Members of the NAP/SET Family of Proteins Interact Specifically with B-Type Cyclins

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Abstract. Cyclin-dependent kinase complexes that contain the same catalytic subunit are able to induce different events at different times during the cell cycle, but the mechanisms by which they do so remain largely unknown. To address this problem, we have used affinity chromatography to identify proteins that bind specifically to mitotic cyclins, with the goal of finding proteins that interact with mitotic cyclins to carry out the events of mitosis. This approach has led to the identification of a 60-kD protein called NAP1 that interacts specifically with members of the cyclin B family. This interaction has been highly conserved during evolution: NAP1 in the *Xenopus* embryo interacts with cyclins B1 and B2, but not with cyclin A, and the *S. cerevisiae* homolog of NAP1 interacts with Clb2 but not with Clb3. Genetic experiments in budding yeast indicate that NAP1 plays an important role in the function of Clb2, while biochemical experiments demonstrate that purified NAP1 can be phosphorylated by cyclin B/p34^{cdc2} kinase complexes, but not by cyclin A/p34^{cdc2} kinase complexes. These results suggest that NAP1 is a protein involved in the specific functions of cyclin B/p34^{cdc2} kinase complexes. In addition to NAP1, we found a 43-kD protein in *Xenopus* that is homologous to NAP1 and also interacts specifically with B-type cyclins. This protein is the *Xenopus* homolog of the human SET protein, which was previously identified as part of a putative oncogenic fusion protein (Von Lindern et al., 1992).

PASSAGE through the eukaryotic cell cycle is controlled by the activity of protein kinase complexes that are composed of an activating subunit (a cyclin) and a catalytic subunit (a cyclin-dependent kinase [Cdk][1]; for reviews see Murray and Hunt, 1993; Norbury and Nurse, 1992). Cyclins were originally identified as proteins that fluctuate in abundance during the cell cycle (Evans et al., 1983), while cyclin-dependent kinases were identified genetically as the products of genes required for cell cycle progression in yeasts (the *CDC28* gene in *S. cerevisiae* and the homologous *cdc2* gene in *S. pombe*) (Hartwell et al., 1974; Nurse and Thuriaux, 1980). An active cyclin-dependent kinase complex was first identified biochemically as purified maturation promoting factor (MPF), an activity from *Xenopus* eggs that can drive cells into mitosis and meiosis (Dunphy et al., 1988; Gautier et al., 1988; Lohka et al., 1988).

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1. *Abbreviations used in this paper*: Cdk, cyclin-dependent kinase; GST, glutathione-*S*-transferase; LB, Luria-Beltrani media; NAP1, nucleosome assembly protein 1.

The cell cycle of the budding yeast *S. cerevisiae* is driven by a single cyclin-dependent kinase called p34^{CDC28}, which is activated during G1 by association with members of the G1 class of cyclins (encoded by three *CLN* genes), and during S phase and mitosis by association with the B-type cyclins (encoded by 6 *CLB* genes) (Epstein and Cross, 1992; Nasmyth, 1993; Reed, 1992; Schwob and Nasmyth, 1993). In vertebrate cells, the cell cycle is driven by a number of related cyclin-dependent kinases (see for example, Meyerson et al., 1992). One of these, called p34^{cdc2}, is activated during mitosis and is most closely related to p34^{CDC28} from *S. cerevisiae*, while the other cyclin-dependent kinases appear to be activated during interphase. There are at least five classes of cyclins in vertebrates, called cyclins A, B, C, D, and E. Cyclin A appears during S phase, the B-type cyclins during mitosis, and cyclins D and E function during G1 (for reviews see Morgan, 1995; Pines, 1993). Recent experiments have revealed that specific Cdk-cyclin complexes are also involved in the phosphorylation of p34^{cdc2} and RNA polymerase, and in the regulation of phosphate metabolism in budding yeast, demonstrating that these complexes can play a wide variety of roles in cell physiology (Fisher and Morgan, 1994; Kaffman et al., 1994; Feaver et al., 1994; Roy et al., 1994).

In budding yeast, activation of p34^{cdc28} by B-type cyclins during G2 induces mitotic spindle assembly and the other

events of mitosis, while activation by G1 cyclins during G1 induces such events as DNA replication and spindle pole body duplication. Thus, a key question concerns how kinase complexes that contain the same catalytic subunit are able to induce such different events at different times during the cell cycle. One possibility is that the many different kinds of cyclins serve to determine the specific functions of cyclin-dependent kinase complexes. A number of experiments support the idea that the cyclin subunits determine the functions of specific cyclin-dependent kinase complexes. For example, cyclin A, but not cyclin B, is found in complexes that include the adenovirus E1A protein, the cellular transcription factor E2F, and an Rb-like protein called p107; and cyclin A is able to form kinase complexes that can bind and phosphorylate p107, while cyclin B is not (Nevins, 1992; Peeper et al., 1993; Pines and Hunter, 1990). Cdk4 can be activated by D cyclins, but not by cyclins A, B1, and E, and kinase complexes formed between Cdk4 and cyclins D2 or D3 can phosphorylate the Rb protein, while complexes formed with cyclin D1 cannot (Kato et al., 1993). Sea urchin cyclin B induces the dramatic destabilization of microtubules that occurs as cells enter mitosis, while bovine cyclin A does not, even though the two cyclins activate H1 kinase activity to the same level (Verde et al., 1992).

In contrast to these observations, a number of experiments have suggested that the different cyclins can perform the same functions. For example, both cyclin A and B can induce *Xenopus* oocyte maturation, chromosome condensation, and nuclear envelope breakdown, and they both form kinase complexes that can phosphorylate histone H1 in a similar manner (Draetta et al., 1989; Minshull et al., 1990; Pines and Hunt, 1987; Swenson et al., 1986; Westendorf et al., 1989). In addition, truncated forms of human cyclins A and B can rescue budding yeast cells that lack G1 cyclin function, suggesting that the different cyclins share considerable functional overlap (Koff et al., 1991; Leopold and O'Farrell, 1991; Lew et al., 1991; Xiong et al., 1991).

To learn more about potential functional differences between the cyclins, we have used affinity chromatography to identify proteins that interact specifically with mitotic cyclins, with the goal of identifying proteins that mediate specific functions of cyclin-dependent kinase complexes. This approach reveals that an evolutionarily conserved family of proteins interacts specifically with B-type cyclins. At least one member of this family is a specific substrate of cyclin B/p34^{cdc2} kinase complexes and is required for the normal function of Clb2 in budding yeast. Our experiments support the idea that cyclins can target the activity of cyclin-dependent kinases to specific substrates, and identify proteins that may mediate specific activities of B-type cyclins.

Materials and Methods

Reagents, Stock Solutions, and Media

Luria-Beltrani media (LB) and YPD media are prepared as described (Sambrook, 1989; No. 1499), and NZ media contains 20 g NZ amine, 10 g yeast extract, 5 gm casamino acids, and 5 g NaCl per liter. PMSF is made up as a 0.1-M stock solution in 100% ethanol and is stored at −20°C. PMSF is added to the Extract buffer just before lysis because it is unstable in water. DNAse I (Sigma Chem. Co., St. Louis, MO) is prepared as a 10-mg/ml stock solution in 50 mM Hepes, 50 mM KCl, 1 mM DTT, and 50% glycerol, and is stored at −20°C. Lysozyme (Sigma) is prepared as a 100-mg/ml stock solution in water, and is stored at −20°C.

Buffers

Phosphate buffered saline (PBS): 14 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.2, 138 mM NaCl, 2.7 mM KCl. LPC Protease Inhibitor Mix: 1 mg/ml leupeptin, 1 mg/ml pepstatin, 1 mg/ml chymostatin dissolved in dimethylsulfoxide. Extract buffer: 50 mM Hepes-KOH, pH 7.6, 50 mM KCl, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM PMSF, 1/1000 LPC Protease Inhibitor mix. Wash buffer: 50 mM Hepes-KOH, pH 7.6, 50 mM KCl, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 10% glycerol, 1/1,000 LPC protease inhibitor mix. Elution buffer: 50 mM Hepes-KOH, pH 7.6, 0.35 M KCl, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 1/1,000 LPC protease inhibitor mix, 10% glycerol. Phosphorylation buffer: 75 mM Hepes-KOH, pH 7.6, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 10% glycerol, 1/1,000 LPC protease inhibitor mix, 0.25 mM ATP, 0.1 mCi/ml [γ ^{32}P] ATP (10 mCi/ml, 3,000 Ci/mmol). Sample buffer: 65 mM Tris-HCl, pH 6.8, 3% sodium dodecyl sulfate (SDS), 5% β-mercaptoethanol, 10% glycerol.

Purification of GST-Cyclin Fusion Proteins

We express cyclins as glutathione-*S*-transferase (GST) fusions in *E. coli* using the vector pGEX.1 (Smith and Johnson, 1988). For purification of GST-cyclins, bacterial cells carrying a particular GST-cyclin expression construct are grown to saturation in approximately 400 ml of LB medium containing 100 mg/ml ampicillin. This culture is then used to inoculate 6 liters of NZ medium containing 100 mg/ml ampicillin. The culture is grown either at room temperature or 30°C until an optical density of approximately one is reached, and the temperature of the culture is then reduced to room temperature, if necessary. IPTG is added to a concentration of 0.1 mM, and the culture is incubated at room temperature for 3–4 h. The cells are harvested by centrifugation, and washed once with 200 ml ice cold PBS and pelleted again. The cells are kept on ice during harvesting, and the final cell pellet is frozen on liquid nitrogen and stored at −80°C.

For purification of GST-cyclins, the frozen cell pellet is thawed by the addition of 5 vol of PBS containing 1 mM EGTA, 1 mM EDTA, 1 mM PMSF, 0.1% Tween 20, and 200 mg/ml lysozyme. The PMSF and lysozyme are added to the buffer just before it is added to the cells. The cell pellet is then stirred for approximately 30 min to break up the pellet and allow lysis to occur, and the cells are then sonicated for several minutes to further promote lysis. KCl and DTT are added to the extract to final concentrations of 0.3 M and 15 mM, respectively. If the extract is fairly viscous at this point, DNAse I and $MgCl_2$ are added to final concentrations of 10 μg/ml and 2 mM, respectively. The extract is dialyzed against 20 vol of PBS containing 0.3 M KCl for 45 min. This step is repeated a total of three times. The dialysis step improves the yield of GST-cyclins approximately twofold, possibly by removing free glutathione in the extract that competes with the glutathione on the column for binding of the GST fusion to the column. The extract is centrifuged at 100,000 *g* for 1 h and the supernatant is loaded onto a 5–10-ml glutathione agarose column at a rate of 5–10 column vol/h. The column is washed at a flow rate of 10 column vol/h with PBS containing 1 mM DTT and 0.3 M KCl until no protein can be detected in the flowthrough (~15 column volumes). The column is eluted with 50 mM Tris (pH 8.1), 0.3 M KCl and 5 mM reduced glutathione (Sigma Chemical Co.), and the presence of protein in the elution fractions is determined by the Bradford assay (Bradford, 1976). The peak fractions are pooled and dialyzed extensively into buffer containing 50 mM Hepes (pH 7.6), 0.25 M KCl, and 30% glycerol. The yield of protein is between 15 and 50 mg, depending on the cyclin fusion protein. The purified protein is frozen on liquid nitrogen and stored at −80°C.

Construction of Affinity Columns

Affinity columns are constructed by coupling 4–6 mg of purified GST-cyclin fusion protein to 1 ml of Affigel 10 (Bio-rad Labs., Richmond, CA). The Affigel 10 is first washed several times with buffer (50 mM Hepes, pH 7.6, 0.25 M KCl) to remove the alcohol in which it is stored. The washes are carried out by resuspending the Affigel 10 beads in 10 vol of buffer, followed by sedimenting the beads in a table top centrifuge. After the Affigel 10 beads have been washed, they are left on ice until 20 min have passed since the wash buffer was first added. This allows partial inactivation of the beads (the activated coupling sites on Affigel 10 beads are slowly inactivated by water) and prevents overcoupling of the protein to the beads. 1 ml of purified protein (at 4–6 mg/ml) is added to the Affigel 10 beads, and the mixture is gently mixed on a rotator. The progress of the

coupling reaction is monitored by periodically pelleting the beads and measuring the protein concentration in the supernatant. When approximately 80% of the input protein has been removed from the supernatant, the reaction is terminated by the addition of 1 M Tris (pH 8) to a final concentration of 25 mM. The coupling reaction generally takes 10–60 min. The beads are transferred to a small column (we use a 3-ml syringe fitted with a polypropylene disk at the bottom), and are washed with elution buffer before being used in an affinity chromatography experiment. For storage, the columns are washed with buffer containing 100 mM Hepes (pH 7.6), 1 mM EGTA, 1 mM $MgCl_2$, 2 mM DTT, and 50% glycerol. The columns are stored at −20°C and can be reused three times without significant changes in their properties. All of the steps described above are carried out at 4°C, except where noted.

Columns containing BSA are made by adding 5 ml of a 10 mg/ml BSA solution to 5 ml of packed Affigel 10. The BSA solution is made up in 50 mM Hepes, pH 7.6, and is dialyzed against the same buffer extensively before being used to make columns. The BSA coupling reaction is started immediately after the Affigel 10 has been washed, and the reaction is allowed to proceed for at least 1 h at room temperature. BSA columns are washed with 50 mM Hepes (pH 7.6), 2 M urea before use and between each use. The columns are stored at 4°C in PBS that contains 0.02% azide, and are reused many times.

Affinity Purification of Yeast Cyclin-binding Proteins

We use yeast cells arrested by nutrient depletion as the starting material for our experiments. Cells are allowed to grow to saturation in YPD media, and are harvested by centrifugation. The cells from several liters of culture are resuspended in 100 ml of 50 mM Hepes (pH 7.6), 50 mM KCl, and are then pelleted in 50-ml disposable conical tubes. The supernatant is removed, and a small hole is made in the bottom of the tube. The plunger from a 60-ml syringe is then used to force a thin stream of yeast out of the hole and directly into liquid nitrogen. This allows rapid freezing of the yeast paste. The frozen cells are stored at −80°C until needed.

Yeast cells are broken open by grinding the frozen paste under liquid nitrogen with a mortar and pestle until a very fine powder is obtained (20–30 min). For the experiment shown in Fig. 2 *B*, we used 15 grams of yeast cells as starting material. The powder is transferred to a small beaker and left at room temperature until it is just beginning to thaw. 35 ml of Extract buffer at room temperature is then added and the extract is stirred until a uniform solution is obtained. The extract is centrifuged at 40,000 rpm in a 50.2Ti rotor for 1 h at 4°C and the supernatant is removed with a pipette, taking care to avoid particulate matter at the bottom of the tube. DTT is added to the supernatant to a final concentration of 1 mM. The protein concentration of the resulting supernatant is ~10 mg/ml.

The extract is first passed through a 5-ml BSA column (see above). This serves as a prefilter to remove particulate matter and proteins that bind nonspecifically to the affinity columns. The BSA column is connected in series to the cyclin affinity column, such that the flowthrough from the BSA column flows directly onto the cyclin column. For the experiment shown in Fig. 2 *B*, we used a 1-ml Clb2 column and a 1-ml Clb3 column, each having a BSA precolumn. The columns are preequilibrated with Extract buffer before loading the crude extract. The cyclin affinity columns are loaded at 8 column vol/h, and are then disconnected from the BSA precolumns and washed with 8 column volumes of Wash buffer at 8 column vol/h. The columns are then eluted with Elution buffer and 0.25-ml fractions are collected. The elution is carried out by pipetting 0.25-ml aliquots of elution buffer directly onto the surface of the column bed. The elution buffer is then allowed to flowthrough the column by gravity and is collected in 1.5-ml plastic tubes. The fractions are assayed for protein qualitatively by mixing 5 μl of each fraction with 45 μl of Bradford reagent on a piece of parafilm. The peak fractions are pooled and protein is precipitated by addition of TCA to a final concentration of 10% from a 100% (wt/vol) stock. After a 10-min incubation on ice, the protein solution is centrifuged for 10 min in a microfuge, and the supernatant is removed. The tube is centrifuged again to remove the remaining liquid from the walls of the tube. The final supernatant is removed and the precipitate is resuspended in gel sample buffer, which is neutralized with the vapor from a cotton swab soaked in ammonium hydroxide. All steps are carried out at 4°C except where noted. The yield of protein from the Clb2 affinity column is ~25 μg.

Affinity Purification of Xenopus Egg Cyclin-binding Proteins

Concentrated extracts from activated *Xenopus* eggs were made as previously described (Murray, 1991). For the experiment shown in Fig. 2 *A*, 2.8 ml of concentrated extract was diluted into 21 ml of Extract buffer. The diluted extract is centrifuged at 50,000 rpm in a 50Ti rotor for 100 min. This unusually long spin is required to completely remove all ribosomes from the supernatant, because ribosomal subunits that remain in the supernatant will bind to the cyclin B columns and obscure the binding of the 60-kD and 40-kD proteins. We do not know whether the binding of ribosomes to the B-type cyclins represents a specific interaction, although the endogenous cyclin B in frog egg extracts has also been observed to bind to ribosomes (Leiss et al., 1992). Our finding that yeast elongation factor 1α binds to Clb2 and Clb3 (Fig. 2 *B*, see the Results section) suggests that the binding of ribosomes to the *Xenopus* B cyclins may be due to an interaction with elongation factor 1α. The remainder of the experiment is carried out as described in the previous section for yeast cyclin affinity columns, except that the extract is loaded onto the affinity columns at 2 column vol/h. The yield of protein from the cyclin B affinity columns is ~30 μg.

Immunoprecipitations

To prepare beads for immunoprecipitation experiments, 10 μg of affinity purified antibody is mixed with 15 μl of protein A–Affigel 10 beads (Bio-rad Labs.) in PBS for 1 h at room temperature. The beads are then washed three times with 0.5 ml of 50 mM Hepes, 1 M NaCl, 0.05% Tween-20, followed by two times with Extract buffer. Yeast extracts are made from cells growing in log phase in YPD media, and the cells are broken open by grinding under liquid nitrogen as described above. For each 0.25 gm of yeast powder, 0.6 ml of Extract buffer is added, and the suspension is then centrifuged for 10 min in a microfuge at 4°C. For each immunoprecipitation, 0.4 ml of extract is mixed with 15 μl of beads containing bound antibody. The immunoprecipitations are mixed gently at 4°C for 2 h, and the beads are then washed four times with 100 vol of Wash buffer containing 0.2 mM DTT. To wash the NAP1 off of the immunoprecipitated CLB2 complexes, the beads are resuspended in 200 μl 50 mM Hepes, 1 M NaCl, and 0.05% Tween-20. The beads are then pelleted, the supernatant is removed carefully to avoid taking any beads, and the protein in the supernatant is precipitated with TCA. This high salt wash allows us to separate the NAP1 from the antibody bound to the beads, which would obscure the NAP1 band on Western blots. The precipitate is resuspended in 30 μl of 1X gel sample buffer, and 15 μl is used for Western blotting experiments to detect NAP1. After the high salt wash, we boiled the beads in sample buffer to release the antibody, and used polyacrylamide gel electrophoresis to confirm that equal amounts of Clb2 and Clb3 antibody were on the beads.

Polyacrylamide Gel Electrophoresis, Western Blotting, and Protein Sequence Analysis

Polyacrylamide gel electrophoresis and Western blotting were carried out as previously described (Anderson et al., 1973; Harlow and Lane, 1988; Towbin et al., 1979). For protein sequence analysis, cyclin-binding proteins were subjected to SDS-PAGE and electroblotted onto PVDF membrane (Immobilon P, Millipore, Bedford, MA). The PVDF blot was stained with Ponceau S and the desired protein band was excised. The PVDF membrane carrying the protein was then destained, incubated with polyvinylpyrrolidone/MeOH to block protein-binding sites, and incubated with 1 μg trypsin in 50 μl of 100 mM Tris-HCl, pH 8.0, 10% acetonitrile, and 1% Triton-X at 37°C for 24 h. Eluted tryptic peptides were subjected to reversed phase HPLC on a narrow bore column and individual peptides sequenced with an ABI gas-phase sequencer (model 475A).

PCR Cloning of the SET cDNA from Xenopus and Drosophila

We initially obtained a partial cDNA clone from the *Xenopus* SET gene using PCR primers based on the peptide sequences that we obtained from the protein (see Results). We then used a nested PCR reaction (Gibbons et al., 1991) to obtain the 5' end of the cDNA, using a *Xenopus* embryo plasmid cDNA library (gift of Jeremy Minshull) as template. To clone the Drosophila homolog of SET, we compared human SET with yeast NAP1 and picked the regions of greatest homology as starting points for designing PCR primers. We eventually used the oligos AA(A/G) AT(A/C/T) CC(A/C/G/T) AA(C/T) TT(C/T) TGG and (G/A)AA CCA (A/C/G/T)GT (G/A)AA (G/A)AA (A/C/G/T)(G/C)(T/A) (C/T)TC(A/C/G/T) GG, corresponding to the peptides KIPNFW and PESFFTWF, for PCR reactions using a *Drosophila* embryo plasmid cDNA library (Brown and Kafatos, 1988) as template. This gave a fragment of the expected size that

had significant homology to human SET, and we used nested PCR reactions to obtain the 5′ and 3′ ends of the cDNA. We used the VENT polymerase (New England Biolabs, Beverly, MA) for the nested reactions, which should minimize the number of errors due to misincorporation. Genbank accession number for the *Drosophila* SET cDNA is U30470.

Deletion of the NAP1 Gene in S. cerevisiae

An EcoR1–Rsa1 fragment of the *NAP1* gene, a Sal1–Xho1 fragment of the *Leu2* gene (from the plasmid YEP13), and an Xho1–HindIII fragment of the *NAP1* gene were successively cloned in the EcoR1–Sma1 sites, Sal1 site and Sal1–HindIII sites, respectively, of the multicloning sites in the pTZ19R vector. This construct was digested with Dra1 and Nde1 and used for disrupting the *NAP1* gene. The gene disruption was verified both by Southern blotting and by immunoblotting for the NAP1 protein with monoclonal and polyclonal antibodies.

Purification of GST-Cyclin/p34^{cdc2} Complexes

We purify activated cyclin/p34cdc2 complexes from *Xenopus* egg extracts according to a procedure modified from the one described by Solomon et al. (1990). Concentrated cytoplasmic extracts are made from electrically activated *Xenopus* eggs as previously described (Murray, 1991) and arrested in interphase by the addition of cyclohexamide to 100 μg/ml. Purified GST-cyclin A or cyclin B is then added to the extract. A total of 300 μg of purified cyclin fusion protein is added to 0.8 ml of extract, but only about half of the total protein in the purified cyclins represents full-length GST-cyclin (see Fig. 1). The extract is incubated at room temperature for 20 min to allow binding of p34^{cdc2} and formation of an activated kinase complex, and is then diluted 1:1 with buffer containing 50 mM Hepes (pH 7.6), 1 mM EGTA, 1 mM $MgCl_2$, 1 M KCl, 1 mM PMSF and 1X LPC. The extract is centrifuged in a microfuge for 10 min before it is loaded onto a 10-ml Biogel P4 desalting column (Biorad Labs.) equilibrated with buffer containing 50 mM Hepes (pH 7.6), 150 mM KCl, 1 mM EGTA, 1 mM $MgCl_2$, 1 mM PMSF, 1:1,000 LPC protease inhibitor mix.

The protein that flows through the column is pooled, brought to 5 ml by addition of the desalting column buffer, and centrifuged at 50,000 rpm in a 70 Ti rotor for 1 h. The supernatant is loaded onto a 1-ml glutathione agarose column at 5 column vol/h, and the column is then washed with 5 column vol of Wash buffer containing 1 M KCl, followed by 3 column vol of Wash buffer. The column is eluted in 0.25-ml fractions with 30 mM Tris (pH 8.1), 75 mM KCl, 1 mM EGTA, 1 mM $MgCl_2$, 10% glycerol, 5 mM reduced glutathione. The fractions are assayed qualitatively for the presence of protein by the Bradford assay, and the peak fractions are pooled, aliquoted, frozen on liquid nitrogen, and stored at −80°C.

Phosphorylation of Xenopus NAP1

The *Xenopus* NAP1 used for phosphorylation assays was further purified by ion exchange chromatography after the cyclin affinity column step. Cyclin affinity chromatography is carried out as described above, except that the cyclin-binding proteins are eluted from the column in a buffer containing 50 mM Tris-HCl, pH 8.1, 350 mM NaCl, 1 mM $MgCl_2$, 10% glycerol, 0.02% Tween-20, 1 mM DTT, and LPC protease inhibitor mix. The eluted protein (approximately 150 μg in 1.5 ml) is diluted to 2 ml with water and loaded onto a 0.4-ml column containing Q sepharose HP (Pharmacia) using the Pharmacia Smart System. The column is equilibrated in 25 mM Tris-HCl, pH 8.1, 250 mM NaCl, 0.5 mM $MgCl_2$, 1 mM DTT, 5% glycerol, 0.02% Tween-20, and is eluted with a gradient starting at 250 mM NaCl and ending at 750 mM NaCl. The total volume of the gradient is 2 ml and 100-μl fractions are collected. Virtually all of the contaminating proteins flowthrough the column under the load conditions, and NAP1 and SET begin to elute at ~0.4 M NaCl. A 10% polyacrylamide gel showing fractions from the NaCl gradient used to elute NAP1 from the Q sepharose column is shown in Fig. 8 *A*. The SET protein elutes slightly later than NAP1.

The fraction containing the most purified NAP1 (marked with an arrow in Fig. 8 *A*) was used for phosphorylation experiments. Each phosphorylation reaction contains 1.5 μl of the purified NAP1 (~0.3 μg), 50 μl of reaction buffer (50 mM Hepes-KOH, pH 7.6, 1 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 0.01% Tween-20, 300 μM ATP, 0.2 μl g32P-ATP [10 mCi/ml, 3,000 Ci/mmol]), and either 9 μl of purified cyclin A/p34^{cdc2} kinase or 4 μl of cyclin B1/p34^{cdc2} kinase complex. The amount of each kinase used is determined empirically to give approximately equal amounts of phosphorylation of histone H1 under the same assay conditions. The reactions are allowed to proceed for 45 min at 25°C, and are terminated by addition of TCA to a final concentration of 10% from a 100% stock. The protein in the reactions is precipitated with TCA as described above, and the entire reaction is analyzed on a 10% polyacrylamide gel.

Immunofluorescence

Affinity purified rabbit polyclonal antibodies against NAP1 were generated using previously described methods (Harlow and Lane, 1988; Kellogg and Alberts, 1992). Immunofluorescence staining with anti-NAP1 antibodies was carried out using previously described methods (Kilmartin et al., 1993; Pringle et al., 1991). We obtained the same results whether we fixed the cells directly in 3.7% formaldehyde for 1 h, or fixed the cells for 5 min in 3.7% formaldehyde after removal of the cell wall.

Yeast Strains

All biochemical experiments were carried out using the protease deficient strain FM135 (*MATa gal1, leu2-3,112 pep4-3 prb1-1122 reg1-501 ura3-52*), with the exception of the immunoprecipitation experiments, which were carried out in the protease deficient strain JB811 (*ura3-52, leu2-3,112, trp1, prb1, pep4-3*). The strain carrying a temperature sensitive allele of CLB2 in a Clb2-dependent background is strain K3080 (*MATa, Δclb1, clb2-ts, clb3::TRP1, clb4::HIS3* [Amon et al., 1993]). The NAP1 gene was deleted in the W303 strain background (*MATa ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100*) to give strain DK96, and a strain carrying deletions of *NAP1, CLB1, CLB3*, and *CLB4* was obtained by crossing DK96 with strain K2652 (*MATa, clb1::URA3, clb3::TRP1, clb4::HIS3*) (Fitch et al., 1992). We obtained five different spores carrying the quadruple deletion, and they all behaved identically. We also obtained the same phenotype by directly knocking out the *NAP1* gene in strain K2652. A strain carrying deletions of CLN1 and CLN3 in the W303 strain background was made by backcrossing JO314-2C (*MATa, cln1::TRP1, cln3::URA3, ade2-1, lys2, leu2, his3D200*) into the W303 strain background three times. The resulting strain was then crossed with DK96 to give a strain carrying deletions for *NAP1, CLN1*, and *CLN3*.

Results

Identification of Cyclin-binding Proteins

We chose to use affinity chromatography to identify cyclin-binding proteins, because this approach should allow detection of proteins that bind only weakly to cyclins, in addition to the proteins that bind tightly (for a theoretical discussion see Scopes, 1988; p. 72–90). To obtain amounts of cyclins sufficient for construction of affinity columns, we expressed cyclins from *Xenopus* and yeast as glutathione-*S*-transferase (GST) fusions in *E. coli*, and then purified the GST-cyclins by virtue of their affinity for glutathione (Smith and Johnson, 1988). Cyclin affinity columns were made by covalently linking the purified GST-cyclins to agarose beads. We have made affinity columns with cyclins A, B1, and B2 from *Xenopus*, and cyclins Clb2 and Clb3 from the yeast *S. cerevisiae*. The purified fusion proteins used for construction of our affinity columns are shown in Fig. 1. All of the fusion proteins show some degree of proteolytic degradation and are slightly contaminated with bacterial proteins of apparent molecular masses 60 and 70 kD. These are likely to be bacterial heat shock proteins.

We carried out affinity chromatography experiments by loading columns with extracts made from either *Xenopus* eggs or yeast cells. After loading the extract, the columns were washed extensively and then eluted with 0.35 M KCl. Fig. 2 *A* shows the results of an experiment in which an interphase *Xenopus* embryo extract was loaded onto affinity columns containing the *Xenopus* cyclins A, B1, and B2. Two prominent proteins of apparent molecular masses 40

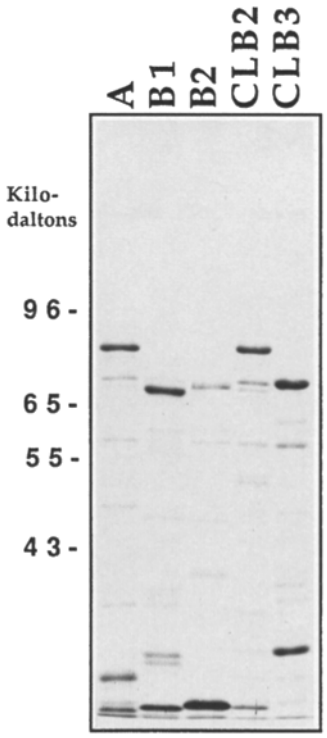

Figure 1. Purified GST-cyclin fusion proteins. 2 mg of each purified GST-cyclin fusion protein was loaded onto a 10% polyacrylamide gel. Several purified fusion proteins have major breakdown products that migrate at about 25 kD. These are likely to correspond to the GST portion of the protein that has been clipped off by endogenous proteases.

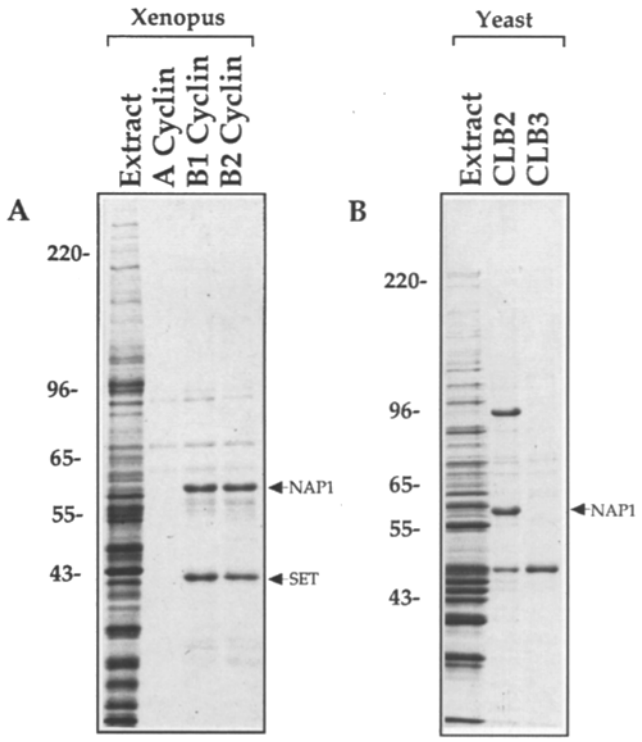

Figure 2. (*A*) Affinity purification of cyclin-binding proteins from *Xenopus* eggs. Cyclin-binding proteins were affinity purified, TCA precipitated, and separated on a 10% polyacrylamide gel. The lane labeled Extract was loaded with 15 μg of the high speed supernatant that was loaded onto the column. The other lanes were loaded with the proteins that elute from cyclin affinity columns with 0.35 M KCl. The lanes labeled B1 cyclin and B2 cyclin were loaded with 3 μg of protein, whereas the A cyclin lane was loaded with an equivalent amount of the elution from the A cyclin. The gel is stained with Coomassie blue. (*B*) Affinity purification of cyclin-binding proteins from *S. cerevisiae*. Cyclin-binding proteins were affinity purified, TCA precipitated, and separated on a 10% polyacrylamide gel. The lane labeled Extract was loaded with 15 μg of the high speed supernatant that was loaded onto the column. The other lanes were loaded with the proteins that elute from Clb2 and Clb3 affinity columns with 0.35 M KCl. The lane labeled Clb2 was loaded with 3 μg of protein, whereas the Clb3 lane was loaded with an equivalent amount of the elution from the Clb3 affinity column. The gel is stained with Coomassie blue.

kD and 60 kD are observed to bind to cyclins B1 and B2, but not to cyclin A. A less prominent protein band at 90 kD, and a group of proteins that migrate just below the 60-kD protein also bind to the B1 and B2 cyclins. There are a number of proteins that interact with all three cyclin affinity columns, but these proteins also bind to columns containing control GST fusion proteins (not shown), and we suspect that these are *Xenopus* proteins that interact with the bacterial heat shock proteins that contaminate the GST fusion proteins. We have found no proteins that bind specifically to cyclin A in this system.

Fig. 2 *B* shows the results of a similar experiment in which an extract made from yeast cells arrested in G1 is loaded onto affinity columns containing Clb2 and Clb3. In this experiment, proteins of apparent molecular mass 90 kD and 60 kD bind Clb2, but not Clb3, while a protein of apparent molecular weight 50 kD binds to both Clb2 and Clb3. The amount of the 90-kD protein that binds to Clb2 is somewhat variable, and the experiment shown in Fig. 2 *B* gave an unusually high yield of the 90-kD protein.

We wanted to determine whether there are additional cyclin-binding proteins that are not released by the 0.35 M KCl elution. For example, $p34^{CDC28}$ and a number of low molecular cyclin-dependent kinase inhibitor proteins interact very tightly with cyclins and can only be released by denaturing conditions (Dunphy et al., 1988; Mendenhall, 1993; Zhang et al., 1993). In our experiments, we have not been able to visualize such proteins directly because elution of the cyclin affinity columns with denaturants releases approximately 100 mg of the bound cyclin and its breakdown products, which obscure proteins smaller than cyclin. We have, however, detected the presence of $p34^{CDC28}$ in denaturing elutions by Western blotting, as expected (data not shown).

A Conserved Cyclin-binding Protein Interacts Specifically with B-Type Cyclins in Xenopus and Yeast

To begin characterizing the proteins that interact with cyclins, we used protein sequencing techniques to obtain a peptide sequence from the 60-kD protein that interacts with Clb2. The peptide that we obtained (TYFYQK) was identical to a sequence of amino acids from a previously described yeast protein called nucleosome assembly protein 1 (NAP1). NAP1 was originally identified as a protein from HeLa cells that promotes nucleosome assembly in vitro, and a monoclonal antibody raised against the purified HeLa cell protein was found to recognize proteins of similar molecular weight in yeast and *Drosophila* (Ishimi et al., 1984, 1985). This monoclonal antibody was used to clone the yeast homolog of NAP1 (Ishimi and Kikuchi,

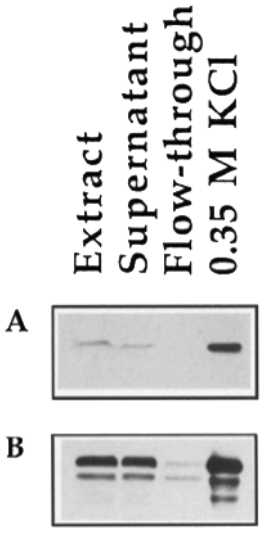

Figure 3. (*A*) The behavior of yeast NAP1 in an affinity chromatography experiment. Fractions from the experiment shown in Fig. 2 *B* were separated on 10% polyacrylamide gel, electroblotted onto nitrocellulose, and probed with anti-NAP1 monoclonal antibody. Lanes: *Crude extract*, 15 μg; *High speed supernatant*, 15 μg; *Column flowthrough*, 15 μg; *0.35 M KCl* elution, 0.3 μg. (*B*) The behavior of *Xenopus* NAP1 in an affinity chromatography experiment. Fractions from the experiment shown in Fig. 2 *A* were separated on 10% polyacrylamide gel, electroblotted onto nitrocellulose, and probed with anti-NAP1 antibody. Lanes: *Crude extract*, 15 μg; *High speed supernatant*, 15 μg; *Column flowthrough*, 15 μg; *0.35 M KCl* elution (from a cyclin B1 affinity column), 0.3 μg.

1991). Although NAP1 promotes nucleosome assembly in vitro, its role in this process in vivo remains unclear.

We used the monoclonal antibody that recognizes NAP1 to confirm that the 60-kD protein that binds to Clb2 is indeed NAP1. The Western blot in Fig. 3 *A* shows that NAP1 is largely depleted from a yeast extract as it flows over a Clb2 affinity column, as expected for a protein that interacts specifically with Clb2. When an extract made from *Δnap1* cells is passed over a Clb2 affinity column, the 60-kD band is no longer present (data not shown). We next used the anti-NAP1 monoclonal antibody in a similar kind of Western blotting experiment to demonstrate that the 60-kD protein that interacts with the *Xenopus* cyclins B1 and B2 is the *Xenopus* homolog of NAP1 (Fig. 3 *B*). In *Xenopus*, the anti-NAP1 monoclonal antibody recognizes a major protein of 60 kD, as well as a number of minor proteins of lower apparent molecular weight. We initially suspected that these lower molecular weight proteins represent proteolytic breakdown products or differently modified forms of NAP1. However, recent experiments in which we have used PCR to clone *Xenopus* homologs of NAP1 indicate that there are at least three homologs of NAP1 in *Xenopus* (Kellogg, D. R., and A. W. Murray, unpublished). The additional protein bands may therefore represent different NAP1 homologs.

To further confirm these results, we tested whether we could detect an interaction between the endogenous cyclins and NAP1. For this experiment, we immunoprecipitated CLB2 and CLB3 from yeast extracts, and used Western blotting to determine whether NAP1 coprecipitates with the cyclins (Fig. 4). The results further confirm that NAP1 interacts with CLB2, but not with CLB3.

These results indicate that the specific interaction between NAP1 and B-type cyclins has been highly conserved during evolution. In support of this, we have also found that *Xenopus* NAP1 interacts with budding yeast CLB2 in affinity chromatography experiments (not shown). This high degree of conservation argues that the interaction between NAP1 and B-type cyclins is biologically relevant,

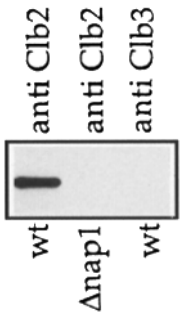

Figure 4. NAP1 coprecipitates with the Clb2 protein. Immunoprecipitations were carried out as described in the Materials and Methods, using extracts made from wild-type or *Δnap1* cells.

rather than the product of an artifactual in vitro association. Genetic evidence that the interaction between B-type cyclins and NAP1 is biologically relevant is presented below.

The SET Protein, a NAP1 Homolog, Binds to the Xenopus B-Type Cyclins

To identify the 43-kD protein that binds to the *Xenopus* B-type cyclins, we obtained two tryptic peptide sequences (QPFFQLR and EFHLNESGDPS) from the protein and used these to design PCR primers for amplification of the coding sequences. We initially obtained a 270-bp DNA fragment that encoded an open reading frame and then carried out further PCR reactions to obtain a larger clone that contains the 5′ end of the cDNA. This partial cDNA encodes a 147–amino acid peptide that is 98% identical to the human SET protein (Von Lindern et al., 1992). Interestingly, the human SET protein is 25% identical to yeast NAP1 (Von Lindern et al., 1992). The SET protein was originally identified as part of a putative oncogene formed by fusion of the SET gene to the CAN gene in a case of acute undifferentiated leukemia (Von Lindern et al., 1992). This fusion results in the production of an mRNA that encodes a SET-CAN fusion protein, and it remains unclear how this fusion protein might contribute to leukemogenesis.

The high degree of conservation that we observed between human and *Xenopus* SET suggested that SET is likely to be found in many organisms. As a first step toward characterizing SET in other organisms, we used PCR to clone the Drosophila SET homolog. Human and Drosophila SET are 55% identical, confirming that SET is a highly conserved protein (Fig. 5). We have not yet found a SET homolog in yeast.

The sequence of tryptic peptides obtained from the 50-kD protein that binds to both Clb2 and Clb3 (Fig. 2 *B*) in-

```
     Human SET: 0 MSAQAAKVSKKELNSNHDGADETSEKEQQEAIEHIDEVQNEIDRLNEQAS
Drosophila SET:   --SVPKRAKLDGAPDGNTS-AAGNNE-ES--L-Q--AC-----A---K--

               51 EEILKVEQKYNKLRQPFFQKRSELIAKIPNFWVTTFVNHPQVSALLGEED
                  --------------K-CYE-----VKR-------S-I------GI-D--E

              101 EEALHYLTRVEVTEFEDIKSGYRIDFYFDENPYFENKVLSKEFHLN....
                  --C--A-NKL--E----------N-H-----------T------SAAA

              151 .ESGD.PSSKSTEIKWKSGKDLTKRSSQTQNKASRKRQHEEPESFFTWFT
                  S-N--W-A-T--P----E--N-L-.LLL-KPYGNK-KRNS-YKT--D--S

              201 DHSDAGADELGEVIKDDIWPNPLQYYLVPDMDDEEGEGEEDDDDDEEEEG
                  -NT-PVN--IA-L----L-----------IEV-..........P-D--D

              251 LEDIDEEGDEDEGEEDEDDDEGEEGEEDEGEDDZ
                  N--N---AFD-...--GE-G----E----.D-K-
```

Figure 5. Comparison of the sequence of the human and Drosophila SET genes. Dashes indicate identical amino acids.

dicate that it is ribosomal elongation factor 1α. Since this is one of the most abundant proteins in yeast (Thiele et al., 1985), it may be retained on the cyclin columns by a weak nonspecific affinity, and more work must be done to determine whether this represents a biologically relevant interaction. It is perhaps worth noting, however, that elongation factor 1α binds to actin in a regulated manner and has microtubule severing activity, suggesting that it may play a more general role in cellular activities (Dharmawardhane et al., 1991; Shiina et al., 1994).

NAP1 Plays an Important Role in Clb2 Function

Since NAP1 interacts with Clb2, one would predict that it is somehow involved in Clb2 function. Analysis of Clb function in budding yeast is complicated by the fact that budding yeast have six different *CLB* genes that carry out overlapping functions, and as many as three of these genes can be deleted with little effect upon cell growth (Fitch et al., 1992; Kuhne and Linder, 1993; Richardson et al., 1992; Schwob and Nasmyth, 1993). Therefore, even if NAP1 is essential for Clb2 function, one might not expect to see any effect of deleting the *NAP1* gene, since *CLB2* itself is not essential. We have circumvented this problem by studying NAP1 function in a strain that has been made dependent upon Clb2 for survival by deleting the *CLB1, CLB3*, and *CLB4* genes.

As a first step towards understanding NAP1 function, we deleted the *NAP1* gene in a wild-type strain and in a strain that is dependent upon Clb2 for survival. Deletion of the *NAP1* gene in a wild-type strain (w303 strain background) has no obvious effect upon cell growth. Although the *Δnap1* strain forms colonies at a normal rate, examination of the cells reveals that they are often found in clumps of connected cells with elongated shapes (Fig. 6, *A* and *B*). Homozygous *Δnap1/Δnap1* diploid cells also exhibit abnormal morphology, and the phenotype appears to be somewhat stronger (data not shown).

Deleting *NAP1* in Clb2-dependent cells has a much more dramatic effect. This quadruple deletion strain (*Δnap1, Δclb1, Δclb3, Δclb4*) is temperature sensitive for growth at 38°C (Fig. 7), and when grown at 30°C forms large clumps of interconnected cells that have an unusual elongated morphology (Fig. 6, *C* and *D*). This phenotype is likely to be due to a defect in Clb2 function, since it is largely rescued by the presence of other Clbs that can compensate for a defect in Clb2 function (e.g., Clb1, Clb3, and Clb4). Several observations confirm this idea. First, the elongated morphology of these cells is quite similar to the morphology of cells that lack Clb1, Clb2, Clb3, and Clb4, which form highly elongated buds because Clb function is required to turn off polarized bud growth that is induced by the G1 cyclins (Amon et al., 1993; Fitch et al., 1992; Kuhne and Linder, 1993; Lew and Reed, 1993; Richardson et al., 1992; Schwob and Nasmyth, 1993). Indeed, the morphology of the *Δnap1, Δclb1, Δclb3, Δclb4* cells is strikingly similar to that seen when cells dependent upon a temperature sensitive Clb2 are grown at a semirestrictive temperature (Fig. 6, *E* and *F*). Deletion of the *NAP1* gene also causes a substantial increase in the temperature sensitivity of the *clb2^{ts}* strain (Fig. 8). These observations indicate that Clb2 function is severely compromised in the absence of NAP1. A detailed analysis of the role of NAP1 in Clb2 function is presented in the accompanying paper.

Strains carrying deletions of *NAP1* and *CLB2* have a phenotype that is similar to that of the quadruple deletion, but somewhat less severe. Because *Δclb2* cells are largely dependent upon *CLB1* (Richardson et al., 1992; Surana et al., 1991), the observation that *Δclb2, Δnap1* cells have a phenotype suggests that NAP1 also interacts with Clb1. Since Clb1 and Clb2 are closely related proteins, this is not unexpected (Surana et al., 1991).

To determine whether NAP1 might play a general role in cyclin function, we also tested the effect of deleting NAP1 in a strain that is compromised for G1 cyclin function. Wild-type yeast have three G1 cyclins, called *CLN1, CLN2,* and *CLN3,* and strains carrying deletions of *CLN1* and *CLN3* are dependent upon *CLN2* for survival. Deletion of *NAP1* in such a Cln2-dependent strain has no obvious phenotype, suggesting that NAP1 is specifically involved in the functions of B-type cyclins.

NAP1 Is Phosphorylated by Cyclin B/p34^{cdc2} Complexes, but Not by Cyclin A/p34^{cdc2} Complexes

To induce the events of mitosis, cyclin-dependent kinase complexes are likely to phosphorylate downstream substrates. To determine whether NAP1 might be such a substrate, we tested whether NAP1 can be phosphorylated by cyclin/p34^{cdc2} kinase complexes in vitro. For these experiments, *Xenopus* NAP1 eluted from a cyclin affinity column was further purified by ion exchange chromatography (Fig. 9 *A*), and then incubated in the presence of purified GST-cyclin B1/p34^{cdc2} complexes or GST-cyclin A/p34^{cdc2} complexes. The results are shown in Fig. 8 *B*. Interestingly, we found that NAP1 is a substrate for cyclin B/p34^{cdc2} complexes, but not for cyclin A/p34^{cdc2} complexes. This is true even under conditions where cyclin A and cyclin B kinase complexes phosphorylate histone H1 to a similar extent. These results demonstrate that NAP1 is a specific substrate of cyclin B/p34^{cdc2} kinase complexes, and lend support to the idea that cyclins can target the activity of p34^{cdc2} to specific substrates. We have not seen phosphorylation of the SET protein in these assays.

If NAP1 is specifically phosphorylated by cyclin B/p34^{cdc2} kinase complexes in vivo, one might expect to see an increase in the level of NAP1 phosphorylation during mitosis. To test this, we grew cells in the presence of ^{32}P and arrested the cell cycle either in G1 phase using the mating pheromone alpha factor, or in mitosis using the microtubule inhibitor benomyl. We then immunoprecipitated NAP1 and determined the amount of phosphate incorporated into NAP1 at the two different cell cycle stages by autoradiography. We found that NAP1 is phosphorylated in vivo, but the total level of phosphate on NAP1 does not increase significantly during the mitotic block (not shown). This result does not rule out the possibility that phosphorylation of a specific site on NAP1 increases during mitosis, even though the bulk level of phosphorylation does not change.

NAP1 Is a Cytoplasmic Protein

NAP1 was originally identified as a protein from HeLa cell extracts that promotes nucleosome assembly in vitro

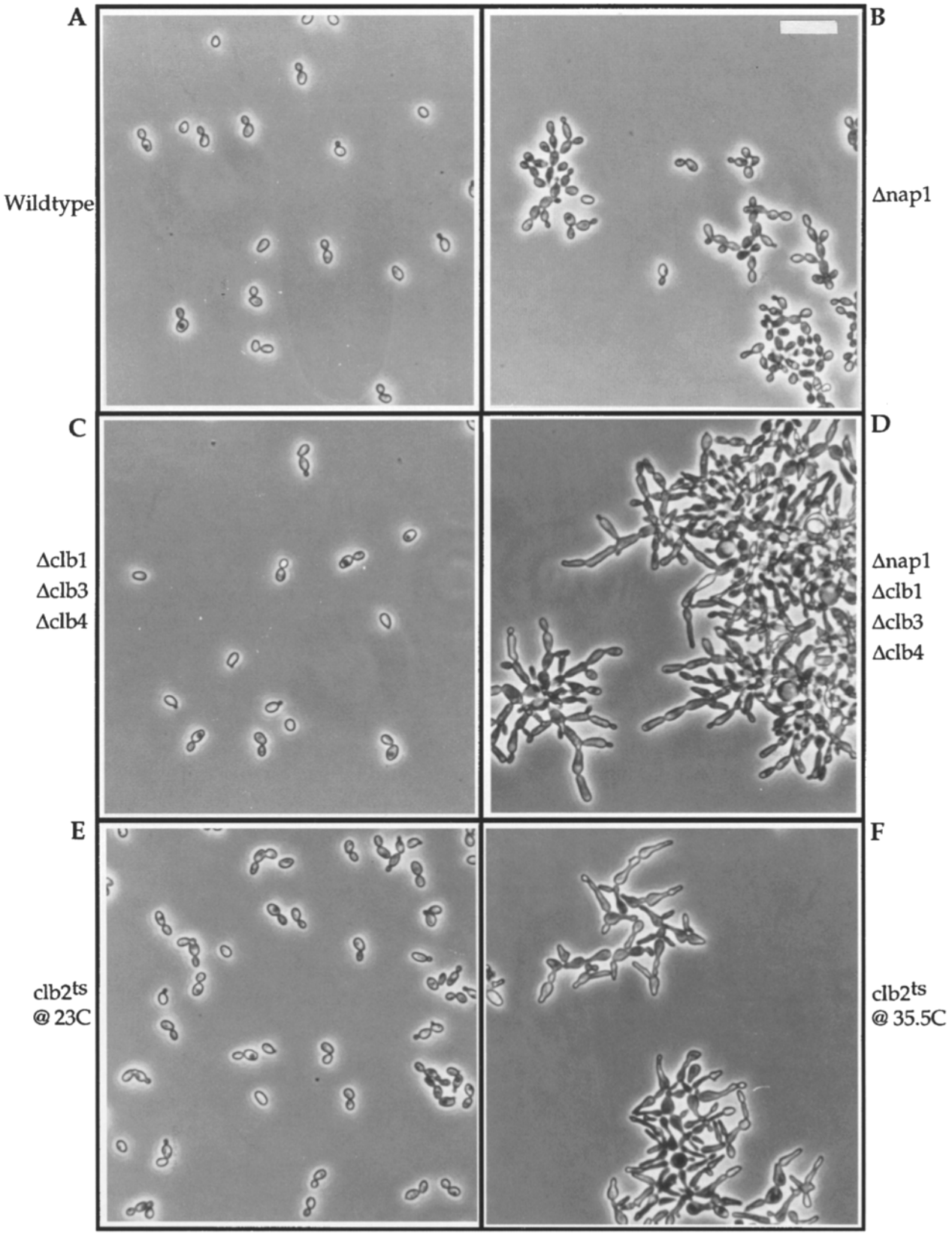

Figure 6. The morphology of yeast cells that lack NAP1 or Clb function. Cells were grown to log phase in YPD media and photographed by phase contrast optics. *A* and *B* compare the morphology of wild-type cells and Dnap1 cells, respectively. *C* and *D* compare the morphology of *Δclb1, Δclb3, Δclb4* cells and *Δnap1, Δclb1, Δclb3, Δclb4* cells. The cells in *A–D* were grown at 30°C. *E* and *F* show a strain carrying a temperature sensitive allele of *Clb2* in a *Clb2*-dependent background (*Δclb1, clb2-ts, clb3::TRP1, clb4::HIS3*) grown at 23°C (*E*) or 35.5°C (*F*).

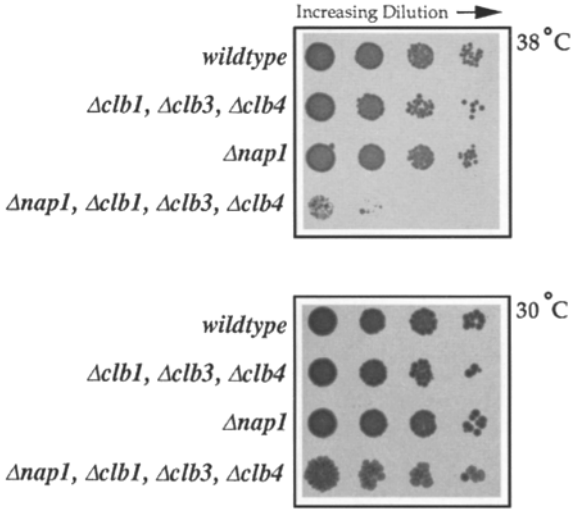

Figure 7. Temperature sensitivity of yeast cells that carry deletions of the *NAP1, CLB1, CLB3*, and *CLB4* genes. A series of 10-fold dilutions of each strain were spotted onto YPD plates and grown at 30°C or 38°C for 2–3 d.

(Ishimi et al., 1984), and subsequent experiments indicate that NAP1 from yeast also has nucleosome assembly activity (Ishimi and Kikuchi, 1991). If NAP1 has a role in nucleosome assembly in vivo, one would predict that it has a nuclear localization. We therefore carried out immunofluorescence experiments to determine the subcellular localization of the NAP1 protein, using a strain carrying a deletion of the NAP1 gene as a control (Fig. 10). We found that NAP1 is primarily a cytoplasmic protein. The strong cytoplasmic staining by anti-NAP1 antibodies made it difficult to determine whether NAP1 is also in the nucleus; in some cells it appeared that NAP1 was excluded from the nucleus, whereas in other cells it appeared that there was some nuclear staining. In either case, the strong cytoplasmic localization of NAP1 indicates that it is unlikely to function only in nucleosome assembly.

Discussion

The Use of Affinity Chromatography to Isolate Novel Cyclin-binding Proteins

We have used affinity chromatography to identify novel proteins that interact specifically with B-type cyclins. In budding yeast, we have found two proteins of apparent molecular masses 60 kD and 90 kD that bind to Clb2, but not to Clb3, whereas in *Xenopus* eggs we have found three proteins of apparent molecular masses 40, 60, and 90 kD that bind to cyclins B1 and B2, but not to cyclin A. Since the GST-cyclins on the affinity columns bind $p34^{cdc2}$ from the extract, we do not know whether the proteins that we have isolated can bind to cyclin in the absence of $p34^{cdc2}$. The cyclin-binding proteins are eluted from the affinity columns with 0.35 M KCl, which indicates that they interact with the cyclins with a relatively low affinity. For comparison, the interaction between $p34^{cdc2}$ and cyclin can only be disrupted with SDS, and other proteins that are known to interact with cyclin/$p34^{cdc2}$ complexes also do so with a relatively high affinity (Dunphy et al., 1988; Mendenhall, 1993; Zhang et al., 1993).

We have found no proteins that bind specifically to the *Xenopus* cyclin A or the yeast cyclin Clb3 under the conditions that we have used for our experiments. There are a number of possible reasons for this result. It may be that the proteins that interact with these cyclins are very rare, and we do not load enough extract onto our affinity columns to detect such proteins. Alternatively, these cyclins may require posttranslational modifications to bind to specific proteins, and therefore fail to bind to the unmodified GST-cyclin on our columns. It is also possible that the GST portion of the cyclin fusion protein interferes with binding, or that the proteins that bind to these cyclins are insoluble in a standard cytoplasmic extract. Since the GST-cyclin A is able to activate H1 kinase activity and induce nuclear envelope breakdown and chromosome condensation in *Xenopus* egg extracts (data not shown), it seems unlikely that it is incorrectly folded when expressed in bacteria.

Cyclin A in mammalian cells has been shown to interact with a number of proteins, including E1A, E2F, p107, p21, and PCNA (Cao et al., 1992; Devoto et al., 1992; Giordano et al., 1989; Peeper et al., 1993; Pines and Hunter, 1990; Zhang et al., 1993; Shirodkar, 1992). Proteins such as p107, E1A, and E2F are involved in transcriptional regulation. Since little transcription occurs before the midblastula transition these proteins are unlikely to interact with regulatory factors such as cyclin A in the early *Xenopus* embryo. This idea is supported by the finding that the E2F present in early *Xenopus* embryos is not associated with the proteins that bind to it in postembryonic cell cycles (Philpott and Friend, 1994). The interaction between cyclin A and proteins such as p21 and PCNA is stable to at least 0.6 M NaCl, and such tight-binding proteins would not be eluted from the column under the conditions that we have used (0.35 M KCl).

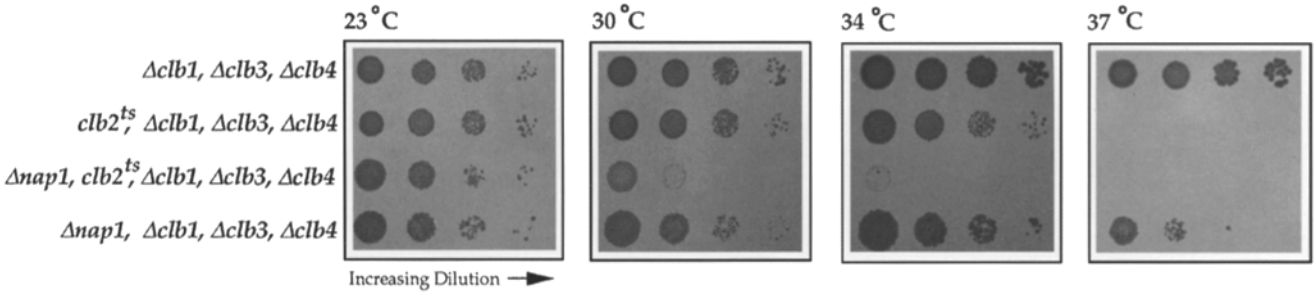

Figure 8. Deletion of the *NAP1* gene increases the temperature sensitivity of a *clb2ts* strain. A series of 10-fold dilutions of each strain were spotted onto YPD plates and grown at 23°C, 30°C, 34°C, or 37°C.

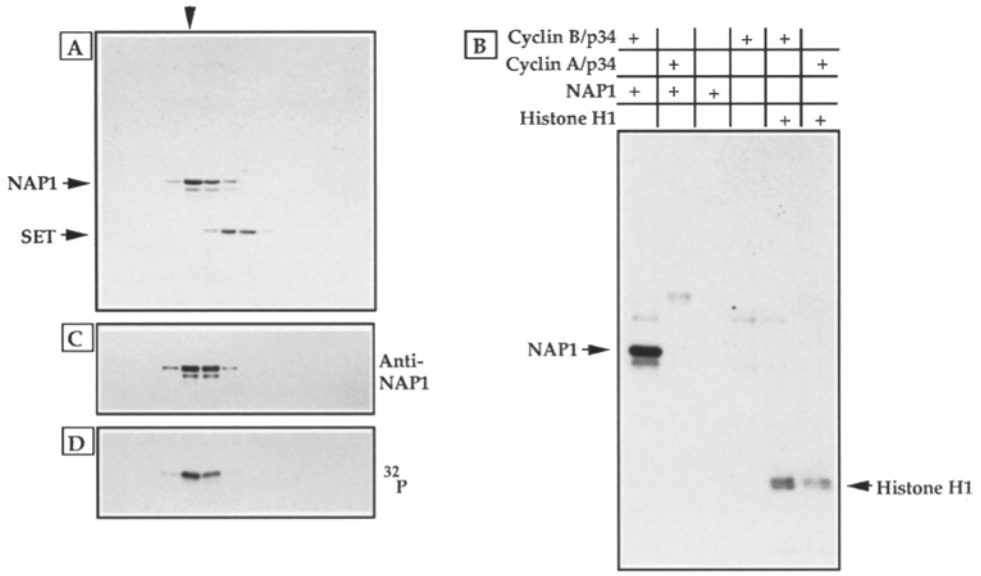

Figure 9. Xenopus NAP1 is specifically phosphorylated by cyclin B/p34^{cdc2} kinase complexes. (*A*) Further purification of NAP1 by ion exchange chromatography on Q sepharose. The elution from a *Xenopus* B1 cyclin column was loaded onto a Q sepharose column and eluted with a salt gradient. This gel shows the fractions from the salt gradient elution that contain NAP1 and SET. The fraction containing the most purified NAP1 (marked with an arrow at the top of the gel) was used for the phosphorylation reactions. (*B*) Phosphorylation of NAP1 by cyclin B/p34^{cdc2} complexes. Reactions were carried out as described in Materials and Methods, and each reaction was analyzed by polyacrylamide gel electrophoresis and autoradiography. The contents of each reaction are indicated at the top of the gel. (*C*) The same fractions from the Q sepharose column shown in *A* were probed with the anti-NAP1 monoclonal antibody. (*D*) The same fractions from the Q sepharose column shown in *A* were used in the phosphorylation assay with cyclin B/p34^{cdc2} complexes. These results show that the protein being phosphorylated by cyclin B/p34^{cdc2} copurifies with NAP1.

The NAP/SET Family of Proteins Interact Specifically with B-Type Cyclins

We have shown that the 60-kD protein that binds to Clb2 is identical to a previously described yeast protein called NAP1. A monoclonal antibody that recognizes NAP1 homologs in many species (Ishimi et al., 1985) allowed us to determine that the *Xenopus* homolog of NAP1 binds to cyclins B1 and B2, but not to cyclin A. These results indicate that the interaction between B-type cyclins and NAP proteins has been highly conserved during evolution.

We also obtained a partial cDNA clone for the 40-kD protein that binds to the *Xenopus* B-type cyclins. The sequence of this partial cDNA reveals that it is the *Xenopus* homolog of the human SET protein, which is ~25% identical to yeast NAP1. The SET gene was initially identified as part of a putative oncogene formed by the fusion of the CAN gene with the SET gene in a case of acute myeloid leukemogenesis (Von Lindern et al., 1992). This fusion results in production of a chimeric mRNA including most of the SET gene fused to the CAN gene. Since the CAN pro-

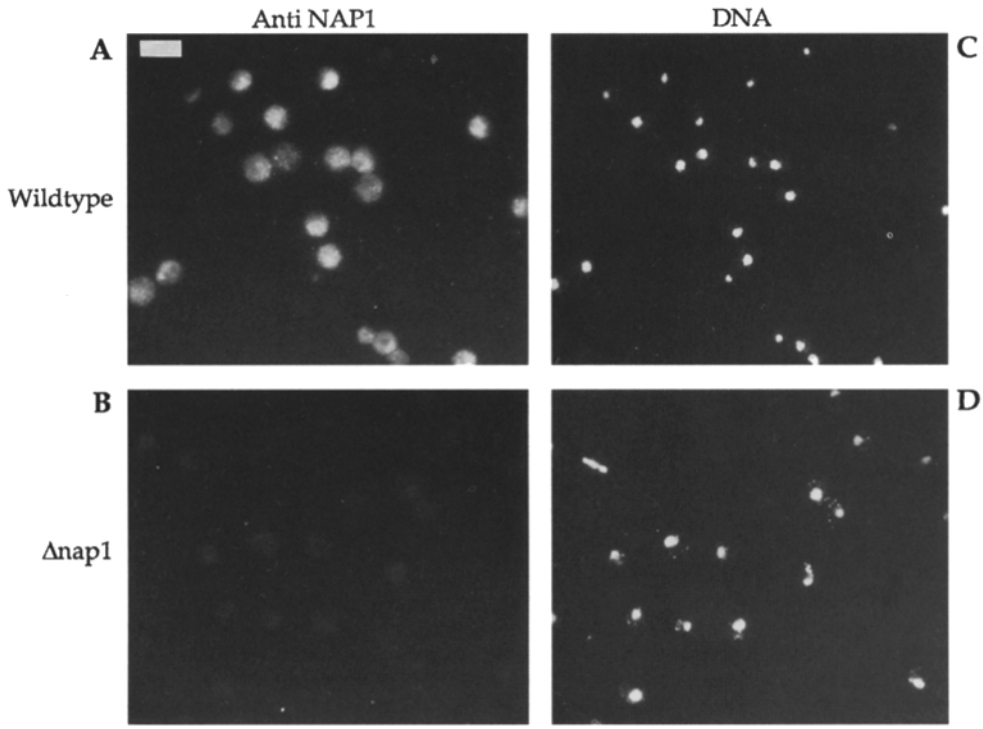

Figure 10. The cytoplasmic localization of NAP1 in yeast cells. The localization of the NAP1 protein was determined using an affinity purified polyclonal antibody, while the distribution of DNA in the same cells was determined with DAPI stain. (*A*) NAP1 staining of wild-type haploid cells. (*B*) A control showing NAP1 staining in *Δnap1* cells. *C* and *D* show DNA staining in the same cells shown in *A* and *B*, respectively. The scale bar represents 10 microns.

tein is also found fused to other proteins in leukemic cells (Von Lindern et al., 1992), it is not clear that the SET protein plays any direct role in leukemogenesis. An intriguing possibility suggested by our results is that fusion to SET targets the CAN protein for inappropriate phosphorylation by cyclin B/p34^{cdc2} kinase complexes.

Our sequence of *Drosophila* SET indicates that SET is a highly conserved protein, being 55% identical between humans and *Drosophila*. The SET protein is found primarily in the nucleus of HeLa cells and is phosphorylated on serine in vivo (Adachi, 1994). Analysis of the expression of SET mRNA in mouse tissues revealed that it is expressed in a wide variety of tissues and at very high levels during embryogenesis. In addition, DNA probes to the 3′ end of the SET coding sequences detect at least 10 different cross-hybridizing bands on a genomic Southern blot, indicating that the SET gene may be part of a large multigene family (Von Lindern et al., 1992). Additional proteins related to NAP1 and SET have been identified in human (Simon et al., 1994), mouse (PIR protein database numbers JS0707 and S16863), sea urchin (Genbank number D21877), *C. elegans* (Genbank number T01616), *Plasmodium falciparum* (Lenstra et al., 1987), and *Arabidopsis thaliana* (Genbank numbers T20922 and U12858). In addition, our preliminary PCR experiments have indicated that there are at least three different NAP1 homologs in *Xenopus*, indicating that NAP1 and SET are members of a growing family of related proteins.

The In Vivo Function of NAP1

NAP1 was originally identified as a protein in HeLa cell extracts that will promote the assembly of nucleosomes onto naked plasmid DNA in vitro (Ishimi et al., 1984). The purified protein was given the name NAP1 (nucleosome assembly protein 1), and a monoclonal antibody raised against it was found to recognize a protein of similar molecular weight in *Drosophila* and yeast (Ishimi et al., 1985). This antibody was used to clone the *S. cerevisiae* homolog of NAP1, which was also found to promote nucleosome assembly in vitro (Fujii-Nakata et al., 1992; Ishimi and Kikuchi, 1991). The relevance of NAP1's ability to drive nucleosome assembly in vitro to its in vivo function is unclear. We found that yeast NAP1 is primarily a cytoplasmic protein, and similar results have been obtained in vertebrate cells (Kikuchi, A., unpublished results). The cytoplasmic localization of NAP1 suggests that it is unlikely to function solely in nucleosome assembly, as such a protein would be expected to be found primarily in the nucleus.

The results presented here demonstrate that NAP1 plays an important role in the function of the yeast mitotic cyclin Clb2. We have studied NAP1 function in a yeast strain that is dependent upon CLB2 for survival, which can be generated by deletion of the genes for additional mitotic cyclins that share functional overlap with Clb2 (i.e., Clb1, Clb3, and Clb4). When *NAP1* is deleted in a Clb2-dependent strain, the cells become temperature sensitive and grow as large clumps of highly elongated cells. This phenotype is similar to the phenotype of cells that partially lack Clb function, and it is largely rescued by the presence of other Clbs that can compensate for defects in Clb2 function. In addition, deletion of the *NAP1* gene substantially increases the temperature sensitivity of a strain that is dependent upon a temperature sensitive allele of *CLB2*. Taken together, these observations demonstrate that NAP1 is somehow required for proper function of Clb2, and a more detailed analysis of the role of NAP1 in Clb2 function is presented in the accompanying paper.

The Substrate Specificity of Cyclin-dependent Kinase Complexes

Many kinases are known to have overlapping or identical substrate specificities when tested in vitro. For example, cyclin-dependent kinases, the MAP kinase family, and glycogen synthase kinase 3 have overlapping substrate specificities in vitro (Nigg, 1991; Peter et al., 1992; Plyte et al., 1992). It seems unlikely, however, that these kinases phosphorylate the same substrates in vivo. What determines the substrate specificities of protein kinases inside cells? The multiplicity of protein kinases and their importance in virtually every aspect of cellular regulation highlight the importance of this question. The finding that NAP1 is a specific substrate of cyclin B/p34^{cdc2} complexes is therefore of particular interest, as it may provide important clues towards understanding kinase specificity.

Several models might explain how NAP1 is phosphorylated specifically by cyclin B/p34^{cdc2} complexes. Binding of NAP1 to the cyclin B/p34^{cdc2} complex might induce a conformational change in NAP1 that exposes a phosphorylation site, or NAP1 might be sterically blocked from occupying the active site of p34^{cdc2} in cyclin A/p34^{cdc2} complexes. Another possibility is that NAP1 is a relatively poor substrate for cyclin-dependent kinases, and that binding to cyclin B increases the effective local concentration of NAP1 and drives the reaction. A less likely hypothesis is that the substrate specificity of the catalytic site on p34^{cdc2} is actually different in cyclin A or cyclin B/p34^{cdc2} complexes. In this view, binding of cyclin B to p34^{cdc2} would induce a conformational change in p34^{cdc2} that creates an active site that can accommodate NAP1, whereas binding of cyclin A does not.

Perhaps there are two kinds of substrates for cyclin-dependent kinases. One kind of substrate can be phosphorylated by multiple cyclin-dependent kinase complexes (e.g., histone H1 or nuclear lamins), while another class can be phosphorylated only by specific cyclin-dependent kinase complexes (e.g., NAP1 and p107). It is also possible that proteins like histone H1 and nuclear lamins are not in vivo substrates of cyclin-dependent kinases. Indeed, the evidence that histone H1 and nuclear lamins are phosphorylated by cyclin-dependent kinases in vivo remains circumstantial, as it is very difficult to prove conclusively that a particular protein is phosphorylated by a particular kinase in vivo.

The Specific Functions of Cyclin-dependent Kinase Complexes

One might imagine two general models that could explain how cyclin-dependent kinase complexes are able to induce different events at different times of the cell cycle. One model would suggest that certain substrates are competent

to be phosphorylated by cyclin-dependent kinases only at particular times during the cell cycle. For example, there may be a mechanism that causes certain proteins to undergo a posttranslational modification at the end of mitosis that renders them competent to be phosphorylated by cyclin-dependent kinases during G1. Other mechanisms would reverse this modification at the end of S phase, and cause the modification of a new group of proteins that could make them competent to be phosphorylated by cyclin-dependent kinases at the beginning of mitosis. One way of regulating phosphorylation at the substrate level would be the existence of specific phosphatases that were only active at certain points in the cell cycle, and would thus prevent the accumulation of phosphate groups on some substrates. In this view, the primary differences in cyclins would involve differences in regulation that determine when they are activated. The finding that expression of truncated forms of human cyclins A, B, C, D, and E during G1 can rescue yeast cells that lack G1 cyclins suggests that any cyclin that can activate $p34^{cdc28}$ kinase activity is sufficient to get cells through G1 (Lew et al., 1991). On the other hand, the finding that expression of G1 cyclins during G2 can induce G1 events, and vice versa (Lew and Reed, 1993) suggests that different cyclins can induce specific events independent of the time during the cell cycle and argues against a substrate availability model. The ability of heterologous cyclins to rescue yeast cells that lack G1 cyclin activity may reflect the ability of the exogenous cyclins to induce expression of other yeast cyclins, such as Clb5 and Clb6, that can induce events downstream of Start.

An alternative model is that the different cyclins act to determine the specific functions of cyclin-dependent kinase complexes. The cyclins could do this by targeting the activity of cyclin-dependent kinase complexes to specific substrates. Our results support this kind of model. We found that NAP1 interacts specifically with B-type cyclins and is phosphorylated by cyclin B/$p34^{cdc2}$ complexes, but not by cyclin A/$p34^{cdc2}$ complexes, clearly indicating that the cyclin subunit can target the kinase activity of $p34^{cdc2}$. In addition, we show in the accompanying paper that NAP1 plays an important role in the specific functions of CLB2/$p34^{cdc28}$ kinase complexes in yeast cells. An ability of the cyclin subunit to target the activity of cyclin-dependent kinases has also been observed in the phosphorylation of p107 and Rb (Kato et al., 1993; Peeper et al., 1993). These results may provide a starting point for understanding how different cyclin-dependent kinase complexes are able to induce different cell cycle events. It seems likely that cells may use several mechanisms to induce different cell cycle events at different times, including the regulation of substrate availability and the targeting of $p34^{cdc2}$ to specific substrates.

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