Cyclin A- and Cyclin E-Cdk Complexes Shuttle between the Nucleus and the Cytoplasm

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Cyclins A and E and their partner cyclin-dependent kinases (Cdks) are key regulators of DNA synthesis and of mitosis. Immunofluorescence studies have shown that both cyclins are nuclear and that a proportion of cyclin A is localized to sites of DNA replication. However, recently, both cyclin A and cyclin E have been implicated as regulators of centrosome replication, and it is unclear when and where these cyclin-Cdks can interact with cytoplasmic substrates. We have used live cell imaging to study the behavior of cyclin/Cdk complexes. We found that cyclin A and cyclin E are able to regulate both nuclear and cytoplasmic events because they both shuttle between the nucleus and the cytoplasm. However, we found that there are marked differences in their shuttling behavior, which raises the possibility that cyclin/Cdk function could be regulated at the level of nuclear import and export. In the course of these experiments, we have also found that, contrary to published results, mutations in the hydrophobic patch of cyclin A do affect Cdk binding and nuclear import. This has implications for the role of the hydrophobic patch as a substrate selection motif.

INTRODUCTION

Successive waves of cyclin-dependent kinase (Cdk) activity control progress through the eukaryotic cell cycle. Cdks are activated by binding a member of the cyclin family and phosphorylation by Cdk-activating kinase. The cyclin-Cdks that have been most strongly implicated in controlling entry into, and progress through, DNA replication are cyclins A and E. Both cyclins bind to Cdk2 (Elledge and Spottswood, 1991; Tsai *et al.*, 1991; Koff *et al.*, 1992), and the levels of both cyclins are strictly regulated throughout the cell cycle, by transcriptional and proteolytic mechanisms. Cyclin E levels are primarily dictated by the rate of its transcription because it is an unstable protein that is rapidly degraded by two different pathways of ubiquitin-dependent proteolysis (Clurman *et al.*, 1996; Won and Reed, 1996; Singer *et al.*, 1999; Wang *et al.*, 1999; Winston *et al.*, 1999; Nakayama *et al.*, 2000). In contrast, cyclin A is stable until cells enter mitosis. Cyclin A levels first start to increase at the beginning of S phase and continue to rise throughout S and G2 phases until prometaphase, when they are rapidly degraded by ubiquitin-de-

pendent proteolysis (Pines and Hunter, 1990; Hunt *et al.*, 1992).

There is extensive evidence to indicate that the activation of cyclin E/Cdk2 leads to the initiation of DNA replication. Cyclin E/Cdk2 activity is maximal at G1/S (Koff *et al.*, 1992), replication of DNA in vitro is dependent on cyclin E/Cdk2 activity (Jackson *et al.*, 1995; Strausfeld *et al.*, 1996; Krude *et al.*, 1997) and, in vivo, cyclin E is essential for DNA replication in *Drosophila* (Sauer *et al.*, 1995). Cyclin A/Cdk2 has also been demonstrated to promote DNA replication (Girard *et al.*, 1991; Pagano *et al.*, 1992; Zindy *et al.*, 1992; Strausfeld *et al.*, 1996). However, cyclin A may be more significant in regulating progression through S phase (Connell-Crowley *et al.*, 1998), perhaps through mediating the nuclear export and degradation of Cdc6 (Saha *et al.*, 1998; Petersen *et al.*, 1999). Furthermore, in *Drosophila* embryos at the cellular blastoderm stage, cyclin E can promote S phase without cyclin A (Knoblich *et al.*, 1994), and cyclin A itself is located in the cytoplasm in S phase and only becomes nuclear during prophase (Lehner and O'Farrell, 1989).

There are also data indicating that at least cyclin A and perhaps cyclin E interact directly with the DNA replication machinery. Immunofluorescence studies have shown that both cyclin A and cyclin E are nuclear proteins (Girard *et al.*, 1991; Pines and Hunter, 1991; Ohtsubo *et al.*, 1995) and that cyclin A can be detected at sites of DNA replication in cells

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(Cardoso *et al.*, 1993; Ohtsubo *et al.*, 1995) and bound to the origin recognition complex in vitro (Romanowski *et al.*, 2000). Therefore, proteins at DNA replication origins are probably physiological substrates of either cyclin A/Cdk2 or cyclin E/Cdk2 (Hua and Newport, 1998). Thus, nuclear localization is crucial to the function of both cyclin A/Cdk2 and cyclin E/Cdk2; indeed, cyclin E has a classical nuclear localization sequence (NLS) that targets it to the nucleus via the well characterized importin- α /importin- β nuclear import pathway (Moore *et al.*, 1999).

More recently, cyclin A- and cyclin E-dependent kinases have been implicated in the control of centrosome replication. Mammalian somatic cell extracts require cyclin A-dependent kinase activity to duplicate centrosomes (Meraldi *et al.*, 1999), whereas *Xenopus* oocytes and early embryos require cyclin E-associated kinase activity (Hinchcliffe *et al.*, 1999; Lacey *et al.*, 1999). Cyclin E-associated kinase also phosphorylates nucleophosmin (Okuda *et al.*, 2000; Tokuyama *et al.*, 2001), a component of the centrosome, causing it to dissociate from the centrosome, which may allow the centrosome to duplicate. Thus, both cyclin E and cyclin A may regulate cell cycle events in both the nucleus and the cytoplasm, but they appear to be exclusively nuclear by immunofluorescence. This raises the question of whether the cyclin-Cdks are more dynamic in living cells or whether their cytoplasmic substrates must first enter the nucleus to be recognized by the kinases.

The behavior of the major mitotic cyclin-Cdk, cyclin B1-Cdk1, sets a precedent for dynamic behavior of cell cycle regulators. Cyclin B1 appears to be exclusively cytoplasmic by immunofluorescence, but this is a reflection of its slow nuclear import being counteracted by more rapid nuclear export. The slow nuclear import of cyclin B1 appears to be mediated by a direct association between cyclin B1 and importin- β , one of the most abundant nuclear import carriers. Cyclin B1 is exported by exportin 1, the primary protein export receptor. It binds directly to exportin 1, and its export is blocked by treating cells with leptomycin B (LMB), which covalently inactivates exportin 1 (Kudo *et al.*, 1999).

In contrast to cyclins E and B1, much less is known about how cyclin A is targeted to the nucleus. Neither cyclin A nor its partner kinase, Cdk2, has consensus NLSs. Using a series of deletion mutants, Nigg and colleagues demonstrated that the nuclear localization of cyclin A correlated with its ability to bind to a Cdk (Maridor *et al.*, 1993). In turn, the cyclin A/Cdk complex is known to bind a variety of proteins with a recognizable NLS, such as p107, E2F1, and the p21 and p27 proteins of the Kip/Cip family. Thus, it was suggested that cyclin/Cdk complexes might "piggy-back" into the nucleus by binding NLS-containing proteins (for discussion, see Galant *et al.*, 1995). A possible precedent for this is the observation that overexpressing members of the Kip/Cip family increase the amount of cyclin D1 imported into nuclei (Diehl and Sherr, 1997; LaBaer *et al.*, 1997). However, cyclin A is still correctly localized to the nucleus in cells lacking p21^{CIP1} (Deng *et al.*, 1995).

In this study, we used live cell imaging to show that cyclin A/Cdk2 and cyclin E/Cdk2 continuously shuttle between the nucleus and the cytoplasm. However, in contrast to cyclin B1/Cdk1, cyclin E and cyclin A are exported more slowly than they are imported, and their export is not inhibited by LMB. Using an in vitro import assay, we show

that cyclin A/Cdk2 import depends on binding to Cdk2 and that it does not piggy-back into the nucleus using NLS-containing proteins. Furthermore, the nuclear import of cyclins A, B1, and E are distinct, raising the possibility that each can be separately regulated at the level of nuclear transport.

MATERIALS AND METHODS

Construction of Chimeras

Chimeras were created between a modified form of green fluorescent protein (GFP)-MmGFP (Zernicka-Goetz *et al.*, 1996) and cyclin A, cyclin E, cyclin B1, and Cdk2. In each case, MmGFP was attached at the carboxyl terminus of the protein. For recombinant baculoviral constructs, the cyclin A and cyclin E fusion proteins were constructed by polymerase chain reaction. The stop codon was removed from the cyclin and replaced with an *EcoRI* site. This was ligated in frame to an *EcoRI* site 5' to the first residue of MmGFP, creating a glutamic acid-phenylalanine linker. Similarly, a four-amino acid linker (CPEF) was created between CDK2 and MmGFP. Cyclin B1 5E-GFP cloned into the pGEX2T expression vector had been previously described (Hagting *et al.*, 1998). Cyclin B1 with MKAIL mutation was fused at the C terminus via a AGAQF linker to the second amino acid of MmGFP. The cDNAs encoding the cyclin A and E fusion proteins were tagged at the 5' with an *XbaI* site and at the 3' with a *BglIII* site and cloned into *XbaI*-*BglIII*-digested pAcAB4 (Belyaev and Roy, 1993). The cDNA encoding cyclin B1-GFP was tagged with a *HindIII* site at the 5' and *XbaI* site at the 3' end and ligated into pCDNA3. The initiating methionine codon of CDK2-MmGFP cDNA was altered to a *NcoI* site (CCATGG) and the cDNA was cloned in frame with the (his)₆ tag of pRSET B (Invitrogen, Breda, The Netherlands). To construct the cyclin E^{R130A} and cyclin E^{R130K} mutants, the cDNA for human cyclin E^{R130A} (a kind gift of by Dr. B. Clurman, FHRC, Seattle, WA) or human cyclin E^{R130K} (E. Sahai and J.P., unpublished results) was altered by polymerase chain reaction to include a 5' *HindIII* site and 3' *EcoRI* site as above and used to replace the wild-type sequence in a pCMX-cyclin E-MmGFP construct. All constructs were sequenced by an ABI automated sequencer (Department of Biochemistry, University of Cambridge, Cambridge, UK).

Protein Expression and Purification

Sf9 cells were infected and coinfecting with recombinant baculovirus encoding human (his)₆-cyclin A, (his)₆-cyclin A-GFP, cyclin E-GFP, and human (his)₆-Cdk2^{K33R}, harvested 48 h postinfection, and lysed as described by Krude *et al.* (1997). Supernatants taken following centrifugation at 100,000 × g were partially purified via their (his)₆-tag using nickel-nitrilotriacetic acid Superflow (Qiagen, Crawley UK). Proteins were then dialyzed against 25 mM HEPES/KOH, pH 7.1, 150 mM NaCl, 5 mM β -mercaptoethanol in a slide-a-lyzer 10-kDa dialysis cassette (Pierce Chemical, Rockford, IL) and then purified by fast-performance liquid chromatography using a Mono-Q ion exchange column (Amersham Pharmacia Biotech UK, Little Chalfont, Buckinghamshire, UK). Cyclin A-GFP and cyclin E-GFP, with or without coexpressed Cdk2, were eluted from the Mono-Q column with 200 mM NaCl. Proteins were then concentrated to a final concentration of ~150 mM using Vivaspin concentrators, 50,000 molecular weight cut-off (Vivascience, Lincoln, UK) and stored in liquid N₂. (his)₆-Cdk2-GFP and (his)₆-Cks1 was expressed in *Escherichia coli* BL21 DE3(pLysS) at 30°C and purified by Ni²⁺ affinity chromatography followed by gel filtration through a Superdex 200 column. Glutathione S-transferase-cyclin B1 5E-GFP was expressed and purified as described by Hagting *et al.* (1998). Rch1, nucleoplasmin, and nucleoplasmin core domain, all tagged at the C terminus with (his)₆, were expressed and purified as described by Görlich *et al.* (1994). Human importin- β was expressed from a pQE60 vector (Qiagen) and purified from a bacterial lysate by anion

exchange and affinity chromatography on an immobilized importin- β -binding domain of *Xenopus* importin- α . Ran/TC4 was expressed with an N-terminal (his)₆-tag and purified as described by Görlich *et al.* (1994). Importin-binding domain (IBB) from *Xenopus* importin- α (gift of D. Görlich) was expressed and purified as described by Görlich *et al.* (1996). *Xenopus* importin- β Δ N 44 (gift of D. Görlich) was expressed and purified as described by Kutay *et al.* (1997). Ran/TC4 Q69L (gift of D. Görlich) was expressed and purified as described by Görlich *et al.* (1997) but with an additional final gel filtration purification step using a Superdex 200 column (Pharmacia Amersham Biotech). Proteins were analyzed using 15% SDS-PAGE, and concentrations were determined using the Coomassie Plus Protein Assay Reagent (Pierce). Fluorescein isothiocyanate-conjugated (FITC)-labeled M9 domain and transportin were kindly supplied by D. Görlich. *Xenopus* extract was prepared as described by Kubota *et al.* (1995). HeLa cell cytosolic extract was prepared by swelling HeLa S3 cells in hypotonic buffer, 20 mM HEPES/KOH, pH 7.6, 20 mM NaCl, 1 mM EDTA, 5 mM β -mercaptoethanol, and protease inhibitor cocktail (Boehringer-Mannheim, Indianapolis, IN) and lysing by Dounce homogenization. After lysis, NaCl in the lysate was immediately adjusted to 150 mM before spinning at $15,000 \times g$ for 15 min. The resulting supernatant was spun at $100,000 \times g$ for 1 h to give a supernatant with a final protein concentration of 8.7 mg/ml, aliquots of which were frozen in liquid nitrogen.

Transfection

Cyclin A-GFP, cyclin E-GFP, and cyclin E^{R130A}-GFP were expressed from the cytomegalovirus early promoter in the pCMX eukaryotic expression vector. Cyclin A and cyclin B1 constructs were expressed from the pCDNA3 expression vector. All transfections were carried out as described by Jackman *et al.* (1995). Subcellular localizations of transfected cyclins were visualized 24 h after transfection using confocal laser microscopy or by time-lapse fluorescence and differential interference contrast (DIC) microscopy. Subcellular localization of myc-cyclin A was carried out using methanol/acetone fixation as described by Pines and Hunter (1991) using the anti-myc 9310 mAb (1:200, 0.2 mg/ml, Santa Cruz Biotechnology, Santa Cruz, CA).

Nuclear Import Assays

Nuclear import assays were carried out as described by Görlich *et al.* (1994). Import reactions were started by adding digitonin-permeabilized HeLa cells to the following reaction mixture: 20 mM HEPES-KOH, pH 7.5, 80 mM potassium acetate, 3 mM magnesium acetate, 250 mM sucrose, an energy-regenerating mixture (1 mM ATP, 100 mM GTP, 10 mM creatine phosphate, 50 mg/ml creatine kinase), 1 mM importin- α (Rch1), 200 nM importin- β , 3 mM RanGDP, 5 mg/ml bovine serum albumin (BSA), and 2 mg/ml nucleoplasmin core (to out-compete nonspecific core binding) or *Xenopus* extract or HeLa cell extract supplemented with energy-regenerating mixture. Nuclear import substrates were used at the following concentrations: cyclin A-GFP/Cdk2^{K33R} and cyclin E-GFP/Cdk2^{K33R}, 5 mM; cyclin A-GFP, cyclin E-GFP and cyclin B15E-GFP, 4 mM; GFP and GFP-Cdk2^{K33R}, 10 mM; tetramethylrhodamine B isothiocyanate (TRITC)-nucleoplasmin, 2 mM. Nuclei were incubated for 30 min at 22 or 37°C, fixed with 4% paraformaldehyde, and centrifuged through a 30% sucrose cushion onto polylysine-coated coverslips. In experiments using importin- β Δ N 44, wheat germ agglutinin, RanQ69L, or IBB, nuclei were preincubated with, or without, these inhibitors in whole-reaction mixtures on ice for 15 min before import substrates were added, and the nuclei warmed to room temperature. Nuclear import was assayed by laser scanning confocal microscopy using a 1024 confocal microscope (Bio-Rad, Hercules, CA), and the relative amounts of fluorescent substrate imported were measured using the Laser Sharp program (Bio-Rad). One hundred cells were counted per time point, and the mean of at least three independent experiments was calculated.

Nuclear Export Assays

To assay nuclear-cytoplasmic shuttling, HeLa cells were microinjected with purified proteins and incubated in DMEM supplemented with 10% calf serum at 37°C/10% CO₂. Cells were fused for 5 min with 50% polyethylene glycol (PEG) 3350/DMEM prewarmed to 37°C, washed five times with phosphate-buffered saline, and analyzed by time-lapse DIC-fluorescence microscopy as described previously (Hagting *et al.*, 1998). To study shuttling in the presence of LMB, cells were pretreated with 20 nM LMB for 1 h, fusions were carried out as above, and LMB was included in the postfusion incubation.

Absorption of Cyclin A/Cdk2 onto Cks1

(His)₆-tagged Cks1 was purified by Ni²⁺-affinity chromatography and coupled onto Ultralink biosupport medium (Pierce). HeLa cells transfected with either pcDNA3-myc-cyclin A or with pcDNA3-myc-cyclin A M210A, L214A, W217A M210A were lysed in 0.1% Nonidet P40, 50 mM Tris pH 8.0, 150 mM NaCl, 0.1 mM EDTA and a protease inhibitor cocktail (Roche Diagnostics, Lewes, East Sussex, UK). Myc-cyclin A was absorbed from a $10,000 \times g$ supernatant by adding 20 μ l of Cks1-linked beads to a 1-ml lysate of 1.5×10^7 cells, incubated for 1 h at 4°C, and then washed four times with lysis buffer. Material absorbed onto beads was resolved by SDS-PAGE and myc-cyclin A associated with Cdk was detected by Western blotting with the anti-myc epitope antibody 9E10.

RESULTS

Both Cyclin A and Cyclin E Shuttle between the Nucleus and the Cytoplasm

Although cyclins A and E appear to be nuclear by immunofluorescence, their localization might be dynamic when analyzed in real time. To study this, we generated fusion proteins between human cyclin A and E and the GFP of *Aequorea victoria*. We have previously described our cyclin A-GFP fusion protein (den Elzen and Pines, 2001); like cyclin A-GFP, the cyclin E fusion protein binds and activates its partner Cdk (Jackman, Kubota, Den Elzen, Hagting, and Pines, unpublished results) and localizes to the nucleus when expressed in tissue culture cells (Figure 1). Therefore, the GFP-fusion proteins can be used as markers for the behavior of the wild-type cyclins.

To analyze whether the nuclear localization of cyclin A- and cyclin E-CDK complexes revealed by immunofluorescence was accurate, or masked more dynamic behavior in living cells, we used a cell fusion assay (Borer *et al.*, 1989) and time-lapse imaging. We injected cyclin A-GFP or cyclin E-GFP into the nucleus of a cell and then fused the cell to its neighbors using PEG (see MATERIALS AND METHODS). As a control we injected TRITC-labeled BSA tagged with an NLS that should not be exported from the nucleus. Any proteins that shuttled between the nucleus and the cytoplasm would enter an uninjected nucleus that shared the cytoplasm with a labeled nucleus. We found that both cyclin A-GFP and cyclin E-GFP visibly began to enter uninjected nuclei ~20 min after cell fusion (Figure 2A), but NLS-BSA remained only in injected nuclei for at least 2 h following fusion (Figure 2B). Cyclin export was not by diffusion because it was blocked by incubating the cells on ice after fusion (Figure 2C). Thus, both cyclin A and E shuttled, and their apparent nuclear localization was a reflection of their nuclear import being more rapid than their export.

Figure 1. Cyclin A-GFP and cyclin E-GFP chimaeras are correctly localized to the nucleus of living cells. Shown are representative fluorescence confocal microscopy images of HeLa cells transfected with cyclin A-GFP (top) or cyclin E-GFP (bottom).

Cyclin A and Cyclin E Export Is Insensitive to LMB

A large number of proteins exported from the nucleus, including cyclin B1, were shown to depend, directly or indirectly, on the nuclear export receptor, exportin 1 by inactivating exportin 1 with LMB (Kudo *et al.*, 1999). Therefore, we analyzed cyclin A- and E-GFP nuclear export by the cell fusion assay in the presence of 20 nM LMB. This showed that both cyclin A and cyclin E were still exported to the surrounding nuclei (Figure 3 and supplementary Figure 9) and indicated that cyclins A and E, unlike cyclin B1 (Hagting *et al.*, 1998; Toyoshima *et al.*, 1998; Yang *et al.*, 1998; Jackman, Kubota, Den Elzen, Hagting, and Pines, unpublished results), do not depend on exportin 1 for their nuclear export. In parallel experiments the same concentration of LMB inhibited the nuclear export of cyclin B1 (supplementary Figure 8).

Cyclin A/Cdk2 Is Actively Imported into Nuclei without Proteins with a Recognizable NLS

The similarities in nuclear export of cyclins A and E raised the possibility that they might also be imported in a similar manner. Cyclin E had been shown to be transported into nuclei by the classical importin- α/β receptors (Moore *et al.*, 1999), whereas cyclin B1 bound directly to importin- β and, unusually, was transported into nuclei independently of the small GTPase, Ran (Takizawa *et al.*, 1999). The cyclin A sequence did not have a classical NLS, and its nuclear localization had been correlated with the ability to bind to its CDK. Thus, it was suggested that the cyclin A-CDK complex piggy-backed into the nucleus by binding to a protein with a NLS (Maridor *et al.*, 1993). To test this hypothesis we

analyzed cyclin A import in vitro. We expressed recombinant cyclin A-GFP with, and without, Cdk2, in baculovirus-infected Sf9 cells, and purified the proteins to homogeneity. We assayed the ability of the purified proteins to be imported into nuclei using the well-characterized in vitro nuclear transport system based on digitonin-permeabilized HeLa cells (Adam *et al.*, 1992). Nuclei were incubated with an energy regenerating mix, plus Ran, NTF2, importin- β and the most abundant subtype of importin- α , Rch1. To eliminate any possible effects of Cdk2 kinase activity on nuclear membrane integrity, we routinely used a kinase deficient mutant of Cdk2, Cdk2^{K33R}. We measured the amount of protein imported into nuclei by confocal fluorescence microscopy and immunoblotted nuclei that had imported the GFP-fusion proteins with an anti-GFP antibody to show that the nuclear fluorescence was from the full-length GFP-fusion proteins.

Using the in vitro import assay, we found that cyclin A-GFP/Cdk2^{K33R} was imported into nuclei in a temperature-dependent and energy-dependent manner (Jackman, Kubota, Den Elzen, Hagting, and Pines, unpublished results, but see Figure 5A), indicating that cyclin A-GFP/Cdk2^{K33R} did not passively diffuse into nuclei. Most importantly, cyclin A/Cdk2 could be nuclearly imported without exogenous NLS-containing proteins, such as p107 or p21 (Figure 4A). To demonstrate that cyclin A-GFP/Cdk2 was imported via nuclear pore complexes, we treated nuclei with a dominant-negative mutant of importin- β (importin- β Δ N 44; Kutay *et al.*, 1997). This mutant binds to the nuclear pore but cannot be released. It strongly blocks facilitated translocation of molecules through the NPC but only mildly inhibits passive diffusion (see Ribbeck and Gorlich, 2001). Importin- β (Δ N 44) blocked the nuclear import of cyclin A-GFP/Cdk2^{K33R} (Figure 4A).

Nuclear Import of Cyclin A Differs from That of Cyclins E and B1

Given that both cyclin A and cyclin E were predominantly nuclear, but both shuttled between the nucleus and the cytoplasm in the presence of LMB, we wished to determine whether they were imported into nuclei in a similar manner. The nuclear import of cyclin E/Cdk2 (and of cyclin E alone) had been shown to require importin- α/β (Moore *et al.*, 1999); therefore, we compared its import in vitro with that of cyclin A. We used purified nuclei in the presence of an energy-regenerating mixture and *Xenopus* extract or HeLa cell extracts to provide all the components necessary for nuclear transport. We assayed the nuclear import of cyclin A-GFP/Cdk2^{K33R} and cyclin E-GFP/Cdk2^{K33R} in the presence of increasing amounts of unlabeled nucleoplasmin, a good importin- α/β cargo, to act as a competitor substrate. TRITC-labeled nucleoplasmin was coincubated with cyclin A-GFP/Cdk2^{K33R} or cyclin E-GFP/Cdk2^{K33R} in the assay to indicate when importin- α/β -dependent nuclear import had been saturated. Increasing the amount of nucleoplasmin in HeLa cell extract or *Xenopus* extract significantly reduced the nuclear import of cyclin E-GFP/Cdk2^{K33R} but did not affect the import of cyclin A-GFP/Cdk2^{K33R} (Figure 4B, a and b). These results were confirmed in competition experiments using purified import components in place of cell extracts; unlabeled nucleoplasmin competed with cyclin E-GFP/Cdk2^{K33R} but did not significantly inhibit the nuclear import

Figure 2. Cyclin A and cyclin E actively shuttle between the nucleus and cytoplasm in a temperature-dependent manner. (A) Cyclins A and E shuttle between nuclei. HeLa cells were injected in the nucleus with 25 mg/ml cyclin A-GFP//CDK2^{K33R} (top) or 35 mg/ml cyclin E-GFP/CDK2^{K33R} (bottom). Cells were fused with PEG 1 h after microinjection. The GFP-chimaeras were detected by fluorescence imaging before and at the indicated time points after injection. (B) NLS-BSA does not shuttle. FITC-conjugated NLS-BSA was injected into HeLa cell nuclei 60 min after cell fusion. Texas Red-conjugated dextran (MW 70,000; 1 mg/ml) was injected at the end of the experiment to show that cells injected with FITC-conjugated NLS-BSA had fused with their neighbors. (C) Cyclin A does not shuttle at 4°C. HeLa cells were fused with PEG, injected in the nucleus with cyclin A-protein, and immediately placed on ice. After 2 h cells were warmed to 37°C. Cyclin A-GFP was detected by fluorescence imaging at the indicated time points after injection

Figure 2 (cont).

of cyclin A GFP/Cdk2^{K33R} (Figure 4B). Moreover, unlabeled cyclin A/Cdk2^{K33R} inhibited the import of cyclin A-GFP/Cdk2^{K33R} but not that of nucleoplasmin or cyclin E-GFP/Cdk2^{K33R} (Figure 4B, d).

These competition assays showed that cyclin A did not enter nuclei in the same manner as cyclin E. Therefore, we compared the nuclear import of cyclins A and E and tested whether it differed from that published for cyclin B1. Wild-type cyclin B1 had been shown to be predominantly cytoplasmic in interphase because it was rapidly exported from the nucleus. Thus, we assayed the import of a mutant form of cyclin B1-5E in which its nuclear export was compromised (Hagting *et al.*, 1998; Takizawa *et al.*, 1999). The nuclear import of cyclin B1-5E was unusual because it did not appear to require GTP hydrolysis by the small GTP-binding protein Ran (Takizawa *et al.*, 1999), nor was it blocked by the Ran Q69L dominant-negative mutant of Ran (Takizawa *et al.*, 1999; Figure 5A). Cyclin B1 import was stimulated by importin- β but insensitive to IBB, the amino-terminal domain of importin- α that binds importin- β and inhibits importin- α -mediated import α/β (Moore *et al.*, 1999; Takizawa *et al.*, 1999; Jackman, Kubota, Den Elzen, Hagting, and Pines, unpublished results). In contrast, we found that cyclin A-GFP/Cdk2^{K33R} import was blocked by RanQ69L and was not stimulated by exogenous importin- β (Figure 5A). Furthermore, IBB almost completely inhibited cyclin A/Cdk2^{K33R} nuclear import in the presence of either purified nuclear import factors (Figure 5B) or cytosol (Jackman, Kubota, Den Elzen, Hagting, and Pines, unpublished results). In these experiments, IBB acted as a specific inhibitor because it blocked the import of the importin- α/β substrate, nucleoplasmin, but not the transportin-dependent import of M9 (Figure 5B). Thus, cyclin A-CDK2 was imported in a manner distinct from either cyclin E or cyclin B1.

Cyclin A Needs to Bind to Cdk2 for Nuclear Import In Vitro

Previous studies using mutant forms of cyclin A transfected into cells showed a correlation between the ability of cyclin A to bind Cdk2 and its import into the nucleus (Maridor *et al.*, 1993). However, this could either have reflected a requirement to bind Cdk2 or have been because the mutant cyclin A was also unable to be recognized by the nuclear import machinery. Therefore, we assayed the import of cyclin A-GFP in the absence of Cdk2^{K33R}. We confirmed that cyclin A-GFP had not copurified with significant amounts of insect cell Cdk2 by immunoblotting the protein preparations with an anti-PSTAIR antibody that recognized insect Cdk1 and Cdk2. We found that cyclin A-GFP could not be imported into nuclei in the absence of Cdk2 and that we could induce cyclin A-GFP import by adding purified Cdk2 (Figure 6A). A Cdk2^{K33R}-GFP (Figure 6A) fusion protein nor GFP alone was not actively imported into nuclei, indicating that cyclin A-GFP/Cdk2^{K33R} was not imported because of a NLS on Cdk2.

Cyclins A and B1, but Not Cyclin E, Need to Bind to Cdk2 for Nuclear Import In Vivo

To confirm this result in vivo, we generated GFP-fusion proteins from mutants of cyclin A that cannot bind to a Cdk. The most well characterized of these have been point mutations of arginine 211 in the sequence MRAIL, part of

Figure 3. Shuttling of cyclin A and cyclin E is insensitive to LMB. HeLa cells were fused and 20 nM LMB was added 40 min later. Nuclei were injected with 35 mg/ml cyclin A-GFP (A) or coinjected with 35 mg/ml cyclin E-GFP/CDK2^{K33R} and 1 mg/ml Texas Red-conjugated dextran (MW 70,000) 20 min after addition of LMB (B). Fluorescence and DIC images were taken at the indicated time points after injection.

helix 1 of the cyclin box, that has been shown to form a buried salt bridge with aspartic acid 240 to stabilize helices 1 and 2 (Jeffrey *et al.*, 1995). Mutating R²¹¹ to K or A prevented cyclin A from binding Cdk2 in vitro (Kobayashi *et al.*, 1992) and, in agreement with our in vitro results, this caused cyclin A to remain in the cytoplasm (Figure 6B). Unlike cyclin A-GFP, cyclin E-GFP did not need to bind its Cdk to become nuclear; cyclin E was

imported into the nucleus in the absence of Cdk2 (Figure 6B), and a cyclin E mutant unable to bind Cdk2, cyclin E^{R130A} (Clurman *et al.*, 1996) was imported into nuclei in vivo (Figure 6B). Cyclin B1 resembled cyclin A in that it was not imported into nuclei in the absence of its Cdk in vivo; mutating R²⁰² in the MRAIL motif to lysine or alanine markedly reduced its import into nuclei of interphase cells treated with LMB (Figure 6B).

Figure 3 (cont).

Mutations in the Hydrophobic Patch Alter the Binding between Cyclin A and Cdk2

We were anxious to exclude the possibility that altering R²¹¹ might have had effects other than preventing Cdk binding. In particular, we thought it might alter the “hydrophobic patch” region on the other face of the MRIL helix that was identified as a potential substrate interaction motif (Schulman *et al.*, 1998; Cross *et al.*, 1999) and bind to substrates with a “Cy” motif (consensus RxL). Therefore, we attempted to distinguish the effects of al-

tering the hydrophobic patch from those due to preventing Cdk binding. We made three mutations in the hydrophobic patch, M210A, L214A, and W217A (Schulman *et al.*, 1998), tagged the hydrophobic patch-mutated protein with the myc epitope, transfected it into tissue culture cells, and analyzed its localization by immunofluorescence. To our surprise, we found that the M/L/W→A hydrophobic patch mutant of cyclin A was cytoplasmic (Figure 7A); as was a GFP-tagged version of this mutant (Jackman, Kubota, Den Elzen, Hagting, and Pines, unpublished results). The hydrophobic patch mutant of cyclin A

Figure 4. Nucleoplasmin and cyclin E-GFP/Cdk2^{K33R} but not cyclin A-GFP/Cdk2^{K33R} compete with one another for their nuclear import. Cyclin A/Cdk2^{K33R} is imported into nuclei in vitro. Permeabilized HeLa cells were coincubated with Rch1, importin- β , Ran GDP, an energy-regenerating mixture, and TRITC-labeled nucleoplasmin (a1 and a2) and cyclin A GFP/Cdk2^{K33R} (b1 and b2). Nuclear uptake in the absence (a1 and b1) or presence of 2.5 μ M importin- β Δ N44 (a2 and b2) after 30 min of incubation at 22°C was visualized by confocal fluorescence microscopy. (B) Competition experiments among cyclin A, cyclin E, and nucleoplasmin. Permeabilized HeLa cells were coincubated in HeLa cell extract (4.4 mg/ml [a]) or *Xenopus* egg extract (3.3 mg/ml [b]), with an energy-regenerating mixture, TRITC-labeled nucleoplasmin, and cyclin A-GFP/Cdk2^{K33R} or cyclin E-GFP/Cdk2^{K33R} and increasing concentrations of unlabeled nucleoplasmin. After 30 min of incubation in HeLa extract at 37°C or *Xenopus* egg extract at 22°C, nuclear uptake of TRITC-labeled nucleoplasmin, cyclin A-GFP/Cdk2^{K33R}, and cyclin E-GFP/Cdk2^{K33R} were

visualized by confocal fluorescence microscopy. c and d, permeabilized HeLa cells were incubated under standard conditions using purified proteins in place of cell extracts, with TRITC-labeled nucleoplasmin, cyclin A-GFP/Cdk2^{K33R}, or cyclin E-GFP/Cdk2^{K33R} in the presence of increasing amounts of unlabeled nucleoplasmin (c) or increasing amounts of unlabeled cyclin A/Cdk2^{K33R} (d). The average amount of each substrate imported into 100 nuclei was calculated in at least two independent experiments and the mean of these values are shown.

was originally described as being nuclear (Schulman *et al.*, 1998), but in that paper the authors had cotransfected the mutant with Cdk2. Therefore, we cotransfected Cdk2 with

the hydrophobic patch mutant and found that there was a substantial increase in the amount of cyclin A in the nucleus (Figure 7A).

Figure 5. Cyclin A-GFP/Cdk2^(K33R) import is not dependent on Rch1/importin- β but is blocked by the IBB. (A) Permeabilized HeLa cells were incubated with cyclin A-GFP/Cdk2^{K33R}, cyclin E-GFP/Cdk2^{K33R}, or cyclin B15E-GFP. Control levels of nuclear import with exogenous Rch1, importin- β , and Ran GDP after 30 min of incubation at 22°C were set to 100%. Import was measured in parallel experiments, without exogenous Rch1, without importin- β , with 60 μ M RanQ69L, or under standard control conditions but with incubation on ice instead of at 22°C. For each experimental condition, the average amount of each substrate imported into 100 nuclei was calculated and the mean of at least two independent experiments is shown. (B) Permeabilized HeLa cells were incubated in import buffer containing Rch1, importin- β , Ran GDP, and an energy-regenerating mixture, with TRITC-labeled nucleoplasmin (top), cyclin A-GFP/Cdk2^{K33R} (middle), or Texas Red-labeled M9 domain (bottom) in the absence (left) or presence (right) of IBB (15 μ M final concentration). In the experiments using TRITC-labeled M9 domain (a3 and b3), the protein was incubated under standard nuclear import assay conditions but with 300 nM transportin instead of Rch1 and importin- β in the reaction mixture. After 30 min of incubation at 22°C, nuclear import was visualized by confocal fluorescence microscopy. Transportin was unable to stimulate cyclin A-GFP/Cdk2^{K33R} import (not shown).

These results indicated either that the hydrophobic patch mutations had blocked nuclear import or that they had interfered with cyclin A binding to its Cdk. Therefore, we tested the ability of the cyclin A hydrophobic patch mutant to bind to Cdk2. In agreement with its intracellular localization, we found that the M/L/W→A hydrophobic patch mutant had a markedly reduced affinity for Cdk2 when transfected into cells (as determined by assaying its ability to bind to CDKs using a p9^{Cks1} affinity column; Figure 7B).

DISCUSSION

Cyclin A-, B1-, and E-Cdk Complexes All Shuttle between the Nucleus and the Cytoplasm

In this paper, we have shown that all the cyclin/Cdk complexes that are essential for S phase and for mitosis are highly dynamic in living cells. The S phase cyclin/Cdks, cyclin E/Cdk2 and cyclin A/Cdk2, are constantly

shuttling between the nucleus and the cytoplasm, although they appear to be nuclear by immunofluorescence. The major mitotic cyclin/Cdk complex, cyclin B1/Cdk1, also shuttles but is predominantly cytoplasmic (Hagting *et al.*, 1998; Toyoshima *et al.*, 1998; Yang *et al.*, 1998). Remarkably, the localizations of the different cyclins appear to be mediated by distinct pathways. The nuclear export of cyclin B1 is more rapid than its import, whereas the converse is true for cyclin A/Cdk2 and cyclin E/Cdk2. In this respect, it is probably significant that the export of cyclins A and E is not sensitive to LMB, in contrast to that of cyclin B1 (Hagting *et al.*, 1998; Toyoshima *et al.*, 1998; Yang *et al.*, 1998). Cyclin A- and cyclin E-Cdk complexes are not the only proteins whose export is not blocked by LMB. Proteins involved in RNA processing and transport, such as hnRNPs with an M9 motif that bind to transportin (Nakielnny and Dreyfuss, 1999), the hnRNP K protein (Michael *et al.*, 1997), and RNA helicase A (Tang *et al.*, 1999),

A Nucleoplasmin

B

Cyclin A (R211K)-GFP

Cyclin E (R130A)-GFP

Cyclin B1-GFP

Cyclin B1(R202K)-GFP

Figure 6. Cyclin A needs to bind a Cdk for nuclear import in vitro and in vivo. (A) In vitro import. Permeabilized HeLa cells were coincubated with Rch1, importin- β , Ran GDP, an energy-regenerating mixture, and TRITC-labeled nucleoplasmin (a1–3), cyclin A-GFP (b1), or cyclin A-GFP that had been preincubated with purified Cdk2^{K33R} for 60 min at 37°C (b2) or a Cdk2^{K33R}-GFP fusion protein (b3). After 30 min of incubation at 22°C, nuclear import was visualized by confocal fluorescence microscopy. (B) In vivo import. HeLa cells were transfected with non-Cdk-binding cyclin mutants; cyclin A^{R211K}-GFP (top left), cyclin E^{R130A}-GFP (top right), wild-type cyclin B1-GFP (bottom left), or cyclin B1^{R202K}-GFP (bottom right) under the control of the cytomegalovirus promoter. Subcellular localizations of the cyclin-GFP chimaeras were visualized 24 h later in the living cells by fluorescence microscopy. Thirty minutes before observation, 20 nM LMB was added to HeLa cells transfected with cyclin B1-GFP constructs to allow the nuclear import of cyclin B1-GFP to be visualized.

Figure 7. Mutations in the hydrophobic patch of cyclin A alter both its binding to Cdk2 and its localization. (A) Localization. HeLa cells were transfected with myc epitope-tagged cyclin A (M210A, L214A, W217A) alone (top) or with CDK2 (bottom). Cells were fixed and processed for immunofluorescence using the anti-myc epitope antibody. (B) Cdk binding. Myc epitope-tagged cyclin A and cyclin A hydrophobic patch mutants were transfected into HeLa cells. Eighteen hours after transfection, cell lysates were incubated with p9^{Cks1}-Sepharose beads. The amounts of myc-cyclin A and myc-cyclin A HPM bound to p9^{Cks1} was compared by immunoblotting using an anti-myc epitope antibody. Top, the amounts of myc-cyclin A and myc-cyclin A hydrophobic patch mutant expressed in each cell lysate.

are also insensitive to LMB. We have not yet identified the region(s) of cyclins A and E that are necessary for their export. However, cyclin A, cyclin E, and CDK2 have no M9 motif or a "K nuclear shuttling domain," and neither cyclin A nor CDK2 have been found associated with RNA, although cyclin E does bind components of the pre-mRNA-splicing machinery (Seghezzi *et al.*, 1998). Recently, β -catenin has been demonstrated to be exported via two distinct pathways, one of which is insensitive to LMB (Eleftheriou *et al.*, 2001; Wiechens and Fagotto, 2001).

Our observation that both cyclin A- and cyclin E-Cdk complexes are able to shuttle between the nucleus and the cytoplasm means that they could directly phosphorylate both nuclear and cytoplasmic substrates in their roles as regulators of both DNA and centrosome duplication (Hinchcliffe *et al.*, 1999; Lacey *et al.*, 1999; Meraldi *et al.*, 1999). Indeed, cyclin A has been detected on centrosomes in prophase cells by immunofluorescence (den Elzen and Pines, 2001; Pagano *et al.*, 1992).

Cyclins A, B1, and E All Have Different Nuclear Import Characteristics

Despite the similarities in their nuclear export, cyclin A/Cdk2 and cyclin E/Cdk2 are imported by different pathways, and both of these are distinct from the pathway that imports cyclin B1. Cyclin E binds to importin- α/β , and cyclin B can bind directly to importin- β (Moore *et al.*, 1999), whereas cyclin A/Cdk2 is transported by a pathway that does not require exogenous importin- α or - β but is energy and temperature dependent and inhibited by both importin- β ($\Delta 78-44$) and wheat germ agglutinin. Moreover, cyclin A/Cdk2^{K33R} does not require additional NLS-containing carrier proteins to piggy-back into the nucleus via the importin- α/β pathway.

In the course of these studies, we also found that mutations in the hydrophobic patch of cyclin A have profound effects on Cdk binding and on the subcellular localization of the cyclin. These mutations have been thought to be specific for a substrate selection region on the cyclin (Schulman *et al.*, 1998; Cross *et al.*, 1999). Thus, it will be important to control for effects on Cdk binding and localization in future studies that use hydrophobic patch mutants to investigate substrate selection.

In vivo, mutants of both cyclin A and mammalian cyclin B1 that cannot bind their Cdk cannot be imported into nuclei. Non-Cdk-binding mutants of chicken cyclin B3 also remain in the cytoplasm (Gallant and Nigg, 1994). However, our results with cyclin B1 appear to be at variance with studies by Moore *et al.* (1999), who found that *Xenopus* cyclin B1 does not need Cdk1 for nuclear import. Moore *et al.* (1999) used cyclin B1 lacking the first 127 amino acids, whereas we used full-length cyclin B1. Thus, it is possible that the amino terminus of cyclin B1 obscures the region to which importin- β binds until the cyclin binds to its Cdk.

The nuclear import of cyclin A-GFP/Cdk2 is not stimulated by additional importin- α , importin- β , or transportin (Jackman, Kubota, Den Elzen, Hagting, and Pines, unpublished results). Thus, cyclin A-GFP/Cdk2^{K33R} is transported via receptors that remain associated with preparations of HeLa cell nuclei. Although this could be importin- β itself, our data indicate that it is likely to be

another member of the importin- β receptor family (Görlich *et al.*, 1997), because cyclin A-GFP/Cdk2^{K33R} does not compete for import with either nucleoplasmin or NLS-BSA under conditions in which importin- β is limiting. If cyclin A/CDK complexes do use importin- β , then the pathway is markedly different from that used to import cyclin B1, because we found that cyclin A/Cdk2 import is blocked by Ran Q69L and IBB, both of which fail to inhibit cyclin B1 transport (Takizawa *et al.*, 1999). We do not think that the receptor is transportin, because cyclin A/Cdk2 import is not stimulated by exogenous transportin, and it is inhibited by levels of IBB at which M9 substrates are still imported.

Alternatively, importin- β might still be the receptor for cyclin A/Cdk2 import, but an adaptor protein, distinct from Rch1 may be required. Such an adaptor would have to remain associated with our preparations of nuclei. We have excluded the importin- α subtype NPI-1, which can import classical NLS-substrates and Stat1 via separate domains (Sekimoto *et al.*, 1997), because a dominant-negative mutant of NPI-1 (NPI-1 78–538, kind gift of T. Sekimoto) does not block cyclin A-GFP/Cdk2 import (Jackman, Kubota, Den Elzen, Hagting, and Pines, unpublished results). A third importin- α subfamily member (importin- α 3/Qip1/hSRP α ; Kohler *et al.*, 1997; Miyamoto *et al.*, 1997; Nachury *et al.*, 1998) is unlikely to be the import receptor for cyclin A/Cdk2 because it has a highly tissue-specific distribution (Nachury *et al.*, 1998).

Cyclin/Cdk Regulation by Nuclear Import and Export

Our results raise the intriguing possibility that different cyclin/Cdks could be regulated by modulating their import and export from the nucleus. In addition to controlling their localization, this might also affect their abundance. For example, cyclin D1 is imported into the nucleus in G1 phase, possibly by piggy-backing on the p21^{Cip1}/p27^{Kip1} Cdk-inhibitors (Diehl and Sherr, 1997; LaBaer *et al.*, 1997), and is then exported in S phase concomitant with phosphorylation by GSK3 and subsequent proteolysis (Diehl *et al.*, 1998). We have not yet determined whether the shuttling of short-lived proteins such as cyclin E and cyclin D1 or of cyclins A and B1 that are unstable in G1 phase cells is also relevant to their proteolysis.

In the early *Drosophila* embryo, cyclin A remains in the cytoplasm throughout S phase and only translocates into the nucleus at prophase (Lehner and O'Farrell, 1989). It will be fascinating to discover whether this behavior reflects changes in the recognition of cyclin A by the nuclear import machinery or a change in the cyclin A/Cdk import machinery itself. Studies of other proteins involved in the G1/S transition, such as the E2F family members (Muller *et al.*, 1997; Verona *et al.*, 1997), as well as components of the DNA replication machinery such as Cdc6 (Saha *et al.*, 1998; Petersen *et al.*, 1999) and the MCM proteins (Chen *et al.*, 1992; Yan *et al.*, 1993), have also revealed strong regulation at the level of their nuclear localization. The identification of the nuclear import mechanisms that are used by specific cyclins, and the elucidation of whether and how these pathways are altered in different phases of the cell cycle, may illuminate a novel aspect of cell cycle control.

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Supplemental Figure 8. LMB treatment of fused cells inhibits cyclin B1 export but not cyclin A shuttling. (A) HeLa cells were injected with an expression construct for cyclin B1-YFP and fused as described in MATERIALS AND METHODS 3 h later. Twenty minutes after fusion, 20 nM LMB was added to the cells. Fluorescence pictures were taken at different time points after LMB addition (top). (B) HeLa cells were fused, and after 20 min, 20 nM LMB was added. Cyclin A-GFP protein was injected into the nucleus 30 min after the addition of LMB, and fluorescence pictures were taken at different time points after injection.

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Supplemental Figure 9. Graphs show two separate experiments quantitating loss and appearance of cyclin A-GFP (fluorescence, arbitrary units, and corrected for background) in nuclei of binucleate fused cells. Black lines with squares show loss of cyclin A-GFP from nuclei into which it was injected. Lines with filled circles show increase in cyclin A-GFP in nuclei not injected with cyclin A-GFP. Movement of cyclin A-GFP between nuclei in two fused cells without LMB treatment (A) and with LMB treatment (B).

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