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9. A bacterially expressed TrpE-HCS26 fusion protein was purified from inclusion bodies by denaturing gel electrophoresis, electroeluted from gel slices, and injected into rabbits (BabCO, Berkeley, CA). Antibodies to HCS26 were affinity-purified as described [B. J. Andrews and I. Herskowitz, *Nature* **342**, 830 (1989)].
10. For immunoprecipitations, cells were grown in logarithmic phase (30°C for CDC28 strains and 25°C for *cdc28-4* strains) to an OD<sub>600</sub> of 0.4 to 0.6. Cells (30 OD<sub>600</sub> units) were collected by centrifugation and resuspended in 1 ml of ice-cold buffer A [50 mM NaCl, 50 mM tris (pH 7.5), 0.1% NP-40, and 0.1 mM EDTA] containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM benzamide, leupeptin (0.1 µg/ml), 1 µM calyculin A, and aprotinin (2 µg/ml). All manipulations were done at 4°C. Resuspended cells were combined with 0.5 ml of glass beads and lysed with three 30-s pulses of a mini beadbeater (Bio-Spec). The lysate was clarified by centrifugation (10 min at 10,000g). Affinity-purified anti-HCS26 or antibody to GST (anti-GST) was added at a 1:100 dilution for 1 hour, and the mixture was then incubated for 45 min with 40 µl of a 1:1 slurry of goat antibodies to rabbit immunoglobulin G coupled to agarose beads (Sigma) suspended in buffer A. Immunoprecipitates were washed three times with 1 ml of buffer A, once with 1 ml of PBS, and once with 1 ml of kinase buffer [50 mM tris (pH 7.5) and 10 mM MgCl<sub>2</sub>].
11. To determine kinase activity in anti-HCS26 immunoprecipitates, we resuspended beads in 50 µl of kinase buffer supplemented with 1 mM dithiothreitol (DTT), 0.5 mM PMSF, aprotinin (2 µg/ml), leupeptin (0.1 µg/ml), and 10 µCi of [γ-<sup>32</sup>P]adenosine triphosphate (ATP). Histone H1 (Boehringer Mannheim) was added at 0.5 mg/ml. Reactions were incubated for 20 min at 30°C (or 20 min at 25°C for the experiment shown in Fig. 2C) and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography.
12. The NH<sub>2</sub>-terminal GST-HCS26 fusion construct (pFHE29) was made by ligating the open reading frame of HCS26 into the polylinker of pGEX1 [D. B. Smith and K. S. Johnson, *Gene* **67**, 31 (1988)]. Expression of GST-HCS26 was induced in logarithmic phase (OD<sub>600</sub> ≈ 0.6) DH5α cells containing pFHE29 by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) followed by incubation for 3 hours at 30°C. Cells were collected; frozen (–70°C); thawed into PBS with lysozyme (0.5 mg/ml), 1 mM EDTA, 1 mM EGTA, and 1 mM PMSF at 4°C; and lysed by sonication (three pulses of 1 min each at setting 35). The lysate was adjusted to 250 mM KCl and 15 mM DTT and clarified by centrifugation (85,000g) for 1 hour. The lysate was loaded onto a glutathione-coupled agarose (Sigma) column and washed extensively with PBS with 250 mM KCl and 0.5 mM DTT, and the GST-HCS26 was eluted with elution buffer [50 mM tris (pH 8.1), 250 mM KCl, and 5 mM reduced glutathione]. Peak fractions were pooled and dialyzed extensively against 50 mM Hepes (pH 7.6), 50 mM KCl, and 30% glycerol. GST-CLB3 and GST were prepared in the same way with the plasmids pGEX-CLB3 and pGEX1, respectively.
13. Logarithmic phase cells (OD<sub>600</sub> ≈ 0.6) were mechanically lysed with glass beads in a mini beadbeater (BioSpec) (two 50-s pulses) in buffer A containing 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, leupeptin (1 µg/ml), and aprotinin (2 µg/ml). Lysates were clarified by centrifugation (10 min at 10,000g). Then 50 µg of purified GST-HCS26 protein was added to 900 µg of lysate containing ATP-regenerating mix [50 mM Hepes (pH 7.5), 40 µM creatine phosphate, creatine phosphate kinase (20 µg/ml), 1 mM MgCl<sub>2</sub>, and 1 mM ATP] and incubated for 15 min at 24°C. GST-HCS26-associated proteins were recovered by adding 50 µl of a 1:1 slurry of glutathione-coupled agarose beads (Sigma) in buffer A and incubating for 1 hour at 4°C. Beads were washed twice with buffer A, once with PBS, and once with kinase buffer. Associated complexes were eluted from the beads by incubation in kinase elution buffer [50 mM tris (pH 7.5), 10 mM MgCl<sub>2</sub>, 1 mM DTT, and 5 mM reduced glutathione] for 15 min at 24°C.
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15. Immunoblotting was done as described [S. M. Murphy, M. Bergman, D. O. Morgan, *Mol. Cell. Biol.* **19**, 5290 (1993)]. Where horseradish peroxidase-coupled secondary antibodies were used, the immunoreactive proteins were visualized with the Enzyme-Linked Chemiluminescence (ECL) Western Blotting Detection Kit (Amersham).
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19. For the analysis of PHO4 kinase activity, samples were incubated in 50 mM tris (pH 7.5), 10 mM MgCl<sub>2</sub>, 100 µM ATP, 800 ng of PHO4, and 10 µCi of [γ-<sup>32</sup>P]ATP. Reactions were incubated for 15 min at 30°C and analyzed by SDS-PAGE and autoradiography. Purified, recombinant PHO4 was a gift of E. O'Shea (18).
20. An in-frame Not I site was added to the 3' end of HCS26 by oligonucleotide-directed mutagenesis. A Not I fragment containing three tandem copies of the HA epitope (YPYDVPDYA) was then ligated into the Not I site to generate HCS26-3XHA. A Hind III-Xho I fragment containing HCS26-3XHA was then ligated into a 2-µm-based plasmid carrying the HCS26 promoter region to generate pFHE40. A, Ala; D, Asp; P, Pro; V, Val; Y, Tyr.
21. Lysates for immunoprecipitations with 12CA5 were prepared as in (13). We added 1 to 7 µg of mAb 12CA5 to lysates (900 µg) and incubated the mixture on ice for 30 min. Protein A-coupled Sepharose beads (Sigma) were then added and the mixture incubated with constant agitation for 1 hour at 4°C. Beads were then washed twice with buffer A, once with PBS, and once with kinase assay buffer.
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## The PCL2 (ORFD)–PHO85 Cyclin-Dependent Kinase Complex: A Cell Cycle Regulator in Yeast

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Cyclin-dependent kinase (cdk) complexes are essential activators of cell cycle progression in all eukaryotes. In contrast to mammalian cells, in which multiple cdk's contribute to cell cycle regulation, the yeast cell cycle is largely controlled by the activity of a single cdk, CDC28. Analysis of the putative G<sub>1</sub> cyclin PCL2 (ORFD) identified a second cyclin-cdk complex that contributes to cell cycle progression in yeast. PCL2 interacted with the cdk PHO85 in vivo and in vitro and formed a kinase complex that had G<sub>1</sub>-periodic activity. Under genetic conditions in which the Start transition was compromised, PHO85 and its associated cyclin subunits were essential for cell cycle commitment. Because PHO85 and another cyclin-like molecule, PHO80, also take part in inorganic phosphate metabolism, this cdk enzyme may integrate responses to nutritional conditions with the cell cycle.

In eukaryotes, cdk's are essential activators of cell cycle transitions. The activity of cdk's is regulated in part by their association with essential positive regulatory subunits called cyclins. Passage through a given transition is thought to require a threshold amount of appropriate cdk activity. In metazoans, multiple cdk's and associated cyclin subunits control the cell cycle (1). In contrast, in the budding yeast *Saccharomyces cerevisiae*, CDC28 is the only cdk enzyme known to be required for cell cycle

progression (2). Specificity of CDC28 function at different points in the cell cycle is achieved through association with different classes of cyclin subunits. Three G<sub>1</sub> cyclins, CLN1, CLN2, and CLN3, are rate-limiting activators of CDC28 in late G<sub>1</sub> that promote irreversible commitment to a new division cycle; this event is called Start and is analogous to the restriction point for mammalian cells (3). The cell cycle machinery is regulated at Start in part by modulation of the abundance of cyclins in response to environmental cues such as glucose availability (4) and mating pheromones (5, 6).

Expression of the genes *CLN1* and *CLN2* (but not *CLN3*) is largely controlled by the activity of two transcription factors, SWI4 and SWI6, which form a heterodimeric complex, SCB-binding factor (SBF), that binds to SWI4,6–cell cycle box

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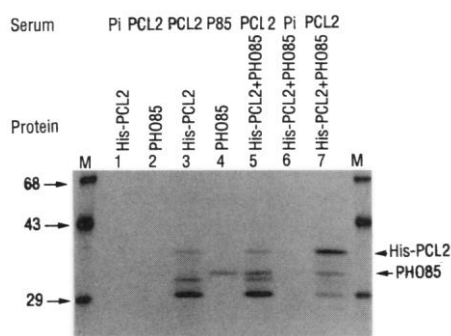
(SCB) sites in the *CLN* promoters (6–10). Mutants lacking SBF can be rescued by overexpression of *CLN1* or *CLN2*, or of *HCS26*, which encodes a cyclin-like protein (8). Another gene with similarity to cyclins, *ORFD*, was discovered by sequencing a region of the yeast genome (11). Expression of *HCS26* and *ORFD*, like that of *CLN1* and *CLN2*, peaks in late  $G_1$  and depends on SBF activity (5, 8, 9, 12–14). *ORFD* and *HCS26* share features not common to all  $G_1$  cyclins and appear to form a distinct subfamily of  $G_1$  cyclins (2, 15, 16). We report that *ORFD* is a cyclin partner for the *PHO85* kinase. Therefore, we propose that *ORFD* be renamed *PCL2* (*PHO85* cyclin 2) [Espinoza *et al.* (14) suggest the name *PCL1* for *HCS26*].

To elucidate the role of *PCL2* in cell cycle progression, we used the yeast two-hybrid protein interaction screen (17–19) to search for proteins that associate with *PCL2*. A library of yeast complementary DNAs (cDNAs) fused to the GAL4 activation domain (AD) was introduced into a yeast strain expressing a fusion of the GAL4 DNA binding domain to *PCL2* (DB-*PCL2*) (20). An AD-cDNA fusion protein that interacts with DB-*PCL2* would bring the GAL4 DNA binding and activation domains into close proximity and induce transcription of reporter genes in the strain. Three of the cDNAs identified in this screen contained in-frame AD-*PHO85* fusion. *PHO85*, a member of the cdk family [51% identity to *CDC28* (21, 22)], has a role in inorganic phosphate metabolism (16, 23) but no known role in cell cycle control. We interchanged the GAL4 domains to produce the fusion proteins DB-

*PHO85* and AD-*PCL2* (20) which, when coexpressed, also activated transcription of the reporter genes (24). We detected no interaction between *PCL2* and *CDC28* suggesting a high selectivity for *PHO85* as the cdk partner of *PCL2* (24). However, *PHO85* did interact with *HCS26* in the two-hybrid system (24). We were able to detect in vitro association of *PHO85* and *PCL2* in two ways (25, 26). First, addition of antibodies to *PCL2* (27) to an in vitro translation reaction containing  $^{35}\text{S}$ -labeled *PHO85* and *PCL2* resulted in the immunoprecipitation of both labeled proteins (Fig. 1). Second, mixing of a reaction containing  $^{35}\text{S}$ -labeled *PHO85* protein with one containing unlabeled *PCL2* allowed immunoprecipitation of the labeled *PHO85* with antibodies to *PCL2* (26).

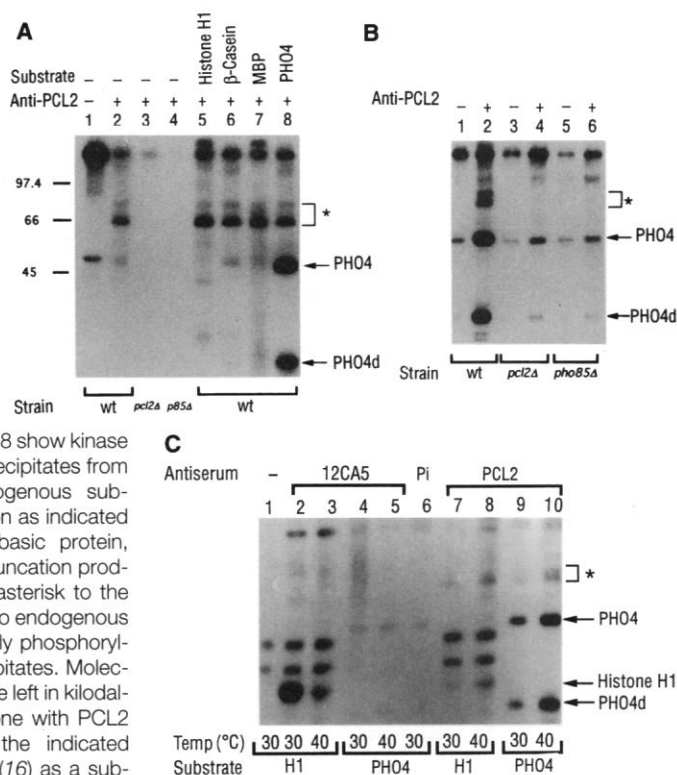
To test whether *PCL2* and *PHO85* form a functional cyclin-cdk complex, we immunoprecipitated *PCL2* from yeast lysates and assayed associated protein kinase activity (28). Two prominent endogenous substrates

were phosphorylated in immunoprecipitates from a wild-type strain (Fig. 2). *PCL2*-associated kinase activity also efficiently phosphorylated purified *PHO4* protein, a known substrate of the *PHO85* kinase (Fig. 2, A and B) (16, 28), but did not phosphorylate histone H1, casein, or myelin basic protein (Fig. 2A). Immunoprecipitates from either *pcl2* or *pho85* deletion strains failed to phosphorylate either *PHO4* or the endogenous substrates (Fig. 2, A and B). To test the specificity of the *PCL2*-cdk interaction, we assayed *CLN2*- and *PCL2*-associated kinase activity in a strain carrying an epitope-tagged *CLN2* allele (5) and the temperature-sensitive *cdc28-13* mutation (29). The *CLN2*-*CDC28* complex showed temperature-sensitive phosphorylation of histone H1 in vitro but did not phosphorylate *PHO4* at either temperature (Fig. 2C). In contrast, *PCL2*-associated kinase immunoprecipitated from the same lysates efficiently phosphorylated *PHO4*, but not histone H1, at both temperatures (Fig. 2C).



**Fig. 1.** Association of *PCL2* and *PHO85* in vitro. Immunoprecipitations were done with in vitro-translated,  $^{35}\text{S}$ -labeled His-*PCL2* and *PHO85* (25). Lane 1, His-*PCL2* + preimmune serum (Pi); lane 2, *PHO85* + antibody to *PCL2* (anti-*PCL2*) (*PCL2*); lane 3, His-*PCL2* + anti-*PCL2*; lane 4, *PHO85* + antibody to *PHO85* (*P85*); lane 5, His-*PCL2* + *PHO85* (translated separately) + anti-*PCL2*; lane 6, His-*PCL2* + *PHO85* (cotranslated) + preimmune serum; and lane 7, His-*PCL2* + *PHO85* (cotranslated) + anti-*PCL2*. Molecular size markers in lanes M are indicated to the left in kilodaltons.

**Fig. 2.** Association of *PCL2* with a *PHO85*-dependent kinase activity that phosphorylates *PHO4*. (A) Kinase assays were done on *PCL2* immunoprecipitates from the strains indicated at the bottom (28). Anti-*PCL2* (+) or preimmune serum (–) was added as shown above the lanes. Lane 1, wild type (wt) + preimmune serum; lane 2, wt + anti-*PCL2*; lane 3, *pcl2Δ* + anti-*PCL2*; and lane 4, *pho85Δ* + anti-*PCL2*. Lanes 5 through 8 show kinase assays on *PCL2* immunoprecipitates from wild-type strains with exogenous substrates added to the reaction as indicated above the lanes (myelin basic protein, MBP). *PHO4d* is a *PHO4* truncation product (16). The bracket and asterisk to the right mark the position of two endogenous products that are specifically phosphorylated in *PCL2* immunoprecipitates. Molecular sizes are indicated to the left in kilodaltons. (B) Kinase assays done with *PCL2* immunoprecipitates from the indicated strains with purified *PHO4* (16) as a substrate. Lane 1, wt + preimmune serum + *PHO4*; lane 2, wt + anti-*PCL2* + *PHO4*; lane 3, *pcl2Δ* with preimmune serum + *PHO4*; lane 4, *pcl2Δ* + anti-*PCL2* + *PHO4*; lane 5, *pho85Δ* with preimmune serum + *PHO4*; and lane 6, *pho85Δ* + anti-*PCL2* + *PHO4*. (C) HA-*CLN2* (5) or *PCL2* were immunoprecipitated from a *cdc28-13* strain grown at the permissive temperature (30°C). Immunoprecipitates were preincubated for 10 min at 30°C or at the nonpermissive temperature (40°C), and kinase assays were done with the immunoprecipitates at both temperatures as indicated. *PCL2* was immunoprecipitated with anti-*PCL2* (27), whereas HA-*CLN2* was immunoprecipitated with the monoclonal antibody 12CA5 (39) as indicated above the lanes. Exogenous substrates were added [histone H1 (H1) or *PHO4*] as shown below the lanes at the indicated temperature. Immunoprecipitation was done with lane 1, no antibody (–); lanes 2 to 5, 12CA5; lane 6, preimmune serum (Pi); and lanes 7 to 10, anti-*PCL2*. The position of migration of phosphorylated *PHO4* and histone H1 is indicated. The slower migrating species above the specific histone H1 band are contaminants in the histone H1 preparation that were phosphorylated in the absence of antibody (lane 1).

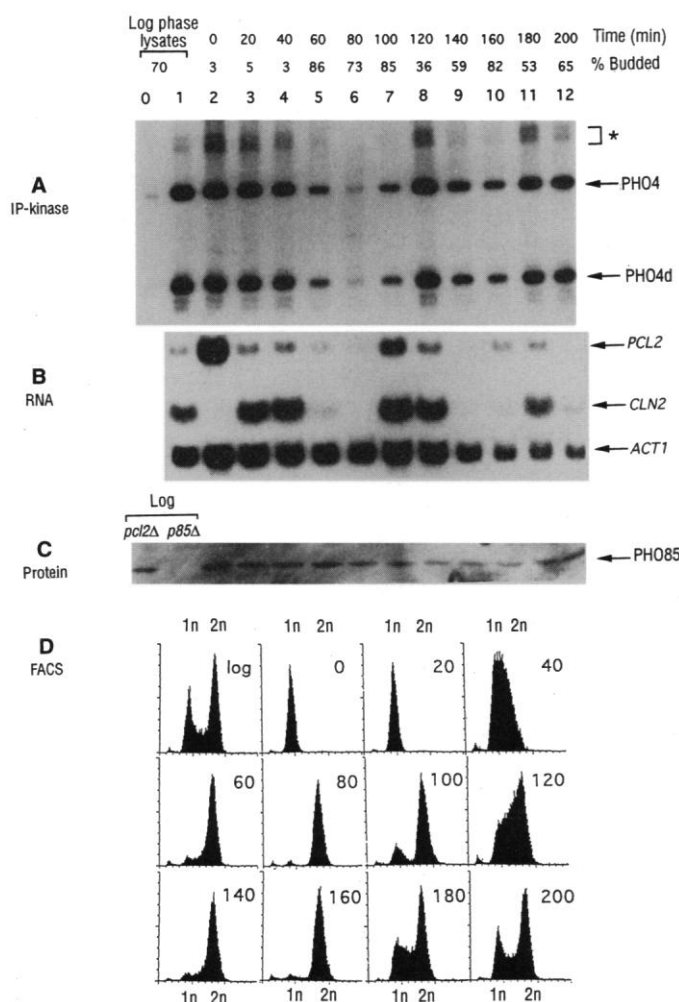


The amount of PCL2 mRNA fluctuates during the cell cycle, peaking at Start (5). To determine if the PCL2-associated PHO85 kinase activity was cell cycle-regulated, we arrested cells at Start, released them into fresh medium, and prepared extracts from samples of the cells as the culture progressed synchronously through the cell cycle (Fig. 3D) (30). PCL2-associated kinase activity fluctuated periodically, peaking in G<sub>1</sub> with the PCL2 transcript (Fig. 3, A and B). In contrast, the amount of PHO85 protein remained constant throughout the cell cycle (Fig. 3C) (31). The PCL2 transcript was induced in response to  $\alpha$  factor, a pheromone that arrests cells at Start (Fig. 3B). The PHO4 kinase activity remained constant during  $\alpha$  factor treatment, whereas phosphorylation of co-immunoprecipitated substrates seemed to increase (Fig. 3A). This result may indicate a substrate-specific change in PCL2-PHO85 kinase activity upon treatment with pheromone. In contrast, CLN1- and CLN2-associated CDC28 activity is absent

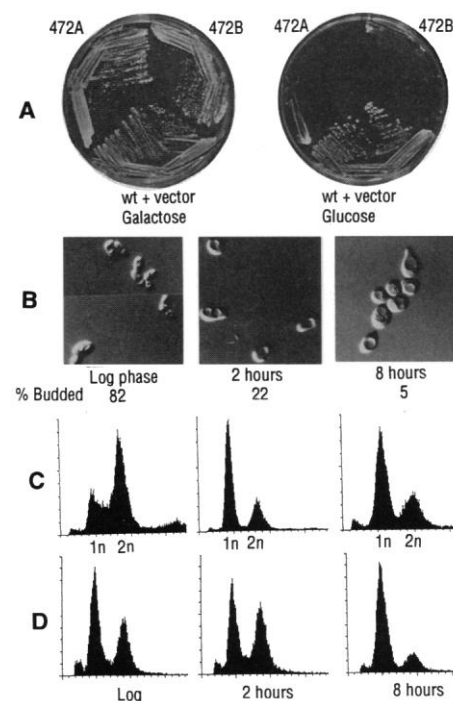
during  $\alpha$  factor arrest (5).

Cells deleted for either CLN1 and CLN2 or PCL2 and HCS26 show no obvious defect in cell cycle control (3, 13). We examined whether all four SBF-regulated genes, CLN1, CLN2, HCS26, and PCL2, formed a functionally overlapping set of cyclins. A *cln1 $\Delta$  cln2 $\Delta$  pcl2 $\Delta$*  strain was mated to a *cln1 $\Delta$  hcs26 $\Delta$  pcl2 $\Delta$*  strain, and diploids were recovered and sporulated (32). Of 50 tetrads dissected, no viable quadruple mutants were recovered (33). We rescued the lethal phenotype of a *cln1 $\Delta$  cln2 $\Delta$  hcs26 $\Delta$  pcl2 $\Delta$*  with a plasmid expressing CLN1 from an inducible GAL promoter and then repressed expression of GAL-CLN1 by adding glucose to the growth medium. Cells accumulated in G<sub>1</sub> with a 1n DNA content within 2 hours of repressing GAL-CLN1 expression. By 4 to 8 hours, most of the cells were large and unbudded and had a 1n DNA content (Fig. 4, A, B, and C). Thus, PCL2 performs a role in G<sub>1</sub> that is functionally redundant with that of other G<sub>1</sub> cyclins.

**Fig. 3.** Cell cycle regulation of PCL2-PHO85 kinase activity. A wild-type strain was arrested with  $\alpha$ -factor ( $\alpha$ -F) and reinoculated into fresh medium (30). Samples were taken at 20-min intervals (as indicated above the lanes) and analyzed for the percentage budded cells (% Budded); kinase activity (28); PCL2, CLN2, and ACT1 mRNA; DNA content (30); and the amount of PHO85 protein (31). (A) PCL2-associated kinase activity in synchronized cell extracts. PHO4 was added as an exogenous substrate in each assay. Lane 0 shows a reaction containing logarithmic phase lysate and preimmune serum. All other reactions are kinase assays on anti-PCL2 immunoprecipitates. Lane 1, logarithmic phase lysate; and lanes 2 to 12, lysates from the indicated time points after  $\alpha$ -F release. (B) Amount of transcript encoding PCL2, CLN2, and ACT1 in the samples used the kinase assays in (A). (C) Protein immunoblot with antibody to PHO85 (27) on extracts from *pcl2 $\Delta$*  and *pho85 $\Delta$*  strains (indicated as Log), and extracts from samples used in kinase assays. (D) DNA content as measured by FACS analysis of logarithmic phase cells and synchronous cells used in (A) to (C). The 1n and 2n peaks and time after release from  $\alpha$ -F are indicated.

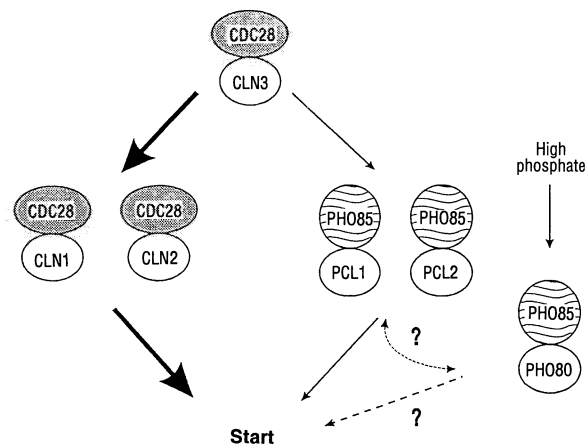


In a similar experiment, we established a role for PHO85 itself at Start. A *cln1 $\Delta$  pho85 $\Delta$*  strain was mated to a *cln2 $\Delta$  pho85 $\Delta$*  strain (34). Diploids were recovered and sporulated, but of 25 tetrads dissected, no *cln1 $\Delta$  cln2 $\Delta$  pho85 $\Delta$*  cells were detected. However, the triple deletion strain was recovered in the presence of a plasmid expressing PHO85 from the GAL promoter; this strain arrested in G<sub>1</sub> when PHO85



**Fig. 4.** Arrest phenotype of cells lacking CLN1, 2-kinase and PCL2, HCS26 (PCL1) or PHO85. (A) The plasmid pGAL-CLN1 rescued the lethality of a *cln1 $\Delta$  cln2 $\Delta$  pcl2 $\Delta$  hcs26 $\Delta$*  strain. A *cln1 $\Delta$  cln2 $\Delta$  pcl2 $\Delta$  hcs26 $\Delta$*  strain (472A and 472B) (32) carrying a plasmid expressing CLN1 from the GAL promoter was streaked on a YEPG (galactose) plate (left) or YEPD (glucose) plate (right) and incubated at 25°C for 4 days. A wild-type strain (BY163, wt) was transformed with the pGAL-CLN1 vector and streaked onto the same plates. (B) Strain BY472A (*cln1 $\Delta$  cln2 $\Delta$  pcl2 $\Delta$  hcs26 $\Delta$  pGAL-CLN1*) was grown in galactose medium, and glucose was added to repress CLN1 expression. Nomarski photos were taken of cells in logarithmic phase and 2 and 8 hours after glucose addition. The percentage budded cells is indicated below the photographs. (C) DNA content as measured by FACS analysis of samples shown in (B). (D) Terminal phenotype of a *cln1 $\Delta$  cln2 $\Delta$  pho85 $\Delta$*  deletion strain. The lethal phenotype of a *cln1 $\Delta$  cln2 $\Delta$  pho85 $\Delta$*  strain was rescued by expression of PHO85 from the GAL promoter (34). The strain was grown in galactose medium, glucose was added to repress PHO85 expression, and samples were taken up to 8 hours. DNA content as measured by FACS analysis of galactose-grown cells (Log) and cells harvested 2 or 8 hours after glucose addition is shown. In panels (C) and (D), the position of cells with G<sub>1</sub> or G<sub>2</sub> DNA contents is indicated by 1n or 2n, respectively.

**Fig. 5.** Model of cdk function at Start.



expression was repressed by addition of glucose to the culture medium (Fig. 4D). Thus, PHO85 is essential for cell cycle progression in the absence of a functional CLN1- and CLN2-CDC28 kinase.

Cells deleted for *PHO85* grow slowly relative to wild-type cells but are otherwise able to complete cell division (21, 22). However, our studies have revealed two potential roles for the PHO85 kinase in cell cycle control. First, the rapid induction of *PCL2* transcription in response to mating pheromone, and the persistence of *PCL2*-PHO85 kinase activity in  $\alpha$ -factor-arrested cells (Fig. 3), suggests that *PCL2*-PHO85 kinase may be involved in pheromone response. Consistent with this idea, *pho85* $\Delta$  strains are supersensitive to  $\alpha$  factor (35). Second, PHO85 was essential for Start in the absence of CLN1- and CLN2-CDC28 kinase activity. Although deletion of *PCL2* in a strain lacking *CLN1* and *CLN2* did not cause cell cycle arrest, deletion of both *HCS26* and *PCL2* did cause  $G_1$  arrest. Thus, like *PCL2*, *HCS26* may function as a cyclin subunit of PHO85 at Start. Indeed, *HCS26* associates with PHO85 (14) in yeast extracts.

The observation that *PCL2*- or *HCS26*-associated PHO85 kinase activity is only essential for cell cycle progression in the absence of CLN1,2-CDC28 kinase activity is consistent with the notion that, like CLN1 and CLN2 (5), *PCL2* and *HCS26* may, together with PHO85, constitute a  $G_1$  cyclin activity that acts downstream of CLN3 (Fig. 5). The catalytic activity provided by *PCL2*- or *HCS26*-PHO85 may become essential only when the activity of other downstream  $G_1$  cyclins is absent. The complete absence of CDC28 function cannot be tolerated even in cells with a functional PHO85 kinase. This may be explained by the substrate specificity of PHO85 which, in kinase assays, is different from that of CDC28 (Fig. 2). The CLN3-CDC28 kinase may phosphorylate essential rate-limiting substrates for Start

that cannot be phosphorylated by the PHO85 kinases.

The PHO85 regulatory system may serve to link certain environmental signals to the cell cycle machinery. PHO85 and the cyclin PHO80 are negative regulators of the acid phosphatase gene, *PHO5* (36). The PHO80-PHO85 cyclin-cdk complex phosphorylates and likely inactivates PHO4, a transcription factor required for induction of *PHO5* (16). PHO80 is similar in sequence to *PCL2* and *HCS26* and thus appears to fall into the same cyclin subclass (16). When complexed with PHO80, PHO85 may be specifically directed toward substrates involved in inorganic phosphate metabolism (PHO4). Although PHO4 is phosphorylated by *PCL2*-PHO85 in vitro, our genetic data suggest that, when complexed with *PCL2* or *HCS26*, PHO85 is targeted to specific substrates that take part in the control of cell cycle progression (Fig. 5).

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20. The *PCL2* coding sequence (11) was isolated by polymerase chain reaction (PCR) and cloned into pAS1 (18) to create a fusion of *PCL2* to the GAL4 DNA binding domain which was then transformed into strain Y153 (18). Y153 containing pAS1-*PCL2* was cotransformed with a yeast cDNA library in vector pACT. Interacting clones were selected as described (18, 19) from a pool of 160,000 yeast colonies. Other GAL4 activation domains (AD) and GAL4 DNA binding domain (DB) fusions were constructed: An AD-*PCL2* fusion was constructed by cloning a *PCL2* fragment from pAS1-*PCL2* into pACTII (19). A DB-*PHO85* fusion was constructed by subcloning the *PHO85* cDNA from one of the AD-*PHO85* library plasmids recovered in the two-hybrid screen into pAS1. AD-*HCS26* and AD-*CDC28* fusions in pACTII were constructed by PCR amplification of *HCS26* and *CDC28* from plasmids.
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25. *PHO85* and *PCL2* were transcribed and translated in vitro in rabbit reticulocyte lysates with or without [<sup>35</sup>S]methionine (TNT kit, Promega). *PCL2* DNA templates consisted of the coding region of *PCL2* cloned into vector pET-HMK (derivative of pET19b, Novagen). *PCL2* protein produced from pET-HMK-*PCL2* carries a polyhistidine tag and heart muscle kinase recognition sequence at the NH<sub>2</sub>-terminus and was used in <sup>35</sup>S-labeled translations of *PCL2* (Fig. 1A). For immunoprecipitation reactions, 5  $\mu$ l of *PCL2*- or *PHO85*-containing translation mixture or both were diluted to 100  $\mu$ l with Buffer D [B. Andrews and L. Moore, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 11852 (1992)]. Reaction mixtures were incubated with 50  $\mu$ l of protein A-Sepharose (100 mg/ml, Sigma) swollen in Buffer D. Sepharose beads were sedimented and affinity-purified antibody to *PCL2* or *PHO85* (27) was incubated with the supernatant by rocking at 4°C for 60 min. Immunoprecipitates were recovered by addition of 50  $\mu$ l of protein A-Sepharose slurry. After incubation for 60 min at 4°C, the immunoprecipitates were washed three times with 1 ml of Buffer D and resuspended in 80  $\mu$ l of 2 $\times$  SDS loading dye. The sample was boiled and centrifuged, and the proteins were separated by polyacrylamide gel electrophoresis (12% gel). The gels were fixed, treated with Amplify (Amersham), dried, and exposed to x-ray film.
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27. Antibody to *PCL2* was prepared with a fusion protein containing the COOH-terminal 114 amino acids of *PCL2* fused to *Escherichia coli* TrpE in plasmid pATH3 [T. Koerner, J. Hill, A. Myers, A. Tzagoloff, *Methods Enzymol.* **194**, 477 (1991)]. Antibody to PHO85 was prepared with a fusion protein containing the PHO85 coding sequence lacking the first three NH<sub>2</sub>-terminal amino acids fused to glutathione-S-transferase in vector pGEX-2T (Pharmacia). TrpE-*PCL2* or Gst-*PHO85* fusion protein eluted from SDS gels was used to immunize rabbits (Faculty of Medicine, University of Toronto). Antisera were affinity purified as described [B. Andrews and I. Herskowitz, *Nature* **342**, 830 (1989)].
28. The following strains were used for kinase assays: BY263 (wild type), *MATa trp1 $\Delta$ 63 ura3-52 lys2-801<sup>+</sup> ade2-107<sup>+</sup> his3 $\Delta$ 200 leu2- $\Delta$ 1*; BY271 (*pcl2 $\Delta$* ) *pcl2 $\Delta$ HIS3*, otherwise isogenic to BY263; BY391a (*pho85 $\Delta$* ), *pho85 $\Delta$ LEU2*, otherwise isogenic to BY263; MT345 (*cdc28<sup>ts</sup>*, CLN2-tag), *MATa cdc28-13 ade1 his3 leu2 met14 ura3 tyr1 cln2::CLN2C-LEU2* [M. Tyers and B. Futcher, *Mol. Cell. Biol.* **13**, 5659 (1993)]. The *pcl2 $\Delta$ HIS3* disruption cassette was constructed by ligation of the *HIS3* gene to a fragment of *PCL2* with an internal deletion of 734 base pairs (bp) that was generated by PCR amplification. The *pho85 $\Delta$ LEU2* disruption allele has

- been described (21). Kinase assays were performed on immunoprecipitated material from 100-ml cultures for 20 min at 30°C as described (37). Kinase reactions also contained 100 ng of purified PHO4 (16), 1 µg of histone H1 (Boehringer Mannheim), 1 µg of β-casein (Sigma), or 1 µg of myelin basic protein (Sigma) as indicated. The background kinase activity associates nonspecifically with protein A-Sepharose beads (16).
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  30. Strain K699a [*a ade2-1 trp1-1 leu2-3,112 his3-11 ura3 can1-100* (5)] was grown in YEPD to  $5 \times 10^6$  cells per milliliter at 25°C. Cells were arrested for 160 min at 25°C with 5 µM α factor (Vetrogen, London, Ontario). Cells were centrifuged, washed with cold YEPD, and reinoculated into fresh YEPD. A sample was taken at this point and every following 20 min and analyzed for RNA, kinase activity, DNA content, and budding as previously described (37). Cells were prepared for fluorescence-activated cell sorting (FACS) analysis on a Becton Dickinson FACSCAN as described (5), and results were analyzed with LYSYS II software (Becton Dickinson). Total RNA was isolated from 10 ml of cells, and 7.5 µg was transferred to nylon membrane and probed as described (37). The probes used were a fragment containing the *PCL2* gene from *pAS-PCL2* (20), a fragment corresponding to the exact *CLN2* open reading frame (38), and a 600-bp Eco RI to Hind III fragment of the *ACT1* gene.
  31. For protein immunoblotting of PHO85, 40 µg of lysate was diluted into SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, boiled, and loaded onto a 10% gel. A chemiluminescence system was used for immunoblot detection (DuPont). PHO85 antiserum was diluted 1:1000; secondary antibodies conjugated to horseradish peroxidase (Bio-Rad) were diluted 1:10,000.
  32. The strains used to construct the quadruple mutant were BY376, *a cln1ΔTRP1 cln2ΔLEU2 pcl2ΔLYS2 his3Δ200 lys2-801<sup>a</sup> ade2-107<sup>o</sup>*; and BY377, *α cln1ΔTRP1 pcl2ΔLYS2 hcs26ΔHIS3 leu2-Δ1 lys2-801<sup>a</sup> ade2-107<sup>o</sup>*. These strains are isogenic to strain BY263 (28). Construction of the *cln1ΔTRP1* and *hcs26ΔHIS3* alleles [(14); J. Ogas, thesis, University of California, San Francisco, CA (1992)] and the *cln2ΔLEU2* allele has been described (38). The *pcl2ΔLYS2* allele was constructed by PCR as described (28). To rescue the quadruple mutant, we transformed plasmid *pGAL-CLN1-URA3-LEU2* [YCPG3 (38)] into strains BY376 and BY377. The transformants were mated, and the quadruple knockout was recovered by dissecting tetrads on galactose medium. BY472A and BY472B are two independent quadruple mutants rescued from this cross. To examine the arrest phenotype, we grew cultures to a density of approximately  $1 \times 10^7$  cells per milliliter in synthetic galactose (SG) medium. A logarithmic phase sample was removed, and 2% glucose was added to the culture to repress expression of *GAL-CLN1*. Samples were taken at the indicated time points (Fig. 4), fixed, and stained as described (30) for FACS analysis. Cells were photographed with Kodak Technical Pan film with a Nikon Microphot FXA microscope equipped with Nomarski optics.
  33. B. Andrews and V. Measday, unpublished data.
  34. The strains used to construct the triple *pho85Δ cln1Δ cln2Δ* mutant were BY442, *a cln2ΔURA3 pho85ΔLEU2 trp1Δ63 lys2-801<sup>a</sup> ade2-107<sup>o</sup> his3Δ200*; and BY438, *α cln1ΔTRP1 cln2ΔURA3 lys2-801<sup>a</sup> ade2-107<sup>o</sup> his3Δ200 leu2-Δ1*. The *cln2ΔURA3* allele is a complete open reading frame deletion of *CLN2* (M. Tyers, unpublished data). The strains were transformed with plasmid *pGAL-PHO85-HIS3*, and the triple mutant was rescued as described (32). The *GAL-PHO85* plasmid was constructed by insertion of a fragment carrying the *PHO85* cDNA from plasmid pSY854 into a *GAL* promoter derivative of pRS313 [R. Sikorski and P. Hieter, *Genetics* **122**, 19 (1989)].
  35. V. Measday, unpublished data.
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  38. J. Hadwiger *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 6255 (1989).
  39. I. Wilson *et al.*, *Cell* **37**, 767 (1984).
  40. We thank H. Espinoza, D. Morgan, and E. O'Shea for discussing unpublished results; E. O'Shea for providing PHO4; B. Dietrich for the pE719-HMK vector; A. Spence for help with microscopy and photography; C. Smith for help with FACS; A. Toh-e for *PHO85* plasmids; S. Elledge for the yeast cDNA library; A. Nieman for helpful discussions; and L. Harrington, A. Spence, and M. Donoviel for comments on the manuscript. V.M. holds a University of Toronto Open Fellowship. J.O. was supported by a fellowship from the Ford Foundation. Supported by grants from the National Cancer Institute (NCI) of Canada with funds from the Canadian Cancer Society and the Terry Fox Run to B.J.A. and M.T. B.J.A. is a scholar of the Medical Research Council of Canada and M.T. is a NCI of Canada Research Scientist.

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## Lymphotactin: A Cytokine That Represents a New Class of Chemokine

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In this study, the cytokine-producing profile of progenitor T cells (pro-T cells) was determined. During screening of a complementary DNA library generated from activated mouse pro-T cells, a cytokine designated lymphotactin was discovered. Lymphotactin is similar to members of both the Cys-Cys and Cys-X-Cys chemokine families but lacks two of the four cysteine residues that are characteristic of the chemokines. Lymphotactin is also expressed in activated CD8<sup>+</sup> T cells and CD4<sup>+</sup>CD8<sup>+</sup> T cell receptor αβ<sup>+</sup> thymocytes. It has chemotactic activity for lymphocytes but not for monocytes or neutrophils. The gene encoding lymphotactin maps to chromosome one. Taken together, these observations suggest that lymphotactin represents a novel addition to the chemokine superfamily.

The pro-T cell is an immature thymocyte subset that is likely to be the final differentiation stage before the onset of T cell receptor (TCR) β chain gene rearrangement (1, 2). These cells are phenotypically characterized by the surface expression of both CD25 and CD44 and by lack of surface expression of CD3, CD4, and CD8. Pro-T cells can produce high titers of interleukin-2 (IL-2), tumor necrosis factor α (TNF-α), and interferon γ (IFN-γ) when activated in vitro with phorbol 12-myristate 13-acetate (PMA), calcium ionophore A23187, and IL-1 (3). We screened a complementary DNA (cDNA) library generated from activated pro-T cells in an attempt to identify new cytokines. Here we describe such a cytokine, which has chemotactic activities that appear to be specific for lymphocytes.

To characterize the cytokine-producing potential of pro-T cells, both in vitro acti-

vated and freshly sorted pro-T cells were analyzed by polymerase chain reaction (PCR). Pro-T cells activated in vitro with PMA, A23187, and IL-1 produced mRNA for IL-2, IFN-γ, TNF-α, granulocyte-macrophage colony-stimulating factor (GM-CSF), and both the p35 and p40 chains of IL-12. No mRNA for IL-4 or IL-10 was detected. Similarly, freshly sorted pro-T cells were shown to contain mRNA for IL-2, IFN-γ, TNF-α, and GM-CSF. Again, no message for IL-4 or IL-10 was detected, and there was no message for either the p35 or p40 chain of IL-12. This common mRNA cytokine profile of freshly sorted pro-T cells and pro-T cells activated in vitro suggests that the pro-T cells are activated in vivo. To further verify the cytokine-producing potential of pro-T cells, a Southern (DNA) blot of an activated pro-T cell cDNA library was probed. IL-2, IL-3, GM-CSF, IFN-γ, and the p40 chain of IL-12 were detected in this library.

During screening of the pro-T cell cDNA library, a clone was isolated (Fig. 1A), the protein translation of which consistently matched a short COOH-terminal segment of Cys-Cys chemokine protein chains in BLAST searches of protein and nucleic acid databases (4). A weaker similarity in this region was also noted with Cys-X-Cys chemokine sequences. Because of its biological activities described below,

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