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Prenylated Isoforms of Yeast Casein Kinase I, Including the Novel Yck3p, Suppress the *gcs1* Blockage of Cell Proliferation from Stationary Phase

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The *GCS1* gene of the budding yeast *Saccharomyces cerevisiae* mediates the resumption of cell proliferation from the starved, stationary-phase state. Here we identify yeast genes that, in increased dosages, overcome the growth defect of *gcs1*Δ mutant cells. Among these are *YCK1* (*CKI2*) and *YCK2* (*CKI1*), encoding membrane-associated casein kinase I, and *YCK3*, encoding a novel casein kinase I isoform. Some Yck3p gene product was found associated with the plasma membrane, like Yck1p and Yck2p, but most cofractionated with the nucleus, like another yeast casein kinase I isoform, Hrr25p. Genetic studies showed that *YCK3* and *HRR25* constitute an essential gene family and that Yck3p can weakly substitute for Yck1p-Yck2p. For *gcs1*Δ suppression, both a protein kinase domain and a C-terminal prenylation motif were shown to be necessary. An impairment in endocytosis was found for *gcs1*Δ mutant cells, which was alleviated by an increased *YCK2* gene dosage. The ability of an increased casein kinase I gene dosage to suppress the effects caused by the absence of Gcs1p suggests that Gcs1p and Yck1p-Yck2p affect parallel pathways.

Saccharomyces cerevisiae cells respond to nutrient depletion by undergoing a regulated cessation of cell proliferation and entering stationary phase, where they exhibit distinctive properties and can be considered to be in a distinct developmental state (reviewed in reference 52). The resumption of cell proliferation from stationary phase that results from the stimulation provided by adequate nutrients involves the restoration of biosynthetic activity, reactivation of the mitotic cell cycle, and reacquisition of the properties of cells in exponential growth.

We have characterized a yeast mutant that is conditionally defective only for the resumption of cell proliferation from stationary phase (15, 16). Mutant cells at the restrictive temperature of 15°C respond normally to the stimulation provided by the resupply of nutrients and exit stationary phase: the mutant cells lose stationary-phase properties such as thermotolerance and resistance to cell wall-lytic enzymes, acquire a gene expression pattern characteristic of actively growing cells, and accumulate cell mass (16, 17, 28). These mutant cells then become blocked after the exit from stationary phase and fail to resume cell proliferation. In marked contrast, mutant cells transferred to the restrictive temperature during exponential cell growth can continue to proliferate indefinitely as long as nutrients are provided. This phenotype is caused by mutations in the *GCS1* gene, whose predicted polypeptide, Gcs1p, contains a novel Zn finger motif that is critical for function. A point mutation in the finger region, as is found in the *gcs1-1* mutant allele, or a *gcs1*Δ null mutation each produces cells with a conditional defect only in the transition from stationary phase to active cell proliferation (28).

Recent findings suggest a role for Gcs1p in intracellular vesicle transport. Gcs1p is similar in sequence to a mammalian protein (10) that can stimulate the GTPase activity of Arf1, a monomeric G-protein that mediates vesicle transport (reviewed in reference 41). We have found that Gcs1p also stimulates Arf GTPase activity in vitro (36), and we show here that *gcs1*Δ mutant cells are impaired in one vesicle-mediated process, endocytosis.

To gain an understanding of functions related to that of Gcs1p, we have identified yeast genes that, in increased dosages, bypass the need for Gcs1p. Two dosage suppressors for the *gcs1*Δ mutation are the previously identified *YCK1* and *YCK2* genes (also called *CKI2* and *CKI1*, respectively), which encode membrane-associated isoforms of casein kinase I (38, 47, 51). An increased *YCK2* gene dosage also alleviates the *gcs1*Δ endocytosis defect. A novel casein kinase I isoform is encoded by another dosage suppressor gene isolated and characterized in this study, *YCK3*, which we show forms an essential gene pair with the *HRR25* casein kinase I gene (11, 26). Further analysis shows that membrane association of casein kinase I is important for suppression of *gcs1* cold sensitivity. Likewise, *CDC55*, encoding a regulatory subunit of protein phosphatase 2A (23), is also a *gcs1* dosage suppressor, suggesting that a balance between protein phosphatase activity and membrane-associated protein kinase activity may be important for this suppression.

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MATERIALS AND METHODS

Strains, plasmids, and culture conditions. The yeast strains used in this study are listed in Table 1; standard procedures were used for strain manipulation (21) and transformation (43), and all gene disruptions were confirmed by Southern analysis. Yeast cells were grown and analyzed as described previously (28). For routine assessment of the *gcs1* mutant phenotype, cells were incubated on complete (but without leucine) solid medium at the permissive temperature of 29°C for several days and then transferred to a fresh portion of the same medium by

TABLE 1. Yeast strains used

Strain	Genotype (reference)	Reference or source
W303-1A	*MAT***a** *leu2-3,112 ura3-1 his3-11,15 trp1-1 ade2-1*	28
W303-1B	*MAT*α *leu2-3,112 ura3-1 his3-11,15 trp1-1 ade2-1*	28
Gwk9A	*gcs1*Δ::*URA3* in W303-1A	28
Gwk9B	*gcs1*Δ::*URA3* in W303-1B	28
Gwd2L	*gcs1-2*::*LEU2* in W303-1A	28
7D	*hrr25*Δ in a W303 haploid	11
YI228	FOA-resistant version of JK111 (47); *MAT***a** *leu2-3,112 ura3-1 his3-11,15 trp1-1 ade2-1 yck1*Δ *yck2*Δ (*TRP1 yck2-2*ts)	This study
YX3	YI228 diploidized with pGAL-*HO* (24)	This study
YX4	YX3 heterozygous for *gcs1*Δ::*URA3*	This study
YX5	*gcs1*Δ::*URA3* in YI228	This study
Grk4-7	*gcs1*Δ::*URA3* in 21R (29)	28
21R-B	*MAT*α version of 21R made by using pGAL-*HO*	This study
Nrk1B	*yck3*::*LEU2* in 21R-B	This study
YX6	*MAT***a**/*MAT*α *leu2-3,112/leu2-3,112 ura3-52/ura3-52 ade1/ade1* +/*gcs1*Δ::*URA3* +/*yck3*::*LEU2*	Grk4-7 × Nrk1B
AHY86	*MAT***a** *leu2 ura3 his3 cdc55*::*LEU2*	23
tpd3.1	*MAT*α *leu2 ura3-52 his3 ade2-1 hml*::*SUP40 hmr*::*SUC2 sir4-1 tpd3-1*	48
Y1378	*MAT*α *leu2 ura3 PPH21 pph22*::*URA3*	48
Y1379	*MAT*α *leu2 ura3 his3 trp1 ade2 pph21*::*LEU2 PPH22*	48

replica plating and incubated at 15°C; growth was assessed after a further 5 to 6 days of incubation.

Plasmid pXW120, isolated from a YEp213 library (a gift from D. Thomas), contains a 5.2-kbp *Sau*3A fragment of yeast genomic DNA inserted into the *Bam*HI site. Plasmids pXW121, pXW122, and pXW123 carry different fragments of pXW120 in YEp351 (25). pJK435-*YCK2* has been described previously (47). pXW126 is YEp351 plus a 2.3-kbp *Sma*I fragment from pB65R7 (38) (provided by M. Carlson), which contains the entire open reading frame of *YCK1*. Plasmid p*YCK1-dK* is a pXW126 derivative with the sequence between two *Bgl*II sites within the *YCK1* kinase domain deleted; p*YCK1-dI* is another internally deleted version of pXW126 lacking sequences between *Fsp*I and *Bsa*AI sites. Plasmid p*YCK1-dC* contains a C-terminally truncated *YCK1* gene constructed by replacing the *Sph*I-*Sma*I region of YEp351 with an ~2-kbp *Sph*I-*Bsa*AI fragment from pXW126.

Plasmid pXW150, another YEp213 library plasmid, contains a 4-kbp region of yeast genomic DNA; pXW151 and pXW152 are fragments of pXW150 in YEp351. An ~4-kbp *Hin*dIII genomic fragment encompassing the *YCK3* gene was also transferred to the episomal *TRP1* plasmid YEplac112 (19) and used to test suppression of the *hrr25*Δ growth defect. A *YCK3* knockout plasmid, pNK-Δ1, was constructed as follows: a 4.3-kbp DNA fragment containing the *YCK3* gene was cloned into pBluescript, the resulting plasmid was cut with *Not*I and *Sma*I and religated after the *Not*I end was filled in, and an internal *Bam*HI-*Bgl*II DNA fragment was then replaced by a 1.7-kbp *HIS3 Bam*HI fragment. pNK-Δ1 was linearized with *Sal*I before transformation. A *yck3*::*LEU2* disruption plasmid was constructed by mini-Tn*3*(*LEU2*) shuttle mutagenesis (27).

For epitope addition, the *YCK3* gene was first modified by adding useful restriction sites (*Sal*I preceding the initiation codon, *Nde*I at the initiation codon, and *Xho*I just after the termination codon) by PCR (51). The resultant 1.7-kbp PCR fragment was digested with *Nde*I and *Xho*I and ligated into the *Nde*I and *Xho*I sites of plasmid pJK435, a derivative of pRS415 (45) that contains the *ADH1* promoter followed by the epitope-coding sequence described by Field et al. (18). The product, pJK435-*YCK3*, encodes a Yck3p in which the N-terminal MSQR. . . is changed to MYPYDVPDYASLGGPMSTHMSQR. . ., which raises the apparent molecular mass to 62.4 kDa.

Plasmid p*HRR25* contains the entire *HRR25* gene in YEp351, and plasmid p*GAL-HRR25* contains *HRR25* expressed from the *GAL10* promoter. Plasmid p*HRR25-CC* encodes a chimeric polypeptide in which the C-terminal 60 amino acids of Yck1p replace the C-terminal 7 amino acids of Hrr25p. Plasmids p*HRR25-YCK2* and p*YCK2-HRR25* contain complementary chimeric genes with the coding sequences of *YCK2* and *HRR25* exchanged at an *Nco*I site. YEp*CDC55* contains the *CDC55* gene in plasmid YEp352 (25), and YEp24-TPD3 contains the *TPD3* gene in plasmid YEp24 (provided by J. Broach and K. York). The *GCS1* knockout plasmid pBN-Δ4 has been described previously (28).

Endocytosis assay. Cells were treated essentially as described by Vida and Emr (49) and photographed with a Leitz Laborlux S microscope. Prior to stimulation with fresh medium, stationary-phase cells were exposed to 80 μM FM 4-64 for 25 min at 0°C (49).

Subcellular fractionation and immunoblotting. Cells of strain W303 harboring pJK435-*YCK3* were grown, lysed, and fractionated by differential centrifugation into pellet (P1, P2, P3, and P4) and supernatant fractions as described previously (47). P1-P2 and P3-P4 were then combined and subjected to density gradient fractionation (47). All fractions were assayed in duplicate for marker enzymes, which included vanadate-sensitive plasma membrane ATPase, cytochrome *c* reductase, cytochrome *c* oxidase, α-D-mannosidase, and GDPase (47); DNA was assayed fluorimetrically (8). Epitope-tagged Yck3p was assayed by immunoblotting with monoclonal antibody 12CA5 (18) and visualized by enhanced chemiluminescence (Amersham, Arlington Heights, Ill.) with sheep anti-mouse immunoglobulin G conjugated to horseradish peroxidase. Images were collected on X-ray film and quantified by laser densitometry (Molecular Dynamics).

RESULTS

Casein kinase I genes *YCK1* and *YCK2* are dosage suppressors of *gcs1*Δ cold sensitivity. To identify genes that, in increased dosages, bypass the need for Gcs1p during the resumption of cell proliferation, mutant cells lacking the *GCS1* gene were transformed with a multicopy yeast genomic library, and plasmids that suppress the cold sensitivity of *gcs1*Δ mutant cells were isolated. Among the 23,000 transformants screened, we identified 5 different dosage suppressor genes in addition to the *GCS1* gene itself. One suppressing sequence was found to be the previously characterized *YCK2* gene (38, 51) (also called *CKI1*), which encodes an isoform of casein kinase I. A subclone of the original suppressing plasmid pXW120 encompassing the entire open reading frame of *YCK2* (pXW123) suppressed the cold sensitivity of *gcs1* mutant cells (Fig. 1A), whereas derivatives containing only portions of *YCK2* did not suppress. Thus, *YCK2* is the dosage suppressor gene in pXW120.

YCK2 forms an essential gene pair with the casein kinase I gene *YCK1* (38, 51) (also called *CKI2*). We obtained *YCK1* and found, as expected from the extensive (~77%) amino acid sequence identity for these casein kinase I enzymes, that *YCK1* in multiple copies also suppresses *gcs1* cold sensitivity (Fig. 1A). Measurements of mRNA levels show that *gcs1*Δ does not alter the expression of *YCK1* and *YCK2* (data not shown), suggesting that an increased dosage of the *YCK1* and *YCK2* genes suppresses *gcs1* cold sensitivity through additional casein kinase I activity.

Genetic interaction between a *gcs1* mutation and a *yck2*-ts mutation. To ascertain whether the normal functions of Yck1p and Yck2p are related to Gcs1p activity, we looked for genetic interactions between *gcs1* and *yck* mutations. *YCK1* and *YCK2* are functionally redundant, so that any genetic interaction among *gcs1* and *yck* mutations might be revealed only if both

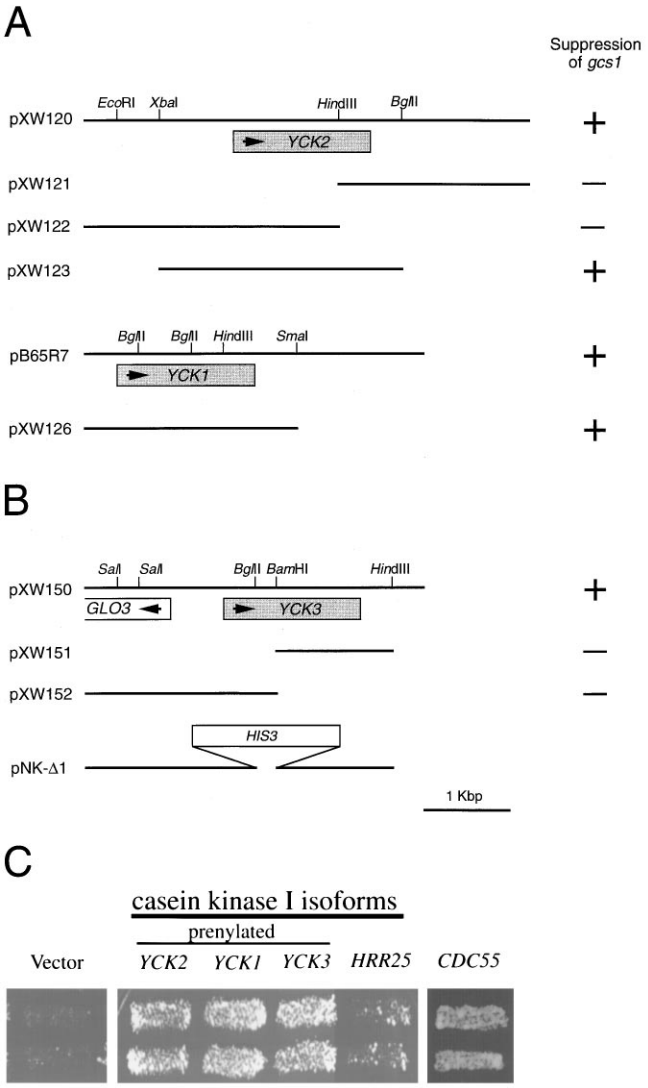


FIG. 1. Suppression of *gcs1*Δ cold sensitivity. (A and B) Suppression by *YCK1*, *YCK2*, and *YCK3* genomic clones. Only yeast sequences are shown; boxes and arrows indicate the positions and 5′→3′ directions of the open reading frames. Hybridization to the yeast lambda phage clone grid (37) showed that *YCK3* is on the right arm of chromosome V between *RAD51* and *BEM2*; the DNA sequence positioned *YCK3* adjacent to *GLO3* (28). (C) Suppression by genes encoding casein kinase I isoforms and by *CDC55*. *gcs1*Δ mutant cells (strain Gwk9A) transformed with high-copy-number plasmids were incubated on selective medium at the permissive temperature of 29°C for several days and then replica plated and incubated at 15°C for 7 days. The vector control is YEp351.

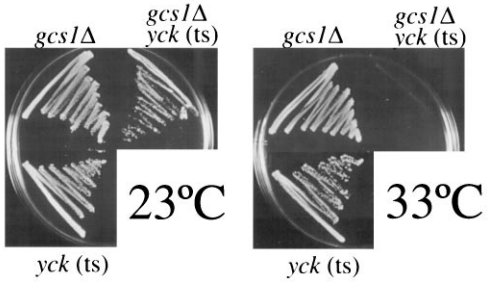


FIG. 2. Gcs1p facilitates the growth of temperature-sensitive *yck* mutant cells. Strains harboring the *gcs1*Δ mutation and/or the *yck1*Δ *yck2-2*ts mutations [*yck* (ts)] were incubated on rich medium for 3 days at the indicated temperatures. *yck* (ts), strain YI228; *gcs1*Δ, Gwk9A; *gcs1*Δ *yck* (ts), YX5.

YCK1 and *YCK2* were mutated. We therefore used strain YI228, which harbors chromosomal *yck1*Δ and *yck2*Δ mutations and is kept alive by a plasmid-borne temperature-sensitive *yck2-2*ts mutant allele, as a starting point. Strain YI228 was diploidized (Table 1), and one *GCS1* gene in the diploid was disrupted by transformation with the *GCS1* knockout plasmid pBN-Δ4 (28). The resulting diploid *yck2*-ts transformant heterozygous for the *gcs1*Δ allele retained the *yck2*-ts phenotype (data not shown). This diploid was sporulated, and tetrads were dissected to obtain *gcs1 yck1 yck2* segregants, which were allowed to germinate at a temperature permissive for both *gcs1*Δ and *yck1*Δ *yck2*Δ *yck2-2*ts. Of the 25 tetrads analyzed, no more than two of the four spores from each tetrad were able to germinate at a permissive temperature (18 tetrads showed a 2:2 segregation pattern, 5 showed 1:3 segregation, and 2 showed 0:4 segregation). All of the viable spores had the genotype of *GCS1 yck1*Δ *yck2*Δ *yck2-2*ts mutant cells, and none had the *gcs1*Δ *yck1*Δ *yck2*Δ *yck2-2*ts genotype (as indicated by the nutritional markers at the *gcs1*Δ and *yck* loci and by the *gcs1*Δ-cs and *yck2-2*ts phenotypes). Since *gcs1*Δ spores germinate and form colonies at a permissive temperature (28), these findings show that either Gcs1p or adequate Yck1p-Yck2p casein kinase I activity is needed for spore germination.

A related but different result was obtained by using transformation to delete the *GCS1* gene from YI228 haploid cells. For this experiment YI228 also harbored a *YCK2* plasmid, which was lost after *GCS1* deletion to yield the *yck1*Δ *yck2*Δ *yck2-2*ts *gcs1*Δ quadruple-mutant cells; identical results were obtained by *GCS1* deletion from cells lacking the *YCK2* plasmid. In both cases the resulting *yck1*Δ *yck2*Δ *yck2-2*ts *gcs1*Δ mutant cells were viable but grew slowly at permissive temperatures. These mutant cells showed the cold sensitivity of the *gcs1*Δ mutation at 15°C and failed to grow at 37°C because of the *yck2-2*ts mutation. More significantly, however, these mutant haploid cells failed to grow at 33°C, a temperature at which both *gcs1*Δ mutant cells and the parental *yck1*Δ *yck2*Δ *yck2-2*ts mutant cells do grow (Fig. 2). These results reveal a synthetic enhancement between the effects of loss of Gcs1p function and the decreased activities of Yck1p-Yck2p and suggest that Gcs1p and the casein kinase I activities of Yck1p-Yck2p are involved in a common process. Since Gcs1p does not contain hallmark protein kinase motifs (28) and is thus not expected to have protein kinase activity, it is unlikely that casein kinase I replaces Gcs1p. Instead, increased casein kinase I activity provided by extra gene copies probably facilitates a parallel pathway that bypasses the need for Gcs1p.

Endocytosis is impaired by *gcs1*Δ and restored by increased *YCK2* gene dosage. In vitro assays show that Gcs1p can stimulate GTP hydrolysis by ARF protein and thus may affect vesicle transport (36). To assess the in vivo involvement of Gcs1p in some aspect of vesicle transport, we monitored endocytosis, using the dye *N*-(3-thiethylammoniumpropyl)-4-(*p*-diethylaminophenylhexatrienyl)pyridinium dibromide (FM 4-64). The uptake of this membrane stain by *S. cerevisiae* cells has been shown to occur via an endocytic route (49). Whereas wild-type cells resuming cell proliferation from stationary phase at 15°C accumulated FM 4-64 in vacuoles, *gcs1*Δ mutant cells tested in the same way were ineffective in this accumulation even after extended incubation (Fig. 3), suggesting that aspects of vesicle transport are impaired in mutant cells that have already exited stationary phase and resumed biosynthetic activity but will not be able to proliferate (16, 17, 28). A multicopy *YCK2* plasmid restored the wild-type pattern of FM

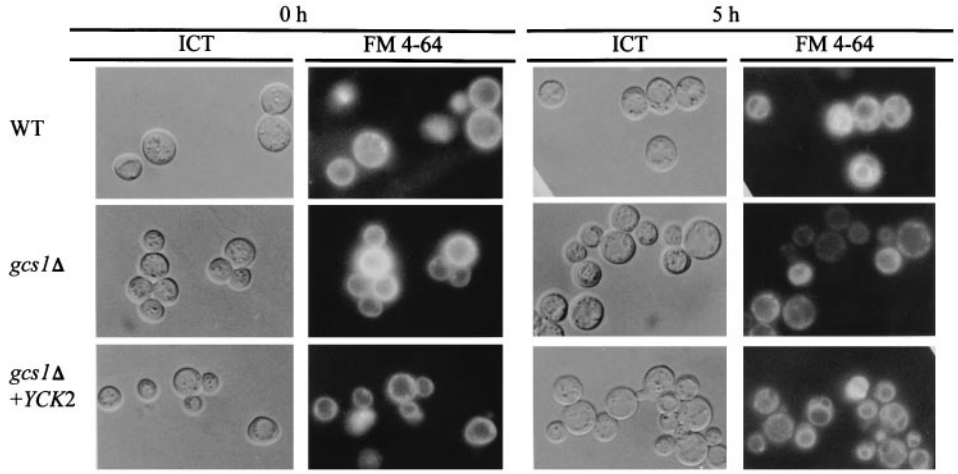


FIG. 3. Suppression of the *gcs1*Δ endocytosis impairment by increased casein kinase I. Stationary-phase cells were incubated with the membrane dye FM 4-64 for 25 min at 0°C and then transferred (time zero) to fresh dye-free growth medium for a further 5-h incubation at 15°C. Dye-bound membranes were visualized by fluorescence (right panels), while cells were imaged by interference contrast transmission (ICT) microscopy (left panels). The movement of plasma membrane FM 4-64 into intracellular vesicles was clearly evident for wild-type (WT) and *YCK2*-suppressed *gcs1*Δ mutant cells by 1.5 h of incubation, while little internalization of dye was seen for *gcs1*Δ cells even after 24 h (data not shown).

4-64 uptake to *gcs1*Δ mutant cells (Fig. 3). Thus, the suppression of *gcs1*Δ cold sensitivity by increased casein kinase I gene dosage is reflected by the restoration of effective vesicle transport.

The protein kinase and C-terminal lipid modification domains are important for suppression of *gcs1* cold sensitivity. Not all casein kinase I enzymes can bypass the need for Gcs1p. Another yeast casein kinase I gene, *HRR25* (26), was not identified as a *gcs1* dosage suppressor, and when tested directly, increased expression of Hrr25p from a multicopy plasmid (pXW321) or an inducible *GAL* promoter (p*GAL-HRR25*) did not suppress *gcs1* cold sensitivity (Fig. 1C). Thus, casein kinase I activity per se is insufficient for *gcs1* suppression; other properties of casein kinase I must be involved.

Both Yck1p and Yck2p have an N-terminal catalytic domain and a prenylation signal at the C terminus, which are joined by a linker region of biased composition. Figure 4 shows that Yck1-dIp, which has part of the linker region deleted, was still effective in suppressing *gcs1* cold sensitivity. In contrast, a derivative lacking the protein kinase domain, Yck1-dKp, was unable to suppress the *gcs1* mutant phenotype, while Yck1-dCp, a derivative lacking the C-terminal prenylation signal, suppressed *gcs1* cold sensitivity so poorly that suppression could be detected only after extended incubation at 15°C. These observations suggest that both the protein kinase and C-terminal prenylation domains are necessary for suppression, while part of the linker region can be deleted without affecting suppression (Fig. 4). The absence of *gcs1* suppression by a C-terminal truncation of *YCK2* (Fig. 1A, pXW122) is consistent with this conclusion. The importance of the C-terminal prenylation domain suggests that membrane localization of casein kinase I is essential for suppression of *gcs1* cold sensitivity.

The importance of a C-terminal lipid modification domain for *gcs1* suppression prompted us to test whether addition of this domain would confer suppression ability on the nonsuppressing casein kinase I isoform Hrr25p, which lacks a C-terminal prenylation signal. We fused the C-terminal 60 residues of Yck1p, including the prenylation signal, to the C terminus of Hrr25p to produce Hrr25-CCp, and we found that Hrr25-CCp expressed from a multicopy plasmid could suppress the cold sensitivity of *gcs1*Δ, although not as effectively as Yck1p or Yck2p (Fig. 4). This weak suppression may reflect inefficient membrane association due to the activity of a nuclear localization signal in the Hrr25p portion of Hrr25-CCp. The nuclear localization signal of Hrr25p has not yet been identified, but the sequence TKKQKY, which is similar to the nuclear localization signal of simian virus 40 large T antigen that functions in *S. cerevisiae* (34), is found at the C-terminal end of the kinase domain. We therefore exchanged the kinase domains of Hrr25p and Yck2p, exploiting *Nco*I sites both in the *HRR25* gene immediately upstream of the TKKQKY-coding sequence and at a homologous location in *YCK2*. The chimeric protein comprising the Yck2p kinase domain fused to TKKQKY and the rest of Hrr25p did not suppress *gcs1*Δ cold sensitivity, although this chimeric protein was effective in restoring normal growth to *hrr25-1* mutant cells (data not shown). In contrast, the chimeric protein Hrr25-Yck2p, with the Hrr25p kinase domain (but not its TKKQKY sequence) fused to the rest of Yck2p (and its prenylation domain), suppressed the *gcs1*Δ phenotype better than did Hrr25-CCp (Fig. 4). These observations suggest that membrane association facilitates casein kinase I suppression of *gcs1* cold sensitivity.

Multicopy *CDC55*, encoding a protein phosphatase 2A regulatory subunit, suppresses *gcs1* cold sensitivity. Yck1p-Yck2p activity is counteracted by protein phosphatase type 2A activity. This conclusion stems from the finding that mutation of the *CDC55* gene, which encodes a regulatory subunit of protein phosphatase type 2A (23), exacerbates the growth impairment of *yck1*Δ *yck2*Δ *yck2-2*ts cells (39). We tested whether altered protein phosphatase 2A activity can exert *gcs1* suppression. Yeast protein phosphatase 2A has three subunits: a catalytic subunit encoded by *PPH21* and *PPH22* (40, 46), an accessory subunit encoded by *TPD3* (48), and the *CDC55*-encoded regulatory subunit (23). Decreasing phosphatase activity by introducing a *pph21* null or *pph22* null mutation did not suppress *gcs1* cold sensitivity (data not shown) (some of these experiments used the *gcs1-2* truncation allele instead of *gcs1*Δ [28]). Because the absence of both the *PPH21* and *PPH22* genes severely impairs yeast cell growth, we were unable to determine whether the *pph21 pph22* double mutant situation suppresses the *gcs1* mutant phenotype. Multicopy expression of

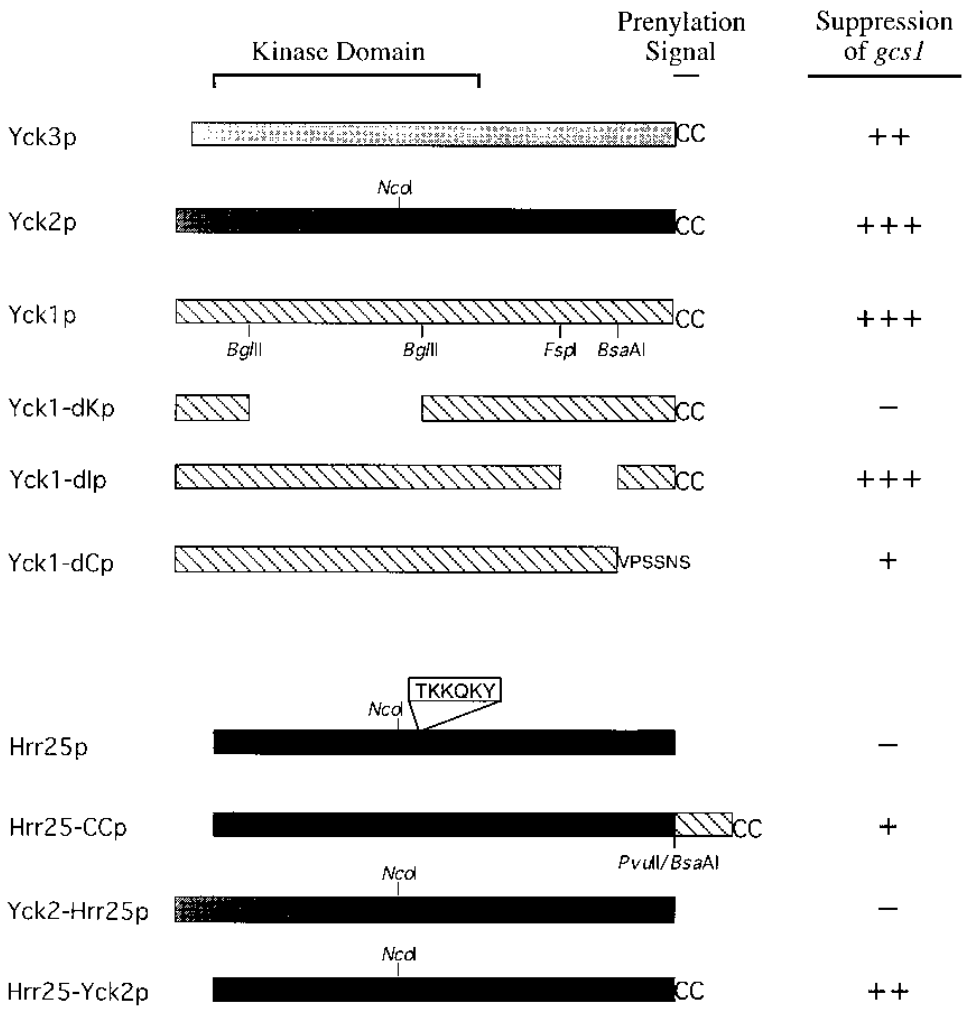


FIG. 4. Suppression of *gcs1*Δ needs a casein kinase I kinase domain and a prenylation signal. Open reading frames are indicated, with restriction sites used for gene constriction marked. CC, C-terminal Cys-Cys motif. Yck1-dCp vector-encoded residues are shown. Suppression by the indicated genes on multicopy plasmids was scored by the extent of growth of transformed cells at 15°C.

the phosphatase 2A subunit encoded by *TPD3* or a decrease of Tpd3p activity with the *tpd3-1* mutation each had no effect on *gcs1* cold sensitivity. In contrast, multicopy expression of the *CDC55* gene suppressed the *gcs1* mutant phenotype (Fig. 1C), suggesting that suppression can be achieved by altering protein phosphatase 2A activity. Our results thus confirm that Cdc55p activity and casein kinase I activities can be related. For some substrates Cdc55p may negatively regulate phosphatase activity (12), suggesting that altering protein phosphatase 2A activity through Cdc55p may suppress *gcs1* cold sensitivity by increasing the phosphorylation state of Yck1p-Yck2p substrates.

The novel casein kinase I gene *YCK3* suppresses *gcs1*Δ cold sensitivity. The significance of casein kinase I activity in *gcs1*Δ suppression was accentuated by the analysis of pXW150 (Fig. 1B), another plasmid isolated as a dosage suppressor of *gcs1*Δ. Subcloning (Fig. 1B) and nucleotide sequence analysis showed that the pXW150 suppressor gene, which we term *YCK3* (EMBL/GenBank accession number X87108), encodes yet another casein kinase I isoform, with a novel structure. The N-terminal region of Yck3p is highly similar to the kinase domains of Yck1p, Yck2p, and Hrr25p (Fig. 5). The Yck3p kinase domain is followed by a serine- and asparagine-rich region, analogous to the proline- and glutamine-rich region of Hrr25p and the glutamine-rich regions of Yck1p and Yck2p, while the C terminus contains a run of cysteine residues that may constitute a signal, similar to those found in Yck1p and Yck2p, for prenylation and membrane association (47). Thus, Yck3p is structurally similar to Yck1p and Yck2p. *YCK3* dosage suppression is also similar to that by *YCK1* and *YCK2*, and the absence of *gcs1*Δ effects on *YCK3* expression (data not shown) suggests that an increased *YCK3* gene dosage also suppresses the *gcs1* mutant phenotype through additional casein kinase I activity.

Lack of genetic interaction between *yck3* and *gcs1* mutations. No effects on haploid cell growth were detected when *YCK3* was inactivated by replacement of an internal fragment with the *HIS3* gene (Fig. 1B). This finding that *YCK3* is not itself an essential gene allowed us to investigate genetic interaction between *yck3* and *gcs1* mutations. Cells harboring *gcs1*Δ were crossed with cells harboring a *yck3*::*LEU2* disruption allele, and the resulting diploid cells were sporulated. The *gcs1*Δ *yck3* spores germinated and except for the *gcs1* cold sensitivity did not exhibit impaired growth. This apparent lack of genetic interaction suggests that Yck3p and Gcs1p are not normally involved in the same process and that suppression of the *gcs1*

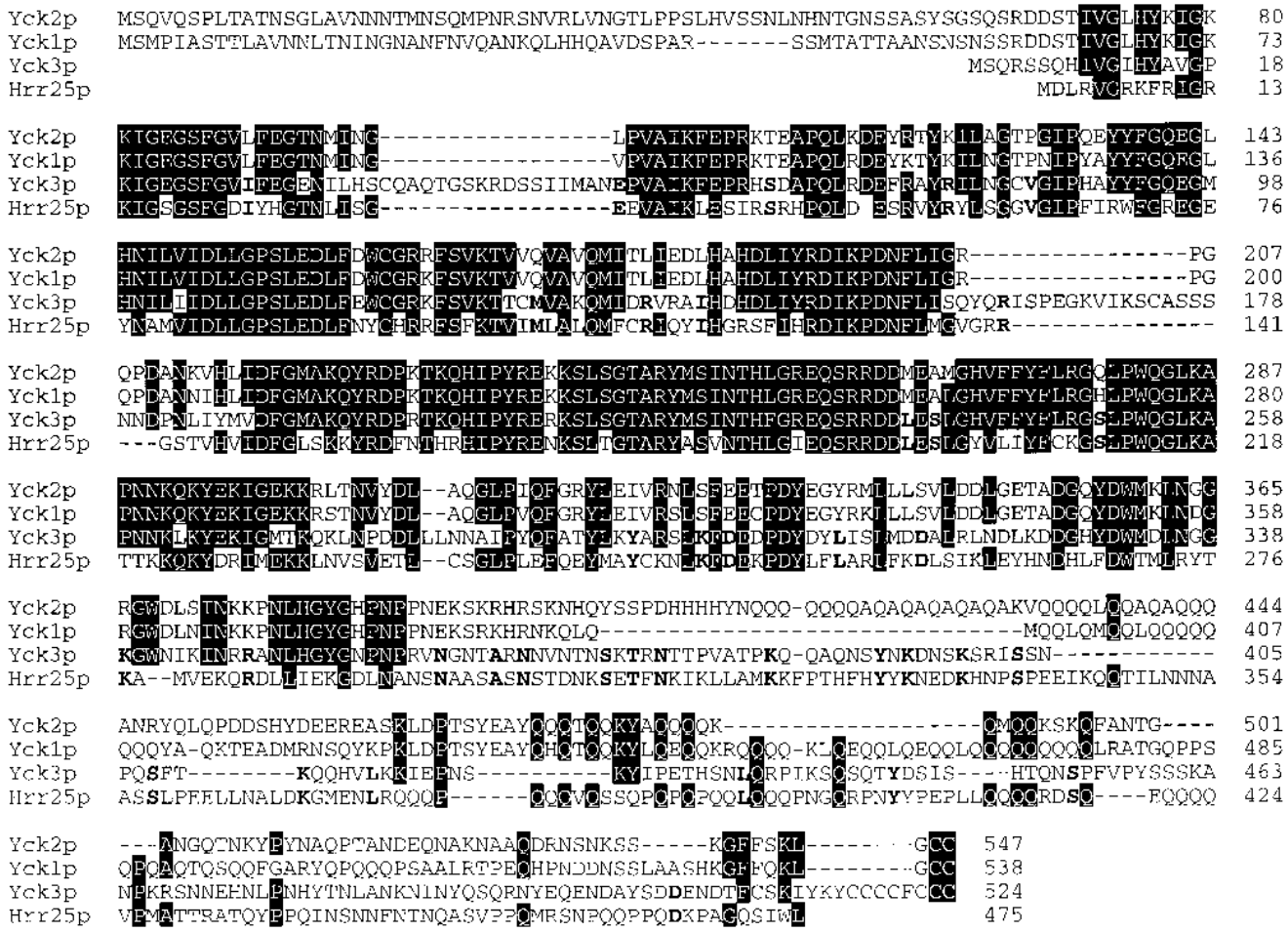


FIG. 5. The *YCK3* gene product, Yck3p, aligned with the Yck1p, Yck2p, and Hrr25p predicted polypeptides by using PileUp (13). White letters on black indicate positions at which at least three proteins have the same amino acid; boldface indicates an identity between Yck3p and Hrr25p. Amino acid positions are indicated on the right.

mutant phenotype by increased Yck3p may be due to increased Yck3p activity augmenting Yck1p-Yck2p activity.

Two casein kinase I families. Yck1p and Yck2p overlap functionally: the loss of either member of that pair has little effect on the cell, but the loss of both Yck1p and Yck2p is lethal (38, 51). Hrr25p is thought to have a different function, because *hrr25* mutations, which cause defects that include slow growth and sensitivity to the DNA-damaging agent methyl methanesulfonate (26), show no synthetic enhancement with either *yck1*Δ or *yck2*Δ mutations (data not shown). We determined by tetrad analysis if there is genetic interaction between *hrr25*Δ and *yck3*Δ and found upon sporulation of a heterozygous diploid strain that *yck3*Δ exacerbates the *hrr25*Δ phenotype: unlike each single mutant, the *hrr25*Δ *yck3*Δ double mutant cells were inviable (data not shown). Furthermore, the *YCK3* gene on a multicopy plasmid was as effective as the *HRR25* gene, or the *Schizosaccharomyces pombe HRR25* homolog *hhp1*$^+$ (14), in suppression of the *hrr25*Δ growth defect (data not shown). Thus, *YCK3* and *HRR25* form a second casein kinase I gene pair with overlapping essential functions.

Prompted by our finding that *YCK3* could be isolated as a multicopy suppressor of the *yck2*-2ts phenotype (Fig. 6A), we examined the relationships between the two casein kinase I gene pairs. An increased dosage of *YCK3* or *HRR25* suppressed the temperature sensitivity of the *yck1*Δ *yck2*Δ *yck2-2*ts mutant strain YI228, although in each case the suppressed cells did not grow as well as wild-type cells (Fig. 6). However, production of Yck3p from a low-copy-number plasmid did not suppress this temperature sensitivity (data not shown), showing that Yck3p can only partially compensate for the loss of Yck1p-Yck2p activity. Similarly, expression of Hrr25p from a low-copy-number plasmid does not suppress the *yck1*Δ *yck2*Δ *yck2-2*ts phenotype (47), perhaps because only a small portion of Hrr25p is localized at the plasma membrane like Yck1p-Yck2p (47). In support of this idea, we found that the Hrr25-CCp chimeric protein, expressed from a multicopy plasmid, suppressed the *yck1*Δ *yck2*Δ *yck2-2*ts mutant phenotype better than did Hrr25p (Fig. 6B). Hrr25-Yck2p and a closely related Hrr25p-Yck2p chimeric protein also suppress the *yck1*Δ *yck2*Δ *yck2-2*ts phenotype (Fig. 6B) (47). Presumably the added C-terminal prenylation signal facilitates this stronger suppression by localizing the chimeric kinase to the site of Yck1p-Yck2p activity. These results suggest that an important feature of *yck1*Δ *yck2-2*ts suppression is membrane association.

Subcellular localization of Yck3p. We determined the membrane association and subcellular distribution of Yck3p. For these studies Yck3p was tagged with the influenza virus hemagglutinin epitope (HA), allowing specific detection with

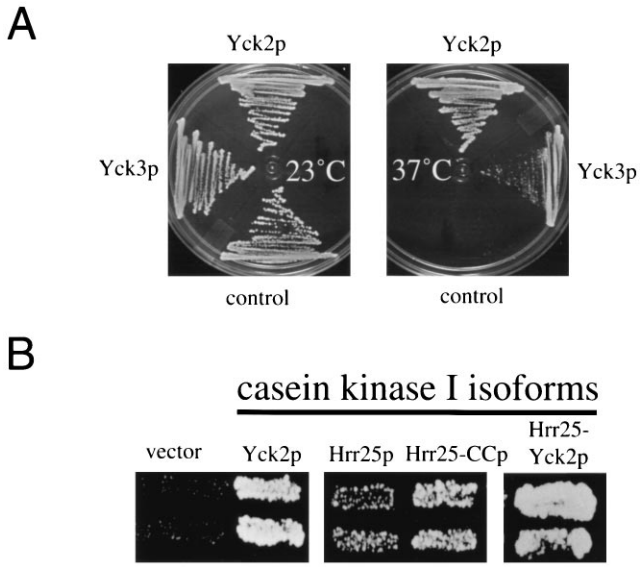


FIG. 6. Dosage suppression of *yck1*Δ *yck2-2*ts. (A) Suppression by *YCK3*. Cells of strain JK111 harboring pJK435-*YCK2*, the high-copy-number plasmid pJK436-*YCK3*, or the vector YEp351 as a control were incubated on rich medium at 23°C (left) or at 37°C (right). (B) A C-terminal prenylation signal improves suppression by Hrr25p. YI228 cells transformed with p*HRR25*, p*HRR25-CC*, p*HRR25-YCK2*, or YEp351 as a vector control were grown at 23°C and then transferred by replica plating and incubated at 37°C.

monoclonal antibody 12CA5 (47), and expressed from the *ADH1* promoter on a low-copy-number plasmid as described in Materials and Methods. This epitope-tagged Yck3p (HA-Yck3p) retained protein kinase activity in vitro and maintained the viability of *yck3*Δ *hrr25*Δ double mutant cells when supplied from the low-copy-number plasmid (data not shown).

Lysates from wild-type cells expressing HA-Yck3p were subjected to differential centrifugation as described previously (47), revealing that Yck3p, like Yck1p, Yck2p, and Hrr25p, associates predominantly (>90%) with the particulate fractions (data not shown). These fractions were then combined (P1-P2 and P3-P4) and resolved by density gradient centrifugation. Under these conditions, HA-Yck3p fractionated into multiple peaks. As shown in Fig. 7A, most (>65%) HA-Yck3p from the low-speed pellets (P1-P2) was recovered at the bottom of the gradient (fraction 20) along with genomic DNA, a marker for cell nuclei. The remaining HA-Yck3p (35%) migrated to the middle of the gradient, consistent with a membranous localization. Most of this HA-Yck3p (25%) comigrated with vanadate-sensitive ATPase, a marker for the plasma membrane; the rest (10%) migrated with a density similar but not identical to that of Golgi membranes (as marked by GDPase).

As shown in Fig. 7B, resolution of the high-speed pellets (P3-P4) confirmed the membrane association of Yck3p. The relative amounts of HA-Yck3p that comigrated with plasma membranes (42%), Golgi membranes (26%), and cell nuclei (7%) are consistent with the enrichment of cell membranes relative to intact nuclei in these fractions. The remaining 25% of the HA-Yck3p became dissociated from particulate material during centrifugation and was recovered near the bottom of gradient.

Of the HA-Yck3p recovered from both gradients, 36% comigrated with intact nuclei, 35% comigrated with plasma membranes, 15% comigrated with membranes that approximated Golgi membranes in buoyant density, and 14% became dissociated from particulate material. Yck3p thus has a wide subcellular distribution, encompassing the narrower distributions of both Hrr25p and Yck1p-Yck2p. The membrane association shown for only some of the HA-Yck3p may account for the weak *yck1*Δ *yck2-2*ts suppression by *YCK3*.

DISCUSSION

The yeast protein Gcs1p facilitates the resumption of yeast cell proliferation from the starved stationary-phase state at 15°C. In the absence of Gcs1p this transition becomes blocked, even though *gcs1*Δ mutant cells exit stationary phase and undergo many of the early responses to the resupply of adequate nutrients that are typical of wild-type cells (16, 28). The *gcs1*Δ blockage is manifested as cold sensitivity for the resumption of cell proliferation and for accompanying endocytotic activity, as shown here. These findings do not indicate whether the impaired endocytosis in *gcs1*Δ mutant cells is functionally related to the inability of these cells to resume cell proliferation from stationary phase. Indeed, we have used endocytosis simply as an indicator of vesicle transport. We note that endocytosis as measured here is also affected by mutations that block the exocytic movement of secretory vesicles from the trans-Golgi network to the plasma membrane (49). The cold sensitivity of endocytosis in *gcs1*Δ mutant cells may therefore reflect an involvement of Gcs1p in vesicle transport, a suggestion bolstered by the resemblance of Gcs1p to a mammalian protein purified as an Arf GTPase-activating protein (10) and by the ability of Gcs1p to stimulate Arf GTPase activity in vitro (36).

The cold sensitivity of *gcs1*Δ mutant cells can be alleviated by increasing the dosage of certain yeast genes, suggesting that increased gene product activity can activate a pathway that bypasses the need for Gcs1p at 15°C. This parallel pathway may, like Gcs1p, also facilitate vesicle transport. The parallel pathway is inherently cold sensitive for function, as indicated by the cold sensitivity of cells relying on this pathway in the absence of Gcs1p. Although this pathway remains undefined, some of its activators have been identified. We show here that increased casein kinase I expression suppresses *gcs1*Δ cold sensitivity for cell proliferation and endocytosis. Yeast casein kinase I enzymes that can activate a Gcs1p bypass pathway are the Yck1p and Yck2p plasma membrane isoforms and the new isoform encoded by the *YCK3* gene described here. These findings point to a role for casein kinase I in vesicle transport. Indeed, mammalian casein kinase I can be found in vesicular structures (4) and purified synaptic vesicles (20). Moreover, preliminary observations indicate that *GCS1 yck1*Δ *yck2-2*ts mutant cells display inefficient endocytosis at 15°C, although the impairment is not as severe as it is for *gcs1*Δ mutant cells (unpublished observations). This observation suggests that casein kinase I also mediates the Gcs1p pathway in addition to activating a parallel pathway, perhaps at a step common to both pathways.

Suppression depends on casein kinase I membrane association. The intracellular localization of casein kinase I potentiates *gcs1*Δ suppression. Yck1p and Yck2p are predominantly associated with the plasma membrane (47, 51), and some of the third suppressing kinase, Yck3p, also cofractionates with plasma membrane (Fig. 7). The C-terminal sequence of Yck1p that specifies membrane association (47) is a necessary structural feature for suppression of *gcs1* cold sensitivity, and suppression by chimeric casein kinase I enzymes is correlated with the presence of membrane association sequences, as seen for chimeric enzymes based on the Hrr25p isoform of casein kinase I. Hrr25p, which is predominantly nuclear (47), cannot provide *gcs1*Δ suppression, but it acquires this ability when

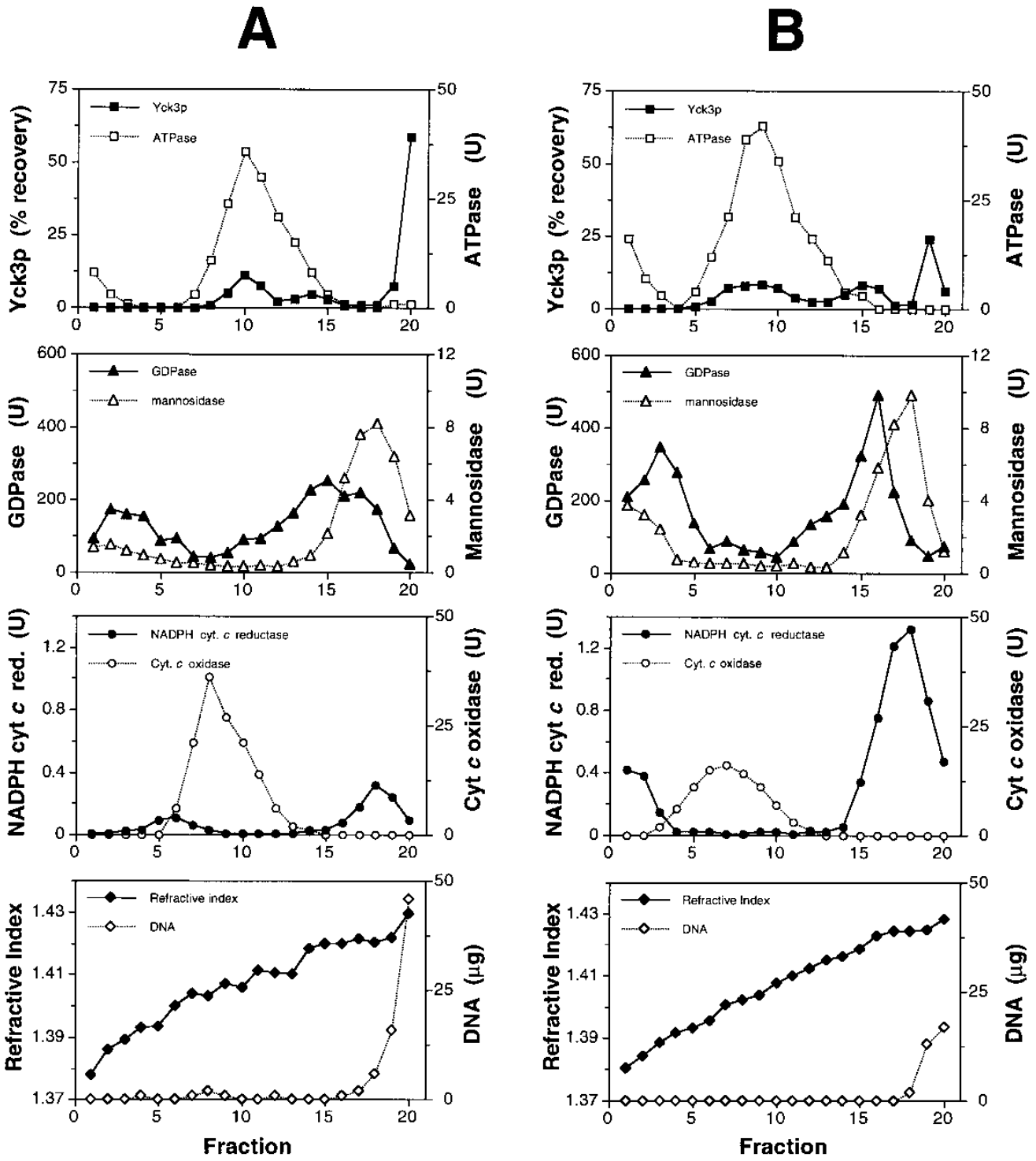


FIG. 7. Subcellular localization of Yck3p. Particulate fractions from wild-type transformants harboring pJK435-*YCK3* were combined and subjected to density gradient centrifugation as described in Materials and Methods. Gradients were fractionated from top (fraction 1) to bottom (fraction 20) and assayed for HA-Yck3p and selected markers. The recovery of HA-Yck3p in each fraction is reported as a percentage of the total amount loaded onto the gradient, whereas marker enzyme activities are expressed in units described previously (47). Cyt *c* red., cytochrome *c* reductase. (A) Resolution of pooled P1-P2 fractions. (B) Resolution of pooled P3-P4 fractions.

fused to the C-terminal membrane association domain of Yck2p. The importance of membrane association for casein kinase I suppression of *gcs1* cold sensitivity supports the idea that casein kinase I may affect membrane processes such as vesicle transport. Figure 8 summarizes the casein kinase I relationships.

Casein kinase I and morphogenesis. Yck1p and Yck2p are also implicated in vesicle transport related to morphogenesis.

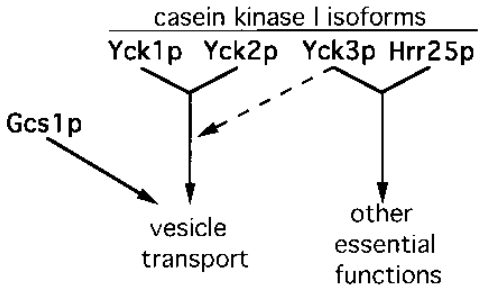


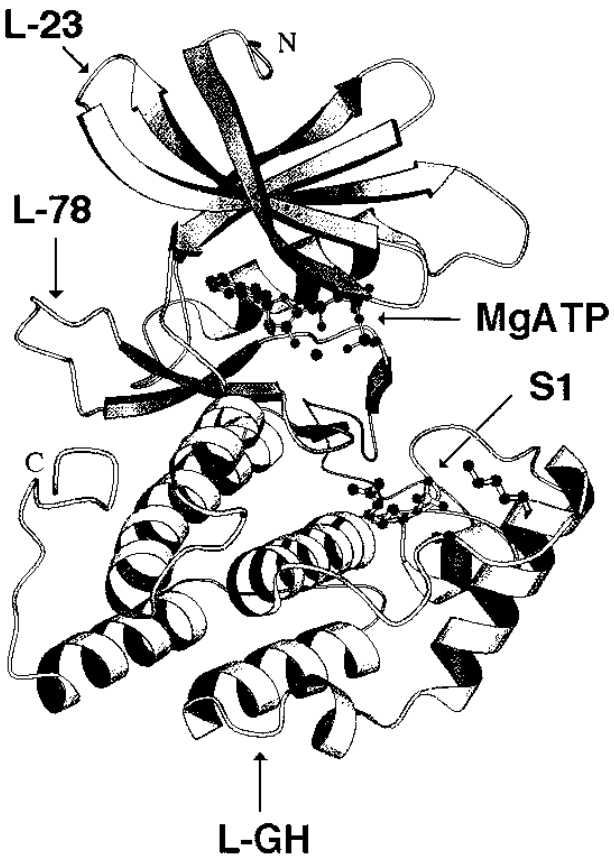
FIG. 8. Functional relationships suggested for Gcs1p and the four *S. cerevisiae* casein kinase I isoforms.

In *yck1*Δ *yck2-2*ts mutant cells at a restrictive temperature, bud initiation and the vectorial transport of vesicular membrane material into the growing bud persist, but two aspects of polarized membrane growth are defective (39). First, the pattern of bud development is aberrant. Insufficient levels of Yck1p-Yck2p lead to hyperpolarized bud growth, showing that the transition from apical to isotropic bud growth (for a discussion, see reference 30) is impaired. Thus, Yck1p-Yck2p may mediate the reorganization that disperses sites of membrane deposition within the bud. Second, these mutant cells are impaired in cytokinesis, the polarized membrane deposition that divides the mother cell cytoplasm from the bud contents. The finding that cytokinesis can take place under some conditions of hyperpolarized bud growth (30) suggests that cytokinesis per se may not depend on the switch from apical to isotropic bud enlargement. Therefore, Yck1p-Yck2p activity may also facilitate the retargeting of membrane growth that drives cytokinesis.

Similar morphogenetic problems are caused by mutation of the B regulatory subunit (Cdc55p) of protein phosphatase 2A (23), and a *cdc55* mutation counteracts the effects of Yck1p-Yck2p, perhaps by increasing protein phosphatase 2A activity to dephosphorylate certain casein kinase I substrates (39). (Likewise, overexpression of the Pph22p catalytic subunit of protein phosphatase 2A produces hyperpolarized buds, although in this situation cytokinesis continues [40]). Our findings support the notion of balanced casein kinase I and protein phosphatase 2A activities: *gcs1* cold sensitivity is suppressed by increasing the dosage of *CDC55*, and thus the abundance of the regulatory subunit Cdc55p, as well as by increasing casein kinase I activity.

Other suppression by Yck1p and Yck2p. An increased *YCK1* or *YCK2* gene dosage permits wild-type cells to grow in media of high salinity without prior adaptation (38). The C-terminal region of Yck2p that specifies membrane association is necessary for this growth, suggesting that phosphorylation at the membrane is important in this effect of casein kinase I. Yeast cells respond to high salinity through the activation of a mitogen-activated protein kinase cascade (2) by a membrane-associated two-component sensor encoded by the *SLN1* and *SSK1* genes (32). Membrane-associated casein kinase I may adapt cells to high-salt conditions through phosphorylation and inhibition of Sln1p (or activation of Ssk1p), thus activating the response pathway. Alternatively, Yck1p-Yck2p could modify another membrane-bound regulator of the mitogen-activated protein kinase cascade (31).

YCK1 and *YCK2* are weak dosage suppressors of growth defects of *snf1* and *snf4* mutant cells (38); Snf1p is a protein kinase necessary for relief of glucose repression (5), while Snf4p is a positive effector of Snf1p function (6, 7). Suppression occurs without a significant increase in extracellular invertase, suggesting that glucose repression is not relieved by increased Yck1p or Yck2p levels. Yck1p-Yck2p may allow hexoses at low concentrations to be metabolized more effectively (38), but more efficient uptake through membrane modification is also consistent with this suppression. Similarly, membrane remodeling may be necessary for the resumption of cell proliferation from stationary phase. The ability of casein kinase I to restore endocytotic function to *gcs1*Δ mutant cells suggests that increased Yck1p-Yck2p activity may facilitate this remodeling process.

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                    β7->              L 78                    β8->
Cki1 (S. pombe) DIKPDNFLg----------rpnskna---------------rniyvvDFG  153
                 |||||||:           |  |                 |:||:|'|
Cki3p           DIKPDNFLIs------qyqrispegkvikscassnndp-----nliymvDFG  186
                 |||||||:          || ||  :|   |: :||     | ::::::||
CKIαL (bovine)  DIKPDNFLmgigrhcnkclesspvgkqkrsmtvstsqqdpefsglnqlfliDFG  180
                ;||| ||||||||||||                          ||||'||
CKIα  (bovine)  DIKPDNFLmgigrhcnk-------                   lfliDFG  151
```

FIG. 9. Insertions in the Yck3p catalytic domain. (Top) The crystal structure (54) of fission yeast Cki1 (50) shows that three inserted sequences in Yck3p, which expand loops L-23, L-78, and L-GH, are predicted to lie adjacent to each other well away from the active site. N and C mark the N and C termini, respectively, of the catalytic domain. The nucleotide substrate in the active-site cleft is also shown. (Bottom) Similarities of the second insertion to that at a similar position in the bovine splice variant αL (42); analogous regions of bovine CKIα (42) and fission yeast Cki1, including Cki1 structural features (β, beta sheet; L, loop), are shown for comparison.

The novel Yck3p isoform. Yck3p, with a molecular weight of 60,234 and an isoelectric point of 9.0, closely follows the domain organization described for the three other budding-yeast casein kinase I isoforms (Fig. 5) (26, 38, 51) but exhibits unique structural features. The Yck3p catalytic domain contains three insertions (Fig. 5). The first of these consists of 17 residues

beginning at position 39. On the basis of the crystal structure of Cki1, a fission yeast casein kinase I isoform (54), this insertion lies in loop L-23, behind the ATP-binding domain (Fig. 9, top). The second insertion comprises approximately 20 residues at residue 154, within loop L-78 in Cki1. In both position and composition this insertion resembles those found in the mammalian splice variants αL (Fig. 9, bottom) (42) and α3 (9). The final insertion, two residues at position 280, expands loop L-GH. The three insertions are adjacent to each other in the folded molecule and form a surface on the back of the protein, well away from the active site. These insertions therefore probably do not influence substrate selectivity but instead modulate Yck3p interactions with regulatory or effector molecules.

The Yck3p C terminus, although hydrophobic in composition and separated from the catalytic domain by a long hydrophilic segment as in Yck1p and Yck2p, is more complex. Instead of a simple CC motif, a consensus sequence for geranylgeranylation that is essential for plasma membrane localization of Yck1p and Yck2p (47), Yck3p terminates with the remarkable sequence CCCCFCCC. The many potential prenylation motifs in this sequence suggest that Yck3p may be modified differently from Yck1p and Yck2p. This feature may in turn affect the subcellular distribution of Yck3p, which does not overlap completely that of any other yeast casein kinase I protein.

Two subgroups of yeast casein kinase I enzymes. The identification of *YCK3* expands the number of *S. cerevisiae* casein kinase I genes to four, which parallels the casein kinase I gene number in the fission yeast *S. pombe* (*hhp1*$^+$, *hhp2*$^+$, *cki1*$^+$, and *cki2*$^+$) (14, 50) and bovine sources (α, β, γ, and δ) (42). *S. pombe hhp1* mutations cause a phenotype that partially overlaps that caused by *hrr25*, suggesting that these proteins might be cognate forms of casein kinase I (14). *hhp2* mutations cause few defects but exacerbate the DNA repair and cell cycle defects of *hhp1* mutations (14). Similarly, *yck3 hrr25* double mutant cells are inviable, suggesting a parallel between *YCK3* and *hhp2*$^+$.

Resumption of cell proliferation from stationary phase differs from outgrowth of a meiotic spore. *gcs1*Δ *yck1*Δ *yck2-2*ts mutant cells grow at permissive temperatures, showing that levels of casein kinase I activity under these conditions are adequate for growth, but spores with the *gcs1*Δ *yck1*Δ *yck2-2*ts genotype fail to form colonies when germinated at a permissive temperature. In the absence of Gcs1p, therefore, the activity of Yck1p-Yck2p in a *gcs1*Δ *yck1*Δ *yck2-2*ts mutant spore is inadequate for germination and outgrowth. We could find no evidence that Gcs1p affects *YCK* gene expression, suggesting that the combined need for casein kinase I activity plus Gcs1p function during outgrowth of a spore is greater than that for mitotic cell growth or for the resumption of cell proliferation from stationary phase.

Analogous differences between outgrowth of spores and mitotic cell growth have been noted by others. Cells devoid of the ubiquitin-conjugating enzyme Ubc1p form tiny colonies upon spore germination, but *ubc1* mutant cells taken from those colonies grow at almost wild-type rates (44): a similar phenotype was found for cells missing Cmk2p, one of two calmodulin-dependent protein kinases (35). Expression of Ssc1p, a mitochondrial hsp70 protein, from the uninduced *GAL7* promoter supports mitotic growth but not the outgrowth of spores (33). Also, mitochondrial mutants that inhibit only the outgrowth of mutant spores have been identified (22). Finally, spore wall mutations can sensitize spores to damage by procedures used for genetic analysis and thereby exert specific inhibition of spore germination (1, 3, 53). Thus, many mechanisms may contribute to the difference seen between (i) spore germination and outgrowth and (ii) the resumption of cell proliferation from stationary phase.

Gcs1p activity at permissive temperatures. The inability of *gcs1* mutant cells to resume proliferation from stationary phase is evident only at low temperatures (15°C); at 29°C, a *gcs1* mutation has no discernible effect on cell growth (16, 28). However, Gcs1p partially alleviates the temperature sensitivity of *yck1*Δ *yck2-2*ts mutant cells, suggesting that Gcs1p functions at normal growth temperatures. This conclusion is consistent with the finding that *gcs1* homozygous diploid cells cannot sporulate at permissive temperatures (16) and extends our appreciation of Gcs1p function to the mitotic cell cycle at normal growth temperatures.

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