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Casein Kinase I-Like Protein Kinases Encoded by *YCK1* and *YCK2* Are Required for Yeast Morphogenesis

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Casein kinase I is an acidotropic protein kinase class that is widely distributed among eukaryotic cell types. In the yeast *Saccharomyces cerevisiae*, the casein kinase I isoform encoded by the gene pair *YCK1* and *YCK2* is a 60- to 62-kDa membrane-associated form. The Yck proteins perform functions essential for growth and division; either alone supports growth, but loss of function of both is lethal. We report here that casein kinase I-like activity is associated with a soluble Yck2–β-galactosidase fusion protein in vitro and that thermolabile protein kinase activity is exhibited by a protein encoded by fusion of a temperature-sensitive *yck2* allele with *lacZ*. Cells carrying the *yck2-2*^{ts} allele arrest at restrictive temperature with multiple, elongated buds containing multiple nuclei. This phenotype suggests that the essential functions of the Yck proteins include roles in bud morphogenesis, possibly in control of cell growth polarity, and in cytokinesis or cell separation. Further, a genetic relationship between the *yck2*^{ts} allele and deletion of *CDC55* indicates that the function of Yck phosphorylation may be related to that of protein phosphatase 2A activity.

Casein kinase I (CK I) is the name given to a Ser/Thr protein kinase activity found in all eukaryotic cells. Extensive characterization of CK I activities from a number of cell types has revealed several distinguishing properties of these activities, including specificity for acidic substrates and independence of classical second messengers (reviewed in reference 41). Classical CK I phosphorylation sites have been defined as acidic amino acids upstream of a Ser or Thr residue (41). However, for one isoform, substrates containing the motif Ser/Thr(PO_4)-X-X-Ser/Thr are recognized with high affinity (10–12), suggesting that a phosphorylated residue can provide a necessary recognition determinant. Such substrate specificity in vivo could link the activity of CK I enzymes with activities of other protein kinases in a synergistic hierarchical mechanism, in which a primary phosphorylation event can generate a recognition site for CK I (31). Thus, CK I activity could be recruited into second messenger-dependent phosphorylation cascades.

CK I activities are found in multiple subcellular compartments (41). Nuclear, cytoplasmic, plasma membrane, and microsomal fractions of various cell types have been found to contain CK I activities. Although historically CK I activity has been associated with a single 34- to 37-kDa subunit, molecular sizes associated with CK I activity vary from 34 to 55 kDa between cell types and even between subcellular fractions (41). The hypothesis that these activities could correspond to discretely encoded isoforms has been confirmed by the recent isolation of multiple related cDNA clones from mammalian tissues. At least four closely related isoforms are encoded in bovine brain (37), and additional representatives of these isoforms have been identified subsequently in other vertebrate cell types (4, 16).

Central roles for CK I phosphorylation in cellular metabolism have long been proposed because CK I activities are so widely distributed and because of the potential variety of cellular processes that could be affected by phosphorylation of its many in vitro substrates (41). Recently one isoform of CK I has been localized to the mitotic spindle of vertebrate cells in a cell cycle-dependent manner, suggesting a role(s) in mitosis (4). However, little other evidence exists to establish the identities of in vivo substrates or the biological processes that are regulated by CK I phosphorylation, and analysis at the molecular level in higher eukaryotes is complicated by the existence of multiple genes encoding products with CK I activity.

We and others previously reported the isolation of the *Saccharomyces cerevisiae* gene pair *YCK1* and *YCK2* (previously called *CKI2* and *CKI1*, respectively), which encode proteins with extensive sequence identity that show striking similarity to bovine and rabbit CK I sequences (32, 44). The predicted Yck protein sequences define a novel larger isoform, 60 to 62 kDa, that shows 50 to 55% amino acid identity with each mammalian isoform for which complete sequence data are available. The isolation of genes encoding CK I in *S. cerevisiae* allows the use of molecular genetic approaches to determine biological function. As a first step in the analysis of Yck function, we and others demonstrated a requirement for Yck function in growth and division of *S. cerevisiae* (32, 44), confirming the long-standing hypothesis of the central importance of CK I in these processes.

We report here the direct demonstration of CK I-like protein kinase activity for the *YCK2* gene product and describe the isolation and properties of the temperature-sensitive *yck2-2*^{ts} mutant allele. The phenotype of *yck2-2*^{ts} cells lacking *YCK1* function at the restrictive temperature suggests that the Yck protein kinases are involved in cellular morphogenesis and completion of cell division. The terminal morphology of *yck2*^{ts} mutants at the restrictive temperature closely resembles the morphogenetic defect of mutants lacking Cdc55 function, which appears to be that of the B regulatory subunit of protein phosphatase type 2A (PP2A) (19). Genetic interaction between *cdc55* and *yck2*^{ts} was tested, and results indicate that the combination is lethal at

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TABLE 1. Yeast strains

Strain	Genotype	Reference or source
LRB264	*MATa his3 leu2 ura3-52 yck1-Δ1::URA3*	32
LRB265	*MATα his3 leu2 ura3-52 yck2-1::HIS3*	32
LRB268	*MATa his3 leu2 ura3-52 yck1-Δ1::URA3 yck2-1::HIS3* [pL2.33]	Segregant from LRB264 × LRB265[pL2.33]
LRB273	*MATa his3 leu2 ura3-52 yck1-Δ1 yck2-1::HIS3* [pL2.33]	5-FOA-resistant derivative of LRB268
LRB500	*MATa/MATα his3/his3 leu2/leu2 ura3-52/ura3-52 yck1-Δ1/+ +/yck2-1::HIS3*	Diploid formed by mating LRB265 × LRB273 followed by curing of plasmid
LRB341	*MATa his3 leu2 ura3-52*	K. Tatchell
LRB343	*MATa his3 leu2 ura3-52 yck2-1::HIS3*	Segregant from LRB363 × LRB265
Ts1	*MATa his3 leu2 ura3-52 yck1-Δ1 yck2-2^{ts}*	Derived from LRB445 by transformation
LRB345	*MATa his3 leu2 ura3-52 yck1-Δ1 yck2-2^{ts}*	Segregant from LRB363 × LRB265
LRB346	*MATα his3 leu2 ura3-52 yck1-Δ1 yck2-2^{ts}*	Segregant from LRB363 × LRB265
LRB445	*MATa his3 leu2 ura3-52 yck1-Δ1 yck2-1::HIS3* [pL2.3]	Segregant from LRB500[pL2.3]
AHY86	*MATa his3 leu2 ura3 cdc55::LEU2*	A. Healy
LR862B	*MATa/MATα his3/his3 leu2/leu2-52/ura3-52 cdc55::LEU2/+ +/yck1-Δ1 +/yck2-2^{ts}*	Diploid formed by mating AHY86 × LRB346
LR8611C	*MATa/MATα his3/his3 leu2/leu2 ura3-52/ura3-52 cdc55::LEU2/+ yck1-Δ1/yck1-Δ1 +/yck2-2^{ts}*	Diploid formed by mating segregants of LR862B

temperatures permissive for either single mutant. This finding suggests a functional relationship between CK I phosphorylation and the activity of PP2A in *S. cerevisiae*.

MATERIALS AND METHODS

Strains, DNA, and genetic analysis. Yeast strains are described in Table 1. Medium composition (rich medium, YEP, or defined synthetic medium with amino acid supplements) and standard genetic procedures for *S. cerevisiae* have been described by Sherman et al. (38). 5-Fluoro-orotic acid (5-FOA; PCR Inc.) was added to synthetic medium at 0.1%. Growth temperatures for yeast cells are indicated.

The low-copy-number (centromere) vectors YCp50 (34) and pRS315 (39) and the multiple-copy (2μm) vector YEp352 (20) were used for expression of *YCK2* in yeast cells. Fusion of *YCK2* sequences to the 5′ end of the *lacZ* coding sequence (Fig. 1) was carried out by insertion of the *YCK2 Xba*I-to-*Hin*dIII restriction fragment into the YEp367 (2μm) and YIp367 (integrative) *lacZ* fusion vectors (27). *YCK2* plasmids used in this study are described in Table 2.

Escherichia coli strains used for cloning were XL1blue (5) and RR1 (26). Bacterial culture, $CaCl_2$ transformation, and recombinant DNA manipulations were carried out as described by Maniatis et al. (26). Restriction and modification enzymes for cloning were used according to the manufacturer's recommendations.

DNA sequence analysis was carried out for both strands of the entire coding region of the *yck2-2^{ts}* allele by using the Sequenase modified T7 DNA polymerase with double-stranded templates as recommended by the manufacturer (U.S. Biochemical) (42). *YCK2*-specific oligonucleotides were synthesized at the University of Wisconsin Biotechnology Center.

Morphological analysis. Yeast cells grown to densities of 1×10^7 to 2×10^7 cells per ml were harvested before and after

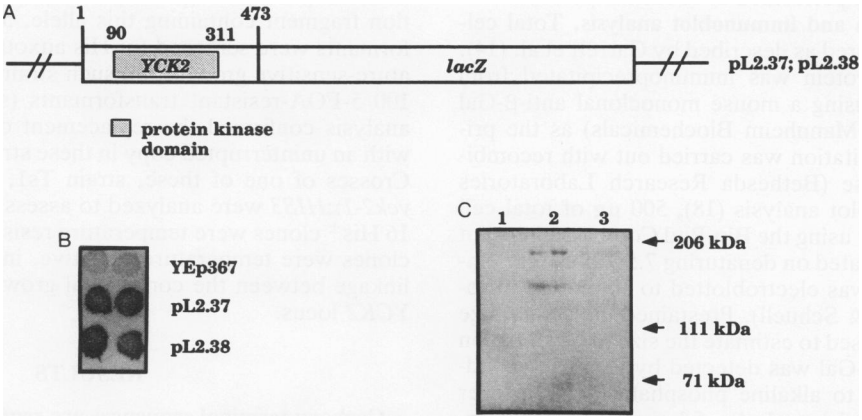

FIG. 1. Expression of Yck2–β-Gal fusion protein. (A) Line drawing of the gene fusion showing the position of the Yck2 protein kinase homology (shaded). Yck2 amino acid numbers are shown above the line drawing. The multicopy and integrative plasmids pL2.37 and pL2.38, respectively (Table 2), contain this gene fusion. (B) Qualitative assay of β-Gal activity for strains with plasmids indicated to the right. (C) Western immunoblot analysis using antibodies directed against β-Gal. Protein extracts were prepared from LRB265 (*YCK1 yck2*) carrying plasmids YEp367 (lane 1), pL2.37 (lane 2), and pL2.38 (lane 3). Arrows to the right indicate migration positions of prestained molecular size markers.

TABLE 2. *YCK2* plasmids[a]

Plasmid	Vector	Relevant functional DNA
pL2.3	YEp352	*URA3*, 2 μm, *YCK2*
pL2.33	pRS315	*LEU2*, CEN6, *ARSH4*, *YCK2*
pL2.33^{ts}	pRS315	*CEN6*, *ARSH4*, *yck2-2^{ts}*
pL2.330	pRS315	*LEU2*, *CEN6*, *ARSH4*, *yck2-2^{ts}*
pL2.331	YEp352	*URA3*, 2 μm, *yck2-2^{ts}*
pL2.37	YEp367	*LEU2*, 2 μm, *YCK2Δ80*
pL2.371	YEp367	*LEU2*, 2 μm, *yck2-2^{ts}Δ80*
pL2.38	YIp367	*LEU2*, *YCK2Δ80*
pL2.6	YEp352	*URA3*, 2 μm, *YCK2Δ80*
pL2.60	YCp50	*URA3*, *CEN4*, *ARS1*, *YCK2Δ80*

[a] Plasmid pLS2.3 was described previously (32). The *YCK2* alleles in plasmids pLS2.37 and pL2.38 are depicted in Fig. 1. *YCK2* and *YCK2Δ80* plasmids were constructed by using restriction endonuclease fragments (*Xba*I and *Sac*I for *YCK2* and *Xba*I or *Eco*RI and *Hin*dIII for *YCK2Δ80*) from pLS2.3. The *Xba*I site lies 620 bp upstream of the *YCK2* coding sequence, the *Hin*dIII site is located at bp 1392 of the *YCK2* coding sequence, and the *Eco*RI and *Sac*I sites lie within vector multiple cloning sites. All *yck2-2^{ts}* plasmids were constructed with similar restriction endonuclease fragments from pL2.33^{ts}, which was derived from pL2.33 by in vitro mutagenesis (Materials and Methods).

a shift in incubation temperature from 23 to 37°C. Cells were fixed with formaldehyde in culture medium as described by Pringle et al. (28). Staining of cells for filamentous actin structures with rhodamine-conjugated phalloidin (Molecular Probes, Inc.) and for chitin deposition with Fluorescent Brightener 28 (Calcofluor; Sigma) have been described by Pringle et al. (28).

Staining with 4′,6-diamidino-2-phenylindole (DAPI; Boehringer Mannheim Biochemicals) to visualize nuclear material was carried out after ethanol fixation (28), and cells were mounted in 1 mM β-mercaptoethanol. Cells were observed and photographed under differential interference contrast and fluorescence, using a Nikon epifluorescence microscope and camera attachment.

β-Gal assay. Qualitative determination of β-galactosidase (β-Gal) activity was carried out with cells grown on nitrocellulose filters placed on synthetic media (3). Cells expressing wild-type Yck2–β-Gal fusion protein were grown at 30°C, and cells expressing yck2-2^{ts}–β-Gal were grown at 23°C and shifted to 37°C 4 h prior to assay. Reaction of cellular β-Gal with 5-chloro-4-bromo-3-indolyl-β-D-galactopyranoside (X-Gal; BiosynthAG) was performed at room temperature.

Immunoprecipitation and immunoblot analysis. Total cellular protein was prepared as described by Garrett et al. (14). Yck2–β-Gal fusion protein was immunoprecipitated from total protein (14) by using a mouse monoclonal anti-β-Gal antibody (Boehringer Mannheim Biochemicals) as the primary antibody. Precipitation was carried out with recombinant protein G-agarose (Bethesda Research Laboratories [BRL]). For immunoblot analysis (18), 500 μg of total cell protein (determined by using the Bio-Rad Coomassie reagent microassay) was separated on denaturing 7.5% polyacrylamide gels, and protein was electroblotted to supported nitrocellulose (Schleicher & Schuell). Prestained molecular size markers (BRL) were used to estimate the size of Yck2 fusion protein. Bound anti-β-Gal was detected by using a secondary antibody coupled to alkaline phosphatase (Boehringer Mannheim Biochemicals) and the 5-bromo-4-chloro-3 indolylphosphate toluidinium–nitroblue tetrazolium color reagent system (BRL).

In vitro phosphorylation assays. For immune complex phosphorylation assays (14), fusion protein immunoprecipitated from 100 to 500 μg of total cellular protein was incubated in the reaction mixture for 5 or 10 min at 23 or 37°C, and phosphorylated products were separated on 10% polyacrylamide gels. For qualitative analysis of phosphorylated products, autoradiography was carried out by using Kodak X-Omat film without signal amplification. For quantitative comparisons of incorporation, counts incorporated into the two major bands corresponding to casein were determined by using a Betagen Betascope. Values obtained after 60 min were adjusted for background values. Linear incorporation of label occurs over this time period (data not shown). EDTA (1 mM) and substrate protein (Sigma) at 80 to 2,000 μg/ml were included as indicated.

Phosphorylation of the CK I-specific synthetic peptide substrate DDDDVASLPGLRRR (D4 peptide [12]; kindly provided by P. Roach) by Yck2–β-Gal fusion protein was carried out by using a P81 (Whatman) filter binding assay (10) with fusion protein immunoprecipitated from 200 μg of total cell protein. Following termination of the reactions, agarose-bound immune complexes were pelleted and supernatants were removed for application to P81 filters. The filters were then placed in Bio-Safe II cocktail (RPI, Inc.) and counted in a Packard Tri-Carb scintillation counter. All reactions were carried out in duplicate.

Isolation of the *yck2-2^{ts}* allele and construction of the *yck^{ts}* mutant. The host strain LRB445 (Table 1) carries null alleles at the *YCK1* and *YCK2* chromosomal loci but remains viable because of the presence of the *YCK2 URA3* plasmid pL2.3. The *YCK2 LEU2* plasmid pL2.33 was mutagenized with hydroxylamine (34), and mutagenized plasmid pools were introduced into LRB445. Leucine prototrophs were screened for conditional growth, with a permissive temperature of 23°C and a restrictive temperature of 36°C, on 5-FOA medium, which does not permit growth of uracil prototrophs (2). Transformants showing conditional growth on 5-FOA medium were tested for temperature-conditional growth on all media after mitotic loss of pLS2.3 at 23°C. Of 5,000 Leu$^+$ transformant colonies screened, a single transformant showed the conditional growth phenotype in the second screen, and plasmid pL2.33^{ts} was recovered from genomic DNA prepared (45) from this strain. We refer to the allele of *YCK2* in pL2.33^{ts} as *yck2-2^{ts}*.

To obtain strains with the *yck2-2^{ts}* allele as the only functional *YCK* copy, the chromosomal *yck2-1::HIS3* allele was replaced with the *yck2-2^{ts}* allele by transforming strain LRB445 (*yck1 yck2* [pL2.3]; Table 1) with a purified restriction fragment containing this allele. 5-FOA-resistant transformants were screened for His auxotrophy and for temperature-sensitive growth. Six such strains were obtained from 100 5-FOA-resistant transformants (see Fig. 5). Southern analysis confirmed the replacement of the disrupted allele with an uninterrupted copy in these strains (data not shown). Crosses of one of these, strain Ts1, with strains carrying *yck2-1::HIS3* were analyzed to assess genetic linkage; 16 of 16 His$^+$ clones were temperature resistant and 20 of 20 His$^-$ clones were temperature sensitive, indicating close genetic linkage between the conditional growth phenotype and the *YCK2* locus.

RESULTS

Carboxy-terminal sequences are required for Yck function. To facilitate study of the properties of the Yck2 protein, a fusion was constructed between the first 467 codons of *YCK2* and the entire *lacZ* gene (Materials and Methods; Fig. 1; Table 2). Although the last 80 codons of *YCK2* are not present in this construction, it contains the entire predicted

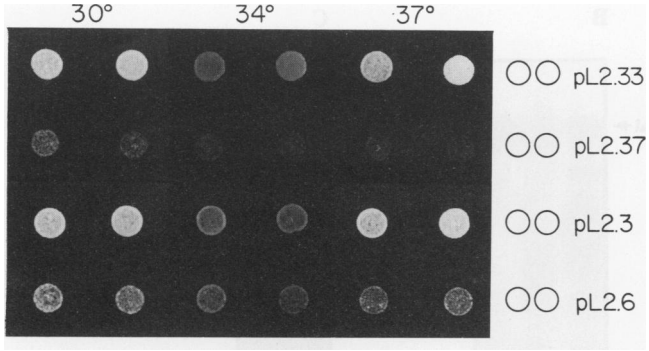

FIG. 2. Growth of strains carrying a truncated *YCK2* allele as the sole functional *YCK* gene. Panels show growth of *yck1 yck2* strains that remain viable as a result of the presence of the plasmid indicated to the right on YEP-glucose plates incubated at the temperatures indicated. Cells were grown to a density of approximately 5×10^7/ml at 30°C, and 5 μl of a 10^{-3} dilution was dropped to yield each spot. Photographs were taken after incubation for 36 h.

protein kinase domain. Strains expressing this fusion from *YCK2* promoter sequences display β-Gal activity in qualitative X-Gal filter assays (Fig. 1). Immunoblots of total yeast protein probed with antibodies directed against β-Gal confirm the production of a fusion protein with an approximate molecular size of 160 kDa only in strains expressing the *YCK2-lacZ* fusion (Fig. 1). An additional, less intense band that comigrated with purified β-Gal was also detected (Fig. 1). Strains expressing *YCK2-lacZ* from a single chromosomal copy (pL2.38) display less β-Gal activity and a smaller amount of 160-kDa protein than do cells carrying the *YCK2-lacZ* fusion in multiple copy (pL2.37; Fig. 1).

The Yck2 fusion protein was tested for biological activity by determining its ability to complement the lethality of *yck1-Δ1 yck2-1::HIS3* (*yck1 yck2*) double-mutant strains. Both multiple-copy (pL2.37) and integrative (pL2.38) fusion plasmids were introduced into the *yck1/+ yck2/yck2* diploid strain LRB500 (Table 1), and meiotic progeny of these transformants were analyzed. When expressed from a multiple-copy (2μm) plasmid, the Yck2–β-Gal fusion protein restores growth to strains lacking a functional chromosomal *YCK* gene, although the growth of such strains is slower than that of the same strain carrying wild-type *YCK2* in low copy number (pL2.33; Table 2; Fig. 2). However, meiotic progeny of the diploid strain LRB500 (Table 1) carrying *YCK2-lacZ* integrated at the *LEU2* locus exhibit exclusively 2:2 segregation for viability (36 of 36 tetrads). Thus, although expression of *YCK2-lacZ* from a multiple-copy plasmid supports growth of strains that otherwise lack Yck function, expression from a single copy does not support growth.

Since fusion of β-Gal could affect the properties of Yck2 protein, similar tests of biological function were carried out with plasmids carrying *YCK2* truncated by 80 codons but not fused to *lacZ*. A multiple-copy plasmid carrying the truncated *YCK2Δ80* allele (pL2.6; Table 2) supports better growth of *yck1 yck2* strains than does a plasmid carrying *YCK2-lacZ* (Fig. 2), indicating impairment of Yck function due to the presence of β-Gal. However, in low copy, *YCK2Δ80* (pL2.60) also fails to support growth. All of 21 tetrads from *yck1/+ yck2/yck2* [pL2.60] diploid strains showed 2:2 segregation for viability, although nonviable colonies reached pinprick size before division ceased. Thus, we conclude that carboxy-terminal sequences of Yck2 protein are required for full biological function.

***YCK2* encodes a CK I-like protein kinase.** To assay protein kinase activity for Yck2, in vitro phosphorylation reactions were carried out with immunoprecipitated Yck2–β-Gal fusion protein. The 160-kDa fusion protein was labeled in this reaction, as were added substrates, including glycogen synthase, casein, or phosvitin (Fig. 3A and B). By contrast, neither bovine serum albumin nor enolase was labeled (data not shown). The phosphorylating activity in immunoprecipitates was competed for by including purified β-Gal in the immunoprecipitation step (Fig. 3A), and no activity was observed when immunoprecipitated protein was from strains not expressing the fusion protein (data not shown). The phosphorylating activity in these reactions resembles that of CK I with regard to requirement for Mg^{2+} (Fig. 3B) (41) and partial inhibition by sodium fluoride (Fig. 3C) (41). We also observed that Mg^{2+} was inefficiently replaced in this assay system by either Ca^{2+} or Mn^{2+} and that Zn^{2+} inhibited labeling even in the presence of an equal amount of Mg^{2+} (data not shown).

The properties of Yck2–β-Gal are similar to those reported for CK I except that the inhibitor CKI 7 (7) failed to inhibit Yck–β-Gal activity significantly in these reactions (data not shown). For a conclusive demonstration that Yck activity is like that of CK I, immunoprecipitated Yck fusion protein was assayed for its ability to phosphorylate a peptide substrate that is specifically recognized by CK I (12). We found that labeled phosphate is incorporated into the peptide substrate in the presence of Yck fusion protein (Fig. 4), indicating that it shares substrate specificity with CK I proteins.

A *YCK2* temperature-sensitive allele results in thermolabile protein kinase activity in vitro. To study the function of the Yck proteins, a temperature-sensitive allele of *YCK2* was generated in vitro (Materials and Methods). A restriction fragment containing the *yck2-2^{ts}* allele was transferred from pL2.33^{ts} to both multiple-copy (2μm; pL2.331) and low-copy-number (CEN; pL2.330) vectors. Each plasmid supported temperature-sensitive growth of *yck1 yck2* double-mutant strains. The *yck2-2^{ts}* allele was transferred to the *YCK2* chromosomal locus in strain LRB445 (Materials and Methods) to study the properties of cells carrying this allele as the sole functional *YCK* copy, and these strains were also temperature sensitive for growth with a restrictive temperature of 35°C.

To determine whether the conditional growth phenotype conferred by *yck2-2^{ts}* could be overcome by an increase in gene dosage, the multiple-copy and low-copy-number *yck2-2^{ts}* plasmids pL2.331 and pL2.330 (Table 2), respectively, were introduced into the *yck1 yck2-2^{ts}* (*yck^{ts}*) strain LRB345 (Table 1). The resulting transformants were tested for growth at 35 and 37°C. Expression of *yck2-2^{ts}* from the multiple-copy plasmid pL2.331 allowed growth at 35°C but not at 37°C (Fig. 5). However, introduction of the low-copy-number plasmid pL2.330 did not raise the minimum temperature at which growth of a chromosomal *yck^{ts}* strain is restricted (Fig. 5). Since the minimum restrictive temperature for growth depends on the dosage of *yck2-2^{ts}*, the mutant protein could retain residual activity at 35°C, at a level that cannot support growth unless the amount of protein expressed is greatly increased.

The complete nucleotide sequence of the *yck2-2^{ts}* allele was determined, and a single alteration was found at nucleotide 558 of the coding region. The resulting amino acid change, at position 187, is His→Tyr. This residue lies within conserved protein kinase subdomain VI and corresponds to His-158 in bovine cyclic AMP-dependent protein kinase α

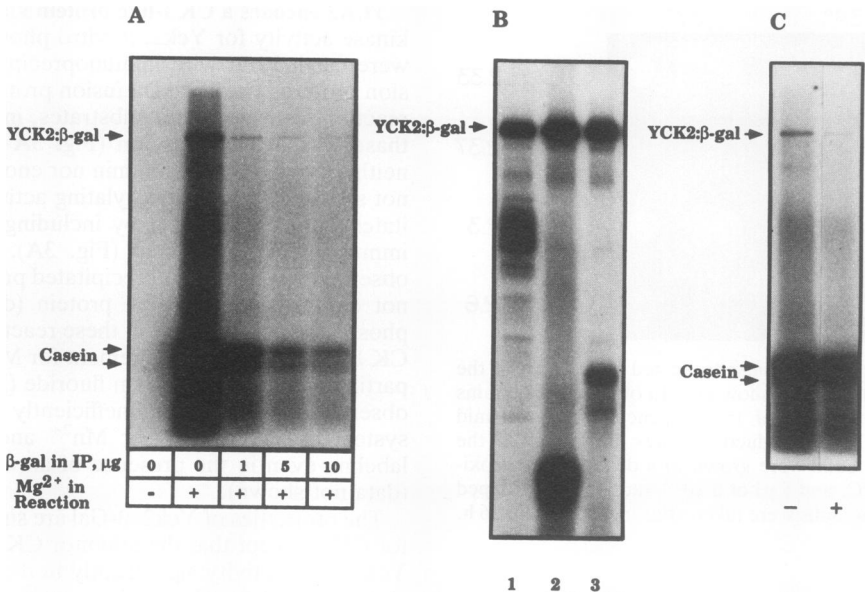

FIG. 3. In vitro demonstration of Yck2–β-Gal protein kinase activity. Shown are autoradiographs of products of phosphorylation assays separated on 10% polyacrylamide gels (Materials and Methods) by using protein immunoprecipitated from strain LRB343 (*YCK1 yck2*) carrying YEp:*YCK2-lacZ* (pL2.37). (A) Fusion protein was immunoprecipitated (IP) from 500 μg of total cell protein with or without addition of purified β-Gal as indicated at the bottom. All reactions were carried out in the presence of casein at 2 mg/ml and EDTA at 1 mM; 5 mM $MgCl_2$ was added as indicated at the bottom. (B) Fusion protein was immunoprecipitated from 200 μg of total cell protein, and reactions were carried out in the presence of substrate as follows: lane 1, glycogen synthase, 120 μg/ml; lane 2, casein, 80 μg/ml; and lane 3, phosvitin, 80 μg/ml. (C) Fusion protein was immunoprecipitated from 200 μg of total cell protein and incubated with casein at 2 mg/ml in the absence (−) or presence (+) of 25 mM NaF.

(cAPKα; 17). All CK I species described, as well as most other Ser/Thr protein kinase types, contain His at this position (17, 37). This residue lies within a region that contains residues involved in catalysis and in substrate recognition (23).

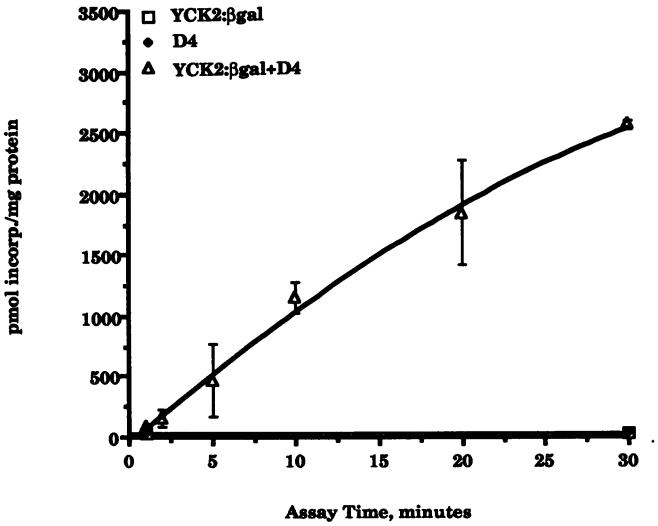

FIG. 4. Phosphorylation of CK I-specific peptide substrate by Yck2 fusion protein. Fusion protein was immunoprecipitated from 200 μg of total cell protein extracted from strain LRB343 (*YCK1 yck2*) containing pL2.37 (*YCK2-lacZ*). Peptide phosphorylation assays (Materials and Methods) were carried out with D4 peptide at 1 mM. Values for incorporation of labeled phosphate into D4 peptide were calculated on the basis of 60% counting efficiency.

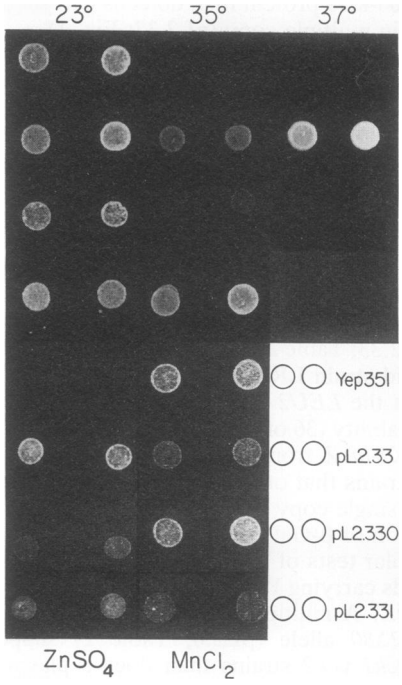

FIG. 5. Phenotype of *yck2-2*^{ts} strains. The plasmids indicated (lower right) were introduced into the *yck*^{ts} strain LRB346 (Table 1), and 5-μl drops of log-phase cultures (approximately 5×10^6 cells per ml) were spotted onto synthetic selective medium incubated at the indicated temperatures as well as onto YEP-glucose containing 17.5 mM $MnCl_2$ and synthetic medium containing 10 mM $ZnSO_4$ incubated at 30°C. Plates were incubated 48 h before photographs were taken.

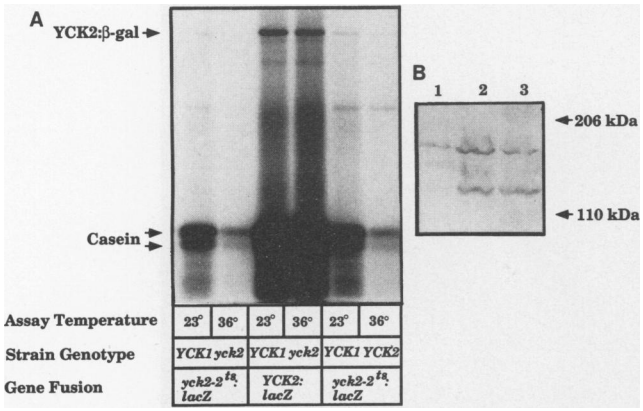

FIG. 6. Temperature-sensitive protein kinase activity of the yck2-2^{ts} fusion protein. (A) Autoradiograph of phosphorylation assay products separated on a 10% polyacrylamide gel (Materials and Methods), with assay conditions for each lane given at the bottom. In each case, fusion protein was immunoprecipitated from 500 μg of total cell protein extracted from cells grown at 23°C. The extra band in the yck2-2^{ts} lanes is seen only for protein expressed from this construction, and the extra bands seen in the wild-type fusion lanes are not present in the yck2-2^{ts} lanes. (B) Western immunoblot analysis of protein extracts prepared from LRB265 (*YCK1 yck2*) carrying pL2.37 (*YCK2-lacZ*) (lane 1) or pL2.371 (*yck2-2^{ts}-lacZ*) (lanes 2 and 3). The protein extract in lane 2 was prepared from cells grown at 23°C, while protein for lane 3 was extracted from cells shifted to 36°C 4 h prior to harvesting.

The location of the *yck2-2^{ts}* conditional allele within the protein kinase catalytic core allowed us to examine the activity of the mutant protein by using a β-Gal fusion. A restriction fragment containing the *yck2-2^{ts}* allele was used to construct a gene fusion with *lacZ* as described for wild-type *YCK2*. Immunoblot analysis was performed by using protein prepared from *YCK$^+$* strains carrying plasmid YEp:*yck2-2^{ts}-lacZ* (pL2.371; Table 2). We found comparable levels of fusion protein in extracts from such strains that were incubated at either 23 or 37°C prior to lysis (Fig. 6). However, the in vitro protein kinase activity of the yck2-2^{ts} fusion protein is thermolabile. Yck2 and yck2-2^{ts} fusion proteins were isolated from *YCK$^+$* strains grown at 23°C, and each was assayed by using casein as a substrate at 23 and 36°C. Wild-type Yck2 fusion protein showed comparable activity at both temperatures (Fig. 6). The activity of the yck2-2^{ts} fusion protein was lower than that of the wild type at 23°C, and at 36°C a further reduction of activity was observed (Fig. 6).

The extent of thermolability of the activity of the mutant protein was quantitated by measuring incorporation of label into casein. The label incorporated into casein in reactions with yck2-2^{ts} fusion protein at 36°C is reduced from that at 23°C by at least 40% (Table 3). Taken together, these results favor the conclusion that the Yck2 fusion protein, rather than a coimmunoprecipitating protein, is responsible for protein kinase activity in these assays. In addition, the conditional growth of *yck^{ts}* cells appears to be due to thermosensitivity of Yck activity.

We examined both the time required for loss of protein kinase activity of the yck2-2^{ts} fusion protein at 36°C and whether the loss of activity is reversible. yck2-2^{ts} fusion protein immunoprecipitated from cells grown at 23°C was assayed at 36°C with and without a preincubation step at 36°C prior to addition of assay components. Incubation of

TABLE 3. Loss of activity of yck2-2^{ts} fusion protein in phosphorylation assays[a]

Preincubation		Assay temp (°C)	pmol of phosphate incorporated/mg of protein[b]
Time (min)	Temp (°C)		
0		24	2.40
0		36	1.16
5	36	36	0.64
10	36	36	0.58
5	36	24	2.10
5, 5	36, 24	24	1.74

[a] Protein was extracted from strain LRB343 (*YCK1 yck2*) carrying pL2.371 (*yck2-2^{ts}-lacZ*) that was grown at 23°C in synthetic selective medium, and fusion protein was immunoprecipitated from 200 μg of total cell protein. Preincubation steps were carried out before addition of assay components. Results are average values from two experiments in which individual values differed by less than 15%.

[b] Calculated assuming 100% counting efficiency and the amount of cell protein from which fusion protein was immunoprecipitated.

yck2-2^{ts} fusion protein at 36°C for 5 min prior to assay at 36°C resulted in 75% activity loss (Table 3). Extension of the preincubation step to 10 min did not result in further reduction of activity (Table 3). The loss of activity is reversible. Fusion protein was preincubated at 36°C for 5 min and either assayed at 24°C or shifted to 24°C for 5 min prior to assay at the same temperature. In each case, activity was at least 75% of that of yck2-2^{ts} fusion protein assayed at 24°C without preincubation at 36°C (Table 3).

Strains lacking Yck activity exhibit cell cycle-related defects. The terminal phenotype of *yck^{ts}* strains arrested at the restrictive temperature was examined microscopically for cells of strain LRB345 (Table 1) at several time points after a shift to 37°C. Although cell division, as measured by separable cells, terminates rapidly at the restrictive temperature, bud initiation, bud growth, and nuclear division continue in these cells for up to 24 h. However, by 2 h after a shift to the restrictive temperature, new buds appear elongated rather than round (Fig. 7). As depicted in Table 4, the proportion of morphologically normal cells in a population shifted to the restrictive temperature decreases rapidly. While 97% of the cells in this population appeared normal prior to the shift, less than 43% appeared normal after 3 h at the restrictive temperature.

Over the next 20 to 24 h, abnormal buds continue to elongate, and initiation of abnormal buds continues, from the mother cell adjacent to the first abnormal bud or at the tip of the elongated bud. By 11 h at 37°C, less than 10% of the cells in this population are normal in appearance. The proportion of cells with a single elongated bud increases at earlier time points but decreases in parallel with an increase in cells with multiple elongated buds (Table 4). The morphology of the latter cells resembles the terminal morphology described previously for nonviable *yck1 yck2* double-mutant cells (31).

Although morphogenesis is clearly defective in *yck^{ts}* cells at the restrictive temperature, nuclear division continues. Multiple DAPI-stained foci are visible both in elongating buds and in mother cells examined 8 h after a shift to 37°C (Fig. 8). Flow cytometry analysis of DNA content confirmed that the DAPI-stained foci represent intact nuclei, since >60% of cells incubated at the restrictive temperature for 8 h contain 4 N or greater DNA content (data not shown).

Although multiple nuclei are present in the elongated buds, continuity of background fluorescence from DAPI stain and continuity of cell wall material indicate that nuclei

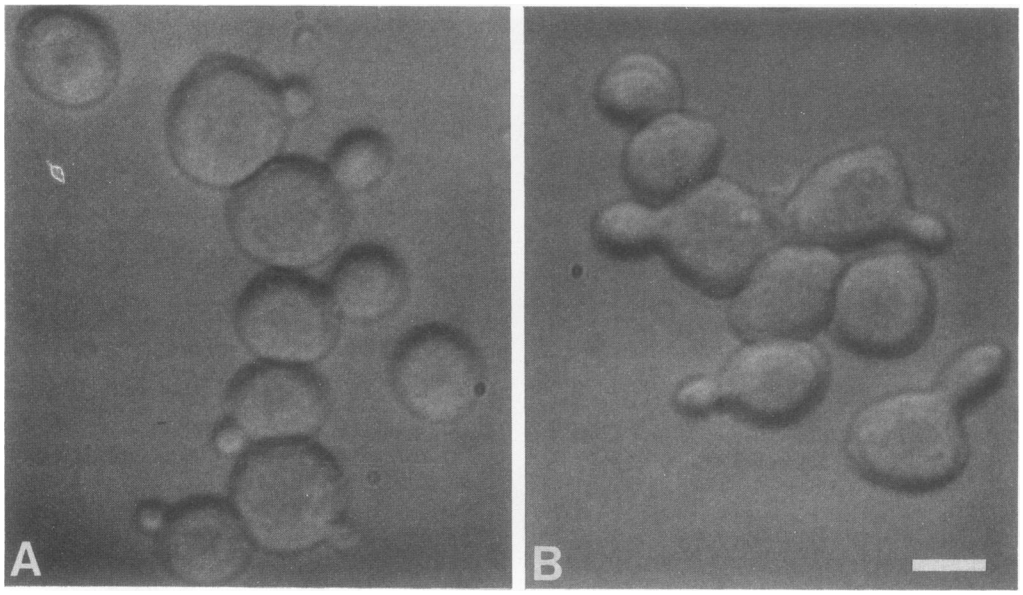

FIG. 7. Early morphogenesis defect due to the *yck2-2*^{ts} mutation. Shown are differential interference contrast images of wild-type cells (A; strain LRB341) and *yck*^{ts} cells (B; strain LRB345) incubated for 2 h at 37°C after growth to log phase at 23°C. The bar corresponds to 2 μm.

are generally within a continuous cellular compartment. Moreover, in most cases, mother cell and elongated bud cannot be micromanipulated apart, nor can parts of an elongated bud be separated at obvious constrictions. These observations suggest that multiple rounds of aberrant budding without cytokinesis or cell separation occur at the restrictive temperature in *yck*^{ts} cells.

The morphological abnormalities of *yck*^{ts} cells are reminiscent of a number of cell division cycle mutants defective in morphogenesis and cytokinesis. The *cdc3*, *cdc10*, *cdc11*, and *cdc12* mutants fail to localize the Cdc3 group of proteins to a ring at the site of bud emergence, and multiple elongated buds result (6, 13, 22). These mutants also fail to form rings of cell wall chitin at the presumptive bud site (13, 22). The *cdc55* mutant and functionally related mutants (19, 33, 43) are also defective in bud morphogenesis and cytokinesis or cell separation, but both the Cdc3 group of proteins and chitin rings are present at the site of bud emergence. In addition, elements of the actin cytoskeleton appear normal in these mutants (19). To determine which mutants *yck*^{ts} cells resemble most closely, actin distribution and cell wall chitin were examined in cells of strain LRB345 (*yck*^{ts}) incubated for 8 h at 37°C. The distribution of filamentous actin in these cells was examined by staining with rhodam-

TABLE 4. Time course of accumulation of abnormal *yck*^{ts} cells at the restrictive temperature[a]

Time after shift (hours)	%Normal cells		% Abnormal cells				Total Cell #
	Unbudded	Budded	Multiple buds	Elongated single bud	Elongated multiple buds	Elongated unbudded	
0	15.4	81.7	2.3	0.3	0	0.3	306
3	25.4	17.5	3.9	37.3	10.5	5.3	228
4	20.9	13.3	3.6	30.5	26.5	5.2	249
6	7.5	5.1	3.5	28.4	50.0	5.5	254
11	5.3	4.2	0	15.9	67.8	6.8	264

[a] Cells (LRB343) were grown in YEPD to log phase at 23°C and then shifted to 37°C, and samples were examined by phase microscope at the indicated times after vigorous vortexing. The numbers shown are from a single representative experiment.

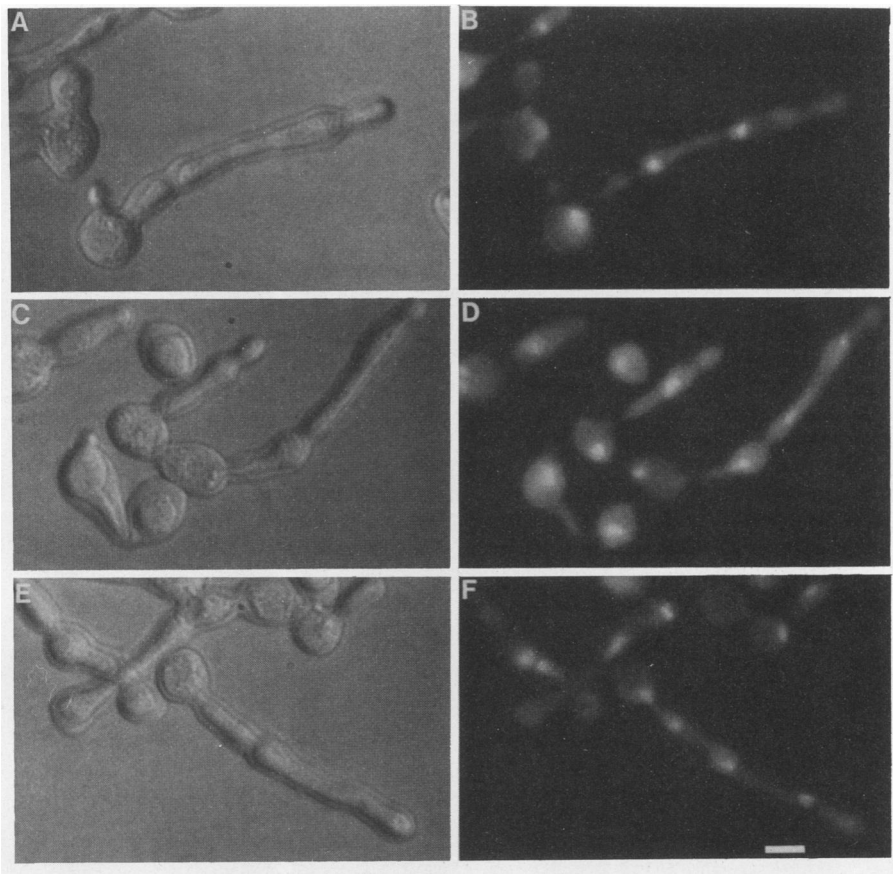

FIG. 8. Nuclear staining of *yck*^{ts} mutant cells. Cells of strain LRB345 were incubated for 8 h at 37°C after growth to log phase at 23°C. Fluorescent images of DAPI-stained cells (B, D, and F) are shown adjacent to corresponding differential interference contrast images (A, C, and E). The bar corresponds to 2 μm.

ine-labeled phalloidin. The major actin cytoskeletal structures in yeast cells are cortical patches most abundant in growing buds and cables extending from mother cells into buds (1, 9, 21). Both structures were observed in *yck*^{ts} cells at the restrictive temperature, although in general both were more abundant than in wild-type cells (Fig. 9).

Chitin deposition in *yck*^{ts} cells at the restrictive temperature was examined by using Calcofluor staining. For wild-type cells, staining is observed almost exclusively in rings formed at the sites of bud emergence. The *yck*^{ts} cells stain intensely with Calcofluor, indicating that chitin deposition is delocalized (Fig. 9). However, bud tips do not stain, indicating that chitin deposition is normal during bud emergence and growth. Rings of staining are observed at junctions of mother cells and buds as well as at sites of constriction along buds (seen most clearly in Fig. 9D and E and also in Fig. 9G and H), although these rings are broader than those seen for wild-type or *cdc55* cells. Thus, the *yck*^{ts} phenotype is similar to the abnormal morphology displayed at low temperature by strains mutant in the *CDC55* gene (19). The major difference in phenotype is the degree of abnormality; the cytokinesis/cell separation defect is more severe in *yck*^{ts} cells than in *cdc55* cells.

Cdc55 and Yck activities may be functionally related. Because of the similar terminal morphologies of strains carrying *cdc55* or *yck2-2*^{ts}, we examined the possibility of genetic interaction between the two mutations. Diploid strains homozygous for *yck1* and heterozygous for *cdc55*:: *LEU2* and *yck2-2*^{ts} were sporulated, and tetrads were dissected. Cells were incubated either at 30°C, the temperature permissive for *cdc55* mutants and semipermissive for *yck*^{ts} cells, or at 23°C, the temperature permissive for *yck*^{ts} cells but semipermissive for *cdc55* cells. In each case, semipermissive indicates that some morphological abnormality is evident under these conditions but that macroscopic growth is observed.

Figure 10A shows the results of 6 days of incubation at 30°C for progeny of one such diploid strain (LR8611C; Table 1). The large colonies are genotypically *CDC55 yck1 YCK2*, whereas smaller colonies are either *cdc55 yck1 YCK2* or *CDC55 yck1 yck2-2*^{ts}. The remainder are nongrowing clones or clones that form pinprick colonies but are not viable upon reculture. We infer from the genotypes of survivors that the nonviable clones correspond to triple-mutant cells. Figure 10B shows micrographs of nonviable meiotic progeny, demonstrating that the *cdc55 yck*^{ts} strains incubated at an intermediate temperature closely resemble either mutant parent at its restrictive temperature. During a second week at 30°C, sibling colonies continued to grow but no further division was observed for the predicted triple mutants. Similar results were obtained with tetrads incubated at 23°C except that the *cdc55 YCK2*$^{+}$ mutant progeny grew very poorly at this temperature. This pattern of viability, seen for 18 of 18 tetrads from diploids of this genotype, is consistent with the idea that loss of *CDC55* function is lethal in combination with decreased Yck activity.

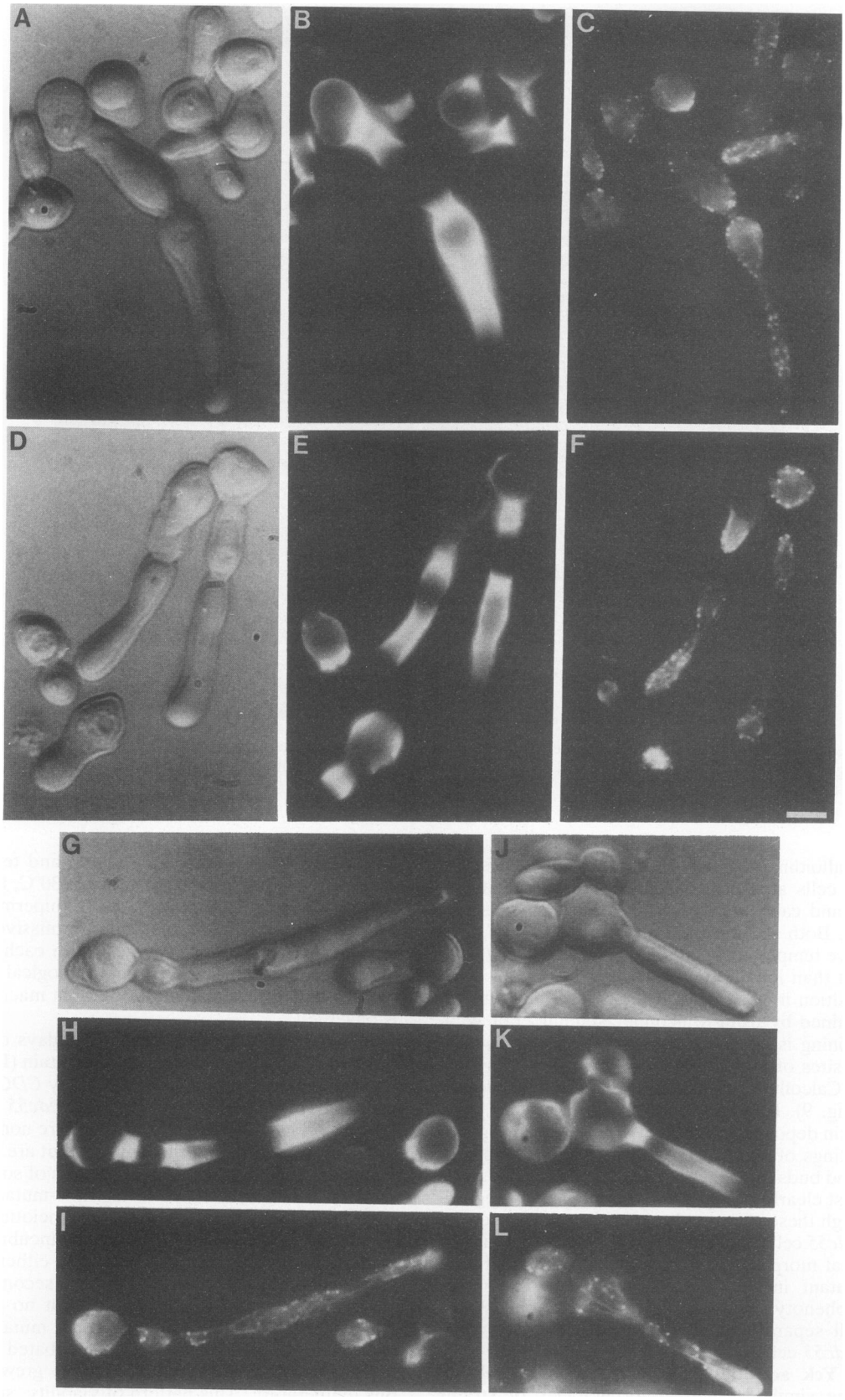

FIG. 9. Terminal phenotype of *yck*^{ts} mutant cells. Cells of strain LRB345 were incubated for 8 h at 37°C after growth to log phase at 23°C. Shown are rhodamine-conjugated phalloidin fluorescence (C, F, I, and L) and Calcofluor (B, E, H, and K) images with corresponding differential interference contrast images (A, D, G, and J). The bar corresponds to 2 μm.

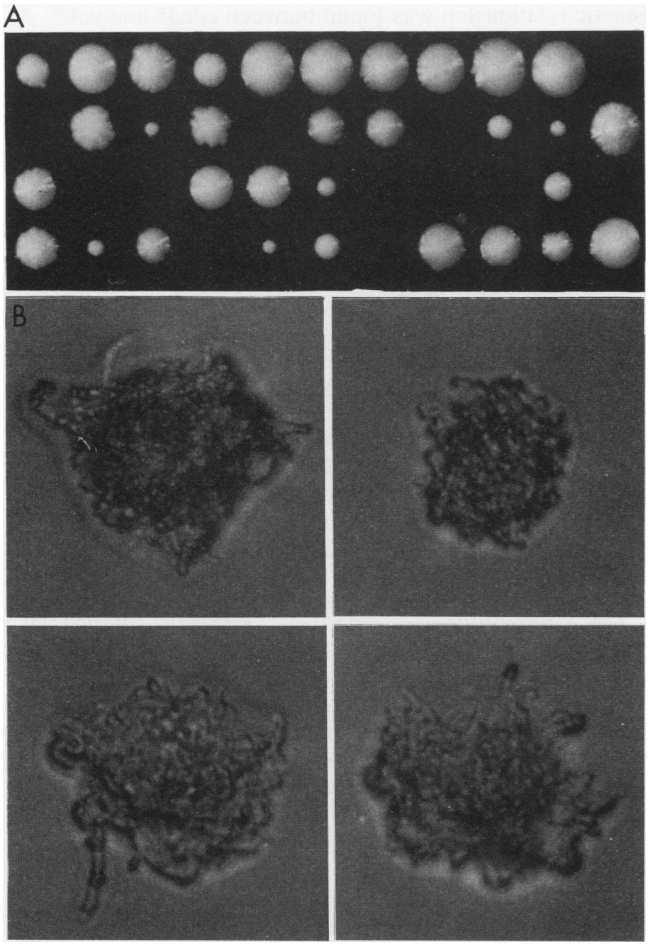

FIG. 10. Evidence that *yck2-2^{ts}* shows synthetic lethality with *cdc55* deletion. (A) Tetrads from the diploid strain LR8611C (*yck1/yck1 yck2-2^{ts}/+ +/cdc55*) incubated for 6 days at 30°C; (B) micrographs of cells at positions devoid of colonies in panel A. Final magnification in lower panels is ×680.

To determine whether the *cdc55* phenotype was affected by altering the dosage of wild-type *YCK*, the diploid strain LRB8611C (described above) was transformed with a multiple-copy *YCK1* or *YCK2* plasmid. Transformants with each plasmid were sporulated for tetrad analysis. Both *cdc55* and *cdc55 yck^{ts}* strains carrying either plasmid were recovered, and all were identical to *cdc55 YCK$^+$* strains lacking *YCK* plasmids with regard to restrictive temperature as well as terminal morphology (data not shown). Thus, increased dosage of *YCK* does not alleviate defects due to loss of *CDC55* function.

Additional traits of the *yck2-2^{ts}* mutant. Since the *YCK2* gene was identified because increased dosage of this gene confers resistance to NaCl (33), the *yck^{ts}* mutant strains were tested for ion-related growth traits. No effect of the *yck2-2^{ts}* mutation was seen on NaCl sensitivity (data not shown). However, compared with related wild-type strains, *yck^{ts}* strains are hypersensitive to Zn^{2+}, since they fail to grow on media containing $ZnSO_4$ at 10 mM. Also, the *yck^{ts}* strains, unlike related wild-type strains, grow in the presence of 17 to 25 mM $MnCl_2$ (Fig. 5). Hypersensitivity to Zn^{2+} and resistance to Mn^{2+} cosegregate meiotically with temperature-sensitive growth, showing that these traits are conferred by the *yck2-2^{ts}* mutation. Also, both defects are corrected by introduction of a wild-type *YCK1* or *YCK2* plasmid (Fig. 5 and data not shown). Both growth defects are associated with loss of Yck activity rather than specifically with *yck2-2^{ts}*, since *yck1 yck2* strains that remain viable as a result of the presence of the truncated wild-type *YCK2Δ80* allele (pL2.6) display both resistance to $MnCl_2$ and hypersensitivity to $ZnSO_4$ (31).

Because the protein kinase activity of the yck2-2^{ts} fusion protein is dramatically reduced even at the temperature permissive for growth, we compared growth rates of *YCK$^+$* strains and *yck^{ts}* strains at both permissive and semipermissive temperatures. The *yck^{ts}* cells had longer doubling times than did wild-type cells at both temperatures. At 23°C, while doubling times for wild-type strains averaged 165 min, those observed for *yck^{ts}* strains averaged 12% longer, 185 min. The increase in doubling time at 30°C was 22% greater than that at 23°C; the average times were 115 min for wild-type cells and 141 min for *yck^{ts}* cells. This increase parallels the increase in the frequency of cells with abnormal morphology in the *yck^{ts}* populations from less than 10% at 23°C to 20 to 30% (500 cells counted for each temperature) at 30°C.

DISCUSSION

We have described here studies with a Yck2–β-Gal fusion protein that confirm that *YCK2* encodes a protein kinase with substrate specificity like that of CK I. Our results are consistent with the extensive amino acid sequence similarity between Yck2 and the CK I proteins (32, 44) and with the results of Wang et al. (44), who isolated the *YCK* genes by using amino acid sequence information obtained from yeast proteins that were purified on the basis of CK I-like properties. Since the Yck2 protein is structurally similar to and functionally redundant with the protein encoded by *YCK1* (32, 44), we infer that both proteins possess this activity.

Our results with the *YCK2-lacZ* fusion and the truncated *YCK2Δ80* allele indicate that the Yck carboxyl terminus plays an important role in biological function. The Yck2 fusion protein, which lacks the carboxy-terminal 80 amino acids, is active as a protein kinase in vitro yet is unable to complement the growth defect of *yck1 yck2* strains. This impairment of function is only partially due to the presence of β-Gal sequences, since the same truncated allele lacking the *lacZ* sequences also fails to support growth in low copy number.

Biological function for the Yck proteins could include both protein kinase activity and proper localization. Our results do not exclude an effect of truncation on protein kinase activity but, in conjunction with other observations, are compatible with a model in which carboxy-terminal Yck sequences are required for proper localization within the cell. Wang et al. (44) observed association of the Yck proteins with a membrane fraction, and the sequence -Gly-Cys-Cys at the extreme carboxy termini of the Yck proteins is identical to a sequence essential for isoprenylation, and membrane localization, of the Ypt1 and Sec4 proteins (36). Accordingly, the partial function of the Yck2Δ80 and Yck2–β-Gal proteins could result from the absence of the -Gly-Cys-Cys sequence and resultant failure to localize to the appropriate membrane compartment. Consistent with this scenario, the Yck2 fusion protein is predominantly cytoplasmic (31). Also, Kuret and colleagues observe that deletion of the carboxy-terminal Cys residues of Yck2 results in a decreased association of the mutant protein with the plasma membrane fraction (24).

This interpretation is also consistent with our observation that overexpression of either truncated Yck2 protein partially rescues biological function, since the ability to overcome defects due to mislocalization by increased expression has been observed for other proteins. For example, overexpression can rescue the biological function of a mutant *RAS2* gene product that fails to undergo isoprenylation, presumably by increasing the intracellular concentration of active Ras2 protein to a level sufficient to allow interaction with its plasma membrane-localized effector, adenylate cyclase (8).

The Yck proteins belong to a novel class of protein kinases, and the *yck2-2^{ts}* allele is the first conditional allele isolated for this class. Since the protein kinase activity of the yck2-2^{ts} fusion protein is thermolabile in vitro, it was possible that the sequence alteration of the *yck2-2^{ts}* allele could provide structural or functional information about the Yck proteins. The His-187 residue altered to Tyr in yck2-2^{ts} lies within the catalytic core and is conserved in most Ser/Thr protein kinases (17). From cAPK crystal structure information (23), the side chain of this His residue in Yck2 could form a salt bridge with that of Asp-264, which could in turn provide stability to the catalytic site. Thus, the *yck2-2^{ts}* allele appears have a reversibly destabilizing effect on the catalytic core that is due to alteration of a structural element shared by protein kinases. Nevertheless, the thermolability of activity due to this mutation appears to be specific to Yck or to the CK I protein kinases. Substitutions at this position in other protein kinase types result in loss of activity that is variable from one protein kinase to another. For example, an identical substitution in the Cdc28 protein kinase resulted in a temperature-sensitive phenotype in vivo (25) but in unmeasurable in vitro protein kinase activity even at a temperature permissive for growth (29). The identical substitution at this position in the v-*mos* protein kinase product resulted in total loss of protein kinase activity as well as biological activity (40). In contrast, substitution of Ala for His at this position in the yeast cAPK catalytic subunit Tpk1 resulted in a slight decrease of activity in vitro (15).

Although the physiological substrates of, and specific biological processes affected by, the Yck protein kinases remain to be determined, the morphological abnormalities of the *yck^{ts}* mutant at the restrictive temperature indicate roles in bud morphogenesis and cytokinesis or cell separation. In the mutant, all buds initiated at the restrictive temperature are elongated, suggesting that bud growth is hyperpolarized. Although the morphology is abnormal, actin cytoskeletal elements and cell wall chitin deposition appear relatively normal in these cells, and the nuclear division cycle continues for at least the first 8 h at the restrictive temperature. The cytokinesis/cell separation defect is observed by 3 h after a shift to the restrictive temperature. Although bud size is often greater than that of the mother cell, cells rarely separate and cannot be micromanipulated apart. The cytokinesis/cell separation block is severe but is not complete and may be related to the formation of an abnormal cell wall rather than to lack of septum formation between mother and bud. Separation of cellular contents is often seen (for example, Fig. 9C and F), although these compartments are always joined by cell wall material (Fig. 9A and D). Interestingly, the cell wall of *yck^{ts}* cells incubated at the restrictive temperature appears thickened (compare Fig. 8C and D and Fig. 9D and F), which may be functionally related to the cell separation defect.

The abnormal morphology of *yck^{ts}* cells at the restrictive temperature resembles that of *cdc55* loss-of-function mutants and functionally related mutants (19, 33, 43), and a genetic relationship was found between *cdc55* and *yck^{ts}*. At temperatures permissive for either mutant alone, the double-mutant strains become inviable after several rounds of division. The terminal morphology of the double mutant at any temperature resembles that of either single mutant at its restrictive temperature. We conclude that loss of Cdc55 function is lethal at any temperature if Yck activity is decreased. While synthetic lethality could simply reflect the effects of additional stress on an already weakly growing strain, neither *cdc55* nor *yck^{ts}* shows synthetic lethality in combination with temperature-sensitive mutations in the *CDC3* and *CDC42* genes (31). Thus, a specific functional relationship between Yck and Cdc55 appears likely.

The sequence of Cdc55 resembles that of the type B regulatory subunit of PP2A, and loss of function is proposed to result in hyperactivity, or nontargeted activity, of the phosphatase (19). Consistent with this notion, overexpression of PP2A catalytic subunit results in a morphogenetic defect similar to that observed for *cdc55* cells (33). Recent results indicate that this phenotype is also shared by mutants in a second PP2A regulatory subunit, type A, encoded in *S. cerevisiae* by the *TPD3* gene (43). Moreover, loss of Tpd3 activity, which also results in hyperactivity of PP2A in vitro, is lethal in combination with loss of Cdc55 activity (43). Thus, the synthetic lethality seen for *cdc55* and *yck^{ts}* suggests a relationship between Yck activity and PP2A activity. An obvious model is that Yck and PP2A have reciprocal phosphorylation and dephosphorylation roles on a single essential substrate. This model may not fully explain our results, since increased dosage of *YCK* fails to suppress the *cdc55* growth or morphogenetic defect. We are currently testing other models, including an effect of Yck phosphorylation on PP2A activity.

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