Cdk1 and Okadaic Acid-sensitive Phosphatases Control Assembly of Nuclear Pore Complexes in Drosophila Embryos

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Submitted July 18, 2005; Revised August 16, 2005; Accepted August 17, 2005
Monitoring Editor: Karsten Weis

Disassembly and reassembly of the nuclear pore complexes (NPCs) is one of the major events during open mitosis in higher eukaryotes. However, how this process is controlled by the mitotic machinery is not clear. To investigate this we developed a novel in vivo model system based on syncytial Drosophila embryos. We microinjected different mitotic effectors into the embryonic cytoplasm and monitored the dynamics of disassembly/reassembly of NPCs in live embryos using fluorescently labeled wheat germ agglutinin (WGA) or in fixed embryos using electron microscopy and immunostaining techniques. We found that in live embryos Cdk1 activity was necessary and sufficient to induce disassembly of NPCs as well as their cytoplasmic mimics: annulate lamellae pore complexes (ALPCs). Cdk1 activity was also required for keeping NPCs and ALPCs disassembled during mitosis. In agreement recombinant Cdk1/cyclin B was able to induce phosphorylation and dissociation of nucleoporins from the NPCs in vitro. Conversely, reassembly of NPCs and ALPCs was dependent on the activity of protein phosphatases, sensitive to okadaic acid (OA). Our findings suggest a model where mitotic disassembly/reassembly of the NPCs is regulated by a dynamic equilibrium of Cdk1 and OA-sensitive phosphatase activities and provide evidence that mitotic phosphorylation mediates disassembly of the NPC.

INTRODUCTION

The double lipid membrane of the nuclear envelope (NE) forms a border between the nucleus and the cytoplasm. The nuclear pore complexes (NPCs) are multiprotein channels located at the fusion points between the outer and inner nuclear membrane. Directional signal-mediated transport of macromolecules and free diffusion of small molecules between the nucleus and the cytoplasm occur through the NPCs. The framework of the NPC has an evolutionary conserved octagonal channel structure spanning the NE and consists of multiple copies of ~30 different proteins called nucleoporins (Rout et al., 2000; Cronshaw et al., 2002), most of which are conserved in higher eukaryotes (Mans et al., 2004). About a third of the nucleoporins contain phenylalanine-glycine (FG) repetitive domains, which are believed to be involved in the selective permeability of the NPC (Weis, 2003; Peters, 2005). In mammals the majority of the FG-nucleoporins are modified with O-linked N-acetyl-d-galactosamine residues (Snow et al., 1987), whose function is still unknown. In addition to NPCs some cell types, such as oocytes and early embryonic cells, also contain mimics of NPCs inserted in cytoplasmic annulate lamellae (AL) membranes (Kessel, 1992). The annulate lamellae pore complexes (ALPCs) have a similar structure and nucleoporin composition as the NPCs (Meier et al., 1995; Cordes et al., 1996; Imreh and Hallberg, 2000; Miller and Forbes, 2000).

During the open mitosis of higher eukaryotes the NPCs (as well as the ALPCs) disassemble into distinct soluble nucleoporin subcomplexes (Macaulay et al., 1995; Cordes et al., 1996; Belgareh et al., 2001), whereas the pore membrane proteins diffuse throughout the ER network (Yang et al., 1997; Imreh and Hallberg, 2000; Daigle et al., 2001). Disassembly of both types of pore complexes is accompanied by phosphorylation of a subset of the nucleoporins (Macaulay et al., 1995; Favreau et al., 1996; Ganeshan and Paraoaik, 2000; Belgareh et al., 2001). At mitotic exit the NPCs sequentially reassemble in the reforming NEs of the daughter nuclei (Bodoor et al., 1999; Belgareh et al., 2001; Daigle et al., 2001).

Mitotic events are controlled by a family of evolutionarily conserved cyclin-dependent kinases (Cdks), which are enzymatically active only in a complex with regulatory cyclin subunits (reviewed in Doree and Hunt, 2002). Cdk1, which forms an active kinase complex with B-type cyclins plays an essential and specific role in triggering the mitotic events (Riabowol et al., 1989; Beckhelling et al., 2003). The activity of Cdks is regulated by the synthesis and destruction of the cyclins as well as by activating and inhibitory phosphorylation (reviewed in Obaya and Sedivy, 2002). Interestingly, some of the nucleoporins phosphorylated during mitosis were shown to be substrates for Cdk1 (Macaulay et al., 1995;
Favreau et al., 1996; Ganeshan and Parnaik, 2000). However, at the moment very little is known about how the mitotic machinery controls disassembly and reassembly of the NPCs.

After fertilization the Drosophila embryo represents a multinucleated cell (syncytium) where embryonic nuclei rapidly (every 10 min) and synchronously divide for 13 rounds without undergoing cytokinesis (Foe and Alberts, 1983). Syncytial embryos contain an excessive pool of maternally contributed nucleoporins, which assemble into NPCs and ALPCs during interphases and disassemble during mitosis (Stafstrom and Staehelin, 1984; Kiseleva et al., 2001; Onischenko et al., 2004). From the 9th division cycle the nuclei are aligned 10–20 μm beneath the embryonal periphery where mitotic divisions can be easily followed directly in living embryos (Foe and Alberts, 1983). Here we used early Drosophila embryos as a model system to dissect the mechanism regulating mitotic disassembly/reassembly of the NPCs.

MATERIALS AND METHODS

Fly Stocks and Collection of Embryos

The Canton-S strain was used as a wild-type in all the experiments. For heat-inducible inactivation of Cdk1 we used the Cdk1ts (A171T) strain (Sigrist et al., 1995) obtained from Dr. Christian Lehner. The mutant fly stock png172 (Shamanski and Orr-Weaver, 1991; Fenger et al., 2000) was obtained from Dr. Terry Orr-Weaver. The embryos were collected at 22°C on agar plates, studied with dry yeast, and harvested according to (Allis et al., 1977). The embryos were batch-dechorionated with Clorox according to (Elgin and Hood, 1973). For time-lapse microcopy of live embryos they were manually dechorionated on a scotch tape.

Immunostaining

Except otherwise mentioned all the procedures were performed at RT. Untreated png embryos (see Figure 3) were fixed in the equal volumes of n-heptane and −70°C methanol. Microinjected png embryos (see Figure 6) were fixed for 20 min in 1:1 mixture of n-heptane and 3.7% paraformaldehyde in phosphate-buffered saline (PBS) pH 7.4, and subsequently freed from vitelline membranes with thin needles. Fixed embryos were permeabilized for 1 h with a blocking solution (0.5% bovine serum albumin [BSA] in PBS containing 0.1% Tween-20) + 0.1% Triton X-100 and subsequently incubated overnight at +4°C in the blocking solution containing RL1 antibodies (Affinity Bioreagents, Golden, CO) diluted 1:5000 or anti-cyclin B antibodies F2F4 (Hybridoma Bank, Iowa City, IA) diluted 1:100, used as primary antibodies. The embryos were subsequently washed and incubated with 0.1 μg/ml WGA-Alexa594 (Molecular Probes, Eugene, OR) and 1:2000 dilution of FITC goat anti-mouse antibodies (Molecular Probes), used as secondary antibodies. For visualization of nuclei the embryos were treated for 1 h with 1 mg/ml RNase-A in PBS-T (0.1% Tween-20 in PBS), followed by staining with 15 μg/ml propidium iodide. The embryos were mounted in Vectashield media (Vector Laboratories, Burlingame, CA) and imaged as described in Microinjections and Time-lapse Microscopy but using Leica PL APO 635/1.32 oil objective (Deerfield, IL).

Western Blotting

The proteins, separated by SDS-PAGE, were transferred to nitrocellulose membranes. The membranes were incubated with anti-cyclin B antibodies diluted 1:300, RL1 antibodies diluted 1:1000, or a mixture of anti-gp210 antibodies AGP-26 and AGP-78 (Hybridoma bank) diluted 1:20 in PBS-T containing 5% dry milk. After washes the membranes were probed with HRP-conjugated goat anti-rabbit or sheep anti-mouse antibodies (Amersham-...
Figure 2. Effects of cycloheximide and cyclin B on disassembly of NPCs and ALPCs in wild-type syncytial embryos. (A) Western blot analysis of cyclin B in embryonal lysates 20 min after microinjection of cycloheximide or buffer. Equal amounts of protein were separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with antibodies specific for Drosophila cyclin B. Ponceau-S staining was used as a loading control. (B and Supplementary Video 2) Time-lapse confocal microscopy of syncytial embryos injected with cycloheximide. WGA-Alexa594-labeled nuclear rims and cytoplasmic foci persisted for more than 20 min, indicating that ALPCs and NPCs failed to disassemble. Note that the embryonic nuclei did not divide, but moved out of the focal plane. This is better appreciated in the corresponding video (Supplementary Video 2). (C–G) EM analysis of cycloheximide-injected embryos. All cycloheximide injected embryos (n = 6) contained typical interphase nuclei (C) with intact NPCs (D) and abundant cytoplasmic AL (E) with normal ALPCs (F). (G) EM morphometric quantification of ALPCs. The vertical bars show the total number of ALPCs in six randomly selected cycloheximide-injected embryos and in four buffer-injected interphase embryos (control), respectively. Note that the embryos injected with cycloheximide contain more ALPCs (mean value, 7.72 × 10^6 ALPCs/embryo) than the buffer-injected interphase embryos (mean value, 4.07 × 10^6 ALPCs/embryo). (H and Supplementary Video 3) Time-lapse confocal microscopy of syncytial embryos injected with cycloheximide together with GST-cyclin B. WGA-Alexa594 displayed a permanent uniform distribution characteristic for mitotic embryos with disassembled pore complexes. (I–K) EM analysis of embryos coinjected with cycloheximide and GST-cyclin B. After 20-min incubation all cycloheximide + GST-cyclin B injected
Microinjections and Time-lapse Microscopy

The embryonic nuclei were purified from fresh 3–7-h old embryos essentially according to Zalokar and Erk (1977), except that for homogenization of embryos in embryos injected with cycloheximide, we microinjected WGA-Alexa594 (0.1 mg/ml), resulting in a final concentration of WGA inside the embryo of ~0001–0005 mg/ml, which is far below the concentrations reported to affect nuclear import and pore complex assembly (Finlay et al., 1987; Dubavalle et al., 1990).

Electron Microscopy and EM-Morphometry

The embryos, fixed and stained en bloc with uranyl acetate according to Zalokar and Erk (1977), were dehydrated with ethanol and embedded individually into Agar-100 according to the standard protocol. The ultrathin sections were stained with uranyl acetate followed by lead citrate and examined with a JEM-100 electron microscope (JEOL, Peabody, MA).

For morphometric analysis, individual embryos were sectioned in the equatorial plane. To determine the total number of ALPCs per embryo, we counted the number of two ALPCs per nucleus in each section and multiplied it by the number of nuclei per embryo. The total number of ALPCs in the embryos was calculated by multiplying V with WD.

In Vitro Assay for NPC Disassembly

The embryonic nuclei were purified from fresh 3–7-h old embryos essentially as described in Fisher et al. (1982), except that for homogenization of embryos and washes of nuclei we used 50 mM Tris-HCl, pH 7.4, and 5 mM MgCl2, and kept on ice. Two microliters of the freshly prepared nuclear suspension was mixed with 30 µl of the reaction buffer (50 mM KCl, 40 mM MgCl2, 10 mM EGTA, 5 mM MgCl2, 2 mM DTT, 0.2% BSA, 20 mM Pipes, pH 7.4, with the addition of 1/20 volume of ATP-regenerating system). The disassembly reaction was performed for 1 h at RT in the presence of 1 µg (10 µl) of active recombinant human Cdk2-cyclin B complex (Upstate Biotechnology, Lake Placid, NY). The centrifugation was centrifuged at 100,000 g in a fixed-angle rotor for 10 min at 4°C. The supernatants were mixed 1:1 with sample buffer. The pellets were resuspended and diluted in the reaction buffer to the equal volumes as supernatants and mixed 1:1 with sample buffer.

RESULTS

Microinjected Fluorescently Labeled WGA Can Be Used as an In Vivo Marker for NPCs and ALPCs in Drosophila Embryos

Previously we have shown (Onischenko et al., 2004) that wheat germ agglutinin (WGA) specifically bound to only one Drosophila NPC protein, NUP58, and perfectly colocalized with two well-established monoclonal nucleoporin marker antibodies (mAb414; Davis and Blobel, 1986 and RL1; Snow et al., 1987). In addition, microinjected gold-labeled WGA specifically decorated the central portion of the NPCs and ALPCs in syncytial embryos analyzed by EM. We were interested in the exploitation of WGA as marker for NPCs and ALPCs in live Drosophila embryos. For this, we microinjected 0.1 mg/ml fluorescently labeled WGA (WGA-Alexa594) into live early embryos and followed its distribution with time by confocal laser scanning microscopy. During interphase syncytial embryos displayed brightly labeled NPCs and cytoplasmic foci (Figure 1A and Supplementary Video 1) in agreement with the distribution of WGA to the NPCs and the ALPCs. The nuclear rim staining and the dotted pattern in the cytoplasm synchronously disappeared during mitosis and reappeared in telophase, consistent with the timing of disassembly and reassembly of NPCs and ALPCs (Staehelin et al., 1984; Onischenko et al., 2004). Usually the staining of the NEs was more intense than the staining of the cytoplasmic foci either because of lower levels of NUP58 or lower accessibility for WGA in the AL membrane stacks. Microinjection buffers containing 0.1 mg/ml WGA-Alexa can be safely used for getting a quick qualitative assessment of disassembly/reassembly of NPC and ALPC in live Drosophila embryos, since we did not observe any abnormalities in nuclear import (Figure 1B), timing of mitotic divisions, or embryonic development (unpublished data). At 0.2 mg/ml some effects on nuclear import and nuclear growth were observed locally at the area of injection.

Mitotic Cyclins Are Required for Disassembly of NPCs and ALPCs in Syncytial Drosophila Embryos

In a previous study we reported that the major fraction of excess nucleoporins in syncytial Drosophila embryos remains soluble and that one of the nucleoporins (p150) is hyperphosphorylated (Onischenko et al., 2004). Because syncytial embryos are known to display high levels of mitotic cyclins and Cdk1 activity (Edgar et al., 1994), we were interested in investigating how mitotic factors influence disassembly/reassembly of pore complexes. We microinjected syncytial embryos with the protein synthesis inhibitor cycloheximide, reported to cause depletion of mitotic cyclins (Edgar et al., 1994). As expected, cycloheximide quickly reduced the level of cyclin B (Figure 2A). Cycloheximide also completely blocked disassembly of NPCs and ALPCs in the injected embryos as shown using time-lapse microscopy (Figure 2B...
and Supplementary Video 2). This conclusion was supported by EM analysis because all cycloheximide-injected embryos (n = 6) contained typical interphase nuclei with intact NPCs (Figure 2, C and D) and abundant ALPCs (Figure 2, E and F). Quantification of ALPCs using EM-morphometry showed that cycloheximide-injected embryos actually contained more ALPCs (about twofold) than interphase control embryos, injected with buffer (Figure 2G). The effect of cycloheximide was primarily due to depletion of mitotic cyclins, since it could be completely reversed by coinjection with nondegradable recombinant Drosophila GST-cyclin B (Su et al., 1998) resulting in persistently disassembled NPCs and ALPCs as judged by time-lapse microscopy of live embryos (Figure 2H and Supplementary Video 3), by EM analysis (Figure 2I and J) and morphometric quantification of ALPCs (Figure 2K). Furthermore, cycloheximide injection also caused massive dephosphorylation of the nucleoporin p150, which is normally dephosphorylated when assembled into NPCs and ALPCs during interphase (Onischenko et al., 2004). The cycloheximide-dependent dephosphorylation of p150 was completely abolished by coinjection of GST-cyclin B (Figure 2L). The results indicate that in normal syncytial embryos disassembly of NPCs and ALPCs is dependent on mitotic cyclins.

Cdk1 Activity Mediates Disassembly of NPCs and ALPCs in Syncytial Drosophila Embryos

To further dissect the mechanism behind pore complex disassembly in Drosophila embryos we used the cyclin-deficient png mutants. As expected, time-lapse movies of buffer-injected png embryos displayed abundant persistently disassembled NPCs and ALPCs as judged by time-lapse microscopy of live embryos (Figure 4A and Supplementary Video 4), which quickly disassembled after microinjection with GST-cyclin B (Figure 4B and Supplementary Video 5). Morphometric analysis of embryos which were fixed and prepared for EM (Figure 4C) showed that the number of ALPCs were 15-fold lower in png embryos injected with GST-cyclin B (mean value, 0.73 × 10⁶ ALPCs/embryo) than that of buffer-injected png control embryos (mean value, 12.1 × 10⁶ ALPCs/embryo). The cyclin B-induced disassembly of ALPCs was completely blocked by coinjection with the specific Cdk inhibitor roscovitine (Figure 4D), indicating that in cyclin-deficient embryos exogenous cyclin B is able to promote disassembly of pore complexes by activating Cdk1 (Campbell et al., 1995; Jacobs et al., 1998).

This led us to investigate the role of Cdk1 in regulation of pore complex disassembly/reassembly. We took advantage of embryos, bearing a point mutation in Cdk1, which makes the enzyme thermosensitive (Sigrist et al., 1995). The activity of Cdk1 can be efficiently blocked in the Cdk1 ts embryos by raising the temperature from 22 to 30°C. Time-lapse movies of WGA-Alexa injected Cdk1 ts embryos showed that at 30°C NPCs and ALPCs did not disassemble (Figure 4E, top row, of ALPCs in png mutants was even higher than in the cycloheximide-injected embryos (for EM-morphometry; see Figure 4C). The large quantity of persistently assembled ALPCs in cyclin-deficient png mutants supports the idea that disassembly of NPCs and ALPCs requires mitotic cyclins.

Figure 3. Characterization of NPCs and ALPCs in png mutant embryos. (A) Confocal images of a 0–4-h old homozygous png mutant embryo fixed and immunostained with WGA and monoclonal RL1 antibodies. In all embryos (n > 100) WGA and RL1 specifically colocalized at the NEs of a few giant nuclei (labeled with “n”) and at multiple large cytoplasmic foci. (B) Fixed embryo double-stained with WGA (green) and propidium iodide (red) to visualize chromatin of giant nuclei. (C–F) EM analysis of png embryos. A portion of the NE of a giant nucleus (C) with apparently intact NPCs (D). The cytoplasm of the embryos was filled with giant stacks of AL (E) containing densely packed ALPCs (F). Scale bars, (A) 20 μm; (B and D) 5 μm; (C and E) 0.5 μm.
Figure 4. Effects of cyclin B and Cdk1 on disassembly of NPCs and ALPCs. (A, B, and D) Time-lapse microscopy of ALPC assembly in png mutant embryos visualized with WGA. Embryos, 0–4 h old, derived from homozygous png mutant females were microinjected with buffer (A), GST-cyclin B (B), or GST-cyclin B + roscovitine (D). ALPCs failed to disassemble in buffer-injected control embryos (A and Supplementary Video 4), but totally dispersed 10 min after injection with GST-cyclin B (B and Supplementary Video 5). (C) EM morphometric analysis of the number of ALPCs in embryos injected with buffer or GST-cyclin B, respectively. Disassembly was completely suppressed in embryos coinjected with GST-cyclin B and the Cdk inhibitor roscovitine (D). (E) Assembly of NPCs and ALPCs in live Cdk1ts mutant embryos was monitored by time-lapse confocal microscopy of WGA distribution. At 30°C, when Cdk1 is inactivated, disassembly of NPCs and ALPCs is suppressed (E, top row, and Supplementary Video 6). By contrast, in Cdk1ts embryos at 22°C (E, middle row, and Supplementary Video 7), as well as in wild-type embryos incubated at 30°C (E, bottom row), NPCs and ALPCs normally disassembled. (F–H) EM analysis showing a typical nucleus (F) of a CDK1ts embryo incubated for 20 min at 30°C and an enlarged view a portion of the NE (G), with intact NPCs (arrows). (H) A representative image of cytoplasmic AL with intact ALPCs (arrows). (I) EM morphometric analysis of five randomly selected embryos from each group, showing that embryos with inactivated Cdk1 contained substantially more ALPC than any of the control embryos, indicating that ALPC disassembly is suppressed. Scale bars, (F) 1 μm; (G and H) 0.5 μm.
and Supplementary Video 6). The inability to disassemble was specifically due to inactivation of Cdk1 because the pore complexes disassembled and reassembled normally in Cdk1ts embryos incubated at 22°C (Figure 4E, middle row and Supplementary Video 7) and wild-type embryos incubated at 30°C (Figure 4E, bottom row). At the EM level all of the Cdk1ts embryos at 30°C (n = 6) had intact NEs with assembled NPCs (Figure 4F and G) and ALPCs (Figure 4H). The quantity of ALPCs in all Cdk1ts embryos at 30°C (n = 5) was substantially higher than in any of the wild-type embryos at 30°C (n = 5) or Cdk1ts mutants at 22°C (n = 5; Figure 4I). On the basis of our findings we conclude that Cdk1 activity is critically required for disassembly of pore complexes in Drosophila embryos.

To find out if the Cdk1 activity is only required to initiate disassembly, or if it is also necessary for keeping pore complexes in a disassembled state during mitosis, we first blocked embryonic division cycles in Cdk1ts mutants or wild-type embryos (control) in mitosis by microinjection of either colchicine (Foe et al., 2000) or GST-cyclin B (Su et al., 1998). After 15 min of incubation, when nuclear divisions were arrested in mitosis and both NPCs and ALPCs were disassembled, we raised the temperature to 30°C and monitored pore complex reassembly by time-lapse microscopy of live embryos. In both cases we observed massive reassembly of the NPCs and ALPCs in Cdk1ts embryos 5–10 min after shifting to the restrictive temperature, whereas wild-type embryos remained unaffected (Figure 5 and Supplementary Video 8). The results show that Cdk1 activity is continuously required in order to keep NPCs and ALPCs disassembled during mitosis.

**Purified Recombinant Cdk1-Cyclin B Complex Is Able to Dissociate Nucleoporins from Purified Embryonic Nuclei In Vitro**

To further dissect the mechanism behind Cdk1-dependent disassembly of pore complexes, we injected one pole of the cyclin-deficient png mutant embryos with GST-cyclin B followed by immediate fixation and immunostaining to study the relation between distribution of cyclin B, ALPCs, and NPCs by immunofluorescence microscopy. The propagation of the cyclin B diffusion zone strictly correlated with the sharp border between diffuse WGA staining and fluorescing...
nuclear rims and cytoplasmic foci (Figure 6), demonstrating that local cyclin B-induced Cdk1 activity is able to promote efficient disassembly of both NPCs and ALPCs.

This led us to examine if Cdk1 activity is able to directly dissociate nucleoporins from pore complexes. For this, we thoroughly purified embryonic nuclei from cytoplasmic components and incubated them with pure recombinant Cdk1-cyclin B and the specific Cdk1 inhibitor roscovitine, as indicated. After centrifugation at 100,000 × g, equal amounts of the supernatant (S) and pellet (P) fractions were separated by SDS-PAGE and analyzed by Western blotting. WGA and monoclonal RL1 antibodies were used to detect nucleoporins, whereas antibodies specific for gp210 were used to control for pelleting of the fraction still associated with the NE membranes. Note that Cdk1 activity was able to induce phosphorylation of p150 (arrows) as well as dissociation of p150 and four other nucleoporins from the nuclear membrane fraction.

Figure 7. Recombinant Cdk1-Cyclin B can phosphorylate p150 and dissociate nucleoporins from the purified Drosophila nuclei in vitro. Purified embryonic nuclei were incubated at room temperature for 0 or 60 min in the absence or presence of human recombinant Cdk1-cyclin B and the specific Cdk1 inhibitor roscovitine, as indicated. After centrifugation at 100,000 × g, equal amounts of the supernatant (S) and pellet (P) fractions were separated by SDS-PAGE and analyzed by Western blotting. WGA and monoclonal RL1 antibodies were used to detect nucleoporins, whereas antibodies specific for gp210 were used to control for pelleting of the fraction still associated with the NE membranes. Note that Cdk1 activity was able to induce phosphorylation of p150 (arrows) as well as dissociation of p150 and four other nucleoporins from the nuclear membrane fraction.

Assemble of NPCs and ALPCs Depends on OA-sensitive Protein Phosphatases(s)

The requirement for persistently active Cdk1 for keeping pore complexes disassembled during mitosis, imply the existence of a reverse acting protein phosphatase(s), which continuously drive reassembly of NPCs and ALPCs. Because two major protein phosphatases, PP1 and PP2A, have been implicated in dephosphorylation of many Cdk1 substrates, we decided to investigate their possible role in driving NPC and ALPC reassembly. For this purpose we microinjected embryos with okadaic acid (OA), which specifically and potently blocks phosphatases PP2A and PP1 (Takai et al., 1987; Bialojan and Takai, 1988). Reassembly of NPCs and ALPCs was prevented in OA-injected Cdk1 ts mutant embryos incubated at 22°C (Figure 8A and Supplementary Video 9) as well as in OA-injected wild type embryos (unpublished data). To exclude the possibility that OA prevents reassembly of the pore complexes by affecting Cdk1 activity, we pretreated Cdk1 ts embryos with OA for 15 min at 22°C to allow complete disassembly and then inactivated Cdk1 at 30°C. This did not induce reassembly of ALPCs and NPCs (Figure 8B and Supplementary Video 10), demonstrating that reassembly of NPCs and ALPCs requires OA-sensitive protein phosphatase activity.

If the OA-sensitive protein phosphatases continuously drive reassembly of pore complexes during mitosis, one
would expect OA treatment to induce disassembly of pore complexes when limited Cdk1 activity is normally insufficient to drive this process. This was indeed the case in png mutant embryos (which are deficient in Cdk1 activity compared with normal embryos) where microinjection of OA immediately induced massive disassembly of ALPCs (Figure 8C, top row). As expected this effect was suppressed by roscovitine (Figure 8C, bottom row). Taken together our findings suggest a model where the activities of Cdk1 and OA-sensitive phosphatase(s) antagonistically regulate disassembly and reassembly of pore complexes during mitosis.

**DISCUSSION**

*Dissection and Reassembly of the NPC*

Most of the available data concerning postmitotic assembly of the NPC have been produced using a powerful in vitro model system based on *Xenopus* egg extracts (Lohka and Masui, 1983). However, complete understanding of this process requires complementary in vivo experimental system(s). Such systems should preferentially be based on one of the available genetic model organisms and should be easy to monitor and manipulate. Developing early *Drosophila* embryos very well fit these requirements. From an experimental perspective syncytial embryos can be regarded as a sample in a test tube containing a pool of excessive maternally contributed nucleoporins, which rapidly and synchronously oscillate between a disassembled state and a state of assembly in NPC and ALPC structures, respectively. Even during interphase in normal syncytial embryos the nucleoporins are only incompletely assembled (Onischenko et al., 2004), making it possible to affect the system in the direction of increased or decreased assembly by manipulation of factors controlling the assembly and disassembly processes. The extent of pore complex assembly can be directly monitored in live embryos injected with fluorochrome-labeled WGA using time-lapse microscopy or visualized by immunofluorescence microscopy, by EM or quantified using EM-morphometric analysis. Assembly of NPCs and ALPCs in this system was sensitive to classical inhibitors of NE formation and pore complex assembly, such as BAPTA, GTPγS, and NEM (unpublished data). Thus, the syncytial *Drosophila* embryo model appears to be compatible with other higher eukaryotic models and, as demonstrated in this article, allows design of experiments to “push” the process of NPC disassembly/reassembly in either direction by microinjection of inhibitors or recombinant proteins or by using mutant fly strains.

*Cdk1 and OA-sensitive Protein Phosphatases Are Mediators of Mitotic Pore Complex Disassembly/Reassembly*

Cdk1 is a key mediator of pore complex disassembly, because selective inactivation of Cdk1 abolished disassembly of NPCs and ALPCs (Figure 4E), whereas activation of Cdk1 induced disassembly of NPCs and ALPCs (Figure 4B). According to our in vitro experiments recombinant Cdk1-cyclin B was able to induce hyperphosphorylation of the *Drosophila* nucleoporin p150 and dissociation of p150 together with at least four other nucleoporins from the NPCs of the purified nuclei. It is tempting to speculate that Cdk1-mediated phosphorylation of nucleoporins disrupts the interactions holding the structure together. This view is supported by the requirement for dephosphorylation of nucleoporins for assembly of pore complexes in vitro (Walther et al., 2003). Although our data do not prove that Cdk1 phosphorylates nucleoporins directly, this view is consistent with the recently reported localization of Cdk1-cyclin B complex at the ALPCs just before the NE breakdown in *Xenopus* oocytes (Beckhelling et al., 2003). Furthermore, in the filamentous fungus *Aspergillus nidulans*, which undergo closed mitosis, phosphorylation of a subset of nucleoporins was also shown to correlate with dissociation from the NPC, whereas the NPC remained functional (De Souza et al., 2004). In this case phosphorylation of nucleoporins is carried out by the downstream serine/threonine kinase NIMA, which is activated by Cdk1. Although overexpression of NIMA apparently can induce NPC disassembly in mammalian cells (Lu and Hunter, 1995), attempts to identify a NIMA homologue in higher eukaryotes have been unsuccessful. It is possible that NIMA has a specialized function in reorganization of pore complexes in organisms undergoing closed mitosis. It should be pointed out that our data do not exclude the possibility that other kinases along with Cdk1 may also be involved in pore complex disassembly.

The reversibility of the *Drosophila* embryo model also allowed us to investigate the role of protein phosphatases in pore complex reassembly. Microinjection of OA at a concentration that specifically inhibits two major protein phosphatases, PP1 and PP2A, induced rapid disassembly of NPCs and ALPCs, suggesting that either or both of these enzymes are involved in postmitotic reassembly of the pore complexes. The involvement of PP1 and/or PP2A in pore complex reassembly is consistent with a number of evidence of their role in promoting postmitotic changes in the cell. For instance, in cultured cells excess PP1 promoted mitotic exit, whereas microinjection of anti-PP1 antibodies led to mitotic arrest (Fernandez et al., 1992). Additionally, PP1 was implicated in postmitotic reassembly of the nuclear lamina (Thompson et al., 1997; Steen et al., 2000), whereas PP2A was shown to play an important role in postmitotic reassembly of the Golgi (Lowe et al., 2000).

Taken together our studies define Cdk1 and OA-sensitive protein phosphatases as the key regulators of pore complex disassembly/reassembly. However this by no means excludes involvement of other factors. Two recent studies performed using the *Xenopus* in vitro assembly system identified Importin-β as a negative regulator of pore complex assembly (Harel et al., 2003; Walther et al., 2003). The latter study also identified Ran-GTP as a positive regulator of this
process possibly acting via release of Importin-β from the nucleoporin subcomplexes (Walther et al., 2003). Consistent with this our preliminary studies indicated that RanT24N and the nucleoporin-binding domain of importin-β both inhibited assembly of NPCPs and ALPCPs in live syncytial embryos (unpublished results).

**Disassembly/Reassembly of Pore Complexes Is Regulated by a Dynamic Equilibrium between the Activities of Cdk1 and OA-sensitive Protein Phosphatases**

The oscillation of pore complexes between a disassembled and an assembled state during syncytial nuclear divisions enables determination of dynamic aspects of pore complex disassembly/reassembly. Using this advantage, we were able to demonstrate that: 1) inactivation of Cdk1 during mitosis results in immediate reassembly of NPCPs and ALPCPs (Figure 5); 2) reassembly of NPCPs and ALPCPs is blocked by OA, a specific PP1/PP2A inhibitor (Figure 7, A and B); 3) in pnmt embryos, displaying persistently assembled NPCPs and ALPCPs (due to low endogenous Cdk1 activity, which is insufficient to induce pore complex disassembly), OA treatment induces immediate disassembly of NPCPs and ALPCPs (Figure 7C). Together these findings suggest a model where disassembly and reassembly of pore complexes during mitosis is regulated by a dynamic equilibrium of the antagonistic actions of Cdk1 and OA-sensitive phosphatases (Figure 9).

**ACKNOWLEDGMENTS**

We thank Dr. Douglass Kellogg for the *Drosophila* GST-cyclin B expression construct, Dr. Christian Lehner for the Cdk1 (A171T) fly strain, and Dr. Terry Orr-Weaver for the png mutant strain. This work was supported by grants (521-2003-5182 and 621-2003-3389) to E.H. from the Swedish Research Council (Sweden) and by grants to E.K. from the Russian Foundation of Basic Research (Russian Federation) and from the Welcome Trust (the United Kingdom).

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