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

Amy F. Roth,¹ Ying Feng,² Linyi Chen,² and Nicholas G. Davis¹,²

¹Department of Surgery and ²Department of Pharmacology, Wayne State University School of Medicine, Detroit, MI 48201

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 autopalmitoylation 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 C2 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

*Abbreviations used in this paper: β-ME, β-mercaptoethanol; CoA, coenzyme A; CRD, cysteine-rich domain; PTase, palmitoyl transferase.
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 NH2-terminal hydrophilic domain, and a DHHC-CRD sequence mapping between transmembrane domains four and five. Homology between Akr1p and Erflp 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<sup>+</sup>) 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 Ras2p(SCIIS)p localizes primarily to intracellular membranes (Bartels et al., 1999). Thus, as with Ras2p, the two COOH-terminal cysteines of Yck2p(CCIIS)p likely are dually lipidated.

As reported previously (Feng and Davis, 2000), Yck2p is mislocalized in akr1Δ cells, localizing like the Yck2p(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 Yck2p(CCIIS)p or Yck2p(SCIIS)p; Yck2p(CCIIS)p still localizes exclusively to the plasma membrane and Yck2p(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.
By analogy to Rab proteins, many of which have COOH-terminal Cys-Cys sequences, the Yck1p/Yck2p COOH-terminal cysteines were suggested to be prenylized, 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 Mr6p 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 \( ^{3}H \)palmitate and the \( ^{3}H \)palmitoyl label was identified and thus available for palmitoylation (protein thio-acylation using the 0.3 M \( ^{3}H \)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.

Figure 2. Akr1p is a PTase. (A) Purified Akr1p, Tri-tagged Akr1p was purifiried from detergent-treated yeast extracts with a sequence of three affinity steps. Purified protein, corresponding to an initial 2 \( \times 10^6 \) 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.

Wild-type cells transformed by either the 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.
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 kDa range (unpublished data).

The three reaction components, the Yck2p substrate, [3H]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 [3H]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 the result of a direct, chemical reaction of the high purity of the Akr1p used, is that the labeling could be the result of a direct, chemical reaction of [3H]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 the result of a direct, chemical reaction of [3H]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 the result of a direct, chemical reaction of [3H]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 the result of a direct, chemical reaction of [3H]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 the result of a direct, chemical reaction of [3H]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 the result of a direct, chemical reaction of [3H]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 the result of a direct, chemical reaction of [3H]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 the result of a direct, chemical reaction of [3H]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 the result of a direct, chemical reaction of [3H]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 the result of a direct, chemical reaction of [3H]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 the result of a direct, chemical reaction of [3H]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 the result of a direct, chemical reaction of [3H]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 the result of a direct, chemical reaction of [3H]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 the result of a direct, chemical reaction of [3H]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 the result of a direct, chemical reaction of [3H]palmitoyl-CoA with the purified protein (Quesnel and Silvius, 1994; Duncan and Gilman, 1996).

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 (Akr1p[DH→AA])p, the other changes the Cys to Ala (Akr1p[C→A])p. Both mutants fail to support the in vivo labeling of Yck2p by [3H]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...
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 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 ack1Δ 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(CCIIS)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 ack1Δ cells (Fig. 1 B). Consistent with this, we also find Ras2p palmitoylation to be unimpaired in ack1Δ 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 musculus, 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 (MATa ura3–52 leu2 his3; Panek et al., 1997). In vivo analyses used both LRB759 and the isogenic NDY1405 as the host AKR1Δ and akr1Δ strains. Akr1p purification was from the ack1Δ 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 ack1Δ, restoring both growth at 37°C (ark1Δ 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 deconvoluted as described previously (Chen and Davis, 2002).

In vivo palmitoylation 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) 3H]palmitic acid (60 Ci/mmol; New England Nuclear) was added to 2×107 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 2×PI (1×PI: 1 mM PMSE and 0.25 μg/ml each of antipain, leupeptin, pepstatin, and chymostatin). Lysates were precipitated (Wessel and Flugge, 1989), 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 1×PI, 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 NH2-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 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 3×HA/FLAG/b-His-tagged Akr1p, under the control of native AKR1 upstream regulatory sequences, was purified from Yck1Δ pep4Δ yeast cells via a three-step affinity purification scheme. For the starting lysate, 2 x 10^9 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 centrifugal spins (1 min, 15,000 g), and then absorbed to 100 μl Ni-agarose for 1 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 MgCl2, 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 3×HA/b-His 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 specific 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 [3H]palmitoyl-CoA (5 μM final), Yck2b 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. [3H]palmitoyl-CoA was synthesized enzymatically from (9,10)[3H]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 [3H]palmitoyl-CoA was estimated to be 60 Ci/mmol.

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