Nuclear Import of Cdk/Cyclin Complexes: Identification of Distinct Mechanisms for Import of Cdk2/Cyclin E and Cdc2/Cyclin B1

Jonathan D. Moore, Jing Yang, Ray Truant,* and Sally Kornbluth

Department of Pharmacology and Cancer Biology, and *Howard Hughes Medical Institute, Duke University Medical Center, Durham, North Carolina 27710

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 NH_2 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)[1]/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'Farell, 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-

Jonathan D. Moore's current address is Imperial Cancer Research Fund, Clare Hall Laboratories, Blanche Lane, South Mimms, Herts, EN6 3LD United Kingdom.

Address correspondence to Sally Kornbluth, Department of Pharmacology and Cancer Biology, Box 3686, C366 LSRC, Research Dr., Duke University Medical Center, Durham, NC 27710. Tel.: (919) 613-8624. Fax: (919) 613-8642. E-mail: kornb001@mc.duke.edu

1. *Abbreviations used in this paper:* Cdk, cyclin-dependent kinase; IBB, importin-β binding; NES, nuclear export sequence; NLS, nuclear localization sequence.

ceptors and then dock at saturable sites on the cytoplasmic face of the nuclear pores (for review see Görlich and Mattaj, 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; Fridell 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 (Fornerod 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 adaptor 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 Cdks 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 Cdks 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 "piggyback" 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-overexpressed 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 amylose beads at 4°C or room temperature for 40–60 min. ^{35}S-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 Cdks 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% [wt/vol] cas-amino acids, 0.1% [vol/vol] 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-PA gel loading dye and separated on 10% SDS-polyacrylamide gels. For detection of ^{35}S-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 cga **gca tgc** gcc cgg gat ccg-3′ and 5′-aat tcg gat ccc ggg cgc atg ctc-3′). pGEX-importin-α was then made by ligating the importin-α encoding SphI-BamHI fragment from pQE70-importin-α (Görlich et al., 1994) to similarly digested pGEX-SB. To construct pGEX-IBB, and pGEX-IBB55, one of two NcoI-HindIII–digested PCR products encoding the NH_2 terminus of importin-α was inserted into similarly digested pGEX-KG (Görlich

et al., 1996b). The same forward primer was used in both PCR reactions (5′-cat atg cac cat cat cat cat cat g**cc atg g**tg ccg acc aca aat gaa gca gat a-3′), the reverse primers were 5′-**aag ctt** aag cat tct ttt ctg gag aga gag aat-3′ for pGEX-IBB and 5′-cgg tac **aag ctt** cac tct tct ggt aag cat aca tt-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 resulting plasmid, pGEX-importin-β, was subsequently used as a template in PCR reactions in which sequences encoding importin-β residues 1–462 were amplified using the forward primer 5′-gag gag **gga tcc** atg gag ctg atc acc att ctc-3′ and reverse primer 5′-cga tgc **gag ctc** taa gca ctg aga ccc tca atc ag-3′. The PCR product was digested with BamHI-SalI and ligated to similarly digested pGEX-KG, yielding pGEX-importin-β.

Expression of GST-cyclin E required that the reading frame of the BamHI site in pGEX-KG be altered to be compatible with our cyclin E clone (pESP11-cyclin E, a kind gift of Dr. T. Hunt). pGEX-KG was digested with BamHI and a self-complementary oligo (5′-gat ctc c**gg atc c**gg a-3′) was inserted to generate pGEX-BamHIFS. A plasmid that encodes a fusion protein consisting of GST linked to *Xenopus* cyclin E (pGEX-cyclin E) was constructed by replacing the multiple cloning site sequences between the BamHI and HindIII sites of pGEX-BamHIFS with a BamHI-HindIII fragment from pEsp11XlE. pGEX-cyclin EΔCT and pEsp11-cyclin EΔCT, plasmids encoding truncation mutants of cyclin E that are incapable of binding to Cdk2 (residues 1–338), were made by replacing the BamHI-HindIII fragment containing the full-length cyclin E coding sequences with a fragment encoding cyclin E residues 1–338 prepared by BamHI-HindIII digestion of a PCR product prepared using pEsp11XlE as a template and primers 5′-gca aaa gca ggg tga cca aag aca-3′ (a sequence proximal to the start of the cyclin E coding sequence in the template) and 5′-ttt tcc ac**a agc tt**a tat ggc cat tgc aaa agg-3′.

To express a GST-cyclin B1 fusion protein, sequences encoding full-length *Xenopus* cyclin B1 were amplified by means of PCR (primers 5′-ggc aca **cca tgg** cgc tac gag tca cca gaa aca tg-3′ and 5′-ggc cgg **aag ctt** cac atg agt ggg cgg gcc at-3′, template pGEM-cyclin B1), cut with NcoI and HindIII, and inserted into similarly digested pGEX-KG to produce pGEX-cyclin B1 (the second residue in cyclin B1 was changed from a Ser to an Ala). A primer introducing BamHI and NcoI sites and a start codon at residue 121 of cyclin B1 (5′-cgg atc **gga tcc** *atg g*tt gat gct gat gat gat ggc aac-3′), along with primers introducing SalI and XhoI sites distal to the native stop codons and one introduced at residue 373 (5′-gca tcg g**c tcg ag***t cga c*tc aca tga gtg ggc ggg cca ttt cca-3′ and 5′-gca tcg g**c tcg ag***t cga c*tc agc tgc tag cat act tgt tct taa cag tca-3′, respectively), were used in PCR reactions with the same template to generate truncation mutants of cyclin B1 encoding residues 121–373 and 121–397 which could be inserted into both BamHI-SalI–digested pGEX-KG to produce GST-cyclin B1 fusion proteins and into an NcoI-XhoI–digested pSP64 variant for in vitro translation of the unfused cyclin.

To express nucleoplasmin (residues 1–200), as well as the nucleoplasmin core region that lacks the bipartite NLS (residues 1–149), we used pET16b-NEB, designed to express *Xenopus* nucleoplasmin with an NH_2-terminal His tag (a gift of Dr. D. Kalininch). To make pET16b-NPcore, a 550-bp fragment encoding residues 1–149 of *Xenopus* nucleoplasmin was amplified by means of PCR using pET16b-NEB (Kalinich et al., 1994) as a template and primers 5′-gaa ggt cgt **cat atg** gct aga att-3′ and 5′-gga ctt **gga tcc** tag gta ccc ccg ggc tag tcg aca gat tct gga tct tct tcc tct tcc tcc tcc tc-3′. The PCR product was digested with NdeI and BamHI and ligated to the large vector fragment derived from NdeI-BamHI digestion of pET16b-NEB. We also generated a plasmid, pET16b-NPcore/IBB, directing the expression of a His-tagged fusion protein consisting of the nucleoplasmin core linked to residues 10–55 of *Xenopus* importin-α (the IBB domain). A PCR reaction was performed with primers 5′-cca gaa tct **gtc gac** atg ccg acc aca aat gaa gca gat-3′ and 5′-gga ctt **gga tcc** tac tct tct ggt aag cat aca tt-3′ and pQE70 importin-α as template. The PCR product was digested with SalI and BamHI and ligated to the vector fragment of similarly digested pET16b-NPcore.

The plasmids encoding GST-Ran and GST-RanG19V fusion proteins (pGEX-Ran and pGEX-RanG19V) were described by Kornbluth et al. (1994). pMBP-Trn which encodes an MBP-human transportin fusion protein and pGEX-4T-M9 which encodes a fusion protein linking the 38–amino acid M9 NLS of human hnRNPA1 to the COOH terminus of GST were described by Fridell et al. (1997). The plasmids encoding the fusion proteins linking the NLS of SV-40 T antigen to the COOH terminus of GST (and the corresponding reversed NLS mutant) were a kind gift of Drs. M. Rexach and G. Blobel.

Recombinant GST-importin-α and GST-importin-β proteins were produced in *Escherichia coli* strain BL21 by induction with 1 mM IPTG for 4–5 h at 25–30°C. Unfused GST and fusion proteins GST-IBB, GST-T antigen-NLS, GST-TNLS(Rev), GST-M9, and GST-Ran were similarly produced, except that inductions were performed at 37°C. GST-*Xenopus* cyclin fusion proteins were also produced in BL21 cells (induced with 0.6 mM IPTG at 18°C for 16–18 h). His-tagged nucleoplasmin derivatives were produced by inducing BL21(de3)pLysS cells with 2 mM IPTG at 37°C for 3 h. In each case, cells were grown at 37°C to an A600 of 0.5–0.9 and then cooled if necessary before addition of IPTG. GST proteins were purified by the method of Solomon et al. (1990) from cells lysed by lysozyme and detergent treatment. Cells containing MBP and nucleoplasmin proteins 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 (Pharmacia) was performed according to the manufacturer's instructions. This was required for the use of Ran, NTF2, importin-α, and importin-β in import assays.

Sf9 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 cyclins B1 and E (all these viruses were a kind gift of Dr. D.O. 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, GST-NLS, and GST-IBB as controls, were dialyzed into PBS (without any sulfhydryl reducing agents) then mixed with a 40:1 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 ^{35}S-labeled in vitro translated *Xenopus* cyclins E, A1, full-length B1, or a truncated B1 mutant (residues 121–397) were mixed with ^{14}C-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 cytoplasmic 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 the radiolabeled proteins were detected by fluorography.

For the digitonin semipermeabilized mammalian cell assay (Adam et al., 1990; Görlich et al., 1994), suspension grown HeLa cells (1 liter, 5–7 × 10^5 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 (nonenzymatic; 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 at −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 10 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 repelleting to allow endogenous transport factors to diffuse out of the cells. The permeabilized cells were then resuspended in 5 ml of PBS, layered on top of a 3-ml 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

contained 10^5 nuclei, an ATP regenerating system consisting of 10 mM creatine phosphate, 1 mM ATP, 500 μM GTP, and 50 μg/ml creatine kinase, 30–100 μg/ml fluorescent import substrate, and, in complete assays, 4 mg/ml egg cytosol or reticulocyte lysate and 6 mg/ml BSA. For assays using recombinant factors, the cytosol was omitted and the BSA concentration increased to 10 mg/ml BSA. Recombinant import factors were added at a final concentration of 100 μg/ml. In some experiments we added wheat germ agglutinin at 0.5 mg/ml or replaced the energy regeneration system with an ATP depletion mix consisting of 1 U/μl hexokinase and 50 mM glucose to block energy-dependent nuclear transport pathways using the nuclear pores. In other experiments we added 350 μg/ml (50 μM) thrombin-cleaved IBB domain (importin-α residues 1–64), 50 μM of a 20–amino acid nucleoplasmin-NLS peptide (CKRPAATKKAGAAKKKKLDK), or 250 μg/ml thrombin-cleaved M9 NLS (50 μM) to competitively inhibit import through the importin and transportin pathways. Assays were started by the addition of nuclei and carried out in the dark at room temperature for 30 min: certain negative control reactions were instead incubated on ice. The nuclei were then fixed by mixing 1:1 with a formaldehyde/sucrose-based fixative containing Hoechst 33258 and examined by fluorescence microscopy.

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 ^{35}S-labeled, in vitro translated *Xenopus* cyclins E, A1, and B1 were individually injected into the cytoplasm of stage VI oocytes along with ^{14}C-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 great 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 (cycB1 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.

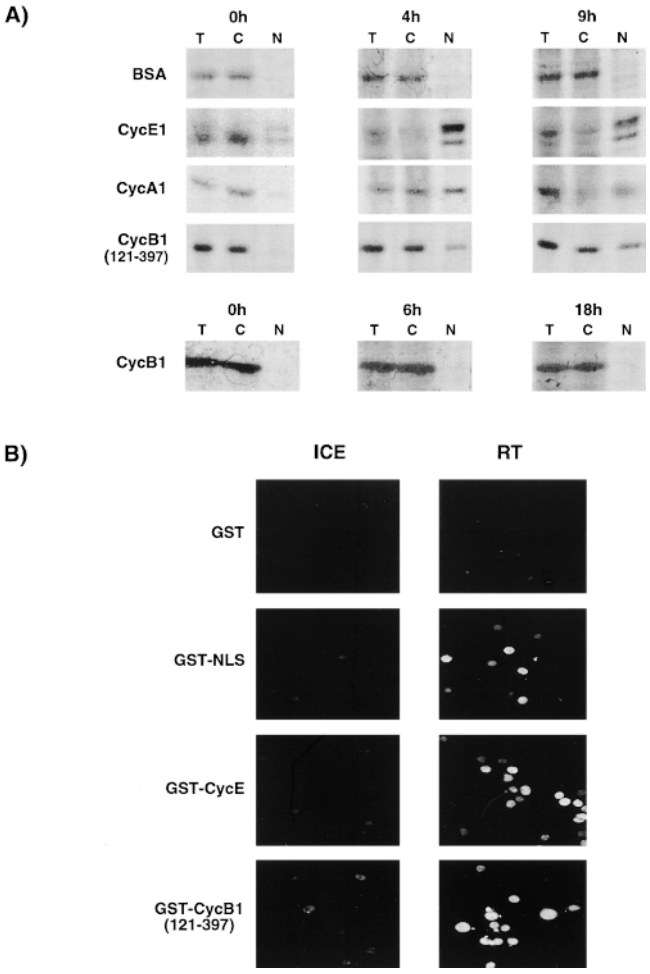

Figure 1. (A) Nuclear import of radiolabeled cyclins in *Xenopus* oocytes. The cytoplasm of 12 stage VI oocytes for each time point was injected with a mixture of ^{14}C-labeled BSA and ^{35}S-labeled in vitro translated *Xenopus* cyclin E, A1, B1, or a B1 truncation mutant (residues 121–397) lacking the NES near the NH_2 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. 1 B) 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.

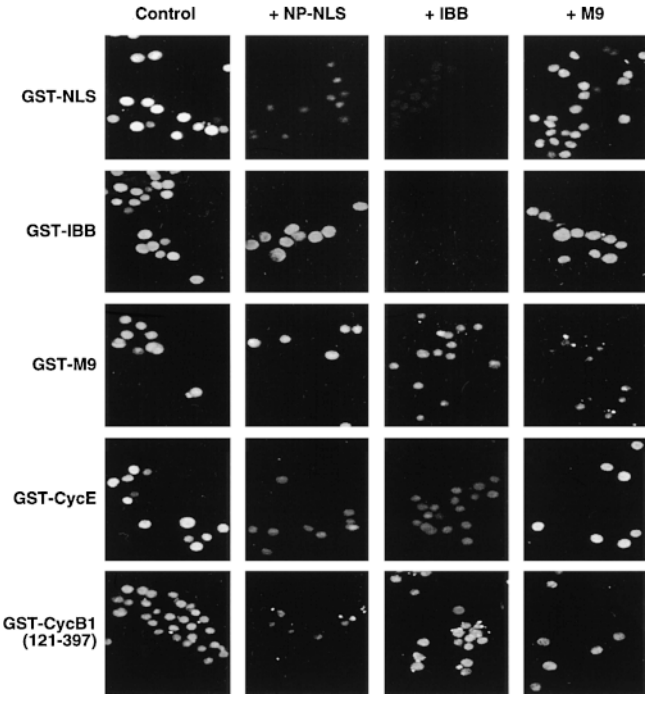

Figure 2. Nuclear import of GST-cyclin E and GST-cyclin B1 (121–397) is blocked by peptides that interfere with importin-mediated transport. Uptake assays into digitonin-permeabilized cells were performed for fluorescein maleimide–labeled GST-NLS, GST-IBB, GST-cyclin E, and GST-cyclin B1 (121–397), and FLUOS-labeled GST-M9 proteins. Assays contained egg cytosol (4 mg/ml final protein concentration), an energy-regenerating system, and either no peptide or one of the following peptides at a final concentration of 50 μM: NP-NLS (the 20–amino acid peptide containing the basic bipartite NLS of nucleoplasmin); IBB, residues 1–64 of importin-α produced by thrombin cleavage of GST-IBB; or M9, the transportin-dependent NLS of human hnRNPA1.

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. 3 A) or with control cytosol

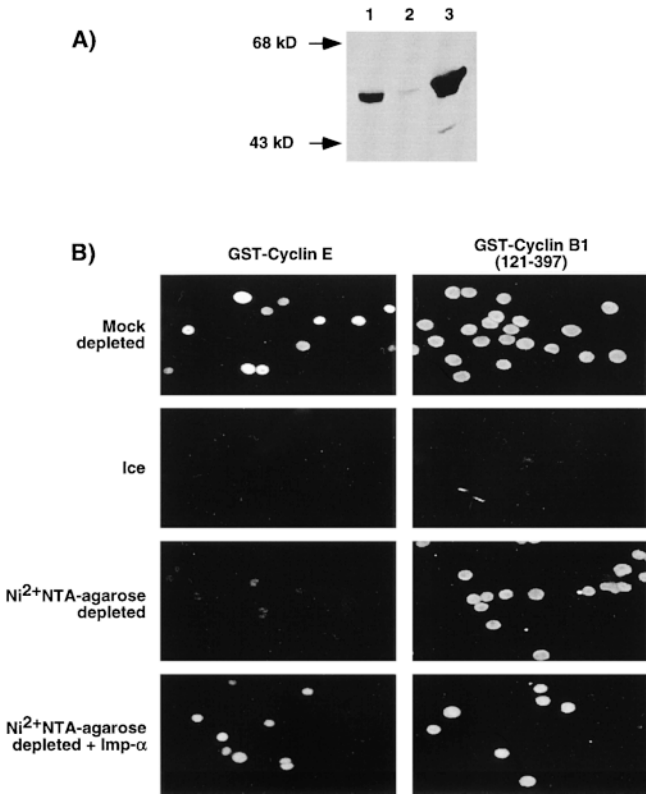

Figure 3. Nuclear import of GST-cyclin E, but not GST-cyclin B1 (121–397), requires importin-α. (A) Ni^{2+}NTA-agarose can be used to deplete importin-α from egg cytosol. Anti–importin-α Western blot for mock (Sepharose CL-4B)-depleted (lane 1), Ni^{2+}NTA-agarose–depleted (lane 2), and Ni^{2+}NTA-agarose–depleted extract to which excess recombinant importin-α (100 μg/ml) had been added back (lane 3). (B) Uptake assays into digitonin-permeabilized cells were performed for fluorescein maleimide–labeled GST-cyclin E and GST-cyclin B1 (121–397) with mock-depleted extract (passed over Sepharose CL-6B beads) at both room temperature and on ice, and at room temperature with Ni^{2+}NTA-agarose–depleted extract. All assays contained the same final protein concentration of extract (4 mg/ml). Recombinant importin-α, 50 μg/ml, was added back to a set of assays performed with Ni^{+2}NTA-agarose–depleted extract to show that the effect of depletion on import was due primarily to the removal of importin-α.

mock-depleted with Sepharose CL-6B. GST-cyclin E import was abolished in Ni^{2+}NTA-agarose–treated extracts, whereas cyclin B1 (121–397) import was not severely affected (Fig. 3 B). Readdition of recombinant importin-α to Ni^{2+}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-

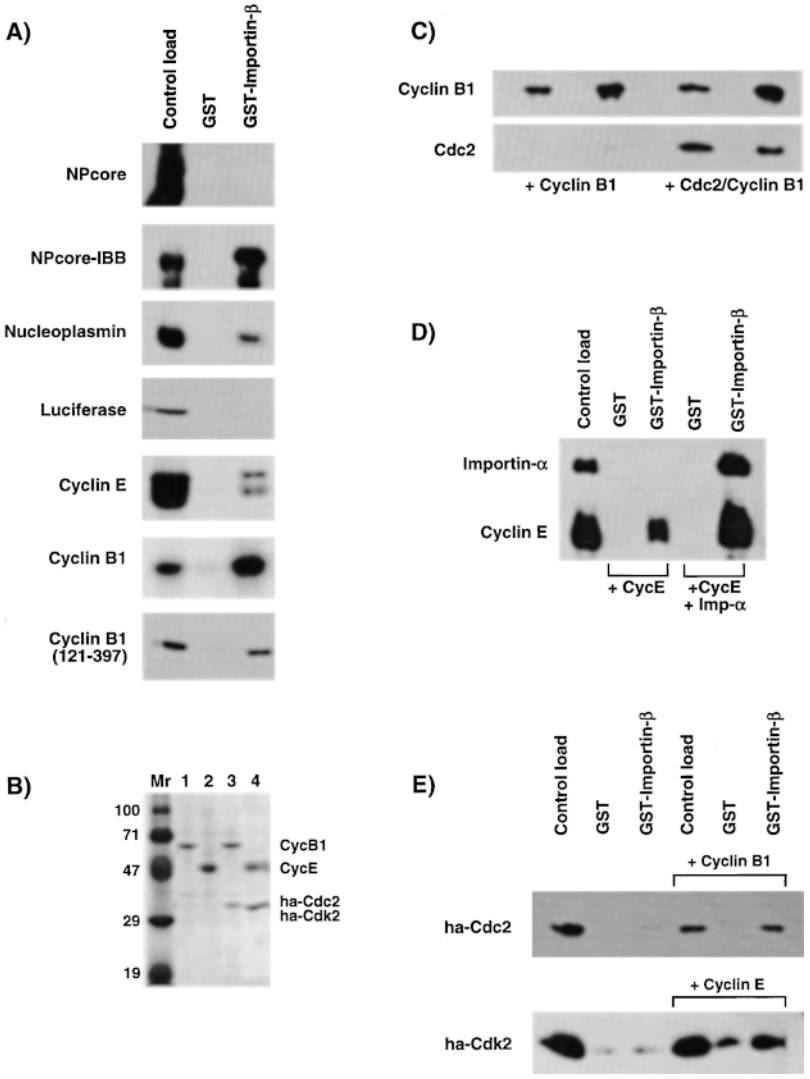

Figure 4. Interaction of *Xenopus* cyclins with importins. (A) Diluted interphase egg cytosol (final protein concentration of 10 mg/ml) was supplemented with 1/50 vol of reticulocyte lysates programmed to produce [^{35}S]methionine-labeled variants of each of the following: *Xenopus* cyclin E, *Xenopus* cyclin B1, the truncated *Xenopus* cyclin B1 (121–397) mutant, or firefly luciferase. For controls, we used the known importin-α/β interactor nucleoplasmin (biotinylated for subsequent detection and added to extracts at 20 μg/ml), a similarly prepared "core" nucleoplasmin lacking an NLS (NPcore), or the core nucleoplasmin linked to the IBB domain of importin-α (NPcore-IBB). After addition of an ATP-regenerating system, extracts were incubated in batches with the GST-importin-β or GST resins. The beads were then pelleted, washed in buffer, and bound radiolabeled proteins were resolved by SDS-PAGE for detection by autoradiography or, in the case of the nucleoplasmins, for blotting with HRP-streptavidin. As egg extracts are rich in the adaptor subunit importin-α, immobilized GST-importin-β should be capable of interacting with basic NLS containing proteins such as nucleoplasmin. 1/10 the amount of supplemented extract used in the binding assay was loaded onto the gel as a control. (B) Coomassie-stained gel showing purity of human His-tagged cyclin E and cyclin B1, plus the same proteins copurified with their Cdc2 and Cdk2 catalytic subunit partners. Each lane contains 10 μg of recombinant protein. (C) The interaction between cyclin B1 and importin-β is direct and is not stimulated by Cdc2. His-tagged human cyclin B1 was purified on Ni^{2+}NTA-agarose beads from overexpressing Sf9 cell lysates. For the purification of Cdc2/cyclin B1 complexes, the cyclin on the Ni^{2+} beads was incubated in Sf9 cell lysates overexpressing human HA-tagged Cdc2. After extensive washing, the immobilized cyclin B1 or Cdc2/cyclin B1 complex was eluted with imidazole (250 mM) then concentrated in Centricon-30 microconcentrators and transferred to the PBS/cas-amino acids/Tween buffer (PAT buffer). The cyclin B1 or Cdc2/cyclin B1 complexes (15–20 μg/ml) were incubated with immobilized GST or GST-importin-β (~1 μg protein/μl of beads). The beads were recovered after extensive washing; the bound proteins were then eluted and separated by SDS-PAGE (along with one-eighth of the relative amount of cyclin or Cdc2/cyclin complexes that had not been incubated with beads) and transferred to PVDF membranes. Cyclin B1 was detected by a murine mAb; Cdc2 was detected with sera raised against the conserved PSTAIRE epitope shared by Cdk2 and Cdc2. Blots probed with anti-PSTAIRE antiserum reveal that the cyclin B1 preparation was contaminated with very small amounts of Cdc2, presumably the native protein from the Sf9 cells. However, we estimate that our Cdc2/cyclin B1 preparation contains 25 times more Cdc2 than the cyclin B1 preparation. (D) Importin-α is required for efficient binding of cyclin E to importin-β. His-tagged human cyclin E (15 μg/ml in 180 μl PAT buffer) was incubated at 4°C with 20 μl of glutathione-Sepharose beads coupled to either GST or GST-importin-β, in the presence or absence of 100 μg/ml importin-α. The beads were recovered by centrifugation, washed extensively, and the bound proteins eluted and separated by SDS-PAGE. 1/10 the relative amount of cyclin E and recombinant importin-α included in the binding assays was also run on the gel as a control. Western blots were probed with antibodies against human cyclin E and *Xenopus* importin-α. (E) Both Cdc2 and Cdk2 require a cyclin partner to bind to importin-β. Extracts were made from Sf9 cells infected with recombinant baculoviruses inducing the overproduction of either human Cdc2 or Cdk2. The Sf9 lysates containing the Cdks were supplemented with 70 μg/ml importin-α and 10 μg/ml affinity-purified His-tagged cyclin B1 (for Cdc2) or E (for Cdk2), then incubated with immobilized GST or GST-importin-β. The human Cdks were detected with a mAb directed against their epitope tags (influenza hemagglutinin). As a control 1/10 of the relative amount of extracts before incubation with beads was loaded onto the gels along with the material eluted from the beads.

not be occupied simultaneously. Under these circumstances, the relative affinities of the NLS/importin-α, cyclin B1, and IBB proteins for importin-β would dictate which of these proteins would bind preferentially. Since the binding of an NLS to importin-α stimulates the importin-α/β interaction (Rexach and Blobel, 1995), it is possible that the intact 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-α bound to different sites on importin-β was to identify an importin-β mutant that could bind to one

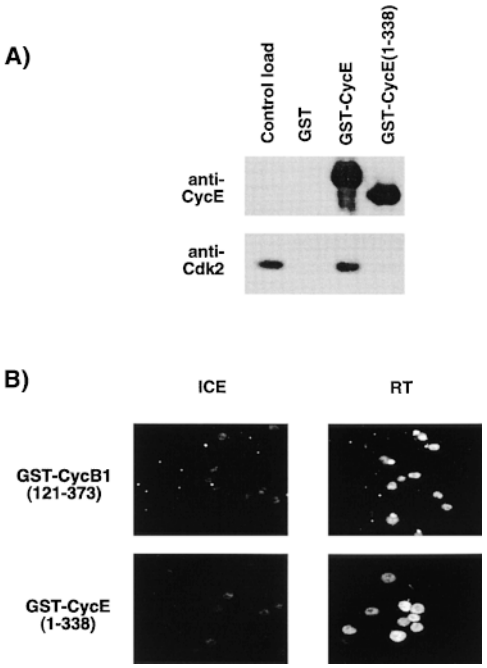

Figure 5. (A) A truncated cyclin E protein (GST-cyclin E 1–338) does not bind Cdk2. Alignment of cyclin B1 with cyclin E, which contains a longer extension beyond the second cyclin fold, revealed that a mutant of cyclin E defective in Cdk2 binding [analogous to the truncated cyclin B1 (121–373) mutant which does not bind to Cdc2] might be made by removal of the COOH-terminal 71 amino acids of cyclin E. Here GST, GST-cyclin E, or GST-cyclin (1–338) proteins immobilized on glutathione-Sepharose beads (20 μl) were incubated with 200 μl of interphase egg cytosol (diluted with PAT buffer to 10 mg/ml final protein concentration). Bead-bound proteins were recovered after pelleting and extensive washing of the beads, eluted, and separated by SDS-PAGE. Western blots were probed with antibodies against *Xenopus* cyclin E or Cdk2. Untreated egg cytosol (1/10 the relative amount used in the binding assays) was also run on the gel to show the loading of Cdk2. Note that endogenous cyclin E from the egg cytosol is not visible on the portion of the gel shown because it is considerably smaller than the cyclin E fusion proteins. (B) COOH-terminal truncated versions of cyclin E and cyclin B1 that are unable to bind to their Cdk partners are imported into nuclei of digitonin-permeabilized cells. *Xenopus* egg cytosol was diluted 1:10 (4 mg protein/ml final concentration) to provide a source of soluble transport factors. Fluorescein-labeled GST-cyclin E (1–338) and GST-cyclin B1 (121–373) were added to permeabilized cell assays at final concentrations of 50–100 μg/ml. Control reactions were performed on ice. These samples were processed together with the samples shown in Fig. 1 B, which provide the wild-type cyclin E and B1 controls.

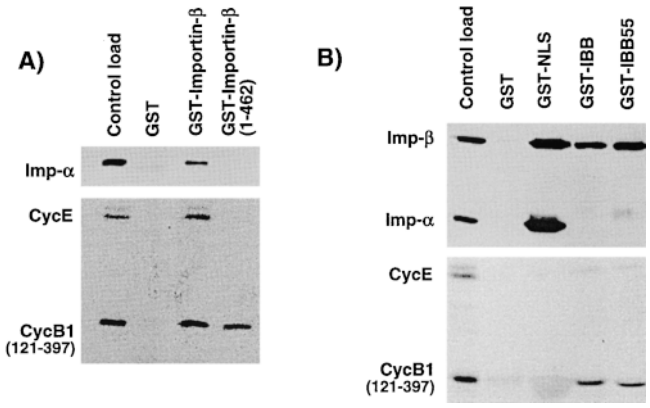

Figure 6. (A) Cyclin B1 (121–397), but not importin-α, is capable of binding to an importin-β truncation mutant containing residues 1–462. Interphase egg cytosol, diluted 1:3 with PAT buffer, was supplemented with 1/50 vol of reticulocyte lysate containing radiolabeled cyclins E and B1 (121–397) and 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.

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 NH_2-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 ^{35}S-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 Cdc2/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, Cdc2 (Fig. 6 B). These data suggest that cyclin B1 and an IBB peptide can bind simultaneously to impor-

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 $p21^{CIP}$ 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,

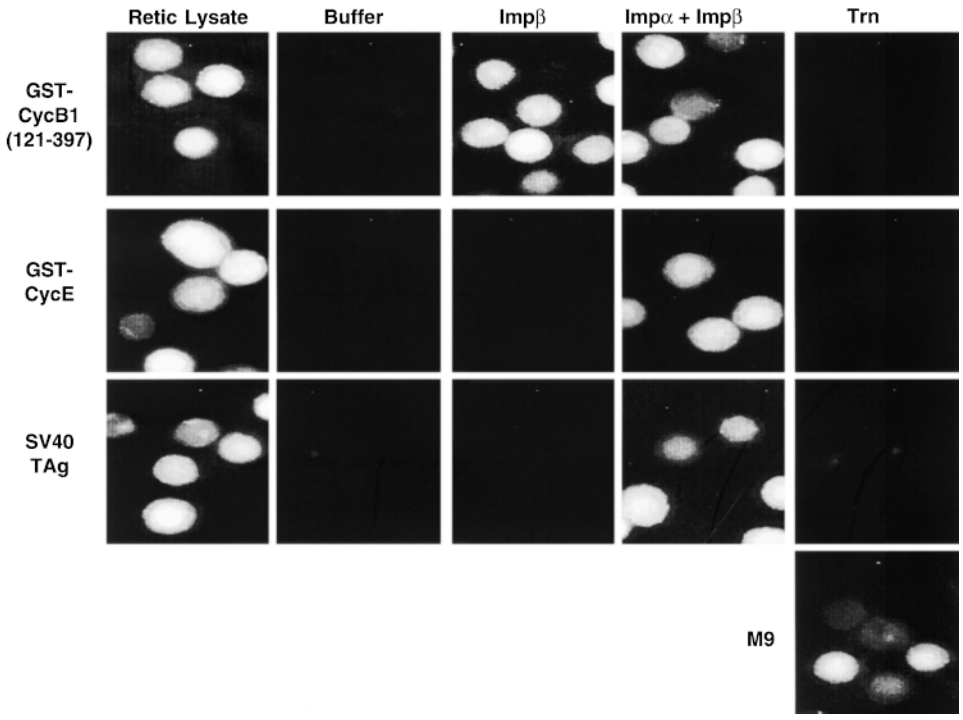

Figure 7. Recombinant importins direct cyclin transport into nuclei from permeabilized HeLa cells. Isolated HeLa cell nuclei from digitonin-permeabilized cells were incubated in the presence of recombinant Ran and NTF2 proteins along with one of the following fluorescently labeled substrate proteins: GST-cyclin B1 (121–397), GST-cyclin E, GST-SV-40 T antigen NLS, or GST-M9. Assays were supplemented with recombinant importin-α, importin-β, or both or with MBP-transportin. In control assays, reticulocyte lysate was used as a source of all soluble transport factors. This was chosen rather than egg cytosol because it had been demonstrated previously to support robust import of transportin cargo in this assay.

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 nucleoplasmin NLS, though the NH_2-terminal 55 amino acids of cyclin E appear basic in character. Basic regions are also present in the NH_2 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 RSRKRK 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 NH_2-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 $p21^{Cip}$ 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.

We thank Dr. B. Cullen for his generous help. We thank E. Bardes for figure preparation and D. Lew for critical comments on the manuscript. We would like to thank Dr. T. Hunt for pESP-Xlcyclin E and Dr. D.J. Donoghue for the *Xenopus* cyclin B1–containing vectors used for in vitro translation. Recombinant baculoviruses encoding human cyclins and Cdks were generous gifts from Dr. D.O. Morgan. Clones of import factors and antiimportin sera were kindly provided by Dr. D. Görlich. Drs. J. Kalinich and D. McClain provided the original nucleoplasmin expression vector and Dr. C. Feldherr antinucleoplasmin antibodies. Finally, Drs. R. Hartley and J. Maller provided us with anti–cyclin B1 antisera. Dr. J. Pines is to be thanked for sharing unpublished observations.

This work was funded by a grant from the American Cancer Society and a postdoctoral fellowship awarded to J.D. Moore by the Leukemia Research Foundation. S. Kornbluth is a Scholar of the Leukemia Society of America.

Received for publication 9 October 1998 and in revised form 8 December 1998.

References

Adam, S.A., R.S. Marr, and L. Gerace. 1990. Nuclear protein import in permeabilized mammalian cells requires soluble cytoplasmic factors. *J. Cell Biol.* 111:807–816.

Baldin, V., J. Lukas, M.J. Marcote, M. Pagano, and G. Draetta. 1993. Cyclin D1 is a nuclear protein required for cell cycle progression in G1. *Genes Dev.* 7:812–821.

Bonifaci, N., J. Moroianu, A. Rudu, and G. Blobel. 1997. Karyopherin β2 mediates nuclear import of a mRNA binding protein. *Proc. Natl. Acad. Sci. USA.* 94:5055–5060.

Chevalier, S., A. Couturier, I. Chartrain, R. Le Guellec, C. Beckhelling, K. Le Guellec, M. Philippe, and C.C. Ford. 1996. *Xenopus* cyclin E, a nuclear phosphoprotein, accumulates when oocytes gain the ability to initiate DNA replication. *J. Cell Sci.* 109:1173–1184.

Chi, N.C., E.J. Adam, and S.A. Adam. 1995. Sequence and characterization of cytoplasmic nuclear protein import factor p97. *J. Cell Biol.* 130:265–274.

Chida, K., and P.K. Vogt. 1992. Nuclear translocation of viral Jun but not cellular Jun is cell cycle dependent. *Proc. Natl. Acad. Sci. USA.* 89:4290–4294.

Corbett, A.H., and P.A. Silver. 1997. Nucleocytoplasmic transport of macromolecules. *Microbiol. Mol. Biol. Rev.* 61:193–211.

Diehl, J.A., and C.J. Sherr. 1997. A dominant negative cyclin D1 mutant prevents nuclear import of cyclin-dependent kinase 4 (CDK4) and it phosphorylation by CDK-activating kinase. *Mol. Cell. Biol.* 17:7362–7374.

Dingwall, C., and R.A. Laskey. 1991. Nuclear targeting sequences: a consensus? *Trends Biochem. Sci.* 16:478–481.

Doye, V., and E. Hurt. 1997. From nucleoporins to nuclear pore complexes. *Curr. Opin. Cell Biol.* 9:401–411.

Fornerod, M., M. Ohno, M. Yoshida, and I.W. Mattaj. 1997. CRM1 is an export receptor for leucine-rich nuclear export signals. *Cell.* 90:1051–1060.

Fridell, R.A., R. Truant, L. Thorne, R.E. Benson, and B.R. Cullen. 1997. Nuclear import of hnRNP A1 is mediated by a novel cellular cofactor related to karyopherin-beta. *J. Cell Sci.* 110:1325–1331.

Fukuda, M., S. Asano, T. Nakamura, M. Adachi, M. Yoshida, M. Yanagida, and E. Nishida. 1997. CRM1 is responsible for intracellular transport mediated by the nuclear export signal. *Nature.* 390:308–311.

Gallant, P., and E.A. Nigg. 1994. Identification of a novel vertebrate cyclin: cyclin B3 shares properties with both A-type and B-type cyclins. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:595–605.

Görlich, D., and I.W. Mattaj. 1996. Nucleocytoplasmic transport. *Science.* 271: 1513–1518.

Görlich, D., S. Prehn, R.A. Laskey, and E. Hartmann. 1994. Isolation of a protein that is essential for the first step of nuclear protein import. *Cell.* 79:767–778.

Görlich, D., S. Kostka, R. Kraft, C. Dingwall, R.A. Laskey, E. Hartmann, and S. Prehn. 1995a. Two different subunits of importin cooperate to recognize nuclear localization signals and bind them to the nuclear envelope. *Curr. Biol.* 5:383–392.

Görlich, D., F. Vogel, A.D. Mills, E. Hartmann, and R.A. Laskey. 1995b. Distinct functions for the two importin subunits in nuclear protein import. *Nature.* 377:246–248.

Görlich, D., N. Pante, U. Kutay, U. Aebi, and F.R. Bischoff. 1996a. Identification of different roles for RanGDP and RanGTP in nuclear protein import. *EMBO (Eur. Mol. Biol. Organ.) J.* 15:5584–5594.

Görlich, D., P. Henklein, R.A. Laskey, and E. Hartmann. 1996b. A 41 amino acid motif in importin alpha confers binding to importin-beta and hence transit to the nucleus. *EMBO (Eur. Mol. Biol. Organ.) J.* 15:1810–1817.

Hagting, A., C. Karlsson, P. Clute, M. Jackman, and J. Pines. 1998. MPF localization is controlled by nuclear export. *EMBO (Eur. Mol. Biol. Organ.) J.* 17:4127–4138.

Hua, X.H., H. Yan, and J. Newport. 1997. A role for Cdk2 kinase in negatively regulating DNA replication during S phase of the cell cycle. *J. Cell Biol.* 137: 183–192.

Izaurralde, E., U. Kutay, C. Vonkobbe, I.W. Mattaj, and D. Görlich. 1997. The asymmetric distribution of the constituents of the Ran system is essential for transport into and out of the nucleus. *EMBO (Eur. Mol. Biol. Organ.) J.* 16: 6535–6547.

Jackman, M., M. Firth, and J. Pines. 1995. Human cyclins B1 and B2 are localized to strikingly different structures: B1 to microtubules, B2 primarily to the Golgi apparatus. *EMBO (Eur. Mol. Biol. Organ.) J.* 14:1646–1654.

Jäkel, S., and D. Görlich. 1998. Importin beta, transportin, RanBP5 and RanBP7 mediate nuclear import of ribosomal proteins in mammalian cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 17:4491–4502.

Jin, P., S. Hardy, and D.O. Morgan. 1998. Nuclear localization of cyclin B1 controls mitotic entry after DNA damage. *J. Cell Biol.* 141:875–885.

Kalderon, D., B.L. Roberts, W.D. Richardson, and A.E. Smith. 1984. A short amino acid sequence able to specify nuclear location. *Cell.* 39:499–509.

Kalinich, J.F., and D.E. McClain. 1994. Rapid isolation of nuclear transport competent *Xenopus* nucleoplasmin produced in *Escherichia coli* strain BL21(DE3). *Protein Expr. Purif.* 5:324–330.

Kelly, B.L., K.G. Wolfe, and J.M. Roberts. 1998. Identification of a substrate-targeting domain in cyclin E necessary for phosphorylation of the retinoblastoma protein. *Proc. Natl. Acad. Sci. USA.* 95:2535–2540.

Knoblich, J.A., K. Sauer, L. Jones, H. Richardson, R. Saint, and C.F. Lehner. 1994. Cyclin E controls S phase progression and its down-regulation during *Drosophila* embryogenesis is required for the arrest of cell proliferation. *Cell.* 77:107–120.

Kornbluth, S., M. Dasso, and J.W. Newport. 1994. Evidence for a dual role for TC4 protein in regulating nuclear structure and cell cycle progression. *J. Cell Biol.* 125:705–719.

Kumagai, A., and W.G. Dunphy. 1995. Control of the Cdc2/cyclin B complex in *Xenopus* egg extracts arrested at a G2/M checkpoint with DNA synthesis inhibitors. *Mol. Biol. Cell.* 6:199–213.

Kutay, U., E. Izaurralde, F.R. Bischoff, I.W. Mattaj, and D. Görlich. 1997. Dominant-negative mutants of importin-β block multiple pathways of import and export through the nuclear pore complex. *EMBO (Eur. Mol. Biol. Organ.) J.* 16:1153–1163.

LaBaer, J., M.D. Garrett, L.F. Stevenson, J.M. Slingerland, C. Sandhu, H.S. Chou, A. Fattaey, and E. Harlow. 1997. New functional activities for the p21 family of CDK inhibitors. *Genes Dev.* 11:847–862.

Lehner, C.F., and P.H. O'Farrell. 1989. Expression and function of *Drosophila* cyclin A during embryonic cell cycle progression. *Cell.* 56:957–968.

Lehner, C.F., and P.H. O'Farrell. 1990. The roles of *Drosophila* cyclins A and B in mitotic control. *Cell.* 61:535–547.

Li, J., A.N. Meyer, and D.J. Donoghue. 1995. Requirement for phosphorylation of cyclin B1 for *Xenopus* oocyte maturation. *Mol. Biol. Cell.* 6:1111–1124.

Li, J., A.N. Meyer, and D.J. Donoghue. 1997. Nuclear localization of cyclin B1 mediates its biological activity and is regulated by phosphorylation. *Proc. Natl. Acad. Sci. USA.* 94:502–507.

Maridor, G., P. Gallant, R. Golsteyn, and E.A. Nigg. 1993. Nuclear localization

of vertebrate cyclin A correlates with its ability to form complexes with cdk catalytic subunits. *J. Cell Sci.* 106:535–544.
Melchior, F., and L. Gerace. 1998. Two-way trafficking with Ran. *Trends. Cell Biol.* 8:175–179.
Melchior, F., B. Paschal, J. Evans, and L. Gerace. 1993. Inhibition of nuclear protein import by nonhydrolyzable analogues of GTP and identification of the small GTPase Ran/TC4 as an essential transport factor. *J. Cell Biol.* 123: 1649–1659.
Michael, W.M., M. Choi, and G. Dreyfuss. 1995. A nuclear export signal in hnRNP A1: a signal-mediated, temperature-dependent nuclear protein export pathway. *Cell.* 83:415–422.
Moore, M.S., and G. Blobel. 1993. The GTP-binding protein Ran/TC4 is required for protein import into the nucleus. *Nature.* 365:661–663.
Moore, M.S., and G. Blobel. 1994. Purification of a Ran-interacting protein that is required for protein import into the nucleus. *Proc. Natl. Acad. Sci. USA.* 91:10212–10216.
Moroianu, J., G. Blobel, and A. Radu. 1995. Previously identified protein of uncertain function is karyopherin alpha and together with karyopherin beta docks import substrate at nuclear pore complexes. *Proc. Natl. Acad. Sci. USA.* 92:2008–2011.
Murray, A.W. 1991. Cell cycle extracts. *Methods Cell Biol.* 36:581–605.
Murray, A.W., M.J. Solomon, and M.W. Kirschner. 1989. The role of cyclin synthesis and degradation in the control of maturation promoting factor activity. *Nature.* 339:280–286.
Neville, M.S., F. Stutz, L. Lee, L.I. Davis, and M. Rosbash. 1997. The importin-beta family member Crm1p bridges the interaction between Rev and the nuclear pore complex during nuclear export. *Curr. Biol.* 7:767–775.
Nigg, E.A. 1997. Nucleocytoplasmic transport: signals, mechanisms and regulation. *Nature.* 386:779–787.
Ohno, M., M. Fornerod, and I.W. Mattaj. 1998. Nucleocytoplasmic transport: the last 200 nanometers. *Cell.* 92:327–336.
Ohtsubo, M., A.M. Theodoras, J. Schumacher, J.M. Roberts, and M. Pagano. 1995. Human cyclin E, a nuclear protein essential for the G1-to-S phase transition. *Mol. Cell. Biol.* 15:2612–2624.
Ossareh-Nazari, B., F. Bachelerie, and C. Dargemont. 1997. Evidence for a role of Crm1 in signal-mediated nuclear protein export. *Science.* 278:141–144.
Paschal, B.M., and L. Gerace. 1995. Identification of NTF2, a cytosolic factor for nuclear import that interacts with nuclear pore complex protein p62. *J. Cell Biol.* 129:925–937.
Pines, J., and T. Hunter. 1991. Human cyclins A and B1 are differentially located in the cell and undergo cell cycle-dependent nuclear transport. *J. Cell Biol.* 115:1–17.
Pines, J., and T. Hunter. 1994. The differential localization of human cyclins A and B is due to a cytoplasmic retention signal in cyclin B. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:3772–3781.
Pollard, V.W., W.M. Michael, S. Nakielny, M.C. Siomi, F. Wang, and G. Dreyfuss. 1996. A novel receptor-mediated nuclear protein import pathway. *Cell.* 86:985–994.
Radu, A., G. Blobel, and M.S. Moore. 1995. Identification of a protein complex that is required for nuclear protein import and mediates docking of import substrate to distinct nucleoporins. *Proc. Natl. Acad. Sci. USA.* 92:1769–1773.
Rexach, M., and G. Blobel. 1995. Protein import into nuclei: association and dissociation reactions involving transport substrate, transport factors, and nucleoporins. *Cell.* 83:683–692.
Riggs, P. 1990. Expression and purification of maltose-binding protein fusions. *In* Current Protocols in Molecular Biology. F.M. Ausebel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl, editors. Green Associates/Wiley Interscience, New York. 16.6.1–16.6.14.
Robbins, J., S.M. Dilworth, R.A. Laskey, and C. Dingwall. 1991. Two interdependent basic domains in nucleoplasmin nuclear targeting sequence: identification of a class of bipartite nuclear targeting sequence. *Cell.* 64:615–623.
Sigrist, S., H. Jacobs, R. Stratman, and C.F. Lehner. 1995. Exit from mitosis is regulated by *Drosophila fizzy* and the sequential destruction of cyclins A, B and B3. *EMBO (Eur. Mol. Biol. Organ.) J.* 14:4827–4838.
Smythe, C., and J.W. Newport. 1991. Systems for the study of nuclear assembly, DNA replication and nuclear breakdown in *Xenopus laevis* egg extracts. *Methods Cell Biol.* 35:449–468.
Solomon, M.J., M. Glotzer, T.H. Lee, M. Philippe, and M.W. Kirschner. 1990. Cyclin activation of $p34^{cdc2}$. *Cell.* 63:1013–1024.
Stade, K., C.S. Ford, C. Guthrie, and K. Weiss. 1997. Exportin 1 (Cr,1p) is an essential nuclear export factor. *Cell.* 90:1041–1050.
Stewart, E., H. Kobayashi, D. Harrison, and T. Hunt. 1994. Destruction of *Xenopus* cyclins A and B2, but not B1, requires binding to p34cdc2. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:584–594.
Toyoshima, F., T. Moriguchi, A. Wada, M. Fukada, and E. Nishida. 1998. Nuclear export of cyclin B1 and its possible role in the DNA damage induced G2 checkpoint. *EMBO (Eur. Mol. Biol. Organ.) J.* 17:2728–2735.
Ullman, K.S., M.A. Powers, and D.J. Forbes. 1997. Nuclear export receptors: from importin to exportin. *Cell.* 90:967–970.
Walter, J., L. Sun, and J. Newport. 1998. Regulated chromosomal DNA replication in the absence of a nucleus. *Mol. Cell.* 1:519–529.
Winters, Z.E., W.M. Ongkeko, A.L. Harris, and C.J. Norbury. 1998. p53 regulates Cdc2 independently of inhibitory phosphorylation to reinforce radiation-induced G2 arrest in human cells. *Oncogene.* 17:673–684.
Yang, J., E.G. Bardes, J.D. Moore, J.S. Brennan, M.A. Powers, and S. Kornbluth. 1998. Control of cyclin B1 localization through regulated binding of the nuclear export factor, Crm1. *Genes Dev.* 12:2131–2143.