The Yeast Casein Kinase Yck3p is Palmitoylated, then Sorted to the Vacuolar Membrane with AP-3-Dependent Recognition of a YXXΦ Adaptin Sorting Signal

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ABSTRACT

Our previous work found the two yeast plasma membrane-localized casein kinases Yck1p and Yck2p to be palmitoylated on C-terminal Cys-Cys sequences by the palmitoyl transferase Akr1p. The present work examines a third casein kinase, Yck3p which ends with the C-terminal sequence Cys-Cys-Cys-Cys-Phe-Cys-Cys-Cys. Yck3p is palmitoylated and localized to the vacuolar membrane. While the C-terminal cysteines are required for this palmitoylation, Akr1p is not. Palmitoylation requires the C-terminal Yck3p residues 463-524, while information for vacuolar sorting maps to the 409-462 interval. Vacuolar sorting is disrupted in \textit{cis} through deletion of the 409-462 sequences and in \textit{trans} through mutation of the AP-3 adaptin complex; both \textit{cis}- and \textit{trans}-mutations result in Yck3p missorting to the plasma membrane. This missorted Yck3p restores 37°C viability to \textit{yck1Δyck2-ts} cells. \textit{yck1Δyck2-ts} suppressor mutations isolated within the \textit{YCK3} gene identify the Yck3p vacuolar sorting signal – the tetrapeptide YDSI, a perfect fit to the YXXΦ adaptin-binding consensus. While YXXΦ signals have a well-appreciated role in the adaptin-mediated sorting of mammalian cells, this is the first signal of this class yet shown to operate in yeast.
INTRODUCTION

The four adaptin complexes, AP-1, AP-2, AP-3, and AP-4, mediate membrane sorting at multiple points within the mammalian Golgi/endosomal system (Bonifacino and Traub, 2003). These protein complexes truly are adaptors – linking the cargo protein to the bud-forming coat proteins. This has been most clearly outlined in clathrin-mediated endocytosis for the AP-2 adaptin complex where the adaptin complex links through dual binding the endocytic cargo and the heavy chain of the clathrin coat. Cargo and coat thus are recruited together, forming a nascent coated pit, which invaginates and is then released into the cytoplasm as a clathrin-coated vesicle.

Each adaptin complex is comprised of a conserved set of four subunits: two large subunits, a β (β1-4) and either a γ, α, δ, or ε, depending on the complex (i.e. AP-1, -2, -3, or -4), a medium subunit μ (μ1-4), and a small subunit σ (σ1-4). AP-2 functions at the plasma membrane to effect plasma membrane uptake, while AP-1, AP-3, and AP-4 function at the Golgi to mediate communication with endosomal compartments and the lysosome. Similar types of signals are recognized by the four complexes, with the signals conforming either to the tyrosine-based consensus YXXΦ (Φ being a bulky, hydrophobic residue) or to the dileucine-based consensus [DE]XXL[LI] (Bonifacino and Traub, 2003).

The yeast Saccharomyces cerevisiae has three adaptin complexes – AP-1, AP-2, and AP-3 (Boehm and Bonifacino, 2002). AP-1 has been suggested to function at the level of the Golgi, acting to maintain the integrity of this organelle through the AP-1/clathrin-mediated retrieval of Golgi proteins that escape to the early endosome (Stepp et al., 1995; Yeung et al., 1999;
Valdivia et al., 2002). Despite the well-defined role for AP-2 in mammalian endocytosis, the function of the yeast AP-2 has proved elusive – deletion of the genes encoding the subunits of the yeast AP-2 complex is without discernible effect on endocytosis, or on any trafficking pathway yet examined (Huang et al., 1999; Yeung et al., 1999). Analysis of yeast AP-3 has proved particularly fruitful, leading to the definition of a new trafficking pathway that connects the Golgi to the vacuole (yeast lysosome) (Cowles et al., 1997a; Stepp et al., 1997). The AP-3-dependent pathway differs from the more well-studied VPS pathway, which also connects Golgi to vacuole, in that transport does not pass through the multivesicular body (MVB) (Cowles et al., 1997b; Piper et al., 1997). The MVB, also known as either the pre-vacuolar compartment or late endosome, is an intermediary compartment, located proximal to the vacuole that functions to sort cargoes destined for the vacuolar lumen away from cargoes targeted to the limiting membrane of the vacuole (Odorizzi et al., 1998). Only two cargo proteins have been yet identified for the yeast AP-3 pathway – the vacuolar phosphatase ALP and the vacuolar t-SNARE Vam3p; both utilize classic dileucine-based signals to instigate their AP-3-mediated sorting to the limiting membrane of the vacuole (Darsow et al., 1998; Vowels and Payne, 1998). Rather than partnering with clathrin, the yeast AP-3 complex is thought instead to link to Vps41p, which has been suggested to provide the coat function (Darsow et al., 2001). AP-3 appears to fill a similar niche in mammalian cells, dileucine- and tyrosine-based signals are used to initiate the sorting from the Golgi to the lysosome (Boehm and Bonifacino, 2002; Bonifacino and Traub, 2003). The issue of whether or not clathrin participates in mammalian AP-3-dependent sorting remains to be settled (Newman et al., 1995; Simpson et al., 1997; Dell'Angelica et al., 1998).
Mutations in the yeast AP-3 subunit genes were first identified in a selection for suppressors of the \( yck1\Delta yck2-ts \) mutations (Panek et al., 1997). Yck1p and Yck2p are homologous and functionally-redundant type I casein kinases that localize to the yeast plasma membrane (Robinson et al., 1993; Vancura et al., 1994). Given that \( yck1\Delta yck2\Delta \) cells are inviable, a conditional \( yck1\Delta yck2-ts \) strain was constructed and was found to be defective both in endocytosis as well as in cell growth and morphogenesis (Robinson et al., 1993; Panek et al., 1997). Suppressors conferring 37\(^\circ\)C growth to the \( yck1\Delta yck2-ts \) strain were found to map to four complementation groups, the four AP-3 subunit genes; suppression also could be achieved through total deletion of the subunit genes, indicating suppression depended on a loss of AP-3 function (Panek et al., 1997). At the time, nothing was known regarding AP-3 function and it was thought, given the Yck1p/Yck2p participation in endocytosis, that this new adaptin complex might also participate in endocytosis. However, while they suppressed the \( yck1\Delta yck2-ts \) endocytosis defect, the AP-3 mutations were themselves not associated with any endocytosis defect. Even with the understanding of AP-3 function in Golgi-vacuole trafficking that has been since gleaned, the mechanism underlying the \( yck1 \ yck2 \) suppression has remained a puzzle.

The present study which focuses on Yck3p, a third and related type I casein kinase, resolves this mystery. Having recently demonstrated that Yck2p is tethered to the plasma membrane through palmitoylation of a C-terminal Cys-Cys sequence (Roth et al., 2002), we were intrigued to find that the Yck3p C-terminal sequence is the striking cysteine-rich sequence CCCCCFCCC. Yck3p, we find, also is palmitoylated. However, rather than localizing to the plasma membrane like Yck1p and Yck2p, Yck3p, instead localizes to the cytoplasmic surface of the vacuolar membrane. The trafficking of Yck3p to the vacuole, we find, is via the AP-3-
dependent, ALP pathway: in \textit{apm3}\textsuperscript{Δ} or in \textit{aps3}\textsuperscript{Δ} cells, deleted for the AP-3 \(\mu\) or \(\sigma\) subunit genes, respectively, Yck3p is missorted to the plasma membrane. This Yck3p missorting might explain the \textit{yck1}\textsuperscript{Δ} \textit{yck2-ts} suppression by AP-3 mutations (Panek \textit{et al.}, 1997): cell surface casein kinase function, presumably deficient in \textit{yck1}\textsuperscript{Δ} \textit{yck2-ts} cells at 37\textdegree C, might be replenished through the diversion of Yck3p to plasma membrane. To test this hypothesis, Yck3(Δ409-462)p, a mutant Yck3p deleted for its AP-3-dependent vacuolar sorting information, was expressed in \textit{yck1}\textsuperscript{Δ} \textit{yck2-ts} cells: Yck3(Δ409-462)p is missorted to the plasma membrane and consequently, 37\textdegree C growth is restored to the \textit{yck1}\textsuperscript{Δ} \textit{yck2-ts} cells. The \textit{yck1}\textsuperscript{Δ} \textit{yck2-ts} suppression phenotype was exploited to isolate point mutations within the Yck3p sorting signal; these mutations identify a classic tyrosine-based YXX\(\Phi\) consensus signal for adaptin-mediated sorting. While such signals are known to participate in the AP-1-, AP-2-, and AP-3-mediated sorting of mammalian cells, this is the first YXX\(\Phi\) signal to be identified in yeast.
MATERIALS and METHODS

Strains

Two strain backgrounds were used in this work. Most of the work was done with the LRB759
\((MAT\alpha \text{ura3-}52 \text{leu2 his3)}\) strain background (Panek et al., 1997). In addition to the wild-type
LRB759 strain, the isogenic \(yck1{-1}::\text{ura3-} yck2{-2}^{ts}\) strain LRB757 (Panek et al., 1997), and the
akr1\(\Delta\) version NDY1405 (Roth et al., 2002) were also used. Other strains (used for Figure 5)
were from the Saccharomyces Deletion Consortium, including the wild-type \(MAT\alpha \text{ura3}\Delta0 \text{leu2}\Delta0 \text{his3}\Delta1 \text{met15}\Delta0\) strain BY4741 and the \(\text{aps3}\Delta, \text{apm3}\Delta, \text{vps2}\Delta, \text{and vps4}\Delta\) deletion
derivatives, in which the relevant ORF is replaced with the \(G418^R\) marker (Research Genetics).
PCR was used for confirming the presence of each deletion within the Consortium strains.

Plasmids

All \(YCK2\) and \(YCK3\) constructs were introduced into yeast on the \(URA3/CEN/ARS\) vector
plasmid pRS316 (Sikorski and Hieter, 1989). A \(YCK3{-p-4xHA/YCK3}\) construct was used for
most of the indirect immunofluorescent experiments. This construct which has four copies of the
HA epitope tandemly inserted immediately following the initiator codon, retains the natural
\(YCK3\) regulatory sequences, both upstream (the 438 bp immediately upstream of the initiator
codon) and downstream (420 bp downstream of the terminator codon) of the \(YCK3\) ORF.
\(GAL1{-p-6xHis/FLAG/HA/YCK3}\), used both in the palmitate-labeling experiment and for some
immunofluorescence, differs in having the \(GAL1\) regulatory sequences in place of the \(YCK3\)
upstream sequences and in having an amino-terminal \(6xHis/FLAG/HA\) tripartite tag instead of
the \(4xHA\) sequence. Two plasmids were used for expression of tagged \(YCK2: YCK2{-p-HA/YCK2}\)
has Yck2p, N-terminally-tagged with a single HA epitope controlled by the 623 bp upstream of the YCK2 ORF, while for GAL1p-6xHis/FLAG/HA/YCK2, Yck2p is N-terminally-tagged with the tripartite 6xHis/FLAG/HA and is controlled by the GAL1 promoter.

The ΔCys truncation was constructed through the insertion of a terminator codon just prior to the C-terminal CCCCCCCC encoding sequence of YCK3. The Δ409-462 mutation is an in-frame deletion of YCK3 codons 409 through 462, with Thr-Arg-encoding a Mlu I site in place of the missing codons. The YCK232 construct encodes a chimeric protein having the N-terminal portion of YCK2 (codons 1 through 396), followed by a portion of YCK3 (codons 409 through 462), and ending with the C-terminal portion of YCK2 (codons 496 through 546). This plasmid was constructed by in vivo recombination in yeast. A 264 bp PCR fragment was generated from a YCK3 template using two 76 nt primers having 51 nt of YCK2 sequence at their 5' ends and 25 nt of YCK3 sequence at their 3' ends to serve as the template-specific primers. The resulting PCR fragment has a core 162 bp of YCK3 sequences (codons 409 through 462) flanked by 51 bp of upstream and downstream YCK2 sequence (upstream YCK2 codons 382-396; downstream YCK2 codons 496-512) to direct the in vivo recombination. The PCR fragment was cotransformed into yeast along with YCK2p-HA/YCK2 plasmid linearized at its unique Mfe I site (located at YCK2 codons 435 and 436). Circularized plasmids repaired through homologous recombination with the PCR fragment were recovered from the Ura+ yeast colonies.

A GAL1p-HA/URA3 construct (single HA epitope tag at the Ura3 N-terminus) on the LEU2/CEN/ARS plasmid pRS315 was the starting point for the HA/URA3/YCK3 fusions. Xho I restriction sites that had been introduced into YCK3 just prior to codon 1, codon 409, codon 462,
and codon 517, were used in ligations to a Sal I introduced just prior to the *URA3* terminator codon.

**YCK3 Suppressors of *yck1Δyck2-ts***

The *YCK3* C-terminal truncation mutation Δ409-524 was constructed in the *YCK3p-4xHA/YCK3* plasmid context using PCR-based methods; the 116 deleted codons in this mutation were replaced by a Mlu I restriction site. Yck3(Δ409-524)p lacks the C-terminal palmitoylation sequences and, like Yck3(ΔCys)p, is expected to be cytoplasmic. A 796 bp fragment of *YCK3* (corresponding to codons 336 to 524 plus 230 bp of downstream sequence), generated from a *YCK3* plasmid template by mutagenic PCR (reactions included 10 mM MnCl₂ in addition to the usual 50 mM MgCl₂ and 80 µM dATP and 400 µM of the other dNTPs) was transformed into *yck1Δyck2-ts* yeast cells together with the Mlu I-linearized *YCK3p-4xHA/YCK3(Δ409-524)* plasmid. Ura⁺ transformants that grew at 37°C were selected. Plasmid DNA was isolated from these cells and the C-terminal region of *YCK3* was sequenced.

**Palmitate Labeling**

*GAL1*-driven *YCK2* and *YCK3* constructs, tagged with amino-terminal 6xHis/FLAG/HA sequences, were introduced both into the *AKR1*⁺ wild-type strain LRB759 and the isogenic *akr1Δ* strain NDY1405. The in vivo labeling with [(9,10)³H]-palmitic acid (60 Ci/mmol; DuPont), along with the subsequent preparation of extracts and the anti-FLAG immunoprecipitation of the labeled Yck kinases were as previously described (Roth *et al.*, 2002).
Quantitation of the Yck protein recovery from the immunoprecipitation by anti-HA Western blotting was used to normalize the amount of sample applied to the SDS-polyacrylamide gel for assessing incorporation of the labeled palmitate.

**Indirect Immunofluorescence Microscopy**

For most experiments, cells that carried HA-tagged *YCK URA3/CEN/ARS* plasmid constructs were cultured in minimal yeast medium lacking uracil. For experiments that utilized *GAL1*-driven constructs, the Yck proteins were expressed from cultures grown in YP medium containing 2% raffinose with a 2 h addition of 2% galactose, followed by a 20 min "chase" period with 3% glucose (giving newly-synthesized proteins the opportunity to attain their endpoint destinations). Cells were fixed, spheroplasted, and otherwise developed for immunofluorescent microscopy as previously described (Chen and Davis, 2002). The HA-tagged proteins were detected using a 1:1000 dilution of the HA.11 mAb (Covance) as the primary antibody, followed by a 1:500 dilution of the Cy3-conjugated goat anti-mouse IgG secondary antibody. Z-stacks of digital images with focal planes spaced at 0.2 µm increments were collected using the 100x objective on a Leica DMRA2 microscope and Hamamatsu Orca ER CCD camera. Images were deconvolved with 25 iterations of Autoquant Auto-Deblur software (Autoquant) and contrast-enhanced with Photoshop (Adobe).
RESULTS

Yck3p Palmitoylation

The Yck3p striking cysteine-rich C-terminal sequence Cys-Cys-Cys-Cys-Phe-Cys-Cys-Cys (CCCCFCCC), suggested the possibility of its palmitoylation. To test this possibility, cells were labeled with $[^3]$H-palmitic acid and Yck3p was immune precipitated via N-terminally-attached epitope tags (Figure 1). Indeed, we found that palmitate label is incorporated into Yck3p. In contrast, Yck3(ΔCys)p, a deletion mutant missing just the cysteine-rich C-terminal eight Yck3p residues, was not labeled, indicating a likely role of some or all of the C-terminal cysteines as palmitoyl acceptors (Fig. 1).

Our recent work showed the polytopic membrane protein Akr1p to be the palmitoyl transferase responsible for the palmitoylation of Yck1p and Yck2p (Roth et al., 2002). Furthermore, our work indicated that cellular palmitoylation is likely carried out by multiple, distinct palmitoyl transferase activities. Indeed, a second yeast activity comprised by Erf2p and Shr5p has been identified as the devoted palmitoyl transferase for yeast Ras protein (Lobo et al., 2002). Here, we examined the dependence of Yck3p palmitoylation on Akr1p function (Fig. 1). As we have shown previously (Roth et al., 2002), the palmitoyl-labeling of Yck2p was fully abolished in akr1Δ cells (Fig. 1). In contrast, Yck3p labeling appeared undiminished. While Akr1p clearly is not required for Yck3p palmitoylation, we cannot rule out that Akr1p participates in some partial capacity, perhaps functioning redundantly with some other palmitoyl transferase.
Yck3p Localizes to the Vacuolar Membrane

The palmitoylation of Yck1p and Yck2p serves to tether them to the plasma membrane (Roth et al., 2002). Here, Yck3p localization was examined using indirect immunofluorescent detection of N-terminal HA epitope tags (Figure 2). Like Yck1p and Yck2p, palmitoylated Yck3p also is membrane-localized, however rather than being localized to the plasma membrane, it localized instead to the limiting membrane of the yeast vacuole – the vacuole being easily visualized by DIC optics as apparent surface depressions (a well-known artifact of this optical method). The Yck3(ΔCys)p mutant, which is not palmitoylated, was mislocalized; it showed a diffuse distribution throughout the cytoplasm and nucleus. We conclude that the palmitoylation of Yck3p serves to tether it to the cytoplasmic face of the vacuolar membrane.

In akr1Δ cells, consistent with its continued palmitoylation, Yck3p was seen to be largely localized to the vacuole membrane (Fig.2). However, this vacuolar membrane localization was somewhat less complete than that seen in the wild-type AKR1+ context; in the akr1Δ cells, in addition to vacuolar membrane staining, some Yck3p localization to cytoplasmic puncta was also apparent (Fig. 2). Thus, while no difference was discerned in the palmitate-labeling of Yck3p in AKR1+ and akr1Δ cells, Akr1p does appear to play some partial role in the Yck3p localization process. A possibility discussed below (see Discussion) is that Yck3p palmitoylation might involve Akr1p acting together with other palmitoyl transferases.

Subtle effects on Yck3p localization also were seen with overexpression from the GAL1 promoter (Fig. 2). While the bulk of the staining was again at the limiting membrane of the vacuole, some Yck3p clearly could be seen also localizing to the surface plasma membrane.
Overexpression may partially overwhelm the normal Yck3p vacuolar sorting mechanism, resulting in some Yck3p escape to the plasma membrane.

Mapping the Vacuolar Sorting Signals of Yck3p

To delimit the Yck3p signals directing both palmitoylation and localization, we constructed a series of chimeric proteins which fuse various C-terminal portions of Yck3p to the C-terminus of Ura3p, a cytoplasmic enzyme of the uracil biosynthesis pathway (Figure 3A). Fusion proteins were expressed from the GAL1 promoter and have the HA epitope tag attached to the N-terminus of the Ura3 sequences to facilitate immunofluorescent detection. The starting Ura3p construct with no fused Yck3p sequences was found to be diffusely distributed through the cytoplasm (our unpublished results). When the entire Yck3 coding sequence was fused to Ura3, the Ura3-Yck3(1-524) fusion was found to localize primarily to the vacuolar membrane (Fig. 3B). Consistent with results reported above for GAL1 promoter overexpression (Fig. 2), the Ura3-Yck3 fusion which was also expressed from the GAL1 promoter, also was partially mislocalized to the plasma membrane. The Ura3-Yck3(409-524) fusion protein localized like Ura3-Yck3(1-524) (Fig. 3B); thus the Yck3 C-terminal 116 residues contains sufficient information for directing both palmitoylation and vacuolar sorting. In contrast, the Ura3-Yck3(462-524) fusion protein localized exclusively to the plasma membrane (Fig. 3B). The failure of vacuolar targeting indicates a requirement for Yck3 409-462 sequences for proper vacuolar sorting – loss of this vacuolar sorting information, resulted in Ura3-Yck3(462-524)p being delivered, presumably by default, to the plasma membrane. The Ura3-Yck3(517-524) fusion protein, having just the C-terminal CCCCCCFFFF sequence as its Yck3 contribution, was
found diffusely distributed throughout the cell cytoplasm (Fig. 3B); this localization was identical to that seen both for the parental Ura3 construct with no fused Yck3 sequences (our unpublished results) and for the unpalmitoylated Yck3(ΔCys)p mutant (Fig. 2). Thus, while the CCCCCCCC sequence is required for palmitoylation, it is not, by itself, a sufficient signal. Instead, palmitoylation apparently requires additional sequences mapping within the C-terminal 63 Yck3 residues (i.e. the sequences required for the membrane localization of the Ura3-Yck3(462-524) fusion protein). We conclude that the Yck3 409-462 sequences are required for vacuolar targeting and 462-524 sequences are required for palmitoylation.

As a further test of the involvement of the 409-462 sequence in vacuolar targeting, a mutant Yck3p having an in-frame deletion of this interval was constructed, Yck3(Δ409-462)p (Figure 4A). The Δ409-462 mutation indeed did perturb Yck3p localization, however the effect was only partial: while some Yck3(Δ409-462)p clearly was mislocalized to the plasma membrane, much still continued to be delivered to the vacuolar membrane (Fig. 4B). Thus, while we can conclude that "efficient" sorting to the vacuole requires elements within the 409-462 interval, the continued delivery of substantial amounts of the Yck3(Δ409-462)p to the vacuole suggests that Yck3p may have additional sorting information, that maps outside of the 409-462 interval. Alternatively, the residual vacuolar transport could represent default transport to the vacuole (Roberts et al., 1992) – default transport being transport that occurs in the absence of sorting information (see Discussion).

To test the sufficiency of the sorting information within the 409-462 interval, we transplanted this sequence into the Yck2p context. The resulting Yck2/Yck3/Yck2 chimeric protein (Yck232p) has the Yck3 409-462 interval in place of Yck2 residues 396 through 496
The Yck2 396-496 interval is a glutamine-rich sequence, located C-terminal to the kinase domain, that is required neither for Yck2p palmitoylation, nor for its transport to the plasma membrane; the \textit{YCK2}(\Delta396-496) allele also fully complements a temperature-sensitive \textit{yck2} allele (Babu \textit{et al.}, 2002) (Roth and Davis, unpublished results). Finally, the C-terminal 50 amino acids of Yck2p retained by the Yck232 chimera suffice to direct Yck2p palmitoylation (Babu \textit{et al.}, 2002)(Roth and Davis, unpublished results).

The addition of the Yck3 sequences to Yck2p resulted in a striking change in localization: rather than being localized to the plasma membrane like Yck2p, Yck232p instead was found to localize predominately to the vacuolar membrane (Fig. 4B). Thus, the Yck3 409-462 interval does contain a sufficient signal for vacuolar sorting. We note, however, that the redirection to the vacuole is not fully complete as some Yck232p also was seen to localize to the surface membrane. This partial localization to the plasma membrane might again be the result of overexpression; the Yck232 chimeric construct is expressed from upstream \textit{YCK2} promoter sequences which may be substantially stronger than the \textit{YCK3} promoter: expressed from their native promoters, Yck2p accumulates to twenty-fold higher levels within the cell than does Yck3p (our unpublished results).

\textbf{AP-3-Dependent Sorting}

In yeast, two distinct trafficking mechanisms have been found to deliver membrane proteins to the vacuolar membrane. The CPY pathway, which transports soluble hydrolases like carboxy-peptidase Y (CPY) from the Golgi lumen to vacuolar lumen, also transports membrane
proteins to the vacuolar membrane (examples being the protease DPAP-B and the vacuolar ATPase). The second pathway for Golgi-to-vacuole integral membrane protein traffic is the ALP or AP-3 pathway. We used mutants separately disabling these two pathways to see which might participate in Yck3p transport to the vacuole. Class E \( vps \) mutants were used to disrupt traffic along the CPY pathway. Acting at the level of the multi-vesicular body (MVB), these mutants accumulate CPY pathway cargoes within so-called class E bodies, aberrant compartments located just adjacent to the vacuole (Conibear and Stevens, 1998; Katzmann et al., 2002). Blockade of the ALP pathway was achieved through deletion of the genes encoding subunits of the AP-3 complex. Two class E mutants, \( vps2\Delta \) and \( vps4\Delta \), were tested – neither showed discernible effects on Yck3p transport. In both mutant strains, Yck3p was found to localize exclusively to the vacuolar membrane; no staining of perivacuolar class E bodies, characteristic of CPY pathway cargoes, was discerned (Figure 5A). This lack of an effect of the class E mutations also excludes scenarios in which Yck3p might access the vacuole via an endocytic route (after first being delivered to the cell surface); endocytic traffic to the vacuole also goes through the MVB and endocytic cargoes consequently also accumulate within the class E bodies of class E \( vps \) mutants (Davis et al., 1993; Piper et al., 1995). To more directly rule out the participation of the endocytic pathway, we assessed Yck3p localization in two strains mutationally disabled for the plasma membrane internalization step of endocytosis, namely \( end3\Delta \) and \( sac6\Delta \) cells (D'Hondt et al., 2000). No impairment in the vacuolar transport of Yck3p was seen in either of these \( end \) mutant strains (our unpublished results). For blockade of AP-3 pathway sorting, again two mutants were tested, \( aps3\Delta \) and \( apm3\Delta \), deleted for the AP-3 \( \sigma \) and \( \mu \) subunit genes, respectively. In both mutant backgrounds, a clear mislocalization of Yck3p to the plasma membrane was observed (Fig. 5A). However, again, as previously observed with Yck3(\( \Delta 409-462 \)p, the missorting was not total: a substantial fraction of the Yck3p continued to
be delivered to the vacuolar membrane (the issue of this residual vacuolar sorting is further explored within the Discussion). Nonetheless, we can conclude that Yck3p depends, at least partially, on the AP-3 pathway for its transport to the vacuole.

We have also examined Yck232p sorting in apm3Δ cells (Fig. 5B). While in wild-type cells, Yck232p localized mainly to the vacuolar membrane, in apm3Δ cells, its sorting to the vacuole was abolished as the chimeric protein localized instead, fully to the plasma membrane (Fig. 5B). Thus, the vacuolar sorting signal(s) that map within the 409-462 interval are fully AP-3-dependent.

Yck3p Missorting to the Plasma Membrane Suppresses yck1Δyck2-ts

As described in the Introduction, disabling mutations within the AP-3 subunit genes were first identified as suppressors of the 37°C inviability of yck1Δyck2-ts cells (Panek et al., 1997). Given the role described here for AP-3 in Yck3p sorting, we wondered if the Yck3p missorting to the plasma membrane might account for yck1Δyck2-ts suppression. Does the Yck3p that is diverted to the plasma membrane in AP-3 mutants help to compensate for the casein kinase deficit of the yck1Δyck2-ts cells? Supporting such an explanation, yck1Δyck2-ts suppression can also be achieved through Yck3p overexpression (Wang et al., 1996), a condition that also results in some Yck3p being diverted to the plasma membrane (Fig. 2).
To test the role of Yck3p missorting in \( yck1\Delta yck2-ts \) suppression, we have assessed the 37\(^\circ\)C growth of a \( yck1\Delta yck2-ts \) strain transformed with plasmids carrying either the wild-type \( YCK3 \) allele or the \( YCK3(\Delta409-462) \) mutant allele. While the addition of the wild-type \( YCK3 \) allele to the \( YCK3^+ yck1\Delta yck2-ts \) strain provided no growth benefit, introduction of the \( YCK3(\Delta409-461) \) allele clearly conferred substantial 37\(^\circ\)C growth to the \( yck1\Delta yck2-ts \) strain (Figure 6). Thus, Yck3p missorting does provide a possible mechanism for \( yck1\Delta yck2-ts \) suppression.

Point Mutants Disabling the Vacuole Sorting Signal Identify a Tyrosine-Based Adaptin Recognition Sequence

The \( yck1\Delta yck2-ts \) suppression by Yck3p missorting suggested a scheme for isolating mutants within the Yck3p sorting signal. Like the \( \Delta409-462 \) deletion, point mutants that inactivate the sorting signal also should confer suppression. Mutations were randomly directed into the C-terminal region of \( YCK3 \) (including the 409-462 interval) by mutagenic PCR and the resulting library of plasmid-borne mutant \( YCK3 \) alleles was introduced into the \( yck1\Delta yck2-ts \) strain with growth at 37\(^\circ\)C selected. From an estimated 40,000 transformants, 14 colonies surviving at 37\(^\circ\)C were selected. Plasmid DNA was recovered from each and mutations were identified by sequencing. While seven of the isolates had multiple changes within the 409-462 interval, the other seven showed changes just at single residues (Figure 7A). The seven single changes all clustered within the four residue sequence YDSI from Tyr\(_{444}\) to Ile\(_{447}\). Suppression
was conferred by changes of Tyr\textsubscript{444} to His, Cys, and Asn, with a change of Ser\textsubscript{446} to Pro, and by changing Ile\textsubscript{447} to Asn. Of the seven mutants with multiple changes within the 409-462 interval, all had one of the mutations within the YDSI tetrapeptide sequence, with Tyr\textsubscript{444} changed to His, Cys, or Asp, or with Ser\textsubscript{446} again changed to Pro.

Localization of three of these mutant Yck3 proteins was assessed by indirect immunofluorescence (Figure 7B). Each showed a missorting defect similar to that seen for Yck3(Δ409-462)p, giving a dual localization – some of the Yck3p sorted to the vacuolar membrane and some missorted to the plasma membrane. The effects of two of these mutations, namely Y444H and S446P, were also assessed within the Yck232 chimeric protein context (Fig. 7C). Here, the effects of the mutations were more dramatic. Rather than being localized to the vacuolar membrane like wild-type Yck232p, the two mutant Yck232 proteins, were found to localize exclusively to the plasma membrane. Thus, introduction of either of these two point mutations fully destroys the AP-3-dependent sorting determinant contained within the Yck3 409-462 interval.

The YDSI tetrapeptide fits nicely to the YXXΦ consensus for tyrosine-based adaptin recognition motifs (Bonifacino and Traub, 2003). To further explore this connection with the adaptin consensus, alanine substitution mutants were constructed at each of the four residues within the YDSI tetrapeptide. Examining the effect of these mutations within the context of the Yck232 construct, we found that localization to the vacuole is abolished by both the Y444A and I447A substitutions, while the D445A and S446A changes were without effect (Fig. 7D). Thus, the Yck3 YDSI fits well with the YXXΦ consensus motif for adaptin-mediated recognition: the
initial Tyr as well as the Φ residue at the Y+3 position are critical, while residues at the Y+1 and Y+2 make little or no contribution.
DISCUSSION

The present work localizes Yck3p to the limiting membrane of the vacuole. This localization requires both palmitoylation for membrane association and the AP-3 pathway for sorting. Blocking the AP-3-mediated sorting either through mutation of the AP-3 complex itself or through mutation of the AP-3-dependent sorting signal resulted in Yck3p missorting to the plasma membrane. This AP-3 involvement in Yck3p sorting suggests an answer to an old puzzle – the suppression of yck1Δyck2-ts by AP-3 disabling mutations (Panek et al., 1997). The answer, our results suggest, is that the Yck3p missorting that occurs in these AP-3 mutants serves to replenish active kinase to the kinase-deficient yck1Δyck2-ts plasma membranes. Exploiting this suppression phenotype, we have isolated mutations within the Yck3p AP-3-dependent sorting signal that identify a classic YXXΦ adaptin-sorting signal.

The Yck3p vacuolar localization suggests the possibility of vacuolar function. Indeed, a recent genomic study in which the yeast deletion collection was screened for vam mutants (aberrant vacuolar morphology) found yck3Δ cells to have fragmented vacuoles (Seeley et al., 2002), i.e. having multiple small vacuoles rather than the one to three large vacuoles found in wild-type cells. vam mutants often show correlated defects in vacuolar homotypic fusion (Wickner, 2002). The same genomic analysis also found yck3Δ cells to have an associated Vps phenotype, i.e. CPY is partially missorted to the extracellular milieu rather than to the vacuolar lumen (Seeley et al., 2002). Tethered to the external surface of the vacuole, Yck3p is well-positioned to modulate both transport into the vacuole, as well as the vacuolar fusion-fission reactions that control vacuolar number and size.
Yck3p Palmitoylation

We previously showed that Yck1p and Yck2p are palmitoylated and that Akr1p is the enzyme responsible for the palmitoyl modification (Roth et al., 2002). Akr1p is one of only two palmitoyl transferases (PTases) to be identified to date from any species. The other PTase, which was identified concurrently with Akr1p, is comprised by two proteins, Erf2p and Shr5p and is apparently devoted to yeast Ras protein palmitoylation (Lobo et al., 2002). The two transferases differ in their specificity for substrate, with Akr1p being devoted to the palmitoylation of Yck1p and Yck2p and Erf2p/Shr5p, to the palmitoylation of the yeast Ras proteins, Ras1p and Ras2p. Erf2p/Shr5p has been localized to the ER and Akr1p has been preliminarily localized to the yeast Golgi (Lobo et al., 2002; Roth et al., 2002). Akr1p and Erf2p both are polytopic integral membrane proteins and both have DHHC cysteine-rich domains (DHHC-CRD), i.e. a novel 58 residue-long, zinc finger-like domain. DHHC-CRD sequences have been identified in a evolutionarily-widespread family of proteins found in all eukaryotic genomes sequenced to date; seven DHHC-CRD proteins have been identified in S. cerevisiae and 28, in man. These proteins all are predicted to be polytopic integral membrane proteins and all have the DHHC-CRD sequence similarly disposed within an inter-transmembrane domain loop segment. The integrity of the DHHC-CRD is required for the activity of both the Akr1p and Erf2p/Shr5p PTases (Lobo et al., 2002; Roth et al., 2002). We have suggested that the DHHC-CRD sequence may be a signature palmitoyl transferase feature, with the DHHC-CRD protein family constituting a diverse family of palmitoyl transferase specificities, needed to accommodate the diverse range of substrates known to undergo palmitoylation (Lobo et al., 2002; Roth et al., 2002).
What PTase mediates Yck3p palmitoylation? As palmitoylation is required for Yck3p membrane attachment, we expect that palmitoylation temporally precedes the AP-3-dependent Golgi to vacuole sorting. Therefore, the cognate PTase for Yck3p should localize either to the Golgi or to a compartment with access to the Golgi (e.g. the ER). The unimpaired Yck3p palmitoylation seen in \textit{akr1}\textDelta cells by [\textsuperscript{3}H]-palmitate labeling (Fig. 1), indicates a participation for some PTase other than Akr1p. Nonetheless, we cannot rule out that Akr1p also participates, perhaps in some partial or redundant capacity. Indeed, consistent with such participation, a subtle impairment to Yck3p localization was noted in \textit{akr1}\textDelta cells – in addition to the usual vacuolar membrane localization, some Yck3p was found to localize in the \textit{akr1}\textDelta cells to cytoplasmic puncta (Fig. 2). This mislocalization is accentuated with \textit{GAL1}-driven Yck3p overexpression in \textit{akr1}\textDelta cells which results in Yck3p being predominantly localized to such extra-vacuolar membranous structures (our unpublished results). Given the seven potential palmitoyl acceptors in the C-terminal CCCFCCCC sequence, the involvement of multiple PTases seems reasonable – Akr1p could be the devoted PTase for some of the modifications, while other cysteines perhaps being modified by other palmitoyl transferases – an incompletely palmitoylated Yck3p might be poorly delivered to the vacuole. Alternatively, Akr1p effects on Yck3p localization could be indirect – perhaps the Akr1p-dependent palmitoylation of some other protein is required for the proper functioning of the Yck3p vacuolar trafficking mechanism.

Akr2p would seem to be the obvious candidate for a collaborating PTase with Akr1p – named for its homology to Akr1p that extends across its entire length and includes both the ankyrin repeats and the DHHC-CRD sequence. However, despite its striking and extensive homology to Akr1p, we have been unable to detect a role for Akr2p in the palmitoylation or
function of any of the Yck proteins (our unpublished results). For Yck3p, no additional localization defect is seen in \textit{akr1Δ akr2Δ} cells, beyond the subtle mislocalization previously noted for \textit{akr1Δ} cells (Fig. 2; B. Sun and N. Davis, unpublished). Future experiments will examine Yck3p palmitoylation in strains having \textit{AKR1} deleted in combination with each of the other five yeast DHHC-CRD genes.

Deletion of the Yck3 C-terminal CCCCCFCCC eliminates both palmitoylation and Yck3p membrane association, suggesting a likely role for these cysteines as the palmitoyl acceptors. We do not expect that this sequence also is prenylated. First, this sequence does not fit to the rigid CaaX prenylation consensus: the fourth residue from the C-terminus, conspicuously, is not a cysteine. Furthermore, while C-terminal CC or CxC sequences can be geranylgeranylated, such prenylation is limited to members of the Rab GTPase family; this Rab protein prenylation requires, in addition to the geranylgeranyl transferase, a second essential participant, Rab escort protein REP (Mrs6p in \textit{S. cerevisiae}), which specifically binds to the generic Rab structure and presents it to the transferase for modification (Jiang and Ferro-Novick, 1994; Zhang and Casey, 1996).

While the Yck3 CCCCCFCCC sequence is required for palmitoylation it is not sufficient. Results from the Ura3-Yck3 fusion protein analysis indicated that additional sequences mapping within the Yck3 C-terminal 62 residues also are required. This is similar to what has been found for Yck2p, where the sequence requirements for palmitoylation have been more extensively analyzed: in addition to the the C-terminal Cys-Cys sequence, palmitoylation also requires sequence elements distributed through the Yck2 C-terminal 50 residues (Babu \textit{et al.}, 2002; Roth
et al., 2002) (A. Roth and N. Davis, unpublished). Thus, it appears that relatively large, perhaps folded protein domains may serve as palmitoylation signals for the Yck kinases.

Residual AP-3-independent Yck3p Transport to the Vacuole

We demonstrated that the AP-3 pathway plays a major role in trafficking of Yck3p to the vacuole. However, it is not absolutely essential for this vacuolar transport. Substantial Yck3p traffic to the vacuole continues when the AP-3 complex is disabled or when the Yck3p AP-3-sorting signal is deleted. This is also true for the two other yeast AP-3 substrate proteins Vam3p and ALP. For both, substantial transport to the vacuole persists when their AP-3 sorting is blocked either through *cis*-mutation of their dileucine sorting signals or through *trans*-mutation of the subunits of the AP-3 complex (Stepp et al., 1997; Darsow et al., 1998; Vowels and Payne, 1998). For Vam3p and ALP, this default, AP-3-independent sorting to the vacuole appears to route predominantly through the CPY pathway (Darsow et al., 1998; Vowels and Payne, 1998). To test if the residual, AP-3-independent, Yck3p sorting to the vacuole might also be via the CPY pathway, we have examined the localization of two AP-3 sorting-defective Yck3p mutants, Yck3(Δ409-462)p and Yck3(Y444H)p, in three different class E *vps* mutant – in *vps2Δ*, in *vps4Δ*, or in *vps27Δ* cells. While these same *vps* mutations showed no effect on the trafficking of wild-type Yck3p (Fig. 5B), the two mutant Yck3 proteins both did show substantial localization to peri-vacuolar class E bodies in each of the three different *vps* mutant strains (our unpublished results). The block imposed by these class E *vps* mutations was not total: in addition to the class E body accumulation, substantial immunostaining of the vacuolar membrane still persisted (our unpublished results). Nonetheless, the class E body localization that was
observed indicates that at least some of the residual, AP-3-independent traffic to the vacuole is through the MVB and perhaps also indicates the CPY pathway is being used for the default transport, as previously suggested for both Vam3p and ALP.

Rather than using the CPY pathway for this secondary trafficking from Golgi to vacuole, a second plausible scenario would involve endocytosis of Yck3p that is first missorted to the plasma membrane due to impaired AP-3 sorting. Like CPY pathway cargoes, endocytic cargoes destined for the vacuole also pass through the MVB and thus also get trapped within the aberrant pre-vacuolar, class E compartments of class E \textit{vps} mutant cells (Davis \textit{et al.}, 1993; Piper \textit{et al.}, 1995). To assess the contribution made by the endocytic pathway to the AP-3-independent, residual sorting, Yck3(Δ409-462)p and Yck3(Y444H)p localization was examined in \textit{end3Δ} and in \textit{sac6Δ} cells – two \textit{end} mutants disabled for the initial plasma membrane uptake step of endocytosis (D'Hondt \textit{et al.}, 2000). The two AP-3 sorting-deficient Yck3p mutants showed a localization in the two \textit{end} mutants qualitatively quite similar to that seen in the isogenic endocytosis-competent, wild-type cell – i.e. a dual localization to both vacuolar and plasma membranes (our unpublished results). Thus, blocking the endocytic pathway does not, by itself, block the residual transport of the mutant Yck3 proteins to the vacuole. Nonetheless, we cannot rule out that such routing of Yck3p to vacuole via the plasma membrane makes some partial contribution to this phenomenon of secondary or default Yck3p transport.

\textbf{YXXΦ Sorting Signal}
The Yck3p AP-3-dependent sorting signal was first mapped to the 409-462 sequence interval. This interval inserted into Yck2p, redirected sorting from the plasma membrane to the vacuole, demonstrating the sufficiency of this interval in signaling vacuolar sorting. Using the \textit{yck1Δyck2-ts} suppression strategy, we isolated point mutations within this interval that abolished function of this signal. These mutations identified the tetrapeptide sequence YDSI, a classic example of a tyrosine-based adaptin sorting signal.

In mammalian cells, the AP-1, AP-2, and AP-3 complexes recognize their cargoes through a remarkably similar set of tyrosine- or dileucine-based signals, with the tyrosine signals based on the YXXΦ consensus and with the dileucine-based signals generally conforming to a [DE]XXXL[LI] consensus. The two signals bind different sites within the adaptin complexes. Of the two, the binding site for YXXΦ signals, located on the surface of the μ subunit, has been more extensively characterized. Crystal structures of mammalian AP-2 μ2 complexed with YXXΦ-containing peptides have shown that the YXXΦ sequence binds in an extended conformation, with the critical Y and Φ sidechains contributing the major contacts, fitting into hydrophobic pockets on the μ surface (Owen and Evans, 1998). Consistent with the primacy of the Y and Φ interactions, most of the changes within the Yck3 YDSI sequence were isolated in these two residues, i.e. the Tyr and the Ile at the Y+3 position. Indeed, only one change was found that did not occur at either of these two residues – the Ser-to-Pro substitution at the Y+2 position (Fig. 7A). While this Ser-to-Pro change abolished function of the signal, a Ser-to-Ala change at the same position had no effect (Fig. 7D). Though the residues at the Y+1 or Y+2 positions are not predicted to contribute key binding interactions, one can easily see from the crystal structure how the rigidifying effect of a Pro substitution at either of these positions might
disrupt interaction of the Y and Φ sidechains with their binding pockets – given the fixed positions of these binding pockets on the μ surface, accomodation of the simultaneous binding of Y and Φ depends on the rotational flexibility of the intervening peptide backbone at the Y+1 and Y+2 positions.

Yck3p is only the third cargo protein yet identified for AP-3-dependent sorting in yeast. The two other yeast AP-3 cargoes yet identified, i.e. ALP and Vam3p, both utilize classic dileucine signals (Darsow et al., 1998; Vowels and Payne, 1998). AP-3-dependent sorting in mammalian cells makes extensive use of both dileucine- and tyrosine-based sorting signals. This first identification of a yeast YXXΦ signal indicates that the adaptin-signal interaction is remarkably well-conserved through evolution. Additional examples of this classic sorting signal likely will be found as more is learned about adaptin-mediated sorting in yeast.
ACKNOWLEDGEMENTS

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REFERENCES


**FIGURE LEGENDS**

**Figure 1.** Yck3p is subject to Akr1p-independent palmitoylation. Yck2 and Yck3 proteins, tagged at their amino-termini with a 6xHis/FLAG/HA sequence, were expressed from the GAL1 promoter in either wild-type (AKR1⁺) cells or in isogenic akr1Δ cells. The ΔCys mutation is a deletion of the last C-terminal eight Yck3p residues, CCCCFC CCC. Following labeling of the cells with [³H]-palmitic acid, extracts were prepared, the tagged kinases were immune precipitated and subjected to SDS-PAGE and fluorography (upper panel). Recovery of the immune-precipitated kinases was assessed by Western blotting using an anti-HA mAb (lower panel).

**Figure 2.** Yck3p localizes to the vacuolar membrane. Indirect immunofluorescent detection of Yck3 proteins through amino-terminal HA epitope tags. The tagged constructs were expressed at normal endogenous levels under the control of YCK3 upstream sequences or in one instance, overexpressed from the GAL1 promoter (GAL1p-Yck3p). Two typical cells for each conditions are shown, with the fluorescent images of the cells on the left and the same cells as visualized by DIC optics shown to the right.

**Figure 3.** Deletional mapping of the Yck3 sequences that direct palmitoylation and vacuolar localization. (A) Schematic of Ura3-Yck3 fusion proteins. At the top is a schematic of Yck3p; the highly-conserved (among the Yck proteins) N-terminal kinase domain is followed by the poorly-conserved C-terminal domain, terminating in the indicated cysteine-rich sequence. The different Ura3-Yck3 fusion protein constructs with the indicated portions of Yck3 fused in-frame to the C-terminus of the cytoplasmic enzyme Ura3p are shown below. The Ura3 amino-terminus
is tagged with a copy of the HA epitope to facilitate immune detection. (B) Indirect immunofluorescent localization of the Ura3-Yck3 constructs. Fusion proteins were expressed from the GAL1p. For each construct, two typical cells are shown, with the fluorescent images of the cells on the left and the same cells visualized by DIC optics shown to the right.

**Figure 4.** The Yck3 409-462 sequence interval harbors a vacuolar sorting signal. (A) Schematic of the different HA-tagged Yck mutant constructs. (B) Indirect immunofluorescent localization of the different HA-tagged mutant Yck proteins. Expression of the Yck2 and Yck3 constructs was directed by the natural YCK2 and YCK3 upstream regulatory sequences. Two typical cells are shown for each construct with the fluorescent images of the cells on the left and the same cells visualized by DIC optics shown to the right.

**Figure 5.** Yck3p traffics to the vacuole via the AP-3 pathway. HA-tagged Yck3p (panel A) or HA-tagged Yck232 (panel B) expressed at normal endogenous levels under the respective control of YCK3 or YCK2 upstream sequences, in either the wild-type Saccharomyces Deletion Consortium strain or in the isogenic Consortium strains individually deleted for the indicated genes. For each mutant strain, two typical cells are shown, with the fluorescent images of the cells on the left and the same cells visualized by DIC optics shown to the right.

**Figure 6.** Yck3p missorting confers 37°C viability to yck1Δ yck2-ts cells. Serial ten-fold dilutions of yck1Δ yck2-ts cells, transformed by the CEN/ARS vector pRS316 or by pRS316 carrying either the wild-type YCK3 or the mutant YCK3Δ409-462 allele, were plated at both permissive (25°C) and non-permissive (37°C) temperatures. Growth of the yck1Δ yck2-ts
transformants is compared to that of the isogenic \( YCK1^+ \ YCK2^+ \) parental strain at the two temperatures.

**Figure 7.** Point mutations within the Yck3 YDSI tetrapeptide disrupts vacuolar sorting. (A) Mutations within the Yck3 YDSI tetrapeptide conferring yck1\( \Delta \) yck2-ts suppression. The ten residue-long Yck3p sequence harboring the YDSI tetrapeptide is shown. Just below are the substitutions identified from the seven mutants having only a single substitution within the 409 to 462 interval. Below this are substitutions within YDSI peptide found among the seven mutants which had more than one amino acid changes within the 409-462 interval. For substitutions that were re-isolated multiple times, the number of times the particular change was found is indicated (in parentheses). (B-D) Effects of YDSI mutations on localization of HA-tagged Yck3 and Yck232 proteins. Localization of wild-type and mutant versions of Yck3p and the chimeric Yck232p, expressed under the control of \( YCK3 \) and \( YCK2 \) promoter sequences, respectively, were detected by indirect immunofluorescent microscopy. For each mutant, two typical cells are shown, with the fluorescent images of the cells on the left and the same cells visualized by DIC optics shown to the right. (B) Effects of the indicated suppressor mutations on Yck3p localization. (C) Effect of suppressor mutations within the Yck232 chimeric protein context. (D) Effect of alanine substitutions at each position within the YDSI tetrapeptide tested on Yck232p localization.
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Ser-Gln-Thr-Tyr-Asp-Ser-Ile-Ser-His-Thr

single changes

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|          | Pro(2) | Pro(4) |

B

Yck3p

wild-type  Δ409-462  Y444H  S446P  I447N

C

Yck232p

wild-type  Y444H  S446P

D

Yck232p

Y444A  D445A  S446A  I447A