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Palmitoylation and the yeast casein kinase yck2

Irene Papanayotou
Wayne State University

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PALMITOYLATION AND THE YEAST CASEIN KINASE YCK2

by

IRENE PAPANAYOTOU

DISSERTATION

Submitted to the Graduate School

of Wayne State University,

Detroit, Michigan

in partial fulfillment of the requirements

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DOCTOR OF PHILOSOPHY

2011

MAJOR: PHARMACOLOGY

Approved by:

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Advisor                                              Date

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DEDICATION

I dedicate this work to my parents, Stavroula and George Papanayotou. You have done your best to give me everything in life, and for this I owe all of my achievements to you. Thank you for your constant love, support, and trust.
ACKNOWLEDGEMENTS

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CHAPTER I

INTRODUCTION

Palmitoylation is a post-translational lipid modification that allows proteins to interact with membranes. In the yeast *Saccharomyces cerevisiae*, the casein kinase Yck2 is palmitoylated twice at its two C-terminal palmitoyl-accepting cysteine residues, by the palmitoyl-transferring enzyme Akr1. Once palmitoylated, Yck2 traffics through the well characterized secretory pathway to the plasma membrane where it participates in many cellular functions, including bud morphogenesis, cytokinesis, nutrient sensing, and receptor internalization. While the hydrophilic Yck2 is presumably synthesized on cytosolic ribosomes, it gains access to the membrane system by interaction with the six transmembrane-spanning Golgi-localized Akr1. Since palmitoylation occurs at membranes and the palmitoyl transferases are localized to membranes, one key question our lab has been asking is, “How does Yck2 locate Akr1?” This is a general problem for exclusively palmitoylated hydrophilic proteins because while other lipid modifications occur either co-translationally or in the cytoplasm and provide an immediate introduction to membranes, Yck2 must somehow traffic from its place of synthesis to Akr1.

To answer this question, a differential centrifugation technique was utilized that produced strikingly distinct phenotypes for palmitoylated Yck2 versus non-palmitoylated Yck2 when assayed from glass bead-generated lysates. Upon detergent treatment, the palmitoylated wild-type Yck2 behaved like a typical integral membrane protein, fractionating to the supernatant. Surprisingly, the non-palmitoylated Yck2 fractionated to the pellet, both in the presence and absence of detergent. Additional analysis
determined this pellet fraction to identify a high molecular-weight aggregate. This distinct fractionation phenotype allowed us to distinguish between palmitoylated and non-palmitoylated forms of Yck2, and we anticipated this link between impaired palmitoylation and aggregation to represent a more general phenomenon, as seen in Huntington’s disease. However, as documented in Chapter III, the aggregation of non-palmitoylated Yck2 occurred in vitro, being artifically induced as a consequence of the forces produced by the vortexing glass beads. Given the widespread use of glass bead lysis within the yeast community, we have published this finding and included analyses that reveal that glass bead-induced aggregation does apply to other, but not all yeast proteins.

Chapter IV continues to ask how Yck2 might gain access to Akr1, by looking at the sequences within Yck2 that play a role in palmitoylation. We hypothesize that the kinase domain would play an important role since it is essential to protein function. To learn about the role of the kinase domain in the palmitoylation of Yck2, mutants that were completely devoid of the kinase or inactivated for kinase activity were characterized. Both types of kinase mutants were deficient for palmitoylation and a more severe deficiency was seen for kinase-deleted Yck2 compared to the kinase-dead mutants, suggesting that while kinase activity is important for palmitoylation, the kinase domain also provides a structural element necessary for efficient palmitoylation. To further explore the structural element of the kinase domain in palmitoylation, the kinase domain of Yck2 was replaced with the kinase domains of other casein kinase proteins. Analysis of the replacement chimeras revealed that some, but not all replacements
sufficed to restore Yck2 activity, suggesting that the Yck2 kinase domain could possess specific elements needed for palmitoylation.

**Protein Lipidation**

Membrane targeting of proteins requires specialized structures made of proteins or lipids that allow for insertion into the hydrophobic environment of the membrane. Proteins with transmembrane spanning domains consist of α-helices or β-sheets that create a hydrophobic interface within the hydrocarbon core of the lipid bilayer. In addition to membrane spanning proteins, lipid attachment of proteins offers an alternative method of membrane association. First described in 1969, the covalent attachment of lipids to proteins is an event that changes the behavior and localization of cytosolic and transmembrane proteins (Braun and Radin 1969). Over time the list of lipid modifications has grown to consist of four major types based on the identity of the lipid attached to the protein: prenylation (farnesylation and geranylgeranylation), fatty acylation (myristoylation and palmitoylation), glypiation (GPI - glycophosphatidylinositol anchoring), and cholesteroylation. These lipid modifications are involved in protein trafficking and proper function, some mediated by the lipid’s affinity for certain micro-domains in the membrane, such as lipid rafts. Lipid rafts are fluctuating regions of sphingolipid, cholesterol, and proteins that form platforms in the plasma membrane (Lingwood and Simons 2010). The current thinking is that anchors made of saturated fatty acids (GPI anchoring, myristoylation, palmitoylation) and sterols will target proteins to the more tightly packed lipid raft region, while unsaturated and branched modifications (farnesylation, geranylgeranylation, O-acylation) will favor the less restrictive non-raft regions. Following the addition of the well-characterized GPI anchor
in the ER, the protein traffics through the secretory pathway to a lipid microdomain at the extracellular face of the plasma membrane (Chatterjee and Mayor 2001). Also, the C-terminal glycine of the secreted morphogen Hedgehog is modified with cholesterol after an auto-proteolytic processing event, following this a palmitate moiety is added to the N-terminal cysteine (Pepinsky, Zeng et al. 1998).

The three common lipid modifications that occur in the cytoplasm are prenylation, myristoylation and palmitoylation. Prenylation is the post-translational addition of the isoprenoid farnesyl (C15) or geranylgeranyl (C20) moieties to C-terminal cysteine(s) by a thioether linkage (Zhang and Casey 1996). Myristoylation is the addition of myristate (14:0) to the N-terminal Gly residue following cleavage of the initiator methionine (Resh 1999). Both prenylation and myristoylation have conserved motifs that participate in directing the modification. In contrast, palmitoylation has no conserved sequence prerequisites other than simply the presence of an available cysteine residue. N-palmitoylation, first shown in Hedgehog, is the irreversible attachment of palmitate to a cysteine at the N-terminus of a protein, followed by a spontaneous rearrangement to form an amide linkage (Resh 1999). S-palmitoylation is the reversible thioester linkage of palmitate (16:0) to cysteines (Smotrys and Linder 2004). The focus of this thesis will be on S-palmitoylation and will hereafter be referred to as palmitoylation.

**Palmitoylation**

Palmitoylation modifies a diverse range of proteins, including guanosine triphosphate (GTP)-binding proteins, enzymes, ion channels, synaptic receptors and scaffolding proteins. Examples include G protein α subunits, small GTPases (H-Ras, N-
Ras, RhoB) non-receptor tyrosine kinases, and transmembrane G protein coupled receptors (e.g. human δ opioid receptor). The Davis lab has used an acyl-biotinyl exchange (ABE) method coupled with a proteomic technique to confirm 12 of the 15 known palmitoyl-proteins and to identify 35 new palmitoyl-proteins in the yeast *Saccharomyces cerevisiae* (Roth, Wan et al. 2006). Palmitoylation occurs on cysteines located throughout proteins. PSD-95 and Gα are palmitoylated at the amino terminal region, while the synaptosomal associated protein SNAP-25 is modified in its internal region. Palmitoylation affects H-Ras and RhoB at the carboxyl terminal region. The juxtamembrane region of various transmembrane proteins are palmitoylated to offer the option of lipid raft partitioning.

The reversible feature of palmitoylation creates an ideal scenario in the cell where proteins can shuttle between the cytoplasm/organelles and the plasma membrane upon extracellular signals. An example of this phenomenon is best shown in the synaptic neuron, where receptors and their accessory proteins react to a multitude of synaptic neurotransmitters within seconds. Many important proteins in the neuron are palmitoylated such as PSD-95, a key scaffolding protein in the post-synaptic neuron involved in anchoring the AMPA type glutamate receptor (El-Husseini Ael, Schnell et al. 2002). Upon glutamate stimulation, depalmitoylation of PSD-95 results in dissociation from the AMPA receptor and subsequent down-regulation of receptors. Our lab has recently added 21 palmitoylated neural proteins, and over 200 palmitoyl-protein candidates, to the previously existing list of 68 (Kang, Wan et al. 2008). This study also characterized a brain-specific splice variant of Cdc42 that is palmitoylated (in contrast to
the somatic prenylated form) and found to play a role in the induction of dendritic spine formation.

**Palmitoyl-Acyl Transferases (PATs)**

Palmitoylation is the only lipid modification whose removal is presumably under dynamic enzymatic control. The enzymes that add palmitate to proteins (palmitoyl-acyl transferases [PATs]) and those that cleave the thioester bond (palmitoyl-protein thioesterases [PPT]) create a cycling mechanism that aids in delivery and stability of the protein at the membrane and eventual removal and recycling. It is not known why certain cysteines are the palmitoyl acceptors; the only requirement known thus far for palmitoylation of the substrate is the presence of this residue. It is also interesting that, palmitoylation targets the same cysteines *in vitro* as *in vivo* (Resh et al. 1999).

Forward genetic screens first identified the PATs for the yeast Ras2 and the yeast casein kinase 2 (Yck2). Deletion of Erf2/Erf4 (effect on Ras function) prevented palmitoylation of Ras2 and similar deletion experiments for Akr1 (ankyrin repeat) resulted in a non-palmitoylated Yck2 (Bartels, Mitchell et al. 1999; Lobo, Greentree et al. 2002; Roth, Feng et al. 2002). Erf2 and Akr1 have four and six membrane spanning regions, respectively, and share a common DHHC domain - a cysteine rich domain with a conserved aspartate-histidine-histidine-cysteine signature motif. Akr1 has been shown to localize to the early Golgi (Huh, Falvo et al. 2003). The DHHC domain is necessary for enzymatic function suggesting this as the catalytic domain. Other DHHC proteins were found to have palmitoylating activity in yeast including Pfa3, Pfa4 and Swf1. Pfa3 and Pfa4 mediate Vac8 and Chs3 palmitoylation, respectively, and Swf1 showed to
palmitoylate the yeast SNARE Tlg1 (Smotrys, Schoenfish et al. 2005; Valdez-Taubas and Pelham 2005; Lam, Davey et al. 2006). Yeast has 7 PATs while humans have 23. Our lab’s recent publication applied the proteomic method to mutant yeast strains that were either singly or multiply deficient for the 7 DHHC PATs (Roth, Wan et al. 2006). The study showed that when six of the seven yeast PATs were deleted, 29 of the 30 surveyed palmitoyl-proteins were not detected by the ABE method. They concluded that the DHHC family of proteins are authentic PATs that catalyze most protein palmitoylation in yeast and have both discrete and overlapping functionalities.

**Palmitoylation and Depalmitoylation**

Despite the large effort that has been made in discovering and characterizing the PATs, much less is known of the enzymes that remove palmitate from proteins. Depalmitoylation is an enzymatic process catalyzed by protein thioesterases. To date, only two thioesterases have been shown to remove fatty acids from proteins, and of the two, only one seems to be involved in the dynamic acylation cycles seen for lipidated intracellular proteins. Acyl protein thioesterase 1 (APT1) has been shown to interact with and deacylate Ras and Gα subunits *in vitro* (Duncan and Gilman 1998). A yeast strain devoid of Apt1, the yeast homolog of APT1, does not undergo deacylation of Gα (Duncan and Gilman 2002). The lack of a consensus sequence for palmitoylation is in line with the observation that there is also no clear consensus for sequences surrounding the thioacyl group that are identified by APT1. Indeed APT1 is very promiscuous in its substrate specificity, but not all palmitoylated proteins are substrates *in vitro*. APT1 has been shown to depalmitoylate multiply modified substrates such as
Ga, which is palmitoylated and myristoylated, and Ras, which is C-terminally farnesylated and palmitoylated. In addition APT1 also affects a list of transmembrane viral proteins that are palmitoylated at the juxtamembrane region, including HA (hemagglutinin) and VSVG viral entry protein (Rose, Adams et al. 1984; Veit, Kretzschmar et al. 1991). Yet, the integral membrane component of caveolae membranes, caveolin-1, is not deacylated by APT1. Moreover palmitoylation of caveolin-1 is not required for proper localization to caveolae (Dietzen, Hastings et al. 1995). Perhaps this is explained simply by the possibility that unidentified thioesterases exist.

**Ras Depalmitoylation/Repalmitoylation Cycle**

The dynamic process of depalmitoylation and repalmitoylation can affect the function and localization of a protein and is seen with the H- and N-Ras (H/N-Ras) acylation cycle. H/N-Ras rapidly cycles between the Golgi and plasma membranes and this cycle is controlled by the cyclical addition and removal of palmitate. Ras is dually modified, first by a farnesyl group, and second by a palmitoyl group. In a dually modified protein, palmitoylation occurs usually second to the other modification. Myristoyl and prenyl-transferring enzymes are cytosolic and can access their hydrophilic substrate proteins soon after synthesis. The initial lipid modification alters the solubility of the protein, and targets it to proximal intracellular membranes. Added by a thioether linkage, farnesylation is static, remaining attached to H/N-Ras throughout its Golgi-PM cycle. The singly farnesyl tether by itself, however, provides a relatively weak membrane association, allowing the protein to equilibrate on and off membranes (Peitzsch and
McLaughlin 1993; Silvius and l'Heureux 1994). Once H/N Ras is farnesylated on the cysteine of its C-terminal CaaX sequence, it is directed from the cytoplasm to the cytosolic surface of the ER where the -aaX is cleaved by an aminopeptidase and the new C-terminus is carboxy-methylated. At this point, H/N-Ras is further palmitoylated (twice for H-, and once for N-Ras) by the ER localized Erf2/Erf4 in the early secretory pathway. The addition of the palmitate stabilizes H/N-Ras’ affinity for membranes and this allows for successful vesicular trafficking to the plasma membrane. At the PM, H/N-Ras is depalmitoylated by APT1, thus membrane association is destabilized, and H/N-Ras can redistribute in all membranes and somehow travel to the Golgi for repalmitoylation (Rocks, Peyker et al. 2005; Dekker, Rocks et al. 2010). The cycle occurs constitutively with an overall time course of about 5-10 minutes (Goodwin, Drake et al. 2005; Rocks, Peyker et al. 2005; Rocks, Gerauer et al. 2010).

Findings from the H/N-Ras acylation cycle and from studies of other lipidated peptides and model membranes using biophysical and fluorescence-based bleaching techniques and live cell imaging are consistent with the bilayer membrane trapping model proposed by Silvius to explain the behavior of lipid-modified proteins (Shahinian and Silvius 1995; Goodwin, Drake et al. 2005; Rocks, Peyker et al. 2005). This hypothesis states that the association of monoacylated or monoprenylated peptides/proteins with the membrane lipid bilayer is very rapidly reversible, even when binding is considered strong thermodynamically. The addition of a second palmitoyl chain suppresses the rapid reversibility and allows the protein to be targeted to a membrane compartment, thus ‘trapping’ it in the bilayer. This stable association would remain
irreversible until the protein is released from the membrane by a controlled, protein-mediated process.

**Yeast Casein Kinase 2 (Yck2)**

Yck2 was originally identified as a multi-copy suppressor of high salt intolerance seen in *Saccharomyces cerevisiae* (Robinson, Hubbard et al. 1992). Yck1 and Yck2 form an essential gene pair. While deletion of either gene does not strongly affect viability, deletion of both genes causes aberrant cell morphology and growth arrest. Yck2 contains 546 amino acids and has a molecular mass of 62 kDa. It is palmitoylated twice at its two C-terminal cysteines by the PAT Akr1. Although redundant palmitoylation activity exists within the PAT family, palmitoylation of Yck2 occurs only in the presence of Akr1 (Roth, Wan et al. 2006). Yck2 and its functionally redundant partner Yck1 are highly similar to each other with an overall amino acid identity of 77%. Yck2 consists of a well conserved Ser/Thr kinase domain from residue 72 - 360. This is followed by a 186 residue-long C-terminal domain (residues 361-546). Aside from the conserved kinase domain, there is sequence similarity between the glutamine-rich regions of Yck1 and Yck2 (residues 465-474). The final conserved segment of both proteins consists of a 12-residue sequence (83% sequence identity). This region is similar to the C-termini of GTP-binding protein family members that are also C-terminally lipid-modified and targeted to the membrane, such as Ypt1 and Sec4 (Rossi, Yu et al. 1991). In addition to these regions, Yck1 and Yck2 have many lysine residues in their C-termini that vary in number and distance from the palmitoyl-accepting cysteines. As in the case of K-Ras
that is singly farnesylated, these positively charged lysines may assist in targeting proteins to membranes (Hancock, Magee et al. 1989).

Yck1 and Yck2 also share functions including bud morphogenesis, cytokinesis, nutrient sensing, and the internalization of plasma membrane permease and pheromone receptors. The phenotype of a temperature-sensitive strain that lacks \( YCK1 \) (\( yck1\Delta \)) function and is defective for \( YCK2 \) (\( yck2-2^{ts} \)) revealed abnormally elongated buds and multiple nuclei per cell, suggesting that Yck1 and Yck2 are involved in cellular morphogenesis and completion of cell division (Robinson, Menold et al. 1993). Expression of Yck1 or Yck2 is required for the glucose-driven induction of hexose transporter (\( HXT1 \)) genes (Moriya and Johnston 2004). Recent studies also implicate an essential role for Yck1/2 in the glucose-sensor mediated pathway. Yck1/2 kinase activity is also required for the phosphorylation of the maltose permease that stimulates permease ubiquitination and internalization from the plasma membrane in an alternative response to glucose sensing (Pasula, Chakraborty et al. 2010). Kinase activity is suspected to play a role in phosphorylating the FUR4-encoded uracil permease, a key event in regulating its endocytosis (Marchal, Haguenauer-Tsapis et al. 2000). In addition to regulating the yeast pheromone \( \alpha \)-factor receptor Ste2, Yck1/2 are required for phosphorylation of the other pheromone receptor Ste3 (Panek, Stepp et al. 1997; Hicke, Zanolari et al. 1998).

**Casein Kinase Family**

The casein kinase I and II families, CK1 and CK2, constitute a large superfamily of serine/threonine kinases that are ubiquitously expressed and are common to all
eukaryotic organisms from yeast to human. They are historically named casein kinases for their ability to phosphorylate acidic substrate recognition sites on the convenient milk protein substrate, casein. While much is known of the CK2 proteins, the CK1 family represents an important group within the superfamily that has been studied to a lesser extent.

There are several mammalian isoforms of CK1 (α, β, γ1, γ2, γ3, δ, and ε) and their various splice variants. CK1 γ1, γ2, and γ3 are presumed to be palmitoylated (Kang, Wan et al. 2008). The growing list of CK1 substrates indicates that these proteins are involved in the regulation of many cellular processes. These substrates include, enzymes, transcription factors, viral oncogenes, cytoskeletal proteins, membrane receptors and associated proteins. Concurrent with this list of substrates, CK1 proteins participate in a myriad of cellular regulatory functions such as, membrane transport, cell morphogenesis, DNA repair, circadian rhythm function, cancer development, cell division, and apoptosis. Most notably, CK1 proteins (α, γ, and ε) have been shown extensively to participate in positive and negative regulatory roles in the Wnt signaling pathway (Swiatek, Tsai et al. 2004; Davidson, Wu et al. 2005; Takada, Hijikata et al. 2005).

The fission yeast *Schizosaccharomyces pombe* has at least five CK1 genes that are divided into two subgroups: one contains Cki1, Cki2, and Cki3, the other contains Hhp1 and Hhp2 (Dhillon and Hoekstra 1994; Wang, Vancura et al. 1994; Kitamura and Yamashita 1998). The primary structures of Cki1, Cki2, and Cki3 share significant sequence identities to the budding yeast casein kinases Yck1 and Yck2. Moreover, *pombe* Cki1 and Cki2 both have shown to suppress the lethality of a yck1-yck2 double
Saccharomyces cerevisiae mutant, suggesting functional homology (Wang, Vancura et al. 1994). Cki1 and Cki2 are non-essential and their function has been difficult to prove, as simultaneous inactivation or over-expression of both proteins does not result in any observable phenotypes. Cki1 has, however, been shown to phosphorylate and subsequently inactivate phosphatidylinositol (4)P 5-kinase, the last enzyme that catalyzes the synthesis of phosphatidylinositol 4,5-bisphosphate. PtdIns(4)P 5-kinase has many roles in the cell, but it is likely that its inactivation of PtdIns(4,5)P\(_2\) plays a role in regulation of vesicular trafficking (Vancurova, Choi et al. 1999). Cki1, Cki2 and Cki3 are likely candidates for palmitoylation by virtue of a cluster of cysteine residues near each C-terminus. The \textit{pombe} Hhp1 and Hhp2 are closely related to the budding yeast casein kinase Hrr25, and are required for proper segregation of chromosomes during meiosis I by phosphorylating the meiosis-specific subunit of cohesin, Rec8 (Rumpf, Cipak et al. 2010). Since chromosome segregation is a conserved cellular action from yeast to human, Hrr25 is also essential for proper chromosome segregation in budding yeast via Rec8 phosphorylation, and CK1 \(\delta\) and \(\varepsilon\) are responsible for this role in mammalian cells (Katis, Lipp et al. 2010).

In the budding yeast \textit{Saccharomyces cerevisiae}, the casein kinase genes have been identified and characterized, namely \textit{YCK1}, \textit{YCK2}, \textit{YCK3}, and \textit{HRR25}. The essential gene pair of \textit{YCK1} and \textit{YCK2} has diverse roles including control of cell growth and morphogenesis, and its activity is linked to the endocytosis of several membrane proteins. Yck1 and Yck2 proteins are very similar to each other, with an amino-acid identity of 77\% and kinase domains that are nearly identical (>90\% sequence identity). Yck3’s localization to the vacuolar surface is provided by both the palmitoylation of its
C-terminal CCCFCCC sequence and the AP-3 dependent signal that identifies a classic YXXΦ adaptin-sorting signal (Sun, Chen et al. 2004). Once at the vacuole, Yck3 regulates vesicle fusion and is required for efficient vacuole inheritance. In an effort to preserve the osmotic environment of the cell, Yck3 has been shown to prevent vacuolar fragments from refusing during hypertonic stress by phosphorylation of Vps41, a subunit of the HOPS vacuole protein sorting complex (LaGrassa and Ungermann 2005).
CHAPTER II
MATERIALS AND METHODS

Strains

Two wild-type yeast strains, namely the $\text{MATa his3}\Delta 1 \text{ leu2}\Delta 0 \text{ ura3}\Delta 0 \text{ met15}\Delta 0$ strain BY4741 (Brachmann, Davies et al. 1998) and the $\text{MATa ura3-52 leu2 his3}$ strain LRB759 (Panek, Stepp et al. 1997) were used. The $\text{akr1}\Delta$ strain used was NDY1405 (Roth, Feng et al. 2002), which is isogenic to LRB759 except for its unmarked $\text{akr1}\Delta$ allele. For complementation analysis, the $\text{MATa his3 leu2 ura3 yck1::ura3 yck2-2}^{\text{ts}}$ strain LRB757 (Panek, Stepp et al. 1997) was used.

Plasmids

Plasmids used in this study were constructed by standard cloning techniques, including $\text{in vivo}$ recombination, restriction fragment assembly, oligonucleotide-directed mutagenesis (Kunkel, Roberts et al. 1987) and PCR. For $\text{in vivo}$ recombination, a PCR fragment was generated from a gene of interest. This fragment was flanked with sequences homologous to a starting vector plasmid, which when linearized by restriction digest, recombined with the PCR-generated fragment. Recombination was carried out in the 288c background strain, BY4741 ($\text{MATa his3}\Delta 1 \text{ leu2}\Delta 0 \text{ ura3}\Delta 0 \text{ met15}\Delta 0$), and plasmids were recovered and transformed by electroporation into the $\text{Escherichia coli (E. coli)}$ TOP10 strain. For oligonucleotide-directed mutagenesis, an oligonucleotide is designed which is complementary to the part of the DNA template (containing the sequence to be changed) but contains an internal mismatch to direct the desired change (point mutation, multiple mutations, insertion, or deletion). In addition,
mutations were designed to either add or remove restriction enzyme digestion sites, allowing for identification by restriction analysis. The *E. coli* strain TB1 was used for amplification and sub-cloning. Restriction enzymes and DNA ligase (New England Biolabs) were used according to the manufacturer’s instructions. PCR amplification was conducted using the high fidelity PCR Phusion kit (New England Biolabs) and a PTC-150 Minicycler (MJ Research). Plasmid DNA was purified for sequence analysis by RNase treatment, followed by ethanol precipitation. DNA sequencing was carried out by either the Wayne State University sequencing center or GENEWIZ®. Unless specified, most plasmids were introduced into yeast on the *URA3/CEN/ARS* vector plasmid pRS316 (Sikorski and Hieter 1989).

Plasmids used in this study are divided by chapter and are described below:

**Chapter III Plasmids**

Many different plasmids were used in this chapter (listed in Table 1). Plasmids for galactose-induced expression were based upon the *GAL1*-p-6xHIS/FLAG/HA/YCK2(wt) construct pND1427 (Roth, Feng et al. 2002). A series of pND1427 derivatives that mutate the Yck2 C-terminal Cys-Cys palmitoyl-acceptors to Ser-Ser [Yck2(SS) (pND1446)], to Cys-Cys-Ile-Ile-Ser [Yck2(CCIIS) (pND1432)], and to Ser-Cys-Ile-Ile-Ser [Yck2(SCIIS) (pND1447)] have been previously described (Roth, Feng et al. 2002). Additional C-terminal Yck2 mutants, included mutation of Cys-Cys to Ser-Cys [Yck2(SC) (pND2631)] and to Cys-Ser [Yck2(CS) (pND2632)]. The Yck2 transmembrane-domain (TMD) allele replaces the Yck2 C-terminal Cys-Cys with the C-terminal 31 residues of
the plasma membrane v-SNARE Snc2 [including the C-terminal Snc2 TMD (pND1131)]. Psr1-Yck2(SS) (pND2167) attaches the N-terminal 20 residues from the dually myristoylated and palmitoylated Psr1 to the N-terminus of Yck2(SS) with the Psr1 sequences separated from the Yck2 sequences by a 3xHA tag. Psr1(Δ2-10)-Yck2(SS) (pND2189) is identical to Psr1-Yck2(SS) except for its deletion of the sequences coding for Psr1 residues Gly₂ through Cys₁₀, thus removing the putative Psr1 myristoyl and palmitoyl acceptors.

The two plasmids for expression of tagged Yck2(wt) (pND1515) or Yck2(SS) (pND1907) from the YCK2 promoter are identical to equivalent GAL₁₉₆-YCK2 plasmids, except for their replacement of the YCK2 promoter in place of the GAL₁ promoter. The YCK2 promoter is a 623 bp sequence immediately upstream of the YCK2 ORF.

The testing of other yeast palmitoyl-proteins relied on GAL₁₉₆-driven expression of epitope-tagged constructs carried on pRS 316. For these constructs the epitope tags were engineered either at the N or C terminus, depending on the protein’s lipidation site, as previously described (Roth, Wan et al. 2006).

For the E. coli expression of Yck2(SS) a pET30a vector (Novagen) for high-level, T7 polymerase-driven expression was used.
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Chapter IV Plasmids

Both the Ura3-Yck2 fusion proteins and the in-frame Yck2 deletion mutants were derived from a series of XhoI restriction site mutations that were introduced into the Yck2 coding sequence all with the same reading frame, such that the coding for two consecutive amino acid residues was replaced by the CTCGAG hexanucleotide sequence, encoding the Leu-Glu dipeptide sequence. XhoI site replacements were made at sequences encoding the ten following Yck2 dipeptides, using standard methods for site-directed mutagenesis (Kunkel, Roberts et al. 1987): Ser_{30}Asn_{31}, Ala_{58}Ser_{59}, Tyr_{120}Arg_{121}, Gly_{207}Gln_{208}, Ser_{396}Lys_{397}, Arg_{461}Glu_{462}, Lys_{495}Gln_{496}, Asn_{503}Gly_{504}, Pro_{510}Tyr_{511}, and Ser_{535}Ser_{536}. In addition, a Leu-Glu-encoding XhoI site was inserted between the Yck2 codons for Met_{1} and Ser_{2}.

Ura3-Yck2 fusion constructs: The Ura3-Yck2 constructs, which have various C-terminal portions of Yck2 attached to the C-terminus of Ura3, all are carried on the LEU2/CEN/ARS vector plasmid pRS315 (Sikorski and Hieter 1989). These fusion proteins were expressed from the inducible GAL1 promoter and, for detection, have the tripartite 6xHis/FLAG/HA epitope tag at the Ura3 N-terminus. They were derived from pND2443, GAL1_p-6xHis/FLAG/HA-URA3(Sal I), which has a Val-Asp-encoding Sal I site added to the URA3 ORF, immediately prior to the translational termination codon. This URA3-Sal I site is engineered to be in the same reading frame as the above YCK2 Xho I sites (sticky end compatible with Sal I sticky end), allowing the in frame fusion of Ura3 to the various C-terminal portions of Yck2. The Ura3-Yck2 fusions were constructed by using Xho I to Kpn I C-terminal Yck2 fragments to replace the pND2443 URA3 downstream Sal I to Kpn I fragment (the Kpn I site is located within the downstream
pRS315 polylinker). For each Ura3-Yck2 fusion, the fusion joint encodes a Val-Glu dipeptide from the joined Sal I/Xho I restriction site.

**Yck2 in-frame deletions:** The Yck2 in-frame deletion mutants (Table 2) were constructed by ligating the relevant fragments at their Xho I sites. For example, the \( \Delta 58-397 \) deletion (pND2441) was derived from ligating the Xho I sites at codons 58-59 to the site at codons 396-397. The mutant product has the deleted codons replaced by the Xho I site-encoded Leu-Glu dipeptide. The deletion mutants were constructed into two different *URA3/CEN/ARS* pRS316-based formats (Sikorski and Hieter 1989), with expression being driven either by the *GAL1* promoter (\( \text{GAL1}_p \)) or by the native *YCK2* promoter (\( \text{YCK2}_p \)). Expression from the *GAL1*\( _p \) results in a ten-fold over-expression of Yck2 facilitating both indirect immunofluorescent microscopy and palmitoylation analysis. The constructs driven by the \( \text{YCK2}_p \) were used primarily for testing complementation. The \( \text{GAL1}_p \)-driven deletion mutants are all derived from the Yck2(wt) construct, pND1427, which has the 6xHis/FLAG/HA epitope tripartite tag at the Yck2 N-terminus. The \( \text{YCK2}_p \)-driven constructs are derived from the Yck2(wt) construct pND1987, which has a dual FLAG/HA epitope tag at the Yck2 N-terminus.

**Yck2-CK1 chimeras:** The Yck2 chimeras contain full kinase-domain replacements with the complete kinase domains from other CK1 proteins. These plasmids were constructed by *in vivo* recombination in yeast. PCR fragments were generated from the respective CK1 templates (*YCK1, HRR25, CKI2, CSGK1G2*) by using two primers having between 52-55 nt of *YCK2* sequence at their 5’ ends and 21-26 nt of the respective CK1 sequence at their 3’ ends to serve as the template-
specific primers. The resulting PCR fragment contained the core CK1 kinase domain sequences flanked by upstream and downstream YCK2 sequence to direct the in vivo recombination. The PCR fragment was cotransformed into yeast along with GAL1p-6xHis/FLAGHA/YCK2(wt) (pND1427) plasmid linearized at its unique Msc I site (located in YCK2 codon 222). Circularized plasmids repaired through homologous recombination with the PCR fragment were recovered from the Ura+ yeast colonies. Proper chimera design was confirmed by DNA sequencing.

Other Yck2 mutants: The di-alanine substitution mutants and the kinase-inactive mutants were derived from pND1427 by standard methods for site-directed mutagenesis (Kunkel, Roberts et al. 1987), with the introduced mutation being confirmed by DNA sequencing.
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Yeast Cultures

Plasmid-transformed yeast were inoculated from selective plates, into either YP-Raf medium (1% yeast extract, 2% peptone, 2% raffinose) or YPD (1% yeast extract, 2% peptone, 2% glucose) for overnight log-phase growth. The next day following appropriate culture dilution and an additional 2-hour period of log-phase growth, cells either were directly harvested (cultures in YPD) or were subjected to a 2-hour galactose-induction period (addition of 2% galactose to YP-Raf cultures) prior to the collection of 4 x 10^8 cells (i.e. 20 A_600 units).

Glass Bead Cell Lysis, Membrane Solubilization, and Centrifugal Fractionation

Cell pellets were resuspended in 0.3 ml of ice-cold Lysis Buffer (LB: 150 mM NaCl, 50 mM Tris/Cl, 5 mM EDTA, pH 7.4) containing 2xPI (1xPI: 1 mM PMSF and 0.25 μg/ml each of antipain, leupeptin, pepstatin, and chymostatin) and then transferred to 2-ml centrifuge tubes (Thermo-Fisher 02-681-344) that contained a 200 μl dry volume of 212-300 mm acid-washed glass beads (Sigma-Aldrich G1277). Cells were then disrupted by five 45-second blasts of vigorous vortexing, interspersed with 2-minute rests of the tube on ice (to minimize heating). Following the final vortexing, the beads were allowed to settle and the supernatant was decanted to a fresh tube. The glass beads were then washed by resuspension into an additional 0.2 ml of LB containing 1xPI, with this supernatant being pooled together with the first. To solubilize cellular membranes, 400 μl of this lysate was adjusted to 1% Triton X-100 (Anatrace APX100) and incubated to 30 minutes at 4°C with rotation. Triton X-100 was omitted from this incubation when unsolubilized membranes were to be collected (Figure 1). Prior to the
high-speed, fractionating centrifugation, lysates were first subjected to a low-speed spin, 30 seconds at 3000 x g, to remove unbroken cells. This supernatant was then diluted to 2.7 ml with LB containing 1xPI and 1% Triton X-100 and centrifuged at 200,000 x g for 30 minutes, yielding S100 and P100 fractions for analysis. Equal portions of the S100, P100, and the total sample (taken prior to centrifugation) were subjected to SDS-PAGE and then Western blotting with anti-HA-HRP (Roche). Some experiments (Figures 1D, 4C, 8C, and 8D) relied on a lower-speed spin (20 minutes, 15,000 x g) to fractionate supernatant from pellet.

For sucrose gradient analysis, Triton X-100 solubilized supernatants were layered onto a 4.5 ml 15-50% sucrose gradient prepared in 1% Triton X-100, 150 mM Tris/Cl, pH 7.5, with a 0.5 ml 80% sucrose cushion in the same buffer. Gradients were centrifuged at 4ºC in a swinging-bucket rotor for 2 hours at 140,000 x g and 300 µl fractions were collected from the tube bottom. Absorbance of each fraction was read at both 260 and 415 nm to identify the gradient positions for both endogenous 80S ribosomes and the 50 µg of bovine hemoglobin which was added to the sample prior to centrifugation.

**Mortar-Pestle Lysis**

5 x 10⁹ cells, collected from log-phase cultures, were resuspended into 0.5 ml of LB containing 2xPI, then quick frozen as droplets into liquid nitrogen. As previously described (Roth, Feng et al. 2002), cells were lysed by grinding in a cold mortar and pestle under liquid nitrogen. The lysate then was subjected to Triton X-100 membrane solubilization and centrifugal fractionation as described above.
**Yck2 Production from *E. coli***

Log-phase 50 ml cultures of Rosetta DE3 strain *E. coli* cells (Merck), transformed by pET30a-derived Yck2(SS) expression plasmids, were induced for 2 hours with 1 mM IPTG. The harvested cells were resuspended into 1 ml of 50 mM Tris, 100 mM NaCl, 1 mM DTT pH 8.0, then incubated with chicken egg white lysozyme (Sigma-Aldrich) at 1 mg/ml for 20 minutes on ice, and then lysed with five 20-second blasts (interspersed with 1-minute ice rests) with a Fisher Scientific 60 Sonic Dismembrator set at power level 3.5. Lysates then were centrifuged at 15,000 x g for 10 minutes at 4°C to yield pellet and supernatant fractions.

**Indirect Immunofluorescent Microscopy**

Cells were fixed, spheroplasted, and otherwise developed for immunofluorescent microscopy as described previously (Sun, Chen et al. 2004). The HA-tagged proteins were detected using a 1 hour room temperature incubation with a 1:1000 dilution of the HA.11 monoclonal antibody (Covance, NJ) as the primary antibody, followed by 1 hour with Cy3-conjugated goat anti-mouse IgG secondary antibody. Images in Chapter III were captured using a Zeiss Axiovert-200 equipped with Perkin-Elmer Ultraview ERS (spinning disk laser confocal). Images in Chapter IV were captured and simultaneously deconvoluted using a Zeiss Axioplan equipped with Apotome structured illumination module (Zeiss).
LC/MS/MS Protein Identification

Following glass bead treatment, proteins were separated by SDS-PAGE and stained. Bands showing differential fractionation to the centrifugal pellet were excised. Peptides were prepared by a stepwise, in-gel process of reduction, alkylation and trypsinization. LC/MS/MS analysis utilized an LTQ-XL mass spectrometer (Thermo-Scientific), with MS2 spectra being searched by Mascot (Matrix Science), simultaneously against yeast forward and reverse protein sequences. The protein with the greatest number of associated spectra per protein size was considered to be the major component of the excised gel band. The EF-3 identification was based on 254 spectral identifications (the next most abundant protein was associated with 48 identifications). The EF-1A identification was associated with 346 spectral identifications (132 for the next most abundant protein).

Immunoprecipitation (IP)

Glass bead lysates were Triton X-100-solubilized, then clarified of unbroken cells by low-speed centrifugation as described above. Equal lysate aliquots then were processed through either “native” or “denatured” IP protocols. For the native IP, the lysate was diluted to 1 ml with LB containing 1xPI, 0.2% Triton X-100, and 0.1% SDS. Samples then were subjected to a 30 minute, 4°C pre-incubation with end-over-end rotation), followed by a 1 minute, 15,000 x g centrifugation to remove particulates, and finally a 16 hour, 4°C binding incubation with 20 µl of anti-FLAG agarose (Sigma-Aldrich). The antibody beads were then subjected to four 1 ml washes and then eluted into 50 µl Urea Sample Buffer (USB: 5% SDS, 8M urea, 40 mM Tris, 0.1 mM EDTA,
pH6.8) containing 1% β-mercaptoethanol (10 minutes at 37°C). Samples for Western analysis were taken at different stages of the IP reaction: 1) just prior to the anti-FLAG agarose addition (the “total” samples), 2) the first supernatant following the anti-FLAG agarose incubation (the “unbound” fraction), and 3) the eluted fraction (“bound”). Equal portions of each sample were loaded onto SDS-PAGE for anti-HA Western analysis. The denatured IPs were processed identically to the native IPs, except for inclusion of a denaturation step, prior to the IP. For this denaturation, lysate proteins were collected by chloroform-methanol precipitation (Wessel and Flugge 1984), then dissolved into 50 ml 2% SDS, 8M urea, 50mM Tris, 1mM DTT, pH 7.4 for a 10 minute incubation at 37°C. The denatured protein samples then were diluted 20-fold into 1 ml LB with 1xPI, 0.2% Triton X-100 and 0.1% SDS and were further processed as described for the native IPs.

**Palmitoylation Analysis**

Palmitoylation was assessed for the different Yck2 mutants using one of two different approaches, either 1) by acyl-biotinyl exchange (ABE) methodology or 2) by metabolic labeling with the alkyne-tagged palmitic acid analog, 17-octadecynoic acid (ODYA; Cayman Chemicals), followed by an in vitro click reaction (see later) with azido-AlexaFluor647.

**ABE detection of palmitoylation:** A scaled-down version of the proteomic ABE protocol was used as has been previously described (Politis, Roth et al. 2005; Roth, Wan et al. 2006). In brief, denatured protein prepared from yeast cells expressing the FLAG/HA-tagged proteins of interest, were processed through the three ABE steps: 1) blockade of free thiols with N-ethylmaleimide, 2) cleavage of the thioester-linked acyl
modifications with neutral pH hydroxylamine, 3) marking of the newly uncovered acylation site cysteinyl thiols with the thiol-specific biotinylation reagent HPDP-biotin (Thermo Scientific). Finally, the protein of interest is purified by anti-FLAG agarose (Sigma Aldrich) immunoprecipitation (IP), then subjected to Western blotting both with anti-biotin-horseradish peroxidase (HRP) (Sigma Aldrich) to detect palmitoylation and with anti-HA-HRP (Roche) to assess protein recovery and expression.

**Click detection of palmitoylation:** The click labeling protocol of Charron et al. (Charron, Zhang et al. 2009) was adapted to yeast. Log-phase yeast cultures expressing FLAG/HA epitope-tagged proteins from the GAL1 promoter, were labeled with 25 μM ODYA (added from 25 mM stock solution in DMSO, stored at -20ºC) for the second hour of the 2 hour galactose induction (see “Yeast Cultures” above). At the end of the labeling period, 10⁸ labeled cells were harvested by centrifugation and resuspended into 100 μl of cold LB (50 mM Tris/Cl, 150 mM NaCl, 5 mM EDTA, pH 7.4) containing 2 mM PMSF and 2xPI. The cell suspension was then lysed by five 45 second intervals of vortexing with a 100 μl volume of acid-washed glass beads. The lysate was decanted to a second tube, with the beads being washed with 150 μl of LB containing 1 mM PMSF and 1xPI, which was pooled with the first lysate. Lysate membranes then were solubilized with the addition of Triton X-100 to 1% and a 30 minute incubation at 4ºC with rotation. Unbroken cells were removed by centrifugation (10,000 x g for 30 seconds), with protein from 150 μl of the cleared lysate then collected by chloroform-methanol precipitation (Wessel and Flugge 1984). The resulting precipitate was resuspended into 25 μl of 4% SDS, 50 mM Tris/Cl, 5 mM EDTA, pH 7.4 and incubated for 10 minutes at 37ºC. FLAG/HA-tagged proteins then were purified from 20 μl of
denatured protein by immunoprecipitation (1 hour, 4°C incubation with anti-FLAG agarose in 800 μl of LB, containing 0.2% Triton X-100, 1mM PMSF and 1xPI). After 3 x 1 ml washes with LB with 0.2% Triton X-100 and 0.1% SDS, bound protein was eluted (5 minutes, 65°C) into 25 μl of 4% SDS, 50 mM triethanolamine, pH 7.4. Twenty microliters of the eluted protein was adjusted to 47 μl with the addition of 27 μl of CB (50 mM triethanolamine, 150 mM NaCl, 0.5% Triton X-100) with 1 mM PMSF and 1xPI. To initiate the click reaction, 3 μl of a mix composed of 5 μl of 10 mM azido-AlexaFluor647 (prepared in DMSO, Invitrogen), 5 μl of 10 mM tris[(1-benzyl-1H-1,2,3-triazol-4y1)methy-1]amine (TBTA, prepared in DMSO), 10 μl of 50 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP, freshly prepared) and 10 μl of 50 mM CuSO₄/5H₂O (freshly prepared) was added. Following 1 hour incubation at room temperature, 100 μl of CB was added and the reaction was terminated by chloroform-methanol precipitation. The protein precipitate was dissolved in 25 μl of SB (8M urea, 5% SDS, 40 mM Tris/Cl, 0.1 mM EDTA, 0.4 mg/ml bromophenol blue, 1% β-mercaptoethanol) and heated at 65°C for 5 minutes prior to SDS-PAGE. AlexaFluor647 labeling was detected by fluorescent scans of the gel using a Typhoon 9200 (GE HealthCare). An identical second gel was analyzed by anti-HA Western blotting to assess the expression of the different mutant proteins.

**Phosphatase Treatment**

Denatured protein extracts were prepared from 1.5 x 10⁷ cells. Harvested cells were rapidly resuspended with 180 μl of cold 1.4 M sorbitol, 25 mM Tris/Cl, and 45 μl of 85% trichloroacetic acid (TCA) prior to being flash frozen on dry ice and stored
overnight at -80°C. The next day, 180 μl of acid-washed glass beads were added and cells were disrupted by 10 minutes of auto-vortexing at room temperature. Then 250 μl of 5% TCA was added to the lysate, then decanted away from the glass beads into a new tube. The glass beads were washed with 300 μl of 5% TCA, with this wash added to the initial lysate. After 20 minutes at 0°C, precipitated protein was collected by centrifugation (10 minutes, 16,000 x g). The dessicated protein pellet was dissolved into 100 μl of Urea Sample Buffer (USB: 5% SDS, 8M urea, 40 mM Tris, 0.1 mM EDTA, pH 6.8) for 10 minutes at 65°C.

To digest with phosphatase, 2 μl aliquots of the final protein extracts were diluted to 1 ml with the addition of PB (20 mM citrate/Na, 50 mM NaCl) and 1xPI. Then 30 μg of potato acid phosphatase (Roche) was added and samples were incubated at 30°C for 1 hour. Finally, 2 μg of BSA was added as carrier and the digested proteins were TCA precipitated, dessicated, and dissolved into 20 μl USB at 37°C for 10 minutes, prior to SDS-PAGE and anti-HA Western analysis.

Complementation Analysis

Single colonies of yeast expressed in the strain LRB757 [MATα his3 leu2 ura3 yck1::ura3 yck2-2ts] carrying URA3/CEN/ARS plasmids for YCK2p-driven expression of different Yck2 mutants were picked from selective, minus-uracil plates into 1 ml of 1xS (0.67% yeast nitrogen base). Three μl aliquots of 10^-1, 10^-2, and 10^-3 dilutions were spotted onto two YPD plates which were incubated for 2 days at 30°C (permissive temperature) and 34°C (non-permissive temperature).
CHAPTER III
PROTEIN AGGREGATION INDUCED DURING GLASS BEAD LYSIS OF YEAST

SUMMARY

The use of glass beads to mechanically disrupt yeast cells is used in a variety of applications, including for the analysis of native protein function (e.g. protein-protein interaction, enzyme assays, membrane fractionations). This chapter describes a surprising case of protein denaturation and aggregation that is induced by this lysis protocol. Most of the analysis focuses on the type I casein kinase Yck2, which normally tethers to the plasma membrane through palmitoylation of its C-terminal cysteines. Interestingly, when yeast cells are subjected to glass bead disruption, non-palmitoylated, cytosolic forms of the kinase denature and aggregate, while membrane-associated forms, whether attached by their native palmitoyl-tethers or by a variety of artificial membrane-tethering sequences, are protected from aggregation and denaturation. A broader look at the yeast proteome shows that while the majority of proteins resist glass bead-induced aggregation, a significant subset does experience such denaturation. Thus, yeast researchers should be aware of this potential artifact when using glass bead lysates to look at native protein function. We have also demonstrated an experimental utility for glass bead-induced aggregation, using its fine discrimination of membrane-associated from non-associated Yck2 forms to discern the palmitoylation states of Yck2 mutants that are partially defective for palmitoylation.
CHAPTER III
INTRODUCTION

The tough exterior yeast cell wall is an obstacle for the biochemist, with access to cellular contents gained only through enzymatic cell wall digestion or through mechanical disruption. For enzymatic digestion, cells are incubated with digestive enzymes for a substantial period of time (15–60 minutes) at temperatures in the range of 25–37 °C, which are conditions compatible with continued biological activity. Thus, the enzymatic approach is not ideal for analyses where one wants to acquire a quick ‘snapshot’ of intracellular processes. For mechanical disruption, the most widely used approach involves the vigorous agitation (vortexing) of the cells in the cold with small glass beads in the 100–600 µm diameter range. Yeast cell lysates produced by such glass bead disruption are widely used for a variety of applications, including the analysis of native protein function for enzyme assays, sub-cellular fractionations, and for analyzing protein complexes. In this first part of my thesis, we report a case where such glass bead disruption of yeast cells leads to a troubling instance of protein aggregation, likely reflecting induced protein denaturation. Given the vast amount of literature and the multitude of scientific advances that have relied upon this fundamental yeast method, it is obvious that for most yeast proteins, glass bead lysis-induced protein denaturation and aggregation is not a problem. Indeed, we find only a relatively small minority of yeast proteins to be susceptible to such induced aggregation. Nonetheless, yeast researchers should be alert to this potential artifact.

As previously described in Chapter I, Yck2, together with its close homologue Yck1, provides an essential yeast function (yck1Δ yck2Δ cells are non-viable). The two
kinases tether to the cytoplasmic surface of the plasma membrane through dual palmitoylation of the C-terminal Cys–Cys dipeptide (Roth, Feng et al. 2002; Babu, Deschenes et al. 2004), where they regulate a variety of cell surface processes, including endocytosis, cell morphogenesis, mRNA localization and nutrient sensing (Robinson, Menold et al. 1993; Panek, Stepp et al. 1997; Hicke, Zanolari et al. 1998; Feng and Davis 2000; Abdel-Sater, El Bakkoury et al. 2004; Moriya and Johnston 2004; Spielewoy, Flick et al. 2004; Paquin, Menade et al. 2007; Pal, Paraz et al. 2008). When palmitoylation is blocked, either through mutation of the C-terminal cysteines or through deletion of the gene encoding their Golgi-localized palmitoyl-transferase, Akr1, the kinases mislocalize to the cytoplasm (Roth, Feng et al. 2002; Babu, Deschenes et al. 2004).

During the course of this study, we found strikingly distinct fractionation phenotypes for palmitoylated Yck2 vs. non-palmitoylated Yck2 when assayed from glass bead-generated lysates. The palmitoylated, wild-type Yck2 behaved like a typical integral membrane protein, fractionating to the membrane pellet, but then to the supernatant with prior detergent treatment. Non-palmitoylated Yck2, we expected, would fractionate as a soluble cytoplasmic protein to the supernatant and thus we were surprised to find it fractionating instead to the pellet, in both the presence and absence of detergent. Additional analyses demonstrated this pellet fractionation to reflect a quantitative segregation of non-palmitoylated Yck2 into a high-molecular-weight protein aggregate.

Our interest in this aggregation of non-palmitoylated Yck2 was sparked by a recent publication connecting impaired palmitoylation to protein aggregation in
Huntington's disease (HD) (Yanai, Huang et al. 2006). Huntingtin, the disease protein of HD, is palmitoylated, and decreased huntingtin palmitoylation was shown to result in its increased aggregation. Thus, in light of our Yck2 results, we wondered if this link between impaired palmitoylation and aggregation might be more general. However, as we document below, rather than occurring within the living yeast cell, this aggregation of non-palmitoylated Yck2 occurs instead in vitro, being artifactually induced during the process of glass bead cell disruption. We conclude this is a consequence of mechanical shear forces produced by the vortexing glass beads. Given the widespread use of glass bead lysis within the yeast community, we felt obliged to investigate this artifact more deeply and have found that glass bead-induced aggregation does indeed apply to other, but certainly not all, yeast proteins.
CHAPTER III

RESULTS

Non-Palmitoylated Yck2 Fractionates to a High Molecular-Weight Aggregate or Complex

To assess the effects of palmitoylation on Yck2 membrane fractionation, glass bead lysates were prepared from cells expressing either wild-type Yck2 or a non-palmitoylated Yck2. Wild-type Yck2 fractionates like an integral membrane protein, fractionating to the P100 membrane pellet, then to the supernatant (S100) when membranes are dissolved with Triton X-100 prior to centrifugation (Figure 1A). For non-palmitoylated Yck2, produced either by mutation of the two C-terminal, palmitoyl-accepting cysteines to serine [Yck2(SS)] or by expression of wild-type Yck2 in the akr1Δ cell context (deleted for the Yck2 palmitoyl-transferase, Akr1), our expectation, based on prior fluorescence-based localization analyses, was for non-palmitoylated Yck2 to fractionate like a soluble cytoplasmic protein, to the supernatant (Vancura, Sessler et al. 1994; Feng and Davis 2000; Roth, Feng et al. 2002; Babu, Deschenes et al. 2004). Instead, we found it fractionated mainly to the pellet, irrespective of whether lysates were pretreated with detergent (Figure 1A). The differing mobilities seen for the palmitoylated and non-palmitoylated Yck2 forms result from differential phosphorylation (Figure 1A) (Roth, Feng et al. 2002). This is an auto-phosphorylation which depends on both high-level Yck2 expression (e.g. GAL1 promoter-driven expression) and Yck2 being properly localized to the plasma membrane (I. Papanayotou, A. Roth and N. Davis, unpublished data). Furthermore, it can occur in trans as shown in Chapter IV; over-produced, plasma membrane-localized Yck2 can phosphorylate kinase-inactivated
mutant versions of Yck2 that are otherwise normally localized to the cell surface (I. Papanayotou, A. Roth and N. Davis, unpublished data). While the gel mobility shift is directly the consequence of phosphorylation, the requirement for surface localization means that this modification is also dependent on palmitoylation. Thus, the non-palmitoylated Yck2(SS) does not show the upper, hyper-phosphorylated band (Figure 1A). We also noticed the non-palmitoylated forms of Yck2 (either Yck2(SS) or Yck2(wt) in the akr1Δ cell context) to be expressed at lower levels than the fully palmitoylated, wild-type Yck2. We suspect that this lowered expression likely reflects that the non-palmitoylated, mislocalized kinase is subject to increased degradative turnover.

To examine the nature of the forces responsible for the Yck2(SS) pellet fractionation, lysates from Yck2(SS)-expressing cells were treated with various chemical treatments prior to the centrifugal fractionation (Figure 1B). Treatments with Triton X-100, NaCl or DTT had no effect on Yck2(SS), since it continued to fractionate to the pellet. However, the two strong protein denaturants, SDS and urea, released Yck2(SS) to the supernatant. To crudely size this Yck2(SS) complex or aggregate, Triton X-100-treated glass bead lysates from Yck2(wt)- or Yck2(SS)-expressing cells were subjected to sucrose gradient analysis (Figure 1C). Yck2(wt), as would be expected following detergent dissolution of membranes, fractionated as a soluble protein to the gradient top. In contrast, Yck2(SS) was seen to distribute throughout the gradient with the bulk of the protein concentrating towards the gradient bottom, indicating its incorporation into an extremely protein aggregates.

The above analyses all relied on Yck2 expression from the strong and inducible GAL1 promoter (GAL1p), resulting in approximately 10-fold over-expression relative to
Figure 1. Non-Palmitoylated Yck2 from glass bead lysates fractionates as a part of a high molecular-weight aggregate. Glass bead lysates, prepared from either wild-type or akr1Δ yeast cells, expressing either Yck2(wt) or the Yck2(SS) mutant, were fractionated by centrifugation. The expressed Yck2 proteins are N-terminally tagged with the 6xHis/FLAG/HA tri-tag, allowing Western blot detection with anti-HA-HRP. (A) Lysates from cells expressing the indicated Yck2 proteins from the GAL1p on CEN/ARS plasmid (2 hour expression) were treated with 1% Triton X-100 for 30 minutes at 4ºC or a parallel mock solubilization (no detergent) and subjected to high-speed centrifugation to yield the supernatant (S) or pellet (P) fractions which were analyzed by anti-HA Western blotting. (B) Glass bead lysates deriving from cells expressing Yck2(SS) from the GAL1p, were subjected to 30-minute treatments with either 1% Triton X-100, 1M NaCl, 10 mM DTT, 1% SDS, or 6M urea (incubations were at 4ºC, except for SDS and DTT which were at 23ºC), then fractionation by high-speed
and finally, anti-HA Western blotting. (C) Lysates from cells expressing either Yck2(wt) or Yck2(SS) from the \textit{GAL1}_p were treated with 1\% Triton X-100 prior to sucrose gradient fractionation. Samples of each gradient fraction were analyzed by anti-HA Western blotting. The positions of the endogenous 80S ribosome and added hemoglobin are indicated. (D) Detergent-treated lysates prepared from cells expressing either Yck2(wt) or Yck2(SS) from the \textit{YCK2} promoter were analyzed as for a panel A except that, in addition, samples taken just prior to the centrifugal fractionation also were analyzed (Σ).
the endogenous YCK2 promoter (YCK2p) expression level. Since aggregation is often promoted at high expression levels, the fractionation behavior of Yck2(SS) was also examined at the normal Yck2 expression level (Figure 1D). We found the fractionation to be unchanged: Yck2(wt) was solubilized into the supernatant, while Yck2(SS) continued to fractionate to the pellet. At this native level of expression, the hyper-phosphorylated species that was seen above for GAL1p driven Yck2(wt), was absent.

**Aggregation of E. coli-produced Yck2 in Mock Glass Bead Lysis Protocol**

Previous analysis of the sub-cellular localization of non-palmitoylated Yck2, either Yck2(SS) or Yck2(wt) in the akr1Δ context, by either GFP-tagging or indirect immunofluorescence, found no obvious visual evidence of aggregation (no intracellular puncta were seen) (Vancura, Sessler et al. 1994; Feng and Davis 2000; Roth, Feng et al. 2002; Babu, Deschenes et al. 2004). Instead, non-palmitoylated Yck2 was found to distribute throughout the cytoplasm and nucleus, being excluded from the membrane-enclosed vacuole (Figures 4B and 9B). Furthermore, when Yck2(wt) or Yck2(SS) was artificially expressed in E. coli, these being cells not thought to utilize protein palmitoylation, we found that a substantial proportion of the Yck2 protein, even when massively over-produced, was both soluble and active (data not shown), indicating that palmitoylation is not intrinsically required for proper Yck2 folding. Taken together, these two findings, (the lack of visual evidence of non-palmitoylated Yck2 aggregation as well as non-palmitoylated Yck2’s ability to properly fold within the E. coli context) raised concerns that, rather than occurring in vivo, non-palmitoylated Yck2 aggregation instead might be occurring in vitro during the cell lysis protocol. To test this possibility, a
Figure 2. Soluble *E. coli*-produced Yck2(SS) is converted to insolubility by the mock glass bead lysis protocol. A supernatant fraction, prepared from Yck2(SS)-overproducing *E. coli* cells was subjected to a mock-up of the yeast glass bead lysis protocol (beads + vortex), or alternatively, either to simple incubation with the glass beads (beads, no vortex), or to no treatment (control). Samples then were treated by the 1% Triton X-100 incubation and centrifugal fractionation described for Fig. 1A.
supernatant fraction derived from *E. coli* over-expressing Yck2(SS) was subjected to conditions replicating our yeast glass bead lysis protocol. We were astounded to see that the repetitive vortexing with glass beads did quite efficiently convert the soluble Yck2(SS) into an insoluble, pellet-fractionating form (Figure 2). As controls, the *E. coli* supernatant was subjected either to just the mock Triton X-100 membrane solubilization step prior to the centrifugal fractionation (Figure 2, ‘control’) or, alternatively, incubated with the glass beads with no vortexing prior to detergent treatment and centrifugation (Figure 2, ‘beads no vortex’). Neither treatment produced insoluble Yck2(SS). We conclude, that pellet fractionation can be artifically induced as part of the glass bead lysis protocol. The finding is that simple incubation with the beads is not sufficient to induce pellet fractionation: thus, denaturation is not being catalyzed on the surface of the glass beads, neither is the denaturation induced by a chemical washing off of the beads.

**More Gentle Cell Lysis Protocol Yields Soluble Yck2(SS)**

We also have used a different yeast cell lysis protocol to assess the solubility of the non-palmitoylated Yck2 produced within the yeast cell. For this, cells were quickly frozen in liquid nitrogen, then ground, also in the presence of liquid nitrogen, with a mortar and pestle. Further processing of the resulting lysate was essentially as for the glass bead method: detergent extraction, then a low-speed centrifugation to remove unbroken cells and finally, the high-speed centrifugation. With these conditions, in contrast to what was seen with glass bead lysis, palmitoylated Yck2(wt) and non-palmitoylated Yck2(SS) were both found to fractionate to the supernatant (Figure 3).
Figure 3. Lysis by the mortar-pestle method yields soluble Yck2(SS). Yeast cells expressing Yck2(wt) or Yck2(SS) from the \textit{GAL1} promoter were lysed by the mortar-pestle method (see Materials and Methods), then subsequently treated to the 1% Triton X-100 solubilization and centrifugal fractionation as described for Fig. 1A.
Thus, this gentler cell lysis protocol yields soluble Yck2(SS), supporting the conclusion that the Yck2(SS) insolubility seen with glass bead lysis (Figure 1) results from protein aggregation artifactually induced by the agitation with glass beads. Note again that Yck2(wt), over-expressed here from the GAL1 promoter, is shown here as a doublet, with the slower-migrating band, not seen for cytoplasmic Yck2(SS), corresponding to the hyper-phosphorylated form.

Protection From Glass Bead-Induced Aggregation by Membrane Association

What accounts for the striking differential susceptibility of palmitoylated and non-palmitoylated Yck2 to glass bead-induced aggregation? Perhaps the palmitoyl modifications help to maintain native Yck2 structure, making the protein more resistant to denaturation. Alternatively, perhaps membrane association somehow confers resistance. We tested the ability of different ectopic membrane tethering sequences to substitute for the wild-type C-terminal Cys-Cys palmitoylation motif in averting the glass bead-induced aggregation. Three different Yck2 forms were constructed (Figure 4A). Yck2(CCIIS) replaced the Cys–Cys dipeptide with the Ras2 C-terminal peptide – CCIIS. Like Ras2, Yck2(CCIIS) was efficiently farnesylated and palmitoylated (Babu, Bryan et al. 2002; Roth, Feng et al. 2002; Babu, Deschenes et al. 2004). Yck2(TMD) replaced the C-terminal Cys-Cys dipeptide with the C-terminal transmembrane domain (TMD) from the plasma membrane v-SNARE Snc2. Psr1–Yck2 fused a 20 residue-long myristoylation–palmitoylation motif from the plasma membrane phosphatase Psr1 to the N-terminus of Yck2(SS). Like Yck2(wt), all three of the tethering mutant Yck2 proteins localized within the cell, predominantly to the plasma membrane (Figure 4B), indicating
Figure 4. Membrane association protects from glass bead-induced denaturation.

(A) Schematics for ectopically-tethered Yck2 mutants. (B) Mutant Yck2 protein intracellular localization analyzed by anti-HA immunofluorescent analysis. The indicated Yck2 proteins were analyzed following a 2 hour period of \textit{GAL1_p} driven expression. (C) Fractionation of mutants in the glass bead lysis-centrifugation protocol. Cells expressing the indicated mutants were lysed, subjected to 1% Triton X-100 solubilization, then fractionated as described for Fig. 1A. The "lipidation-minus" control for Psr1-Yck2(SS) has a deletion of the N-terminal consensus sequences for myristoylation and palmitoylation, removing Psr1 residues 2 through 10 (\(\Delta 2-10\)). Lacking a capacity for membrane association, Psr1(\(\Delta 2-10\))-Yck2(SS) is converted to insolubility by the glass bead lysis protocol.
avid membrane tethering. Finally, all three ectopic membrane tethers also provided complete protection from glass bead-induced aggregation: like Yck2(wt), all three fractionated to the centrifugal supernatant fraction from Triton X-100-solubilized glass bead lysates (Figure 4C). In contrast, the Psr1(Δ2–10)–Yck2-(SS) control construct, deleted for the Psr1 myristoyl and palmitoyl acceptors, fractionated, like Yck2(SS), to the pellet. Thus, attachment to the membrane appears to be the key to averting glass bead-induced aggregation. One plausible explanation is that membrane-associated proteins may be protected by their incorporation into the vesicles that are produced through the glass bead disruption of cellular membranes. Enclosure within vesicles might allow Yck2 to survive the denaturing actions of the glass beads. With the subsequent Triton X-100 treatment, these proteins then would be released to the supernatant as soluble proteins.

To test the protective role of membrane association, Triton X-100 was included during the glass bead lysis step (Figure 5). The inclusion of detergent caused a significant portion of Yck2(wt) to now fractionate to the pellet, indicating that it had been made susceptible to the denaturing actions of the beads. Presumably, the portion of the Yck2(wt) protein population that did get converted to insolubility was the fraction that was released from vesicles during the glass bead agitation step.
Figure 5. Inclusion of detergent during glass bead disruption reduces the protection from aggregation afforded by membrane association. Glass bead lysates were prepared from yeast cell expressing GAL1p driven Yck2(wt) or Yck2(SS), either by our typical protocol which adds Triton X-100 subsequent to glass bead disruption, or by an alternative protocol which included 1% Triton X-100 during the cell disruption step (+ Triton). Subsequent sample processing was as described for Fig. 1A, except that no additional detergent was added during the membrane solubilization incubation for the “+ Triton” samples.
**Temperature vs. Mechanical Denaturation**

Glass bead-induced aggregation presumably results from protein denaturation. Denaturation could result from increased temperatures produced by the frictional actions of the vortexing glass beads or, alternatively, directly from mechanical shear forces generated by the whirring glass beads. Heat production in glass bead lysis protocols is typically minimized by resting lysates on ice between vortexing blasts. Nonetheless, some temperature elevation is unavoidable. To test the role of temperature in our aggregation phenomenon, a soluble Yck2(SS)-containing supernatant fraction from a high-speed centrifugation of a mortar–pestle lysate was subjected to either the mock glass bead lysis protocol or, alternatively, to 10 minute incubations at different elevated temperatures (30 °C, 40 °C or 65 °C). Following these treatments, a second, high-speed centrifugation was used to yield supernatant and pellet fractions for analysis (Figure 6A, upper panel). As expected, the mock glass bead lysis efficiently converted Yck2(SS) into a pellet-fractionating aggregate. The effects of temperature, by contrast, were less impressive: a minor amount of Yck2(SS) aggregation was apparent following the 10 minutes, 40°C incubation, while incubation at high temperature (65°C), not surprisingly, resulted in a total conversion of Yck2(SS) into its aggregate form. Neither temperature (40°C or 65°C) is likely to be attained during our glass bead lysis protocol. Thus, we suspect that the denaturation of non-palmitoylated Yck2 was instead the product of the mechanical actions of the vortexing glass beads. Both the vortexing and the glass beads were required for Yck2(SS) aggregation, since in mock glass bead lysis experiments like those of Figure 6A (upper panel), where the soluble protein extracts were subjected to vortexing in the absence of glass beads, no
Figure 6. Tests of the contribution of elevated temperature to glass bead-induced aggregation. (A) Comparison of the effects of elevated temperature to those of glass bead agitation on the aggregation of both Yck2(SS) (upper panel) and on bulk yeast proteins (lower panel). A supernatant fraction prepared by high-speed centrifugation (no Triton X-100 present) of a mortar-pestle lysate from yeast cells expressing \textit{GAL1p} driven Yck2(SS), was subjected to a variety of treatments, including either a mock glass bead lysis (glass beads), or 10 minute incubations at the indicated temperatures. Samples then were subjected to a second high-speed centrifugation yielding S100 and P100 fractions that were analyzed either by anti-HA Western blotting (upper panel) or by SDS-PAGE and Coomassie staining (total protein, lower panel). Two proteins that show differential fractionation to the pellet from the glass bead-treated sample, identified by LC/MS/MS as both being translational elongation factors, namely the 116 kDa EF-3 (Yef3) and the 50 kDa EF-1A (Tef1/2) are indicated by asterisks (lower panel) (B) Lysates were prepared from yeast cells expressing \textit{GAL1p} driven Yck2(wt) or Yck2(SS) either by our standard glass bead lysis protocol (5 x 45 second vortexings interspersed with 1 minute slushy ice bath rests) or by a revised protocol intended to minimize heating (45 x 5 second vortexings interspersed with 1 minute slushy ice bath rests). Subsequent lysate processing and fractionation (1% Triton X-100 solubilization and centrifugation) was as described for Fig. 1A.
aggregation was seen (data not shown).

As an additional test of the potential role of thermal denaturation in the generation of the Yck2(SS) glass bead aggregates, the glass bead protocol was altered to further minimize heating (Figure 6B). In addition to our typical protocol, in which cells are glass bead-disrupted with five 45-second vortexing blasts interspersed with 1 minute ice rests, the cell disruption was divided into 45 x 5 second blasts, interspersed by 1 minute rests in a slush ice bath. Even with this new protocol, with heating further minimized, Yck2(SS) continued to fractionate to the pellet, indicating again that the mechanical forces generated by the vortexing glass beads play the predominant role in the Yck2(SS) denaturation that leads to aggregation.

Susceptibility of Other Yeast Proteins to Glass Bead-Induced Aggregation

The above experiment tested the effects of temperature and glass beading on Yck2(SS) solubility (Figure 6A, upper panel), and also tracked the behavior of total yeast cytosolic protein by subjecting the total centrifugal fractions to SDS–PAGE and Coomassie staining (Figure 6A, lower panel). Looking at the entire proteome of soluble yeast proteins, we found that, while the majority of proteins resisted glass bead-induced aggregation, a few of these highly-abundant yeast proteins quite clearly were converted to insolubility. The two protein species most prominently showing differential fractionation to the pellet were identified by LC–MS/MS as the 50 kDa translation elongation factor EF-1\(\alpha\) (the identical proteins Tef1 and Tef2) and the 116 kDa translation elongation factor 3 (Yef3) (indicated by asterisks in the lower panel of Figure 6A). Interestingly, Tef1/2 and Yef3, which are not homologous, do interact and
collaborate in the feed and removal of amino acyl-tRNAs to and from the ribosome as each amino acid is added to the growing polypeptide. We assume that the identification of these two proteins, is largely a consequence of their high abundance within the yeast cell. Proteins of lower abundance that might also be converted to insolubility would not be identified by this analysis; identifications were limited to just those proteins showing differential fractionation as visualized by Coomassie SDS–PAGE (i.e. highly abundant yeast proteins). Nonetheless, it is worth noting that, like Yck2, Tef1/2 and Yef3 both bind and hydrolyse nucleotide triphosphates—Tef1/2 is a G protein and Yef3 an ATPase.

We also have tested a number of other yeast palmitoyl proteins for glass bead-induced aggregation (Figure 7). Consistent with the analysis above (Figure 6A, lower panel), only a minority of the tested proteins showed evidence of aggregation. Proteins showing similar glass bead-induced aggregation behavior to Yck2 included the two palmitoylated Yck2 homologs, Yck1 and Yck3 (Figure 7). Like Yck2, mutant, non-palmitoylated versions of Yck1 and Yck3 aggregated following glass bead lysis, while the palmitoylated, wild-type forms of these two proteins were fully protected from the induced aggregation, presumably a consequence of their membrane associations. Likewise, similar behavior was found for the yeast sphingosine kinase Lcb4. Aggregation was induced for a non-palmitoylated Lcb4 mutant \([\text{Lcb4} \ (C43S,C46S); (\text{Kihara, Kurotsu et al. 2005})]\), as well as when expressed in \(a k r1\Delta\) cells (where palmitoylation is substantially reduced), but not for the wild-type palmitoylated form (Figure 7). However, the bulk of the palmitoyl proteins tested such as Ras2, Psr1, Vac8, Ykl047w, Ypl199c and Ypl236c, showed little evidence for induced aggregation when tested as either lipidated or non-lipidated proteins (Figure 7) (Roth, Wan et al. 2006;
Figure 7. Testing other yeast palmitoyl-proteins for glass bead-induced aggregation. The indicated yeast palmitoyl-proteins expressed from the GAL1₀ both in their native lipidated state as well as in a mutant, non-lipidated state were assessed for potential aggregation in the glass bead lysis protocol described for Fig. 1A. For most of these proteins (namely Yck2, Yck1, Yck3, Ras2, Vac8, Psr1, and Lcb4), the wild-type, lipidated form was compared to a mutant version with the lipidation sites specifically removed. These mutations are for Yck2: C545S,C546S [Yck2(SS)]; for Yck1: C537S,C538S [Yck1(SS)]; for Yck3: Δ517-524 [removes the eight C-terminal residues, including the seven putative palmitoyl-accepting cysteines]; for Ras2: Δ318-322 [removes the five C-terminal residues (the dually palmitoylated and prenylated pentapeptide CCIIS)]; for Vac8: Δ2-7 [an in frame deletion of Gly₂ through Cys₇ (removes the myristoylation site as well as the three cysteinyl palmitoyl-acceptors)]; for Lcb4: C43S,C46S [mutation of the two palmitoyl-acceptors]. In addition, for some of these palmitoyl-proteins known to depend on Akr1 for their palmitoylation (namely Lcb4, Ykl047w, Ypl199c, and Ypl236c), the akr1Δ cell context was used to assess fractionation of the lipidation-minus form.
Subramanian, Dietrich et al. 2006). Thus, the differential susceptibility to mechanical denaturation seen for Yck2 and the handful of other non-palmitoylated kinases tested does not broadly extrapolate to non-lipidated versions of other lipidated proteins.

**Glass Bead Lysates in IPs**

Glass bead lysates are frequently used for co-immunoprecipitation (co-IP) analysis to identify or confirm protein–protein interactions. Thus, we have examined the IP behavior of Yck2 non-palmitoylated and palmitoylated forms from glass bead lysates under both ‘native’ and ‘denatured’ conditions. Anti-FLAG-agarose, which reacts with the FLAG epitope of 6×His/FLAG/HA tri-tag marking the N-termini of both Yck2(wt) and Yck2(SS), was used. For the ‘native’ condition, our protocol paralleled that used for the above centrifugal fractionations. The glass bead lysates were subjected to Triton X-100 solubilization and then applied to the anti-FLAG-agarose. For the ‘denatured’ condition, total protein, precipitated from the lysates, was dissolved into a small amount of SDS-containing buffer at 37°C for 10 minutes prior to its dilution into the IP-compatible buffer. Binding to anti-FLAG-agarose was assessed through anti-HA Western blotting of both the bound and unbound fractions. Under the denaturing condition, both palmitoylated and non-palmitoylated Yck2 proteins showed efficient binding to the anti-FLAG-agarose (Figure 8). In contrast, for the native IP, only palmitoylated Yck2(wt) showed efficient binding. The two non-palmitoylated forms, either Yck2(SS) or Yck2(wt) expressed in the PAT-deficient *akr1Δ* context, showed only a partial binding (Figure 8). Equivalent results were seen when the same experiment was performed using Ni-agarose (binds to the N-terminal 6×His sequence) as affinity absorbent (data not shown). We additionally found
Figure 8. Yck2 immunoprecipitation from glass bead lysates. Glass bead lysates were prepared and 1% Triton X-100 solubilized from cells expressing GAL1p driven Yck2(wt) or Yck2(SS) as described for Fig. 1A. The detergent solubilized lysates then were divided with half diluted directly into the IP reaction with anti-FLAG agarose (“nat.”) and with the other half being subjected to denaturation prior to the anti-FLAG IP (“den”). Following the IP incubation, equivalent portions of the fractions of the eluted (B, bound), the unbound fraction (U), as well as the initial input sample (Σ) were analyzed by anti-HA Western blotting.
that non-palmitoylated forms of the Lcb4 sphingosine kinase, [Lcb4(C43S, C46S) or Lcb4(wt) expressed in the akr1Δ cell context], also failed to bind to immuno-absorbent when assessed under ‘native’ conditions from glass bead lysates (data not shown). The failure of these proteins to be properly immunoprecipitated from glass bead lysates presumably reflects the occlusion of the epitope tags within the aggregate. Thus, for some proteins, glass bead-induced aggregation may also confound analysis of protein–protein interaction via co-IP analysis.

**Glass Bead-Induced Aggregation Provides Insights About Fractional Yck2 Palmitoylation States**

Prior work has shown that the individual mutation of the two Yck2 C-terminal cysteines, Yck2(SC) and Yck2(CS), impairs Yck2 localization and function (Babu et al., 2004), suggesting that normally, both cysteines are palmitoylated. We confirmed this here, using acyl–biotinyl exchange (ABE) chemistry to follow palmitoylation. We found that Yck2(SC) and Yck2(CS) were both palmitoylated: as one might expect, both showed palmitoylation levels that were somewhat lower than those seen for wild-type Yck2(CC), which retained both palmitoyl-acceptors (Figure 9A). This analysis also included Yck2(CCIIS), which was expected to be modified by just a single palmitoyl moiety as well as Yck2(SCIIS), which lacks the palmitoyl-accepting cysteine and was thus expected to be only farnesylated, not palmitoylated (Figure 9A). An examination of the cellular localization of these mutants found that Yck2(CCIIS), like wild-type Yck2 (CC), localized to the cell surface, while Yck2(SCIIS) showed reduced surface localization balanced by increased localization to intracellular membranes (Figure 9B).
Figure 9. Use of the glass bead aggregation to analyze the fractional palmitoylation of singly-lipidated Yck2 mutants. (A) Palmitoylation of the singly-lipidated Yck2 mutants. Protein extracts prepared from cells expressing either Yck2(wt) or the indicated mutant versions under the \textit{GAL1}p (2 hour expression) were analyzed for palmitoylation using the ABE method (see Materials and Methods). Palmitoylation is assessed as the intensity of the anti-biotin Western blot signal relative to signal derived from immunoprecipitated Yck2 protein (anti-HA). (B) The sub-cellular localization of the indicated Yck2 mutant proteins was analyzed by anti-HA indirect immunofluorescence as described for Fig. 4B. (C) Cells expressing the indicated Yck2 mutant proteins from the \textit{GAL1}p were subjected to glass bead lysis/fractionation described for Fig. 1A. (D) Palmitoylation of supernatant vs. pellet fractionated populations. Following the glass bead lysis and centrifugal fractionation described for Fig. 8C, supernatant and pellet fractionated proteins were subjected to ABE palmitoylation analysis.
The difference in membrane avidities provided by one vs. two lipid tethers is considerable. Consequently, singly-lipidated proteins rapidly equilibrated onto and off of membranes, precluding their capacity to exploit classic vesicular transport mechanisms for localization. The result was that singly-lipidated proteins were seen to localize randomly to a variety of cellular membranes (Shahinian and Silvius 1995; Rocks, Peyker et al. 2005). Nonetheless, the localizations seen for singly-palmitoylated Yck2 (SC) and Yck(CS) were quite different from that observed for singly-lipidated Yck2 (SCIIS) (Figure 9B). Yck2(SC) and Yck(CS) both showed little evidence of any membrane association, with only small amounts detectable at surface and intracellular membranes, and with the bulk of both proteins localizing diffusely through the cytoplasm, with a presentation not grossly dissimilar to that seen for cytoplasmic Yck2 (SS).

Analysis of these mutants in our glass bead lysis/fractionation protocol showed the typical behaviors for Yck2(CC) and Yck2(SS) (Figure 9C) - following detergent solubilization, Yck2(CC) fractionated to the supernatant, while Yck2(SS) fractionated to the pellet. Similar to Yck2(CC), Yck2(CCIIS) and Yck2(SCIIS) both fractionated mainly to the supernatant, a finding consistent with the predominant membrane localizations seen for these proteins by immunofluorescence (Figure 9B). Interestingly, Yck2(SC) and Yck2(CS) both showed intermediate phenotypes, showing fractionations divided between both the supernatant and the pellet (Figure 9C). The different gel mobilities of the different Yck2 mutants (Figure 9A and 9C), a reflection of their differing phosphorylation and thereby of their different localization to the plasma membrane, was also well correlated. Yck2(CC) and Yck2(CCIIS), which both localized primarily to the
surface, showed the heaviest phosphorylations (slowest mobility), while Yck2(SCIIS), which is only partially surface-localized (Figure 9B), showed an intermediate mobility shift. In contrast, Yck2(SC) and Yck2(CS) both looked quite under-phosphorylated, showing mobilities that were essentially indistinguishable from the cytoplasmic Yck2 (SS). These Yck2(CS) and Yck2(SC) results—the intermediate fractionation, the reduced phosphorylation and the largely cytoplasmic localizations—together indicated inefficient membrane tethering, with membrane associations substantially reduced relative to the singly-lipidated Yck2(SCIIS).

What explains the less efficient tethering of Yck2(SC) and Yck2(CS), relative to Yck2(SCIIS)? It is not that the Yck2(SCIIS) farnesyl lipid provides stronger membrane tethering than do the Yck2(SC) and Yck(CS) palmitates. The opposite is true. Membrane avidity of palmitoyl tethers is greater than that of farnesyl tethers (Shahinian and Silvius 95). One plausible explanation, however, is that Yck2(SC) and Yck2(CS) palmitoylation is inefficient. Maybe substantial subpopulations of these two proteins fail to receive any palmitoylation. To investigate this, the membrane-associated and cytosolic subpopulations were fractionated from one another via our glass bead lysis/fractionation protocol. Then, palmitoylation on the two sub-populations was assessed by ABE. This analysis clearly showed that these two fractionated subpopulations of Yck2 (CS) and Yck2(SC) did, in fact, differ considerably in their palmitoylation, with substantially more palmitoylation seen for supernatant-fractionated protein than for the pellet-fractionated protein (Figure 9D). Thus, for both mutants, the membrane-associated population was palmitoylated and the nonmembrane-associated population, which accounted for a substantial fraction (greater than 50%) of the total for both
mutants being largely unpalmitoylated. Thus, both the SC and CS mutations were substantially more disruptive to palmitoylation than had been previously appreciated (Babu, Deschenes et al. 2004). The removal of either one of the two palmitoyl-acceptor sites substantially impaired palmitoylation at the remaining cysteine. Therefore, the fractionation provided by this artifactual aggregation has led to an important new conclusion regarding the Yck2 recognition elements that specify its palmitoylation.
CHAPTER III

DISCUSSION

Here we have described a troubling artifact that can confuse yeast analyses that rely on glass bead lysis. Some proteins, when subjected to this mode of cell lysis, denature and aggregate, potentially confounding analyses of native function. While this induced aggregation appears to apply to just a subset of yeast proteins, the overall scope of the problem was left open by our analysis. Our attempt at addressing this issue proteomically demonstrated susceptibility for two additional proteins, Tef1/2 and Yef3 (Figure 6A, lower panel). However, this analysis was limited in that it allowed susceptibility to be tested only for the most highly abundant yeast proteins. Thus, for the vast majority of the proteins comprising the yeast proteome, susceptibility remains unprobed. Nonetheless, an extrapolation from this limited, abundant-protein test set would suggest that, for the vast majority of yeast proteins, glass bead-induced denaturation is likely not a major concern. Glass bead lysis is, and hopefully will continue to be, an extremely valuable and convenient tool for the yeast researcher. Nevertheless, we felt it important that the yeast researcher be made aware of this potential artifact.

What distinguishes the yeast proteins that are susceptible to glass bead-induced aggregation? Our results suggest that the mechanical shear forces produced by the vortexing glass beads likely cause the denaturation driving this aggregation. The proteins identified in this chapter as undergoing this glass bead-induced aggregation, namely Yck2, its homologues Yck1 and Yck3, the sphingosine kinase Lcb4 and the two translation elongation factors identified (Tef1/2 and Yef3), may all be proteins that are
easily altered structurally. Protein kinases as well as lipid kinases, may be particularly susceptible to forces of mechanical denaturation. Interestingly, a deletion analysis of the Yck2 sequences that contribute to aggregation identified the kinase domain to be the main driver of Yck2 aggregation. Mutants retaining the kinase domain were susceptible to glass bead-induced aggregation, while mutants deleted for this domain were not (B. Sun and N. Davis, unpublished data).

The strikingly different behavior of palmitoylated and non-palmitoylated Yck2 in our glass bead lysis/fractionation protocol is curious. Protection from aggregation, we have shown, requires an association of Yck2 with membranes (Figures 4 and 5). We suggest that with the glass bead disruption of membranes, membrane-tethered Yck2 becomes encapsulated within newly formed vesicles that confer protection from the whirring glass beads. The discrete nature of this protection, where almost all of membrane-associated Yck2 and virtually none of cytosolic Yck2 is protected, suggests that all of the mechanically-generated vesicles are orientated so that the cytosolic, Yck2-harboring membrane face is orientated towards the vesicle interior. While this is possible, perhaps a more convincing explanation is that the major default structure produced by mechanical disruptions is multilamellar vesicles (MLVs), structures in which multiple layers of membranes are enclosed within membranes. MLVs tend to be the default membrane structure produced by a variety of means, with unilamellar vesicle production typically requiring specialized methodologies (Matsuoka and Schekman 2000). The membranes-within-membranes MLV structure minimizes the amount of membrane surface that is exposed to the external surroundings. Thus, in addition to the possibility of protein denaturation, investigators also may want to consider the possibility
the MLVs may be the major membrane structures produced by glass bead disruption. Such structures might complicate membrane fractionation analyses as well as in vitro analyses of membrane proteins, like channels or transporters.

Finally, we show that glass bead-induced aggregation, with its specific discrimination of Yck2-soluble from membrane-bound forms, can be of some experimental utility, allowing us to discern the fractional associations of different Yck2 mutants with membranes. This is of particular value when examining palmitoylation. Palmitoylation labeling methods, whether through metabolic labeling with [3H]-palmitate or through the in vitro chemical exchange of biotin moieties at palmitoylation sites (ABE), do not well discern the fraction of the total protein population that is palmitoylated; it is generally unclear for a protein that is found to be palmitoylated, whether 100% or 1% of the protein population is modified by the attached lipid. For instance, palmitoylation analysis of Yck2(SC) and Yck2(CS) both showed palmitoylation levels somewhat lower than wild-type Yck2(CC) (Figure 9A). The level of this reduction seemed roughly in line with expectations for proteins having one vs. two attached palmitates. However, coupling this ABE analysis to glass bead lysis/fractionation revealed that a substantial proportion of both the Yck2(SC) and Yck2(CS) protein populations (>50%), resisted palmitoylation altogether. Apparently, the bulk of these two mutants fail to be recognized by their cognate palmitoyl-transferase Akr1, thus providing an important new insight regarding the nature of the Yck2 palmitoylation signal - efficient recognition of the Yck2 substrate depends on both C-terminal cysteines being present. This novel approach for discriminating membrane-bound from unbound Yck2 may find additional utility in future analyses of the Yck2 palmitoylation signal, and also
for following the kinetics of Yck2 palmitoylation and membrane-association in vivo. It is presently unclear whether this fractionation approach might apply more widely beyond Yck2. In any case, despite this minor utility, our main goal with this study was to alert yeast colleagues to the troubling possibility of aggregation when employing glass bead lysis.
CHAPTER IV

THE ROLE OF THE KINASE DOMAIN IN THE PALMITOYLATION OF YCK2

SUMMARY

Chapter III has described the surprising artifact that resulted from the use of the glass bead lysis method for lysis of yeast cells. While this initially was a troubling finding, we were fortunately able to inform the yeast community of this artifact, and we were able to apply our differential centrifugation technique to Yck2 produced from glass bead lysis to determine the fractional palmitoylation states of the protein and related mutants. Here, we continue to explore the early events that precede palmitoylation to find the mechanism by which palmitoyl proteins synthesized as soluble cytoplasmic proteins, are delivered to the membrane localized PATs. More specifically, we were interested in the role of the kinase domain on palmitoylation and we hypothesized that the kinase domain possessed signals within its sequence to influence palmitoylation and that kinase activity itself is required for palmitoylation. Our analysis finds that both kinase structure and activity are necessary for proper palmitoylation of Yck2. Analysis of various kinase-inactive missense mutants revealed that kinase activity participates in efficient palmitoylation and localization. In addition, analysis of Yck2 chimeric proteins whose kinase domains were replaced with those of other CK1 proteins, revealed that a generic feature does not exist in kinase domains for palmitoylation. Rather, we conclude that Yck2 contains a signal in the kinase domain that presents itself as a result of the proper folding of the kinase. Thus, Yck2 contains multiple factors that participate in palmitoylation, with the kinase domain play one of the major roles.
CHAPTER IV

INTRODUCTION

Proteins associate with membranes to effect many key biological processes like signal transduction and membrane trafficking. Some proteins may access membranes peripherally by protein-protein interaction. Others may also possess specialized domains that allow for membrane interaction such as polybasic domains or plekstrin homology domains that can interact with charged phospholipids. Another class of integral membrane proteins, transmembrane domain-containing proteins, as seen for G-protein coupled receptors (GPCRs), contain regions that actually traverse the lipid bilayer. More widespread, protein modification with fatty acids is a universal feature commonly used by the cell to anchor a variety of proteins to membranes.

Three common lipid modifications that occur in the cytoplasm are prenylation, myristoylation, and palmitoylation. The addition of any single lipid tether to a protein increases its hydrophobicity, thereby allowing membrane interaction, however this increase in hydrophobicity may not be sufficient to stably tether the protein for the required duration. Thus, there are combinations of lipid tethers that work in conjunction to offer more stable membrane interactions as well as provide trafficking mechanisms for desired targeting, as described previously in the bilayer membrane trapping model (Shahinian and Silvius 1995). For example, the combinations of prenylation and palmitoylation as seen for H-Ras and N-Ras (H/N-Ras) and the yeast Ras2 proteins, and myristoylation and palmitoylation, as seen for the yeast vacuolar membrane protein Vac8, result in an ordered pathway of lipidation where palmitoylation occurs second to the other modification.
The sequential lipid modification process is best demonstrated by the H/N-Ras proteins, that undergo a highly orchestrated process taking the proteins on a journey through various sub-cellular compartments. H/N-Ras ends with the amino acid sequence CaaX, where C stands for cysteine, a for an aliphatic amino acid, and X for any amino acid. A farnesyl or geranylgeranyl moiety is added to the cysteine of the CaaX motif in the cytosol, which directs Ras to the cytosolic surface of the ER where the -aaX is cleaved and the remaining C-terminus is carboxy-methylated. At this point in the yeast ER, Ras is palmitoylated by the membrane protein Erf2/Erf4 in the early secretory pathway, then traffics to the plasma membrane where it further displays a controlled cycling mechanism between the Golgi and the cell surface.

With regard to proteins that tether to membranes exclusively by palmitoyl tethers, the pathway by which the protein takes to interact with the membrane associated DHHC palmitoyl acyl transferase (PAT) is unknown. While the soluble prenylation enzymes, farnesyl or geranylgeranyl transferases, or the myristoylation enzyme, myristoyl transferase, are found in the cytosol, PATs are found in membranes. Thus, an important question we initially sought to answer is how a soluble protein like Yck2, that is synthesized in the cytosol, is delivered to its transmembrane, Golgi-localized PAT, Akr1.

To answer this question, we have characterized the palmitoylation signal for Yck2. Our work, which is currently being prepared for publication (Roth, Papanayotou, and Davis, in preparation), shows that the Yck2 palmitoylation signal is surprisingly complex, with two separate domains contributing. Of the two domains, the C-terminal conserved 10 amino acids that include the palmitoyl-accepting cysteines are of greatest importance. This small highly conserved region is part of the sequences that map within
the unstructured 150 residue-long C-terminal domain. Finally there is evidence that the kinase domain itself offers a crucial role. The goal of these studies, described in this chapter, has been to characterize the role of the kinase domain in the palmitoylation of Yck2. we find a critical role for kinase activity as indicated by the characterization of several kinase-inactive Yck2 mutants and kinase-replaced Yck2 mutants.

Yck2 is one of four CK1s expressed in yeast, the others being Yck1, Yck3, and Hrr25. Since Yck1 and Yck2 are functionally redundant partners, it is no surprise that they are most similar to each other in structure, with an overall amino acid identity of 77%. Comparison of the amino acid sequence alignment of these two is presented in Figure. 10 and reveals a conserved organization. Both sequences begin with Met-Ser followed by a ~60 hydrophilic amino acid stretch that is weakly conserved (~25% identity). This region is followed by a common 287 residue protein kinase domain that is nearly identical in the two proteins (>90% identity). This homology is abruptly ended with a 186 residue-long C-terminal region that shows very little sequence homology. Furthermore, it is predicted to be the most flexible region of these proteins. Early within the C-terminal sequences there is a small body of positively charged residues that perhaps plays a similar role to that of the polybasic region of K-Ras, assisting in targeting proteins to membranes by virtue of a cluster of positively charged residues that interact with the negatively charged membrane bilayer. Also within these unstructured C-terminal regions, each sequence contains two glutamine-rich (Q-rich) regions that do not display any structural alignment or predicted function. The final segments of Yck1 and Yck2 consist of a 10 residue-long sequence that is greatly conserved (83% sequence identity) and ends in the two palmitoyl-accepting cysteines.
Figure 10. Multiple sequence alignment of the redundant yeast kinases Yck1 and Yck2. This sequence alignment using Clustalw computer software shows conserved sequences highlighted in blue. Yck1 and Yck2 are highly conserved (among Yck proteins) with an overall amino acid identity of 77%. Two regions that display high levels of conservation are the N-terminal kinase domain, and a small 10 residue domain that includes the C-terminal palmitoyl-accepting cysteines. The C-terminal region between these highly conserved domains shows very little sequence homology, and is predicted to be highly unstructured.
CHAPTER IV

RESULTS

Ura3-Yck2 Fusion Proteins Reveal A Minimal C-terminal Palmitoylation Domain

Previous studies from our laboratory using a series of Ura3-Yck2 fusions first pointed towards a possible role of the Yck2 kinase domain in Yck2 palmitoylation. To initially characterize the sequence requirements for palmitoylation of Yck2, the yeast gene URA3, which codes for a cytosolic enzyme involved in the biosynthesis of uracil, was fused to C-terminal segments of YCK2 (Figure 11A). These Ura3-fusions were characterized by analysis for palmitoylation using the acyl-biotinyl exchange (ABE) methodology (Figure 11B) and for sub-cellular localization by indirect immunofluorescent microscopy (IIF) (Figure 12). The fusion containing the entire length of Yck2 [(Ura3-Yck2(1-546))] showed robust palmitoylation by ABE and proper localization to the plasma membrane by IIF. These results also indicated that Ura3, a cytosolic enzyme, did not dominate the behavior of the newly constructed protein. Figures 11B and Figure 12 clearly demonstrate that exclusion of up to the first 60 residues of Yck2 did not seem to affect the robust palmitoylation and strong surface localization as seen for Ura3-Yck2 (30-546) and Ura3-Yck2(60-546). However, as the deletions proceeded to delete parts of the kinase domain, roughly consisting of residues 70 - 360, there was a decrease in the palmitoylation signal seen by ABE. Moreover, the IIF data showed a divided localization between the cytoplasm and the surface, beginning with Ura3-Yck2(120-546) and ending with Ura3-Yck2(503-546) (Figure 12). As the Ura3-Yck2 fusions became shorter by deletions taken at the N-terminus, palmitoylation and localization became
abnormal displaying a weak, or in some cases showing no anti-biotin signal seen for Ura3-Yck2(512-546) and Ura3-Yck2(535-546).

We noticed a significant breakpoint with the fusion that retained the last 44 residues from the Yck2 C-terminus [Ura3-Yck2(503-546)]. This fusion showed clear evidence of palmitoylation and partial surface localization, whereas Ura3-Yck2(512-546) showed neither palmitoylation nor discernible surface localization. Thus, while the C-terminal 44 residues of Yck2 contained a signal that is sufficient for palmitoylation, it is apparent that these sequences were not capable of directing the efficient palmitoylation and surface localization as seen for full length Ura3-Yck2(1-546). Undoubtedly, other parts of Yck2 beyond the minimal 503-546 domain also contribute to directing efficient palmitoylation.

The vast difference in localization and palmitoylation between the Ura3-Yck2 fusions containing the complete kinase domain and those missing part or all of the kinase domain provided to us the first indication that the kinase domain plays an important role in the palmitoylation of Yck2. This finding initially sparked our interest into the primary structure of Yck2 and its connection with palmitoylation. In an effort to elucidate this connection, our goal was to conduct a project of detailed analysis of the sequences in Yck2 that play a role in palmitoylation. Previous work has found the last 10 amino acids as well as other C-terminal sequences affecting palmitoylation of Yck2. Our work and the focus of this chapter involves the contributions of the kinase domain to palmitoylation, localization, and function of Yck2. We sought to ask, “Do sequences that compose the kinase domain simply offer a structural component that contributes to palmitoylation, or is kinase activity also a prerequisite for proper palmitoylation and/or
localization?” This latter portion of this dissertation is an attempt to answer these questions.

**Evaluation of Kinase-Inactive Mutants**

To explore the effects of Yck2 kinase activity on palmitoylation, we developed and investigated the behavior of various mutants that were devoid of kinase activity. We initially compared the behavior of a Yck2 protein that is deleted for the residues involving the kinase domain, Yck2(Δ58-397), with that of two kinase-inactive missense mutants, Yck2(K105R) and Yck2(D218A). The K105R mutant changes the conserved Lys105, in the conserved subdomain III of the kinase domain, which has been recognized as essential for maximum activity. This Lys helps anchor and orient ATP by interacting with the α- and β-phosphates. The D218A mutant changes the Asp218 of the highly conserved DFG triplet in subdomain VII of the kinase magnesium binding loop, to Ala (REF).

Kinase activity from Yck1 and/or Yck2 is a prerequisite for proper cell viability. To ensure that these kinase-dead mutants were indeed free from any kinase activity, their ability to complement a yeast strain devoid of YCK1 and temperature-sensitive for YCK2 (yck1Δ yck2-ts) was tested (Figure 13A). This strain grows normally at the permissive temperature of 20°C, however it does not grow at the non-permissive temperature of 34°C, without the introduction of a functional Yck1 or Yck2 protein. The mutant strain was transformed with plasmids carrying either the wild-type YCK2 allele or the kinase-inactive mutant alleles. The addition of the kinase-inactive mutants to the yck1Δ yck2-ts strain provided absolutely no growth benefit at 34°C for all samples. This verified that the kinase-dead mutants possessed no activity to complement growth.
Figure 11. Deletional mapping of the Yck2 sequences that direct palmitoylation and plasma membrane localization. (A) Schematic of Ura3-Yck2 fusion proteins with the indicated portions of Yck2 fused in-frame to the C-terminus of the cytoplasmic enzyme Ura3. The Ura3 amino terminus is tagged with a copy of the HA epitope to facilitate immune detection. (B) ABE analysis of Ura3-Yck2 fusion proteins. Constructs expressed under the inducible promoter \textit{GAL1} in wild-type cells were subjected to the ABE protocol as described in “Material and Methods”. The Ura3-Yck2 was subjected to anti-biotin Western analysis (top) and anti-HA Western analysis (bottom).
Figure 12. Indirect immunofluorescent localization of the Ura3-Yck2 constructs. Fusion proteins were expressed under the inducible \textit{GAL1} promoter (2 hour expression). Proteins were detected by an anti-HA monoclonal antibody as primary antibody, and then a Cy3-conjugated donkey anti-mouse secondary antibody.
Previously described in Chapter III, we have noticed a phenomenon of Yck2 auto-phosphorylation when expressed from the high-level promoter \textit{GAL1} and when properly localized to the plasma membrane. We found utility for this hyper-phosphorylated mobility shift to indirectly report on surface localization. Unfortunately, since this phosphorylation is an auto-phosphorylation, it is not available to report on surface localization for these kinase-inactive mutants. However, since the hyper-phosphorylation is dependent on the kinase being active, we were able to confirm kinase inactivity by adding phosphatase enzyme to protein samples driven by the \textit{GAL1} promoter, and analyzing by Western blotting (Figure 13B).

The fully functional wild-type Yck2(wt) showed almost its entire protein population as a hyper-phosphorylated band which collapsed to a lower molecular-weight band upon phosphatase treatment (Figure 13B). The non-palmitoylated Yck2(SS) was used as a negative control since it was mislocalized to the cytoplasm and thus did not display the hyper-phosphorylated phenomenon. The kinase-deleted Yck2(Δ58-397) was also used as a control, since it lacked the kinase domain entirely and surely possessed no activity. The addition of phosphatase to the K105R and D218A kinase-inactive mutant extracts did not cause a band collapse of any kind. This indicated that a hyper-phosphorylated species of protein did not exist for the kinase-dead mutants, verifying that these mutants were inactive.
Figure 13. Kinase-inactive Yck2 mutants do not provide proper Yck2 function. (A) Serial 10-fold dilutions of yck1Δyck2ts cells, transformed by the CEN/ARS vector pRS316 or by pRS316 carrying either the wild-type YCK2 or the kinase-inactive mutants D218A or K105R, were plated at both permissive (20°C) and non-permissive (34°C) temperatures. (B) Wild-type Yck2, the Yck2(SS) non-palmitoylated mutant, the kinase inactive Yck2(K105R) and (D218A) mutants, and the kinase deleted Yck2 (Δ58-397) were introduced into wild-type yeast cells on single-copy plasmids (pRS316) under the inducible GAL1p promoter. Protein extracts were prepared with a portion of the sample digested with phosphatase and equal amounts left untreated. Western analysis was performed with antibody directed against the N-terminal HA epitope tag.
Palmitoylation of Kinase-Inactive Mutants

To directly determine the palmitoylation status of the kinase-dead mutants described above, we used the ABE palmitoylation analysis to show that they were all indeed palmitoylated by detection using antibody to biotin as the palmitoylation signal (Figure 14). To compare levels of palmitoylation between samples, the ABE result (the α-biotin signal) was quantified for each protein normalized to the total amount of recovered sample (the α-HA signal) (Figure 14B). This quantitation showed that Yck2 deleted for its kinase domain, Yck2(Δ58-397), had reduced palmitoylation of more than 50% relative to wild-type Yck2(wt). Both of the kinase-dead mutants, D218A and K105R, were also defective for palmitoylation, but to a lesser extent. Results suggest that the kinase domain of Yck2 provides more than simply a structural function for the protein. The presence of the kinase domain contributes to the palmitoylation of Yck2 and moreover the ability of the kinase to possess activity further increases palmitoylation.

ABE Analysis Reveals Palmitoylated and Non-Palmitoylated Protein Sub-Populations

To further explore the effects of the kinase domain on the palmitoylation of Yck2, we analyzed the kinase-inactive mutants alongside Yck2 proteins with in-frame C-terminal deletions that begin at residue 396 and extend to residue 511, using the ABE palmitoylation method (Figure 15). However this ABE analysis differed from the previous analysis in Figure 11B and Figure 14 in that it is based upon gel mobility shifts for the samples Yck2(Δ58-397), Yck2(Δ396-496), and Yck2(Δ396-511). This analysis also
included minus-hydroxylamine (-HAM) control samples. When HAM was omitted from the ABE protocol, the thioester palmitoyl-linkages should have remained intact and uncleaved, and thus remained unavailable to the HPDP-biotin labeling reagent. Running the sample on SDS-PAGE gels and using anti-HA antibody to probe Western blots for the entire population of protein (palmitoylated and non-palmitoylated) resulted in a subtle shift. This subtle shift was due to the added biotin moieties (429 Daltons/moiety).

The kinase deleted Yck2(Δ58-397) was shown as a single band when detected by anti-biotin antibodies (Figure 14A), but as a doublet in the anti-HA Western blot (Figures 14A and 15A). When detected by α-HA antibodies, the upper band in the Western blot represented the protein population of Yck2(Δ58-397) that possessed the attached biotin moiety, and thus represented palmitoylated protein. Results suggest that only a partial population, and not the entire population of Yck2(Δ58-397) was palmitoylated. The addition of β-mercaptoethanol (β-ME) to the sample cleaved the disulfide bond between the protein’s cysteine and the HPDP-biotin, resulting in the band collapse (Figure 15A). Naturally, the non-palmitoylated Yck2(SS) version showed no mobility shift.

The Yck2(Δ396-496) deletion, which removes the N-terminal two-thirds of the 150 residue-long C-terminal domain, including both glutamine-rich sequences, has previously shown both wild-type palmitoylation and surface localization (Roth, A. and Davis, N., unpublished). When subjected to ABE analysis, the α-HA Western blot showed that the entire population shifted to one of a higher molecular weight upon HAM treatment (Figure 15A), suggesting that all of the protein was modified with the biotin moiety. For the Yck2(Δ396-511), for which only a sub-population was palmitoylated and
Figure 14. Palmitoylation of Yck2 kinase defective mutants. (A) Wild-type yeast cells expressing wild-type or kinase defective Yck2 mutants carried on single-copy pRS316 vector plasmids driven by the GAL1 promoter were analyzed for palmitoylation using the ABE method (see Materials and Methods). Immunoblot analysis using antibody to biotin assessed palmitoylation and immunoprecipitated Yck2 protein was assessed by immunoblotting with antibody to HA. (B) Crude measure of palmitoylation from this experiment by using ImageJ software. The α-biotin signal was quantified for each protein normalized to the α-HA signal (the total amount of protein recovered).
Figure 15. Yck2 mutants that are inactivated or deleted for the kinase domain are partially palmitoylated. To assess palmitoylated sub-populations of Yck2 mutants, a modified ABE protocol was used where biotinylated samples were subjected to Western blot analysis and probed with antibody to HA, demonstrating a doublet phenomena in some cases. (A) GAL1p driven Yck2(Δ58-397), its non-palmitoylated -SS version and the in frame deletions (Δ396-496) and (Δ396-511) were expressed in wild-type cells and subjected to ABE analysis (described in Materials and Methods). Upon treatment with hydroxylamine (HAM) and biotin-HPDP, palmitoylated protein populations are represented as a doublet, with the lower bond comigrating with unpalmitoylated forms of Yck2 [(Δ58-397), SS]. Cleavage of thiol bonds with β-mercaptoethanol collapses the doublet, confirming the biotinylation of the slower migrating species. (B) The modified ABE protocol was applied to the fully palmitoylated Yck2(Δ396-496), its non-palmitoylated -SS mutant, and K105R and D218A versions. To eliminate mobility differences due to phosphorylation, ABE samples were treated with phosphatase prior to SDS-PAGE.
surface-localized (Roth, A. and Davis, N., unpublished), only the hyper-phosphorylated sub-population (the population that was palmitoylated and surface-localized) showed the HAM-dependent shift. Thus, the biotinylation appears to be very efficient in the context of the ABE experiment, with palmitoylated protein displaying a full shift on a Western blot probed with α-HA. This method has proved to be useful to identify sub-protein populations that are palmitoylated versus non-palmitoylated.

We also used this band-shift discrepancy to identify if palmitoylated and non-palmitoylated protein populations of the kinase-inactive mutants Yck2(K105R) and Yck2 (D218A) exist. Unfortunately, the ABE biotinylation of full-length Yck2 did not result in shifted mobility (data not shown). However, since the shift was seen for the fully palmitoylated and surface-localized Yck2(Δ396-496), we tested the kinase-dead mutants within this context. Yck2 mutants were made containing the Δ396-496 in-frame deletion and either a kinase-dead mutation, (K105R) or (D218A), or the non-palmitoylated Yck2(SS) mutation. In order to prevent any confusion when discerning between biotinylated proteins with hyper-phosphorylation, all samples were treated with phosphatase enzyme before gel electrophoresis (Figure 15B). Wild-type Yck2 (Δ396-496) showed a gel shift that was easily identified upon the addition of HAM, indicating that the ABE-induced upward shift was due to the biotinylation of the fully palmitoylated Yck2 (Δ396-496). This shift clearly was not seen for the non-palmitoylated Yck2(SS) version. The same analysis applied to the two kinase-dead mutants revealed that only a portion of the total protein populations of both mutants were palmitoylated, with a significant portion remaining non-palmitoylated. This evidence suggests that only sub-populations of the kinase deleted Yck2(Δ58-397) and kinase-dead Yck2[(D218A)
and (K105R)] were palmitoylated, and that there are subsequent separate sub-populations that are devoid of palmitoylation.

**Kinase-Inactive Mutants Yield Different Sub-Cellular Localizations**

To determine the sub-cellular localization of the kinase-dead Yck2 mutants, we used IIF (Figure 16). While wild-type Yck2(wt) properly localized to the plasma membrane, Yck2(Δ58-397) and Yck2(K105R) showed divided localizations consisting of surface localized and cytoplasmic populations. Yck2(Δ58-397) was more severely mislocalized to the cytoplasm than Yck2(K105R) and this result is in line with the concurrent palmitoylation results that showed Yck2(K105R) palmitoylation levels to be higher than those of Yck2(Δ58-397). In contrast, Yck2(D218A) revealed strong surface localization and little mislocalization despite a level of palmitoylation very similar to Yck2(K105R). The difference in localization between the two kinase-dead missense mutants does not seem to coincide with their similar palmitoylation levels, and may be explained by some yet-to-be discovered phenomenon.

**Phosphorylation in Trans Indirectly Reports on Cellular Localization**

We have previously demonstrated that Yck2 hyper-phosphorylation provides a useful metric of Yck2 surface localization (data not shown). Since hyper-phosphorylation can occur in *trans*, we developed a situation where over-expression of Yck2(wt) acted in *trans* to hyper-phosphorylate the sub-populations of kinase-dead Yck2 mutants that underwent palmitoylation and delivery to the plasma membrane (Figure 17). For this analysis, epitope-tagged (HA) mutant Yck2 (kinase-dead or kinase-
deleted) proteins were expressed from their native YCK2 promoter in cells that simultaneously over-expressed untagged Yck2(wt) from the over-expressing GAL1 promoter.

In the control cell context that lacks the over-expressed Yck2(wt), neither Yck2 (wt) nor any of the kinase mutants showed evidence of hyper-phosphorylation since the hyper-phosphorylation phenomenon is not seen for Yck2 proteins expressed at endogenous levels. In the over-expression cell context, hyper-phosphorylated shifts were seen for each of the tested samples except for Yck2(SS), which displays a completely cytoplasmic localization. Consistent with its localization and palmitoylation status, Yck2(wt) was efficiently hyper-phosphorylated, showing almost an entire protein shift to the hyper-phosphorylated species. For both Yck2(K105R) and Yck2(D218A), approximately half of the protein populations were hyper-phosphorylated and for Yck2 (∆58-397) and even smaller fraction was hyper-phosphorylated.

These results remain consistent when considering the previous ABE experiment that showed the palmitoylation of the kinase-dead mutants was weaker than wild-type levels, but much stronger than the kinase-deleted Yck2(∆58-397 levels) (Figure 14). In addition the palmitoylated sub-populations of both kinase-dead mutants were greater than that of the kinase-deleted mutant (Figure 15). These results again reinforce the previous notion that while kinase structure is essential for robust palmitoylation of Yck2, it is not the only prerequisite. Kinase activity also plays a very important role in palmitoylation.
Figure 16. Kinase inactive mutants yield various sub-cellular localizations. Indirect immunofluorescent detection of Yck2 kinase inactive mutants. Proteins were expressed on single-copy CEN/ARS plasmids under the inducible GAL1p (2 hour expression) and were detected by an anti-HA monoclonal antibody as primary antibody, and then a Cy3-conjugated donkey anti-mouse secondary antibody.
Analysis of Additional Kinase-Dead Mutants

Surprisingly, we were not able to resolve the palmitoylation results with the different sub-cellular localizations of the kinase-dead mutants. In an attempt to sort out this problem, we created and analyzed three additional kinase-dead mutants. The mutants Yck2(D195A) and Yck2(N200A) mutate the invariant residues Asp195 and Asn200 that lie within a consensus motif that is likely the catalytic loop of the kinase. The mutant Yck2(G85A) replaces the invariant Gly85 of the conserved glycine-rich loop that has been shown to accommodate a non-transferable phosphate of ATP.

To ensure that these mutants were null for kinase activity, we tested them using the same two criteria previously used to test kinase-dead mutants, complementation and hyper-phosphorylation. To test for complementation of the mutant strain that is null for *YCK1* and temperature sensitive for *YCK2* (*yck1Δ yck2-ts*), the strain was transformed with plasmids carrying either wild-type Yck2(wt) or the kinase-dead alleles and grown at the permissive (20°C) and non-permissive temperatures (34°C). The mutant strain would require the presence of an active Yck1 or Yck2 to grow at the non-permissive temperature, as seen when Yck2(wt) complemented the strain (Figure 18A). Introduction of the kinase-dead alleles to the strain did not result in complementation of the strain at the non-permissive temperature. This reveals that the additional Yck2 kinase-dead mutants do not possess any kinase activity to sustain cell growth.

To further confirm kinase activity of the additional kinase-dead mutants, protein extracts from samples over-expressed by the *GAL1* promoter were subjected to phosphatase enzyme-treatment prior to running on a SDS-PAGE gel and transferred to
Figure 17. Hyper-phosphorylation of kinase mutants of Yck2 occurs in \textit{trans} by over-produced Yck2(wt). Over-produced plasma membrane-localized Yck2(wt) can phosphorylate the kinase-inactivated mutant Yck2 population that is localized to the surface. Wild-type yeast cells (LRB759) expressing wild-type or kinase mutant versions of N-terminally HA-tagged Yck2 [expressed under the native YCK2\textsubscript{p} on a single-copy plasmid (pRS315)] simultaneously with either an untagged overproduced (GAL1\textsubscript{p}) version of Yck2(wt) (pND2650) or the equivalent empty plasmid control. Following a 2 hour galactose induction period, denatured protein extracts were prepared and analyzed by SDS-PAGE and anti-HA Western blotting. Equal protein loading was confirmed by staining nitrocellulose with Ponceau Red stain (data not shown).
a Western blot (Figure 18B). The addition of phosphatase enzyme did not cause a band collapse to a smaller molecular-weight band in any of the kinase-dead samples when compared to Yck2(wt) and Yck2(SS). This further validates that the kinase-dead mutants Yck2(D195A), Yck2(N200A), and Yck2(G85A) are indeed free of any kinase activity.

In an attempt to resolve the disparity between the sub-cellular localizations of the previous kinase-dead mutants Yck2(K105R) and Yck2(D218A), IIF was used to determine the localizations of the additional kinase-dead mutants, Yck2(D195A), Yck2(N200A) and Yck2(G85A) (Figure 19). Two of the three mutants, Yck2(D195A) and Yck2(G85A) showed a divided localization between the cell surface and the cytoplasm, looking very similar to Yck2(K105R), but not as defective as kinase-deleted Yck2(Δ58-397). Conversely, Yck2(N200A) was localized fully to the surface, looking very similar to Yck2(wt) and Yck2(D218A). These results are confusing in that they are not consistent, displaying various localizations yet showing palmitoylation results that are similar. Thus, while some kinase-inactive Yck2 mutants alter the intracellular localization of Yck2, other mutants have no effect and display wild-type localization. Presently, we are uncertain to the reason to this behavior.

Overall, the analyses of the kinase-dead mutants reveal that kinase activity significantly contributes to Yck2 palmitoylation. However, kinase activity is clearly not the whole story, since substantially more extreme defects are seen for kinase-deleted Yck2(Δ58-397). In addition to providing kinase activity, the kinase domain also offers more than just a structural role when contributing to the behavior of Yck2.
Figure 18. Analysis of additional kinase-inactive Yck2 mutants.
(A) Serial 10-fold dilutions of yck1Δyck2^{ts} cells, transformed by the CEN/ARS vector pRS316 or by pRS316 carrying either the wild-type YCK2, or the kinase-inactive mutants G85A, D195A, or N200A were plated at both permissive (20°C) and non-permissive (34°C) temperatures. (B) Wild-type Yck2, the Yck2(SS) non-palmitoylated mutant, and the kinase inactive Yck2(D195A), N200A, and G85A mutants, were each introduced into wild-type yeast cells on single-copy plasmids (pRS316) under the inducible GAL1_p promoter. Protein extracts were prepared with a portion of the sample digested with phosphatase and equal amounts left untreated. Western analysis was performed with antibody directed against the N-terminal HA epitope tag.
Kinase domain replacements

Considering the important role documented above for the kinase domain and for kinase activity of Yck2, we wondered if generic features of the kinase domain might be recognized for palmitoylation. Although there is rich diversity in the kinase superfamily of homologous proteins, there are also highly conserved common structural features in kinases that are involved in the handling of the nucleotide triphosphate. We decided to create Yck2 kinase-replacement proteins using related palmitoylated and non-palmitoylated CK1 casein kinase regions as the replacement kinase domain and investigated whether these new domains were able to provide a similar palmitoylation function.

Four chimeras were created to precisely replace the Yck2 kinase domain with the kinase domain from the four different yeast and mammalian CK1s. The four replacement CK1s, described in the Introduction chapter, are: Yck1 - the functionally redundant partner to Yck2, Hrr25 - a non-palmitoylated *Saccharomyces cerevisiae* CK1, Cki2 - the evolutionarily distant yeast *Schizosaccharomyces pombe* CK1, and CSNK1G2 - the human γ2 isoform CK1. Like Yck2, Yck1 localizes to the plasma membrane due to Akr1-mediated palmitoylation of its C-terminal Cys-Cys dipeptide. Hrr25, which has shown to be involved in DNA repair, localizes to the cytoplasm and nucleus. Hrr25 lacks C-terminal cysteines and is presumably not palmitoylated. Although neither human CSNK1G2 nor *S. pombe* Cki2 have been extensively studied, both are predicted to be palmitoylated on cysteines that map to their C-termini (Kang, Wan et al. 2008). CSNK1G2, one of three γ mammalian CK1s, has been linked to Wnt
Figure 19. Indirect immunofluorescent detection of additional Yck2 kinase inactive mutants. Additional kinase inactive Yck2 mutants were expressed on single-copy CEN/ARS plasmids under the inducible \textit{GAL1}_{p} (2 hour expression) and were detected by an anti-HA monoclonal antibody as primary antibody, and then a Cy3-conjugated donkey anti-mouse secondary antibody.
signaling (Davidson, Wu et al. 2005) and is a palmitoyl-protein candidate in rodent brain (Kang, Wan et al. 2008). Cki2 is a cytoplasmic kinase that may contribute to the regulation of cell morphology (Wang, Vancura et al. 1994).

The sequence elements comprising the CK1 kinase domain are well defined and well conserved. Based on the crystal structure of the kinase domains for the *S. pombe* Cki2 and the human CSNK1G2, the highly conserved kinase domain for Yck2 has been mapped to residues 72 - 359 (Xu, Carmel et al. 1995; Longenecker, Roach et al. 1996). The multiple sequence alignment of the four kinases and Yck2 is shown in Figure 20 using the ClustalW2 online computer program (www.ebi.ac.uk/clustalw/). The residues highlighted in blue represent conserved sequence and those in orange represent semi-conserved sequence within the alignment. The four chimeras were designed to retain the N-terminal and C-terminal unstructured domains of Yck2 (residues 1-71 and 362-546 respectively) with the replacement kinase domain being introduced in place of the Yck2 kinase domain. Kinase domain definitions were the same for all the proteins, including Yck2, as shown in the multiple sequence alignment. The domain begins with the conserved residues Val-Gly (VG) and ends with Asp-Trp (DW).

**Activity of kinase domain replacements**

We first examined the ability of the CK1 chimeras to complement the *yck1Δ yck2-ts* strain. Previous analyses indicated that complementation of a strain devoid for *YCK1* and temperature-sensitive for *YCK2 (yck1Δ yck2-ts)* required kinase activity, as shown when the kinase-dead mutants failed to complement. Complementation also required the capacity to associate with membranes (non-palmitoylated and mislocalized Yck2
**Figure 20. Multiple sequence alignment of CK1 proteins.** Yck2 in a multiple sequence alignment with CK1 candidates chosen for chimera construction. Alignment performed using Clustalw computer software. Conserved sequences are highlighted in blue, while semi-conserved sequences (residue changes that maintain similar structure) are highlighted in orange.
Transformation of the chimeras into the temperature-sensitive strain showed a robust complementation for the Yck2-Yck1-Yck2 and Yck2-Cki2-Yck2 chimeras, weak but significant growth for the Yck2-Hrr25-Yck2 chimera, and no complementation for the Yck2-CSNK1G2-Yck2 chimera (Figure 21).

Given the close correlation between the hyper-phosphorylation phenotype with both palmitoylation and surface localization, the phosphorylation status of the chimeras was examined (Figure 22). Phosphatase-treated protein extracts of the chimeras, driven by the over-expressing GAL1 promoter, were analyzed by SDS-PAGE and Western blotting. The results remained consistent with those of the previous complementation experiments (Figures 13B and 18B), meaning that the same chimeras that provided complementation of the yck1Δyck2-ts strain also exhibited hyper-phosphorylation. The chimeras containing the kinase domains of Yck1, Hrr25, and Cki2 each displayed a hyper-phosphorylated population that collapsed to a lower, unphosphorylated population upon treatment with phosphatase enzyme. The human CSNK1G2 chimera, however, did not show any hyper-phosphorylation, and presumably possessed no auto kinase activity.

We examined the sub-cellular localization of the chimeras by IIF and saw that the results remained in line with the previous complementation and hyper-phosphorylation findings. The Yck1 and Cki2 chimeras showed surface localizations indistinguishable from Yck2(wt), while the CSNK1G2 chimera was very weakly localized to the plasma membrane and was predominantly present in the cytoplasm, like Yck2(Δ58-397) which lacks the kinase domain altogether (Figure 23). Mutation of the two C-terminal cysteines to serines, of the Yck2-Yck1-Yck2 chimera resulted in mislocalization to the cytoplasm,
Figure 21. Complementation analysis of Yck2 chimeras. Serial 10-fold dilutions of yck1Δyck2ts cells, transformed by the CEN/ARS vector pRS316 or by pRS316 carrying either the wild-type YCK2, or Yck2 proteins with kinase replacements from various CK1 family member proteins. Transformants were plated at both permissive (20°C) and non-permissive (34°C) temperatures.
similar to Yck2(SS). The Hrr25 chimera displayed unusual surface-localized, punctate structures, suggesting a propensity of this chimera to cluster or aggregate. Elimination of palmitoylation of the Hrr25 chimera by substitution of the palmitoyl-accepting cysteines to serines of the C-terminal Yck2 sequences, resulted in the chimera mislocalizing to the cytoplasm. Interestingly, the absence of palmitoylation for this chimera also removed the punctate structures, suggesting that palmitoylation might have enabled the chimeras to interact with one another. Furthermore, replacement of the C-terminal palmitoyl-accepting cysteines of the Yck2-Hrr25-Yck2 chimera with the C-terminal transmembrane domain of the yeast v-SNARE Snc2 stably anchored the chimera to the plasma membrane and restored the presence of punctate structures at the surface.

**Palmitoylation of kinase domain replacements**

The membrane localization of the chimeras was indirectly observed using our atypical membrane fractionation technique previously described in Chapter III, where we found that glass bead-lysis of yeast cells resulted in the mechanical denaturation and aggregation of cytosolic forms of Yck2 [e.g. Yck2(SS)], but not membrane-associated Yck2(wt) (Papanayotou, Sun et al. 2010). Membrane-associated Yck2(wt) was protected from mechanical denaturation due to its inclusion within membrane vesicles produced by the cellular disruption. This technique allowed us to distinguish between membrane-associated and non-associated forms of Yck2. Using this approach, three of the four chimeras, namely Yck1, Hrr25, and Cki2, were found to fractionate like Yck2(wt) largely to the supernatant (Figure 24). Furthermore,
Figure 22. Phosphatase analysis of Yck2 chimeras. Wild-type Yck2 and the Yck2 kinase-replacement chimeras were transformed into wild-type yeast cells on single-copy plasmids (pRS316) under the inducible GAL1p. Protein extracts were prepared with equal amount of sample digested with phosphatase enzyme and equal amount of sample left untreated. Western analysis was performed with antibody directed against the N-terminal HA epitope tag.
Figure 23. Indirect immunofluorescent localization of Yck2 chimeras. Proteins were expressed on single copy CEN/ARS plasmids under the inducible GAL1p (2 hour expression) and were detected by an anti-HA monoclonal antibody as primary antibody, and then a Cy3-conjugated donkey anti-mouse secondary antibody.
replacement of the palmitoyl-accepting cysteines to serines caused these chimeras to fractionate to the pellet like Yck2(SS). Results suggest that like Yck2(wt), the Yck1, Hrr25 and Cki2 chimeras were efficiently palmitoylated. In contrast, the CSNK1G2 chimera showed an intermediate fractionation between the supernatant and the pellet, which is consistent with its failure to complement, ability to mislocalize by IIF, and failure to display a hyper-phosphorylated band by Western blot.

To directly determine the palmitoylation status of the chimeras, we used a click chemistry-based palmitoylation protocol, rather than the ABE-based protocol used previously. The click-based approach, which relies on metabolic incorporation of an alkyne-tagged fatty acid into the protein of interest (Charron, Zhang et al. 2009), offers simpler and more convenient sample processing than does ABE, yielding results roughly comparable to ABE (Figure 25). The click analysis revealed that all of the chimeras were indeed palmitoylated, with those containing Yck2, Hrr25, and Cki2 kinase domains showing strong palmitoylation signals almost similar to that of wild-type palmitoylation.

In addition to analyzing the palmitoylation status of the chimeras, we have examined the effects of mutation of the two phenylalanines 539 and 540 of Yck2. Both phenylalanine residues participate in the highly conserved C-terminal region that is essential for Yck2 palmitoylation (A. Roth and N. Davis, unpublished). Mutation of Phe539,Phe540 dipeptide to Ala539,Ala540 resulted in an incredibly large defect of palmitoylation for wild-type Yck2(wt), which was also severely mislocalized and under-phosphorylated (Figure 16 and A. Roth and N. Davis, unpublished). Applied to the chimeras, the FF to AA mutation resulted in a severe defect of palmitoylation for
Figure 24. Use of glass bead aggregation to analyze the fractional palmitoylation of the Yck2 chimeras. Glass bead lysates prepared from wild-type yeast cells expressing wild-type Yck2, the Yck2 chimeras, or their non-palmitoylated -SS counterparts. The expressed Yck2 proteins possess the tripartite epitope tag 6xHis/FLAG/HA and are under the GAL1p. Protein extracts were fractionated by differential centrifugation and were analyzed by Western blotting and antibody to HA.
Figure 25. Palmitoylation of Yck2 chimeras. Click chemistry-based approach to determine palmitoylation. Various Yck2 constructs and their di-alanine substitution mutants (FF to AA) were tested. Proteins tagged with 6xHis/FLAG/HA were expressed on single copy plasmids (pRS316) under the inducible GAL1p (2 hour expression). Cells were labeled with ODYA after the first hour of galactose induction, for one hour. Resulting lysates were immunoprecipitated with FLAG agarose and the click reaction was initiated for one hour. The samples were precipitated and analyzed by SDS-PAGE and Western blotting. The fluorescent signal (palmitoylation) was detected by using a Typhoon 9200 fluorescent scanner. Protein expression was detected by probing Western blot with antibody to HA.
chimeras with Yck1 and Cki2 kinase domains, and resulted in complete abolishment of palmitoylation for those with Hrr25 and CSNK1G2 kinase domains. Thus, the F539,F540 dipeptide clearly provides a crucial role in directing palmitoylation.
CHAPTER IV

DISCUSSION

Two previous analyses of the Yck2 sequence elements that directed both palmitoylation and surface localization found the Yck2 C-terminal 48 residues to be a sufficient palmitoylation signal (Babu, Bryan et al. 2002; Babu, Deschenes et al. 2004). Our Ura3-Yck2 results support this conclusion. The Ura3-Yck2 analysis found the Yck2 C-terminal 43 residues to be minimally required for palmitoylation (Figure 2). While these 43 C-terminal residues were sufficient to direct palmitoylation, they did not provide complete palmitoylation, indicating that other elements beyond this region also make important contributions in directing palmitoylation. This chapter showed the missing element to be the kinase domain, which we found plays a pivotal role.

The present work found that the \textit{Saccharomyces cerevisiae} type I casein kinase Yck2 showed defects in palmitoylation when the kinase domain in its entirety was either removed or inactivated. Complete removal of the kinase domain resulted in palmitoylation defects that were much more severe (Figure 5). Point mutations in the kinase domain that disabled activity resulted in lesser palmitoylation defects, with greater than 50% of the protein becoming palmitoylated (Figures 5 and 6). While the palmitoylation results remained consistent for the kinase-inactive Yck2 mutants, their sub-cellular localizations surprisingly were not consistent; some mutants exhibited localizations similar to wild-type Yck2, and others resembled localizations similar to Yck2 completely devoid of its kinase (Figure 7). Furthermore, this discrepancy in localization of the various kinase-inactive mutants could not be pinpointed to mutations in the N-terminal lobe vs. the C-terminal lobe of the kinase domain structure, or resolved
by analysis of other kinase-dead mutants (Figure 10). An in-frame replacement of the Yck2 kinase domain with the kinase domains of close and distantly related CK1 proteins revealed that while some replacements were successful, not all CK1 kinase domains were sufficient to restore Yck2 functionality (Figure 12) and localization (Figures 14 and 15. Thus, Yck2’s kinase domain contributes structural and functional roles to the trafficking and palmitoylation of Yck2.

A Structural Role For the Kinase Domain

The finding that Yck2(Δ58-397) was much more deficient for palmitoylation than the kinase-dead mutants Yck2(K105R) and (D218A) may suggest that the kinase domain possesses structural elements that are essential for optimal palmitoylation. In this scenario the properly folded kinase domain could contain recognition sequences for identification by the Golgi-localized Akr1, or by some unidentified factor involved in the delivery of Yck2 to Akr1. However if these recognition elements are essential for palmitoylation, we would expect Yck2(Δ58-397) to be completely non-palmitoylated due to the abolishment of these sequences, which is not the case. Instead Yck2(Δ58-397) had less than half of its protein population palmitoylated, arguing toward a multi-signal palmitoylation model. The deficiency in palmitoylation of the kinase-inactive mutants compared to wild-type palmitoylation levels could be explained by some impairment in folding, either due to the residue-change or to the requirement of a phosphorylation-dependent conformational change which would rely on activity of the kinase for palmitoylation. Arguing against the first reason, both mutations have been widely used
to fully inactivate the function of other kinases, so although possible, it seems unlikely that a single residue change would completely re-configure the folded kinase structure.

With regard to the kinase domain CK1-replacement chimeras, it was a surprise to see that the human replacement, CSNK1G2, failed to provide proper kinase function (Figures 12 and 13), because the kinase domains of the CK1 proteins selected were well defined and strongly conserved. However all of the chimeras showed some level of palmitoylation, which agrees with the idea that there are recognition elements within the kinase domain that participate in somehow gaining the protein access to palmitoylation. These recognition elements are most likely conserved within the CK1 kinase domain.

**A Phosphorylation Role For the Kinase Domain**

While the intact structure of the kinase domain has shown to be important for Yck2 palmitoylation, it is not the only requirement. The impairment of palmitoylation for the kinase-inactive mutants K105R and D218A also suggests that kinase activity is a requirement for efficient palmitoylation of Yck2. In this scenario, kinase activity of Yck2 is needed for phosphorylation of itself and/or accessory protein(s) that could result in an induced conformational change needed for trafficking or for interaction with Akr1. There is one serine in the conserved C-terminal 10 residue-long domain and three additional serines adjacent to this region; auto-phosphorylation of these serines could promote an interaction with Akr1. Our results indicate that the auto-phosphorylation of Yck2 is a relatively late-event, occurring at the plasma membrane; however, the non-palmitoylated Yck2(SS) showed a subtle level of phosphorylation (Figure 9B), indicating that some low level of phosphorylation may also occur early. The different localizations
of the kinase-inactive mutants is peculiar, especially since both the palmitoylation levels and the *trans* hyper-phosphorylation levels of the K105R and D218A mutants are similar. We remain unclear about this discrepancy.

### A Combined Model

What role does the kinase domain play in palmitoylation? Does the domain provide a structural element to the protein that is necessary for proper folding and interaction, or is kinase activity needed for palmitoylation? Despite an extensive effort, the mechanism by which the kinase domain promotes palmitoylation remains unclear. We predict that the requirement for palmitoylation falls in between the possibilities posed above, with important roles being played both by Yck2-mediated phosphorylation and by protein-protein interaction elements exposed on the folded kinase surface.

Overall, the search for the palmitoylation signal in Yck2 has resulted in a complex, multi-signal model. There are two major elements that contribute to the palmitoylation of Yck2: 1) The 186 residue-long, poorly-conserved, unstructured C-terminal domain, save for a 10 residue-long, conserved C-terminal domain which includes the palmitoyl-accepting cysteines, 2) the kinase domain, described in detail in this chapter.

An extraordinary result was seen for the mutation of both phenylalanines, 539 and 540, in the conserved C-terminal 10 residue-long domain, to alanines. The addition of this mutation resulted in severe palmitoylation defects for wild-type, kinase-inactive, and kinase-replaced Yck2, indicating the importance of the conserved portion of the C-terminal domain to palmitoylation. The remaining, non-conserved C-terminal 176
Figure 26. A multi-signal model for how Yck2 might promote Akr1-mediated palmitoylation. The kinase domain and the 10 amino acids of the C-terminus are shown as potentially interacting with two separate Akr1 domains, the ankyrin repeats and the active site DHHC loop, respectively. The poorly conserved C-terminal domain (minus the C-terminal 10 residues) is predicted to be intrinsically disordered and suggests to serve as a flexible linker, accommodating simultaneous interaction of the kinase domain and the C-terminal 10 residues with Akr1.
sequences are predicted to be intrinsically disordered. Intrinsically disordered protein domains have been found to serve as linkers between structured proteins. They also can provide exposed peptide sequences that allow for protein-protein interaction. Each of these two functions could contribute to the functions of the kinase domain and the C-terminal conserved domain in promoting palmitoylation of Yck2.

A possible model envisions Yck2 interacting with Akr1 at two separate points of interest, with the 186 residue-long unstructured C-terminal region acting as a flexible linker between the kinase domain and the C-terminal conserved domain (Figure 26). The C-terminal conserved domain, which contains the palmitoyl-accepting cysteines and the important phenylalanine residues, may interact with the putative Akr1 active site, a conserved zinc finger-like 50 residue-long DHHC domain. Connected by the unstructured linker, the kinase domain may interact with the N-terminal ankyrin repeat domain of Akr1. There is no evidence of this interaction; however, work with the mammalian ortholog of Akr1, HIP14, showed that HIP14 ankyrin repeats play an important role in substrate recognition (Huang, Sanders et al. 2009). The flexible unstructured protein linker would allow both the kinase domain and the C-terminus to interact with Akr1 at distinct, but separate sites.

This above model provides an explanation for the kinase domain in palmitoylation, but does not shed any light on the issue of how Yck2 traffics to Akr1. An ideal scenario would have the C-terminal unstructured region contain recognition elements for chaperones and/or accessory proteins to assist in folding and gaining access to the Golgi-localized Akr1.
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The *Saccharomyces cerevisiae* type I casein kinase Yck2 is tethered to the cytosolic face of the plasma membrane through palmitoylation of its two C-terminal cysteines. Yck2 palmitoylation is mediated by the Golgi-localized palmitoyl transferase Akr1. Yck2 is a cytosolic protein and is presumably synthesized on cytosolic ribosomes. While the trafficking events following palmitoylation of Yck2 at the Golgi are well characterized, the initial trafficking events after synthesis remain unknown. We sought to elucidate these early events of how Yck2 might travel to the Golgi-localized Akr1 after it is synthesized.

Our initial experimental analysis of Yck2 utilized glass bead cell lysates. The use of glass beads to mechanically disrupt yeast cells is used in a variety of applications, including for the analysis of native protein function (e.g. protein-protein interaction, enzyme assays, membrane fractionations). We describe a surprising case of protein denaturation and aggregation that is induced by this lysis protocol. Interestingly, when yeast cells are subjected to glass bead disruption, non-palmitoylated, cytosolic forms of
the kinase denature and aggregate, while membrane-associated forms, whether attached by their native palmitoyl-tethers or by a variety of artificial membrane tethering sequences, are protected from aggregation and denaturation. A broader look at the yeast proteome shows that while the majority of proteins resist glass bead induced aggregation, a significant subset does experience denaturation. Thus, yeast researchers should be aware of this potential artifact when using glass bead lysates to look at native protein function. We have also demonstrated an experimental utility for glass bead-induced aggregation, using its fine discrimination of membrane-associated from non-associated Yck2 forms to discern the palmitoylation states of Yck2 mutants that are partially defective for palmitoylation.

The second portion of this dissertation continues to explore the early events that precede palmitoylation to find the mechanism by which palmitoyl proteins synthesized as soluble cytoplasmic proteins, are delivered to the membrane localized palmitoyl transferases. More specifically, we were interested in the role of the kinase domain on palmitoylation and we hypothesized that the kinase domain possessed signals within its sequence to influence palmitoylation and that kinase activity itself is required for palmitoylation. Our analysis finds that both kinase structure and activity are necessary for proper palmitoylation of Yck2. Analysis of various kinase-inactive missense mutants revealed that kinase activity participates in efficient palmitoylation and localization. In addition, analysis of Yck2 chimeric proteins whose kinase domains were replaced with those of other CK1 proteins, revealed that a generic feature does not exist in kinase domains for palmitoylation. Rather, we conclude that Yck2 contains a signal in the kinase domain that presents itself as a result of the proper folding of the kinase. Thus,
Yck2 contains multiple factors that participate in palmitoylation, with the kinase domain play one of the major roles.
AUTOBIOGRAPHICAL STATEMENT

IRENE PAPANAYOTOU

EDUCATION

**Ph.D Pharmacology**
September 2004 - February 2011
Wayne State University, School of Medicine, Detroit, MI

**Bachelor of Science in Biochemistry**
September 2000 - May 2004
Saginaw Valley State University, University Center, MI

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