Abstract. Reversible phosphorylation of nuclear proteins is required for both DNA replication and entry into mitosis. Consequently, most cyclin-dependent kinase (Cdk)/cyclin complexes are localized to the nucleus when active. Although our understanding of nuclear transport processes has been greatly enhanced by the recent identification of nuclear targeting sequences and soluble nuclear import factors with which they interact, the mechanisms used to target Cdk/cyclin complexes to the nucleus remain obscure; this is in part because these proteins lack obvious nuclear localization sequences. To elucidate the molecular mechanisms responsible for Cdk/cyclin transport, we examined nuclear import of fluorescent Cdk2/cyclin E and Cdc2/cyclin B1 complexes in digitonin-permeabilized mammalian cells and also examined potential physical interactions between these Cdks, cyclins, and soluble import factors. We found that the nuclear import machinery recognizes these Cdk/cyclin complexes through direct interactions with the cyclin component. Surprisingly, cyclins E and B1 are imported into nuclei via distinct mechanisms. Cyclin E behaves like a classical basic nuclear localization sequence–containing protein, binding to the α adaptor subunit of the importin-α/β heterodimer. In contrast, cyclin B1 is imported via a direct interaction with a site in the NH₂ terminus of importin-β that is distinct from that used to bind importin-α.

Key words: importins • cyclins • Cdk • nuclear import • mitosis

Cyclin-dependent kinase (Cdk)/cyclin complexes perform cardinal roles in the eukaryotic cell division cycle by phosphorylating key cellular substrates at the appropriate times. Since many important Cdk targets, including Rb and the lamins, are localized to the nucleus, active Cdk/cyclin complexes must have access to the nuclear compartment. The subcellular distribution of several Cdk/cyclin complexes has been characterized in both vertebrate and invertebrate cells; some are constitutively nuclear, some are localized to distinct structures in the cytoplasm, and others are nuclear at some times during the cell cycle and cytoplasmic at others (for example, see Lehner and O'Farrell, 1989, 1990; Pines and Hunter, 1991; Baldin et al., 1993; Jackman et al., 1995; Ohtsubo et al., 1995; Sigrist et al., 1995). However, little is yet known concerning the dynamics of these localization patterns or the molecular mechanisms underlying the observed distributions.

In this study, we have examined the nuclear import of Cdk2/cyclin E and Cdc2/cyclin B1 which, in combination, are sufficient to drive alternating cycles of S and M phase in Xenopus egg extracts. These two complexes show evolutionarily conserved, contrasting patterns of nuclear localization. In both Drosophila embryos and cultured human cells, Cdk2/cyclin E is consistently concentrated in the nucleus (Knoblich et al., 1994; Ohtsubo et al., 1995), whereas Cdc2/cyclin B1 is retained in the cytoplasm in interphase, entering the nucleus at the earliest stages of mitosis (Lehner and O'Farrell, 1990; Pines and Hunter, 1991). Recent findings indicate that the interphase cytoplasmic localization of vertebrate cyclin B1 is dependent on nuclear export (Hagting et al., 1998; Toyoshima et al., 1998; Yang et al., 1998). Cyclin B1 is continually imported into the nucleus, but is exported at a faster rate. Intriguingly, the interphase cytoplasmic localization of cyclin B1 appears to be important in preventing inappropriate mitosis in the presence of damaged DNA (Jin et al., 1998; Toyoshima et al., 1998).

Nucleocytoplasmic trafficking of proteins and RNAs occurs through nuclear pores. Proteins targeted for the nucleus first interact in the cytoplasm with soluble import re-
ceptors and then dock at saturable sites on the cytoplasmic face of the nuclear pores (for review see Görlich and Mattejat, 1996; Corbett and Silver, 1997; Doye and Hurt, 1997; Nigg, 1997; Ullman et al., 1997; Ohno et al., 1998). Next, the importins, together with their cargo, translocate through the pore for delivery to the nuclear interior. The translocation and delivery of cargo depends on the small ras-related GTPase Ran and its binding partner NTF2 (Melchior et al., 1993; Moore and Blobel, 1993, 1994; Paschal and Gerace, 1995; Görlich et al., 1996a; Izaurralde et al., 1997; Melchior and Gerace, 1998).

Several nuclear transport factors containing Ran-GTP binding domains have been identified recently. Although these soluble nuclear transport receptors share regions of homology, individual receptors are specialized for particular classes of cargo: For example, importin-β (or karyopherin-β1) imports proteins that contain classical basic nuclear localization sequences (NLSs) (Kalderon et al., 1984; Dingwall and Laskey, 1991; Robbins et al., 1991); transportin (or karyopherin-β2) can recognize a 38-amino acid glycine-rich sequence termed M9 and transports a subset of hnRNp proteins (Chi et al., 1995; Görlich et al., 1995a; Michael et al., 1995; Moroianu et al., 1995; Radu et al., 1995; Pollard et al., 1996; Bonifaci et al., 1997; Fридell et al., 1997); and Crm1 serves as a receptor for the nuclear export of proteins containing a leucine-rich nuclear export sequence (NES), including cyclin B1 (Forester et al., 1997; Fukuda et al., 1997; Neville et al., 1997; Ossareh-Nazari et al., 1997; Stade et al., 1997; Hagting et al., 1998; Toyoshima et al., 1998; Yang et al., 1998). Transportin and most other import/export factors interact directly with their cargo, but importin-β interacts with basic NLS-containing proteins via a 55–60-kD adapter subunit, importin-α (or karyopherin-α) (Görlich et al., 1994, 1995b; Moroianu et al., 1995).

At present, little is known concerning the mechanism of Cdk/cyclin complex nuclear import. It is not known if the Cdk/cyclin complex components are directly recognized by the import machinery or which of the import pathways is used; no sequence identifiable as a basic NLS is present in the primary sequences of vertebrate Cdns or cyclins. Analysis of chicken cyclin A deletion mutants showed that the sequences required for nuclear localization corresponded with those required for binding to Cdk2 (Maridor et al., 1993), providing circumstantial evidence that vertebrate Cdns might influence Cdk/cyclin nuclear import. However, a mutant form of human cyclin D1 can prevent Cdk4 from localizing to the nucleus, suggesting that vertebrate cyclins help to determine the subcellular localization of their kinase subunit partners (Diehl and Sherr, 1997). It has also been suggested that Cdk/cyclin complexes gain access to the nucleus by binding to substrates or regulators that do contain recognizable NLSs; that is via a “piggy-back” mechanism (Maridor et al., 1993; Pines and Hunter, 1994). Indeed, the Cdk/cyclin inhibitor, p21, was able to restore Cdk4/cyclin D1 nuclear localization when co-over-expressed with the mutant cyclin D1 noted above (Diehl and Sherr, 1997).

In this work we establish the molecular requirements for nuclear import of the S phase promoting Cdk2/cyclin E and M phase promoting Cdc2/cyclin B1 complexes. We present two primary findings. First, the cyclin component of both complexes confers the ability to bind to components of the importin heterodimer; the Cdk catalytic subunits are dispensable for these interactions. Second, cyclin E and cyclin B1 bind to import factors in different ways; cyclin E binds to the importin-α adaptor subunit as if it contained a conventional basic NLS, while cyclin B1 binds directly to importin-β, but at a site distinct from that used for importin-α binding. In addition, our data suggest that the nuclear import of both cyclins E and B1 occurs through these importin-mediated pathways.

Materials and Methods

Preparation of Xenopus Egg Extracts

Interphase egg extracts and the ATP-regenerating cocktail added to these extracts were prepared as described previously (Murray, 1991; Smythe and Newport, 1991). Egg cytosol for protein binding and transport assays was produced by centrifugation of crude interphase extracts for 70 min at 200,000 g. Depletions of cytosol were carried out essentially as described by Görlich et al. (1994).

Bead Binding Assays

Binding assays were typically performed by mixing 100 µl of egg cytosol (in some cases diluted two- to fourfold with extract buffer) with 10–20 µl of recombinant protein linked to glutathione agarose or amyllose beads at 4°C or room temperature for 40–60 min. 35S-labeled proteins were produced by coupled in vitro transcription/translation (TNT system; Promega) using translabel (New England Nuclear). Reticulocyte lysates were usually mixed 1:20 with diluted egg extract for binding assays. Where necessary, biotinylated nucleoplasmin, NP-core, or NP-core-importin-β binding (IBB) were added to extracts at a final concentration of 20 µg/ml. In some experiments, low speed egg extracts or extracts from SF9 cells overexpressing Cdns were used instead. Beads were spun out of the extracts by centrifugation for 3 min at 2,000 g at 4°C and then washed three times with 1 ml of XB (Murray et al., 1989).

Assays to detect interactions between purified factors were carried out in PAT buffer (PBS, pH 7.2, 1% [w/v] cas-amino acids, 0.1% [v/v] Tween 20) instead of diluted extract. Binding assays were carried out at 4°C with 15 µl of immobilized protein on beads (~1 µg protein/µl packed beads) in final volumes of 200–500 µl. Where appropriate, importin-α was added to 70–100 µg/ml, cyclins to 15–20 µg/ml, and Cdk subunits to ~10 µg/ml. After a 45-min incubation, beads were pelleted and washed six times with 1 ml of PAT buffer.

Bead-bound proteins were eluted with SDS-PAGE gel loading dye and separated on 10% SDS-polyacrylamide gels. For detection of 35S-labeled proteins, gels were soaked in 0.5 M sodium salicylate in 20% methanol, 5% acetic acid then dried and fluorographed. Otherwise, the separated proteins were transferred to PVDF membranes. Biotinylated proteins were detected with streptavidin-HRP conjugate. Other proteins were detected with unconjugated mouse or rabbit primary antibodies followed by HRP-conjugated goat antibody directed against the primary species used. Primary antibodies used in this study included commercial murine monoclonals against human cyclins E and B1 and rabbit antibodies raised against importin-α and -β (a kind gift of D. Görlich). Enhanced chemiluminescence was used to detect HRP activity.

Construction of Recombinant Plasmids and Expression of Recombinant Proteins

To express full-length importin-α, we first constructed pGEX-SB, a modified version of GEX-KG (a GEX2T derivative with a polyglycine linker at the thrombin cleavage site) which introduces a novel SphI site whose ATG encodes a methionine residue in frame with the GST fusion tag. This was constructed by digesting pGEX-KG with BamHI and EcoRI and inserting a pair of complementary oligonucleotides containing a SphI site (5’-gat ega gca tgc gcc gat gca cgc-3’ and 5’-aat tcg gat ccc ggg cgc atg ctc-3’). pGEX-importin-α was then made by ligating the importin-α encoding Sphl-BamHI fragment from pQE70-importin-α (Görlich et al., 1994) to similarly digested pGEX-SB. To construct pGEX-IBB55, one of two NeoI-HindIII-digested PCR products encoding the NH2 terminus of importin-α was inserted into similarly digested pGEX-KG (Görlich et al., 1996b).

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express a fusion protein consisting of GST linked to human importin-β, a 1.8-kb partial NcoI-HindIII fragment from pQE60 importin-β (Görlich et al., 1995a) was ligated to NcoI-HindIII-digested pGEX-KG. The GST-fusion proteins were subsequently produced, by the method of Solomon et al. (1990) from cells lysed by lysozyme and detergent treatment. Cells containing MBP and nucleoplasmin derivatives were lysed by sonication (after lysozyme treatment) and the proteins purified using the fusion tags as described (Riggs, 1990; Kalinich and McClain, 1994). Where appropriate, cleavage of GST-fusion proteins with thrombin (Pharmacon) was performed according to the manufacturer’s instructions. This was required for the use of Ran, NT2f, importin-α, and importin-β in import assays.

St9 cells were grown at 27°C in spinner flasks in Grace’s insect cell medium. They were infected with a multiplicity of infection of 50 of supernatant from a lytic viral culture and grown for a further 40–48 h at 27°C to allow the induction of recombinant human HA-tagged Cdc2 and Cdk2, and His-tagged cyclin B1 and E (all these viruses were a kind gift of Dr. D. Morgan). Cells were harvested and stored at −80°C. Extracts, cyclins, and Cdk/cyclin complexes were prepared as described by Kumagai and Dunphy (1995).

For nuclear import assays, the GST-Xenopus cyclin fusion proteins, as well as GST-NLS, GST-IBB, and GST-Ran as controls, were dialyzed into PBS (with 0.1% Nonidet P40) then mixed with a 200:1 molar ratio of fluorescein maleimide (dissolved in DMSO as a 20 mM stock). The mixtures were incubated at room temperature in the dark for 2 h to allow for the formation of fluorescein conjugates, unbound reactive dye was then quenched with 50 mM β-mercaptoethanol and removed by dialysis against a buffer containing 20 mM Hepes-KOH, 100 mM KCl, 200 mM sucrose, 1 mM DTT, and a protease inhibitor cocktail, pH 7.5. The conjugates were flash frozen in liquid nitrogen and stored at −80°C. The GST-M9 fusion protein was labeled with FLUOS as described by Fridell et al. (1997).

Nuclear Import Assays

For oocyte transport assays, reticulocyte lysates containing 35S-labeled in vitro translated Xenopus cyclins E, A1, full-length B1, or a truncated B1 mutant (residues 121–397) were mixed with 14C-labeled BSA. Stage VI oocytes were then injected with 40 nl of this radiolabeled protein mix and then manually dissected into cytoplasmic and germinal vesicle (nuclear) fractions immediately after injection and after 4 or 9 h of incubation at 18°C. Oocytes which had been injected into the nucleus (as judged by the presence of the hemoglobin pigment in this fraction) were discarded. The cyttoplasmic and nuclear fractions from five cytoplasmically injected oocytes were then separately pooled and separated on 10% SDS-polyacrylamide gels, along with a total lysate derived from five cytoplasmically injected oocytes. The gels were soaked in 0.5 M sodium salicylate, dried, and then exposed to X-ray film. The radiolabeled protein mix was detected by autoradiography of the gel.

For the digitonin semipermeabilized mammalian cell assay (Adams et al., 1990; Görlich et al., 1994), suspension grown HeLa cells (1 liter, 5 × 10^6 cells/ml) were harvested by centrifugation at 200 g, washed once with 200 ml of PBS, then resuspended in 50 ml of cell dissociation solution (nondenaturing, Sigma Chemical Co.). After 5 min at 37°C, cells were pelleted at 200 g and resuspended in a final volume of 12 ml of PBS + 10% DMSO. Cells were frozen slowly to −70°C in 0.5-ml aliquots and stored in liquid nitrogen (they were usable for import assays for at least 6 mo). For an import experiment, an aliquot was thawed rapidly at 37°C, washed twice with 100 ml PBS, then incubated in 2.5 ml of cell dissociation solution at 37°C for 5 min. Cells were then pelleted at 200 g, resuspended in 1 ml of transport buffer (20 mM Hepes-KOH, 100 mM potassium acetate, 0.5 mM magnesium acetate, 0.25 mM EGTA, and 2 mM DTT, pH 7.4; Adam et al., 1990) then mixed with 1 ml of transport buffer containing 100 μg/ml digitonin (Calbiochem) to give a final concentration of 50 μg/ml. After 8 min on ice, 10 ml of ice-cold transport buffer was added to stop permeabilization and the cells were pelleted at 200 g. They were then washed twice with 10 ml of transport buffer, in each case allowing 10 min on ice before repelling to stop the addition of transport buffer. Oocytes contaminated with the nuclei that were not permeabilized were then resuspended in 5 ml of PBS, layered on top of a 1.5 M sucrose cushion (50% wt/vol) in PBS and pelleted at 500 g for 5 min. After a final wash in 10 ml transport buffer, the permeabilized cells (~3 × 10^6) were resuspended in 0.3 ml transport buffer. Nuclear import assays

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et al., 1996b). The same forward primer was used in both PCR reactions (5′-cat atg cac cat cat cat cat cac gag gac tgt gag ccg acc aac aat gaa gca gat a-3′). The reverse primers were 5′-aag ctt cac ttc tca ttc gta ctc ggg gaga gag aag aat-3′ for pGEX-IBB and 5′-ggc aag cag ctc tga ctc ctc ttc gtc ttc gtt gca atc cag atc aa-3′ for pGEX-IBB55. To express a fusion protein consisting of GST linked to human importin-β, a 1.8-kb partial NcoI-HindIII fragment from pQE60 importin-β (Görlich et al., 1995a) was ligated to NcoI-HindIII-digested pGEX-KG. The GST-fusion proteins were subsequently produced, by the method of Solomon et al. (1990) from cells lysed by lysozyme and detergent treatment. Cells containing MBP and nucleoplasmin derivatives were lysed by sonication (after lysozyme treatment) and the proteins purified using the fusion tags as described (Riggs, 1990; Kalinich and McClain, 1994). Where appropriate, cleavage of GST-fusion proteins with thrombin (Pharmacon) was performed according to the manufacturer’s instructions. This was required for the use of Ran, NT2f, importin-α, and importin-β in import assays.

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Results

The enormous size of the *Xenopus* oocyte has made it one of the systems of choice for nuclear transport studies. Microinjection of radiolabeled transport cargo into either the nuclear or cytoplasmic compartment, followed by manual oocyte dissection, allows rapid quantitation of nuclear import and export rates. As a starting point for examining the pathways responsible for nuclear import of Cdk/cyclin complexes, we followed the fate of radiolabeled cyclins injected into the cytoplasm of stage VI oocytes. Since Cdks are present in excess in the oocyte, each injected cyclin should bind to its cognate Cdk. Full-length 35S-labeled, in vitro translated *Xenopus* cyclins E, A1, and B1 were individually injected into the cytoplasm of stage VI oocytes along with 14C-labeled BSA as a control. The nuclear and cytoplasmic fractions were then manually separated immediately, 4 or 9 h after injection. As shown in Fig. 1 A, BSA remained in the cytoplasm for at least 9 h after injection, while cyclins E and A1 translocated to the nucleus. Within 4 h, virtually all of the cyclin E and 50% of the cyclin A1 had translocated to the nucleus; the greatest majority of cyclin A1 was nuclear by 9 h. In contrast, cyclin B1 injected into the cytoplasm remained there for at least 18 h, consistent with its normally interphase cytoplasmic localization. A truncated form of cyclin B1 protein, which eliminates the NES (cyclin B1 121–397), entered the nucleus, albeit more slowly than cyclins E and A1; somewhat less than 50% of the injected protein had accumulated in the nucleus 9 h after injection. These data suggest that the nuclear import rates of all three cyclins are different, raising the possibility that they are recognized by the import machinery with different affinities or that distinct import pathways are used. Indeed, when we added fluorescein-conjugated, recombinant GST-cyclins B1 and E to *Xenopus* egg extracts containing ~1,000 nuclei/μl, fluorescent cyclin E was clearly transported into nuclei more efficiently than cyclin B1, even after removal of the cyclin B1 NES (data not shown). We selected these cyclins, the fastest and slowest for nuclear import, for further study. Interestingly, these cyclins form complexes with different catalytic subunits. Moreover, in combination, Cdk2/cyclin E and Cdc2/cyclin B1 complexes are fully capable of driving multiple rounds of S phase and M phase in *Xenopus* egg extracts.

Figure 1. (A) Nuclear import of radiolabeled cyclins in *Xenopus* oocytes. The cytoplasm of 12 stage VI oocytes for each time point was injected with a mixture of 14C-labeled BSA and 35S-labeled in vitro translated *Xenopus* cyclin E, A1, B1, or a B1 truncation mutant (residues 121–397) lacking the NES near the NH2 terminus. Immediately after injection, or after 4, 6, 9, or 18 h of incubation at 18°C, half of the oocytes were manually dissected into nuclear and cytoplasmic fractions. Samples were processed as described previously (Yang et al., 1998) and fractions representing the total, cytoplasmic, or nuclear fractions of three oocytes were separated by SDS-PAGE and detected by autoradiography. (B) Cyclin transport into the nuclei of digitonin-permeabilized cells. Import assays were performed essentially as described in Materials and Methods with interphase *Xenopus* egg cytosol diluted 1:10 (4 mg protein/ml final concentration) to provide a source of soluble transport factors. Fluorescein-labeled GST or GST-fusion proteins [GST-NLS, GST-cyclin E, and GST-cyclin B1 (121–397)] were added to assays at final concentrations of 50–100 μg/ml. Roscovitine (20 μM) was included in the assays to prevent activation of Cdc2/cyclin B1 complexes and consequent nuclear envelope breakdown. Control reactions were performed on ice. At least eight fields were examined visually for each assay. The degree of nuclear fluorescence showed little variation from field to field. Identical exposures were taken for the assays performed on ice and at room temperature.

Importin-binding Peptides Interfere with Cyclin Nuclear Import

In a first step towards analyzing the mechanism of cyclin nuclear import, we labeled bacterially produced cyclins B1 (121–397) and E with fluorescein maleimide and moni-
tored the nuclear import of these fluoresceinated cyclins using interphase egg cytosol as a source of soluble import factors and digitonin-permeabilized HeLa cells as a source of intact nuclei. As for the positive control GST-NLS protein, import of cyclins required incubation at room temperature (Fig. 1B) and was blocked by the lectin wheat germ agglutinin (data not shown). The negative control GST protein was not detectably imported into nuclei.

To investigate the possible involvement of known soluble transport factors in cyclin import, we preincubated the interphase egg cytosol with competitor peptides designed to prevent importins or transportin from interacting with their cargo. When the interphase egg cytosol used in the digitonin-permeabilized cell import assay was preincubated with peptide encoding the classical NLS from nucleoplasmin (50 μM), fluoresceinated cyclin B (121–397), cyclin E, and control GST-NLS proteins were all prevented from entering nuclei (Fig. 2). These data suggested that cyclins might use the well-known importin-α/β pathway for nuclear import. However, when we used a peptide encoding the IBB domain of importin-α (importin-α residues 1–64) in similar experiments, we found that import of cyclin E, but not cyclin B1 (121–397), was impaired. A peptide encoding the M9 NLS blocked import of only GST-M9 protein and did not interfere with cyclin import. These data suggest that nuclear import of both cyclins E and B1 proceed by importin-dependent, transportin-independent pathways. However, the fact that GST-NLS and GST-IBB proteins interfered differentially with import of cyclins E and B1 suggested, again, that these cyclins might use different pathways for nuclear import.

**Nuclear Import of Cyclin E, but Not Cyclin B1, Requires Importin-α**

Since the basic NLS peptide blocked cyclin import, we wished to investigate whether importin-α was required for nuclear import of either cyclin B1 or cyclin E. For this purpose, nuclear import assays were performed in which the egg cytosol was replaced with cytosol depleted of importin-α (by passage through a column of Ni-NTA-agarose; see Görlich et al., 1994) (Fig. 3A) or with control cytosol.
mock-depleted with Sepharose CL-6B. GST-cyclin E import was abolished in Ni2+NTA-agarose–treated extracts, whereas cyclin B1 (121–397) import was not severely affected (Fig. 3 B). Readability of recombinant importin-α to Ni2+NTA-agarose–depleted extracts restored GST-cyclin E nuclear import, though not quite to the levels observed in the mock-depleted extract. These data indicate that cyclin E nuclear import proceeds via an importin-α–dependent mechanism, whereas, surprisingly, given its inhibition by NLS peptide, cyclin B1 import does not.

**Cyclins E and B1 Bind to Importins**

Since the above data indicated that cyclin E nuclear import required importin-α, we wished to determine whether cyclin E could interact physically with the importin-α/β heterodimer used to import classical NLS-containing proteins. For this purpose, resins linked to GST or GST-importin-β were incubated in interphase Xenopus egg cytosol supplemented with in vitro translated, radiolabeled cyclins, pelleted, washed, and resolved by autoradiography. As shown in Fig. 4 A, cyclin E behaved like the NLS-containing control protein, nucleoplasmin (biotinylated for detection with HRP-streptavidin), binding specifically to importin-β. Similar interactions were observed using a nucleoplasmin variant in which the NLS had been removed and replaced with the IBB domain of importin-α (NPcore-IBB), whereas the NPcore alone, or luciferase, both lacking nuclear localization signals, did not bind any transport factors. The interaction of nucleoplasmin with GST-importin-β beads indicates that importin-α binding proteins are capable of interacting (presumably indirectly via importin-α provided by the extract) with immobilized importin-β in this system. Indeed, given the requirement for importin-α in cyclin E nuclear import (Fig. 3 B), we suspected that importin-α from the extract mediated cyclin E–importin-β interactions (a point we will return to below). Since importin-α did not appear to be required for cyclin B1 nuclear import, we were surprised to find that the importin-β resin was also able to retrieve radiolabeled cyclin B1 from egg extracts (Fig. 4 A). These data suggested that cyclin B1 might bind directly to importin-β.

**Cyclins Bind Directly to Importins in the Absence of Cdks**

To determine whether the interaction between cyclins and importin-β was direct, we produced recombinant His-tagged variants of cyclins E and B1. The purity of these preparations is shown in Fig. 4 B: any contaminating bound factors were likely present at very low stoichiometries. We found that GST-importin-β bound directly to His-tagged cyclin B1 incubated in the absence of cytosol (Fig. 4 C); cyclin B1 actually interacted less well with importin-β in the presence of excess importin-α (data not shown). In contrast, binding of purified cyclin E to importin-β was vastly increased in assays supplemented with recombinant importin-α (Fig. 4 D). Addition of recombinant Cdc2 or Cdk2 did not enhance the importin-β binding of their cognate cyclins (Fig. 4 C and data not shown). These data show that cyclins E and B1 interact with the importins in distinct manners and demonstrate that cyclin-importin interactions are direct; no piggyback from a cross-linking factor is required. They also suggest that Cdks are not required for cyclin–importin interactions.

It remained possible that Cdks could also interact independently with importins. To determine if this was the case, baculovirus-produced HA-tagged Cdk2 or Cdc2 proteins were incubated with importin-β beads (in the presence of importin-α) in the absence of cytosol. Neither Cdk2 nor Cdc2 interacted significantly with importins under these conditions, but addition of the cognate cyclins dramatically increased these interactions (Fig. 4 E).

**Cyclins Do Not Require Cdks for Nuclear Import**

The experiments above suggested that nuclear import of Cdk/cyclin complexes might be promoted by direct interaction of the cyclin subunits with importins. However, previously published studies have indicated that the ability of mutant cyclins A and B3 to be imported into nuclei correlated with their ability to interact with Cdks (Maridor et al., 1993; Gallant and Nigg, 1994). Thus, it remained possible that Cdks might be in some way required for effective cyclin nuclear import. To determine if this was the case, we produced a recombinant mutant of cyclin B1 previously reported to lack the ability to interact with Cdc2 (cyclin B1 121–373; Stewart et al., 1994) and an analogous mutant of cyclin E unable to bind Cdk2 (cyclin E 1–338; Fig. 5 A). Both of these cyclins bound to importins as efficiently as their wild-type counterparts (data not shown). Moreover, fluoresceinated variants of these proteins were imported efficiently into nuclei of digitonin-permeabilized cells (Fig. 5 B; for the purposes of comparison, note that the control cyclin B1 and cyclin E import assays from this experiment are shown with the same exposure time in Fig. 1 B). These data suggest that Cdk binding is entirely dispensable for cyclin nuclear import, in accordance with our observation that the major determinants of importin binding reside within the cyclin subunit of the cyclin/Cdk complex. As predicted by these data, we have found that depletion of all of the Cdk2 and >90% of the Cdc2 from egg cytosol using p13 beads had no deleterious effect on nuclear import of either cyclin E or cyclin B1 (data not shown).

**Cyclin B1 Interacts with Importin-β at a Site Distinct from That Used To Bind Importin-α**

Given the above data demonstrating direct interactions between cyclin B1 and importin-β, it was unclear why, in our initial experiments (Fig. 2), the NLS (presumably complexed to importin-α from the egg cytosol), but not the IBB peptide, could interfere with cyclin B1 nuclear import. Indeed, we also found that addition of the NLS peptide to egg cytosol impaired retrieval of added radiolabeled cyclin B1 on an importin-β resin (data not shown). One interpretation of these data is that cyclin B1 binds to a site on importin-β distinct from that used for binding the IBB domain of importin-α. If so, two possible models could account for the fact that NLS, but not IBB peptide, inhibited productive cyclin–importin-β interactions: the small IBB peptide and cyclin B1 are able to bind simultaneously to importin-β, but the larger 60-kD NLS/importin-α complex, binding to the same site as the IBB peptide, sterically prevents simultaneous binding of cyclin B1; or, the importin-α and cyclin B1 binding sites on importin-β can-
action (Rexach and Blobel, 1995), it is possible that the in-

tact importin-α molecule bound to the NLS peptide would compete more effectively with cyclin B1 for importin-β binding than would the isolated IBB domain.

The most direct way to test the hypothesis that cyclin B1 and importin-β bound to different sites on importin-β was to identify an importin-β mutant that could bind to one
protein, but not the other. Kutay et al. (1997) have shown that the IBB domain of importin-α requires residues 286–876 of importin-β for binding, while Ran-GTP and certain nucleoporins bind to the NH₂-terminal half (residues 1–462) of the importin-β protein. As shown in Fig. 6 A, cyclin B1 bound to both full-length and 1–462 importin-β. Consistent with a requirement for importin-α in the cyclin E–importin-β interaction, the 1–462 mutant could bind neither importin-α from the egg cytosol nor cyclin E (Fig. 6 A).

These data confirm that cyclin B1 binds to a different site on importin-β from that used by the IBB domain of importin-α. Very recently, Jäkel and Görlich (1998) identified an NLS in ribosomal protein L23 that binds in a Ran-GTP–sensitive manner to the first 462 amino acids of importin-β, indicating that such interactions can be relevant to nuclear transport processes.

To determine whether the IBB domain and cyclin B1 could interact with importin-β simultaneously, we immobilized the recombinant GST-IBB domain on beads, incubated these beads in egg cytosol supplemented with 35S-labeled cyclin B1 (121–397), and asked whether cyclin B1 could bind to the IBB domain resin. Indeed, as shown in Fig. 6 B, we found that cyclin B1 was able to bind to the GST-IBB beads, presumably via the importin-β that was also bound from the extract. As expected, the GST-IBB beads did not bind importin-α, nor did they bind radiolabeled cyclin E. Identical results were obtained using a variant of the IBB domain lacking a potential Cdk2/ cyclin B phosphorylation site at residues 60–64 (GST-IBB55), arguing against the possibility that cyclin B1–IBB interactions were mediated via enzyme–substrate interactions of the cyclin B1 binding partner, Cdk2 (Fig. 6 B). These data suggest that cyclin B1 and an IBB peptide can bind simultaneously to impor-

Figure 6. (A) Cyclin B1 (121–397), but not importin-α, is capable of binding to an importin-β truncation mutant containing residues 1–462. Interphase egg cytosol, diluted 1:3 with PAT buffer, was supplemented with 1/50 vol of reticulocyte lysate containing radiolabeled cyclins E and B1 (121–397) and incubated at 4°C with glutathione-Sepharose beads containing immobilized GST, GST-importin-β (full-length), or GST-importin-β (1–462) for 40 min. The beads were recovered by centrifugation and after extensive washing, bound proteins were eluted and separated by SDS-PAGE (along with 1/10 vol of the cyclin-extract mixture that had not been incubated with beads). Western blots were probed for endogenous importin-α, while radiolabeled cyclins E and B1 (121–397) were detected by autoradiography of dried gels. (B) Evidence that cyclin B1 and the IBB domain of importin-α can bind simultaneously to importin-β. The same cyclin E and B1 (121–397)–supplemented egg cytosol as used above was incubated with glutathione-Sepharose beads coupled to GST, GST-NLS, GST-IBB, or GST-IBB55 fusion proteins. Bead-bound proteins were recovered and separated (along with a control representing 10% of the input protein), as before. Western blots were probed with antibodies against importin-α and -β to detect endogenous proteins bound to the beads; the radiolabeled cyclins were detected by autoradiography.
tin-β. Interestingly, cyclin B1 (121–397) did not bind to the importin-α/importin-β complexes that associated with the immobilized GST-NLS resin. When a basic NLS–importin-α complex is bound to importin-β, steric hindrance may make the additional binding site unavailable for cyclin B1. These binding assays thus provide a plausible explanation for the ability of the NP-NLS to block cyclin B1 nuclear import.

Identification of Nuclear Import Mechanisms for Cyclins E and B1

The binding assay data, peptide competitions, and permeabilized cell assays lead to the conclusion that cyclin B1 is imported into nuclei via direct interactions with importin-β and that cyclin E uses the importin-α/β heterodimer. Moreover, these import reactions are not dependent upon Cdks. As a final, definitive test of these conclusions, we performed nuclear transport assays using digitonin-permeabilized cells and entirely recombinant import factors. All assays contained recombinant Ran and its accessory factor, NTF2. Fluoresceinated GST-cyclin E, like GST fused to the NLS of SV-40 T antigen, was imported into nuclei only in the presence of both recombinant importin-α and -β, while cyclin B1 (121–397) required only importin-β (Fig. 7). The full lysate (here reticulocyte lysate) containing all factors imported both cyclins; recombinant transportin could support nuclear import of only the GST-M9 protein. These results establish that cyclin B1 and cyclin E use distinct importin-mediated pathways for nuclear import.

Discussion

Several recent results have highlighted the importance of the nucleocytoplasmic distribution of Cdk/cyclin complexes to cell cycle control. Three recent studies using egg extracts imply that accumulation of Cdk2/cyclin E in the nucleus is required both for initiating DNA replication (Chevalier et al., 1996) and preventing re-replication (Hua et al., 1997; Walter et al., 1998). The ability of Cdc2/cyclin B1 to drive M phase entry in oocytes is dependent on phosphorylation of serine residues on cyclin B1 that promote its nuclear localization (Li et al., 1995, 1997). Moreover, altering the normal subcellular localization of the Cdc2/cyclin B1 complex has been shown to abrogate the DNA damage checkpoint (Jin et al., 1998). Therefore, elucidating the mechanisms used for Cdk/cyclin nucleocytoplasmic trafficking has been an important objective for cell-cycle researchers. Here, we have used a combination of solution binding and nuclear import assays to uncover mechanisms for the nuclear import of Cdc2/cyclin B1 and Cdk2/cyclin E.

At the start of this work, we expected to find that Cdk subunits would provide essential information for the recognition of Cdk/cyclin complexes by the nuclear import machinery. Two studies by the Nigg laboratory had shown that cyclin A and B3 mutants that failed to bind Cdk2 also lacked the nuclear localization of the wild-type proteins (Maridor et al., 1993; Gallant and Nigg, 1994). We also anticipated that Cdk/cyclin complex recognition by the import machinery might not be due to bona fide NLSs on the Cdk/cyclin complexes themselves, but rather, might require a piggyback ride from an interacting factor such as p21CIP or Cdc25. Indeed, it has been demonstrated recently that elevating p21 expression could promote Cdk4 nuclear localization (Diehl and Sherr, 1997).

Surprisingly, however, our results indicate that the two Cdk/cyclin complexes we examined interact directly with the best understood nuclear import factors, importin-α and -β. In fact, the Cdk subunits are dispensable for cyclin E and B1-importin binding and nuclear import. Moreover,
neither Cdk2 nor Cdc2 can interact with components of the importin heterodimer in the absence of these cyclins. Cyclins E and B1 (121–397) thus act as nuclear targeting subunits for their partner kinases. Remarkably, though both cyclins E and B1 are imported via importin-dependent pathways, the molecular details of the interactions are very different. Cyclin E behaves as if it contains a classical basic NLS, using the α adaptor subunit to interact with importin-β; in contrast, cyclin B1 binds directly to importin-β, but at a site distinct from that used to bind importin-α. It is possible that other cyclins (e.g., A, B3) use distinct import mechanisms, perhaps ones that are dependent upon Cdks.

Nuclear Import of Cyclin E

If cyclin E contains a basic NLS, as suggested by its behavior in solution binding and import assays, which sequences constitute the NLS, and why has it not been detected before? A cursory examination of the Xenopus cyclin E sequence reveals no lysine-rich sequences reminiscent of either the SV-40 T antigen or nucleoplasmic NLS, though the NH2-terminal 55 amino acids of cyclin E appear basic in character. Basic regions are also present in the NH2 termini of A and B type cyclins but these have been found to be involved in cyclin degradation, rather than nuclear import. If both lysines and arginines are considered, then the sequence RSRKRRK between residues 24 and 29 of all vertebrate cyclin E proteins might be considered a candidate NLS. This sequence is very similar to the basic NLS that controls the nuclear localization of the v-jun oncogene (Chida and Vogt, 1992). However, in a recent alanine scanning mutagenesis of human cyclin E, it was found that conversion of three adjacent basic residues within this sequence to alanine did not perturb the nuclear localization of cyclin E (Kelly et al., 1998). Thus, either that sequence does not serve as an NLS or the determinants of cyclin E nuclear localization are more complex; perhaps multiple redundant sequences or piggyback mechanisms can contribute to cyclin E localization in vivo. We are currently performing experiments to address these issues.

Nuclear Import of Cyclin B1

Solution binding assays, peptide competition studies, and permeabilized cell transport assays all support the idea that cyclin B1 is imported into nuclei via a direct interaction with importin-β at a site distinct from that used by the IBB domain of importin-α. Since cyclin B1 can interact with importin-β in the absence of Cdc2, it may be possible to define the sequences on cyclin B1 which comprise the cyclin B1 NLS. Indeed, sequences critical for nuclear export lie entirely within the cyclin B1 component of the Cdc2/cyclin B1 complex and these have been identified precisely (Hagting et al., 1998; Yang et al., 1998). However, it is possible that the cyclin B1 sequences which contact importin-β do not lie within a discrete, contiguous amino acid stretch; if so, it may be difficult to identify the primary sequences specifying cyclin B1 nuclear localization. While IBB-like NLSs that interact with importin-β tend to have some common features (e.g., a concentration of arginine residues), our data suggest that cyclin B1 interacts with importin-β in a manner different from that of the IBB domain. Very recently, Jäkel and Görlich (1998) have identified a 32- amino acid sequence in the ribosomal protein L23 that, like cyclin B1, interacts with a site present in the NH2-terminal half of importin-β (residues 1–462). This NLS is very basic in character, a feature that is not shared by any part of the cyclin B1 sequence between residues 121 and 373. In addition, the L23 NLS also interacts with transportin, RanBP5, and RanBP7, all of which can stimulate its import into permeabilized cells. Therefore, whether or not cyclin B1 and the L23 NLS bind to the same site on importin-β, the L23 NLS probably uses a broader array of import mechanisms than cyclin B1, since cyclin B1 import can be abolished by perturbations of the importin-dependent pathway.

Why Do Different Cdk/Cyclin Complexes Use Distinct Pathways To Enter the Nucleus?

While importin-β is responsible for transporting both Cdk2/cyclin E complexes and Cdc2/cyclin B1 complexes across the nuclear pore, the cyclin subunits interact with importin-β in distinct manners. Why do different Cdk/cyclin complexes use distinct import pathways? In all likelihood, the different cell cycle roles of these complexes impose different constraints on their nucleocytoplasmic transport. One implication of the recent finding that cytoplasmic retention of cyclin B1 relies on nuclear export (Hagting et al., 1998; Toyoshima et al., 1998; Yang et al., 1998) is that, during interphase, the rate of cyclin B1 nuclear export must exceed its nuclear import rate. Addition of a strong, importin-α-binding NLS to cyclin B1 results in its nuclear localization, apparently overwhelming the capacity of export mechanisms to maintain cyclin B1 in the cytoplasm (Pines and Hunter, 1994; Li et al., 1997; Jin et al., 1998). At the time of entry into mitosis, the cyclin B1 export rate is decreased and cyclin B1 accumulates in nuclei; the rate of cyclin B1 nuclear import must not be so slow that this relocalization does not occur (Yang et al., 1998). These biological imperatives predict that the import rate of cyclin B1 will be moderate, a result in accordance with our finding that cyclin E import is considerably faster than that of cyclin B1 (approximately fivefold faster based on quantitation of data in Fig. 1 A). Indeed, altering cyclin B1 subcellular localization interferes with proper DNA damage–induced checkpoint regulation, suggesting that localizing Cdc2/cyclin B1 to the cytoplasm in interphase helps to prevent premature mitosis. Thus, a weak NLS (which competes poorly with importin-α-NLS for importin-β binding) may be necessary to maintain moderate rates of cyclin B1 import and proper cell cycle regulation.

We see no compelling reason why a weak NLS would need to bind directly to importin-β, rather than to the importin-α subunit. However, it is possible that there are cooperative binding reactions between importin-α -β, and transport substrates which make these complexes form more readily than dimeric importin-β–transport substrate complexes.

The importin pathways may not be solely responsible for nuclear import of Cdk2/cyclin E and Cdc2/cyclin B1 under all circumstances. Interestingly, in the course of performing our binding assays, we were able to detect interactions between transportin and full-length cyclin B1 (data
not shown). Because the cyclin B1 121–397 mutant used in most of our assays did not bind to transportin (data not shown) or enter nuclei in the presence of recombinant transportin (Fig. 7), we do not think transportin interactions are critical for cyclin B1 import. However, since transportin is a shuttling factor, it could conceivably contribute to cyclin B1 nuclear export. Indeed, we did detect some residual cyclin B1 export after inhibition of CRM1-dependent export with leptomycin B (Yang et al., 1998). With regard to alternative transport pathways for cyclins, we note that elevation of p21S^G^1 levels within cells can promote the nuclear localization of not only Cdk4/cyclin D complexes (Diehl and Sherr, 1997; LaBaer et al., 1997), but also Cdc2/cyclin B complexes (Winters et al., 1998). This suggests that piggyback mechanisms may play some role in Cdk/cyclin nuclear import in vivo. We speculate that the precipitous accumulation of cyclin B1 in the nucleus of vertebrate cells just before mitosis might result from both a downregulation of nuclear export (Yang et al., 1998) and an upregulation in the rate of nuclear import; perhaps a piggyback mechanism involving binding to a basic NLS-containing protein (such as the Cdc25 phosphatase) might provide a boost in the slow importin-β binding mechanism described in this paper. The physiological importance of these mechanisms remains to be determined and will likely require the identification and mutation of cyclin NLSs.

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