Grapes(Chk1) prevents nuclear CDK1 activation by delaying cyclin B nuclear accumulation

Anne Royou, Derek McCusker, Douglas R. Kellogg, and William Sullivan

Department of Molecular Cell and Developmental Biology, University of California, Santa Cruz, Santa Cruz, CA 95064

Introduction

Entry into mitosis is characterized by a dramatic remodeling of nuclear and cytoplasmic compartments. These changes are driven by the activity of CDK1 associated with mitotic cyclins, notably cyclin B (CycB; Morgan, 2006). Activation of CDK1 requires sufficient levels of CycB and the removal of CDK1 inhibitory phosphorylation. CDK1 phosphorylation is controlled by conserved kinases Wee1 and Myt1 and by the phosphatase Cdc25. The rapid onset of CDK1 activation at the end of G2 is driven by inactivation of Wee1 and activation of Cdc25. Active CDK1 contributes to these changes in Wee1 and Cdc25 activity, thus establishing a positive feedback loop that drives cells into mitosis (Ferrell, 2002; Morgan, 2006).

In spite of these insights, little is known about the mechanisms by which nuclear and cytoplasmic CDK1 activities are coordinated. Resolving this issue requires determining whether nuclear and cytoplasmic CDK1 pools are differentially regulated. Support for a differential regulation comes from the finding that subcellular localization of CycB plays a role in regulating CDK1 activity. In vertebrates, CycB is predominantly cytoplasmic at interphase because of Crm1-mediated nuclear exclusion (Hagting et al., 1998; Yang et al., 1998). At prophase, CDK1–CycB nuclear translocation is triggered by the phosphorylation of CycB on its cytoplasmic retention signal (Ookata et al., 1997; Li et al., 1997; Hagting et al., 1999; Takizawa and Morgan, 2000).

Controlling the subcellular localization of CycB may also be involved in checkpoint function. Un-replicated or damaged DNA results in the activation of the conserved S-phase checkpoint kinase 1 (Chk1), which inhibits Cdc25 and activates Wee1 (Furnari et al., 1997; Peng et al., 1997; Sanchez et al., 1997; Lee et al., 2001). Consequently, the S-phase checkpoint delays the cell cycle in interphase by Chk1-mediated inhibition of CDK1 (Walworth, 2001; Melo and Toczyski, 2002). However, expression of CDK1ΔT, a version of CDK1 lacking the phosphorylation inhibitory sites, only partially bypasses the interphase arrest induced upon DNA damage (Jin et al., 1996). This arrest is fully bypassed by coexpressing CDK1ΔT and nuclear-targeted CycB (Heald et al., 1993; Jin et al., 1998). These studies imply that prevention of CycB nuclear localization is one of the mechanisms by which the S-phase checkpoint delays nuclear entry into mitosis. Thus, the coordination of cytoplasmic and nuclear mitotic entry likely involves control of CycB subcellular localization as well as the CDK1 phosphorylation state.

The late syncytial nuclear cycles of the Drosophila melanogaster embryo are regulated by levels of CycB and S-phase protein synthesis inhibitor, injection of CycB accelerates nuclear envelope breakdown and mitotic remodeling of the cytoskeleton. Upon activation of the Grapes(checkpoint kinase 1) (Grapes(Chk1))-dependent S-phase checkpoint, increased levels of CycB drives cytoplasmic but not nuclear mitotic events. Grapes(Chk1) prevents nuclear CDK1 activation by delaying CycB nuclear accumulation through Wee1-dependent and independent mechanisms.
checkpoint activity (Edgar et al., 1994; Fogarty et al., 1997; Sibon et al., 1997; Stiffler et al., 1999; Price et al., 2000; Stumpff et al., 2004; Crest et al., 2007). These cycles provide an excellent system to address the role of CycB subcellular localization in driving cytoplasmic and nuclear mitotic events during normal and S-phase checkpoint–activated conditions. We addressed this question by taking advantage of our ability to inject functional CycB in the syncytial embryo at precise times during the cell cycle and to monitor its effects on multiple cytoplasmic and nuclear events. Increasing the level of CycB during early interphase of cycle 13 induces premature nuclear envelope breakdown (NEB) and the reorganization of the cytoskeleton. Upon activation of the S-phase checkpoint, increased levels of CycB drives cytoplasmic but not nuclear mitotic events. We demonstrate that the S-phase checkpoint protects the nucleus from active cytoplasmic and nuclear mitotic events. We will refer to the recombinant protein as CycB.

Nuclear CDK1 activity is thought to promote chromosome condensation and NEB (Lamb et al., 1990; Peter et al., 1990; Enoch et al., 1991). Therefore, nuclear mitotic events were defined by chromosome condensation (monitored with GFP-H2Av) and NEB (monitored by nuclear infusion of injected rhodamine-conjugated tubulin). The central regions of embryos were injected with either 71 μM GST or 65 μM CycB at the onset of interphase of nuclear cycle 13. After GST injection, NEB occurred almost synchronously throughout the embryo at 14.3 min after injection, and the chromosomes entered anaphase shortly after NEB (Fig. 1 A, GST). After CycB injection, the timing of NEB was greatly accelerated near the site of injection compared with NEB in areas more distant from the site of injection (Fig. 1 A, CycB, white outlines). Premature NEB occurs as a wave probably caused by the slow diffusion of CycB from the injection site. These observations indicate that CycB injection at 65 μM is sufficient to induce a local activation of CDK1, which triggers premature NEB and spindle assembly. This result is consistent with work demonstrating that exogenous CycB promotes premature disassembly of the nuclear pore complex in syncytial Drosophila embryos (Onischenko et al., 2005). Although CycB induced premature NEB, it did not promote premature chromosome condensation before NEB. At NEB, the level of chromosome condensation was much greater in the control embryo than in the CycB-injected embryo (Fig. 1 B, top row). In most cases, once NEB occurred in the area near the site of CycB injection, the chromosomes rapidly condensed and, by metaphase, achieved the same state of condensation as control embryos (Fig. 1 B, bottom row).

To determine whether new rounds of CycB synthesis are required for each mitotic cycle, we investigated the effect of injecting CycB after treatment with the protein synthesis inhibitor cycloheximide (CHX). Injection of CHX at metaphase induced a cytoplasmic and nuclear interphase arrest in the next cycle (Fig. 1 C, left). Exogenous CycB locally overcame the CHX-induced interphase arrest as indicated by NEB and rapid spindle formation (Fig. 1 C, right; and Video 1, available at http://www.jcb.org/cgi/content/full/jcb.200801153/DC1). The data suggest that new rounds of CycB synthesis are necessary to drive the next mitotic cycle. We determined by single embryo Western that the amount of injected CycB was equivalent to the total level of endogenous CycB at metaphase of cycle 13. Taking into account the limited diffusion of the injected CycB, we estimate that the 65 μM of exogenous CycB at the center of the gradient was less than fivefold the level of endogenous CycB at mitosis (see Materials and methods).

### Injection of CycB prematurely drives the cytoplasm into mitosis

We next determined whether injected CycB prematurely drives the cytoplasm into mitosis. We addressed this issue by examining the dynamics of three cytoplasmic proteins fused with GFP that undergo dramatic changes in subcellular distribution during the cortical cycles: nonmuscle myosin II regulatory light chain ([RLC]; RLC-GFP; Fig. 2, A and D; Royou et al., 2004), moesin (GFP-moesin; Fig. 2 B and Video 2, available at http://www.jcb.org/cgi/content/full/jcb.200801153/DC1; Edwards et al., 1997), and Nuclear fallout (GFP-Nuf; Fig. 2 C and Video 3; Riggs et al., 2007).

In all embryos, CycB was injected at the onset of cycle 13 interphase. We monitored the timing of cytoplasmic marker dynamics and NEB in areas close to and distant from the site of injection. We will refer to these as injected and control areas, respectively. In control areas, RLC-GFP concentrated at the cortex throughout interphase and dispersed just before NEB. This dispersion occurred 12 min after the onset of interphase (n = 23, average deviation [AVD] = 1.8; Fig. 2, A and D; and see Fig. S1 for quantification of the RLC-GFP signal, available at http://www.jcb.org/cgi/content/full/jcb.200801153/DC1). In CycB-injected areas, RLC-GFP dispersed prematurely, 4 min after interphase onset (n = 23, AVD = 1.8; Fig. 2, A and D, blue outlines). Interestingly, in both areas, RLC-GFP disappearance always preceded NEB (Fig. 2, A and D, orange outlines). A previous study has shown that RLC-GFP dispersion is driven by CDK1 activity (Royou et al., 2002). Therefore, our results indicate that high levels of CycB promote premature cytoplasmic as well as nuclear CDK1 activation.

GFP-moesin, a marker for F-actin, reorganizes from caps at interphase into furrows in mitosis (Edwards et al., 1997). By focusing 5 μm below the plasma membrane, only furrows that have extended to this depth are visualized (Fig. 2 B). In control and CycB-injected regions, F-actin furrows progressed to this depth at 11.5 min and 3.5 min after injection, respectively (Fig. 2 B, blue outlines). These experiments indicate that exogenous CycB drives premature reorganization of the actin cytoskeleton.
To confirm that the effect of CycB was not confined to actin and myosin, we monitored the effect of CycB injection on Nuf, a Rab11 effector that exhibits a cell cycle–dependent association with the microtubule-organizing center (MTOC; Riggs et al., 2003; Cao et al., 2008). We find that GFP-Nuf is concentrated at the MTOC through interphase of cycle 13 in the control area. Upon CycB injection, Nuf prematurely disperses from the MTOC (Fig. 2 C; blue outlines). Collectively, these observations indicate that high levels of CycB induce local cytoplasmic CDK1 activation and trigger remodeling of the cytoskeleton.

**CycB drives cytoplasmic events independently of the nuclear cycle**

In the aforementioned studies, we observed that cytoplasmic mitotic events precede nuclear mitotic events. This observation raises the possibility that cytoplasmic and nuclear CDK1 activities are differentially regulated by CycB levels. To explore this possibility, we monitored nuclear (NEB) and cytoplasmic (myosin dispersion) mitotic events after injecting CycB at a lower concentration of 32 μM (Fig. 2 D). In six out of eight injected embryos, although CycB induced premature myosin dispersion, it did not induce premature NEB (Fig. 2 D). This uncoupling between the cytoplasmic and nuclear CDK1 activation rate suggests that different mechanisms regulate these two activities.

To further examine independent activation of cytoplasmic and nuclear CDK1, we took advantage of the prolonged interphase of cycle 14. In a process known
However, the timing of NEB was not coordinated with the timing of myosin dispersion. Although CycB injection induced myosin dispersion promptly (4.6 ± 0.6, n = 5; Fig. 3, A and C), NEB was delayed and occurred 5.5 min after myosin had dispersed (10.1 ± 2.1, n = 5; Fig. 3, A and C). This uncoupling between the cytoplasmic and nuclear cycle was even more dramatic when CycB was injected 6 – 9 min after the onset of cellularization. Injection of CycB at this later time failed to induce NEB (Fig. 3, B and C), yet the cytoplasm entered mitosis rapidly after CycB injection (Fig. 3, B and C, blue outlines; and Fig. S2).

CycB also induced the reorganization of GFP-moesin into as cellularization. The mechanisms that trigger cellularization are not understood. To determine whether CycB is limiting for driving CDK1 activation at cycle 14, we injected CycB at different times during cellularization and monitored cytoplasmic and nuclear CDK1 activation. Cytoplasmic CDK1 activation was monitored by examining RLC-GFP, GFP-moesin, and GFP-Nuf dynamics (Fig. 3). Nuclear CDK1 activity was measured by monitoring NEB (when the cytoplasmic markers invade the nucleoplasm). When injected early during cellularization (3 – 5 min after nuclear envelope formation [NEF]), CycB triggered both cytoplasmic and nuclear entry into mitosis (Fig. 3, A and C, blue and orange outlines, respectively; and Video 4, available at http://www.jcb.org/cgi/content/full/jcb.200801153/DC1). However, the timing of NEB was not coordinated with the timing of myosin dispersion. Although CycB injection induced myosin dispersion promptly (4.6 ± 0.6, n = 5; Fig. 3, A and C), NEB was delayed and occurred 5.5 min after myosin had dispersed (10.1 ± 2.1, n = 5; Fig. 3, A and C). This uncoupling between the cytoplasmic and nuclear cycle was even more dramatic when CycB was injected 6–9 min after the onset of cellularization. Injection of CycB at this later time failed to induce NEB (Fig. 3, B and C), yet the cytoplasm entered mitosis rapidly after CycB injection (Fig. 3, B and C, blue outlines; and Fig. S2). CycB also induced the reorganization of GFP-moesin into...
mutants and normal embryos, we injected the embryos simultaneously with CHX and Aph. This provides time to perform the injections in the exceedingly short cell cycles of the checkpoint-compromised embryos. In wild-type embryos, the results are the same for injecting Aph alone or a combined injection of Aph and CHX (unpublished data). For both a wild-type and a grp mutant, only the combined injections are described.

In all experiments presented in Fig. 4, CHX and Aph were injected together at mitosis of cycle 12 in embryos expressing RLC-GFP, which allowed us to simultaneously monitor cytoplasmic and nuclear CDK1 activity during early cellularization. Moreover, these experiments show that increasing the level of CycB is sufficient to induce an additional syncytial cycle. They demonstrate that CycB remains limiting for cytoplasmic and, to a lesser extent, nuclear CDK1 activation during early cellularization. Furthermore, they provide evidence that CDK1 can be activated in the cytoplasm independently of its activation in the nucleus.

The Grapes (Grp)-dependent S-phase checkpoint protects the nucleus from cytoplasmic CDK1 activity

We next addressed the effects of the S-phase checkpoint activity on cytoplasmic and nuclear CDK1 activation. To do so, we observed the effects of CycB injections in syncytial embryos arrested in interphase by injecting the DNA replication inhibitor aphidicolin (Aph). When Aph is injected during mitosis of cycle 12, it activates the S-phase checkpoint and induces a prolonged cytoplasmic and nuclear interphase arrest at the next cycle (unpublished data).

We next addressed whether CycB can overcome the S-phase checkpoint-induced cytoplasmic and nuclear interphase arrest. To perform this analysis in checkpoint-compromised

furrows and the dispersion of Nuf from the MTOC (Fig. 3 B, blue outlines; and Video 5). However, there were no cases in which NEB occurred. CycB injected 15 min after the onset of cellularization had no effect on the progression of nuclear or cytoplasmic events ($n = 6$; unpublished data). These experiments show that increasing the level of CycB is sufficient to induce an additional syncytial cycle. They demonstrate that CycB remains limiting for cytoplasmic and, to a lesser extent, nuclear CDK1 activation during early cellularization. Furthermore, they provide evidence that CDK1 can be activated in the cytoplasm independently of its activation in the nucleus.
consistent premature cytoplasmic and nuclear entry into mitosis at the site of injection (Fig. 4, C and D).

These results support our previous conclusion that cytoplasmic CDK1 activity is independent of its activity in the nucleus. In addition, they suggest that the S-phase checkpoint is more effective at inhibiting CDK1 in the nucleus than in the cytoplasm.

CycB has a dynamic localization through the syncytial cycle and associates with kinetochores before NEB and during mitosis.

Given that the nucleus can be protected from active cytoplasmic CDK1–CycB by the Grp-dependent S-phase checkpoint, this raises the possibility that the checkpoint operates by regulating CycB nuclear import (Jin et al., 1998). To pursue this idea, we

Finally, in 5 out of 15 embryos, neither cytoplasmic nor nuclear CDK1 activity was detected (Fig. 4 B*).

An explanation for the different results upon early and late injection of CycB into CHX + Aph–arrested embryos is that the S-phase checkpoint is activated in late but not in early injected embryos. We propose this idea because live analysis using a Grp-GFP fusion protein reveals that Grp is dispersed in the cytoplasm during mitosis and accumulates in the nucleus upon NEF (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200801153/DC1). Thus, sufficient time after NEF is required to establish a functional S-phase checkpoint. To test this hypothesis, we performed the same experiments in the S-phase checkpoint–compromised mutant grp (Fig. 4 C). As with wild-type embryos, CHX + Aph arrested the nuclei and cytoplasm of grp embryos in interphase (unpublished data). In contrast to wild type, CycB injected late into grp embryos triggered consistent premature cytoplasmic and nuclear entry into mitosis at the site of injection (Fig. 4, C and D).

CycB injections overcome the S-phase checkpoint–induced cytoplasmic cell cycle arrest, and to a lesser extent, nuclear cell cycle arrest. (A–C) Wild-type (WT, A–B) or grp mutant embryos (C) expressing RLC-GFP were injected with CHX and Aph together (CHX + Aph) at metaphase of cycle 12. The embryos were injected with CycB at the onset of interphase [A, early CycB injection] or 10 min after the onset of interphase [B–C, late CycB injection]. The schematic describes the injection and imaging sequence. Arrowheads mark the sites of injection. Blue outlines mark areas with cytoplasmic CDK1 activity, and orange outlines mark areas where NEB occurs. Time is given in minutes/seconds. Bars, 20 μm. (D) Timing of cytoplasmic and nuclear CDK1 activation was measured as described in Fig. 2. Data are represented as mean ± AVD. n, number of embryos injected.
analyzed the subcellular localization of CycB in living embryos by injecting rhodamine-labeled CycB (CycB-R).

We injected a low concentration of 13 μM CycB-R at the onset of interphase of cycles 12 and 13 in embryos expressing RLC-GFP (Fig. 5 A), the kinase Polo fused with GFP (GFP-Polo; Fig. 5 B; Moutinho-Santos et al., 1999), or the centromere-associated protein Cid fused with GFP (GFP-Cid; Fig. 5 C; Schuh et al., 2007). At this concentration, CycB-R did not induce premature entry into mitosis in five out of six wild-type embryos. CycB-R accumulates at the centrosome and is excluded from the nucleus during interphase, in agreement with our previous observations (Huang and Raff, 1999). During early prophase, before NEB, CycB-R maintains its association with the centrosome and accumulates in discrete puncta within the nucleus, indicating that the recombinant CycB used in these experiments is efficiently imported into the nucleus (Fig. 5 A, arrows). After NEB, CycB-R is primarily associated with the bipolar spindle, consistent with observations in other cell types (Pines and Hunter, 1991; Yang et al., 1998). In addition, a strong signal was also detected on the metaphase plate (Fig. 5). CycB-R colocalized with kinetochore GFP-Polo before NEB and throughout mitosis (Fig. 5 B, arrows). The CycB-R puncta in the nucleus before NEB colocalized with GFP-Cid (Fig. 5 C, arrows). This colocalization is observed during mitosis until late anaphase. Collectively, these observations suggest an association of CycB-R with the kinetochore that is initiated before NEB. The specific localization of CycB-R into kinetochores before NEB provides a means to monitor CycB nuclear import.

The S-phase checkpoint prevents CycB nuclear accumulation by maintaining CDK1 in an inactive state

To determine whether the S-phase checkpoint prevents nuclear CDK1 activation by controlling CycB subcellular localization, we injected CycB-R after CHX + Aph treatment into wild-type and grp mutant embryos expressing RLC-GFP (Fig. 6). We assayed three different CycB-R concentrations (100, 56, and 13 μM). We monitored the induction of cytoplasmic and nuclear CDK1 activity by observing cortical myosin dispersion and NEB, respectively. We also monitored the timing of nuclear CycB-R localization. Embryos were injected with CHX + Aph in mitosis of cycle 12 followed by injection of CycB-R 10 min after the onset of cycle 13 interphase (Fig. 6, schematic). In wild-type embryos injected with 100 μM CycB-R, the cytoplasm entered mitosis, as indicated by the disappearance of the RLC-GFP signal from the cortex (Fig. 6 C, compare the second row with the third row). In contrast, injection of 56 or 13 μM CycB-R in the grp mutant triggered...
Moreover, in mammalian cultured cells, Wee1 can protect the nucleus from active cytoplasmic CDK1 (Heald et al., 1993). To test whether a threshold of nuclear CDK1 activity is required to promote CycB nuclear accumulation, we performed the same experiment as described in the previous paragraph in wee1 mutant embryos. We monitored the accumulation of CycB-R in the nucleus and its effect on cytoplasmic and nuclear CDK1 activation. The injection of CHX + Aph during the previous mitosis induced an interphase arrest for at least 10 min in a wee1 background. Injection of 100, 56, or 13 μM CycB-R provoked a rapid cytoplasmic and nuclear entry into mitosis (Fig. 6, D, H, and I, blue and orange outlines, respectively). The first sign of CycB-R nuclear accumulation before NEB was detected at 2.2 min and 3.7 min after injection of 56 μM and 13 μM CycB-R, respectively (Fig. 6, D and I, red arrow).

These data indicate that the Grp S-phase checkpoint prevents mitotic entry by delaying nuclear CycB accumulation. It may be that a threshold of nuclear CDK1 activity is required to trigger CycB import. Activation of the S-phase checkpoint may prevent nuclear CDK1 from reaching this threshold. Support for this idea comes from experiments in *Xenopus laevis*, demonstrating that Chk1 phosphorylates and activates Wee1, a CDK1 inhibitor (Lee et al., 2001). Moreover, in mammalian cultured cells, Wee1 can protect the nucleus from active cytoplasmic CDK1 (Heald et al., 1993).

To test whether a threshold of nuclear CDK1 activity is required to promote CycB nuclear accumulation, we performed the same experiment as described in the previous paragraph in wee1 mutant embryos. We monitored the accumulation of CycB-R in the nucleus and its effect on cytoplasmic and nuclear CDK1 activation. The injection of CHX + Aph during the previous mitosis induced an interphase arrest for at least 10 min in a wee1 background. Injection of 100, 56, or 13 μM CycB-R provoked a rapid cytoplasmic and nuclear CDK1 activation (Fig. 6, E–H,

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**Figure 6.** The S-phase checkpoint delays nuclear CycB accumulation and CDK1 activation by a Grp and Wee1-dependent mechanism. [A–E] Wild-type (A and C), grp (B and D), and wee1 (E) embryos expressing RLC-GFP (cyan) were injected with a mix of Aph and CHX (CHX + Aph) at metaphase of cycle 12. 10 min after the onset of the next interphase, they were injected with CycB-R (red) at the specified concentrations. The schematic describes the injection and imaging sequence. Time is given in minutes/seconds [see Videos 6, 7, and 8 for A, B, and E, respectively, available at http://www.jcb.org/cgi/content/full/jcb.200801153/DC1]. The right column of each panel is an enlargement of the area outlined with the white box shown on the first image of the left column. However, for A and B, the areas are at a focal plane 2 μm above the focal plane shown in the left columns. Blue outlines mark the areas with cytoplasmic CDK1 activity, and orange outlines mark the areas undergoing NEB. [C and D] Red arrows point to the nuclear localization of CycB-R. n, number of embryos injected. Bars: (left columns) 20 μm; (right columns) 5 μm. [F–H] 100 (F), 56 (G), and 13 μM (H) CycB-R was injected 10 min after the onset of interphase in CHX + Aph–treated embryos. The timing of cytoplasmic and nuclear CDK1 activation was determined as described in Fig. 2. (I) The timing of CycB-R accumulation in nuclei was determined when CycB-R appeared at kinetochores in at least three nuclei. [F–I] Data are represented as mean ± AVD. n, number of embryos injected.

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and Roscovitine (Meijer et al., 1997). We performed the same experiments as described in Fig. 6, monitoring CycB nuclear import in wild-type, 
\textit{grp}, and \textit{wee1} embryos but with CDK1 maintained inactive through injection of CDK1 inhibitors (Fig. 7). In \textit{grp} and \textit{wee1} mutant embryos injected with CHX + Aph, CycB-R triggered rapid myosin dispersion from the cortex and NEB (Fig. 6, D–H). In contrast, the addition of Roscovitine or RO-3306 before CycB-R injection prevented both myosin dispersion and NEB (Fig. 7, B and C). This demonstrates that these compounds efficiently inhibit cytoplasmic and nuclear CDK1 activity.

In wild-type embryos treated with CHX + Aph and RO-3306, no nuclear CycB-R signal was detected in three out of seven embryos for up to 15 min. The first signs of CycB-R in the nuclei were detected at a mean of 12.9 min \((n = 7);\) Fig. 7 D). A similar result was obtained with Roscovitine; no nuclear CycB-R signal was detected in 5 out of 10 wild-type embryos injected with Roscovitine showed nuclear CycB-R accumulation before 15 min, which is the time limit of each video for the Roscovitine assay. Therefore, the mean timing of nuclear CycB-R import represents only half of the embryos injected. (E) The table summarizes the results in Figs. 6 and 7. (F) The schematic presents a model for the control of CycB nuclear import. CycB nuclear accumulation is inhibited by Grp and activated by nuclear CDK1 activity, which is itself inhibited by Grp and Wee1.
Wee1 (Fig. 7 F).

mulation of CycB through a separate mechanism not shared by nuclear CDK1 in an inactive state, Grp prevents nuclear accumulation of CycB-R in the cytoplasm early in interphase because the S-phase checkpoint is activated in the cytoplasm before its nuclear activation (Heald et al., 1993; Jin et al., 1996; De Souza et al., 2000; Draviam et al., 2001; Jackman et al., 2003).

Increased levels of CycB induce premature cytoplasmic and nuclear CDK1 activation

In this study, we directly address the role of CycB in driving the syncytial divisions in the Drosophila embryo by demonstrating that increased levels of CycB are sufficient to trigger nuclear and cytoplasmic mitotic entry. Unlike more conventional cell cycles, the maternal pool of CycB is not fully degraded during these syncytial mitotic divisions; therefore, it was not clear whether CycB was limiting for CDK1 activation (Edgar et al., 1994). Our finding that inhibition of protein synthesis induced a prolonged interphase in wild-type and S-phase checkpoint–compromised embryos indicates that new rounds of protein synthesis are required to drive the late syncytial cycles. Of more significance is the fact that injection of CycB into these interphase-arrested embryos efficiently drives cytoplasmic and nuclear entry into mitosis. Our data imply that, even though CycB is not fully degraded at mitosis, new rounds of CycB synthesis are required to trigger the next mitosis. Support for this idea comes from the fact that increasing the maternal copies of CycB affects the timing of entry into mitosis during the late syncytial cycles (Ji et al., 2004). It may also be that the pool of endogenous CycB is maintained in a form that prevents CDK1 activation through posttranslational modifications and that the exogenous CycB lacks these modifications.

Careful analysis of the timing of cytoplasmic and nuclear mitotic events in the last syncytial cycle reveals that initiation of chromosome condensation and reorganization of the cytoskeleton occur before NEB. We have found that, even in the absence of protein synthesis, exogenous CycB can efficiently coordinate the reorganization of the cytoskeleton with NEB. However, exogenous CycB does not preserve the order of the nuclear mitotic events because the chromosomes did not condense before NEB. There are three maternal mitotic cyclins in a Drosophila embryo, cyclin A, B, and B3, with overlapping functions (Jacobs et al., 1998). One interesting idea is that the proper ratio of the three mitotic cyclins is required to maintain the order of the nuclear events.

We have observed that increased levels of CycB at the onset of cellularization during cycle 14 drive an additional round of syncytial mitosis. A recent study has shown that the knockdown of the three mitotic cyclins with RNAi blocks the syncytial cycles but does not affect the timing of cellularization (McCleland and O’Farrell, 2008). Both findings are compatible with the idea that low levels of CycB are required for the correct timing of cellularization. Earlier work demonstrated that inhibition of zygotic transcription also prevents cellularization and drives an additional round of syncytial mitosis (Edgar et al., 1996). Collectively, these results fit a model in which cellularization is triggered by thresholds of high levels of as yet unknown zygotic gene products and low levels of CycB. Although injecting CycB within the first 9 min of cycle 14 prevents cellularization, injecting CycB after this time point has no effect on cellularization. This suggests a narrow time window in which the embryo is not fully committed to cellularization.

CycB injections uncouple the cytoplasmic and nuclear cycles

Our results also demonstrate that CDK1 can be activated in the cytoplasm independent of its activation in the nucleus. The prolonged interphase of nuclear cycle 14 provided us with an opportunity to determine the effects of CycB injections later in interphase. Early injections of CycB (0–5 min after NEF) prematurely drive nuclear and cytoplasmic events. However, CycB injections later in interphase (6–9 min after NEF) drive the cytoplasm but not the nuclei into mitosis. Specifically, we observe myosin dispersion while the nuclear envelope remains intact. Given that myosin dispersion requires high CDK1 activity (Royou et al., 2002), these studies indicate that CDK1 is activated in the cytoplasm but not in the nucleus. This result is consistent with the observation that the cytoplasm continues to cycle in enucleated frog embryos (Wasserman and Smith, 1978). It is also in accord with studies in mammalian cultured cells and Xenopus egg extracts, indicating that the CDK1–CycB complex is activated in the cytoplasm before its nuclear activation (Heald et al., 1993; Jin et al., 1996; De Souza et al., 2000; Draviam et al., 2001; Jackman et al., 2003).

The S-phase checkpoint component Grp prevents nuclear CDK1 activation

In performing CycB injection in embryos treated with Aph and CHX, we observed that the effects of CycB on nuclear and cytoplasmic CDK1 activation varied depending on the timing of injection. CycB injections during early interphase drive both cytoplasmic and nuclear CDK1 activation, whereas later injections induced only cytoplasmic CDK1 activation. Moreover, this nuclear protection from premature cytoplasmic CDK1 activity upon late CycB injection depends on the S-phase checkpoint component Grp. Our observation that Grp must be imported into the nucleus at the beginning of interphase raises the possibility that time is required for sufficient quantities of Grp to accumulate in the nucleus and create a functional S-phase checkpoint. Injection of CycB induces mitosis in both the nuclei and cytoplasm early in interphase because the S-phase checkpoint is not fully established. However, later in interphase, Grp has accumulated sufficiently in the nucleus to completely prohibit nuclear CDK1 activation.

Discussion

Increased levels of CycB induce premature cytoplasmic and nuclear CDK1 activation

In this study, we directly address the role of CycB in driving the syncytial cycles of the Drosophila embryo by demonstrating that increased levels of CycB are sufficient to trigger nuclear and cytoplasmic mitotic entry. Unlike more conventional cell cycles, the maternal pool of CycB is not fully degraded during these syncytial mitotic divisions; therefore, it was not clear whether CycB was limiting for CDK1 activation (Edgar et al., 1994). Our finding that inhibition of protein synthesis induced a prolonged interphase in wild-type and S-phase checkpoint–compromised embryos indicates that new rounds of protein synthesis are required to drive the late syncytial cycles. Of more significance is the fact that injection of CycB into these interphase-arrested embryos efficiently drives cytoplasmic and nuclear entry into mitosis. Our data imply that, even though CycB is not fully degraded at mitosis, new rounds of CycB synthesis are required to trigger the next mitosis. Support for this idea comes from the fact that increasing the maternal copies of CycB affects the timing of entry into mitosis during the late syncytial cycles (Ji et al., 2004). It may also be that the pool of endogenous CycB is maintained in a form that prevents CDK1 activation through posttranslational modifications and that the exogenous CycB lacks these modifications.

Careful analysis of the timing of cytoplasmic and nuclear mitotic events in the last syncytial cycle reveals that initiation of chromosome condensation and reorganization of the cytoskeleton occur before NEB. We have found that, even in the absence of protein synthesis, exogenous CycB can efficiently coordinate the reorganization of the cytoskeleton with NEB. However, exogenous CycB does not preserve the order of the nuclear mitotic events because the chromosomes did not condense before NEB. There are three maternal mitotic cyclins in a Drosophila embryo, cyclin A, B, and B3, with overlapping functions (Jacobs et al., 1998). One interesting idea is that the proper ratio of the three mitotic cyclins is required to maintain the order of the nuclear events.

We have observed that increased levels of CycB at the onset of cellularization during cycle 14 drive an additional round of syncytial mitosis. A recent study has shown that the knockdown of the three mitotic cyclins with RNAi blocks the syncytial cycles but does not affect the timing of cellularization (McCleland and O’Farrell, 2008). Both findings are compatible with the idea that low levels of CycB are required for the correct timing of cellularization. Earlier work demonstrated that inhibition of zygotic transcription also prevents cellularization and drives an additional round of syncytial mitosis (Edgar et al., 1996). Collectively, these results fit a model in which cellularization is triggered by thresholds of high levels of as yet unknown zygotic gene products and low levels of CycB. Although injecting CycB within the first 9 min of cycle 14 prevents cellularization, injecting CycB after this time point has no effect on cellularization. This suggests a narrow time window in which the embryo is not fully committed to cellularization.

CycB injections uncouple the cytoplasmic and nuclear cycles

Our results also demonstrate that CDK1 can be activated in the cytoplasm independent of its activation in the nucleus. The prolonged interphase of nuclear cycle 14 provided us with an opportunity to determine the effects of CycB injections later in interphase. Early injections of CycB (0–5 min after NEF) prematurely drive nuclear and cytoplasmic events. However, CycB injections later in interphase (6–9 min after NEF) drive the cytoplasm but not the nuclei into mitosis. Specifically, we observe myosin dispersion while the nuclear envelope remains intact. Given that myosin dispersion requires high CDK1 activity (Royou et al., 2002), these studies indicate that CDK1 is activated in the cytoplasm but not in the nucleus. This result is consistent with the observation that the cytoplasm continues to cycle in enucleated frog embryos (Wasserman and Smith, 1978). It is also in accord with studies in mammalian cultured cells and Xenopus egg extracts, indicating that the CDK1–CycB complex is activated in the cytoplasm before its nuclear activation (Heald et al., 1993; Jin et al., 1996; De Souza et al., 2000; Draviam et al., 2001; Jackman et al., 2003).

The S-phase checkpoint component Grp prevents nuclear CDK1 activation

In performing CycB injection in embryos treated with Aph and CHX, we observed that the effects of CycB on nuclear and cytoplasmic CDK1 activation varied depending on the timing of injection. CycB injections during early interphase drive both cytoplasmic and nuclear CDK1 activation, whereas later injections induced only cytoplasmic CDK1 activation. Moreover, this nuclear protection from premature cytoplasmic CDK1 activation upon late CycB injection depends on the S-phase checkpoint component Grp. Our observation that Grp must be imported into the nucleus at the beginning of interphase raises the possibility that time is required for sufficient quantities of Grp to accumulate in the nucleus and create a functional S-phase checkpoint. Injection of CycB induces mitosis in both the nuclei and cytoplasm early in interphase because the S-phase checkpoint is not fully established. However, later in interphase, Grp has accumulated sufficiently in the nucleus to completely prohibit nuclear CDK1 activation.
How does Grp protect the nucleus from active cytoplasmic CDK1? In response to damaged or incompletely replicated DNA, Chk1 phosphorylates Cdc25, altering its relative import/export rates such that it remains cytoplasmic (Furnari et al., 1997; Peng et al., 1997; Sanchez et al., 1997; Takizawa and Morgan, 2000). Additionally, Chk1 has been shown to phosphorylate and activate Wee1 (Lee et al., 2001). Although these findings remain to be demonstrated in Drosophila, it provides an explanation for the Grp-dependent protection of the nucleus from active cytoplasmic CDK1. Grp maintains the inhibitory tyrosine phosphorylation on nuclear CDK1 via activation of Wee1 and exclusion of Cdc25 from the nucleus. Support for this idea comes from a study in mammalian cultured cells in which overexpression of Wee1 prevents NEB in the presence of high cytoplasmic CDK1 activity (Heald et al., 1993). The fact that increased levels of cytoplasmic CycB overrides the cytoplasmic but not the nuclear S-phase checkpoint inhibitory signal suggests that the S-phase checkpoint is more efficient at inhibiting CDK1 in the nucleus. One idea is that the level of S-phase checkpoint activity is higher in the nucleus than in the cytoplasm. This notion is supported by experiments in binucleate yeast and sea urchin embryos in which one of the two nuclei has altered DNA. Entry into mitosis is delayed in the nucleus with altered DNA with respect to the other normal nucleus and the common cytoplasm (Sluder et al., 1995; Demeter et al., 2000). In addition, our observations, as well as others’, that Grp and Wee1 are predominantly nuclear during S phase support this idea (Heald et al., 1993; Purdy et al., 2005).

**Grp and Wee1 delay CycB nuclear accumulation**

The subcellular localization of CycB plays an important role in regulating nuclear CDK1 activity (Takizawa and Morgan, 2000). Specifically, it has been shown in mammalian cultured cells that CycB is predominantly cytoplasmic during the G2 arrest caused by DNA damage, and a nuclear-targeted CycB can partially bypass this arrest (Smeets et al., 1994; Jin et al., 1998). To test the hypothesis that the S-phase checkpoint affects CycB subcellular localization, we injected CycB-R and monitored its dynamics after inhibition of DNA replication in wild-type embryos and embryos lacking Grp or Wee1. In a normal cycle, we observed that CycB-R is imported into the nucleus and forms discrete puncta, which colocalize with GFP-Polo and GFP-Cid, suggesting an association of CycB-R with kinetochores before NEB. This provides an excellent means to monitor CycB nuclear accumulation.

In embryos with high S-phase checkpoint activity, CycB nuclear accumulation was impaired. In contrast, in wee1 mutant embryos, CycB rapidly accumulated in the nucleus. One explanation is that in the absence of Grp, Wee1 activity is low, resulting in high nuclear CDK1 activity, which in turn promotes CycB import. Consistent with this hypothesis, we also found that CycB-R is rapidly imported into the nucleus and promotes NEB in wee1 mutant embryos. Interestingly, the observation that high levels of CycB efficiently triggered cytoplasmic CDK1 activation but failed to localize in the nucleus reveals that cytoplasmic CDK1 activity is not sufficient to promote CycB nuclear import.

**Grp also delays CycB nuclear accumulation by a mechanism independent of Wee1**

Significantly, our observation that Grp-mediated inhibition of CycB nuclear import is independent of both Wee1 and the state of nuclear CDK1 activity reveals an additional mechanism by which the S-phase checkpoint prevents nuclear CDK1 activation. Monitoring CycB nuclear import in Aph and CHX-treated wild-type wee1 and grp mutant embryos in which CDK1 is maintained in an inactive state reveals that CycB is rapidly imported into the nucleus only in the grp mutant. These results indicate a novel mechanism by which the S-phase checkpoint prevents mitotic entry: a Grp-dependent but Wee1-independent mechanism that prevents CycB nuclear accumulation. A satisfying aspect of this result is that it readily explains why bypassing interphase arrest caused by DNA damage not only requires a constitutively active form of CDK1 but also nuclear localized CycB (Jin et al., 1998). It would be of interest to determine whether Grp inhibits nuclear import of other cyclins as well.

The observation that CycB rapidly accumulates in the nucleus in the wee1 mutant where Grp is fully active contrasts with our results that Grp delays CycB nuclear import. One idea is that, in the absence of wee1, the active form of nuclear CDK1 associated with basal levels of CycB promotes CycB (and more likely the CDK1–CycB complex) nuclear import, which in turn increases nuclear CDK1 activity. This triggers a positive feedback loop that efficiently bypasses the Grp inhibitory signal.

**Model for the regulation of cytoplasmic and nuclear CDK1 activity**

This study provides a model to explain the differential regulation of cytoplasmic and nuclear CDK1 in a normal cycle. We speculate that, at interphase, the slow rate of CycB synthesis in the cytoplasm provides sufficient time for cytoplasmic CDK1 inhibitors, such as Wee1 and Myt1, to prevent cytoplasmic CDK1 activation (Price et al., 2000; Stumpf et al., 2004; Jin et al., 2005). However, nuclear CDK1 inhibition is maintained by controlling CycB nuclear accumulation. At interphase, the rate of CycB nuclear translocation is low because of inhibition of nuclear CDK1 by Wee1 and Grp-dependent inhibition of CycB transport. Upon completion of S phase, Grp and Wee1 activity decreases. This triggers cytoplasmic CDK1 activation and initiation of cytoplasmic mitosis. This decrease in Grp and Wee1 activity also allows the abrupt CycB nuclear translocation catalyzed by nuclear CDK1 activity, thus increasing CDK1 activity to a level sufficient for triggering NEB.

**Materials and methods**

*Drosophila* stocks

The fly stocks GFP-H2Av (Clarkson and Saint, 1999), GFP-moesin (Edwards et al., 1997), RLC-GFP (Royou et al., 2004), GFP-Nuf (Riggs et al., 2007), GFP-Polo (Moutinho-Santos et al., 1999), and GFP-Cid (Schuh et al., 2007) were used in these studies. The GFP embryos were derived from a male homozygote for the *grp* null allele (Fogarty et al., 1994). The wee1 embryos were derived from females carrying the null allele *d wee1* 1st over Df(2L) dwee1 1nd deficiency (Price et al., 2000).

**GFP-Grp cloning**

GFP-Grp-bearing transgenics were constructed by ligating a full-length *grp* cDNA into pEGFP-C3 (Clontech Laboratories, Inc.) upstream of the GFP.
construct. The GFP-Grp subcloned was ligated into the Germ-10 transformation vector. Transgenics were generated by standard methods.

**CycB purification**

Two independent GST-CycB purifications were performed and produced identical results. CycB cDNA was cloned at its N-terminal to GST in pGEX-1 vector. The recombinant protein was overexpressed and purified from 4 liters of XLI-10 Escherichia coli grown in 2XYT (trypose, yeast extract, and NaCl). The culture was grown at RT until it reached an OD of 0.8. The cells were induced with 100 μM IPTG for 3 h at 26 °C. The pellets were ground in liquid nitrogen for 15 min. The powder was dissolved in 5 vol PBS that contained 0.5% Tween 20, 1 mM PMSE, and 1 mM NaCl. The solution was sonicated three times for 20 s. 10 mM DTT was added to the solution before its ultracentrifugation at 40,000 rpm for 1 h in a 60.7T rotor (Beckman Coulter). The supernatant was loaded onto a 9-mL glutathione-agarose column (Sigma-Aldrich) for a period of 2–4 h. The column was extensively washed with PBS, pH 7.4, 1 mM NaCl, 0.05% Tween 20, and 0.5 mM DTT and rinsed with a 1-vol column with wash buffer without Tween 20. The protein was eluted with 50 mM Tris, pH 8.1, containing 500 mM KCl and 5 mM of reduced glutathione. The fractions were pooled and dialyzed extensively into 50 mM Hepes, pH 7.6, 500 mM KCl, and 30% glycerol. In all of the experiments shown in Figs. 1–4, CycB was injected at a concentration of 65 μM. A recent study used such purified recombinant GST-CycB and has demonstrated that it retains its ability to bind and activate CDK1 in the presence of 65 μM DTT and 0.5% Tween 20.

Two independent GST-CycB purifications were performed and produced identical results. CycB cDNA was cloned at its N-terminal to GST in pGEX-1 vector (Edgar et al., 1994). Another recent study has shown that GST-CycB is resistant to degradation in Xenopus embryo extracts (Su et al., 1998). Our studies have focused on mitotic entry, not exit, so the fact that it is not degraded should not affect the interpretation of our experiments.

**CycB labeling**

CycB was coupled with NHS-rhodamine (Thermo Fisher Scientific) in a 1:4 ratio. The solution was mixed gently and sat at RT for 75 min. The solution was loaded onto a 3-mL Sephacryl G-25 column (GE Healthcare). The column was washed with 50 mM Hepes, pH 7.6, 500 mM KCl, and 30% glycerol. The absorbance of the fractions was measured with a Nanodrop (Eppendorf). The stoichiometry of the labeling was calculated as 0.6 mol NHS-rhodamine per mole of CycB.

**Microscopy**

Microscopy was performed at RT with an inverted microscope (DMIRB; Leica) equipped with a scanning laser confocal imaging system (TCS NT; Leica). Images were acquired with a 63× lens and confocal software (version 2.61; Leica). All embryos were monitored at three different z positions every 30 s for various amounts of time. Each image in all figures represents a single focal plane.

**Microinjection**

The embryos were prepared for microinjection as follows. They were dehydrated and covered with halocarbon oil. The embryos were injected at the center lengthwise and one third heightwise toward the surface. The same hole was used for double and triple injections. The embryos were injected at the center lengthwise and one third heightwise toward the surface. The same hole was used for double and triple injections. The embryos were monitored within 30 s after injection. Injection of CycB at 31.6 μM did not have a significant effect on timing of cytoplasmic and nuclear entry into mitosis in wild-type embryos (n = 6 for each concentration). Aphi and Chk (Sigma-Aldrich) were used at final concentrations of 300 μM and 1 mg/ml, respectively, in 2% DMSO and injected either on their own or mixed together during mitosis. CycB-R was injected at 13, 56, or 100 μM. Roscovitine and RO-3306 (EMD) were injected at 10 μM in 100% DMSO. Injection of 100% DMSO was used as a control. Rhodamine-conjugated tubulin (cytoskeleton) and Cy5-labeled histone (provided by E. Homola, University of Alberta, Edmonton, Canada) were injected at interphase of the previous cycle. The quantification of RLC-GFP signal was performed by drawing two regions of interest of the same size, one near the site of CycB injection and one in a region not affected by CycB injection. The mean pixel intensity for each region was calculated using the Leica software.

**Single-embryo Western blot and determination of the amount of injected CycB**

RLC-GFP embryos were prepared for microinjection as described in the previous paragraph. They were monitored with the confocal microscope until they reached interphase of cycle 13. They were injected with 0.5 mM colchicine and monitored until they reached metaphase. Each embryo was either collected and boiled in 10 μl of sample buffer or injected with 65 μM CycB and immediately collected and boiled in 10 μl of sample buffer. The samples were loaded on a 10% acrylamide gel and transferred onto the nylon membrane, and the membrane was probed with anti-CycB to reveal endogenous CycB and GST-CycB. Membranes were also probed with anti-GFP (Roche) to reveal RLC-GFP as a loading control. As expected, the injected embryo extract reveals an additional higher molecular weight GST-CycB band (Fig. 1 D, injected CycB). The intensities of the endogenous and injected CycB bands were estimated to be equivalent. Because CycB is injected at the center of the embryo lengthwise and diffuses very slowly through a fifth of the embryo, we estimated that, at the center of the gradient, the injected CycB is <10-fold the amount of endogenous CycB at mitosis of cycle 13.

**Online supplemental material**

Fig. S1 shows RLC-GFP signal intensities over time in control and CycB-injected areas of embryos previously injected or not injected with CHX and Aph. Fig. S2 shows a sagittal view of an embryo expressing RLC-GFP injected with CycB 8 min after the onset of cellularization. Fig. S3 shows the dynamics of Grp-GFP from cycle 13 through cycle 14 in an embryo previously injected with rhodamine-tubulin. Video 1 corresponds to the embryo presented in Fig. 1 C. Video 2 corresponds to the embryo presented in Fig. 2 B. Video 3 corresponds to the embryo presented in Fig. 2 C. Video 4 corresponds to the embryo presented in Fig. 3 B. Video 5 corresponds to the embryo presented in Fig. 6 A. Video 6 corresponds to the embryo presented in Fig. 6 B. Video 8 corresponds to the embryo presented in Fig. 6 E. Video 9 corresponds to the embryo presented in Fig. 7 B, where only CycB-R is visualized. Video 10 corresponds to the embryo presented in Fig. 7 C, where only CycB-R is visualized. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200801153/DC1.

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