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 sub-domains, 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, Gox 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) (Duphny 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 (Berthuaume 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 C2H 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 Erp2p 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 lipid modification.

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 Yck2p(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. Yck2p(CCIIS)p, a second Yck2p mutant lacking the Ras2p palmitoyl-accepting cysteine, was also constructed. In wild-type (**AKR1**Δ) cells, we find that Yck2p(CCIIS)p localizes like wild-type Yck2p, exclusively to the plasma membrane (Fig. 1 A). Yck2p(SCIIS)p, which we presume is farnesylated (it retains the CaaX consensus), also localizes to the plasma membrane (Fig. 1 A). Yck2p(SCIIS)p, which we presume is farnesylated (it retains the CaaX consensus), also localizes to the plasma membrane (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 exclusively 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 exclusively to intracellular membranes (Fig. 1 A).

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 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 Mrsp6p 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 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 [3H]palmitic acid and subjected to anti-FLAG IP and then SDS-PAGE, as for Fig. 1 B.

We have also examined the palmitate labeling of Yck2(CCIIS)p and Yck2(SCIIS)p. Consistent with Ras2p lipiddation, 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 Yck2p palmitoylation (Fig. 1 A), Akr1p, we find, also is not required for Yck2p(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 [3H]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 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 × 10^7 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 [3H]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–AKR1 construct with a HA-tagged Yck2p construct (Fig. 1 B) or by an analogous GAL1–AKR1 construct with a COOH-terminal HA/FLAG/6xHis tag sequence were labeled with [3H]palmitic acid and subjected to anti-FLAG IP and then SDS-PAGE, as for Fig. 1 B.
contaminants, not copurifying subunits. Similarly, no co-

purifying proteins were seen even on gels that allow vis-

ualization 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 purifica-

tion, PTase activity assayed from both the crude initial frac-

tions and from the final purified preparation remains strictly

nonhomologous. To explore the possibility that the

DHHC-CRD may indeed be a signature PTase feature.

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 en-

hancing effect of ATP is seen reproducibly on the in vitro

palmitoylation of Yck2p (Fig. 3 C). This indicates that 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 addi-
tion of the palmitoyl moiety to substrate proteins (Quesnel

and Silvius, 1994; Duncan and Gilman, 1996). Clearly, a

deep look at the ATP role in Akr1p-mediated palmito-
ylation 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 intro-
duced 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]). Both mutants fail to support the

in vivo labeling of Yck2p by \[^{3}H\]palmitic acid (Fig. 3 A). A

furthermore, the two Akr1p mutants are themselves not

dependent 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 localiza-
tion. Akr1p is found to localize intracellularly to discrete cy-

toplastic 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 endo-
cytosis-defective end4Δ or end4-1 mutant cell contexts,

indicating that the endocytic route is not required for Akr1p

delivery to this intracellular locale. Definitive identification

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 sur-

prising 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

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The Yck2p COOH-terminal di-cysteine 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–membrane 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 aer1Δ 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 cysteiny1 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 aer1Δ cells (Fig. 1 B). Consistent with this, we also find Ras2p palmitoylation to be unimpaired in aer1Δ cells (unpublished data). Furthermore, the erf2Δ and shf5Δ 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 NDY140S as the host AER1+ and aer1Δ stains. Akr1p purification was from the aer1Δ 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 aer1Δ, restoring both growth at 37°C (aer1Δ cells have reduced viability at 37°C) and a wild-type cell morphology (aer1Δ 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 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)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 μg/ml each of antipain, leupeptin, pepstatin, and chymostatin. Lysate proteins were precipitated (Wessel and Flugge, 1987), resuspended in 0.5 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 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.

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 aer1+ 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.
isolated from the wild-type yeast plasma membrane (Fig. 1B, 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 kinasedead D218A versions of Yck2p as in vitro substrates. An HA-tagged Yck2/D218Ap 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 Δakr1Δ pep4Δ yeast cells via a three-step affinity purification scheme. For the starting lystate, 2 × 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 centrifugation spins (1 min, 15,000 g), and then absorbed to 100 μl of anti-FLAG M2 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 and 300 μg/ml FLAG peptide (Sigma-Aldrich). For the second affinity step, the 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 was used a 5-min 0°C incubation in 1 ml of SL containing 0.3% Triton X-100 and 300 μg/ml FLAG peptide (Sigma-Aldrich). For the 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 protein, COOH-termially 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 sample 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), 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. [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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