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The yeast DHHC cysteine-rich domain protein Akr1p is a palmitoyl transferase

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Protein palmitoylation has been long appreciated for its role in tethering proteins to membranes, yet the enzymes responsible for this modification have eluded identification. Here, experiments in vivo and in vitro demonstrate that Akr1p, a polytopic membrane protein containing a DHHC cysteine-rich domain (CRD), is a palmitoyl transferase (PTase). In vivo, we find that the casein kinase Yck2p is palmitoylated and that Akr1p function is required for this modification. Akr1p, purified to near homogeneity from yeast membranes, catalyzes Yck2p palmitoylation in vitro, indicating that Akr1p is itself a PTase. Palmitoylation is stimulated by added ATP. Furthermore, during the reaction, Akr1p is itself palmitoylated, suggesting a role for a palmitoyl-Akr1p intermediate in the overall reaction mechanism. Mutations introduced into the Akr1p DHHC-CRD eliminate both the trans- and auto-palmitoylation activities, indicating a central participation of this conserved sequence in the enzymatic reaction. Finally, our results indicate that palmitoylation within the yeast cell is controlled by multiple PTase specificities. The conserved DHHC-CRD sequence, we propose, is the signature feature of an evolutionarily widespread PTase family.

Introduction

Many signaling proteins tether to membrane sites through lipid modifications, i.e., palmitoylation, myristoylation, or prenylation. Palmitoylation, the thioesterification of cysteine by palmitic acid, often directs the modified protein to the plasma membrane; indeed, often to plasma membrane subdomains, i.e., lipid rafts and caveolae that serve as dedicated sites of signal transduction and/or cellular entry/exit (Brown and London, 2000; Campbell et al., 2001; Zacharias et al., 2002). The list of palmitoylated proteins includes Ras and Rho G proteins, nonreceptor tyrosine kinases (e.g., Fyn, Lyn, Lck, and Yes), caveolin, Gα and Gγ subunits of heterotrimeric G proteins, G protein–coupled receptors, nitric oxide synthases, the SNAP-25 component of the plasma membrane SNARE complex, and many viral coat proteins (e.g., HIV and influenza) (Dunphy and Linder, 1998; Resh, 1999).

The enzymes that catalyze the prenyl and myristoyl protein modifications, i.e., the prenyl and myristoyl transferases, have been well characterized and are conserved from yeast to man. These enzymes are attractive as potential drug targets. Prenyl transferase inhibitors that block Ras protein farnesylation are under investigation as anticancer agents (Prendergast, 2000). Although drug targeting of palmitoylation should have similar potential, given the many key signaling proteins that rely on this modification, no palmitoyl transferase has been yet identified from any species. Attempts at palmitoyl transferase (PTase)* purification have been thwarted, in large part, by the integral association of these activities with cellular membranes (Berthiaume and Resh, 1995; Dunphy et al., 1996). Furthermore, a prominent nonenzymatic reaction of palmitoyl coenzyme A (CoA) directly with the protein substrate (Quesnel and Silvius, 1994; Duncan and Gilman, 1996) clouds the ability to assay PTase activity. A genetic approach in yeast, screening for the functions that participate in yeast Ras2p palmitoylation, identified two genes, *SHR5* and *ERF2* (Bartels et al., 1999). *SHR5* encodes a hydrophilic 26.5-kD protein with no informative sequence homology, and *ERF2* encodes a 41-kD membrane protein with four predicted transmembrane domains and a 50-residue-long DHHC cysteine-rich domain (CRD), a variant of the C_2H_2 zinc finger domain (Putilina et al., 1999), defined by the core Asp-His-His-Cys (DHHC) tetrapeptide sequence. Though *erf2Δ* and *shr5Δ* strains were found to be partially defective for Ras2p palmitoylation, other phenotypes suggested

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*Abbreviations used in this paper: β-ME, β-mercaptoethanol; CoA, coenzyme A; CRD, cysteine-rich domain; PTase, palmitoyl transferase.

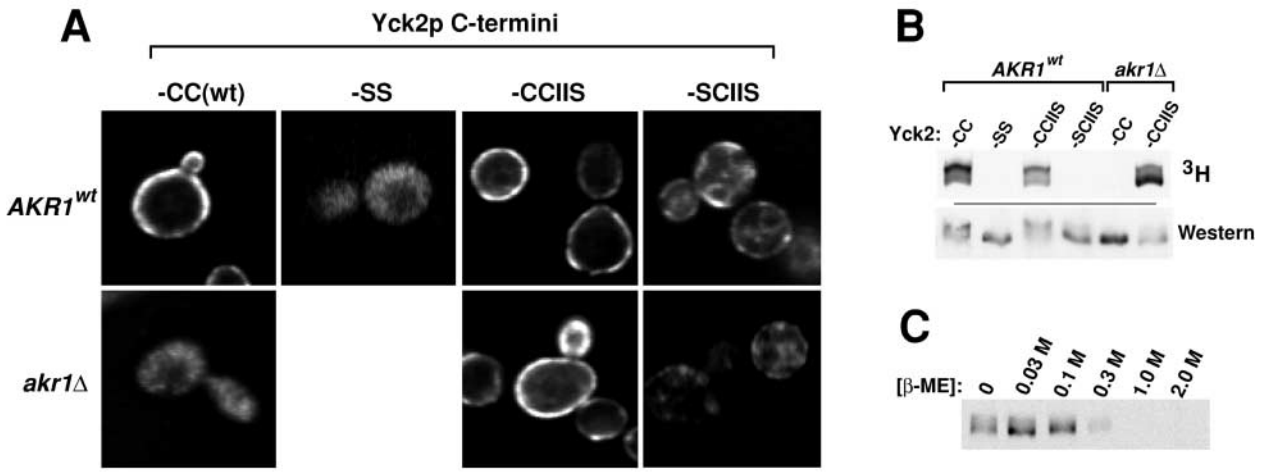

Figure 1. **Akr1p is required for Yck2p palmitoylation.** Wild-type Yck2p or one of three Yck2p mutants, having the COOH-terminal Cys-Cys (CC) replaced by SS, CCIIS, or SCIIS, all NH_2-terminally tagged with a 6xHis/FLAG/HA sequence and under the inducible control of the *GAL1* promoter were introduced into wild-type (*AKR1*$^+$) yeast cells or isogenic *akr1Δ* cells on single-copy plasmids (pRS316 based). (A) Subcellular localization of wild-type and mutant Yck2 proteins in *AKR1*$^+$ and *akr1Δ* cells. Cells were subjected to a 2-h period of galactose (2%)-induced expression, followed by a 20-min period of glucose (3%)-mediated repression, time in which the newly synthesized kinases can achieve their final subcellular destinations. Detection of the Yck2 kinases used an anti-HA mAb as primary antibody, and then a Cy3-conjugated donkey anti–mouse secondary antibody. (B) [^{3}H]palmitate labeling of wild-type and mutant Yck2 proteins in *AKR1*$^+$ and *akr1Δ* cells. Cells were cultured and labeled with [^{3}H]palmitic acid as described in the Materials and methods. Labeled Yck2p recovered by anti-FLAG IP was subjected to SDS-PAGE, fluorography, and autoradiography (top). To assess Yck2p recovery, a second portion of the anti-FLAG IP sample was subjected to anti-HA Western analysis (bottom). The differing gel mobilities are a consequence of differential phosphorylation; phosphatase treatment of wild-type Yck2p–containing extracts from *AKR1*$^+$ cells shifts Yck2p gel mobility to a position coincident either with Yck2(SS)p or with wild-type Yck2p from *akr1Δ* cells (unpublished data). (C) Release of palmitate label from Yck2p by β-ME. Yck2p, labeled in vivo by [^{3}H]palmitic acid and purified by anti-FLAG IP, was incubated for 10 min at 100°C in 2% SDS, 10% glycerol, 62.5 mM Tris, pH 6.8, containing the indicated concentrations of β-ME.

that the primary defect might instead be in Ras trafficking (Bartels et al., 1999). The work described below linking yeast Akr1p, a second DHHC-CRD protein, to palmitoylation suggests a general role for members of the DHHC-CRD protein family in palmitoylation.

AKR1 encodes an 86-kD protein with six predicted transmembrane domains, six ankyrin repeat sequences mapping to the NH_2-terminal hydrophilic domain, and a DHHC-CRD sequence mapping between transmembrane domains four and five. Homology between Akr1p and Erf2p is limited to the DHHC-CRD sequence. Our previous work demonstrated Akr1p to be required for the proper localization of the type I casein kinase Yck2p to the yeast plasma membrane (Feng and Davis, 2000). The membrane association of Yck2p and of its functionally-redundant partner kinase, Yck1p, depends apparently on lipid modification of COOH-terminal Cys-Cys sequences (Vancura et al., 1994). Significantly, essentially the same Yck2p localization defect is seen in *akr1Δ* cells as is seen with cis mutation of the Yck2p COOH-terminal cysteines; both mutations result in the kinase being mislocalized to the cytoplasm (Feng and Davis, 2000), an indication of possible Akr1p function in the Yck2p lipid modification process.

Results and discussion

We have constructed a Yck2p mutant that has the COOH-terminal pentapeptide lipidation site of yeast Ras2p; sequences encoding the tripeptide Ile-Ile-Ser were appended to the Yck2p COOH terminus, generating Yck2(CCIIS)p. Yeast Ras2p, like mammalian H- and N-Ras counterparts, is dually modified; the more COOH-terminal of the two cysteines (part of the CaaX prenylation consensus) being farnesylated and the adjacent cysteine, palmitoylated. Yck2(SCIIS)p, a second Yck2p mutant lacking the Ras2p palmitoyl-accepting cysteine, also was constructed. In wild-type (*AKR1*$^+$) cells, we find that Yck2(CCIIS)p localizes like wild-type Yck2p, exclusively to the plasma membrane (Fig. 1 A). Yck2(SCIIS)p, which we presume is farnesylated (it retains the CaaX consensus), also localizes to cellular membranes, but largely to intracellular membranes (Fig. 1 A). These localizations are consistent with those reported for the analogous Ras2p forms; wild-type Ras2p (CCIIS COOH terminus) localizes to the plasma membrane, whereas the mutant Ras2(SCIIS)p localizes primarily to intracellular membranes (Bartels et al., 1999). Thus, as with Ras2p, the two COOH-terminal cysteines of Yck2(CCIIS)p likely are dually lipidated.

As reported previously (Feng and Davis, 2000), Yck2p is mislocalized in *akr1Δ* cells, localizing like the Yck2(SS)p cis mutant lacking the COOH-terminal dicysteine, diffusely throughout the cytoplasm (Fig. 1 A). In contrast, no effect of the *akr1Δ* mutation can be discerned on the localization of either Yck2(CCIIS)p or Yck2(SCIIS)p; Yck2(CCIIS)p still localizes exclusively to the plasma membrane and Yck2(SCIIS)p still to the cell's internal membrane system (Fig. 1 A). Thus, addition of the IIS tripeptide to Yck2p allows the Akr1p requirement to be bypassed.

What is the Yck2p lipid modification? Potentially, cysteines can accept either prenyl or palmitoyl modifications.

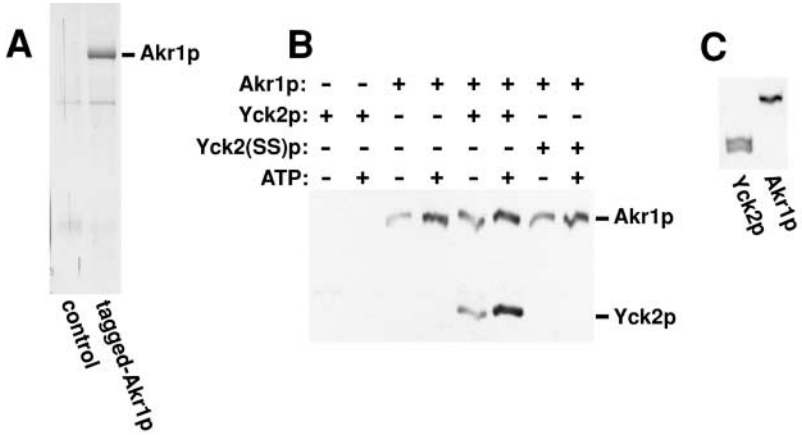

Figure 2. **Akr1p is a PTase.** (A) Purified Akr1p. Tri-tagged Akr1p was purified from detergent-treated yeast extracts with a sequence of three affinity steps. Purified protein, corresponding to an initial 2×10^9 cells, was subjected to SDS-PAGE and silver staining. As a control, extracts from isogenic cells expressing the untagged, wild-type Akr1p were mock purified and stained in parallel. (B) In vitro palmitoylation. Reactions contain [^{3}H]palmitoyl-CoA and, as indicated in the figure, 1 mM ATP, Yck2 substrate proteins purified from *E. coli*, and the tagged Akr1p purified from yeast. After a 60-min 30°C incubation, reactions were subjected to SDS-PAGE, fluorography, and autoradiography to assess protein labeling. The two labeled protein species were identified to be Akr1p and Yck2p. (C) Akr1p is palmitoylated in vivo. Wild-type cells transformed by either the *GAL1*-driven 6xHis/FLAG/HA-tagged Yck2p construct (Fig. 1) or by an analogous *GAL1–AKR1* construct with a COOH-terminal HA/FLAG/6xHis tag sequence were labeled with [^{3}H]palmitic acid and subjected to anti-FLAG IP and then SDS-PAGE, as for Fig. 1 B.

By analogy to Rab proteins, many of which have COOH-terminal Cys-Cys sequences, the Yck1p/Yck2p COOH-terminal cysteines were suggested to be prenylated, specifically geranylgeranylated (Vancura et al., 1994). Arguments against the likelihood of Yck1p/Yck2p prenylation have been discussed previously (Feng and Davis, 2000). Most notably, unlike the CaaX consensus, the COOH-terminal Cys-Cys sequence is not a sufficient prenylation signal (Khosravi-Far et al., 1992); the geranylgeranylation of this sequence in Rab proteins depends absolutely on the accessory protein REP in mammalian cells and Mrs6p in yeast, which recognizes the generic Rab tertiary structure and acts to present the Rab COOH-terminal dicysteine to the geranylgeranyl transferase for modification (Zhang and Casey, 1996). Given the Akr1p–Erf2p connection, Erf2p having been isolated for its participation in Ras2p palmitoylation (Bartels et al., 1999), we decided to first concentrate on the possibility of Yck2p palmitoylation. Cultures expressing wild-type or mutant Yck2 proteins were labeled with [^{3}H]palmitic acid and the Yck2 proteins were immune precipitated (Fig. 1 B). Wild-type Yck2p is indeed found to be labeled (Fig. 1 B). This labeling is abolished both by the Yck2p CC→SS cis mutation and by the *akr1Δ* trans mutation (Fig. 1 B). Thus, Yck2p is palmitoylated and Akr1p is required for this palmitoylation.

We have also examined the palmitate labeling of Yck2(CCIIS)p and Yck2(SCIIS)p. Consistent with Ras2p lipidation, we find that Yck2(CCIIS)p is palmitoylated and Yck2(SCIIS)p is not (Fig. 1 B). Furthermore, in line with Akr1p's dispensability for Yck2(CCIIS)p surface localization (Fig. 1 A), Akr1p, we find, also is not required for Yck2(CCIIS)p palmitoylation (Fig. 1 B). Thus, we conclude that Akr1p function is not required for all palmitoylation within the cell. Akr1p may be limited in its "specificity," being supplanted by other functionalities when the Ras2p COOH-terminal lipidation signal is used.

The thioester linkage of palmitoylation is chemically labile and can be cleaved by a number of relatively weak nucleophiles, including hydroxyl ions, thiols, and hydroxylamine. To test if the Yck2p labeling is consistent with palmitoylation, the stability of the Yck2p label to trans thiol displacement by β-mercaptoethanol (β-ME) was assessed. Consistent with susceptibilities reported for other palmitoylated proteins (Bizzozero, 1995), partial loss of the Yck2p tritium label is seen with the 0.3 M β-ME treatment and more extensive loss at 1 M and 2 M (Fig. 1 C).

Is Akr1p the Yck2p PTase? Testing this required developing a system for analyzing Yck2p palmitoylation in vitro. To serve as the in vitro substrate, Yck2p was overproduced and purified from *Escherichia coli*. The COOH-terminal dicysteine of the *E. coli*–produced Yck2p should be unmodified and thus available for palmitoylation (protein thio-acylation has not been described in bacteria). The source of the palmitoyl label was [^{3}H]palmitoyl-CoA, the presumed donor of the palmitoyl moiety in vivo (Berthiaume and Resh, 1995; Dunphy et al., 1996). Finally, as the potential PTase to be tested, we have affinity-purified Akr1p, COOH-terminally tagged with the tripartite 3xHA/FLAG/6xHis sequence, from detergent-extracted yeast membranes. FLAG and 6xHis sequences were used for the affinity bindings, whereas the HA sequence was used for following the purification by Western blotting. We opted against overexpressing the tagged Akr1p (hoping to preserve native stoichiometries within potential multisubunit complexes). Buffers were supplemented with exogenous lipids (from bovine liver) to avoid the complete delipidating extraction of Akr1p into detergent micelles, a concern given the large volumes of detergent-containing buffer used for washing the Akr1p-bound resins. Although both the Ni-agarose and the anti–FLAG-agarose proved to be efficient binders of the tagged Akr1p, either step alone resulted in only a partial purification of tagged Akr1p. The best purification was achieved by coupling three affinity steps together in sequence: anti–FLAG-agarose, and then Ni-agarose, and finally, again, anti–FLAG-agarose. The result is Akr1p purified to near homogeneity, presenting as one major species on a silver-stained SDS–polyacrylamide gel (Fig. 2 A). Overdevelopment of the silver stain reveals a light background comprised of other proteins (unpublished data), however, these background proteins are all also found to be equivalently present in the mock-purified samples derived from the control yeast extracts lacking the tagged Akr1p construct; thus, these background proteins are fortuitous

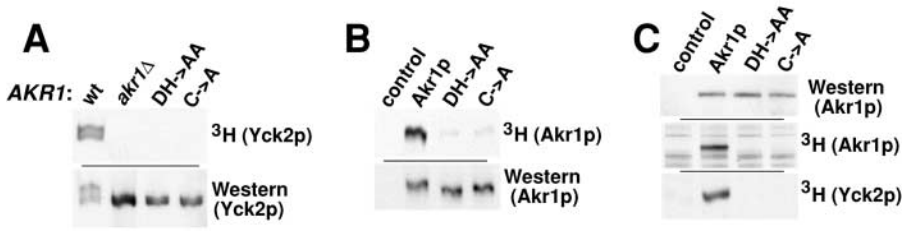

Figure 3. **Akr1(D543A,H544A)p and Akr1(C546A)p (DH→AA and C→A, respectively) are unable to promote palmitoylation.** (A) Mutant *akr1* alleles fail to support the in vivo palmitoylation of Yck2p. Strains with the *akr1* missense alleles replacing chromosomal *AKR1*, in addition to an isogenic *akr1Δ* and wild-type *AKR1*$^+$ strain, were transformed by the *GAL1–6xHis/FLAG/HA–YCK2* plasmid construct (Fig. 1 B). Cells were cultured, labeled with [^{3}H]palmitic acid, and processed for anti-FLAG IP (top). Yck2p recovery from the anti-FLAG IP was assessed by anti-HA Western blotting (bottom). (B) Mutant Akr1 proteins are not palmitoylated in vivo. *AKR1*$^+$ yeast cells transformed by plasmid constructs having either a *GAL1*-driven, HA/FLAG/6xHis-tagged, wild-type *AKR1* (Fig. 2 C) or the equivalent DH→AA or C→A mutant versions were cultured, labeled with [^{3}H]palmitic acid, and then subjected to anti-FLAG IP (top). Akr1p recovery after anti-FLAG IP was assessed by anti-HA Western blotting (bottom). (C) Mutant Akr1 proteins do not promote palmitoylation in vitro. Wild-type and DH→AA and C→A mutant Akr1 proteins having COOH-terminal 3xHA/6xHis tag sequences were partially purified from yeast via Ni-agarose. Recoveries from Ni-agarose of the wild-type and mutant Akr1 proteins were compared by anti-HA Western analysis (top). One portion of the labeled proteins from each in vitro palmitoylation reaction using the different Akr1 proteins was analyzed directly by SDS-PAGE to assess Akr1p autopalmitoylation (middle), and a second portion was subjected, before SDS-PAGE, first to anti-FLAG IP to isolate the FLAG-tagged Yck2 substrate protein (bottom).

contaminants, not copurifying subunits. Similarly, no copurifying proteins were seen even on gels that allow visualization of very low molecular weight proteins, down to the 5–10 kD range (unpublished data).

The three reaction components, the Yck2p substrate, [^{3}H]palmitoyl-CoA, and Akr1p, were coincubated and the palmitoyl label was found to be transferred to Yck2p (Fig. 2 B). This labeling was fully Akr1p dependent and required the Yck2p COOH-terminal dicysteine; the CC→SS mutant Yck2p substrate was not labeled. Given the high purity of the Akr1p preparation used (Fig. 2 A), we conclude that Akr1p is a PTase. Akr1p by itself is apparently sufficient for activity. We find no evidence for a multisubunit complex. Indeed, during the course of its three-step affinity purification, PTase activity assayed from both the crude initial fractions and from the final purified preparation remains strictly proportionate to the level of Akr1p that is present (unpublished data); thus, key activity-enhancing or inhibitory subunits are not being removed during purification.

Two outcomes of the in vitro palmitoylation reaction were unexpected. First, in addition to the labeling of Yck2p, Akr1p also is found to be strongly labeled. Second, an enhancing effect of ATP is seen reproducibly on the in vitro palmitoylation of both Yck2p and Akr1p. With regard to the Akr1p autopalmitoylation, one concern, especially given the high purity of the Akr1p used, is that the labeling could be the result of a direct, chemical reaction of [^{3}H]palmitoyl-CoA with the purified protein (Quesnel and Silvius, 1994; Duncan and Gilman, 1996). Arguing against this, Akr1p labeling remains strong and specific even in reactions that use extremely crude Akr1p preparations, having Akr1p as $<$1% of the partially purified sample (unpublished data). Furthermore, experiments below show the Akr1p autopalmitoylation to be abolished by mutations in Akr1p that abolish activity (Fig. 3). To follow up on the in vitro Akr1p palmitoylation, we have also tested for Akr1p palmitoylation in vivo. We find that Akr1p is indeed efficiently labeled by [^{3}H]palmitic acid in vivo (Fig. 2 C). The Akr1p autopalmitoylation, we believe, may provide a clue regarding the underlying enzymatic mechanism; perhaps palmitoylation proceeds via a two-step mechanism, with the palmitoyl moiety being transferred first from palmitoyl-CoA to the PTase and then, in a second step, to the final substrate protein.

The effect of ATP on the in vitro palmitoylation is surprising in several respects. First, previous analyses of crude mammalian PTase activities reported no ATP requirement (Berthiaume and Resh, 1995; Dunphy et al., 1996). Second, the Akr1p sequence lacks a discernible nucleotide binding or hydrolysis domain. Third, the need for ATP is unclear; palmitoyl-CoA is a "high energy" reactant, capable under certain experimental conditions of direct, uncatalyzed addition of the palmitoyl moiety to substrate proteins (Quesnel and Silvius, 1994; Duncan and Gilman, 1996). Clearly, a deeper look at the ATP role in Akr1p-mediated palmitoylation is warranted.

The conserved DHHC-CRD sequence provided a first connection between Erf2p (Ras2p palmitoylation) and Akr1p (Yck2p palmitoylation); otherwise, Akr1p and Erf2p are nonhomologous. To explore the possibility that the DHHC-CRD sequence might constitute a core element of a PTase activity domain, two missense mutations were introduced into the Akr1p DHHC-CRD, specifically into the core DHHC tetrapeptide, which, in Akr1p, is diverged to Asp-His-Tyr-Cys (DHYC). One mutant changes the Asp-His to Ala-Ala (Akr1[DH→AA]p), the other changes the Cys to Ala (Akr1[C→A]p). Both mutants fail to support the in vivo labeling of Yck2p by [^{3}H]palmitic acid (Fig. 3 A). Furthermore, the two Akr1p mutants are themselves not palmitoylated either in vivo (Fig. 3 B) or in vitro (Fig. 3 C, middle). Finally, neither Akr1p mutant supported detectable in vitro palmitoylation of Yck2p (Fig. 3 C, bottom). Thus, the core DHYC tetrapeptide is required for both the auto- and transpalmitoylation activity of Akr1p, suggesting that the DHHC-CRD may indeed be a signature PTase feature.

Finally, we report a preliminary analysis of Akr1p localization. Akr1p is found to localize intracellularly to discrete cytoplasmic puncta (Fig. 4), a presentation grossly similar to that of yeast Golgi apparatus or early endosome. Essentially the same punctate Akr1p presentation is found in the endocytosis-defective *end3Δ* or *end4-1* mutant cell contexts, indicating that the endocytic route is not required for Akr1p delivery to this intracellular locale. Definitive identification

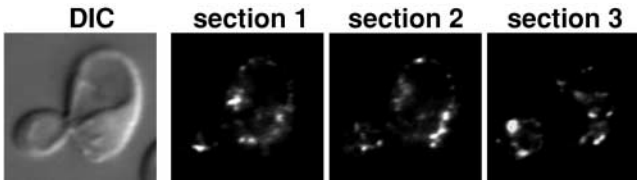

Figure 4. **Indirect immunofluorescent localization of Akr1p.** Akr1p COOH-terminally tagged with a 3xHA sequence and under control of native *AKR1* upstream regulatory sequences was introduced into wild-type *AKR1*$^+$ yeast cells on a single-copy vector plasmid (pRS316 based). Three deconvolved optical sections of the same cell are shown together with the cell visualized by DIC.

of this intracellular organelle will await Akr1p colocalization with appropriate organelle-specific marker proteins.

The Yck2p COOH-terminal dicysteine is required for its palmitoylation, both in vivo and in vitro, and we believe that it is the acceptor site for two added palmitoyl moieties. Two lipid moieties generally are required for stable protein–bilayer interactions (Dunphy and Linder, 1998; Resh, 1999). For many palmitoylated proteins, palmitoylation occurs secondarily to some primary lipidation event, either prenylation or myristoylation; the primary lipid modification provides the hydrophobicity for the initial interaction with cellular membranes. For newly synthesized Ras2p, addition of a farnesyl moiety to the COOH-terminal Cys within the COOH-terminal pentapeptide CCIIS targets the Ras protein to the ER; palmitoylation of the adjacent Cys occurs subsequently (Powers et al., 1986; Deschenes and Broach, 1987; Fujiyama et al., 1987). Several facts argue against a similar dual lipidation scenario for Yck2p. First, signals for prenylation and myristoylation are well defined and Yck2p lacks any such signal. Second, in the absence of the Yck2p PTase activity, i.e., in *akr1Δ* cells, Yck2p behaves like a fully unmodified protein, localizing like the CC→SS Yck2 mutant, diffusely through the cytoplasm with no hint of membrane interaction (Fig. 1 A). This contrasts with the clear membrane localization seen for Yck2(SCIIS)p, which is apparently modified by a single farnesyl moiety (Fig. 1 A). Thus prenylation, we feel, is unlikely. Nonetheless, it may well be that other fatty acid moieties, in addition to or instead of palmitic acid, are added to Yck2p in thioester linkage. Indeed, medium chain fatty acids in addition to the 16-carbon palmitoyl moiety, including either the 14-carbon myristate or the 18-carbon stearate, can be found thioesterified to some cysteinyl acceptors in place of, or sometimes in addition to, the typical palmitoyl moiety (Resh, 1999). In fact, it has been suggested that this lipid modification is more appropriately termed "protein S-acylation" rather than the usual, but too specific, "protein palmitoylation." Which fatty acids get esterified to substrate could reflect either the specificity of the modifying PTase or the cellular availability of the different acyl-CoAs.

Finally, our results imply that multiple PTase specificities control palmitoylation within the cell. Indeed, the existence of at least one additional PTase is inferred from the unimpaired palmitoylation of Yck2(CCIIS)p in *akr1Δ* cells (Fig. 1 B). Consistent with this, we also find Ras2p palmitoylation to be unimpaired in *akr1Δ* cells (unpublished data). Furthermore, the *erf2Δ* and *shr5Δ* mutations, which impair palmitoylation of Ras2p (Bartels et al., 1999), have no effect on Yck2p palmitoylation (unpublished data). Differing from the discrete motifs that specify myristoylation and prenylation, palmitoylated cysteines are found in quite a wide variety of sequence contexts (Dunphy and Linder, 1998; Resh, 1999). Accommodating such substrate diversity may require multiple PTase specificities. Over 120 DHHC-CRD–containing proteins have been identified through the genomic sequencing in *Saccharomyces cerevisiae*, *Drosophila melanogaster*, *Caenorhabditis elegans*, *Mus musculum*, *Homo sapiens*, and *Arabidopsis thaliana*, with 23 examples from *H. sapiens* and 7 from *S. cerevisiae*. All are predicted to be polytopic membrane proteins with the DHHC-CRD locating between membrane-spanning segments. Erf2p and Akr1p, the only two members of this family for which there is any functional information, both are now linked to protein palmitoylation. Is the DHHC-CRD protein family a family of palmitoyl transferases?

Materials and methods

Yeast strains

The yeast strains used in this work are isogenic with LRB759 (*MATα ura3–52 leu2 his3*; Panek et al., 1997). In vivo analyses used both LRB759 and the isogenic NDY1405 as the host *AKR1*$^+$ and *akr1Δ* stains. Akr1p purification was from the *akr1Δ pep4Δ* strain NDY1547. The *pep4Δ* mutation blocks activation of vacuolar proteases, eliminating a potential source of contaminating protease activity.

Tagged AKR1 constructs

The tagged Akr1p constructs used herein have combinations of various epitope and/or affinity tags fused to the Akr1p COOH terminus. None of the tags were found to impact Akr1p function; all tagged alleles fully complement *akr1Δ*, restoring both growth at 37°C (*akr1Δ* cells have reduced viability at 37°C) and a wild-type cell morphology (*akr1Δ* cells are large and multinucleate with hyperelongated buds) (unpublished data). Tagged constructs were introduced into yeast on the single-copy, centromeric vector pRS316 (Sikorski and Hieter, 1989), with expression controlled either by the native *AKR1* upstream regulatory sequences or by the inducible *GAL1* promoter, as indicated in the figure legends.

Indirect immunofluorescence microscopy

Cells, cultured as described in the figure legends, were fixed and then treated with primary and secondary antibodies (Chen and Davis, 2002). Z-series of digital images of the fluorescent cells were collected at 0.25-μm intervals and then deconvolved as described previously (Chen and Davis, 2002).

In vivo palmitate labeling

To inhibit endogenous fatty acid synthesis, cerulenin (Sigma-Aldrich) was added to 3 μg/ml 1 h before the initiation of the 2-h galactose (2%) induction period. 1 h into the galactose induction period, 1 mCi [(9,10)^{3}H]palmitic acid (60 Ci/mmol; New England Nuclear) was added to 2×10^7 cells in a 10-ml culture volume. After a 1-h labeling period, cells were collected by centrifugation and disrupted by glass bead lysis in 0.2 ml cold TBS (100 mM NaCl, 50 mM Tris, pH 8.0) containing 2xPI (1xPI: 1 mM PMSF and 0.25 μg/ml each of antipain, leupeptin, pepstatin, and chymostatin). Lysate proteins were precipitated (Wessel and Flugge, 1984), resuspended in 50 μl of 8 M urea, 2% SDS, 100 mM NaCl, 50 mM Tris, pH 8.0, and then incubated for 10 min at 37°C. The labeled proteins were then diluted into 1 ml of IPB (50 mM Tris/Cl, pH 8.0, 100 mM NaCl, 2 mM EDTA, 0.1% Triton X-100) with 1xPI, and immunoprecipitated with 20 μl of anti-FLAG M2 mAb-conjugated agarose (Sigma-Aldrich) for 2 h at 4°C. After four 1-ml washes in IPB containing 0.1% SDS, bound proteins were eluted at 37°C for 10 min into 20 μl of 8 M urea, 5% SDS, 40 mM Tris/Cl, pH 6.8.

Yck2 substrate proteins

Yck2p NH_2-terminally tagged with a 6xHis/FLAG/HA sequence was overproduced in *E. coli* using the pET expression system (Novagen) and isolated by Ni-NTA-agarose (QIAGEN) affinity chromatography from clarified cell lysates. The *E. coli*–produced Yck2p was found to be heavily phosphorylated (unpublished data); in fact, more heavily phosphorylated than Yck2p

isolated from the wild-type yeast plasma membrane (Fig. 1 B, bottom). This phosphorylation was abolished with introduction of the kinase-inactivating D218A mutation into the conserved DFG sequence of Yck2p, indicating it to result from the overproduced kinase activity (i.e., Yck2p autophosphorylation). Because of concerns that the unnaturally heavy phosphorylation might interfere with our analysis in vitro, we opted to exclusively use kinase-inactivated D218A versions of Yck2p as in vitro substrates. An HA-tagged Yck2(D218A)p was found to localize in yeast like the wild-type kinase, exclusively to the cell surface (unpublished data).

Affinity purification of Akr1p

A COOH-terminally 3xHA/FLAG/6xHis-tagged Akr1p, under the control of native *AKR1* upstream regulatory sequences, was purified from *akr1Δ pep4Δ* yeast cells via a three-step affinity purification scheme. For the starting lysate, 2×10^{10} cells were harvested from log-phase cultures, resuspended in 5 ml cold TBS containing 1 mM DTT and 2xPI, and then frozen as droplets in liquid nitrogen. The frozen cell droplets were then subjected to 10 min of grinding with mortar and pestle under liquid nitrogen. The lysate, which remained frozen throughout the grinding process, was thawed on ice and an additional 2 ml of TBS containing 1 mM DTT and 5xPI was added. Membranes were then solubilized with gentle mixing for 30 min at 4°C in the presence of 1% Triton X-100 (Anatrace). The lysate was divided into 10 1-ml aliquots, clarified by two sequential centrifuge spins (1 min, 15,000 *g*), and then absorbed to 10 30-μl portions of the anti-FLAG M2 mAb-agarose for 2 h at 4°C. The bound resin was washed with four 1-ml aliquots of cold SL (50 mM Hepes, 150 mM NaCl, 140 mM sucrose, 1 mM DTT, 0.5 mg/ml bovine liver lipids [Avanti Polar Lipids], pH 8.0) containing 1% Triton X-100. Elution used a 30-min 0°C incubation with 100 μl of SL containing 0.3% Triton X-100 and 300 μg/ml FLAG peptide (Sigma-Aldrich). For the second affinity step, the 10 elution fractions were combined and absorbed to 200 μl Ni-NTA-agarose for 1 h at 4°C. Washes were as for the anti-FLAG step, except that the SL contained 0.3% Triton X-100. Elution from Ni-agarose used a 5-min 0°C incubation in 1 ml of SL containing 0.3% Triton X-100 and 0.25 M imidazole. For the third and final affinity step, the Ni-agarose eluant was absorbed to 100 μl of anti-FLAG agarose for 2 h at 4°C. Washes were as described above for the Ni-agarose step, except that the SL was buffered to pH 7.5 rather than to pH 8.0. The final elution was into 250 μl of pH 7.5 SL containing 0.1% Triton X-100 and 300 μg/ml FLAG peptide.

To assess the PTase activity of the mutant Akr1 proteins, the Akr1 proteins, COOH-terminally tagged with a 3xHA/6xHis sequence and under control of native *AKR1* upstream regulatory sequences, were partially purified via a single Ni-agarose step protocol. Lysates were prepared as described above for the three-step purification except that the volumes and starting cell number were scaled down 10-fold. The detergent-treated lysates were bound to 200 μl of Ni-agarose for 1 h at 4°C, washed with SL containing 1% Triton X-100, and then eluted with a 5-min 0°C incubation in 500 μl SL containing 0.1% Triton X-100 and 0.25 M imidazole.

In vitro palmitoylation

The 50 μl in vitro palmitoylation reaction contained 5 μCi of [^{3}H]palmitoyl-CoA (5 μM final), Yck2 substrate protein at 0.33 μM, 1 mM ATP, 50 mM MES, pH 6.4, 0.2 mg/ml bovine liver lipids, and, finally, 10 μl of the affinity-purified Akr1p. After 1 h at 30°C, reaction proteins were precipitated (Wessel and Flugge, 1984) and subjected to SDS-PAGE. [^{3}H]palmitoyl-CoA was synthesized enzymatically from [(9,10)^{3}H]palmitic acid (60 Ci/mmol; New England Nuclear), CoA, and ATP using acyl-CoA synthase (Sigma-Aldrich) and purified as previously described (Dunphy et al., 1996). The synthesis was highly efficient, with >95% conversion of palmitic acid to palmitoyl-CoA. The final specific activity of the [^{3}H]palmitoyl-CoA was estimated to be 60 Ci/mmol.

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Note added in proof. New work from Lobo et al. (Lobo, S., W.K. Greentree, M.E. Linder, and R.J. Deschenes. 2002. *J. Biol. Chem.* 10.1074/jbc.M206573200) demonstrates that the yeast DHHC-CRD protein Erf2p, acting together with Erf4p (Shr5p), is a palmitoyl transferase with specificity for a farnesylated Ras2 substrate protein.

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