roughex down-regulates G₂ cyclins in G₁

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Cell cycle arrest in G₁ at the onset of patterning in the *Drosophila* eye is mediated by *roughex*. In *roughex* mutants, cells accumulate Cyclin A protein in early G₁ and progress into S phase precociously. When *Roughex* is overexpressed in S/G₂ cells, Cyclin A is mislocalized to the nucleus and degraded, preventing mitosis. Whereas *Roughex* inhibits Cyclin A accumulation, Cyclin E down-regulates *Roughex* protein in vivo. *Roughex* binds to Cyclin E and is a substrate for a Cyclin E-Cdk complex in vitro. These data argue that *Roughex* inhibits Cyclin A accumulation in early G₁ by targeting Cyclin A for destruction. In late G₁, *Roughex* is destabilized in a Cyclin E-dependent process, releasing Cyclin A for its role in S/G₂.

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Most somatic cell cycles consist of four stages—S phase, where DNA synthesis occurs; M phase (mitosis), where chromosomes segregate and cell division takes place; and two intervening gap phases, G₁ and G₂. Progression through the various cell cycle stages occurs largely through the activity of different isoforms of cyclin-dependent kinases (Cdks) and their associated positive regulatory subunits, the cyclins (Cycs) (Norbury and Nurse 1992; Morgan 1995). In addition to positive controls that ensure the specific activation of Cyc-Cdk complexes during each stage of the cell cycle, regulatory mechanisms prevent their activity in inappropriate stages. Cyclin expression is regulated in part at the level of transcription, with mRNA levels of most cyclins peaking at specific times during the cell cycle (Sherr 1994). In addition to transcriptional controls, Cycs are regulated at the post-translational level. This is illustrated most dramatically by the massive destruction of G₂ cyclins by ubiquitin-dependent proteolysis as cells progress through mitosis (King et al. 1994). In budding yeast, proteolysis of G₂ cyclins persists in G₁, preventing the accumulation of G₂ cyclins (Amon et al. 1994), whereas in both fission and budding yeasts the activity of G₂ Cyc-Cdk complexes is prevented in G₁ by cyclin kinase inhibitors (Ckis) (Schwob et al. 1994; Correa-Bordes and Nurse 1995). In this paper we consider the regulation of the *Drosophila* G₂ cyclin, CycA, in G₁.

Several lines of evidence indicate that CycE-Cdk complexes have a key role in regulating the G₁/S transition

in higher eukaryotic cells. CycE-Cdk2 activity increases in late G₁. In *Drosophila* and mammalian cells, overexpression of CycE shortens G₁ and advances entry into S phase (Ohtsubo and Roberts 1993; Knoblich et al. 1994; Resnitzky et al. 1994; Richardson et al. 1995). In *Drosophila*, *cycE* mutants arrest cell cycle progression in G₁, and down-regulation of CycE is required for G₁ arrest (Knoblich et al. 1994; Richardson et al. 1995). CycA-Cdk2 also may have a role in S-phase entry in mammalian cells. CycA-Cdk2 activity appears toward the end of G₁, peaks during S phase, and can activate DNA replication in extracts from G₁ cells (D'Urso et al. 1990). In cultured human cells, inactivation of CycA blocks entry into and progression through S phase (Girard et al. 1991; Pagano et al. 1992), although its ectopic expression can drive precocious entry into S phase (Resnitzky et al. 1995). However, in *Drosophila*, a CycA requirement for S-phase entry has not been demonstrated.

CycA also is required for mitosis. Like CycB, the canonical G₂ cyclin, CycA mRNA or protein added to *Xenopus* oocytes or egg extracts can activate the G₂ Cdk, Cdc2, inducing events such as nuclear envelope breakdown and chromatin condensation (Swenson et al. 1986). In *Drosophila*, mutations in *cycA* arrest embryonic cell cycle progression in G₂ (Lehner and O'Farrell 1989). In late G₂, mitosis is triggered by the activation of a CycB-Cdc2 complex by Cdc25 phosphatase, encoded by the *string* (*stg*) gene in *Drosophila* (Russell and Nurse 1986; Edgar and O'Farrell 1989; Kumagai and Dunphy 1991; Gabrielli et al. 1992).

The *Drosophila* compound eye is well suited to the study of cell cycle regulation in higher eukaryotes. The eye develops from a columnar epithelium called the eye

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imaginal disc. During the third and final stage of larval development, a wave of differentiation sweeps across the eye disc anteriorly. The front of this wave is marked by a depression in the disc epithelium, the morphogenetic furrow (MF). Ahead of the MF, cells are unpatterned and undifferentiated, and progress through the cell cycle asynchronously. All cells become synchronized in G_1 beginning just anterior to the MF (Thomas et al. 1994), such that cells at the anterior edge of the MF are in early G_1 . As cells proceed through G_1 , their positions shift posteriorly relative to the MF. Cells emerging from the posterior edge of the MF either become postmitotic and differentiate into neurons, or up-regulate CycE before entering a final synchronous S phase (Richardson et al. 1995). As cells transit S and G_2 , CycA and CycB proteins accumulate. Hence, progression through the cell cycle can be visualized as a continuum from the anterior edge of the MF extending posteriorly (for summary diagram, see Fig. 2A, below). Specific mutations disrupting G_1 progression in the MF can be used as a genetic background in which to search for additional genes regulating cell cycle progression (Thomas et al. 1994; Dong et al. 1997).

Cells become synchronized in G_1 through the activa-

tion of the Cdc25 phosphatase, encoded by *stg*, and the product of the *roughex* (*rux*) locus, which encodes a novel protein of unknown biochemical function (Thomas et al. 1994). A burst of Stg expression occurs in a band of cells just anterior to the MF (the "synchronization domain") under the control of the patterning gene *hedgehog* (Alphrey et al. 1992; Heberlein et al. 1995). Stg drives G_2 cells through mitosis and into G_1 . G_1 cells are prevented from entering S phase by the product of the *rux* locus. In *rux* mutants, cells enter S phase precociously in early G_1 . In this paper, we show that Rux inhibits entry into S phase by preventing accumulation of CycA protein during G_1 . We show further that CycE promotes down-regulation of Rux, allowing accumulation of CycA for its function in S and G_2 .

Results

Rux binds to CycE

Because *rux* inhibits and CycE promotes S phase in the MF, we reasoned that *rux* may inhibit CycE-Cdk activity. This hypothesis was supported initially by the observation that Rux interacts physically with CycE (Fig.

Figure 1. Interaction of Rux with CycE. (A) Specific interaction between Rux and CycE in yeast two-hybrid interaction mating. X-gal indicator plates containing mating exconjugates from yeast strains expressing LexA fused to human Cdk6 (lane 1), the amino terminus of Bicoid (lane 2), the *Drosophila* CIP-KIP family Cdk inhibitor Dacapo (Cdi4) (Finley and Brent 1994; de Nooij et al. 1996; Lane et al. 1996) (lane 3), or Rux (lane 4), mated to strains expressing an activation domain fused to *Drosophila* Cycs or Cdks (as indicated), or to no protein (vector). Interactions between fusion proteins resulted in a dark-staining colony. CycE box refers to a fragment containing residues 193–517 of *Drosophila* CycE (Richardson et al. 1993). (B) GST-Rux binds *Drosophila* CycE in vitro. An aliquot (5 μ l) of in vitro-translated [35 S]methionine-labeled CycA (A, lanes 1,4), CycE (E, lanes 2,5) or Stg (S, lanes 3,6) was incubated with glutathione-agarose beads containing bound GST (lanes 1–3) or GST-Rux fusion protein (lanes 4–6). Bound proteins were fractionated by SDS-PAGE and visualized by autoradiography. An aliquot (1.5 μ l) of in vitro translation product is shown in lanes 7–9. (C) Rux does not inhibit *Drosophila* CycE-Cdc2c or CycA-Cdc2 histone H1 kinase activities and is itself a substrate in vitro. Kinase assays were performed as described in Materials and Methods. Extracts from stage 11 *Drosophila* embryos were prepared for immunoprecipitation as described (Knoblich et al. 1994). Protein A-Sepharose beads coated with affinity-purified polyclonal rabbit antibodies against *Drosophila* CycE (2 μ g, lanes 2,3), *Drosophila* CycA (1 μ g, lanes 4,5), or nonimmune serum (lane 1) were incubated with extracts (200 μ g of protein). Washed beads were assayed for kinase activity. Reactions contained 2 μ g of histone H1 and no added GST-Rux protein (lanes 1,2,4) or 1.2 μ g of partially purified GST-Rux fusion protein (lanes 3,5). For comparison, the addition of only 5 ng of Dacapo to similar assays completely inhibits the histone H1 kinase activity of CycE-Cdc2c (de Nooij et al. 1996; Lane et al. 1996). The doublet seen in lane 5 corresponds to a proteolytic fragment of GST-Rux generated during the kinase assay.

1A). Binding of Rux to CycE was detected in a variation of a yeast two-hybrid screen in which a strain carrying CycE fused to an activation domain was mated to a panel of strains harboring various cell cycle and signaling proteins, including Rux, fused to LexA. Subsequently, Rux–LexA was tested for interactions with other Cycs and Cdk2s and was shown to interact specifically with *Drosophila* and human CycE and a fragment of CycE containing the “cyclin box” (Draetta 1990). It did not interact with *Drosophila* Cycs C, D, and J, or *Drosophila* Cdk2s Cdc2 or Cdc2c (Fig. 1A). We were unable to test whether Rux interacted with CycA in the two-hybrid system because CycA expression is toxic to yeast (R.L. Finley and R. Brent, unpubl.). The fact that Rux binds directly to CycE but not to CycA was demonstrated in vitro (Fig. 1B). ^{35}S -Labeled *Drosophila* CycE or CycA were incubated with agarose beads linked to GST–Rux or GST alone, and bound proteins were analyzed by SDS-PAGE. GST–Rux also bound human CycE in extracts prepared from Rat-1 cells expressing human CycE (data not shown).

We next asked whether the addition of Rux protein inhibited the kinase activity of *Drosophila* or human CycE–Cdk2 complexes. CycE–Cdc2c or CycA–Cdc2 immunoprecipitated from stage-11 embryo extracts were not significantly inhibited by GST–Rux protein (Fig. 1C), however GST–Rux served as an efficient substrate for phosphorylation by both Cyc–Cdk complexes. Similarly, GST–Rux or histidine-tagged Rux had little effect on histone H1 phosphorylation by purified recombinant human CycE–Cdk2 (or CycA–Cdk2) complexes (data not shown). Therefore, Rux binds CycE but does not inhibit CycE–Cdk activity directly.

Rux inhibits CycA- but not CycE-dependent S phases

To test whether *rux* acts as a negative regulator of *cycE* in vivo, we first assessed whether the *rux* phenotype (i.e., ectopic S-phase entry in the MF; Fig. 2B,C) could be dominantly suppressed by mutations in *cycE*. Neither a *cycE* deficiency nor a strong point mutation dominantly suppressed the *rux* S-phase phenotype. In addition, the *rux* phenotype was unaffected in larvae homozygous for a weak loss-of-function *cycE* allele (H. Richardson, pers. comm.; data not shown). Finally, ectopic S phases driven by Hs–*cycE* expression in the eye disc (Knoblich et al. 1994; Richardson et al. 1995) were not suppressed by co-expression of Hs–*rux* (data not shown) under conditions in which Hs–*rux* expression inhibited ectopic S phases induced by Hs–*cycA* (see below).

We next assessed the effects of Rux on CycE-induced S phases in the developing embryo. CycE has been shown to be necessary and sufficient to drive cells into S phase in embryonic somatic cell cycles (Knoblich et al. 1994; Duronio and O’Farrell 1995). Rux was overexpressed in alternating stripes in Hs–*cycE* embryos using *prd*–GAL4 and UAS–*rux* transgenes (Brand and Perrimon 1993). CycE expression was induced and entry into S phase was monitored by 5-bromo-2-deoxyuridine (BrdU) labeling. The fraction of BrdU-positive cells in the Rux-expressing

and nonexpressing stripes was identical, indicating that, as in the eye disc, Rux does not inhibit entry into S phase triggered by ectopic CycE (Fig. 2F). Finally, high levels of Rux did not inhibit the normal endoreduplication in salivary gland cells (data not shown). Endoreduplication is known to be CycE-dependent (Knoblich et al. 1994) and is inhibited readily by ectopic expression of Dacapo, a *Drosophila* CIP/KIP family Cdk inhibitor (de Nooij et al. 1996; Lane et al. 1996; C.F. Lehner, unpubl.). Therefore, *rux* does not appear to inhibit CycE-dependent processes in vivo. The physiological significance of the interaction between CycE and Rux is considered in the last section of the results (see Fig. 6, below).

Genetic studies revealed that *rux* inhibits CycA-dependent processes. In screens for dominant suppressors of the *rux* rough-eye phenotype, we identified three regulators of Cdk activity—*cycA*, *stg*, and *regulator of cyclin A-1* (*rca1*) (Thomas et al. 1994; Dong et al. 1997). Because *Drosophila* Stg activates mammalian CycA–Cdk complexes in vitro (Gu et al. 1992) and *rca1* encodes an upstream positive regulator of CycA (Dong et al. 1997), we considered whether *rux* suppresses entry into S phase by preventing ectopic activation (either directly or indirectly) of a CycA–Cdk complex in the G_1 domain within the MF. Consistent with this interpretation, overexpression of CycA from a heat-inducible transgene (Hs–*cycA*) mimicked the *rux* mutant phenotype (Fig. 2B), showing extensive induction of S-phase cells just anterior to and within the MF (Fig. 2D). In addition, as in *rux*, a broad domain of BrdU-labeled cells was observed extending throughout the normally quiescent region of the disc posterior to the MF (data not shown). Coexpression of Hs–*rux* resulted in suppression of the ectopic S phases induced by Hs–*cycA* in all discs assayed ($n = 10$; Fig. 2E). Interestingly, the wild-type pattern of asynchronous S phases anterior and synchronous S phases posterior to the MF were not inhibited by overexpression of Rux. Therefore, ectopic CycA expression can drive G_1 cells into S phase, and coexpression of Rux inhibits this phenotype. We conclude that *rux* acts as a negative regulator of CycA- but not CycE-dependent S phases.

Ectopic Rux inhibits mitosis

Because purified GST–Rux neither binds to CycA nor inhibits CycA–Cdk activity (Fig. 1), a series of experiments were pursued to evaluate the relationship between CycA and Rux in vivo. We tested the effect of misexpressing Rux under the control of the eye-specific GMR enhancer (GMR–*rux*), which drives expression in all cells beginning in late G_1 in the MF and continuing to the posterior edge of the disc (Ellis et al. 1993; Hay et al. 1994; Fig. 6D, below). S phases posterior to the MF were not inhibited by GMR–*rux* (Fig. 3A). However, a marked decrease in the number of dividing cells was observed; some 21 (± 9) mitotic figures were detected in wild-type discs in the region behind the MF compared with 1 (± 1) in GMR–*rux* ($n = 8$ for both wild-type and GMR–*rux*). Similarly, in embryos expressing UAS–*rux* under the control of *prd*–GAL4, cell division in the Rux-expressing

Figure 2. CycA-induced S phases are inhibited by Rux. (A) A schematic representation of cell division in the eye imaginal disc. During eye development the morphogenetic furrow (MF, arrow) moves anteriorly across the eye disc. Anterior to the MF, cell division proceeds asynchronously, depicted by scattered S-phase cells (brown circles), mitotic figures and broad expression of CycA, CycB, and CycE (off-white). Just anterior to the MF, *stg* mRNA (blue) is expressed in a band defining the synchronization domain. Approximately 1–2 cell diameters posterior to the onset of *stg* expression, *cycD* mRNA also is expressed in a stripe (stippled region), defining early G₁ (Finley et al. 1996). Within the MF, groups of cells segregate into preclusters, the precursors of photoreceptor cells. In late G₁, *cycE* mRNA is expressed in cells that subsequently enter a synchronous S phase. CycA and CycB expression occurs in S/G₂ cells posterior to the MF. (B–E) BrdU labeling and subsequent immunohistochemical detection of S-phase nuclei in the developing eye disc. Anterior is to the right. In wild type (C), all cells in the MF (arrow, B–E) are in G₁. In *rux*⁸ (B) and in heat shock-induced Hs-*cycA* eye discs (D), most if not all cells in the MF are in S phase. (E) Hs-*rux* inhibits Hs-*cycA*-induced S phases. Hs-*rux* does not inhibit the normal pattern of S phases anterior or posterior to the MF. (F,G) Rux does not inhibit CycE-induced S phases in the embryo. Embryos in which CycE is expressed ubiquitously under heat shock control express Rux in alternating segments. These were labeled with BrdU (F) or stained with anti-tubulin antibodies (G). Rux-expressing segments (left of the broken line) flanking Rux-nonexpressing segments (right of the broken line) are shown. Whereas Rux expression does not reduce the proportion of cells induced into S phase by CycE in each segment, it inhibits M phase (data not shown) resulting in a lower cell density and larger nuclei in the Rux-expressing stripes. Scale bar, 25 μ m (B–E) and 7.5 μ m (F,G).

stripes arrested during G₂ of cycle 15 in some epidermal cells and during G₂ of cycle 16 in the remaining cells, resulting in a lower cell density (Fig. 2G; data not shown). Using an Hs-*rux* transgene, an average of 149 (± 53) mitotic figures was observed in heat-pulsed wild-type embryos, whereas no mitotic figures were seen in the Hs-*rux* embryos after a brief heat pulse in G₂ of cycle 16. Therefore, ectopic Rux blocks mitosis.

Several lines of evidence are consistent with Rux not only inhibiting mitosis but also leading to alterations in S-phase regulation subsequent to the mitotic block. In embryos ubiquitously expressing UAS-*rux* under the control of an *armadillo*-GAL4 transgene (a gift of J.P. Vincent, Medical Research Council, Cambridge, UK), a fraction of cells entered into another S phase without a preceding mitosis (i.e., endoreduplication; data not shown). Rux overexpression also induces endoreduplication cycles in the wing disc. UAS-*rux* was expressed under the control of the enhancer trap line 30A (Brand and Perrimon 1993), which results in expression of Rux in a proximal ring in wing imaginal discs during a period of continuous proliferative growth. DNA labeling revealed a severe reduction in the number of cells in the expression domain, reflecting inhibition of mitosis, and a marked increase in nuclear size, consistent with endoreduplication (Fig. 3D,E). In the eye, an increase in BrdU incorporation and larger nuclei were consistently observed after heat-induced expression of Hs-*rux* (Fig. 3B,C). Whether this reflects increased rates of DNA synthesis, multiple initiations, or activation of additional

origins is not known. Conversion of mitotic cycles into endoreduplication cycles has been described previously in *cycA*, *cdc2*, and *rca1* mutants (Sauer et al. 1995; Hayaishi 1996; Dong et al. 1997; K. Weigmann and C.F. Lehner, in prep.). We propose that ectopic Rux converts the mitotic cycle into an endoreduplication cycle by down-regulating CycA-associated kinase activity.

Rux controls CycA accumulation

Striking defects in the level and subcellular distribution of CycA were observed in cells ectopically expressing high levels of Rux (Fig. 4A,B,E–G; and Fig. 6, below). In the eye, dividing cells lose their connection to the basal lamina and mitotic nuclei are found at the extreme apical surface of the disc epithelium, immediately below the peripodial membrane (Tomlinson 1985). In wild type, CycA accumulates in the cytoplasm of cells with basally located nuclei. As nuclei rise apically on entry into mitosis, CycA localizes transiently to the nucleus and then rapidly disappears (Lehner and O'Farrell 1989; Whitfield et al. 1990). In apical focal planes, this expression pattern is revealed as a reticular network of thin cytoplasmic processes surrounding the unstained differentiating nuclei (Fig. 4A). In GMR-*rux*, there was a marked decrease in the overall number of CycA-expressing cells, and no cytoplasmic CycA staining was detected in apical focal planes (data not shown). Instead, CycA accumulated transiently in basally located nuclei of cells behind the MF (Figs. 4B and 6E, below). More-

Figure 3. Ectopic Rux expression does not inhibit normal S phases and promotes endoreduplication. BrdU labeling of eye (A–C) and wing (D,E) imaginal discs. (A–C) Anterior is to the right. (A) A GMR-*rux* eye disc. Rux expression commences at the posterior edge of the MF (arrow) and extends posteriorly (see Fig. 5). S phase is not inhibited by ectopic Rux. (C) After heat shock treatment, S-phase nuclei (arrowhead) posterior to the MF in Hs-*rux* are more strongly labeled and appear larger than non-heat-shocked controls (B). (D) The 30A-GAL4 enhancer trap line drives UAS-*rux* expression in a portion of the proximal ring of the wing imaginal disc. Fewer cells with much larger nuclei are seen in this region (left of broken line). (E) A corresponding region from a wild-type wing disc shows nuclei of uniform size. The marked increase in nuclear size in D is consistent with conversion of mitotic to endoreduplication cell cycles. Scale bar, 10 μ m (A,D,E) and 5 μ m (B,C).

over, these did not show features of cells that are entering mitosis (e.g., condensed mitotic chromosomes; B.J. Thomas, unpubl.). In strains expressing high levels of Rux (attributable to position effects related to the transposon insertion site), CycB-positive cells were never seen (Fig. 6F, below). However, when Rux was expressed at lower levels, CycB also was localized to the nucleus (data not shown). Conversely, in *rux* mutants CycA protein levels were increased beginning in the region just anterior to the MF and extending posteriorly, corresponding to cells in early G_1 (Fig. 4C,D).

Similar results were obtained in embryos expressing UAS-*rux* with *prd*-GAL4. Immunolabeling revealed that CycA (Fig. 4E–G), CycB and CycB3 (data not shown) proteins were shifted to a predominantly nuclear localization and eventually became undetectable in Rux-expressing cells. F. Sprenger and P. O’Farrell (pers. comm.) also have shown that, in embryos, a CycA mutant protein lacking the destruction box motif accumulated to high levels in the nucleus in response to ectopic Rux expression. In conclusion, we propose that Rux controls CycA levels by promoting its nuclear localization and thereby its rapid degradation.

Rux is expressed anterior to the MF and is down-regulated in cells that re-enter S phase

The spatiotemporal pattern of Rux expression is consis-

tent with its role in negatively regulating CycA levels in early G_1 . Because Rux is expressed at very low levels, it has not been possible to detect endogenous expression of the gene during development (B.J. Thomas and S.L. Zupursky, unpubl.). As a step toward assessing the normal pattern of Rux expression, localization of a Rux-*lacZ* protein fusion was determined. Genomic DNA containing the *rux* open reading frame and 910 bp of upstream sequences were fused in-frame to the *Escherichia coli lacZ* gene and introduced into flies by P-element-mediated transformation. This transgene rescued the *rux* mutant phenotype (data not shown), indicating that it is expressed in at least those cells that require Rux normally. Expression of Rux-*lacZ* was monitored using a monoclonal antibody to LacZ (Fig. 5A).

High levels of expression were observed in the nuclei of cells in a band just anterior to the MF. Lower and variable levels were seen within the MF, whereas behind the MF expression increased. Therefore, Rux is ubiquitously expressed in early G_1 cells just anterior to the MF. In *rux* mutants, it is these cells that accumulate CycA and enter S phase precociously. Rux-*lacZ* protein is down-regulated in late G_1 cells within the MF, whereas behind the MF Rux-*lacZ* expression increases in many

Figure 4. Rux regulates the subcellular distribution and level of CycA protein. (A–D) Eye disc and (E–G) embryo. (A) Apical focal plane. A wild-type eye disc stained with anti-CycA antibody. The stained cytoplasm of cells between developing photoreceptor cell clusters forms a reticular pattern in apical focal planes. (B) Basal focal plane. The GMR enhancer drives Rux expression posterior to the MF. This leads to a marked decrease in CycA levels and localization of CycA to the nuclei of basally localized cells. No CycA staining is seen in apical focal planes. (D) In *rux*⁸, increased CycA protein is seen in the MF, and overall CycA levels are higher than in wild type (C). (E) In the embryo, Rux overexpression (using *prd*-GAL4 driving a UAS-*rux* transgene) led to mislocalization of CycA to the nucleus. (F) CycA is eventually lost from the Rux-expressing cells (left of the broken line). CycA appears in the cytoplasm of cells that do not ectopically express Rux (right of the broken line). (G) Hoechst 33258 staining of DNA of the same region as D. (A–D) Anterior is to the right. Arrow indicates MF. Scale bar, 25 μ m (A–D), 2.5 μ m (E), and 5 μ m (F,G).

Figure 5. Rux is expressed anterior to the MF and down-regulated in cycling cells behind the MF. (A) Rux expression assessed using a genomic rescue transgene tagged with *lacZ* (*Rux-lacZ*, see text) and an antibody to LacZ. *Rux-lacZ* expression is low in the nuclei of many cells in the region of asynchronous divisions in the most anterior region of the disc. Rux protein is up-regulated in a stripe corresponding to the region of G_1 synchronization anterior to the MF (early G_1). Rux protein is down-regulated in the MF (late G_1) and reaccumulates in most cells posterior to the MF. (Inset) Rux expression in a *Hs-rux* disc anterior to the MF shows nuclear staining of the Rux antigen. (B) The GMR enhancer drives Rux expression commencing at the posterior edge of the MF and continuing to the posterior edge of the disc, as detected with an anti-Rux monoclonal antibody. Rux is initially seen in the nuclei of most cells, but is down-regulated in cycling cells (basally located nuclei) between clusters of differentiating neurons (apically located nuclei, arrowhead). An apical focal plane is shown. (C) A *GMR-lacZ* control shows that LacZ remains expressed in both cycling and differentiating cells. A basal focal plane is shown. Arrow indicates position of MF (A) and posterior edge of the MF (B,C). Anterior is to the right. Scale bar, 20 μ m (A, inset) and 10 μ m (B,C).

cells and remains detectable in them to the posterior edge of the disc. However, studies with the native Rux protein indicate that the persistence of *Rux-lacZ* in cycling cells is a consequence of the increased stability of the LacZ moiety relative to Rux itself (see below).

Although Rux cannot be detected in tissue when expressed under its own promoter, it can be detected using an anti-Rux monoclonal antibody in discs in which it is ectopically expressed using the *GMR-rux* and *Hs-rux* transgenes. In *GMR-rux*, Rux protein is observed initially in the nuclei of most cells at the posterior edge of the MF. Just behind the MF, Rux protein remains at high levels within the nuclei of differentiating photoreceptor cells but is rapidly down-regulated in the nuclei of S/ G_2 cells. As a control, *GMR-lacZ* expression persists to variable levels in all cells behind the MF (Fig. 5B,C) (Ellis et al. 1993; Hay et al. 1994). A similar pattern is observed after heat induction of *Hs-rux*. Rux is initially expressed

in all cells anterior and posterior to the MF. By 6 hr after heat induction, Rux protein becomes localized to a stripe of cells anterior to the MF and in differentiating neurons behind the MF. During this time, Rux disappears from cycling cells both anterior and posterior to and late G_1 cells within the MF (data not shown). In summary, studies with the *Rux-lacZ* fusion indicate that the onset of Rux expression occurs in early G_1 . Using *GMR-rux*, Rux protein was shown to be down-regulated in S/ G_2 cells. Therefore, high levels of Rux correlated with low levels of CycA and conversely, low levels of Rux correlated with high levels of CycA.

CycE inhibits Rux accumulation

Rux rapidly disappears from cycling cells at the posterior edge of the MF where the level of CycE increases. That CycE directly binds to Rux and that Rux is phosphorylated by CycE-Cdk2 in vitro, raised the intriguing possibility that CycE-Cdk activity inhibits accumulation of Rux in these cells. To assess this possibility, the *Hs-cycE* construct was introduced into a *GMR-rux* background and the levels of Rux, CycA, and CycB proteins were monitored after a heat pulse (Fig. 6). Overexpression of CycE, (Fig. 6G) but not of CycA (i.e., via *Hs-cycA*; data not shown), inhibits Rux protein accumulation. A marked increase in both CycA and CycB staining also was observed (Fig. 6H,I). Reversion of the Rux inhibition was not complete; much of the CycA remained in the nucleus and mitosis was only partially restored (data not shown). These results suggest that CycE targets Rux for destruction in cells that re-enter S phase behind the MF.

Figure 6. CycE inhibits Rux. Wild-type discs were stained for Rux (A,D,G), CycA (B,E,H), and CycB (C,F,I) proteins. (A) The wild-type pattern of Rux is undetectable. (D) The GMR enhancer drives expression of Rux commencing at the posterior edge of the MF and extending posteriorly. *GMR-rux* inhibits the accumulation of CycA (E) and CycB (F). Heat-shock-induced expression of CycE (*Hs-cycE*) in a *GMR-rux* background inhibits accumulation of Rux (G) and restores CycA (H) and CycB (I) staining. Posterior edge of MF is indicated with arrow. Anterior is to the right. Scale bar, 30 μ m.

Therefore, during normal eye development, accumulation of CycE in late G_1 cells may down-regulate Rux protein. This, in turn, facilitates the accumulation of CycA, which is required in subsequent cell cycle stages.

Discussion

In both unicellular and multicellular organisms, the coordination of cell cycle progression and development is critical. Analysis of the relationship between these processes provides insights into mechanisms by which the developmental program controls the cell cycle, as well as the ways in which the cell cycle impinges on development. A critical step in *Drosophila* eye development is the synchronization of cells in G_1 within the MF; failure to maintain G_1 leads to defects in pattern formation (Thomas et al. 1994). In *rux* mutants, cells enter S phase precociously just anterior to the MF. As we show here, in addition to Stg, Rux protein is markedly up-regulated within this region. It is likely that it is the coordinate expression of Stg and Rux under the control of patterning genes that regulates G_1 synchronization (Thomas and Zipursky 1994; Heberlein et al. 1995). Whereas the function of Stg/Cdc25 in driving the G_2 /M transition is well established from studies in fission yeast (Russell and Nurse 1986), frogs (Gabrielli et al. 1992), and *Drosophila* (Edgar and O'Farrell 1989), little was known previously about the processes regulated by *rux*.

In earlier studies we showed that loss-of-function mutations in both *cycA* and *stg* are dominant suppressors of *rux* (Thomas et al. 1994). This raised the possibility that early entry into S phase was a consequence of ectopic activation of a CycA–Cdk complex by the elevated Stg levels in the synchronization domain. Indeed, mammalian CycA–Cdk2 complexes can be activated in vitro by Stg (Gu et al. 1992). That ectopic expression of CycA drives G_1 cells in the MF into S phase, and that overexpression of Rux prevents this, is consistent with this notion. In *rux* mutants, elevated levels of CycA protein were observed in early G_1 . During normal eye development an inverse correlation is observed between Rux and CycA levels. In the asynchronously dividing cells anterior to the MF, CycA levels are high and Rux levels are low. Immediately anterior to the MF, CycA protein disappears where Rux is expressed at high levels. Behind the MF, CycA reaccumulates in cells entering S phase. It is in these cells that Rux protein declines rapidly.

The results of ectopic Rux expression are consistent with a role for Rux in controlling CycA stability. Ectopic expression both in the embryo and eye disc led to a block in mitosis and a marked decrease in CycA protein levels coupled to its nuclear localization. Ectopic Rux not only led to a block in mitosis, but to endoreduplication cycles. This is consistent with inhibition of CycA. CycA is not expressed in the normal endoreduplication cycles in the fly embryo and loss-of-function *cycA* mutants not only fail to undergo mitosis of cell cycle 16, but enter an endoreduplication cycle (Lehner and O'Farrell 1989; Sauer et al. 1995; K. Weigmann and C.F. Lehner, in prep.). Although levels of *stg* mRNA and CycB protein

also are misregulated in *rux* (Thomas et al. 1994), several lines of evidence are consistent with the cell cycle phenotype resulting from altered CycA levels. First, heat shock-induced expression of CycA, but neither Stg nor CycB (B.J. Thomas, S.L. Zipursky, and C.F. Lehner, unpubl.), phenocopies loss-of-function *rux* mutations. Second, overexpression of Rux not only inhibits mitosis but also leads to endoreduplication, a phenotype associated with loss-of-function *cycA* mutations but not mutations in *cycB* or *stg*. Third, although *stg* mRNA is up-regulated in *rux*, *stg* mRNA is not altered by overexpression of *rux*. And finally, the *rca1* gene, which was isolated as a strong suppressor of *rux*, positively regulates *cycA* (Dong et al. 1997).

rux also regulates CycA post-transcriptionally in meiosis. *rux* males are sterile—whereas meiosis I and meiosis II occur normally, haploid germ cells attempt a third division with nuclear envelope breakdown and abnormal chromosomal segregation. As in mitosis, CycA and the germ-line isoform of Cdc25, *twine*, act as dominant suppressors of this phenotype (Gönczy et al. 1994; Thomas et al. 1994), and Rux affects CycA levels during meiosis (Gönczy et al. 1994). In wild-type premeiotic G_2 cells, CycA is found in both the nucleus and cytoplasm. In *rux* mutants, increased CycA protein accumulates in the cytoplasm but is excluded largely from the nucleus. Conversely, a modest increase in *rux* gene dosage also leads to nuclear localization of CycA and a block in meiosis II in premeiotic G_2 cells. Therefore, during both meiosis and mitosis, Rux controls CycA levels, presumably by controlling CycA stability. Immunolocalization studies raise the intriguing notion that Rux may control CycA stability by regulating its subcellular distribution.

Whereas genetic studies argue that *rux* negatively regulates CycA levels, Rux was shown to bind CycE but not CycA, both in vitro and in yeast. Furthermore, Rux does not inhibit the activity of either CycA– or CycE–Cdk complexes. Because CycE expression precedes the up-regulation of CycA protein at the posterior edge of the MF (i.e., in late G_1), we assessed the possibility that CycE negatively regulates Rux in vivo. Overexpression of CycE reversed the effects of Rux overexpression. This led to a marked decrease in Rux, an increase in CycA and CycB protein levels and a restoration of mitosis. We are currently assessing whether phosphorylation by CycE–Cdk regulates Rux stability.

The control of CycA activity in G_1 cells in the MF shows striking similarities to the control of G_2 cyclins during G_1 in yeast. Degradation of the G_2 cyclin, CLB2, in *Saccharomyces cerevisiae* continues in early G_1 prior to Start, overexpression of CLB2 protein in G_1 drives cells into S phase, and the CLB2 degradation pathway is inhibited by G_1 cyclins in late G_1 (Amon et al. 1994). In *Schizosaccharomyces pombe*, the Rum1 protein has a key role in regulating the G_2 Cyc–Cdk complex, Cdc13–Cdc2, in G_1 . Genetically, Rum1 shares several features with *rux*. Rum1 overexpression inhibits mitosis and leads to endoreduplication. Conversely, loss-of-function *rum1* mutations shorten G_1 and lead to premature entry into S phase (Correa-Bordes and Nurse 1995). This is ac-

accompanied by increased levels of Cdc13–Cdc2 in G_1 . Whereas Rum1 inhibits the activity of Cdc13–Cdc2 (Correa-Bordes and Nurse 1995), it may also promote proteolytic degradation of the cyclin subunit (P. Nurse, pers. comm.). Recent studies in mammalian cells also show that Cyclin B is unstable in G_1 (Brandeis and Hunt 1996). Therefore, studies from yeast, mammalian cells, and flies argue that negative regulation of G_2 cyclins in G_1 is a fundamental feature of cell cycle control.

Despite its critical role in regulating CycA in G_1 cells within the MF, *rux* is not an essential gene. This may reflect the existence of redundant mechanisms controlling the activity of G_2 Cyc–Cdk complexes in G_1 , as described for yeast and mammalian cells. For instance, whereas CLB2 proteolysis is active in early G_1 , CLB2/CDC28 activity also is inhibited by the Cki Sic1 (Schwob et al. 1994). In mammalian cells, not only is CycB proteolysis active in early G_1 , CycB transcription also is down-regulated (Brandeis and Hunt 1996). Redundant mechanisms control CycA activity in the embryo, as supported by the observation that Hs–*cycA* expression drives cells into S phase more readily in *rux*-mutant than wild-type embryos (N. Yakubovitch and P. H. O'Farrell, pers. comm.). Given the importance of normal cell cycle progression for cell viability, perhaps it is not surprising that multiple regulatory mechanisms have evolved to ensure the precise control of specific Cyc–Cdk complexes at the appropriate cell cycle stage. The importance of a single mechanism may be revealed by specific constraints imposed on the development or physiology of different tissues. For instance, as a consequence of the cell cycle synchronization mechanism in the eye disc, an abnormally high level of the Cyc–Cdk activator Stg–Cdc25 is induced, thereby revealing the importance of *rux*-mediated down-regulation of CycA in these cells. As we have shown recently, this unique feature of cell cycle regulation in the developing eye provides a powerful genetic system for identifying genes regulating CycA levels and/or activity (Dong et al. 1997).

Materials and methods

Fly strains and genetics

A description of genetic markers and balancers used can be found in Lindsley and Zimm (1992). The *rux*⁸ null allele was described previously (Thomas et al. 1994). The Hs–*rux* and GMR–*rux* constructs were made by inserting a 1.3-kb *EcoRI* fragment containing the entire *rux* cDNA into the *EcoRI* site of pCaSpeR-hs (Piotto 1988) and pGMR (Hay et al. 1994), respectively. UAS–*rux* was made by cloning a 1.1-kb *EcoRI*–*XbaI* fragment containing the 5'-untranslated region and *Rux* coding sequence up to the translation termination codon into pUAST (Brand and Perrimon 1993). The transcription termination and polyadenylation sites are provided by the vector. The *Rux*–*lacZ* protein fusion was constructed by synthesizing the *Rux* coding region up to the translation termination codon and 910 bp of upstream regulatory sequences by PCR from *rux* genomic clone pRX16. The 5' primer was 5'-CATGGATCCATGCTGCTGAA-TGCTGGAATGCTGTCC-3'. The 3' primer was 5'-CATGGG-ATCCAAACGCATCCGCCTTGGTCCCATCGCACG-3'. The *Bam*HI sites used for cloning are underlined in both primers. An

in-frame protein fusion to *lacZ* was made by cloning the *rux* *Bam*HI fragment and a *Bam*HI–*EcoRI* fragment from pC4- β -gal (Thummel et al. 1988) into pCaSpeR-2 (Piotto 1988). The Hs–*cycE* (Knoblich et al. 1994; Richardson et al. 1995), Hs–*cycA* (Lehner et al. 1991) and *prd*–GAL4 (Brand and Perrimon 1993) strains were described previously.

Immunohistochemistry

The *Rux* monoclonal IgG antibody IC12-H9 was made by immunizing mice with a bacterially-produced full-length *Rux*–GST fusion protein. Monoclonal supernatants were used at a dilution of 1:5. CycA polyclonal serum and monoclonal antibodies to CycA and CycB were a generous gift of P. O'Farrell (University of California, San Francisco). The CycA serum was diluted 1:100 before use. The CycA and CycB monoclonal antibodies were preabsorbed against fixed adult heads and used at a final dilution of 1:5. The anti- β -galactosidase monoclonal antibody (Promega) was used at 1:500. HRP-conjugated anti-mouse or anti-rabbit secondary antibodies (Bio-Rad) were used at 1:50. Biotinylated anti-rabbit and anti-mouse secondary antibodies and avidin-FITC (Vector labs) were used at 1:200. Heat shock of wandering third-instar larvae was done by placing larvae in *Drosophila* medium into an eppendorf tube plugged with cotton and incubating for 1 hr at 37°C in a Multi-Block Heater (Baxter). Larvae were allowed to recover for 2 hr at room temperature before dissection. For heat shock of embryos, eggs were collected on apple juice agar plates for 1 hr at 25°C and aged appropriately. Plates were transferred to a 37°C humidified chamber for 30 min, and returned to 25°C for the desired recovery period. Confocal microscopy was done using a Bio-Rad MRC 1024.

Two hybrid interaction matings

Interaction mating assays were performed essentially as described (Finley and Brent 1994). The “prey” strains were EGY48 (*MAT α his3 ura3 trp1 LYS2 leu2::3Lexop–LEU2*) containing pJG4-5 (Gyuris et al. 1993) expressing activation-tagged *Drosophila* Cycs or Cdks. LexA fusion “bait” plasmids were derived from pEG202 (Estojak et al. 1995). Interaction matings were done by streaking haploid yeast containing either plasmid individually onto selective medium and allowing growth for two days at 30°C. Bait and prey grids were replica-mated by pressing both plates to the same replica velvet and lifting the impression with a single YPD plate. Mating was allowed to proceed for one day at 30°C and mated diploids were then replica-plated to two selective X-Gal indicator plates to select for diploids and score for interactions.

In vitro binding and kinase assays

For in vitro binding, 5 μ l of reticulocyte translate (Promega) was added to the 0.4-ml binding buffer [20 mM HEPES–KOH (pH 7.7) 150 mM NaCl, 0.1% NP-40 10% glycerol, 20 mM NaF, 0.5 mM Na vanadate, 1 mM PMSF, 2 μ g/ml leupeptin, and 2 μ g/ml of aprotinin] with 10 μ l of glutathione–agarose containing bound GST (100 μ g) or GST–*Rux* (20 μ g) and rotated for 2 hr at 4°C. The beads were washed four times with 0.5 ml of binding buffer before electrophoresis and autoradiography.

Histone H1 kinase assays were carried out in 25 μ l of kinase buffer [50 mM Tris–HCl (pH 7.6), 10 mM MgCl₂, 1 mM DTT, 10% glycerol, 100 μ g/ml BSA] supplemented with 10 μ M ATP, 5 μ Ci of [γ -³²P]ATP, and 2 μ g of histone H1 (Sigma). Reactions were initiated by the addition of ATP, incubated at 30°C for 20 min, and stopped by the addition of 5 μ l of 5 $\times$ gel sample buffer before analysis by SDS-PAGE and autoradiography.

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