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Cla4p, a *Saccharomyces cerevisiae* Cdc42p-Activated Kinase Involved in Cytokinesis, Is Activated at Mitosis†

BENJAMIN K. BENTON,[1]‡ ARTHUR TINKELENBERG,[1]§ ISABEL GONZALEZ,[2] AND FREDERICK R. CROSS[1]*

The Rockefeller University, New York, New York 10021,[1] and Institute for Molecular Pathology, A-1030 Vienna, Austria[2]

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Yeasts have three functionally redundant G_1 cyclins required for cell cycle progression through G_1. Mutations in *GIN4* and *CLA4* were isolated in a screen for mutants that are inviable with deletions in the G_1 cyclins *CLN1* and *CLN2*. *cln1 cln2 cla4* and *cln1 cln2 gin4* cells arrest with a cytokinesis defect; this defect was efficiently rescued by *CLN1* or *CLN2* expression. *GIN4* encodes a protein with strong homology to the Snf1p serine/threonine kinase. Cla4p is homologous to mammalian p21-activated kinases (PAKs) (kinases activated by the rho-class GTPase Rac or Cdc42). We developed a kinase assay for Cla4p. Cla4p kinase was activated in vivo by the GTP-bound form of Cdc42p. The specific activity of Cla4p was cell cycle regulated, peaking near mitosis. Deletion of the Cla4p pleckstrin domain diminished kinase activity nearly threefold and eliminated in vivo activity. Deletion of the Cla4p Cdc42-binding domain increased kinase activity nearly threefold, but the mutant only weakly rescued *cla4* function in vivo. This suggests that kinase activity alone is not sufficient for full function in vivo. Deletion of the Cdc42-binding domain also altered the cell cycle regulation of kinase activity. Instead of peaking at mitosis, the mutant kinase activity exhibited reduced cell cycle regulation and peaked at the G_1/S border. Cla4p kinase activity was not reduced by mutational inactivation of *gin4*, suggesting that Gin4p may be downstream or parallel to Cla4p in the regulation of cytokinesis.

The *Saccharomyces cerevisiae* G_1 cyclins, Cln1p, Cln2p, and Cln3p, are functionally redundant activators at START of the Cdc28p cyclin-dependent kinase (13, 58). Despite this genetic redundancy, Cln1p and Cln2p may have functions distinct from Cln3p and may promote cell cycle progression through different mechanisms (6, 15–17, 37). Previously, we and others screened for mutants that might identify pathways that were selectively affected by each of the specific Cln/Cdc28p complexes (6, 16). These screens identified bud emergence (6, 16), DNA synthesis (69), and cytokinesis (6, 15) as pathways subject to G_1 cyclin control. In the cytokinesis-deficient class, *erc19*, *cdc12*, *cla6*, and *cla4* were synthetically lethal in the presence of deletion of both *cln1* and *cln2*.

S. cerevisiae CLA4 encodes a protein with strong homology to p21-activated kinases (PAKs). PAKs are serine/threonine kinases that are activated when bound to the GTP-bound form of Cdc42p and Rac1p (7, 31, 40–43, 53, 63, 68). Cla4p contains a Cdc42p-binding domain, a pleckstrin homology (PH) domain, and a serine/threonine kinase domain with homology to other PAK kinase domains (15). In addition to being synthetically lethal in the presence of deletion of both *CLN1* and *CLN2*, *cla4* is synthetically lethal in cells missing another PAK homolog, *STE20* (15). As with the *cln1 cln2 cla4* synthetic lethal mutants, *cla4 ste20* double mutants arrest with a cytokinesis defect.

The Rho-class GTPases are involved in a multitude of cellular processes, both in yeast cells and in mammalian cells (for reviews, see references 24 and 59). In mammalian cells, Cdc42, Rac, and Rho proteins are required for filopodium formation, membrane ruffling, and stress fiber formation, respectively (48, 60, 61). Additionally, there appears to be a hierarchy of function affecting the actin cytoskeleton, in which Cdc42p works on Rac1p, which works on Rho protein (48). Another major function of these GTPases is in various signal transduction cascades (9, 10, 12, 26, 44, 54, 65, 72). Cdc42p and Rac1p are involved in the jun kinase pathway and activate serum response factor. Rho also activates serum response factor by a mechanism independent of that affected by Cdc42 and Rac (26). These Rho-class GTPases also play a role in oncogenesis (29, 57), cell cycle progression (10, 52), and possibly the etiology of AIDS (14, 40, 63). In *S. cerevisiae*, Cdc42p is required for cytoskeletal organization and bud formation and for the pheromone signal transduction pathway (1, 28, 65, 72). PAKs are probable effectors of Cdc42p and Rac1p in many cellular processes. Likewise, both Ste20p and Cla4p may be Cdc42p effectors of cytoskeletal rearrangements that occur during cytokinesis (15).

To further examine the role of Cla4p and Cdc42p in cytokinesis, we developed a kinase assay involving epitope-tagged Cla4p and studied the regulation of its activity. We also constructed PAK and PH domain deletions in Cla4p and studied their functional and biochemical properties. (In this paper, we designated the Cdc42p-binding domain of Cla4p the PAK domain, following the convention of Cvrckova et al. [15].)

MATERIALS AND METHODS

Media and reagents. YPD, YPGAL, and minimal media were made by standard techniques (4). Yeast transformations were performed as described previously (4). Bovine myelin basic protein (MBP) and 4′,6-diamidino-2-phenylindole (DAPI) were from Sigma. Mouse monoclonal antibody 9E10 (c-myc) and protein G-Sepharose were from Santa Cruz Biotechnology.

Yeast strains. The yeast strains used in this study are listed in Table 1.

Plasmid constructions. The *cla4* null plasmid (pBB119) was constructed by replacing the *Pvu*II-*Swa*I fragment internal to the *CLA4* open reading frame with a TRP1-Kan^r cassette (2). The *cla4* null allele deletes amino acids (aa) 4 to 769 of the 842-aa deduced open reading frame of Cla4p. This *cla4* null allele was

* Corresponding author. Mailing address: The Rockefeller University, 1230 York Ave., New York, NY 10021. Phone: (212) 327-7685. Fax: (212) 327-7923. E-mail: fcross@rockvax.rockefeller.edu.

† This work is dedicated to A.B.B.

‡ Present address: Cadus Pharmaceutical Corp., Tarrytown, NY 10591.

§ Present address: Institute for Human Nutrition, Columbia University, New York, NY 10032.

TABLE 1. Yeast strains used in this study

Strain	Genotype
BF264-15D background	
2507-5B	*MAT*α *cln1 cln2 CLN3 leu2*::*LEU2*::*GAL*::*CLN1 CLA4*
2507-5B(CM1)	*MAT*α *cln1 cln2 CLN3 leu2*::*LEU2*::*GAL*::*CLN1 cla4*::*CLA4*::*myc*::*URA3*
1354-11C	*MAT***a** *cln1 cln2 CLN3 ere10-1 leu2*::*LEU2*::*GAL*::*CLN1*
3129-1B	*MAT*α *cln1 cln2 CLN3 erc47-1 leu2*::*LEU2*::*GAL*::*CLN1*
3129(CM2)	*MAT*α *cln1 cln2 CLN3 erc47-1 leu2*::*LEU2*::*GAL*::*CLN1 cla4*::*CLA4*::*myc*::*URA3*
2330(gin4ΔA)-7D	*MAT*α *cln1 cln2 CLN3 gin4*::*URA3 leu2*::*LEU2*::*GAL*::*CLN1*
3126-4B	*MAT*α *cln1 cln2 CLN3 erc19-1 leu2*::*LEU2*::*GAL*::*CLN1*
3127-1B	*MAT*α *cln1 cln2 CLN3 erc32-1 leu2*::*LEU2*::*GAL*::*CLN1*
3128-6C	*MAT*α *cln1 cln2 CLN3 erc46-1 leu2*::*LEU2*::*GAL*::*CLN1*
3137(cla4ΔB)-3C	*MAT*a *cln1 cln2 CLN3 cla4*::*TRP1 leu2*::*LEU2*::*GAL*::*CLN1*
3137(cla4ΔB)-IB	*MAT*α *cln1 cln2 CLN3 cla4*::*TRP1* leu2::*LEU2*::*GAL*::*CLN1*
BYC4ΔPAK	*MAT*α *cln1 cln2 CLN3 cla4*::*TRP1 pURA3*/*cla4ΔPAK*::*myc leu2*::*LEU2*::*GAL*::*CLN1*
BYC4ΔPH	*MAT*α *cln1 cln2 CLN3 cla4*::*TRP1 pURA3*/*cla4ΔPH*::*myc leu2*::*LEU2*::*GAL*::*CLN1*
BYC4WT	*MAT*α *cln1 cln2 CLN3 cla4*::*TRP1 pURA3*/*CLA4*::*myc leu2*::*LEU2*::*GAL*::*CLN1*
1255-5C	*MAT***a** *CLN1 CLN2 CLN3 CLA4*
1255(134.7)	*MAT***a** *CLN1 CLN2 CLN3 CLA4 pURA3*/*cla4ΔPAK*::*myc*
1255(135.7)	*MAT***a** *CLN1 CLN2 CLN3 CLA4 pURA3*/*cla4ΔPH*::*myc*
1255(131.1)	*MAT***a** *CLN1 CLN2 CLN3 CLA4 pURA3*/*CLA4*::*myc*
DLY657[b]	*MAT***a** *cdc24-1 CLA4*
DLY657(CM1)	*MAT***a** *cdc24-1 cla4*::::*CLA4*::*myc*::*URA3*
DLY679[b]	*MAT***a** *cdc42-1 CLA4*
DLY679(CM2)	*MAT***a** *cdc42-1 cla4*::*CLA4*::*myc*::*URA3*
1242(CM2)	*MAT***a** *cln1 cln2 cln3 cla4*::*CLA4*::*myc*::*URA3 leu2*::*LEU2*::*GAL*::*CLN2*
1608(CM3)	*MAT***a** *cln1 cln2 cln3 cla4*::*CLA4*::*myc*::*URA3 leu2*::*LEU2*::*GAL*::*CLN3*
W303-1a background	
K2944-1B[d]	*cdc15-2 CLA4*
501(CM1)	*cdc15-2 cla4*::*CLA4*::*myc*::*URA3*
501(138)E	*cdc15-2 CLA4*::*cla4ΔPAK*::*myc*::*URA3*
501(139)B	*cdc15-2 CLA4*::*cla4ΔPAK*::*myc*::*URA3*
501(134.7)	*cdc15-2 CLA4 pURA3*/*cla4ΔPAK*::*myc*
501(135.7)	*cdc15-2 CLA4 pURA3*/*cla4ΔPH*::*myc*
501(131.1)	*cdc15-2 CLA4 pURA3*/*CLA4*::*myc*

[a] *trp1-1a leu2-3,112 ura3 ade1 his2.*
[b] Obtained from D. Lew.
[c] *MAT*a *bar1*::*ura3-ns ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 ssd1.*
[d] Reference 51.

unable to rescue *cln1 cln2 erc10-1* (*cla4*). The allele can be released for one-step gene replacement (62) by digestion with *Afl*II and *Eco*RI.

The myc-tagged *CLA4* (23) contains 9 myc epitope repeats (32) fused to the carboxyl terminus of Cla4p. This tag was then used for all other *CLA4* constructs by rescuing the tag onto a CEN plasmid containing *CLA4* (pATL3). Plasmid pATL3 was digested at the unique *Swa*I site 222 bp upstream of the *CLA4* stop codon and cotransformed with a fragment containing the carboxyl terminus of the myc-tagged CLA4 into a *cln1 cln2 cla4*::*TRP1 GAL*::*CLN1* strain. Plasmids were rescued from yeast that had a myc-tagged *CLA4* [pATL3(CM3)]. The epitope tagging was checked by immunoblotting (see below).

The *CLA4* gene was subcloned into a pRS416 derivative (11, 64) missing a *Not*I site. pATL3 was digested with *Afl*II, filled with Klenow fragment, and digested with *Eco*RI. The 3.4-kb fragment containing *CLA4* was inserted into the *Sma*I-*Eco*RI sites of the pRS416 derivative to give pBB130. The sites from *Kpn*I to *Cla*I in the multiple-cloning site were eliminated by digestion with both enzymes, filling with Klenow fragment, and religating (pBB130ΔCK). *CLA4* was then myc tagged by substituting a 2.0-kb *Sal*I-*Eco*RI fragment from pATL3(CM3) for the same untagged fragment in pBB130ΔCK (pBB131).

pBB131 was then used for subsequent construction of *cla4* deletion mutants. In-frame deletions of the PH domain (aa 62 to 178 [pBB135]) and the Cdc42-binding (PAK) domain (aa 184 to 242 [pBB134]) were constructed by splice extension PCR (27) with the proofreading Vent polymerase (New England Biolabs). Mutants were sequenced across the splice to verify the constructions. PAK mutants were subcloned into an integrating vector by inserting a *Hin*dIII-*Eco*RI fragment from pBB134 into pRS406 (pBB138 and pBB139). These tagged mutants were integrated into the *cdc15-2* strain by transforming *Swa*I-digested plasmids. Successful tagging was verified by immunoblotting.

The 4.8-kb *Mlu-Kpn*I fragment containing *GIN4* was subcloned into pRS414 to give pBB133. pBB137.11 is a replacement of the 2.2-kb *Nco*I-*Eco*RI fragment internal to *GIN4* with the *URA3*-Kan^r cassette (2). This makes a null allele of *GIN4* that deletes aa 19 to 762 from the 1,142-aa protein encoded by the deduced open reading frame. This allele is unable to rescue *cln1 cln2 erc47-1* (*gin4*). The null allele can be released for one-step gene replacement by digestion with *Eco*RI and *Sal*I.

Strain constructions. *cla4* and *gin4* null strains were generated by replacing one of the wild-type alleles for each respective gene in strain 2330d3.1 by one-step gene replacement, sporulating the diploids, and dissecting tetrads on YPGal. Null strains were identified by scoring the selectable marker used to disrupt the gene and were verified by Southern blot analysis (4).

DAPI staining. The cells were fixed and stained with DAPI as described by Benton et al. (6).

Immunoprecipitation of myc-tagged Cla4p. A 50-ml portion of culture was harvested at an optical density at 660 nm of 0.8 to 1.0 and resuspended in 8 ml of ice-cold buffer B-T (50 mM Tris [pH 7.5], 100 mM NaCl, 50 mM NaF, 1 mM sodium orthovanadate, 10 mM EDTA). All subsequent manipulations were done at 4°C. The cells were centrifuged at 2,000 rpm for 5 min in Beckman GPR centrifuge, the supernatant was removed, and the pellet was resuspended in 1 ml of buffer B-T and transferred to iced microcentrifuge tubes. The cells were pelleted and resuspended in 250 μl of buffer B (0.1%T) (buffer B-T with 0.1% Triton X-100) containing protease inhibitors (10 μg each of pepstatin and leupeptin per ml and 0.5 mM phenylmethylsulfonyl fluoride), and 300 μl of acid-washed glass beads was added. The samples were vortexed for 45 s and quick-spun for 10 s in a microcentrifuge, and the pellets were resuspended. This was repeated three times. The final supernatant was transferred to a new microcentrifuge tube on ice. The beads and pellets were extracted once more with 250 μl of buffer B (0.1%T) with protease inhibitors and quick-spun, and the supernatants were pooled. The extracts were cleared by centrifugation in a microcentrifuge for 3 min. A small portion of the supernatant was diluted in 2× sample buffer (4) to assay total Cla4p in the extracts, and the rest was transferred to a new microcentrifuge tube containing 400 ng of 9E10 monoclonal antibody (which recognizes the myc epitope). The antibody was incubated with the extracts for 1 h on ice. The extracts were centrifuged for 3 min, and the supernatants were transferred to 25 μl of protein G-Sepharose previously washed in buffer B (0.1%T). The antibody-treated extracts were rotated with the beads for 1 h. The beads were then pelleted, the supernatant was aspirated, and the beads were washed four times with buffer B (0.1%T) and once with 1.3× PKB (65 mM Tris [pH 7.5], 130 mM NaCl, 13 mM $MgCl_2$, 1.3 mM $MnCl_2$). After the final wash, the beads were resuspended in 25 μl of 1.3× PKB and kept on ice.

Kinase assay. Bead suspension (15 μl) was added to iced tubes containing 2 μl of 100 μM ATP, 1 μl of myelin basic protein (5 mg/ml), 1 μl of water, and 1 μl of [γ-^{32}P]ATP (final concentrations, 50 mM Tris [pH 7.5], 100 mM NaCl, 10 mM $MgCl_2$, 1 mM $MnCl_2$, 10 μM ATP, 10 μCi of [γ-^{32}P]ATP, 5 μg of MBP). The tubes were incubated at 30°C for 20 min. The reactions were stopped by adding 20 μl of 2× sample buffer and heating to 95°C for 5 min. Then 2× sample buffer was added to the rest of the immunoprecipitates and also heated. The kinase assay mixtures were electrophoresed on sodium dodecyl sulfate (SDS)–15% polyacrylamide and transferred to Immobilon P (Millipore) with a semidry blotter (Hoefer) in transfer buffer (see below), and the membrane was exposed to autoradiography. Cell cycle synchronizations, used to determine the cell cycle regulation of kinase activity, were performed with *cdc15-2* strains as described by Oehlen and Cross (50).

Immunoblots. Proteins from whole-cell extracts or immunoprecipitates were heated in sample buffer at 95°C for 5 min before being loaded for polyacrylamide gel electrophoresis (6% polyacrylamide). After electrophoresis, the separated proteins were transferred to Immobilon P with a Hoefer electroblotter at 0.5 mA for 1 h in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol). The membranes were blocked in Super Blotto (0.5% Triton X-100, 0.1% Tween 20, 0.5% bovine serum albumin, 2.3% low-fat dried milk, and 0.02% azide in phosphate-buffered saline [PBS]) for 90 min at room temperature or overnight at 4°C. After being briefly rinsed three times in PBS–0.2% Tween 20 (PBST) and washed once for 10 min in PBST, the membranes were incubated for 1 h with agitation at room temperature with a 1:7,500 dilution of 9E10 mouse monoclonal antibody in PBST–2% milk. The membranes were briefly rinsed three times in PBST and washed twice more for 10 min each in PBST. A 1:1,000 dilution of sheep anti-mouse immunoglobulin G conjugated to horseradish peroxidase (Amersham) in PBST–2% milk was added to the membranes and incubated with shaking for 1 h at room temperature. After this incubation, the membranes were briefly rinsed three times with PBST and subjected to three 10-min washes in PBST. The membranes were then prepared for chemiluminescence with the Pierce kit as specified by the manufacturer. Protein amounts were estimated from the intensity on a series of carefully time exposures.

Cdc42p binding assay. Cells (120 optical density at 660 nm units) were harvested, and extracts were made as for the immunoprecipitations, except that NBM-T (50 mM Tris [pH 7.5], 100 mM NaCl, 10 mM $MgCl_2$, 1 mM sodium orthovanadate) was used for the harvest and NBM (NBM-T with 0.5% Triton X-100 and the same protease inhibitors as were used for immunoprecipitation extraction) was used for extracting proteins. Glutathione-*S*-transferase-Cdc42p was prepared from baculovirus-infected Sf9 pellets (73) (a generous gift of D. Lew) by lysing five plates of infected cells with lysis buffer (20 mM Tris [pH 7.5], 5 mM $MgCl_2$, 0.1% Triton X-100, 1 mM dithiothreitol, protease inhibitors, 5 μM GDP) and incubating with 150 μl (packed-bed volume) of glutathione agarose for 15 min at 4°C. The beads were pelleted and washed five times in wash buffer plus NaCl (20 mM Tris [pH 7.5], 2.5 mM $MgCl_2$, 100 mM NaCl, 1 mM dithiothreitol, 0.5 μM GDP) and three times in wash buffer (the same as above except without NaCl) and resuspended in 500 μl of wash buffer. The GST-Cdc42p-bound beads were loaded with the appropriate nucleotide in loading buffer [25 mM Tris (pH 7.5), 5 mM $MgCl_2$, 1 mM EDTA, 2 mM dithiothreitol, 400 mM $(NH_4)_2SO_4$, 5 μM either GDP or GTPγS] for 45 min at room temperature. Incubation under these conditions loads nucleotide onto at least 50% of the GST-Cdc42p molecules (37a). The loaded beads were washed three times in loading wash buffer (20 mM Tris [pH 7.5], 10 mM $MgCl_2$, 5 μM appropriate nucleotide) and resuspended in wash buffer. Beads loaded with each nucleotide were added to half of each extract and incubated on a rotator at 4°C for 2 h. The beads were pelleted and washed four times with PBSTM (PBS [pH 7.5], 0.05% Triton X-100, 10 mM $MgCl_2$). After being washed, the beads were boiled in sample buffer and loaded on a 6% polyacrylamide gel. Immunoblotting was as described above.

RNA extraction and blotting. RNA was extracted and Northern blotting was performed as described by Oehlen and Cross (50).

RESULTS

Cla4p and Gin4p, two kinases involved in cytokinesis. We previously reported a screen for mutations that were arrested when the G_1 cyclins *CLN1* and *CLN2* were deleted (leaving *CLN3*) but were rescued by expression of *CLN1* from the inducible *GAL1* promoter (6). One class of these *erc* (elevated requirement for CLNs) mutants arrested with a cytokinesis defect when in the presence of *CLN3* alone. Because this screen was not saturated as indicated by the prevalence of complementation groups with only one mutant, we further screened for *erc* mutants and identified three more complementation groups, in addition to *erc10* and *erc19*, that had a cytokinesis defect (Fig. 1). These cytokinesis complementation groups were of two phenotypic classes: those in which expression of *CLN1* from the *GAL1* promoter rescued inviability and

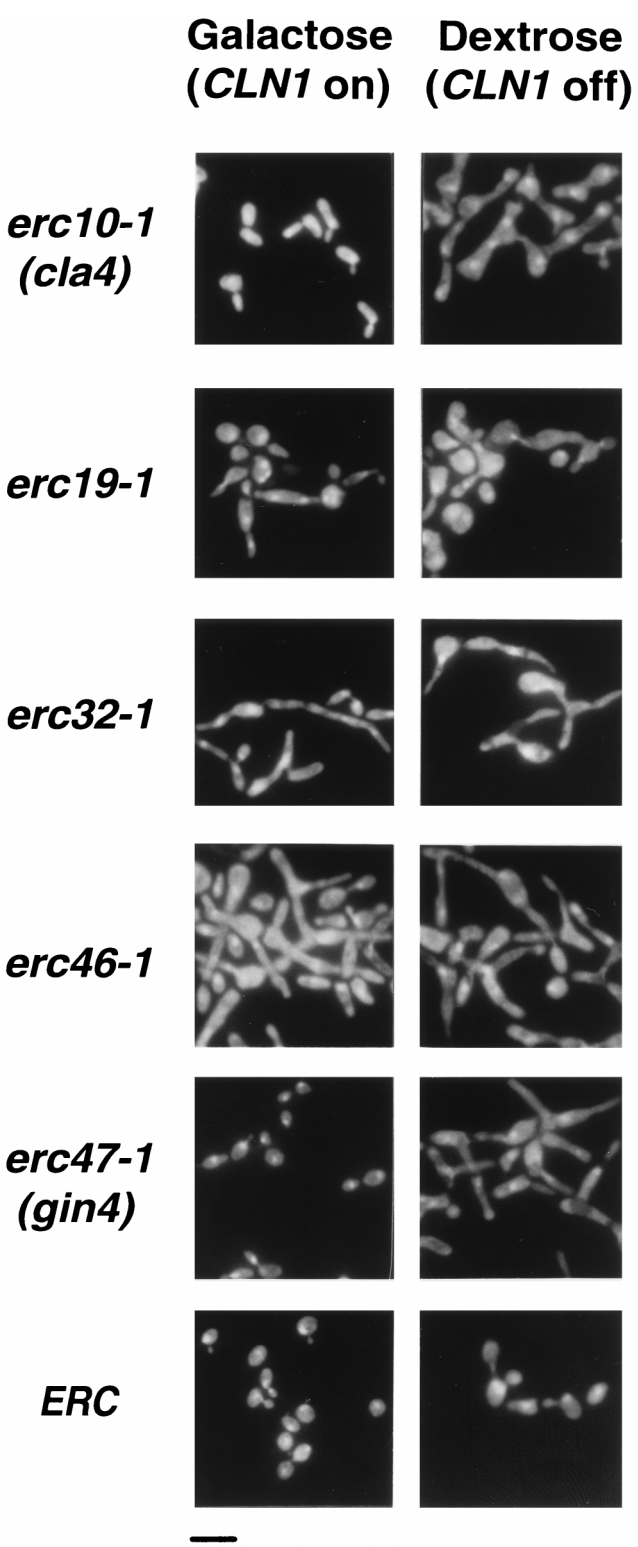

FIG. 1. *cln1 cln2 erc* mutants with a cytokinesis defect. Strains were grown to the mid-log phase in galactose (*CLN1* on), and half of each culture was shifted to dextrose for 10 h (*CLN1* off). All cultures were grown at 38°C except *erc10*, which was grown at 30°C. The cells were fixed, and DNA was stained with DAPI. The strains used were 1354-11C (*erc10-1*), 3126-4B (*erc19-1*), 3127-1B (*erc32-1*), 3128-6C (*erc46-1*), 3129-1B (*erc47-1*), and 2507-5B (wild-type *ERC*). All strains are *cln1 cln2 CLN3 GAL1*::*CLN1*. Bar, 10 μm.

returned the mutant morphology to normal (*erc10* and *erc47*), and those in which *CLN1* could rescue inviability but not morphology (*erc19*, *erc32*, and *erc46*). We further pursued those that were rescued for both viability and morphology, reasoning that the phenotypes caused by these mutations were specifically reversed by *CLN1* expression. *CLN1* expression may rescue the other mutants by allowing them to survive with a cytokinesis defect instead of correcting the defect, and thus the rescue may be less specific.

Genomic libraries were transformed into both *erc10* and *erc47*, and candidate genes were isolated. *CLA4* rescued *erc10*, and meiotic linkage analysis confirmed that *ERC10* was *CLA4* (5; see also complementation data in references 6 and 15). A null allele of *CLA4* had the same phenotype as *erc10*, i.e., *cla4* null *cln1 cln2* strains arrested with a cytokinesis defect, and the strains were rescued for both viability and morphology by CEN plasmids containing *CLN1* or *CLN2* (5). This indicates that *CLN1* and *CLN2* bypass the requirement for Cla4p function in cytokinesis. *CLA4* encodes an 842-aa protein with homology to Ste20p (15), a serine/threonine kinase required for pheromone signal transduction (34). Cla4p and Ste20p contain another region of homology that they also share with PAKs of higher eukaryotes (8) (see Introduction). Cla4p contains a PH domain that is not present in Ste20p. Pleckstrin homology domains have been implicated in both phospholipid binding and protein-protein interactions (22, 36, 45). Cla4p and Ste20p kinases are functionally redundant in cytokinesis (15).

ERC47 was identified as *GIN4* by complementation and meiotic linkage (no recombinants between *erc47-1* and *gin4*::*URA3* in 23 tetrads). *GIN4* was also identified in a similar screen by Cvrckova and Nasmyth (15) as *CLA6* (23). Null alleles of *GIN4* have the same Erc$^-$ phenotype as *erc47-1* (temperature-sensitive growth; branched, multinucleate morphology at all temperatures) and can be rescued by CEN plasmids containing *CLN1* or *CLN2* (5). This indicates that *CLN1* and *CLN2* can also bypass the requirement for *GIN4* in cytokinesis. *GIN4* also encodes a serine/threonine kinase with significant similarity to Snf1p, a kinase involved in glucose repression, and to a kinase of unknown function, Yc1024p. Further evidence that Gin4p kinase is involved directly in cytokinesis comes from the finding that *gin4* and *cdc12* are synthetically lethal (38), since *CDC12* encodes one of the septins that are believed to be structural components of the cytokinesis ring in *S. cerevisiae* (for a review, see reference 39). In contrast to the synthetic lethality seen between *cla4* and *ste20*, we saw no effect of the *ste20* null allele on the *cln1 cln2 erc47-1* (*gin4*) *GAL1*::*CLN1* phenotype, when grown on either galactose (*CLN1* on) or dextrose (*CLN1* off). Additionally, *gin4 cla4* double mutants are viable (38). These genetic data suggest that *GIN4* is not required for Cla4p or Ste20p function; thus, Gin4p may play a distinct role in cytokinesis.

Cla4p may be a PAK in vivo. To further characterize the regulation of Cla4p, we developed a kinase assay. Cla4p was fused at the carboxyl terminus with a ninefold repeat of the 9E10 myc epitope (23). Cla4p immunoprecipitated from cell extracts with the 9E10 monoclonal antibody was assayed for the ability to phosphorylate myelin basic protein (MBP), an efficient PAK substrate (42, 71).

PAKs are activated by binding the GTP-bound form of either Cdc42p or Rac1p. In *S. cerevisiae*, no Rac1p homologs are known, suggesting that Cdc42p would be the activator of Cla4p. To ask if Cla4p is a PAK in vivo, we used the *cdc42-1* and *cdc24-1* temperature-sensitive mutations. In the *cdc42-1* strain, Cdc42p levels are reduced compared to those in the wild type, even at the permissive temperature (74), while in the *cdc24-1* strains, Cdc42p is most probably found predominantly

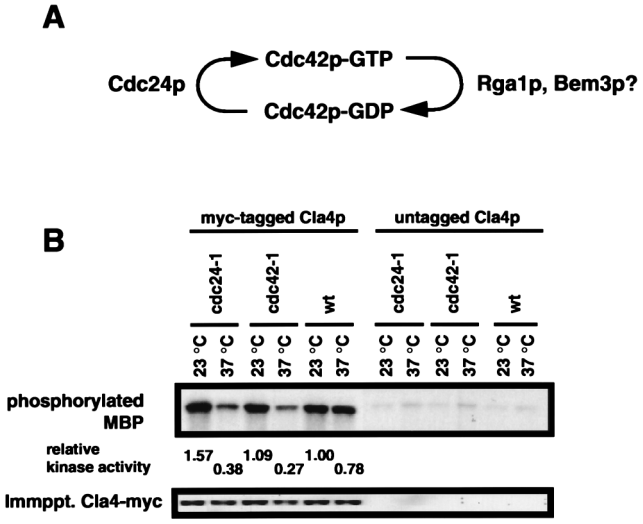

FIG. 2. Cla4p kinase is a PAK in vivo. (A) Cdc42p is a small GTPase that alternates between an active GTP-bound and an inactive GDP-bound form. The intrinsic GTPase activity is thought to be stimulated by the GTPase-activating proteins, Rga1p and Bem3p (66, 73). Cdc24p is the guanine nucleotide exchange factor that reloads Cdc42p with GTP. Mutants with mutations in *CDC24* can no longer reactive Cdc42p. (B) Kinase activity of Cla4::myc immunoprecipitations (Immppt.) from strains lacking GTP-Cdc42p. Strains were grown at 23°C and then arrested at 37°C for 2.5 h. Extracts were made, myc-tagged Cla4p was immunoprecipitated, and MBP was used as a substrate to determine kinase activity at 30°C. Relative kinase activity was determined by normalizing the amount of MBP phosphorylation with the amount of Cla4::myc protein in the immunoprecipitates and making values relative to wild-type strains at 23°C.

in the GDP-bound or inactive form at the nonpermissive temperature (73). The kinase activity of Cla4p immunoprecipitates from both the *cdc42-1* and the *cdc24-1* strains grown at the nonpermissive temperature (37°C) was reduced three- to fourfold compared to the activity under permissive conditions (23°C) (Fig. 2). Although the molecular nature of the *cdc42-1* mutation is unknown, the small amount of Cdc42p at the permissive temperature is sufficient for cell cycle progression. Shifting to the nonpermissive temperature decreases Cdc42p function in vivo and correlates with a reduction in Cla4p kinase activity. In the *cdc24-1* mutant, the amount of active Cdc42p-GTP is reduced at the nonpermissive temperature, and this also correlates with a reduction in Cla4p kinase activity. Together, these data are consistent with at least a partial requirement for GTP-bound Cdc42p in Cla4 kinase activity. The effect of the *cdc24-1* and *cdc42-1* mutations on Cla4p kinase are not extreme, but the residual Cla4p kinase activity at the nonpermissive temperature of the mutants could be due to residual Cdc42p or Cdc24p or to a basal, Cdc42p-independent Cla4p kinase activity.

Functional-domain mapping of Cla4p. *cla4* genes with deletions of either the PAK domain (*cla4*Δ*PAK*) or the pleckstrin homology domain (*cla4*Δ*PH*) were introduced on low-copy-number plasmids into *cln1 cln2 cla4 GAL1*::*CLN1* cells. Turning off *GAL1*::*CLN1* in control vector transformants resulted in a drop in viability of approximately 1,000-fold at 30°C and >10,000-fold at 38°C (Fig. 3B). Wild-type *CLA4* rescued viability at both temperatures. *cla4*Δ*PH* did not rescue *cln1 cln2 cla4* and actually inhibited residual growth at 30°C. Expression of *cla4*Δ*PAK* rescued *cln1 cln2 cla4* at 30 but not 38°C. Although viability was restored at 30°C in the *cla4*Δ*PAK* transformants, morphological rescue was incomplete (the cells were tubular with multiple nuclei). The mutant and wild-type Cla4p

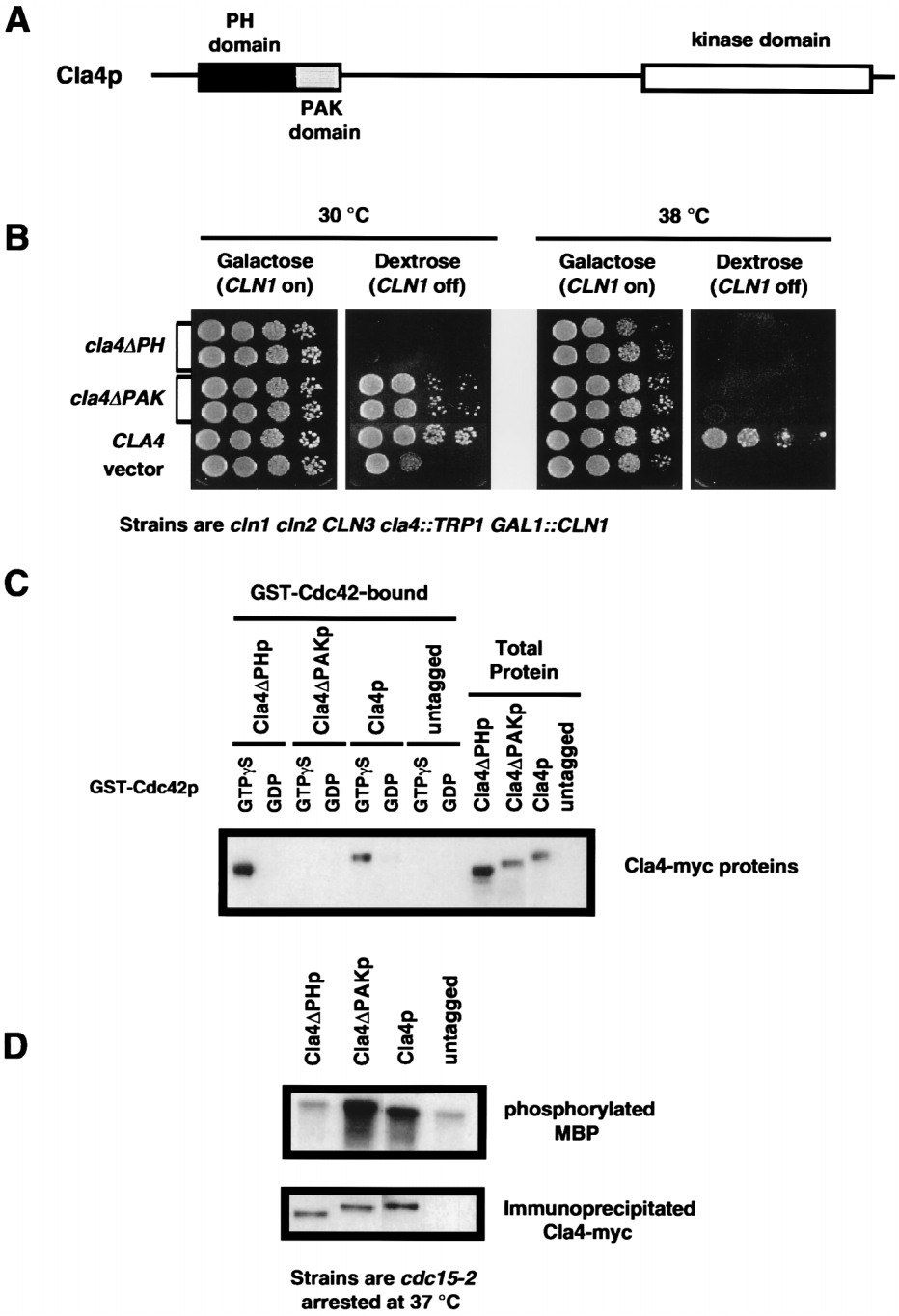

FIG. 3. Requirement of the PAK and PH domains for Cla4p function. (A) Schematic of Cla4p denoting the locations of the PAK, PH, and kinase domains. (B) Ability of PAK or PH deletion mutants to rescue a *cln1 cln2 cla4* strain. (3137cla4ΔB)-1B was transformed with CEN plasmids containing the indicated forms of myc-tagged Cla4p and spot diluted (10-fold dilutions) onto the indicated media. The strains were grown for 3 days at the indicated temperatures. Strains are PH (BYC4ΔPH), PAK (BYC4ΔPAK), CLA4 (BYC4WT) and vector [(3137cla4ΔB)-1B transformed with pRS416]. (C) Affinity of PAK and PH deletion mutants for Cdc42 protein. Extracts were made from 1255(135.7), 1255(134.7), 1255(131.1),and 1255-5C transformed with pRS416 (all grown at 30°C) and incubated with beads containing GST-Cdc42p loaded with the indicated nucleotide. Proteins bound to the beads were subjected to SDS-polyacrylamide gel electrophoresis and immunoblotted with the 9E10 antibody, which recognizes the myc epitope on Cla4::myc. A sample of total protein from each extract was also processed. (D) Kinase activity of Cla4 deletion mutant proteins isolated at the *cdc15* mitotic block. Strains BOY501(135.7), BOY501(134.7), BOY501(131.1), and BOY501 (untagged) were grown in minimal medium at 23°C and then arrested at 37°C for 2.25 h. Extracts were made, the myc-tagged Cla4p was immunoprecipitated and the kinase activity of the immunoprecipitates was determined at 30°C.

levels were roughly comparable (5). Thus, both the PAK and PH domains are required for full Cla4p function in vivo.

To investigate domain requirements for Cdc42p binding, we expressed the myc-tagged mutants in a wild-type yeast strain and made protein extracts. These extracts were then incubated with GST-Cdc42p-bound agarose beads that had been preloaded with GTPγS or GDP. The amount of myc-tagged Cla4p that bound to each form of GST-Cdc42p beads was deter-

mined by immunoblotting. Wild-type Cla4p and Cla4ΔPHp bound GTPγS-Cdc42p with similar efficiency but did not bind GDP-Cdc42p (Fig. 3C). Thus, the PH domain is unnecessary for binding activated Cdc42p. In contrast, Cla4ΔPAKp was unable to bind either form of Cdc42p. These and previous data (15) demonstrate that the PAK domain is both necessary and sufficient for binding to activated Cdc42p.

We immunoprecipitated the myc-tagged mutant or wild-type proteins and assayed their kinase activity. We used extracts from cells blocked in mitosis (using the *cdc15-2* telophase block) because this is the time when wild-type Cla4p kinase activity is highest (see below). Cla4ΔPHp had a 2.5-fold decrease in kinase activity compared to wild-type Cla4p (Fig. 3D). Surprisingly, the kinase activity of Cla4ΔPAKp was nearly threefold higher than the wild-type Cla4p kinase. These data suggest that the PAK domain is a negative regulator of Cla4p kinase activity and that binding of activated Cdc42p to this domain alleviates this inhibition. However, Cla4ΔPAKp was much less efficient than wild-type *CLA4* at in vivo rescue of *cla4* function (see above). Thus, high kinase activity may not be sufficient for full in vivo Cla4p function.

Cell cycle regulation of Cla4p kinase activity. To investigate possible cell cycle regulation of Cla4p kinase, we myc tagged Cla4p in a synchronizable *cdc15-2* strain (56, 67) to determine kinase activity through the cell cycle. Cla4p kinase activity was strongly periodic, with essentially constant Cla4p protein levels (Fig. 4A to C). Cla4p kinase activity was maximal at the telophase block and then dropped about sevenfold coincident with completion of cytokinesis and entry into the G_1 phase. The activity rose again late in the budded interval and fell again following cytokinesis. Since *cln1 cln2 cla4* cells and *ste20 cla4* cells show a cytokinesis defect, Cla4p kinase is activated at a time in the cell cycle when it may be required.

Because of the initial identification of *cla4* as a synthetic-lethal mutation in combination with *cln1 cln2* (6, 15; also see above), we were interested to determine if *CLN* function was required for the regulation of Cla4p kinase activity. However, this was not the case. A peak of Cla4p kinase activity late in the budded interval of the cell cycle was observed with *cln1 cln2 cln3* strains synchronized by depletion and synthesis of *CLN2*, *CLN3*, or *CLB5* (Fig. 5) by using a *cln* block/release protocol (13). These results indicate that there is no specific *CLN* requirement for Cla4p kinase regulation and indeed that periodicity of Cla4p kinase activity is observed under conditions of constitutive Cln2p, Cln3p, or Clb5p expression. Thus, it is unlikely that the rise or fall of Cla4p kinase activity is directly triggered by Cln2p-, Cln3p-, or Clb5p-associated kinase activity.

Does Cdc42p binding contribute to cell cycle regulation of Cla4p kinase activity? Since Cdc42p binding to the Cla4p PAK domain is required for wild-type kinase function, we investigated whether Cdc42p played a role in the cell cycle regulation of Cla4p kinase activity. Since the mutant Cla4ΔPAKp cannot bind Cdc42p yet has kinase activity, we assayed the effect of the PAK domain deletion on cell cycle regulation of the kinase. The gene encoding myc-tagged Cla4ΔPAKp was integrated in a *cdc15-2* strain that also had an untagged wild-type Cla4p. After synchronization and release of the cells from the *cdc15* mitotic block, samples were taken at various cell cycle positions, the mutant Cla4ΔPAKp was immunoprecipitated, and the kinase activity of the immunoprecipitates was assayed. In comparison to wild-type Cla4p (Fig. 4B and C), cell cycle regulation of the kinase activity of Cla4ΔPAKp was significantly reduced and the timing of the peak was shifted to earlier in the cell cycle, near the G_1/S border (Fig. 4D and E). (The large error bars in Fig. 4D are due to variability in the timing

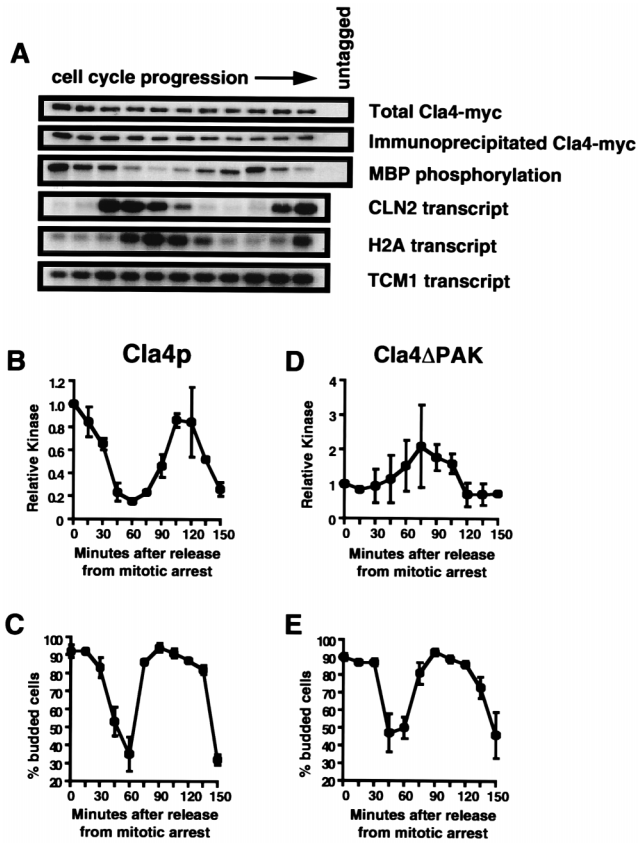

FIG. 4. Cell cycle regulation of Cla4p kinase activity. (A) *cdc15-2* cells with myc epitope-tagged Cla4p (BOY501[CM1]) were arrested at mitosis (37°C) and then released. The cells were harvested at 15-min intervals and processed for RNA extraction and for Cla4::myc immunoprecipitation and blotting, and the kinase activity of Cla4p immunoprecipitates was determined. The cell cycle position was determined by the cell cycle transcript CLN2, which peaks at START, and the S-phase transcript H2A. TCM1 is the loading control for the RNA blots. (B) Relative kinase activity of Cla4p at each point in the cell cycle from the data in panel A and a separate experiment; the mean and standard deviation are indicated. (C) Percent budded cells is another indicator of cell cycle position. (D and E) As in panels B and C but averaging the results of three experiments with *cdc15-2* cells expressing myc-tagged Cla4ΔPAKp [BOY501(138)E, BOY501(139)B].

and extent of the peak mutant kinase activity. In three experiments, the kinase activity peaked at 75 min with a 3.8-fold increase in relative kinase activity from the *cdc15* block, at 90 min with a 2.2-fold relative kinase activity, and at 60 min with 1.4-fold relative kinase activity.) These data indicated that the PAK domain is required for proper cell cycle regulation of Cla4p kinase. In addition, deletion of the PAK domain may have uncovered another cell cycle regulation of Cla4p kinase which peaks at G_1/S.

The alteration of cell cycle regulation of Cla4ΔPAKp kinase suggested the possibility that Cdc42p binding is involved in Cla4p kinase regulation. To examine this, we assayed Cla4p kinase activity in the presence of a constitutively activated Cdc42p at the *cln1 cln2 cln3* block. The G12V mutant Cdc42p is not susceptible to the action of a GTPase-activating protein, and thus the protein is locked in a GTP-bound, or active, form (74). Overexpression of the *CDC42val12* in three experiments stimulated kinase activity at least 2.5-fold in cells blocked before Start by depletion of *cln1*, *cln2*, and *cln3* (Table 2). The inactive *CDC42ala118* (74) and wild-type *CDC42* (74) alleles had a minimal effect (Table 2). Thus, Cdc42p is an activator of

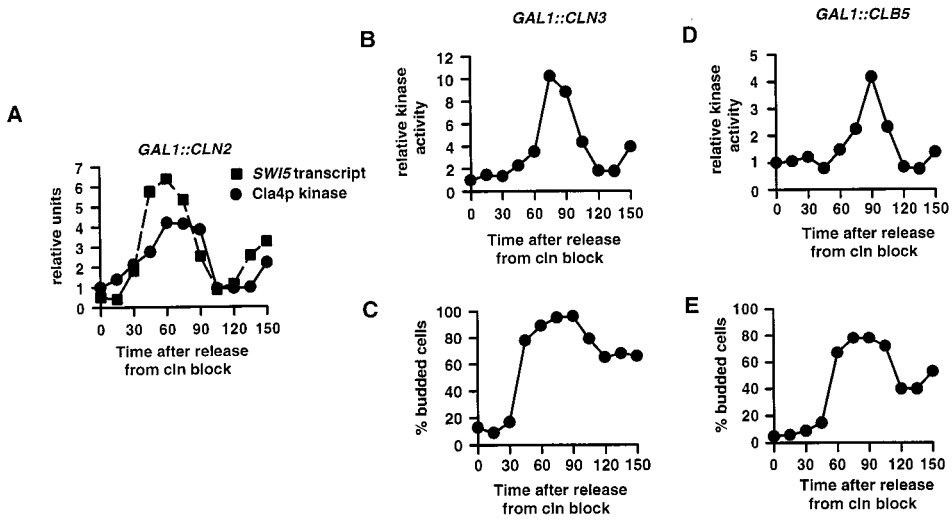

FIG. 5. Cell cycle regulation of Cla4p kinase activity is not dependent on specific G_1 cyclin function. Strains were arrested at the *cln* block by a switch from YEPGal to YEPRaffinose for 2.5 h and released by the addition of galactose to 3%, as described previously (13). (A) Cla4p kinase activity in strain 1242(CM2): *cln1 cln2 cln3 GAL1*::*CLN2*. In this experiment, buds are difficult to measure because of a cytokinesis defect in cells overexpressing *CLN2*. *SWI5* transcription was used instead as a marker of cell cycle position. *SWI5* transcription is cell cycle regulated, peaking at G_2/M (47). (B and C) Cla4p kinase activity (B) and budded cells (C) in strain 1608(CM3): *cln1 cln2 cln3 GAL1*::*CLN3*. (D and E) Cla4p kinase activity (D) and budded cells (E) in strain 2181(CM2): *cln1 cln2 cln3 GAL1*::*CLB5*.

Cla4p even in unbudded cells and the G_1 cyclin-dependent kinases are not required for activation of Cla4p kinase activity by Cdc42p. Finally, we observed a modest (~75%) stimulation of Cla4p kinase immunoprecipitated from *cdc24*- or *cdc42*-blocked cells by recombinant GTPγS-Cdc42p (5).

GIN4 is not required for Cla4p kinase activity. Genetic data (see above) indicated nonredundant roles for *GIN4* and *CLA4* in cytokinesis. To further investigate this, we assayed Cla4p kinase activity in *cln1 cln2 erc47-1* (*gin4*) *GAL1*::*CLN1* cells. *cln1 cln2 erc47-1* (*gin4*) *GAL1*::*CLN1* cells were grown in galactose (*CLN1* on), and half the culture was shifted to dextrose (*CLN1* off) for 4 h to allow cell cycle arrest due to the *gin4* mutation. Epitope-tagged Cla4p was immunoprecipitated from both cultures, and the kinase activity was assayed. Cla4p kinase activity was not decreased by the *erc47-1* (*gin4*) mutation; in fact, Cla4p kinase activity in the *cln1 cln2 erc47-1* mutant was consistently (but somewhat variably) higher (Fig. 6) (5). This suggests that *GIN4* is not required for Cla4p kinase activity; Gin4p kinase may work parallel to or downstream of Cla4p.

TABLE 2. Constitutively activated Cdc42p stimulates Cla4p kinase activity

Plasmid	Relative Cla4p kinase activity[a] in:		
	Expt 1	Expt 2	Expt 3
CDC42val12	19.77	2.69	2.61
CDC42ala118	2.40	0.87	0.93
CDC42	4.76	1.07	1.66
Vector	1.00	1.00	1.00

[a] Values are the specific activities of the Cla4p kinase relative to the specific activity of Cla4p kinase in *cln*-blocked vector transformants for each experiment. Strains are *cln1 cln2 cln3 MET3*::*CLN2*. Cultures were grown in ScRaff without methionine, which represses *CLN2* transcription from the *MET3* promoter. Asynchronous cultures were shifted into YPRaff (which contains methionine) for 2.5 h to repress *CLN2* transcription and to allow arrest at the *cln* block. *CDC42* genes were driven from the *GAL1* promoter and are on CEN plasmids. The vector is pRS416. Transcription of the *CDC42* genes was induced by adding 3% galactose to *cln*-blocked cultures and growing them for an additional 1.5 h. The cells were then harvested for the kinase assay.

DISCUSSION

Cla4p is a functional PAK; possible roles of the PAK and PH domains. By sequence analysis, Cla4p kinase is a member of an evolutionarily conserved class of kinases, the PAKs. These kinases associate with and are activated by GTP-bound forms of either Cdc42p or Rac1p through a conserved PAK domain (8, 42). The PAK domain of Cla4p is necessary and sufficient for binding to GTP-Cdc42p (15; also see above). Cla4p immunoprecipitated from cells with lower levels of GTP-Cdc42p had significantly lower kinase activity. Conversely, mutants of Cdc42p that are locked into the GTP-bound form activated Cla4p at least 2.5-fold. Compared to the reported activation of other PAKs by GTP-Cdc42p (7, 31, 40–43, 53, 63, 68), Cla4p activation by GTP-Cdc42p was modest. For example, immunoprecipitated human Pak1p is activated 40-fold when coexpressed with a mutant human Cdc42p similar to the *S. cerevisiae* mutant we used here (7). Recently, Cla4p-associated kinase was reported to be unstimulated by recombinant Cdc42p-GTP-S, with myosin-I as a substrate (70).

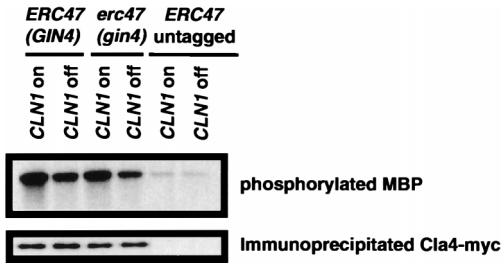

FIG. 6. Gin4p may not be required for Cla4p kinase activity. Epitope-tagged Cla4p was immunoprecipitated from strains 2507 (CM1) (*cln1 cln2 ERC*+) and 3129 (CM2) [*cln1 cln2 erc47-1* (*gin4*)] grown in the indicated media at 38°C for 4 h. The kinase activity of the Cla4::myc immunoprecipitates was determined at 30°C with MBP as a substrate. All strains are *cln1 cln2 CLN3 GAL1*::*CLN1*.

It is unclear why Cla4p shows low stimulation of its activity by Cdc42p compared to other members of the PAK family.

Deletion of the PAK domain in Cla4p reduces the ability to rescue a *cln1 cln2 cla4* strain but causes hyperactivation of Cla4p kinase. This suggests that the PAK domain inhibits the Cla4p catalytic domain and that GTP-Cdc42p activates Cla4p by binding the PAK domain and relieving this inhibition. Thus, the PAK domain may be required for full Cla4p function for reasons other than kinase activation by Cdc42p. Binding of Cla4p to GTP-Cdc42p not only may activate kinase activity but also may localize Cla4p to a particular part of the cell or allow other activators to bind Cla4p. Recently, it was shown that deletion of the Ste20p PAK domain did not eliminate kinase activity but altered subcellular localization (35, 55), which would be consistent with this hypothesis. However, we have detected a diffuse cytoplasmic localization for wild-type Cla4 (5, 23), which is different from the localization of wild-type Ste20p to the bud or shmoo tip (35, 55). Still, we cannot rule out regulated localization of a subpopulation of Cla4p, e.g., Cdc42p-associated Cla4p.

This speculation may define a new role for Cdc42p in cell cycle regulation. Mutants with mutations in *CDC42* display a bud emergence defect (1, 28). The bud normally emerges at the G_1/S border, defining a role for Cdc42p at this point in the cell cycle. However, it may also be required for activation and targeting of a kinase that works much later in the cell cycle, i.e., in cytokinesis. Cdc42p steady-state protein levels remain unchanged throughout the cell cycle, but the localization of the protein changes (75). In G_1, Cdc42p concentrates at the future site of bud emergence, after which it is found exclusively in the bud. Since most of the Cdc42p is in the bud, this may also be the major locale of activated Cla4p.

Deletion of the Cla4p PH domain lowered its kinase activity and eliminated its ability to rescue *cln1 cln2 cla4* and *ste20 cla4* cells. The pleckstrin domain of other proteins is critical for protein-protein interactions, for protein-membrane interactions, and for binding to phosphatidylinositol 4,5-bisphosphate (22, 25, 36, 45). Mutations in *S. cerevisiae PIK1*, which codes for a nuclear phosphatidylinositol 4-kinase, result in a cytokinesis defect (20, 21). Thus, binding of phosphatidylinositol 4,5-bisphosphate to the Cla4p pleckstrin homology domain may regulate the kinase. The PH domain may also regulate the association of Cla4p with its target through protein-protein interactions. These ideas on PH domain function in Cla4p are only speculative at present.

G_1 cyclins, cytokinesis, Cla4p, and Gin4p. Since *CLA4* was identified in a screen for pathways regulated by the G_1 cyclin-dependent kinases (Cln/Cdc28p), we examined whether Cla4p kinase activity was cell cycle regulated. We were surprised to find that Cla4p kinase was up-regulated near mitosis. This is much later than the time in the cell cycle when the Cln/Cdc28p kinases are maximally active. Our results do not show any specific G_1 cyclin-Cdc28p requirement for Cla4p kinase regulation, since cell cycle regulation of Cla4p kinase is observed even in the absence of all three G_1 cyclins (when they are bypassed by overexpression of the *CLB5* S-phase-specific B-type cyclin [19]; also see above). Our results on the involvement of the mitotic B-type cyclins in Cla4p kinase regulation are somewhat equivocal, but significant Cla4p kinase activity can be detected in cells blocked in G_2 by depletion of *CLB1-4* (5) with the *clb1,3,4-del clb2-ts* strain described by Amon et al. (3). Cla4p kinase also did not require Gin4p, a kinase identified here that is also involved in cytokinesis, or Cdc15p, a kinase required for the completion of telophase (56, 67). This report is the first to demonstrate cell cycle regulation of a PAK kinase. However, the connection of this regulation to the cyclin-dependent kinase activities that ultimately drive cell cycle progression is still unclear (46).

The cell cycle regulation of the Cla4ΔPAKp kinase activity was altered with respect to wild-type Cla4p. The magnitude of cell cycle regulation was reduced, and instead of peaking near mitosis, the kinase activity peaked near the G_1/S border. These data suggest that binding of GTP-Cdc42p to the PAK domain of Cla4p is involved in the cell cycle regulation of Cla4p kinase. In G_1, GTP-Cdc42p may not be bound to the PAK domain, and thus this domain inhibits Cla4p catalytic activity. After bud emergence, GTP-Cdc42p may bind to Cla4p through the PAK domain, alleviating the inhibition of the PAK domain on catalytic activity. After cytokinesis, GTP-Cdc42p could dissociate from Cla4p, resulting in low catalytic activity. Alternatively, association of Cla4p with GTP-Cdc42p may occur transiently at the G_1/S border, which would place Cla4p in a "primed" state for activation (transient association of huPAK65 with Cdc42p or Rac1p is sufficient to maintain kinase activity [43]). Then other factors present at G_2/M (e.g., those that interact with the pleckstrin homology domain) would activate the primed Cla4p kinase activity. To test these speculative models further, an assay for the binding of endogenous Cdc42p to Cla4p through the cell cycle would be required. In Ste20p, autophosphorylation of a serine residue conserved in Cla4p is required for catalytic activity (71), but autophosphorylation is not dependent on association with Cdc42p or Rac1p (35, 55). Thus, it would be interesting to know whether autophosphorylation of Cla4p is another contributor to the cell cycle regulation of Cla4p kinase activity.

The observation that *cla4* and *gin4* mutants do not arrest owing to cytokinesis defects unless both *CLN1* and *CLN2* are missing strongly suggests a role for these G_1 cyclins in cytokinesis. At first this seems paradoxical, because the G_1 cyclin-dependent kinases are active only in G_1/S whereas cytokinesis occurs much later in the cell cycle. However, events required for cytokinesis could be set up in G_1 to be used later. For example, the septin ring is formed in late G_1 at a time when Cln1,2/Cdc28p is active (30). This suggests that these cyclin-dependent kinases may regulate septin ring formation. In support of this is the identification of a *cln1 cln2 cdc12* mutant that can be rescued by expression of *CLN2* (15) (*CDC12* encodes a septin ring component). Second, *gin4* was also identified as a synthetic lethal mutation with *cdc12* (38). Another possible target of *CLN1* and *CLN2* in cytokinesis is the redundant PAK, *STE20. cla4* mutants give a cytokinesis defect either when both *CLN1* and *CLN2* are missing or when the redundant PAK *STE20* is missing. This suggests that Cln1,2/Cdc28p may bypass the requirement for Cla4p in cytokinesis by activating Ste20p. Ste20p may also be the target for Cln2/Cdc28p repression of pheromone signalling (49, 50). Cln1,2/Cdc28p may therefore repress the mating-factor pathway by recruiting Ste20p from the mating-factor pathway to a distinct pathway required for cytokinesis. By analogy, a functional homolog of Gin4p may also be regulated by the G_1 cyclin-dependent kinases Cln1,2/Cdc28p, since these cyclin-dependent kinases bypass the requirement for *GIN4*, possibly by activating an alternative cytokinesis component.

A model for the role of the G_1 cyclins in bud morphogenesis and cytokinesis is illustrated in Fig. 7. Cln1,2/Cdc28p play a direct role in initiating bud emergence, while Cln3/Cdc28p requires the function of Bud2p to promote bud emergence (6, 16, 17). Cln1,2/Cdc28p (and Cln3/Cdc28p?) may then be involved in septin ring formation, which is required for later cytokinesis. Cln1,2/Cdc28p may also activate Ste20p (and perhaps a Gin4p functional homolog?) at this time. After bud emergence, Cla4p becomes activated by binding to GTP-

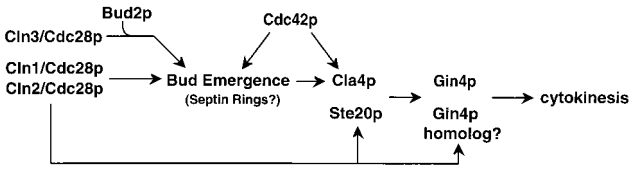

FIG. 7. Model for Cln/Cdc28p activation of morphogenesis in budding yeast. See the text for a discussion.

Cdc42p, which, along with activated Ste20p and Gin4p, may lead to cytokinesis.

A role for the Rho class of small GTPases like Cdc42p in cytokinesis is not limited to yeast. Mutations in a *Rac* homolog in *Dictyostelium* lead to a cytokinesis defect (33). Overexpression of a mutant Cdc42p locked in the GTP-bound form in mammalian fibroblasts also leads to cytokinesis defects (18). The effectors of these small GTPases are unknown in these cases; perhaps Cla4p homologs may regulate cytokinesis in other eukaryotes.

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