Akr1p-dependent Palmitoylation of Yck2p Yeast Casein Kinase 1 Is Necessary and Sufficient for Plasma Membrane Targeting*

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The Yck2 protein is a plasma membrane-associated casein kinase 1 isoform that attaches to membranes via palmitoylation of its C terminus. We have demonstrated that Yck2p traffics to the plasma membrane on secretory vesicles. Because Akr1p, the palmitoyl transferase for Yck2p, is located on Golgi membranes, it is likely that Yck2p first associates with Golgi membranes, and then is somehow recruited to budding plasma membrane–destined vesicles. We show here that residues 499–546 are sufficient for minimal Yck2p palmitoylation and plasma membrane localization. We previously described normal plasma membrane targeting of a Yck2p construct with the final five amino acids of Ras2p substituting for the final two Cys residues of Yck2p. This Yck2p variant no longer requires Akr1p for membrane association, but targets normally. We have generated the C-terminal deletions previously shown to affect Yck2p membrane association in this variant to determine which residues are important for targeting and/or modification. We find that all of the sequences previously identified as important for plasma membrane association are required only for Akr1p-dependent modification. Furthermore, palmitoylation is sufficient for specific association of Yck2p with secretory vesicles destined for the plasma membrane. Finally, both C-terminal Cys residues are palmitoylated, and dual acylation is required for efficient membrane association.

The budding yeast Saccharomyces cerevisiae encodes four casein kinase I (CK1)† protein kinase isoforms: Yck1p, Yck2p, Yck3p, and Hrr25p (1–5). These four enzymes are strongly conserved with their higher eukaryotic counterparts, exhibiting greater than 50% amino acid identity through their catalytic domains (6). Yck1p and Yck2p are encoded by an essential gene pair (3, 5) whose functionally redundant products are involved in a number of cellular processes, including bud morphogenesis (7), cytokinesis (7), nutrient sensing (8), and the internalization of plasma membrane permease (9), and pheromone receptors (10, 11). The more abundant Yck2p is a 62-kDa kinase that is tightly associated with the inner leaflet of the plasma membrane (12). Yck2p biological function depends on its membrane association, because subcellular localization is necessary and sufficient for defining the functions of the yeast CK1 isoforms (1, 12–14). Like Yck1p, Yck2p terminates with the sequence -Cys-Cys, which is essential for its membrane association and function (7, 12, 15).

Yck2p plasma membrane localization requires the Akr1 protein (16), a polytopic integral membrane protein originally identified by genetic and two-hybrid analyses as a component in the pheromone response pathway (17–19). At least one of the two terminal Cys residues is palmitoylated by the Akr1 protein (20). Akr1p protein acyltransferase activity depends on its cysteine-rich domain (CRD), which contains a conserved Asp-His-His-Cys (DHHC) motif (20–22). The DHHC-CRD may be a hallmark of protein acyltransferase activity, because the only two protein acyltransferases characterized in yeast, Akr1p and the Erf2p-Erf4p complex, both possess this motif (20, 21), mutation within it abolishes protein acyltransferase activity (20, 21), and the DHHC motif is highly conserved in multiple proteins from yeast to humans (22–24).

In addition to the well characterized C-terminal -Cys-Cys sequence, Yck2p plasma membrane localization requires sequences within the final 48 residues of the C-terminal domain (CTD) of the enzyme. Deletions within this region impair Yck2p plasma membrane targeting (14). Furthermore, although Yck2p does not possess any known signal sequence, the kinase requires secretory pathway function for its subcellular translocation to the periphery of the cell (14). It is currently not known whether the Yck2p CTD is required for palmitoylation, trafficking, or both. For example, the palmitoylated SNAP-25 protein is thought to utilize a neuronal factor to facilitate its secretory pathway-dependent plasma membrane localization (25, 26). Alternatively, the palmitoyl moiety itself could provide a Yck2p plasma membrane targeting signal. The acylation of a number of proteins is sufficient to direct their subcellular localization (27, 28), and palmitoylation-deficient H- and N-Ras proteins fail to move from internal membranes to the plasma membrane (29, 30).

We show here that the C-terminal 48 Yck2p residues represent the minimal region required for targeting to the plasma membrane. We also demonstrate that these residues (499–546) are sufficient for Yck2p palmitoylation and that acylation itself is sufficient for plasma membrane targeting of Yck2p. Finally, we show that both C-terminal Cys residues are palmitoylated and that modification of both is required for efficient plasma membrane targeting.

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Abbreviations used are: CK1, casein kinase 1; CRD, cysteine-rich domain; CTD, C-terminal domain; DHHC, Asp-His-His-Cys; DRM, detergent-resistant membrane; ER, endoplasmic reticulum; GFP, green fluorescent protein; PIC, protease inhibitor cocktail; PMSF, phenylmethylsulfonyl fluoride; ts, temperature-sensitive.
Palmitoylation Directs Targeting of Yck2p

**EXPERIMENTAL PROCEDURES**

DNA Manipulation—Escherichia coli strains DH5α and XL1-Blue (Stratagene) were used for plasmid amplification and subcloning. Restriction enzymes (Promega; American Allied Biochemicals) and DNA ligase (New England Biolabs) were used according to the manufacturer’s instructions. Plasmid DNA was purified either by a rapid boiling method (31) or with the Perfectprep Plasmid Mini kit (Eppendorf). PCR amplification was carried out with Bio-X-Act DNA polymerase (Bioline), and site-directed mutagenesis was carried out using the QuickChange oligonucleotide-directed mutagenesis system (Stratagene) with Pfu Turbo or Pfu Ultra enzyme using a PerkinElmer Life Sciences 9600 or a GeneAmp 2400 (Applied Biosystems) thermocycler. Plasmid DNA purified by the rapid boiling method was further purified for DNA sequence analysis by RNase treatment, followed by precipitation with polyethylene glycol 8000 (32). DNA sequence analysis of cloned DNA was performed by RNase treatment, followed by precipitation with polyethylene glycol 8000 (32). DNA sequence analysis of cloned DNA prepared by the rapid boiling method was further purified for DNA sequence analysis by RNase treatment, followed by precipitation with polyethylene glycol 8000 (32). DNA sequence analysis of cloned DNA prepared by the rapid boiling method was further purified for DNA sequence analysis by RNase treatment, followed by precipitation with polyethylene glycol 8000 (32). DNA sequence analysis of cloned DNA prepared by the rapid boiling method was further purified for DNA sequence analysis by RNase treatment, followed by precipitation with polyethylene glycol 8000 (32). DNA sequence analysis of cloned DNA prepared by the rapid boiling method was further purified for DNA sequence analysis by RNase treatment, followed by precipitation with polyethylene glycol 8000 (32). DNA sequence analysis of cloned DNA prepared by the rapid boiling method was further purified for DNA sequence analysis by RNase treatment, followed by precipitation with polyethylene glycol 8000 (32). DNA sequence analysis of cloned DNA prepared by the rapid boiling method was further purified for DNA sequence analysis by RNase treatment, followed by precipitation with polyethylene glycol 8000 (32).

**TABLE I**

| Plasmid | Characteristics | Source/reference |
|---------|-----------------|-----------------|
| pHRP45  | Ycp50, GALI promoter, GFP::ycyk2(C546S) ORF | This study |
| pHRP46  | Ycp50, GALI promoter, GFP::ycyk2(C455S) ORF | This study |
| pJB4-4  | pUC183 EcoRI, GFP::YCK2 ORF | (33) |
| pJB9    | Ycp50, GALI promoter | (33) |
| pJB10   | Ycp50, GALI promoter, GFP::YCK2 ORF | (33) |
| pJB2    | Ycp50, GALI promoter, GFP::ycyk2(C485S) ORF | (33) |
| pl2.99  | pUC183 EcoRI, YCK2 ORF | (33) |
| plR10   | pUC183 SalI, GFP::YCK2 ORF | (14) |
| pl250   | pUC183 EcoRI, GFP::YCK2-CCIIS ORF | This study |
| pl251   | Ycp50, GALI promoter, GFP::YCK2-CCIIS ORF | (14) |
| pl260   | pUC183 EcoRI, yck2(499–546) ORF | This study |
| pl261   | pUC183 EcoRI, GFP::ycyk2(499–546) ORF | This study |
| pl262   | Ycp50, GALI promoter, GFP::ycyk2(499–546) ORF | This study |
| pPB5.1  | pUC183 EcoRI, yck2(C456S) ORF | This study |
| pPB5.2  | pUC183 EcoRI, yck2(C454S) ORF | This study |
| pPB6    | pUC183 EcoRI, GFP::ycyk2(C456S) ORF | This study |
| pPB7    | pUC183 EcoRI, GFP::ycyk2(C456S) ORF | This study |
| pPB27   | pUC183 EcoRI, GFP::YCK2A 519–522 ORF | This study |
| pPB28   | pUC183 EcoRI, GFP::YCK2A 523–527 ORF | This study |
| pPB34   | Ycp50, GALI promoter, GFP::YCK2A 519–522 ORF | This study |
| pPB35   | Ycp50, GALI promoter, GFP::YCK2A 523–527 ORF | This study |
| pPB9    | pUC183 SalI, GFP::YCK2A 499–518-CCIIS ORF | This study |
| pPB95   | Ycp50, GALI promoter, GFP::YCK2A 499–518-CCIIS ORF | This study |
| pPB102  | pUC183 SalI, GFP::YCK2A 519–522-CCIIS ORF | This study |
| pPB105  | Ycp50, GALI promoter, GFP::YCK2A 519–522-CCIIS ORF | This study |
| pPB107  | pUC183 SalI, GFP::YCK2A 523–527-CCIIS ORF | This study |
| pPB110  | Ycp50, GALI promoter, GFP::YCK2A 523–527-CCIIS ORF | This study |
| pPB112  | pUC183 SalI, GFP::YCK2A 528–540-CCIIS ORF | This study |
| pPB115  | Ycp50, GALI promoter, GFP::YCK2A 528–540-CCIIS ORF | This study |
| pPB123  | pUC183 SalI, GFP::yck2A 528–540 ORF | This study |
| pPB126  | Ycp50, GALI promoter, GFP::yck2A 528–540 ORF | This study |
| pPB141  | pUC183 EcoRI, GFP::ycyk2-CCIIS ORF | This study |
| pPB142  | Ycp50, GALI promoter, GFP::ycyk2-CCIIS ORF | This study |
| pPB147  | pUC183 SalI, GFP::yck2A 499–518-CCIIS ORF | This study |
| pPB148  | Ycp50, GALI promoter, GFP::yck2A 499–518-CCIIS ORF | This study |
| pPB151  | Ycp50, GALI promoter, GFP::yck2A 499–518-CCIIS ORF | This study |
| pPB153  | Ycp50, GALI promoter, GFP::yck2A 523–527-CCIIS ORF | This study |
| pPB155  | pUC183 SalI, GFP::yck2A 519–522-CCIIS ORF | This study |
| pPB156  | pUC183 SalI, GFP::yck2A 523–527-CCIIS ORF | This study |
| pPB158  | Ycp50, GALI promoter, GFP::yck2A 523–527-CCIIS ORF | This study |
| pPB160  | Ycp50, GALI promoter, GFP::yck2A 523–527-CCIIS ORF | This study |
| pPB168  | pUC183 SalI, GFP::yck2A 499–518 ORF | This study |
| pPB169  | Ycp50, GALI promoter, GFP::yck2A 499–518 ORF | This study |

To construct the yck2(499–546) allele, a PCR product was generated that encoded only the final 48 amino acids of Yck2p. The PCR product, with a BamHI site at the 5′-end and SalI site at the 3′-end, was amplified from template pl2.99 (YCK2 ORF in pUC183EcoRI) with primers yck2(499–546)F and yck2(499–546)R (Table II). Purified product was digested with BamHI and SalI and cloned into similarly digested pUC183EcoRI, yielding plasmid pl2.60. The GFP ORF was then inserted in-frame as an EcoRI fragment upstream of the YCK2 sequence as described previously (33), yielding plasmid pl2.61. Finally, the GFP::yck2(499–546) allele was cloned on a BamHI-Sall fragment into pJB9 for expression from the GALI promoter, yielding plasmids pHRP45 and pHRP46.

**Yeast Strains—**Yeast strains used for this work are listed in Table III. All LRB strains are closely related and differ only at the indicated loci. Yeast cells were cultured in standard media (34). Rich media (yeast extract, peptide) and synthetic media (yeast nitrogen base and amino acid supplement) were prepared with 2% carbon sources. Yeast transformation was carried out by a LiOAc procedure (35) modified in two ways. Cells were grown in standard rich media or synthetic media to an OD at 600 nm of 0.6–0.9, and calf thymus DNA was used as carrier DNA because it produced the highest efficiency for strains of this genetic background. YCK2 alleles were tested for function by testing for
2% to induce GFP fusion expression. After a 3-h induction period, cells were harvested by centrifugation at 4 °C for 3 min at 450 g of cleared lysate by incubation in 5 ml of TNET (50 mM KPO4, pH 7.5, 10 mM NaN3) and resuspended in 750 μl of cold lysis buffer (0.8 M sorbitol, 1 mM EDTA, 1 mM PMSF) containing 1× PIC. Samples were lysed manually using pre-chilled pestle tissue grinders (Thomas Scientific) on ice and transferred to pre-chilled microcentrifuge tubes (Beckman Coulter) and centrifuged at 200,000 × g for 3 min at 450 × g. An aliquot of each cleared lysate was retained as the total (T) fraction. The remainder was transferred to 13 × 51 mm polycarbonate ultracentrifuge tubes (Beckman Coulter) and centrifuged at 200,000 × g for 1 h at 4 °C in a Beckman Coulter TL-100 ultracentrifuge using a TLA 100.3 rotor. The supernatant was retained as the soluble (S) fraction. Membrane pellets were resuspended in a volume of lysis buffer containing 1× PIC equal to the supernatant, solubilized, and retained as the membranous (M) fraction. Sample buffer (60 μl Tris-HCl, pH 6.8, 1% SDS, 10% sucrose, 0.05% bromphenol blue) was added to T, C, and M samples prior to freezing or lyophilization. Protein samples were separated by electrophoresis through pre-cast 4–20% Tris-glycine denaturing SDS-polyacrylamide gels (Bio-Rad) using the Criterion SDS-PAGE apparatus (Bio-Rad). For fluorographic analysis, gels were fixed in isopropanol:water:acetic acid (25:65:10), treated with Amplify fluorographic reagent (Amersham Biosciences) as per manufacturer’s instructions, dried, and exposed to film at –80 °C for indicated times. For immunoblot analysis of immunoprecipitated samples, gels were blotted to nitrocellulose, probed with monoclonal antiserum against GFP (BD Biosciences/Clontech), and visualized with horseradish peroxidase-conjugated secondary antisera (Calbiochem) and the ECL chemiluminescence kit (Amersham Biosciences). For immunoblot analysis of subcellular fractionation samples from an akr1Δnakr1Δ genetic background, A9298 polyclonal antiserum against GFP was used for detection. To assess efficiency of fractionation, blots were stripped and reprobed for H+ plasma membrane complementation of the temperature-sensitive growth and morphology of strain LBR951 (yck1Δ yck2Δ). In Vivo Palmitate Labeling and Immunoprecipitation—Cells harboring galactose-inducible plasmid constructs were grown in synthetic selective media containing 2% raffinose as carbon source to logarithmic phase in 10-ml culture volumes. Non-transformed cells were similarly induced from a wild-type genetic background, affinity-purified polyclonal antiserum against GFP was used for detection. To assess efficiency of fractionation, blots were stripped and reprobed for H+ plasma membrane.
FIG. 1. The final 48 residues of Yck2p are sufficient to direct palmitoylation and plasma membrane targeting. A, schematics of GFP-Yck2p fusion variants. Numbering is that of Yck2p. B, localization of wild-type and deletion GFP fusion proteins depicted in A. Transformants of wild-type strain LRB906 or akr1 deletion strain LRB1028 carrying plasmids encoding the indicated fusion proteins were grown to log phase in selective synthetic media containing 2% raffinose as a derepressing carbon source, induced with 2% galactose for 120 min, and cells were photographed using the GFP-selective filter set 41001. Bar, 3 μm. C, cells of strains LRB937 (sec23-1), LRB933 (sec14-3), and LRB934 (sec9-4) carrying pL262 (pGAL1:GFPyck2(499–546)) were grown to log phase at 24°C in selective 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 120-min incubation at the indicated temperature. Bar, 3 μm. D, 1H]palmitate labeling of wild-type and mutant GFP fusion proteins depicted in A. Cultures of wild-type strain LRB906 carrying pGAL1:GFPyck2(499–546) were grown to log phase at 24°C in synthetic medium under derepressing conditions and labeled as described under “Experimental Procedures.” For mock treatment, cultures were grown in synthetic complete medium. Labeled protein was recovered by anti-GFP IP, subjected to SDS-PAGE, and visualized by immunoblotting (left panel) or fluorography (right panel; 36-day exposure). Quantitation of palmitoylation incorporation normalized to the corresponding immunoblot protein band is shown in the inset.

**RESULTS**

**The Yck2p Residues 499–546 Are Necessary and Sufficient for Plasma Membrane Targeting via the Secretory Pathway—** Deletion analysis within the Yck2p CTD revealed that one or more sequences within residues 499–546 are necessary for plasma membrane targeting (14). To determine if this short sequence is sufficient to direct Yck2p to the plasma membrane, we examined a deletion mutant, GFP-yck2(499–546)p, in which only these final 48 residues are appended to GFP (Fig. 1A). We found that this segment of the Yck2p CTD is sufficient for GFP plasma membrane localization in a wild-type genetic background, although membrane association and plasma membrane localization appear less efficient than for the wild-type fusion (Fig. 1B, upper panels). Significantly, GFP-yck2(499–546)p membrane association is lost in an akr1Δ/akr1Δ strain (Fig. 1B, lower panels).

Vesicle-mediated secretory pathway function is required for full-length Yck2p plasma membrane targeting (14). To test whether it is required for GFP-yck2(499–546)p targeting, we introduced the pGal:GFPyck2(499–546) plasmid (pL262) into each of three conditional secretory pathway-defective (sec) mutants. These temperature-sensitive (ts) mutations block secretory traffic at discrete steps upon a shift to restrictive temperature (37°C) while 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 (sec23 (37)); trafficking through the Golgi (sec14 (38)); and fusion of secretory vesicles with the plasma membrane (sec9 (39)).

Induction of GFP-yck2(499–546)p synthesis in each sec mutant at permissive temperature (24°C) resulted in plasma membrane localization (Fig. 1C), but localization is less efficient than was observed for wild-type GFP-Yck2p (14). However, after 120 min of induction at restrictive temperature (37°C), cells of each of the sec mutants showed only punctate intracellular fluorescence (Fig. 1C). Thus, like the wild-type GFP-Yck2 protein, the GFP-yck2(499–546) protein is targeted to the plasma membrane via the secretory pathway.

To verify that GFP-yck2(499–546)p is palmitoylated in vivo, we immune precipitated the protein from cleared lysates of cells labeled with 1H]palmitic acid and performed both immunoblot analysis and fluorography. As shown previously (20), GFP-Yck2p, but not GFP-yck2(C545,546S)p, is palmitoylated in vivo (Fig. 1D). Like GFP-Yck2p, palmitate also is incorporated into the GFP-yck2(499–546)p mutant in vivo (Fig. 1D). As with plasma membrane localization, palmitoylation incorporation is lower for the GFP-yck2(499–546)p mutant than for wild-type GFP-Yck2p. Only a fraction of the GFP-yck2(499–546)p fusion is palmitoylated in vivo during an equivalent time of labeling.
suggesting that less efficient plasma membrane localization results from less efficient palmitoylation. Nevertheless, the final 48 residues of Yck2p are sufficient for palmitoylation in vivo and are both necessary and sufficient to direct kinase targeting to the plasma membrane, an Akr1p-dependent process that requires classical secretory pathway function.

**Yck2p Plasma Membrane Targeting Specifically Requires Acylation**—The fact that residues 499–546 are necessary and sufficient to target Yck2p to the plasma membrane via the classic secretory pathway can be explained in two ways. First, this region may contain elements that mediate specific protein-protein interactions that allow the kinase to associate with plasma membrane-destined secretory vesicles. Second, because this sequence is sufficient to direct Akr1p-mediated palmitoylation of the kinase (Fig. 1), all or part of the sequence could be responsible for interaction with Akr1p. These possibilities are not necessarily mutually exclusive: some residues could be involved in targeting while others may be important for modification. To distinguish between these possibilities, we turned to two sets of Akr1p-independent YCK2 alleles.

In one set, the C-terminal -Cys-Cys sequence was replaced with the C-terminal Ras2p pentapeptide -Cys-Cys-Ile-Ile-Ser (Fig. 2A), which directs both farnesylation at its ultimate Cys residue by farnesyl transferase (40, 41) and palmitoylation at its upstream Cys residue (20). In the second set, the -Cys-Cys was replaced by a mutated form of the Ras2p signal sequence in which the penultimate Cys residue is mutated to a Ser residue, whereas the CAAX box consensus remains intact (-Ser-
Cys-Ile-Ile-Ser). This mutated signal is farnesylated but not palmitoylated (42, 43). Roth et al. (20) demonstrated that the Yck2CCIIISp variant is palmitoylated even in the absence of Akr1p, whereas the yck2CCIIISp variant is not palmitoylated in either wild-type or akr1Δ strains. To define the role that the Yck2p C-terminal 48 residues play in plasma membrane targeting and/or Akr1p binding and palmitoylation, we generated CTD deletions in the context of these two Ras2p farnesylation signals.

Unlike the palmitoylated wild-type GFP-Yck2p fusion, we found that the farnesylated GFP-yck2CCIIISp variant, which lacks the acylation site adjacent to its CAAX consensus, fails to localize to the plasma membrane in a wild-type genetic background (Fig. 2B, compare panels 1 and 2). GFP-yck2CCIIISp is capable of membrane association, however, because GFP signal was observed to decorate intracellular membranes. GFP-yck2Δ499–518p tested in the context of the -CCIIIS consensus also localizes uniformly on internal membranes, exhibiting a cellular distribution identical to that of the full-length -CCIIIS variant (Fig. 2B, compare panels 2 and 5). This endomembrane localization pattern is similar to that reported for the analogous non-palmitoylated Ras2CCIIISp mutant (42, 43). All other deletion mutants behaved in a similar manner. 

GFP-Yck2CCIIISp, which is both farnesylated and palmitoylated, localizes to the plasma membrane in both wild-type and akr1Δ strains. To test the biological function of the deletion-carrying Yck2p farnesylation variants, we expressed each variant in the background (Fig. 2, compare panels B-3 and C-2) (14). More importantly, all deletion mutants carrying the -CCIIIS signal exhibited plasma membrane localization of the GFP signal (Fig. 2B). Because palmitoylation of the Yck2CCIIISp variant was reported to be independent of Akr1p, we tested whether the AKR1 genotype affects the localization of the deletion mutants. All of the deletion mutants carrying the -CCIIIS signal localized to the plasma membrane in an akr1Δ null mutant (Fig. 2C). These results suggest that all residues defined by deletion analysis as important for Yck2p localization are vital only for Akr1p-mediated palmitoylation. Consequently, the -CCIIIS variants could be targeted to the plasma membrane by a different mechanism than the wild-type Yck2 protein. Although we observed Sec-dependent plasma membrane targeting for GFP-Yck2CCIIISp previously (14), we tested whether each deletion mutant requires the secretory pathway for plasma membrane association. Like GFP-yck2Δ499–518p (Fig. 3A), all deletion mutants exhibited plasma membrane localization in cells incubated at permissive temperature. However, no plasma membrane-associated fluorescence was detected at restrictive temperature (Fig. 3B). This confirms that normal targeting of GFP-Yck2p is retained even with Akr1p-independent modification and further supports the premise that Yck2p residues 499–546 are required specifically for Akr1p-mediated palmitoylation of wild-type Yck2p.

Essential Yck Function Requires Plasma Membrane Localization—To test the biological function of the deletion-carrying Yck2p farnesylation variants, we expressed each variant in the yck1Δ yck2-2Δ yck3Δ yck4Δ temperature-sensitive strain. We found that all -CCIIIS variants are able to complement the cellular growth defect (Fig. 4, upper panels), thus providing Yck function. Conversely, none of the -CCIIIS variants were able to support cell growth (Fig. 4, lower panels). Hence, the endomembrane localization of the exclusively farnesylated -CCIIIS variants (Fig. 2), coupled with their failure to complement the conditional growth defect, indicates that plasma membrane localization is specifically required for the essential Yck biological function(s).

Palmitoylation of Both Yck2p C-terminal Cys Residues Contributes to Efficient Plasma Membrane Localization—Yck2p biological function depends on the ability of the enzyme to associate with the plasma membrane, which in turn requires palmitoylation of the C-terminal cysteine residues (7, 12, 20). To determine whether each Cys is modified by Akr1p, and whether modification of both is required for plasma membrane targeting, we examined two point mutant GFP-Yck2 fusion proteins: GFP-yck2(Cys545Ser)p and GFP-yck2(Cys546Ser)p (Fig. 5A). As previously shown, GFP-yck2(Cys545,546Sp) or GFP-yck2(SS)p, which lacks both C-terminal cysteine residues and is therefore not modified, remains cytosolic (Fig. 5B). GFP-yck2(Cys545Ser)p or GFP-yck2(SC)p also appears to be soluble (Fig. 5B). On the other hand, a detectable fraction of the GFP-yck2(Cys546Ser)p mutant, or GFP-yck2(CS)p, associates with membranes, including the plasma membrane (Fig. 5B). The minor fraction of GFP-yck2(CS)p that localizes to the plasma membrane exhibits the specific enrichment at small buds and at the bud neck during cytokinesis (Fig. 5B, arrows) that is characteristic of wild-type Yck2p (33). Membrane association of this mutant, as for the wild-type Yck2 protein, is lost in an akr1Δ genotype (Fig. 5B, panels at right).

The presence of some GFP-yck2(SC)p at the plasma membrane suggests that a singly palmitoylated Yck2p is capable of targeting but incapable of efficient membrane association. To more accurately assess the ability of each mutant to associate with cellular membranes, whole cell lysates were prepared from cells expressing either wild-type or mutant GFP-Yck2p, separated into membranous and soluble fractions by centrifugation at 200,000 × g, and analyzed by immunoblotting with antibody directed against GFP. Although the GFP-yck2(SC)p appears to be completely soluble by epifluorescence (Fig. 5B), both GFP-yck2(SC)p and GFP-yck2(SS)p are distributed between the soluble and membranous pools from wild-type cells (Fig. 5C). This is in contrast to the entirely membrane-associated wild-type GFP-Yck2p and largely cytosolic GFP-yck2(SS)p.
Palmitoylation Directs Targeting of Yck2p

Yck2p residues 499–546 are necessary and sufficient for palmitoylation and targeting to the plasma membrane via the classic secretory pathway (Fig. 1). Deletion within this region fails to affect Yck2p targeting in the context of consensus sequences that direct Akr1p-independent acyl and prenyl modification (Fig. 2), indicating that it is required explicitly for Akr1-dependent targeting. The simplest explanation of these results is that this small portion of the Yck2p C-terminal domain may act as an Akr1p binding sequence. Both Yck1p and Yck2p require Akr1p for membrane association and secretory pathway function for plasma membrane targeting (14). However, although the C-terminal sequences of both isoforms are Gln-rich, only their final 12 residues share significant sequence identity (3, 5). Therefore, we propose that secondary structural features are instrumental in Yck1p and Yck2p recognition by Akr1p.

Although the Yck2p CTD is generally devoid of predicted secondary structure and was not included in the crystalized protein (44), structural predictions reveal a high propensity for residues 519–527 to adopt an a-helical configuration. We found that GFP-yck2Δ519–527p is inexplicably unstable and fails to associate with cellular membranes (14). To determine whether the removal of only one or a few specific residues is responsible for the GFP-yck2Δ519–527p phenotype, we created and examined five smaller deletions throughout this nine-residue sequence.2 Only the GFP-yck2Δ519–525p and GFP-yck2Δ523–527p mutants exhibited any mislocalization phenotype.2 These observations provide further evidence of a mechanism in which secondary structure plays a role in Akr1p-mediated palmitoylation; with respect to residues 519–527, only deletions that are large enough to disrupt the putative a-helix reproduce the mislocalization defect. Interestingly, it has also been suggested that secondary structure may play some role in the modification of the vacuolar CK1 isoform Yck3p, which is palmitoylated throughout its C-terminal stretch of multiple Cys residues by an as yet unidentified protein acyltransferase (13). Secondary structure may be involved in SNAP-25 modification as well. A domain nestled between two a-helices is both necessary and sufficient for syntaxin-independent SNAP-25 palmitoylation and plasma membrane localization (25, 26). Nevertheless, further examination of how Yck2p secondary structure facilitates Akr1p-mediated palmitoylation is required.

At present, only Yck1p and Yck2p have been characterized as Akr1p protein acyltransferase substrates (20). However, it is unlikely that Akr1p binds only to Yck1p and Yck2p. Two-hybrid analysis has established interactions not only between Akr1p and Yck1p/Yck2p (45), but also between Akr1p and plasma membrane proteins of the mating signal transduction pathway (17–19), as well as the ER/Golgi membrane protein Gsc1p (18). Moreover, Akr1p possesses six ankyrin repeat protein sequence motifs (18), which may act as scaffolding modules to mediate protein–protein interactions (46). Perhaps secondary structure within Yck2p residues 499–546 is required for recognition by Akr1p. Alternatively, residues within this region and upstream could be required for binding to an unidentified accessory factor that facilitates Golgi membrane association, Akr1p binding, Yck2p palmitoylation, or some combination of
these events. Sucrose gradient fractionation data confirm preliminary immunofluorescence analysis of a tagged Akr1p (20), placing it at the Golgi membrane. Thus, Yck2p palmitoylation most likely occurs at the Golgi. However, internal GFP-Yck2p accumulation was observed in the sec23 mutant strain (Fig. 3B), which exhibits a block in vesicle budding from the ER at restrictive temperature. An accessory factor could account for this surprising result. Blocking all subsequent secretory traffic at the ER membrane halts Golgi-related steps and could inhibit translocation of an accessory factor to the Golgi for Yck2p binding or processing. Endogenously expressed Yck2p complexed with pre-existing accessory factor would preclude newly synthesized GFP-Yck2p from binding to accessory factor at the Golgi, as newly synthesized and non-complexed accessory factor would accumulate at the ER membrane. Alternatively, cycling of free accessory factor between the ER and Golgi would lead to its accumulation at the ER in the sec23 mutant, because this block halts transport to the Golgi. The future identification of any additional Akr1p protein acyltransferase substrates may clarify our results, but at present, it is not clear whether an unidentified accessory factor or Akr1p itself recruits Yck2p to the Golgi.

A model in which an accessory factor aids in some aspect of Yck2p modification and plasma membrane localization also could explain why residues 499–546 are only minimally sufficient for Akr1p-dependent acylation. We previously described a fusion of the final 186 residues of Yck2p to GFP (GFP-yck2p/H9004–360p) that targets normally to the plasma membrane (i.e. localization requires classic secretory pathway function) but shows increased soluble signal relative to full-length GFP-Yck2p (14), suggesting that palmitoylation of GFP-yck2p/H9004–360p is less efficient than wild-type GFP-Yck2p. Even more GFP-yck2p(499–546)p fails to associate with membranes than GFP-yck2p/H9004–360p. Thus, it appears that targeting becomes increasingly effective as more sequence is appended to the final 48 residues of Yck2p. Perhaps upstream sequence is required for interaction with an accessory factor that promotes Golgi membrane association or proper presentation of residues 499–546 to Akr1p; the absence of these sequences would eliminate accessory factor binding, thereby reducing Yck association with Akr1p to a random event and reducing the efficiency of yck2p(499–546)p palmitoylation. Although it is formally possible that catalytic activity could be involved, we have observed efficient plasma membrane association of a kinase-dead Yck2p mutant.2

Our results indicate that the localization of Yck2p specifically to the plasma membrane is strictly required for biological function (Figs. 2 and 4). It was reported that a deletion mutant

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3 L. C. Robinson, unpublished observations.

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**Fig. 5. Both Yck2p C-terminal Cys residues contribute to efficient membrane localization and function.** A, schematics of GFP-Yck2 fusion proteins with mutations to the C-terminal -Cys-Cys sequence. Numbering is that of Yck2p. B, localization of wild-type and mutant GFP Yck2 fusion proteins depicted in A. Transformants of wild-type strain LRB906 or akr1 deletion strain LRB1028 with plasmids encoding the indicated GFP-Yck2 fusion proteins were grown, induced, and photographed as described for Fig. 1B. Bar, 3 μm. C, wild-type LRB906 cells expressing the fusion proteins depicted in A were grown in synthetic medium under derepressing conditions to log phase. GFP fusion expression was induced, and cells were fractionated into total (T), soluble (S), and membranous (M) fractions as described under “Experimental Procedures.” Immunoblot analysis was performed with antibodies directed against GFP and Pma1p. D, growth tests of yck1Δ yck2–2ts strain LRB951 expressing the fusion proteins depicted in A were performed as described for Fig. 4.
lacking the C-terminal -Cys-Cys sequence (yck2ΔCCp) is sufficient to rescue the conditional lethality phenotype of the yck1Δ yck2Δ-2ts strain (12). However, we have found that even overexpression of the yck2ΔSS/SS allele in this strain fails to rescue both morphological and cellular growth defects at restrictive temperature (14, 33). Our recent work illustrated a correlation between plasma membrane localization and biological function (14), and we have presented additional evidence to substantiate this link (Fig. 4). Thus, Yck1p and Yck2p must localize specifically to the plasma membrane to execute their essential cellular functions.

Our analysis of fluorescent protein fusions demonstrates that acylation is necessary and sufficient to target Yck2p to the plasma membrane. Only dually lipidated Yck2p with at least one palmitoyl-accepting cysteine is able to specifically and efficiently localize to the plasma membrane (Figs. 2 and 5). For membrane-associated cytosolic proteins, palmitate is found attached close to myristic acid, prenyl groups, or in the absence of other types of modification (27, 28, 47). Co-translationally myristoylated Src family kinases and G protein-coupled receptors are also palmitoylated (48–51). Thus, the palmitoylation of a number of proteins provides for stable membrane association and, at times, a subcellular targeting signal.

Similarly, we found that, unlike wild-type GFP-Yck2p, both GFP-yck2ΔCys546Serp and GFP-yck2ΔCys546Serp fail to exclusively associate with cellular membranes (Fig. 5), suggesting that Yck2p is dually palmitoylated in vivo. In addition, GFP-yck2ΔCys546Serp is capable of limited plasma membrane localization. Perhaps the modification of the terminal Cys546 residue additionally directs Yck2p plasma membrane targeting. Although it is not readily apparent what might designate the palmitoylation of Cys546 (but not Cys546) as a targeting signal, palmitoylation may direct Yck2p incorporation into membrane microdomains destined for the plasma membrane. It has been shown that protein acylation, but not prenylation, promotes clustering into sphingolipid and sterol-rich membrane microdomains, also known as lipid rafts or detergent resistant membranes (DRMs) (52–54). Furthermore, dually palmitoylated Cys residues are required for raft association of the neuronal protein GAP-43 (55) and of the T cell adaptor protein LAT (56). The direct demonstration of Yck2p incorporation into DRMs is required to definitively state that Yck2p achieves plasma membrane localization by way of lipid rafts. However, our results support a model in which Yck2p plasma membrane targeting is directed by acylation. First, newly synthesized GFP-Yck2p is recruited to the cytosolic face of the Golgi membrane either by an as yet unidentified accessory factor or by Akr1p itself. Our results suggest that both Cys546 and Cys546 are palmitoylated prior to normal kinase translocation (Figs. 5 and 6). Palmitoylated GFP-Yck2p may then partition into sphingolipid and ergosterol-rich membrane microdomains, which are destined for vesicle-mediated transport to the plasma membrane. Finally, vesicle fusion at the peripheral membrane delivers GFP-Yck2p to the cytosolic face of the plasma membrane. Localization via lipid rafts would account for the strict requirements of palmitoylation and secretory pathway function. A raft targeting model also is consistent with the observation that Yck2p cannot be solubilized by nonionic detergents such as Triton X-100 (12), because incorporation of palmitoylated Yck2p into DRMs would confer such resistance. In addition, the saturated acyl tails of tightly packed DRM sphingolipids constitute an ideal environment for palmitoyl groups (53). This model could apply generally to the targeting of palmitoylated proteins.

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