on an 8% SDS-polyacrylamide gel, transferred to nitrocellulose, and probed as described previously (Harlow and Lane 1988). Immunoprecipitation was carried out using monoclonal antibodies to dE2F (Hao4) and dDP (Yun3). Western blots were probed with mouse polyclonal antisera raised against dE2F, dDP, and RBF. Anti-RBF antibodies were raised against the histidine-tagged protein. For kinase assays, immune complexes of anti-cyclin E or anti-Dmcdc2c were collected from *Oregon R* embryo extracts and were incubated with 2 μ g of purified recombinant RBF in the presence of 50 mM HEPES (7.0), 10 mM MgCl₂, 5 mM MnCl₂, 1 mM DTT, and 5 μ Ci of [γ -³²P]ATP for 30 min at 30°C.

Transfections and CAT assays

Transfections of SL-2 cells and CAT assays were carried out as described previously (Dynlacht et al. 1994). The E2F expression and reporter constructs were described in Dynlacht et al. (1994). CCAAT/enhancer-binding protein (C/EBP) constructs are from J. Olesen and T. Maniatis (Harvard University, Cambridge, MA). The RBF expression vector contained the full-length cDNA cloned into the pBSAct expression vector (gift from J. Hagler, Harvard University, and T. Maniatis).

Fly stocks

Five independent transgenic lines P[w⁺; GMR] were generated for GMRRBF. Transgenic lines were generated following P-element-mediated germ-line transformation with a pGMR (Hay et al. 1994) construct containing the full-length RBF open reading frame. The pGMR construct was coinjected with the pU-ChsΔ2-3 helper plasmid into w¹¹¹⁸ embryos. Injection and transformation were carried out as described in Spradling and Rubin (1982) and Rubin and Spradling (1982). Four of the single-insertion GMR lines on the third chromosome were recombined pair-wise; similar phenotypes were observed for all four recombinant lines derived. The phenotype of one of the recombinant lines (GMRRBF⁴) on chromosome III was shown.

All fly crosses were performed at 25°C. Stocks used were GMRRdE2F²dDP² (Du et al. 1996) *Df(2R)59(AB)* (Knoblich and Lehner 1993), *cdc2^{E10}* (Stern et al. 1993), *Df(2R)H81* (Stern et al. 1993), *CycA⁵* [synonym *l(3)neo114*; Lehner and O'Farrell 1989], and *l(2)35Dd^{AR95}* (Knoblich et al. 1994).

Eye analysis

Samples for SEM were prepared as described in Kimmel et al. (1990). Sectioning of fly heads was carried out as described by Tomlinson and Ready (1987), with the modifications described by Carthew and Rubin (1990) and Hariharan et al. (1995). Retinas from 40-hr-old pupae were stained with TRITC-conjugated phalloidin and visualized by confocal microscopy.

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