Plasma membrane localization of the Yck2p yeast casein kinase 1 isoform requires the C-terminal extension and secretory pathway function

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Summary

The *S. cerevisiae* Yck2 protein is a plasma membrane-associated member of the casein kinase 1 protein kinase family that, with its homolog Yck1p, is required for bud morphogenesis, cytokinesis, endocytosis and other cellular processes. Membrane localization of Yckp is critical for its function, since soluble mutants do not provide sufficient biological activity to sustain normal growth. Yck2p has neither a predicted signal sequence nor obvious transmembrane domain to achieve its plasma membrane localization, but has a C-terminal -Cys-Cys sequence that is likely to be palmitoylated. We demonstrate here that Yck2p is targeted through association with vesicular intermediates of the classical secretory pathway. Yck2p lacking C-terminal Cys residues fails to associate with any membrane, whereas substitution of these residues with a farnesyl transferase signal sequence allows *sec*-dependent plasma membrane targeting and biological function, suggesting that modification is required for interaction with early secretory membranes but that targeting does not require a particular modification. Deletion analysis within the 185 residue C-terminus indicates that the final 28 residues are critical for membrane association, and additional sequences just upstream are required for proper plasma membrane targeting.

Key words: Casein kinase 1, GFP fusion proteins, Protein targeting, *AKR1*

Introduction

Casein kinase I (CK1) protein kinases comprise a large subfamily of acidic serine/threonine-specific protein kinases that is strongly conserved from yeast to humans (Gross and Anderson, 1998). CK1 activities are generally monomeric, suggesting that these enzymes lack regulatory subunit(s). Although a mammalian CK1α on erythrocyte membranes is regulated by membrane content of phosphatidylinositol 4,5-bisphosphate (Brockman and Anderson, 1991), CK1 activities in general are not dependent on second messengers such as cAMP, diacylglycerol and calcium (Tuazon and Traugh, 1991). CK1 enzymes appear to be regulated in two major ways. First, substrate sites may be generated by phosphorylation of upstream residues, which suggests that CK1 enzymes are involved in second messenger-dependent phosphorylation cascades (Roach, 1990). The second mechanism is the spatial restriction of specific isoforms to distinct subcellular compartments.

The budding yeast *Saccharomyces cerevisiae* encodes four CK1 isoforms: Yck1p, Yck2p, Yck3p and Hrr25p (DeMaggio et al., 1992; Hoekstra et al., 1991; Robinson et al., 1992; Wang et al., 1992; Wang et al., 1996). These four enzymes are strongly conserved with their higher eukaryotic counterparts, exhibiting greater than 50% amino acid identity through their catalytic domains. Hrr25p is a nuclear protein that is important for DNA replication and repair (DeMaggio et al., 1992; Hoekstra et al., 1991). Yck3p shares some function with Hrr25, as deletion of *YCK3* in an *hrr25* genetic background is lethal (Wang et al., 1996). However, Yck3p is distributed throughout the cell (Wang et al., 1996). *YCK1* and *YCK2* form a functionally redundant gene pair that is essential for viability (Robinson et al., 1992; Wang et al., 1992). Deletion of either gene does not strongly affect cells, but deletion of both genes causes aberrant cellular morphology and growth arrest (Robinson et al., 1992; Wang et al., 1992).

The Yck1p and Yck2p protein kinases are involved in numerous cellular processes, including bud morphogenesis (Robinson et al., 1993), internalization of plasma membrane permeases (Marchal et al., 2000) and pheromone receptors (Hicke et al., 1998; Panek et al., 1997), and cytokinesis (Robinson et al., 1993). Yck2p is a 62 kDa protein that is tightly associated with the plasma membrane (Vancura et al., 1994), and biological function depends on membrane association. CK1 isoforms generally comprise an N-terminal kinase domain and a highly divergent C-terminal domain that often is responsible for localization (Gross and Anderson, 1998). Subcellular localization is necessary and sufficient for defining the functions of the yeast isoforms (Wang et al., 1996). For example, the nuclear isoform Hrr25p complemented *yck1 yck2* mutant strains when Hrr25p was engineered to contain the Yck2p -Cys-Cys site at its C-terminus (Wang et al., 1996). Conversely, a chimeric protein comprised of the Yck2p

Table 1. Strain list

Strain	Genotype	Source/reference
HPY225	*MAT***a**/*MATα his3/his3 leu2/leu2 ura3-52/ura3-52 end4-1/+*	Diploid strain from a cross of RH1597 (*end4-1*) by LRB759
LRB756	*MATa his3 leu2 ura3-52 yck1-Δ1::ura3 yck2-2^{ts}*	Robinson et al., 1993
LRB758	*MAT***a** *his3 leu2 ura3-52*	Panek et al., 1997
LRB759	*MATα his3 leu2 ura3-52*	Sibling of LRB758
LRB906	*MAT***a** *his3 leu2 ura3-52*	Meiotic progeny of a cross between LRB758 and LRB759
LRB932	*MATα his3 leu2 ura3-52 sec4-2*	Derived from Yeast Genetic Stock Center strain HMSF13 by four serial backcrosses to LRB758
LRB933	*MATα his3 leu2 ura3-52 sec14-3*	Derived from Yeast Genetic Stock Center strain HMSF169 by four serial backcrosses to LRB758
LRB934	*MATα his3 leu2 ura3-52 sec9-4*	Derived from Yeast Genetic Stock Center strain HMSF143 by four serial backcrosses to LRB758
LRB936	*MATα his3 leu2 ura3-52 sec18-1*	Derived from Yeast Genetic Stock Center strain HMSF176 by four serial backcrosses to LRB758
LRB937	*MATα his3 leu2 ura3-52 sec23-1*	Derived from Yeast Genetic Stock Center strain HMSF190 by four serial backcrosses to LRB758
LRB951	*MATα his3 leu2 ura3-52 yck1-Δ1::ura3 yck2-2^{ts}*	Meiotic progeny of a cross between LRB756 and LRB759
LZY103	*MAT***a**/*MATα his3/his3 leu2/leu2 ura3-52/ura3-52 akr1::kan^r/akr1::kan^r*	R. Deschenes, University of Iowa, Iowa City, IA
PBY050	*MAT***a** *his3 leu2 ura3-52 end4-1*	Meiotic progeny of strain HPY225
PBY052	*MAT***a** *his3 leu2 ura3-52 sec23-1 end4-1*	Meiotic progeny of a cross between LRB937 and PBY050

catalytic domain and an Hrr25p region, including its nuclear localization signal, complements an *hrr25* null mutant (Vancura et al., 1994).

Although Yck1p and Yck2p C-termini share very little overall sequence identity, both contain Gln-rich sequences (Robinson et al., 1992; Wang et al., 1992) and a 12 residue C-terminal sequence with 83% sequence identity that terminates with the sequence -Cys-Cys. The two C-terminal Cys residues are essential for Yck2p membrane association and function (Robinson et al., 1993; Vancura et al., 1993; Vancura et al., 1994). Experiments to determine the nature of Yck2p membrane association demonstrated that Yck2p is solubilized only by a combination of salt and nonionic detergent or the use of sodium dodecyl sulfate (Vancura et al., 1993). These findings, in conjunction with the presence of the Cys-Cys motif, led to the proposal that Yck2p is tethered to the inner leaflet of the plasma membrane via prenylation (Vancura et al., 1993). However, the enzymology of the relevant enzyme argues against this possibility (Desnoyers et al., 1996), and it was reported recently that Yck2p localization requires the Akr1 protein (Feng and Davis, 2000) and that Yck2p is a substrate for palmitoyl transferase activity of Akr1p (Roth et al., 2002). It is likely that both terminal Cys residues are modified in this way.

We have been interested in how Yck2p is targeted specifically to the plasma membrane. Palmitoylated proteins with no other lipid modification are generally plasma membrane associated, but there are few examples where the targeting mechanism is understood. Palmitoyl transferase activities have been observed in plasma membrane fractions and to a lesser degree in Golgi membranes (Resh, 1999). However, only recently have proteins with palmitoyl transferase activity been identified, and their distributions within the cell are often at internal membranes (Lobo et al., 2002; Roth et al., 2002). Two potential mechanisms of plasma membrane association of palmitoylated proteins are that they first associate with the plasma membrane and become modified there, or that they are modified at an internal membrane and then targeted to the plasma membrane. The SNAP-25 protein, for example, is thought to utilize the secretory pathway for targeting (Gonzalo and Linder, 1998; Loranger and Linder, 2002). The palmitoyl moiety itself could provide a plasma membrane targeting signal, since palmitoylation-deficient Ras proteins fail to move from internal membranes to the plasma membrane (Apolloni et al., 2000; Choy et al., 1999).

We previously reported that Yck2p is differentially enriched at sites of polarized secretion during the cell cycle (Robinson et al., 1999). The localization pattern we observed resembles that of proteins directed to the plasma membrane via the classical secretory pathway. Here, we report that secretory function, including ER-Golgi trafficking, is necessary for Yck2p plasma membrane localization. Furthermore, we show that the two terminal Cys residues are necessary but insufficient for proper Yck2p targeting; C-terminal sequences upstream of the Cys residues are required to direct GFP-Yck2p plasma membrane targeting.

Materials and Methods

Yeast strains used for this work are listed in Table 1. All LRB, HPY and PBY strains are closely related or differ only at the *YCK* loci. *sec* mutant strains derived from Yeast Genetic Stock Center strains by serial backcrosses were chosen for Gal$^+$ phenotype. Yeast were cultured in standard media (Sherman et al., 1986). Rich media (yeast extract, peptone) and synthetic media (yeast nitrogen base and amino acid supplement) were prepared with 2% carbon source. *YCK2* alleles were tested for function by testing for complementation of the temperature-sensitive growth and morphology of strain LRB951 (*yck1 yck2^{ts}*). Yeast transformation was carried out by a LiOAc procedure (Gietz et al., 1992), modified in the following two ways. Standard rich media or synthetic media were used to grow cells to OD at 600 nm of 0.6-0.9. Calf thymus DNA was used as carrier DNA because it produced highest efficiency transformation for strains of this genetic background.

DNA manipulation

E. coli strains DH5α and XL1blue were used for plasmid amplification and subcloning. Restriction enzymes (Promega; American Allied Biochemicals), Klenow fragment of DNA polymerase 1 (Promega) and DNA ligase (New England Biolabs) were used according to manufacturer's recommendations. Plasmid DNA was purified either by an alkali lysis method or by a rapid boiling preparation (Taylor et al., 1993). For DNA sequence analysis, either preparation was further purified by RNase treatment followed by precipitation from polyethylene glycol 8000. PCR amplification was carried out with Bio-X-Act polymerase (Bioline) using a Perkin-Elmer 9600 or a GeneAmp 2400 (Applied Biosystems) thermocycler. DNA sequence analysis of cloned PCR products and of mutagenesis products was carried out either manually, using the Sequenase (US Biochemical) dideoxy chain

Table 2. Plasmid list

Plasmid	Characteristics	Source/reference
pUCΔSal	pUC19 lacking *Sal*I site	This study
pJB9	YCp50, *GAL1* promoter	This study
pJB1	YCp50, *GAL1* promoter, *GFP:YCK2* ORF	Robinson et al., 1999
pJB2	YCp50, *GAL1* promoter, *GFP:yck2*Cys^{545,546}Ser	Robinson et al., 1999
pJB4-4	pUC19ΔEco, *GFP:YCK2* ORF	Robinson et al., 1999
pJB6.3	pRS316, *GFP:yck2*Cys^{545,546}Ser	This study
pLR10	pUC19ΔSal, *GFP:YCK2*	This study
pL2.35	YEp352, *GFP:YCK2*	This study
pL2.99	pUC19ΔEco, *YCK2*	Robinson et al., 1999
pL210	pUC19ΔEco, *yck2Δ2-360*, ORF	This study
pL211	pUC19ΔEco, *GFP: yck2Δ2-360*, ORF	This study
pL212	YCp50, *GAL1* promoter, *GFP: yck2Δ2-360* ORF	This study
pL221	pUC19ΔEco, *GFP: yck2Δ2-360*	This study
pL222	pRS316, *GFP: yck2Δ2-360*	This study
pL230	pUC19ΔEco, *GFP:yck2Δ397-532*	This study
pL240	YCp50, *GAL1* promoter, *GFP:YCK2/YCK1* ORF	This study
pL241	pUC19ΔSal, *GFP:YCK2/YCK1*	This study
pL242	pRS316, *GFP:YCK2/YCK1*	This study
pL250	pUC19ΔEco, *GFP:YCK2-CIIS* ORF	This study
pL251	YCp50, *GAL1* promoter, *GFP:YCK2-CIIS* ORF	This study
pPB2	pRS316, *GFP:YCK2*	This study
pPB4.1	pUCΔEco, *GFP:yck2Δ519-527*	This study
pPB5	pUCΔEco, *GFP:yck2Δ471-498*	This study
pPB15	YCp50, *GAL1* promoter, *GFP:yck2Δ519-527*	This study
pPB16	YCp50, *GAL1* promoter, *GFP:yck2Δ471-498*	This study
pPB18	pUC19ΔSal, *YCK2*	This study
pPB19	pUC19ΔSal, *YCK2* with *Bam*HI site at ORF 5′ end	This study
pPB21	pUC19ΔSal, *YCK2* with *Sal*I site at ORF 3′ end	This study
pPB23	pUC19ΔSal, *YCK2*; *Bam*HI and *Sal*I sites flank ORF	This study
pPB24	pUC19ΔSal, *GFP:yck2Δ519-527*	This study
pPB25	pUC19ΔSal, *GFP:yck2Δ471-498*	This study
pPB51	pUC19ΔSal, *GFP:yck2Δ413-444*	This study
pBF1	pRS316, *GFP:yck2*Cys^{545,546}Ser	This study
pBF2	pRS316, *GFP:yck2Δ519-527*	This study
pSJ13	pRS316, *GFP:yck2Δ528-540*	This study
pSJ14	YCp50, *GAL1* promoter, *GFP:yck2Δ528-540*	This study
pSJ15	YCp50, *GAL1* promoter, *GFP:yck2Δ499-518*	This study
pSJ16	pRS316, *GFP:yck2Δ499-518*	This study
pSJ23	pRS316, *GFP:yck2Δ397-532*	This study

termination method, or by automated sequencing (Iowa State University DNA Sequencing and Synthesis Facility or Retrogen).

Construction of plasmids and mutant alleles

Plasmids used for this work are listed in Table 2. High copy plasmid pL2.35 (YEp352:*GFP:YCK2*) was constructed by cloning the *Xba*I-*Sac*I fragment, containing the *GFP:YCK2* fusion gene with flanking sequences, from pL2.991 [pUCΔEco:*GFP:YCK2* (Robinson et al., 1999)] into YEp352 (2μ, *URA3*).

The pJB9 plasmid was constructed to allow expression of *YCK* variants from the *GAL1* promoter. An *Eco*RI-*Bam*HI *GAL1* promoter fragment was digested from plasmid pGal-*CLB5* (kindly provided by C. Wittenberg) and cloned directly into YCp50. *YCK* variant open reading frames (ORFs) were generated with 5′ *Bam*HI and 3′ *Sal*I sites, and these sites were used for cloning all *YCK2* ORF variants into pJB9.

The pPB23 plasmid was constructed as a cassette vector to place any *GFP:YCK2* variant ORF in the context of natural *YCK2* flanking sequences. The *Sal*I site of pUC19 was destroyed by *Sal*I digestion followed by a fill-in reaction with Klenow fragment, yielding pUC19ΔSal. The *YCK2* gene with flanking sequences was cloned into pUC19ΔSal on an *Xba*I-*Sac*I fragment, yielding plasmid pPB18. A *Bam*HI site was introduced into pPB18 following the *YCK2* ATG codon by inverse PCR with primers PB13 and PB14 (Table 2), yielding plasmid pPB19. A *Sal*I site was also introduced into pPB18 following the *YCK2* stop codon by inverse PCR using primers PB15 and PB16 (Table 2), yielding plasmid pPB21. Finally, the pPB21 *Hin*dIII-*Sac*I fragment with the introduced *Sal*I site was swapped into pPB19, yielding plasmid pPB23.

The *yck2Δ2-360* allele encodes residues 361 to 546, lacking all catalytic domain-encoding sequences. This allele was generated by PCR using primers Y2CORF1A and Y2ORF2X (Table 3) on template pL2.99. The product was cloned into pUCΔEco (Robinson et al., 1999) to yield pL210. After correct *YCK2* sequence was confirmed, the F64L S65T GFP allele was cloned into the *Eco*RI site following the initiating ATG of *yck2Δ2-360*, yielding plasmid pL211. The GFP fusion gene was then cloned into pJB9 for expression from the *GAL1* promoter, yielding plasmid pL212. The *Bam*HI-*Sal*I fragment was also swapped into pPB23 to place *GFP:yck2Δ2-360* under control of the *YCK2* promoter, yielding plasmid pL221. The *Xba*I-*Sac*I fragment containing the fusion allele was cloned into the low copy vector pRS316, yielding plasmid pL222.

The *yck2Δ397-532* mutant allele, lacking all but the final 14 residues of the C-terminal domain (CTD), was constructed by inverse PCR. Primers YCK-CG1 and YCK-CG4 (Table 3) were designed to add *Hin*dIII sites after codons 396 and 532, respectively. PCR with these primers on template pJB4-4 (*GFP:YCK2* in pUCΔEco) resulted in a linear product with terminal *Hin*dIII sites lacking codons 397 to 532. This product was digested with *Hin*dIII and religated to yield plasmid pL230. The entire *yck2Δ397-532* allele was digested from this plasmid with *Xba*I and *Sac*I and cloned into pRS316 to yield plasmid pSJ23.

Table 3. PCR and mutagenic oligonucleotide primers

Primer name	Sequence
Y2CORF1A	5′ CCCGGATCCATGGAATTCAAGCTGAATGGTGGCCGTGG 3′
Y2ORF2X	5′ GCTCGAGGTCGACCTAACAGCATCCTAG 3′
YCK-CG1 (*yck2Δ397-532*)	5′ CGCAAGCTTAACAAATCATCGAAAGG 3′
YCK-CG4 (*yck2Δ397-532*)	5′ CGCAAGCTTACTTCTATGCCTTTTTG 3′
PB5 (*yck2Δ519-527*)	5′ CAACCAACGGCGAACCAAGATAGAAATAG 3′
PB6 (*yck2Δ519-527*)	5′ CTATTTCTATCTTGGTTCGCCGTTGGTTG 3′
PB13	5′ TGCGGATCCATGGAATTCTCTCAAG 3′
PB14	5′ TGCGGATCCTTTTGGAAAACTATTTTC 3′
PB15	5′ TCTAGAGTCGACAATAGAAAACGGAGG 3′
PB16	5′ TCT AGA GTC GAC CTA ACA GCA TCC TAG 3′
Y2ORF1G1	5′ CGAGGATCCATGGAATTCATGAGTAAAGG 3′
Y2CAAX2 (*YCK2*-CIIS)	5′ CAAAGTCGACCTAAGAGATTATACAGCATCCTAGCTTAC 3′
Y2DGANG-F (*yck2Δ499-518*)	5′ CCAAACAGTTTGCCGACGAACAAAACGC 3′
Y2DGANG-R (*yck2Δ499-518*)	5′ GCGTTTTGTTCGTCGGCAAACTGTTTGG 3′
Y2DQDRN-F (*yck2Δ528-540*)	5′ GCTAAAAACGCAGCGAGTAAGCTAGGATGC 3′
Y2DQDRN-R (*yck2Δ528-540*)	5′ GCATCCTAGCTTACTCGCTGCGTTTTTAGC 3′
Y2/1 MID 5′	5′ CTACTTGCATGCAACAAACAGCTCCAAATG 3′
Y2/1- MID 3′	5′ CCTACCGCATGCTCTATGCCTTTTTG 3′
Y2/1-3′	5′ CCACTCGAGGTCGACTTAGCAACAACCTAA 3′

Mutant alleles encoding C-terminal deletions were constructed using the QuikChange mutagenesis kit (Stratagene) with pJB4-4 (*GFP:YCK2* ORF fusion in pUCΔEco) as template. Primers are listed in Table 3. In each case, the mutant *GFP*:*YCK2* ORF was cloned into pJB9 for expression from the *GAL1* promoter. For expression from the *YCK2* promoter, each mutant ORF was cloned into pPB23. The entire allele then was cloned on an *Xba*I-*Sac*I fragment into pRS316. The *GFP:YCK2-CIIS* ORF was generated by PCR using primers Y2ORF1G1 and Y2CAAX2 (Table 3) on template pJB4-4. The PCR product was digested and the resulting fragment was ligated into pUC19ΔEco. Among products with correct sequence was plasmid pL250. The *YCK2* ORF was excised from pL250 and cloned into pJB9 for expression from the *GAL1* promoter, yielding plasmid pL251.

To construct the *YCK2/YCK1* chimera, two separate PCR products were generated that encode GFP fused to the Yck2p catalytic domain and the Yck1p C-terminal domain. The *YCK2* product, with *Bam*HI site at the 5′ end and *Sph*I site at the 3′ end, was generated on template pLR10 (*GFP:YCK2* in pUC19ΔSal) with primers Y2ORF1G1 and Y2/1-mid5′. The *YCK1* product, with *Sph*I site at the 5′ end and *Sal*I site at the 3′ end, was generated using primers Y2/1-mid3′ and Y2/1-3′ with pLJ721 [*YCK1* in YEp352 (Robinson et al., 1992)] as template. Purified products were digested with *Bam*HI and *Sph*I (*YCK2* fragment) or *Sph*I and *Sal*I (*YCK1* fragment). The resulting fragments were ligated together in the presence of *Bam*HI and *Sal*I-digested pJB9. Ligation products with intact *YCK2* and *YCK1* sequences included pL240. For expression from the *YCK2* promoter, the GFP fusion ORF was swapped into pPB23, yielding pL241. The *Xba*I-*Sac*I fragment containing the chimera with *YCK2* flanking sequences was cloned into pRS316, yielding plasmid pL242.

Protein analysis

For induction of GFP fusion expression from the *GAL1* promoter, cells were first grown in synthetic media containing 2% raffinose as carbon source. Galactose was then added to cultures to a final concentration of 2%, and cultures were incubated at indicated temperature, removing samples for microscopic observation and/or for protein isolation at times indicated.

For immunoblot analysis, total protein extracts were prepared by TCA precipitation (Davis et al., 1993) from cells grown to OD at 600 nm of 0.5-0.9. Protein extracts were separated by electrophoresis through pre-cast denaturing SDS-polyacrylamide gels (Bio-Rad and Fisher Scientific). Gels were blotted to nitrocellulose and blots were probed with affinity-purified antiserum against GFP (kindly provided by J. N. Davis, Louisiana State University Health Sciences Center, Shreveport). Horseradish peroxidase-conjugated secondary antisera (Sigma) and the ECL chemiluminescence kit (Amersham) were used to detect the primary antibody. To control for loading, blots were stripped and reprobed for phosphoglycerate kinase (PGK), using a monoclonal antibody (Molecular Probes).

Fluorescence microscopy

Microscopy to examine GFP fluorescence in live cells was performed using an Olympus AX70 Provis microscope equipped for differential interference contrast optics and epifluorescence, as described previously (Robinson et al., 1999).

Results

Yck2p targeting to the plasma membrane requires secretory pathway function

The distribution of Yck2p within the plasma membrane changes during the cell cycle, being enriched specifically at small buds and at the bud neck at cytokinesis (Robinson et al., 1999). These sites are associated with polarized secretion (Botstein et al., 1997). Therefore, we investigated whether Yck2p utilizes the secretory pathway for transport to the plasma membrane. To monitor the localization of Yck2p within live cells over time, we used inducible *GFP*:*YCK2* fusion constructs. *GFP:YCK2* fusion alleles were placed under the control of the galactose-inducible *GAL1* promoter in a low copy (centromere-based) vector (Robinson et al., 1999).

Induction of GFP-Yck2p synthesis in a wild-type strain was monitored by immunoblot analysis and fluorescence microscopy at intervals after addition of galactose. As shown in the immunoblot in Fig. 1A, the fusion protein is below detection level in cells grown on glucose (left panel, lane 1) and on the derepressing carbon source raffinose (t=0 minutes; right panel). Following addition of galactose to derepressed cultures, GFP-Yck2p is detectable at low levels after 30 minutes; after 90 minutes the level of fusion protein is comparable with the steady-state level in cells expressing the fusion protein from the *YCK2* promoter at its endogenous chromosomal locus (Fig. 1A, compare lane 2, left panel, with 90 minutes lane in right panel). Further induction results in levels comparable with those in cells expressing the fusion

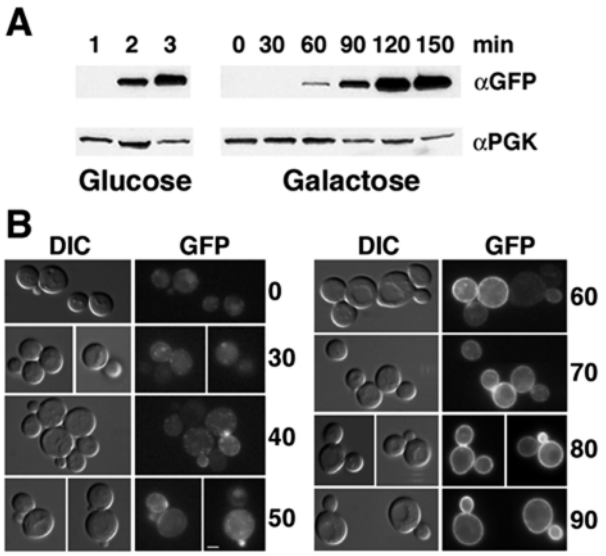

Fig. 1. GFP-Yck2p induction results in accumulation of intracellular punctate fluorescence before localization to the plasma membrane. (A) Induction of GFP-Yck2p was monitored by immunoblot analysis (Panek et al., 1997) using an affinity-purified antiserum against GFP. Detection in extracts from glucose-grown cells is shown in the left panels, for pJB1 (pGal:*GFP:YCK2*; lane 1) in wild-type strain LRB939, for GFP-Yck2p expressed from a chromosomal copy under the control of the *YCK2* promoter in strain LRB913 (lane 2), and from a high copy (2μ) plasmid (pL2.35; lane 3) in LRB939. For the right panels, cells were grown at 30°C in synthetic medium containing 2% raffinose (derepressing conditions) and galactose was added to 2% at the 0 minute time point. Extracts were prepared from culture aliquots taken at the time points indicated. Antiserum against phosphoglycerate kinase (PGK) was used to reprobe the blots for loading control. (B) DIC and fluorescence images of living cells treated as in B were captured at the indicated time points. Bar, 2 μm.

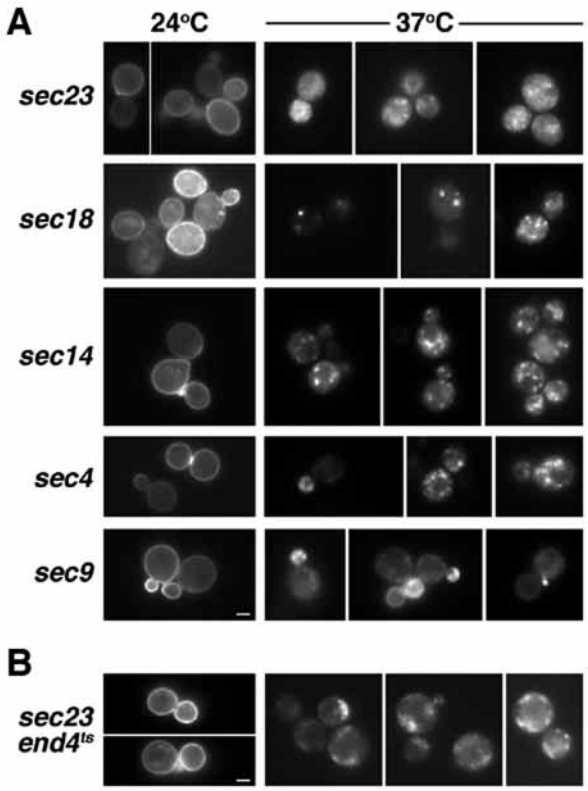

Fig. 2. GFP-Yck2p fails to reach the plasma membrane in secretory pathway-defective strains. Cells of strains LRB936 (*sec18*), LRB937 (*sec23-1*), LRB933 (*sec14-3*), LRB932 (*sec4-2*), LRB934 (*sec9-4*) (A) and PBY052 (*sec23-1 end4-1*) (B) carrying pJB1 (pGAL1:*GFP:YCK2*) were grown to log phase at 24°C in synthetic media containing 2% raffinose as a derepressing carbon source. Galactose was added to 2% and cultures were split, with half of each culture incubated at permissive (24°C) and restrictive (37°C) temperatures. Fluorescence images of live cells were captured after 90 minutes incubation at the indicated temperature. Bars, 2 μm.

protein from a high copy (2μ) plasmid (Fig. 1A, compare lane 3 with the 120 minutes time point).

Microscopic observation of GFP-Yck2p induction revealed a similar time course. Fluorescent signal detectable above background was observed first at 30 minutes of induction (Fig. 1B). At time points up to 60 minutes, GFP fluorescence was mainly cytosolic and punctate, but was brightest at sites of polarized growth, below the membrane of small buds and the bud necks of dividing cells. At 60 minutes, the plasma membrane was labeled in 40-50% of the cells (n=200). At 90 minutes of induction, cells resembled those expressing the fusion from the *YCK2* promoter at steady state. All detectable fluorescence was plasma membrane associated and was enriched at small bud membranes and at the necks of dividing cells. These results are consistent with the idea that Yck2 protein associates with internal membranes early after synthesis and remains associated with membrane-limited compartments during targeting to the plasma membrane.

To test directly whether vesicle-mediated secretory pathway function is required for GFP-Yck2p targeting to the plasma membrane, we introduced the pGal:*GFP*:*YCK2* plasmid (pJB1) into each of eight conditional secretory pathway-defective [*sec* (Novick et al., 1980)] mutants. These temperature-sensitive (ts) mutations block secretory traffic at discrete steps upon shift to restrictive temperature but protein synthesis continues, resulting in accumulation of protein at the affected compartment. The *sec* mutants used here cause vesicle trafficking blocks at the following steps: vesicle budding from the ER [*sec12*, *sec23* (Barlowe et al., 1994)]; consumption of ER-derived vesicles at the Golgi [as well as fusion of vesicles at all subsequent steps; *sec18* (Eakle et al., 1988)]; trafficking through the Golgi [*sec14* (Bankaitis et al., 1989)]; and docking and fusion of secretory vesicles with the plasma membrane [*sec1* (Egerton et al., 1993); *sec4*, *sec8* (TerBush and Novick, 1995); and *sec9* (Brennwald et al., 1994)]. The location of fluorescence was examined after induction of GFP-Yck2p synthesis at permissive and restrictive temperature in each mutant.

Induction of GFP-Yck2p synthesis in each mutant at permissive temperature resulted in plasma membrane localization (Fig. 2A shows *sec18*, *sec23*, *sec14*, *sec4* and *sec9*). However, after 90 minutes of induction at the restrictive temperature, cells of each of the *sec* mutants showed only punctate intracellular fluorescence. The internal fluorescence patterns at restrictive temperature were generally consistent with patterns predicted by the nature of the blocks, although *sec23* cells did not always show perinuclear fluorescence

consistent with ER accumulation. Perinuclear fluorescence was observed in fewer than 20% of *sec23* cells, with the majority of cells showing bright dots. This pattern is more reminiscent of endosomal labeling than of ER structures. Since the length of the shift to restrictive temperature is relatively long, it was possible that we were not observing a primary result of the *sec23* block in these cells, but instead, a secondary effect on recycling from the plasma membrane, as documented in this mutant for the plasma membrane SNARE protein Snc1p (Lewis et al., 2000). If this were the case, it would suggest that Yck2p is modified at the plasma membrane but is rapidly recycled, and that our *sec* blocks revealed only a block to this recycling.

To test this possibility, we performed an identical shift with a strain mutant for both *sec23* and *end4*. Endocytosis is blocked in the *end4-1* mutant at 37°C (Raths et al., 1993), which is also the restrictive temperature for *sec23-1*. If the Yck2 protein is associated with early secretory membranes after synthesis rather than with endosomal or Golgi membranes after internalization from the plasma membrane, we expected that we should observe a pattern identical to that observed for cells of the single *sec23* mutant. By contrast, if recycling results in the pattern observed for the single *sec23* mutant, we expected to observe plasma membrane accumulation in the double *sec23 end4* mutant. As shown in Fig. 2B, double mutant cells appear identical to single *sec23* mutant cells at both 24 and 37°C, indicating that the block to ER exit likely results in accumulation of GFP-Yck2p on an early secretory membrane. Perinuclear fluorescence may not be observed often in these cells at least partly as a result of changes to organization of early secretory membranes that occur after a prolonged shift to restrictive temperature (Lewis et al., 2000).

Hydrophobic modification of Yck2p is required for secretory membrane association

Plasma membrane association and biological function of Yck2p and GFP-Yck2p require the two terminal Cys residues (Robinson et al., 1999; Robinson et al., 1993; Vancura et al., 1994). There is now strong evidence that the modification on one or both Cys residues is palmitate, with addition catalyzed by the Akr1 protein (Roth et al., 2002). By two hybrid analysis, Akr1p has been reported to interact not only with plasma membrane proteins of the mating signal transduction pathway (Givan and Sprague, 1997; Kao et al., 1996; Pryciak and Hartwell, 1996), but also with Gcs1p (Kao et al., 1996), which functions at ER and/or Golgi membranes (Poon et al., 1999). Thus, Yck2p could be modified at a secretory membrane, or could associate with a targeting protein in an unmodified state and transit to the plasma membrane for modification.

Preliminary immunofluorescence analysis of a tagged Akr1p suggested location on intracellular membranes (Roth et al., 2002), but the identity of the membrane(s) is not yet clear. If Yck2p requires modification before association with secretory membranes, then no association of a Yck2-Cys^{545,546}Ser mutant protein should occur. To test this, we expressed the GFP-Yck2-Cys^{545,546}Ser fusion protein (Fig. 3) in early (*sec23*) and late (*sec9*) acting *sec* mutants. In neither case was the GFP-Yck2-Cys^{545,546}Ser protein obviously associated with intracellular structures at permissive or restrictive temperature (Fig. 4; GFP-Yck2-Cys^{545,546}Ser panels). Thus, interaction of Yck2p with secretory membranes requires the C-terminal Cys

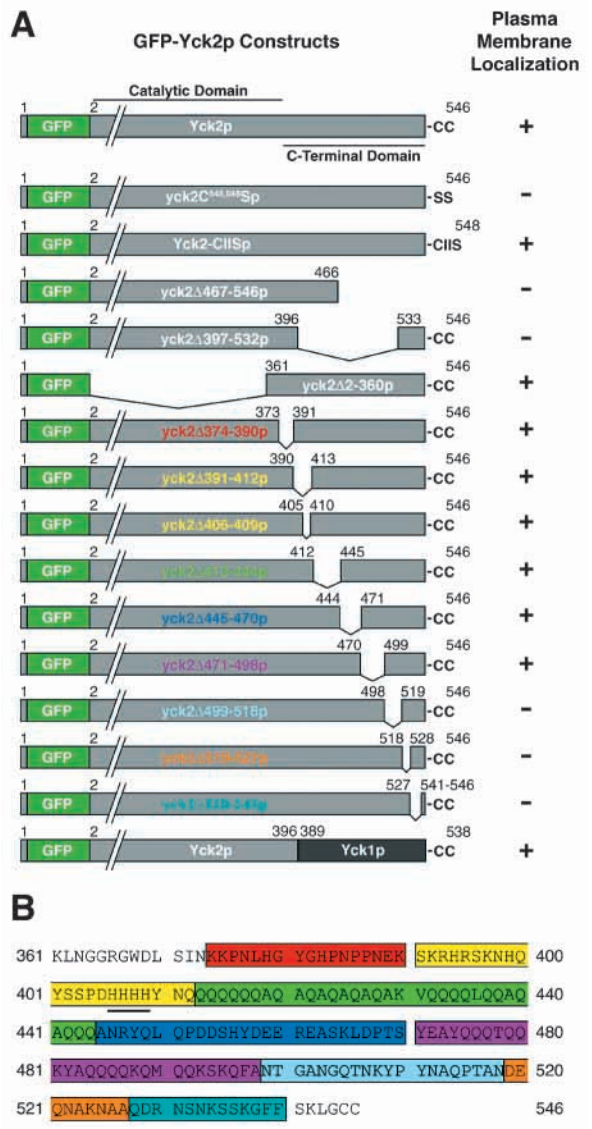

Fig. 3. GFP-Yck2p deletion mutants. (A) Illustration of full length GFP-Yck2 fusion protein, C-terminal substitution mutants (Cys^{545,546}Ser; CIIS), C-terminal truncation mutant, C-terminal deletion mutant with intact -Cys-Cys motif, catalytic domain deletion mutant, and consecutive smaller C-terminal deletions. GFP portions are shown in green and Yck2p portions in gray. Plasma membrane localization (+) or mislocalization (–) is indicated to the right of each fusion protein. Color coding for smaller deletions matches that in panel B. (B) Yck2p C-terminal amino acid sequence with deleted regions indicated as follows: yck2Δ374-390p in red; yck2Δ391-412p in yellow, yck2Δ406-409p with underscore; yck2Δ413-444p in green; yck2Δ445-470p in dark blue; yck2Δ471-498p in violet; yck2Δ499-518p in light blue; yck2Δ519-527p in orange; yck2Δ528-540p in turquoise.

residues, which are probably modified before targeting through the pathway.

As mentioned previously, Yck2p is probably modified by Akr1-mediated palmitoylation. Palmitoylation affects protein-protein interactions between a number of partners (Dunphy and Linder, 1998; Resh, 1999), so Yck2p targeting could require palmitoylation at the C-terminus. We tested whether a CAAX

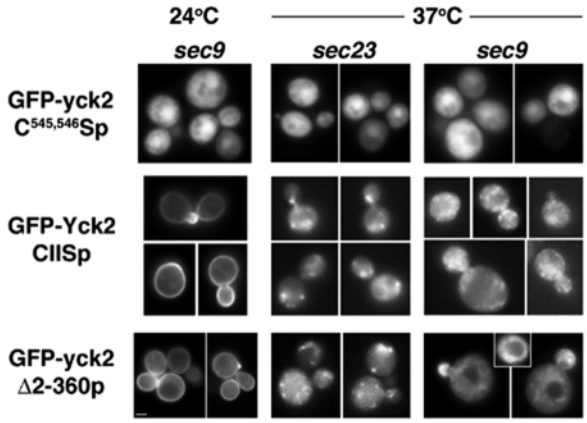

Fig. 4. Localization of Yck2p C-terminal variants in *sec* mutants at permissive and restrictive temperatures. Cultures of strains LRB936 (*sec18*) and LRB934 (*sec9*) carrying p*GAL1* plasmids expressing the indicated Yck2p variants were grown and treated as described for Fig. 2, except that the induction period was 120 minutes before images were captured. Bar, 2 μm.

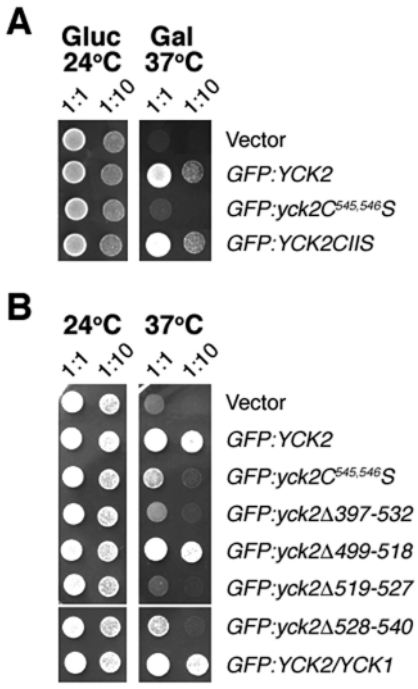

Fig. 5. Complementation analysis of Yck2p C-terminal variants. (A) Galactose-inducible plasmids were introduced into *yck^{ts}* strain LRB951 and cultures were grown in selective medium containing 2% raffinose as the carbon source, and then plated onto medium containing 2% galactose. (B) Plasmids encoding the indicated Yck2p variants were introduced into the *yck^{ts}* strain LRB951. Transformants were grown to mid-log phase in media selective for plasmid retention and 5 μl drops of each culture and of 1/10 diluted cultures were plated onto selective agar media at the indicated temperature. Plates were incubated for 24 (glucose) or 48 (galactose) hours at 24°C or at 37°C before photographing.

signal sequence that directs addition of a single farnesyl group by the farnesyl transferase can substitute for the -Cys-Cys sequence. We used -Cys-Ile-Ile-Ser, the Ras2p terminal sequence, to direct farnesyl modification. The GFP-Yck2-CIISp protein (Fig. 3) provides Yck function when expressed from the Gal promoter, as indicated by complementation of the temperature-conditional *yck^{ts}* mutant (*yck1-Δ1 yck2-2^{ts}*; Fig. 5A). Although there is some vacuolar membrane fluorescence, probably due to overexpression, the GFP-Yck2-CIIS protein localizes primarily to the plasma membrane (Fig. 4, GFP-Yck2-CIIS 24°C panels), consistent with the complementation data.

To confirm that modification of the Yck2-CIIS variant differed from that of wild-type Yck2p, GFP-Yck2-CIISp was expressed in strain LZY103, which is deleted for *AKR1*. While GFP-Yck2p is not detectably present at any membrane in this strain, GFP-Yck2-CIISp is plasma membrane associated in LZY103 cells, as it is in wild-type cells (Fig. 6). These results confirm that the Yck2-CIIS protein does not require Akr1p function for membrane association.

Interestingly, plasma membrane localization of the GFP-Yck2-CIIS fusion protein is blocked in secretory mutants at restrictive temperature (37°C; *sec23* and *sec9* cells are shown in Fig. 4), indicating that this protein can associate with secretory membranes and probably follows a similar trafficking route to wild-type Yck2p. These results show that different C-terminal modification does not abolish plasma membrane targeting of Yck2p via the secretory pathway, and further indicate that Yck2p sequences in addition to the two Cys residues determine the targeting route.

The Yck2p C-terminal third is necessary and sufficient for modification and targeting

To determine which Yck2p sequences are important for modification and for targeting via the secretory pathway, we carried out deletion analysis. We first expressed an N-terminally truncated GFP-Yck2 fusion protein that lacks the entire catalytic domain but retains the wild-type C-terminal 185 residues (GFP-yck2Δ2-360; Fig. 3). It was reported previously that replacing the C-terminal sequences of Hrr25 with those of Yck2p resulted in a fusion protein that could function as Yck2p (Wang et al., 1996). As predicted by these results, the GFP-yck2Δ2-360 fusion protein is targeted to the plasma membrane (Fig. 4, GFP-yck2Δ2-360, 24°C panels). As observed for the full-length Yck2 protein, targeting of the truncated yck2Δ2-360 protein requires secretory pathway function. Both *sec9* and *sec23* cells incubated at restrictive temperature show only internal fluorescence for this mutant (Fig. 4, GFP-yck2Δ2-360, 37°C panels). These results demonstrate that the sequences sufficient for secretory membrane association and plasma membrane targeting are contained within the C-terminal third of the Yck2 protein.

To determine whether the entire 185 amino acid C-terminal sequence is required for targeting, we deleted 137 amino acids beyond the catalytic domain, moving up the final 14 amino acids to the end of the catalytic domain (*yck2Δ397-532*; Fig. 3). The final 14 amino acids were left because they include the two Cys residues and also residues shared with Yck1p. The resulting GFP fusion protein fails to complement the *yck^{ts}* conditional mutant (Fig. 5B), and the majority of its fluorescence is not associated with the plasma membrane. As shown in Fig. 7, fluorescence from this fusion protein was

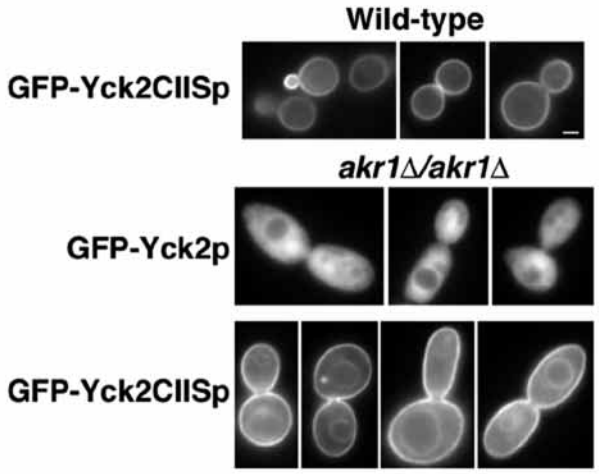

Fig. 6. A Yck2p variant carrying an FTase signal instead of the -Cys-Cys signal does not require Akr1p for membrane association. Plasmids pJB1 (pGal:*GFP:YCK2*) and pL251 (pGal:*GFP:YCK2-CIIS*) were introduced into strains LRB906 (wild-type) and LZY103 (*akr1Δ*/*akr1Δ*). Transformants were grown to mid-log phase at 30°C in selective medium with 2% raffinose as the carbon source, then galactose was added to 2% and cultures were incubated for 2 hours before fluorescence images were captured. Bar, 2 μm.

generally soluble signal. Thus, residues within the C-terminus, in addition to the terminal Cys-Cys sequence and the 12 residues upstream, are probably necessary for modification.

Sequences proximal to the terminal Cys residues are required for modification and targeting

Aside from Gln-rich sequences, the C-terminal domain of Yck2p shares no obvious sequence similarity with known proteins, and the *S. pombe* homolog Cki1p was crystallized without this domain (Carmel et al., 1994; Xu et al., 1995). Therefore, we made a series of consecutive deletions throughout the C-terminal 185 residues to uncover sequences important for modification and/or localization (Fig. 3). We deleted a sequence shared with Yck1p (*yck2Δ374-390*), a sequence that includes a group of four consecutive His residues (*yck2Δ391-412*), the four His residues separately (*yck2Δ406-409*), the two Gln-rich tracts (*yck2Δ413-444*, and *yck2Δ471-498*), the sequences between these Gln-rich tracts (*yck2Δ445-470*), a sequence that could form an α-helix (*yck2Δ519-527*), and two additional regions close to the terminal Cys residues (*yck2Δ499-518* and *yck2Δ528-540*).

Each deletion allele was expressed as a GFP fusion from the *GAL1* and *YCK2* promoters in wild-type and *yck*^{ts} strains, and products were assayed for localization and for complementation of loss of Yck activity, respectively. We expected one of three outcomes for effect on localization: no effect, indicating that the sequence lost is dispensable for modification and targeting; increased cytosolic fluorescence, suggesting that the deleted sequence is important or necessary for modification; or accumulation on internal membranes, suggesting that the deleted sequence is important for targeting to secretory membranes. The latter two outcomes are not mutually exclusive, so we expected that mutants could exhibit both defects, which could indicate roles for such sequences in both modification and targeting. To classify the mutants, all were

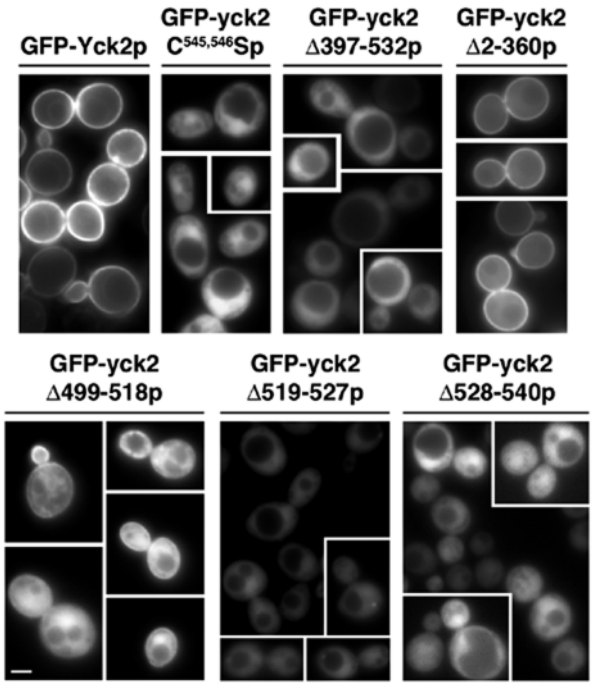

Fig. 7. GFP-Yck2p C-terminal deletion mutants are mislocalized. Galactose-inducible plasmids encoding the indicated fusion proteins were introduced into wild-type strain LRB906 by transformation. Cells were grown on solid selective medium containing galactose as a carbon source at 30°C overnight, and were suspended in selective medium for capture of fluorescence images. Bar, 2 μm.

compared to the GFP-Yck2Cys^{545,546}Ser mutant protein and to GFP-Yck2p trapped internally by imposition of a *sec* block.

None of the deletions closest to the catalytic domain, including the region shared with Yck1p and the two Gln-rich tracts, affected membrane association, plasma membrane targeting or biological function (L.C.R., R.T.C., B.M.F. and P.B., unpublished). Thus, these sequences are dispensable for modification and targeting. However, the three deletions removing sequences closest to the terminal Cys residues, *yck2Δ499-518*, *yck2Δ519-527* and *yck2Δ528-540*, each impaired plasma membrane association. The most upstream of these sequences appears important for both modification and targeting (Fig. 7). Cells from log phase cultures expressing GFP-yck2Δ499-518p showed mainly soluble and particulate internal fluorescence, but some fluorescence was observed at the plasma membrane. This mutant allows growth of the *yck*^{ts} strain at restrictive temperature (Fig. 5B), possibly due to accumulation of the mutant protein at the plasma membrane over time. Up to 50% of cells taken from overnight plate cultures showed approximately equal levels of plasma membrane and internal fluorescence (L.C.R., unpublished).

The nine codon deletion in *yck2Δ519-527* had the most dramatic effect on the resulting protein. This sequence appears to be essential for modification (as demonstrated by complete lack of visible membrane-associated fluorescence; Fig. 7), and the mutant fails to complement the temperature-sensitive (ts) growth of the *yck*^{ts} strain (Fig. 5B). However, the sequence is essential for accumulation of Yck2 protein. The GFP-yck2Δ519-527 protein was detected at low levels by fluorescence microscopy. We confirmed the low level of protein

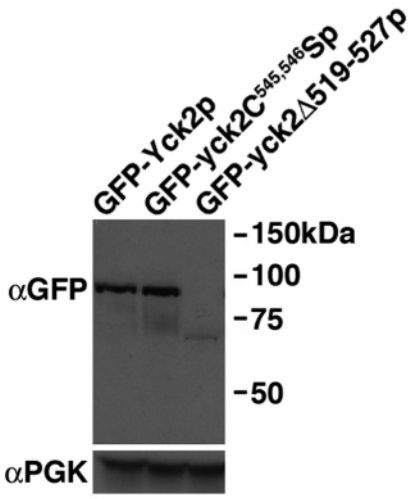

Fig. 8. GFP-yck2Δ519-527p fails to accumulate to normal levels. Immunoblot showing steady-state GFP-Yck2p, GFP-yck2-$C^{545,546}$Sp and GFP-yck2Δ519-527p levels in wild-type (LRB906) cells transformed with low-copy plasmids encoding the indicated fusion proteins. Protein samples were separated and transferred to nitrocellulose as described in Materials and Methods. Membranes were probed independently with αGFP and αPGK antibodies.

relative to wild-type GFP-Yck2p or other C-terminal deletion mutants by immunoblot (Fig. 8). Since the gene is expressed from the *GAL1* promoter, we attempted to determine the half-life of the mutant protein by promoter shut-off experiments, but the mutant protein failed to accumulate to reasonable levels during the pulse. Thus, either the message or the protein is unstable. Therefore, although the fluorescence pattern of GFP-yck2-Δ519-527p suggests loss of modification, the lack of protein accumulation compromises clear interpretation of the results.

Deletion of a 13 amino acid sequence close to the C-terminus (yck2Δ528-540p) gave results consistent with loss of sequences important for both targeting and modification. When expressed from *YCK2* sequences carried in a low copy plasmid, GFP-yck2Δ528-540p fails to complement the *yck^{ts}* mutant for ts growth or for morphology at restrictive temperature (Fig. 5B; L.C.R. and S.L.J., unpublished). Plasma membrane fluorescence of GFP-yck2Δ528-540p is dramatically reduced in favor of both soluble and particulate cytosolic fluorescence (Fig. 7).

The Yck1p C-terminus directs the Yck2p catalytic domain to the plasma membrane via the secretory pathway

Although Yck1p and Yck2p are functionally redundant, it has not been demonstrated directly that Yck1p is plasma membrane localized. Further, although Yck2p and Yck1p share high sequence similarity in the catalytic domain, and the C-terminal sequences of both proteins are Gln-rich, the C-terminal domains are divergent in sequence (Fig. 9A). Only the final 12 amino acids are markedly similar in sequence (83% identity). These include the two terminal Cys residues, and six of the residues defined by the *yck2Δ528-540* allele as required for both modification and targeting. The additional sequences

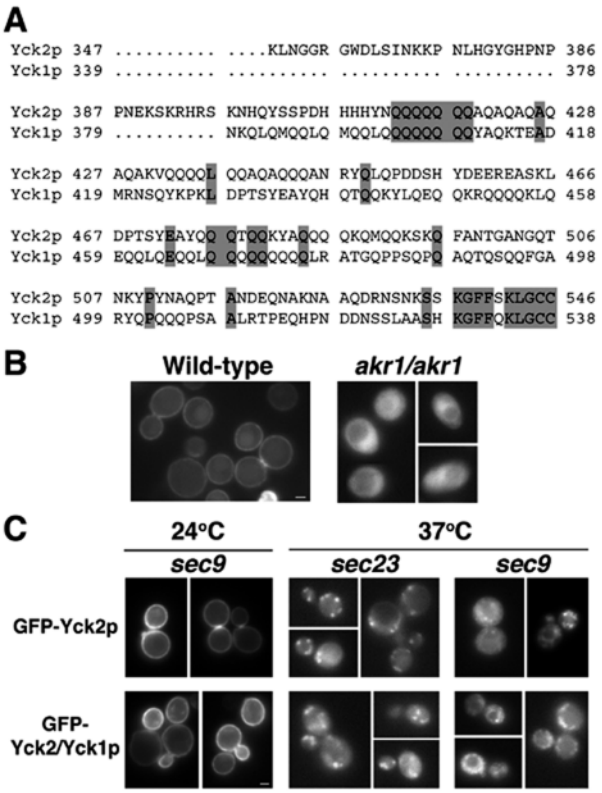

Fig. 9. A GFP-Yck2/Yck1p chimera is modified and targeted to the plasma membrane similarly to GFP-Yck2p. (A) Sequence alignment of Yck2p and Yck1p C-terminal sequences. The sequences swapped in the Yck2/Yck1p chimera are shown. (B,C) Plasmid pL240, expressing GFP-Yck2/Yck1p upon galactose induction, was introduced into wild-type strain LRB906 and *akr1Δ/akr1Δ* strain LZY103 (B) and into *sec9*, *sec14* and *sec23* strains (C). (B) Cells were grown to log phase in selective medium containing 2% raffinose as the carbon source, then galactose was added to 2% and cells were incubated at 30°C for 120 minutes before photographing. (C) Cells were grown and treated as described for Fig. 2 except that the induction period was 120 minutes. Bar, 2 μm.

in the Yck2p C-terminus that are important for Yck2p membrane association and plasma membrane targeting are not present in Yck1p, and the Gln-rich sequences of Yck2p do not appear to influence targeting greatly. These observations raise two questions, whether Yck1p is modified in the same *AKR1*-dependent manner as Yck2p, and whether Yck1p is targeted to the plasma membrane via the same route as Yck2p.

Because a GFP fusion to the N-terminus of Yck1p was neither functional nor readily detectable (L.C.R., unpublished), we generated a chimera between Yck1p and Yck2p (see Materials and Methods; Fig. 3A). GFP and the first 396 residues of Yck2p, including the entire catalytic domain, were fused to the C-terminal domain of Yck1p (residues 389 to 538). This chimera was functional as a Yck protein, as demonstrated by complementation of the *yck^{ts}* mutant when expressed from the *YCK2* promoter (Fig. 5B). Consistent with this result, fluorescence of the chimera was detected at the plasma membrane (Fig. 9B, left panel), as for the wild-type GFP-Yck2p fusion.

We tested whether the GFP-Yck2/Yck1p chimera requires Akr1p for membrane association by introducing a low copy plasmid containing the chimeric gene into an *akr1Δ/akr1Δ*

strain. As shown in Fig. 9B (right panel), fluorescence of the chimera in this strain is not associated with the plasma membrane (or any membrane), as for the wild-type GFP-Yck2 fusion protein in the *akr1* strain, or for the GFP-Yck2-$Cys^{545,546}$Ser fusion protein in any strain. Thus, the Yck1 protein also appears dependent on Akr1p for modification that allows membrane association.

We also tested whether the divergent Yck1p C-terminus is sufficient for plasma membrane targeting via the secretory pathway. Plasmid pL240, containing the chimera behind the *GAL1* promoter, was introduced into *sec23* and *sec9* strains. Induction of expression at permissive and non-permissive temperatures was carried out as for the GFP-Yck2 protein. As expected, the GFP-Yck2/Yck1p chimera is plasma membrane-associated in the *sec9* mutant at its permissive temperature (Fig. 9C), as it is in the *sec23* mutant at permissive temperature (L.C.R., unpublished). However, in neither *sec* mutant did the chimera reach the plasma membrane at restrictive temperature (Fig. 9C). Thus, targeting of this chimera is identical to that of intact GFP-Yck2p, even though there is no obvious sequence similarity in regions of the C-terminus required for Yck2p plasma membrane targeting.

Discussion

Our results demonstrate that the Yck2p protein kinase, which requires a terminal -Cys-Cys sequence for peripheral plasma membrane localization, is targeted to the cell surface via association with membranes of the secretory pathway. These results provide a clear and simple explanation for the cell-cycle-specific enrichment of Yck2 protein at sites of polarized growth; it is targeted there by polarized secretion. The topology of the Yck2 protein at the plasma membrane, anchored by the C-terminal modification and facing the cytosol, predicts its association with the cytosolic face of secretory membranes. Our results suggest a model in which Yck2p is recruited to an early secretory membrane for modification, then is directed through the vesicle-mediated secretory pathway by interaction with as yet unknown factors.

The Yck2p C-terminal Cys residues are essential for membrane association. Our results with the farnesylated Yck2-CIISp variant demonstrate that hydrophobic modification at the C-terminus is sufficient to allow membrane association. It was proposed that the two terminal Cys residues on Yck2p are modified by geranylgeranylation, based on the conditions needed to extract Yck2p from membranes and on the fact that the only other proteins that carry two terminal Cys residues, Rab proteins, are modified on both Cys residues by the GGTase type II. However, there is no direct evidence for this, and there is now strong evidence that Yck2p is modified by palmitoylation. The Yck2 protein is neither labeled with palmitate nor membrane associated in cells lacking the Akr1 protein (Feng and Davis, 2000; Roth et al., 2002). We have demonstrated here that the Yck1p C-terminal domain also confers dependence on Akr1 for membrane association. The Akr1 protein shares sequence similarity with the Erf2 protein, which is required for palmitoylation of Ras2p (Bartels et al., 1999), and Yck2p can be palmitoylated in vitro in an Akr1p-dependent fashion (Roth et al., 2002). These results indicate that one or both of the Cys residues of both Yck1p and Yck2p are palmitoylated.

Yck1p and Yck2p provide additional examples of palmitoylated proteins targeted to the inner surface of the plasma membrane. However, unlike some palmitoylated proteins (Resh, 1999), it is not likely that Yck2p modification occurs at the plasma membrane. Our results show that secretory membrane association of Yck2p occurs soon after synthesis. The presence of the terminal Cys residues is required for this association, suggesting that palmitoylation is a prerequisite for association. The possibility that the Cys residues are required in and of themselves for membrane association, for example, as recognition determinants for a membrane targeting factor, is not ruled out by our data. However, the observations that GFP-Yck2-CIISp is targeted normally and retains full biological function argue against this possibility.

The apparently normal targeting of the Yck2-CIISp variant also addresses another issue. Palmitoylation has been demonstrated to influence protein-protein interactions (Dunphy and Linder, 1998; Resh, 1999), and so it seemed possible that substitution of a farnesylation signal on Yck2p could impair secretory pathway targeting. This does not appear to be the case, arguing that modification serves to promote membrane association rather than targeting for Yck2p. Finally, the Yck2-CIISp results, along with the results of our deletion analysis, indicate that sequences in the C-terminus other than the Cys residues are important for plasma membrane targeting. The Yck2p sequences that are required for modification and targeting lie within the final 47 residues of the protein, with the final 28 residues most important for modification. There is no known consensus sequence for palmitoylation, and database searches with the Yck2p terminal 28 residue sequence yielded no significant matches.

Two C-terminal deletion mutants had the strongest effect on Yck2p membrane association. The GFP-yck2Δ519-527 protein is not visible at any membranes, but this variant is present at very low abundance, and lack of modification may reflect a very short half-life of the protein. We have no explanation for the low abundance of this mutant protein. GFP-yck2Δ519-527 does not contain any known degradation signal, and the possibility that lack of modification affects stability is ruled out by the observations that the $Cys^{545,546}$Ser mutant protein, and the wild-type protein in the *akr1* mutant, accumulate to wild-type levels. The GFP-yck2Δ528-540 protein, by contrast, demonstrates marked impairment of membrane association without effect on protein levels. Very little of this protein is detectable on internal membranes and biological function is lacking. It is likely that residues recognized by the palmitoyl transferase lie within this deleted region.

The *yck2Δ499-518* deletion shows very little effect on biological function or protein levels, but the mutant protein is less efficiently transported to the plasma membrane. There is also an increased pool of apparently soluble protein for this variant. Therefore, this protein could be impaired for modification and/or targeting.

Yck1p and Yck2p are functionally redundant (Robinson et al., 1992; Wang et al., 1992) and a chimera with Yck2p catalytic domain and Yck1p C-terminal domain is functional and is targeted to the plasma membrane as is intact Yck2p. However, outside the catalytic domain, the two proteins share little in common aside from C-terminal Gln-rich sequences. Beyond the last protein kinase subdomain, only the first eight

and the final 12 residues share identity. Shared residues closest to the Cys residues, deleted in yck2Δ528-540p, are required for membrane association, probably for modification. The residues deleted in yck2Δ499-518p are unique to Yck2p and are important for plasma membrane targeting. These residues are similar neither to sequences in the Yck1p tail nor to any other protein sequence from yeast or higher eukaryotes. Therefore, we predict that some sequence in the Yck1p C-terminus is functionally similar to this Yck2p sequence. Structure rather than primary sequence could be most important for recognition and targeting, but the C-terminus overall, and this sequence in particular, has little predicted secondary structure. Identification of targeting factor(s) and definition of a sequence in Yck1p that functions similarly to the Yck2p sequence deleted in yck2Δ499-518p will be the first steps in determining how such factor(s) can recognize two very different sequences.

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