Transmembrane Topology of the Protein Palmitoyl Transferase Akr1*

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The two recently identified protein acyl transferases (PATs), Akr1p and Erf2p/Erf4p, point toward the DHHC protein family as a likely PAT family. The DHHC protein family, defined by the novel, zinc finger-like DHHC cysteine-rich domain (DHHC-CRD), is a diverse collection of polytopic membrane proteins extending through all eukaryotes. To define the PAT domains that are oriented to the cytoplasm and are thus available to effect the cytoplasmically limited palmitoyl modification, we have determined the transmembrane topology of the yeast PAT Akr1p. Portions of the yeast protein invertase (Suc2p) were inserted in-frame at 10 different hydrophilic sites within the Akr1 polypeptide. Three of the Akr1-Suc2-Akr1 insertion proteins were found to be extensively glycosylated, indicating that the invertase segment inserted at these Akr1p sites is luminaly oriented. The remaining seven insertion proteins were not glycosylated, consistent with a cytoplasmic orientation for these sites. The results support a model in which the Akr1 polypeptide crosses the bilayer six times with the bulk of its hydrophilic domains disposed toward the cytoplasm. Cytoplasmic domains include both the relatively large, ankyrin repeat-containing N-terminal domain and the DHHC-CRD, which maps to a cytosolic loop segment. Functionality of the different Akr1-Suc2-Akr1 proteins also was examined. Insertions at only 4 of the 10 sites were found to disrupt Akr1p function. Interestingly, these four sites all map cytoplasmically, suggesting key roles for these cytoplasmic domains in Akr1 PAT function. Finally, extrapolating from the Akr1p topology, topology models are proposed for other DHHC protein family members.

Many proteins, particularly many signaling proteins, rely on covalent lipid modifications, either prenylation, myristoylation, or palmitoylation, for membrane attachment. Being limited to the cytoplasmic compartment, these lipid modifications serve to tether proteins to the cytoplasmic surface of cellular membranes. The enzymes that mediate myristoylation and prenylation, i.e. the myristoyl and prenyl transferases, are soluble cytoplasmic proteins. In contrast, the palmitoyl transferases, i.e. the protein acyl transferases (PATs),¹ the first examples having been identified only recently, are predicted to be polytopic membrane proteins (1, 2). To gain insight into the PAT domains that are available cytoplasmically to mediate this lipid modification, we have determined the transmembrane topology of the prototypical PAT, the yeast protein Akr1p.

The first PATs were identified through recent work in the yeast Saccharomyces cerevisiae. Akr1p was identified as the PAT for the two plasma membrane-localized, type I casein kinases, Yck1p and Yck2p, whereas Erf2p and Erf4p were found to collaborate for the palmitoylation of the yeast Ras proteins, Ras1p and Ras2p (1, 2). The two yeast PATs are substrate-specific. Although Ras2p is mislocalized from the plasma membrane in erf2Δ or in erf4Δ cells, the plasma membrane localization of Yck2p remains unperturbed. Likewise, in akr1Δ cells, Yck1p and Yck2p mislocalize, whereas Ras2p plasma membrane localization remains unaffected (1). Akr1p localizes to the Golgi (1, 3), whereas Erf2p and Erf4p localize to the endoplasmic reticulum (4, 5). Palmitoylation of Yck1p and Yck2p is fully dependent on Akr1p in vivo, being abolished in an akr1Δ cell context. Furthermore, when purified to apparent homogeneity, Akr1p alone promotes the in vitro transfer of the palmitoyl moiety from palmitoyl-CoA to the Yck2 substrate protein, indicating that no accessory subunits are required for Akr1 PAT activity (1). In contrast, Erf2p and Erf4p appear to collaborate for Ras2p palmitoylation, both in vivo and in vitro (2, 4), suggesting that these two proteins may be co-subunits of the Ras PAT. As the 86-kDa Akr1p is notably larger than either the 41-kDa Erf2p or the 27-kDa Erf4p, Akr1p may represent an evolutionary melding of the two functions into a single polypeptide.

Although the two PATs do not share extensive sequence homology, Akr1p and Erf2p are both predicted to be polytopic membrane proteins, and both contain the 51-residue-long novel zinc finger-like sequence, the DHHC cysteine-rich domain (DHHC-CRD). The DHHC-CRD sequence defines a diverse protein family extending through all eukaryotes; yeast has seven DHHC proteins, whereas 23 have been identified from the human genome. Aside from the DHHC-CRD, DHHC proteins share little sequence homology. Nonetheless, all are predicted to be polytopic membrane proteins, and all have the DHHC-CRD sequence analogously positioned between predicted transmembrane domains (TMDs). Beyond the two PATs, Akr1p and Erf2p, little is known regarding the biochemical function of other DHHC family members. An intriguing possibility is that these proteins may all be PATs. As mutation of conserved DHHC-CRD residues abolishes PAT activity for both Akr1p and Erf2p (1, 2), the DHHC-CRD sequence, the single element preserved throughout this family, could represent the conserved elements of the PAT active site. The substantial diversity embodied within this family perhaps accommodates the diverse range of substrate proteins that are known to be palmitoylated (6–8).

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¹ The abbreviations used are: PAT, protein acyl transferase; CRD, cysteine-rich domain; TMD, transmembrane domain; HA, hemagglutinin; HRP, horseradish peroxidase; mAb, monoclonal antibody; GST, glutathione S-transferase.
Several mammalian DHHC proteins have been identified in two-hybrid searches. GODZ was identified by its interaction with the glutamate receptor α1 subunit (9); HIP14, the human orthologue of Akr1p, was identified through its interaction with huntingtin, the disease protein of Huntington’s disease (10); and Aph2 was identified through its interaction with c-Abl tyrosine kinase (11). GODZ and HIP14 have both been localized to the Golgi, whereas Aph2 has been localized to the endoplasmic reticulum (9–12). Consistent with a general DHHC protein role in protein palmitoylation, a recent study of the invertase-encoding gene (13). The vector backbone for pND1436 is the yeast, S. cerevisiae, Akr1p C-terminally tagged by three iterations of the HA epitope. Through this approach, we identified a stretch of six ankyrin repeats that localize to its N-terminal hydrophilic domain. The two known Akr1p subdomains, the type I casein kinases Yck1p and Yck2p, presumably, are presented to Akr1p from the cytoplasm. When Yck palmitoylation is blocked, either through cis-mutation of their C-terminal, palmitoyl-accepting Cys-Cys dipeptides or through trans-mutation of the Akr1 PAT (i.e. akr1Δ), the kinases, rather than tethering to the plasma membrane, instead are left to distribute diffusely throughout the cytoplasm. To identify the Akr1p domains potentially available for substrate interaction and catalysis, we have determined, with the analysis below, a transmembrane topology for Akr1p.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains**

The yeast strains used in this work (Table I) are isogenic to LRB759 (MATα ura3–52 leu2 his3) (13) except at the Akr1 locus. The new strains were constructed by the previously described two-step gene replacement strategy (14).

**Plasmids**

**AKR1-SUC2-AKR1 Constructs**—The starting point for the AKR1-SUC2-AKR1 constructs was a GAL1p-URA1 (3×HA/FLAG/His) plasmid pND1436, which expresses from upstream GAL1 regulatory sequences, Akr1p C-terminally tagged by three iterations of the HA epitope, a single FLAG epitope downstream, and then finally, at the C terminus, 6 histidine residues (1). The vector backbone for pND1436 is the yeast, single copy CEN/ARS/URA3 vector plasmid pRS316 (15). Insertion of the invertase-encoding SUC2 sequence into Akr1 utilizing a strategy of in vivo gap repair in which the GAL1p-URA1 (3×HA/FLAG/His) plasmid, linearized at the insertion site, was recircularized through in vivo homologous recombination with a chimeric DNA fragment consisting of the SUC2 sequence, flanked by 51 bp of upstream and downstream AKR1 homology. The first step in this process was to introduce, by oligonucleotide-directed mutagenesis (16), unique SmaI restriction sites at the desired insertion sites within the AKR1 coding sequence of pND1436. Ten sites, sites A–J (see Fig. 3A), were chosen, with the 6-bp CCGGGA sequence being inserted between codons Met471 and Val481 (site A), Cys482 and Arg526 (site B), Tyr545 and Ser546 (site C), His547 and Leu553 (site D), Arg574 and Met575 (site E), Thr581 and Phe582 (site F), Val583 and Arg584 (site G), Lys585 and Asn586 (site H), and Lys586 and Ser587 (site I). Insertion site J is located just C-terminally of the 3×HA/FLAG/His tri-tag sequence. The AKR1-SUC2-AKR1 chimeric DNA fragments for gap repair of the SmaI-linearized plasmids were generated by PCR. The upstream PCR primer consisted of the 51 bp of the AKR1 sequence immediately upstream of the insertion point followed at the 3′ end by 19 bp of the SUC2 sequence that serves as the primer for the invertase segment. Likewise, the downstream primer had the 51 bp of the AKR1 sequence immediately downstream of the insertion point followed by the 19 bp of the SUC2 sequence, serving as the downstream primer of the invertase segment to be inserted. The PCR template was the SUC2-containing plasmid pH883 (17). The PCR product together with the appropriate SmaI-linearized plasmid were co-introduced into LRB759 yeast cells, and Ura+ transformants were selected. Plasmids isolated from the yeast transformants were amplified in Escherichia coli, and the fidelity of the gap repair was assessed by restriction analysis. Two series of AKR1-SUC2-AKR1 constructs were made. The first series inserted a 46-residue-long invertase segment, invertase residues Glu666–Tyr693, this invertase segment, like the one used by Glustrung and Ljungdahl (18), includes the three NXS/T sites at residues 97–99, 111–113, and 118–120, which are known to be used for N-linked glycosylation in secreted invertase (19). The second series utilized the entire mature form of secreted invertase from Thr22 to the C-terminal side of the invertase signal peptide cleavage site) to the C-terminal invertase residue Lys732. The yeast strains used in this work (Table I) are isogenic to LRB759 (MATα ura3–52 leu2 his3) (13) except at the Akr1p locus. The new strains were constructed by the previously described two-step gene replacement strategy (14).

**Analysis of N-linked Glycosylation**

Wild-type yeast cells (LRB759), transformed by plasmids for GAL1p-driven protein expression, were cultured overnight to log phase in YPR (1% yeast extract, 2% peptone, 2% raffinose). The addition of galactose to 2% initiated a 2-h period of protein expression, which was terminated by the subsequent addition of glucose to 3%. Following a final 20-min growth interval, 4 × 10⁶ cells were collected by centrifugation and resuspended in 0.1 ml of ice-cold lysis buffer (1 M sorbitol, 25 mM Tris/Cl, pH 6.8, 0.1 mM EDTA) containing 1% (w/v) Triton X-100 and an equal volume of 2-mercaptoethanol. The cell lysate was collected by centrifugation (10 min, 13,000 rpm, 4 °C) and the protein pellets were washed with 1 ml of acetone at room temperature, and then, following desiccation, 100 µl of sample buffer (5% SDS, 8 M urea, 40 mM Tris/Cl, pH 6.8, 0.1 M EDTA containing 1% β-mercaptoethanol) was added, and the pellets were dissolved with a 10-min, 37 °C incubation interspersed with vigorous vortexing. Six microtiter plates of each sample was then subjected to SDS-PAGE, Western blotting, and finally, development with the anti-HA mAb conjugated with horseradish peroxidase (HRP) (Roche Applied Science).
and purifies palmitoylated proteins from complex protein extracts. In contexts, our modification is a proteomic adaptation that biotinylates sequences exceeding the hydrophobicity threshold for service as TMDs.

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Biotin Replacement of Protein Acyl Modifications

A modification of the Drisdel and Green protocol (20), an in vitro exchange of protein acyl modifications for a biotinylated compound, was used to assess Yck2p palmitoylation in the different akr1 mutant cell contexts. Our modification is a proteomic adaptation that biotinylates and purifies palmitoylated proteins from complex protein extracts. In brief, total protein prepared from the different akr1 mutant cells expressing N-terminally His/FLAG/HA tri-tagged Yck2p from the GAL1 promoter were subjected to the Drisdel and Green acyl-biotin exchange reactions (20). First, free thiols were blocked with N-ethylmaleimide; second, thioesters (i.e. cysteinyl-acyl linkages) were cleaved with 1M iodoacetamide; and finally, thiols, newly exposed by the hydroxylamine treatment, were modified with a thiol-specific biotinylation reagent. Biotin-HPDP (Pierce), which disulfide-bonds to thiols, was substituted for the biotin-BMCC (Pierce) used by Drisdel and Green (20). Following biotinylation, epitope-tagged Yck2p was immune-precipitated with M2 anti-FLAG mAb-agarose and subjected to SDS-PAGE by Western analysis either with avidin-HRP (Pierce, Rockford, IL) to detect Yck2p biotinylation or with anti-HA-HRP to detect total recovery of the His/FLAG/HA-tagged Yck2 protein.

Akr1p Multimerization Test

NDY1405 cells were doubly transformed by the AKR1–3xHA/FLAG/His/CEN/ARS/LEU2 and the Akr1p multimerization test plasmids pRS516 under the control of the GAP1 promoter in the presence or absence of tunicamycin, a drug that blocks N-linked glycosylation (21). Indeed, tunicamycin treatment does substantially alter gel mobility for Ste2p, whereas those introduced into cytoplasmic domains should be unavailable to the luminaly localized glycosyl transferases and thus should remain unglycosylated. Prior to embarking on this analysis, we first examined the usage of the four NXS/T sites that are naturally present in the Akr1p sequence (Fig. 1). A C-terminally, epitope-tagged Akr1p was expressed from the GAP1 promoter in the presence or absence of tunicamycin, indicating that the four Akr1p NXS/T sites in the glycosylation sites being donated from segments of the secreted and heavily glycosylated yeast protein invertase (Ste2p). NXS/T sites introduced into the luminaly oriented domains of Akr1p would be expected to be glycosylated, whereas those introduced into cytoplasmic domains should be unavailable to the luminaly localized glycosyl transferases and thus should remain unglycosylated. To assess Akr1p topology, we made a series of Akr1p constructs that inserted a 46-residue-long segment of invertase, containing three NXS/T sites known to be utilized for glycosylation (19). At seven different sites known within the AKR1 open reading frame (Fig. 3A, positions A, C, D, E, F, G, and H). This strategy had been successfully employed by Gilstring and Ljundahl (18) to assess topology of the yeast general amino acid permease Gap1p. Gel mobility of the seven Akr1p insertion proteins was compared (Fig. 3B). Our expectation, based on the published Gap1p analysis (18), was to see up-shifted gel mobilities for those constructs for which the

RESULTS

Akr1p Hydropathy Analysis—Akr1p hydropathy analysis finds numerous sequences with hydrophobicity sufficient for service as TMDs (Fig. 1). In addition to the five strongly hydrophobic peaks, there are three sequences of marginal hydrophobicity, at residues 182–201, 554–568, and 661–680, that could potentially serve as TMDs, particularly within the context of a polytopic membrane protein. A further uncertainty relates to the broad bifurcated peak, corresponding to residues 324–361, a 38-residue stretch of uncharged residues with the somewhat polar sequence SHINP, centrally embedded at residues 342–346. Does the 324–361 correspond to one or two TMDs? Thus, sequence information alone predicts anywhere from 5–9 TMDs.

Analysis of Akr1p-Akr1p Complementation—As an experimental approach to the topology problem, we have opted to examine the usage of Anf-X/Ser/Thr (NXS/T) N-linked glycosylation sites introduced into the different Akr1p hydrophilic domains, the glycosylation sites being donated from segments of the secreted and heavily glycosylated yeast protein invertase (Ste2p). NXS/T sites introduced into the luminaly oriented domains of Akr1p would be expected to be glycosylated, whereas those introduced into cytoplasmic domains should be unavailable to the luminaly localized glycosyl transferases and thus should remain unglycosylated. To assess Akr1p topology, we made a series of Akr1p-Akr1p constructs that inserted a 46-residue-long segment of invertase, containing three NXS/T sites known to be utilized for glycosylation (19), at seven different sites known within the AKR1 open reading frame (Fig. 3A, positions A, C, D, E, F, G, and H). This strategy had been successfully employed by Gilstring and Ljundahl (18) to assess topology of the yeast general amino acid permease Gap1p. Gel mobility of the seven Akr1p insertion proteins was compared (Fig. 3B). Our expectation, based on the published Gap1p analysis (18), was to see up-shifted gel mobilities for those constructs for which the

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FIG. 1. Akr1p hydropathy analysis. Upper panel, a hydropathy plot derived from the Akr1 protein sequence using TopPred II (27). Sequences exceeding the hydrophobicity threshold for service as TMDs are indicated in black. Lower panel, an Akr1p schematic with the strongly hydrophobic sequences indicated in black and with the more marginally hydrophobic sequences indicated in gray. The positions of the DHHC-CRD, the six ankyrin repeats, and the four potential NXS/T glycosylation sites (asterisks) are also indicated.

FIG. 2. Analysis of N-linked glycosylation for Akr1p and Ste2p. Cells expressing HA epitope-tagged versions of Akr1p or of Ste2p were inducibly expressed from the GAL1 promoter. Thirty minutes prior to the galactose addition, cells either were treated with tunicamycin at 20 μg/ml or were mock-treated. Following a 2-h expression period, protein extracts were prepared, and the extracts were subjected to SDS-PAGE and Western analysis with anti-HA mAb. In addition to being glycosylated, Ste2p is also subject to both phosphorylation and ubiquitination, which likely accounts for the residual heterogeneity of the tunicamycin-treated protein.

Akr1p Transmembrane Topology

The different SUC2 insertion alleles and several N- and C-terminal AKR1 deletion alleles were tested for their ability to restore 37 °C growth to akr1Δ cells. The different mutant alleles were introduced into NDK1405 on the single copy URA3 yeast vector plasmid pRS516 under the control of the natural AKR1 upstream regulatory sequence. Suspension of the NDK1405 transformants were diluted and spotted on uracil-lacking, minimal medium plates that were incubated for 2 days prior to growth at 37 °C.
Akr1-Suc2-Akr1 proteins with either the small, 46-residue invertase proteins have the large 511-residue invertase inserts. Induction of Akr1p or Akr1-Suc2-Akr1p expression. Akr1-Suc2-Akr1 were treated with (−) or the large, 511-residue invertase inserts (C). HA-tagged Akr1-Suc2-Akr1 proteins were expressed and detected by anti-HA mobility of Akr1-Suc2-Akr1-(H)p is due to Western blotting as described for Fig. 2. Akr1-Suc2-Akr1-(H)p is due to N-linked glycosylation. Cells were treated with (+) or without (−) tunicamycin 30 min prior to the induction of Akr1p or Akr1-Suc2-Akr1p expression. Akr1-Suc2-Akr1 proteins have the large 511-residue invertase inserts.

Fig. 3. Analysis of Akr1-Suc2-Akr1 proteins. A, invertase insertion sites. Positions of the 10 invertase insertions are indicated within the Akr1 protein sequence. As in Fig. 1, strongly hydrophobic sequences are designated by black boxes, and more marginally hydrophobic sequences are designated by gray boxes. B and C, gel mobility assessed for Akr1-Suc2-Akr1 proteins with either the small, 46-residue invertase inserts (B) or the large, 511-residue invertase inserts (C). HA-tagged Akr1-Suc2-Akr1 proteins were expressed and detected by anti-HA Western blotting as described for Fig. 2. wt, wild type. D, retarded mobility of Akr1-Suc2-Akr1-(H)p is due to N-linked glycosylation. Cells were treated with (+) or without (−) tunicamycin 30 min prior to the induction of Akr1p or Akr1-Suc2-Akr1p expression. Akr1-Suc2-Akr1 proteins have the large 511-residue invertase inserts.

inserted invertase sequences were luminally oriented. Surprisingly, no substantial mobility differences were seen among the seven Akr1-Suc2-Akr1 proteins; all migrated to essentially the same position, the position of the unglycosylated Akr1-Suc2-Akr1 protein (Fig. 3B). For Akr1-Suc2-Akr1-(D) and -(F), a minor subpopulation of the protein was found to diffusely migrate with retarded mobility (Fig. 3B). This rather subtle effect likely reflects a low level of heterogeneous glycosylation as this more slowly migrating protein smear is abolished with expression of the insertions in tunicamycin-treated cells (data not shown). Nonetheless, this minor glycosylation effect is far more subtle than the striking glycosylation shifts seen for analogous Gap1-Suc2-Gap1 insertion constructs (18). Perhaps for the Akr1p structure, luminal segments are less well exposed and are thus less available to glycosyltransferases; indeed, glycosylation site usage is known to correlate with site availability during protein folding (22). Another possibility is that luminally oriented NX(S/T) sites of the Akr1-Suc2-Akr1 proteins are, in fact, glycosylated but simply with smaller, and thus less easily detectable, oligosaccharide moieties than those attached to Gap1p. Indeed, the elaboration of the N-linked core oligosaccharide varies tremendously for yeast glycoproteins; secreted proteins and proteins of the yeast cell wall generally have highly elaborated oligosaccharides with up to 100 mannose moieties added per oligosaccharide, whereas the glycosyl modifications of Golgi and vacuolar constituents generally are much less elaborate (23).

In hopes of magnifying the glycosylation effect, a second series of Akr1-Suc2-Akr1 constructs with virtually the entire invertase coding sequence inserted was generated. The 511-residue-long invertase portion used corresponds to mature invertase in its entirety (minus the N-terminal signal peptide) with its 10 N-linked glycosylation sites. This invertase segment was inserted at 10 Akr1p sites: the seven positions utilized for the smaller, 46-residue Suc2 inserts plus three additional sites (Fig. 3A, sites B, I, and J). More striking gel mobility differences were seen for these second generation Akr1-Suc2-Akr1 proteins (Fig. 3C). Retarded mobilities consistent with glycosylation were evident for insertions at positions D, F, and H but not for insertions at positions A, B, C, E, G, I, and J. To test whether the up-shifted mobilities result from N-linked glycosylation, two of the Akr1-Suc2-Akr1 proteins were expressed in tunicamycin-treated cells. Indeed, tunicamycin does eliminate the Akr1-Suc2-Akr1-(H) up-shift, causing it to comigrate with the presumably unglycosylated Akr1-Suc2-Akr1-(E) (Fig. 3D).

We also note, for several of the insertion proteins, the presence of a secondary, minor gel band on the Western blot in addition to the major gel band (Fig. 3C). For instance, for Akr1-Suc2-Akr1-(D) and -(H), whereas the bulk of the protein runs at the glycosylated position, a minor species is also apparent for both, migrating at the position of non-glycosylated Akr1-Suc2-Akr1 protein. Similarly, for Akr1-Suc2-Akr1-(E), in which the bulk of the protein migrates to the unglycosylated position, an additional faint band is also apparent at the glycosylated position. This minor Akr1-Suc2-Akr1-(E) species is apparently glycosylated since it is both lost in tunicamycin-treated cells (Fig. 3D) and also collapses to the unglycosylated position when extracts are treated prior to Western analysis with endoglycosidase H to remove N-linked oligosaccharides (data not shown). The existence of such minor species suggests that at least some of the insertion proteins can adopt alternative topologies in which domains that are normally cytoplasmic are instead oriented to the luminal compartment, or conversely, where luminal domains are instead oriented to the cytoplasm. Most likely, the 511-residue invertase segment inserted at these three Akr1 sites (i.e. at sites D, E, or H) is somewhat disruptive to the process by which Akr1p is assembled into the membrane, resulting in a low level of misinsertion. However, these minor electrophoretic species, even when most prominent, as for Akr1-Suc2-Akr1-(D) and -(H) insertions (Fig. 3C), represent less than 5% of the total Akr1-Suc2-Akr1 protein. Therefore, in developing an Akr1p topology model, we have concentrated on the glycosylation status of just the most prominent, major electrophoretic species, presumably representing the bulk topology for each insertion construct.

Akr1p Topology Model—Of the 10 Akr1-Suc2-Akr1 constructs examined, only three, Akr1-Suc2-Akr1-(D), -(F), and -(H), were found to be extensively glycosylated, indicating that just three sites (i.e. D, F, and H) are luminally oriented. The remaining seven constructs are not extensively glycosylated, indicating that the corresponding Akr1p sites (i.e. A, B, C, E, G, I, and J) all are cytoplasmic. A topology model derived from these results is shown in Fig. 4. Akr1p is found to have six TMDs with the bulk of the polypeptide being oriented to cytoplasm. Indeed, only three short connecting loop domains are
luminally oriented. The cytoplasmically oriented domains are: a large 323-residue-long N-terminal domain that includes the ankyrin repeats; two loop domains, one of which harbors the DHHC-CRD; and finally a 206-residue-long C-terminal domain. The various segments of marginal hydrophobicity noted previously (Fig. 1, gray boxes) are not TMDs, whereas the extended, 38-residue-long hydrophobic segment (residues 324–361) corresponds apparently to two TMDs linked by an extremely short hydrophilic loop sequence.

Functionality of the Akr1-Suc2-Akr1 Proteins—To assess functionality, the different AKR1/SUC2-AKR1 alleles were tested for akr1Δ complementation. Like many other of the yeast functions participating in endocytic and/or vacuolar trafficking, AKR1 gene deletion results in temperature sensitivity; thus, akr1Δ cells, although viable at 30 °C, are inviable at 37 °C. This 37 °C requirement for AKR1 appears to be a requirement for the Akr1 PAT function since akr1Δ missense alleles with substitutions of conserved DHHC-CRD residues both fail to support Yck2p palmitoylation in vivo and in vitro (1) and also fail to support 37 °C viability (data not shown).

Complementation of the 10 AKR1/SUC2-AKR1 alleles with the large, 511-residue invertase insertion was tested first. akr1Δ cells, harboring the different insertion alleles on single copy, centromeric plasmids, were plated either at 25 or at 37 °C (Fig. 5A). Six of the constructs, namely insertions at sites A, D, F, H, I, and J, complemented, fully restoring 37 °C growth to the akr1Δ cells. The remaining four insertion alleles, B, C, E, and G, failed to restore 37 °C growth. Interestingly, all four of these non-complementing insertions map to cytoplasmic domains; B and C map within the large N-terminal cytosolic domain, whereas E and G map to the two cytosolic loops. In contrast, insertions into the three luminal loop domains, i.e. sites D, F, and H, apparently do not disrupt Akr1p function (Fig. 5A).

The functionality of the seven AKR1/SUC2-AKR1 alleles having the smaller, 46-residue invertase segment inserted were also examined (data not shown). Results with the smaller insertions were quite consistent with those for the larger insertions; A, D, E, F, and H complemented, whereas C and G failed to complement (data not shown). The one difference between the two sets of complementation data relates to insertions at position E, within one of the two cytosolic loops; although Akr1p function apparently is not abolished with insertion of the smaller 46-residue invertase segment (data not shown), function is lost with insertion of the larger invertase segment at the same site.

In addition, several N- and C-terminal AKR1 deletion alleles have been constructed and tested for complementation (Fig. 5B). Again, restoration of 37 °C growth to akr1Δ cells was assessed. The less severe deletions at both the N and the C termini restored growth, whereas the more severe Δ2–295 N-terminal and Δ615–764 C-terminal deletions failed to complement. We note for the non-complementing Δ2–295 allele that the deletion extends into the ankyrin repeat sequence, whereas the deletions of the complementing N-terminal deletion allele, Δ2–21 and Δ2–70, do not encroach upon this sequence. Together with the non-complementation of the AKR1/SUC2-AKR1(B) allele (Fig. 5A), this suggests a likely requirement for the ankyrin repeats in Akr1p function.

PAT Function of Akr1/Suc2-Akr1 Proteins—Although we argue above that the restoration of 37 °C growth to akr1Δ cells likely represents a restoration of Akr1p PAT function, such a complementation assay is obviously quite an indirect measure of PAT function. As a more direct assessment of PAT function, we have examined several AKR1/SUC2-AKR1 insertion alleles for their support of Yck2p palmitoylation in vivo. Rather than using the standard method for assessing palmitoylation, i.e. examining the in vivo incorporation of label from [3H]palmitic acid into the protein of interest (Yck2p in this case), we have opted to use instead a recently published protocol involving a chemical exchange of the protein acyl modifications for an introduced labeling compound (20). For this, protein is subjected to three sequential chemical treatments. First, free thiols are exhaustively blocked with N-ethyl-maleimide. Second, palmitoyl moieties are released from modified cysteines through specific hydroxylamine cleavage of the thioester linkage. Finally, the thiols newly exposed by the hydroxylamine treatment (i.e. the cysteine residues that were palmitoylated) are marked with a labeled thiol reagent. This method is more robust than the in vivo palmitate labeling approach and quite specific for detection of palmitoyl modifications (20). Yck2p palmitoylation was examined in strains utilizing three of the different AKR1/SUC2-AKR1 insertion alleles (511-residue-long Suc2 insertions) in place of chromosomal AKR1 (Fig. 6). As a control, palmitoylation of a mutant Yck2p with its C-terminal palmitoyl-accepting Cys-Cys sequence mutated to Ser-Ser, i.e. complementation assay is obviously quite an indirect measure of PAT function.
depending on Yck2p transport to the plasma membrane.3

that the Yck2p hyperphosphorylation is compartment-specific,
teins comigrate (1) (data not shown). Indeed, we have found
instead a consequence of phosphorylation; following phospha-
tion on gel mobility, the more slowly migrating species is
coherent gel band. However, when palmitoylated, an addi-
tion of Yck2p, immune-precipitated from the biotinylated protein extracts with anti-FLAG mAb-agarose,
was subjected to Western analysis both with avidin-HRP to detect
biotinylation and with anti-HA-HRP to assess Yck2p recovery.

Yck2(SS)p, also was tested. Consistent with previous analyses
of Yck2p palmitoylation by standard in vivo [3H]palmitate
labeling (1, 24), the new methodology finds Yck2p to be palmit-
oylated when isolated from wild-type cells but not when iso-
lated from akr1Δ cells (Fig. 6); furthermore, the mutant
Yck2(SS)p, which lacks the palmitoyl-accepting cysteines, also
failed to get biotinylated (Fig. 6). Of the three Akr1-Suc2-Akr1
alleles tested, insertions at sites D and F supported
Yck2p palmitoylation, whereas the G insertion allele did not.
These results are fully consistent with the complementation
analysis; the D and F insertion alleles restored 37 °C growth to
the akr1Δ cells, whereas the G insertion allele did not (Fig. 5A).
Thus, for D and F, and likely also for A, H, I and J, the
invertase insertions do not abolish Yck2p PAT function.

In addition, we note a striking effect on Yck2p gel mobility
shift that correlates with palmitoylation status (Fig. 6, lower
panel). In the three contexts in which Yck2p fails to be palmit-
oylated, i.e. for Yck2(SS)p and for wild-type Yck2p in either
akr1Δ or Akr1-Suc2-Akr1(G) cells, Yck2p runs as a single
coherent gel band. However, when palmitoylated, an
additional, more prominent, slower migrating species is also seen.
Rather than reflecting a direct effect of the palmitoyl
modification on gel mobility, the more slowly migrating species is
instead a consequence of phosphorylation; following phospha-
tase treatment, palmitoylated and non-palmitoylated Yck2 pro-
teins comigrate (1) (data not shown). Indeed, we have found
that the Yck2p hyperphosphorylation is compartment-specific,
depending on Yck2p transport to the plasma membrane.3

Akr1p Multimerization Test—In our published analysis of
the Akr1p-associated PAT activity, Akr1p was purified to ap-
parent homogeneity; we found no evidence for other stoichio-
metric components that co-purified (1). Thus, unlike Erp2p and
Erp4p, which do co-purify and which appear to behave as co-
subunits for Ras palmitoylation, for Akr1p, no accessory pro-
teins appear to be required. Nonetheless, the active PAT could
still be composed of multiple copies of the Akr1 polypeptide. To
test for such homo-multimerization, we co-expressed two Akr1
proteins, differing in the sequences fused at their C termini.
Akr1–3xHA/FLAG/His has the tri-tag sequence previously
used in the affinity purification of the active Akr PAT (1).
Akr-GST/3xHA has at its C terminus the 223-residue bacterial
glutathione S-transferase (GST) followed by three iterations of
the HA epitope tag. Both constructs fully complement akr1Δ,
restoring 37 °C growth (data not shown), and the two proteins
can be easily differentiated by their quite different gel mobili-
ties (Fig. 7). Lysates, prepared from akr1Δ cells expressing the
two proteins either separately or together, were subjected to
anti-FLAG affinity purification under the native conditions
previously used to maintain Akr1 PAT activity (1), were subjected to immune precipitation (IP) with
anti-FLAG mAb-agarose followed by anti-HA Western blotting. As controls, cells singly transformed by the Akr1–3xHA/FLAG/His-expressing and Akr1-GST/3xHA-expressing plasmid were processed in parallel.

**DISCUSSION**

Our results indicate that the Akr1 polypeptide chain crosses
the bilayer six times with its major hydrophilic domains oriented
to the cytoplasm (Fig. 4). Cytoplasmic domains include the
ankyrin repeat-containing, 323-residue-long N-terminal domain,
the 166-residue-long C-terminal domain, and two inter-TMD
loop domains, one of which contains the DHHC-CRD sequence.
The luminal exposed domains consist of three relatively short
loop segments. Such a topology, in which the bulk of the protein
is accessible from the cytoplasm, fits well with the known biology
for Akr1p. Its two known substrates, the kinases Yck1p and
Yck2p, are expected to be presented to Akr1p from the cytoplasm;
Yck1p and Yck2p are synthesized initially as soluble, cytoplasmic
proteins, and indeed, when their palmitoylation is blocked, they
are found to diffusely distribute through the cytoplasm (1, 25).
The acyl donor for the palmitoylation reaction, palmitoyl-CoA,
given its amphiphilic nature, might be expected to dissolve in
membranes and thus gain access to the PAT through the bilayer.
However, it has been argued that the overwhelming bulk of the
acyl-CoA pool is sequestered within the cytoplasm by the abun-
dant cytoplasmic protein, acyl-CoA-binding protein (26), or
Acb1p in yeast. By shielding the acyl-CoA hydrophobic acyl tail
from the aqueous environment, acyl-CoA-binding protein is
thought to potentiate acyl-CoA transport through the cytoplasm.
Thus, like the substrate proteins, palmitoyl-CoA may also access
the PAT from the cytoplasm. Finally, the finding that the Akr1p
DHHC-CRD sequence is cytoplasmically oriented suggests that

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3 A. Roth and N. Davis, unpublished information.
catalysis, i.e. the addition of the acyl moiety to the target cysteine residue, may also be mediated by cytoplasmic domains. The role for the DHHC-CRD in catalysis was suggested by previous findings made for both Akr1p and Erf2p that mutation of conserved DHHC-CRD residues abolished PAT activity in vivo and in vitro (1, 2). In addition to fitting well with the biology of Akr1p, the presented topology, with the bulk of the PAT polypeptide oriented to the cytoplasm, also more generally fits with palmitoylation as a cytoplasmically limited protein modification (6–8).

A central role for the cytoplasmic Akr1p domains is also supported by the analysis of the function of the different Akr1-Suc2-Akr1 insertions. These mutations insert either 46 or 511 residues at different sites within the Akr1 polypeptide and thus have great potential for disrupting Akr1 function. Although some insertions do abolish Akr1p function, surprisingly, many do not. Indeed, in addition to withstanding fusions to both the N and the C termini, insertions at multiple internal sites also appear not to disrupt Akr1p function. Perhaps most significantly, all of three luminally oriented insertions, i.e. the insertions into the three luminally oriented loop domains of Akr1p, remain grossly functional. In fact, two of the three luminal insertions were tested for their in vivo support of Yck2p palmitoylation and were found to be fully functional; no loss of Akr1 PAT function was detected. The four insertions that were found to disrupt function all mapped to cytoplasmic domains (sites B, C, E, and G). At site E, within the more N-terminal of the two cytoplasmic loop domains, different results were found for the small versus the large Suc2 insertions; function was disrupted by the large, but not by the small, Suc2 insertion. Thus, by this crude mutational analysis, the Akr1p cytoplasmic domains appear to play a more critical role in Akr1p function than do the luminal domains.

How does the Akr1p topology model (Fig. 4) fit with the hydropathy analysis (Fig. 1)? First, the three segments of marginal hydrophobicity, residues 182–201, 554–568, and 661–680 (Fig. 1, gray boxes of the Akr1p schematic), apparently do not serve as TMDs. Second, the relatively broad, 38-residue-long 324–361 hydrophobic sequence harbors two TMDs. Central within this extended hydrophobic segment is a short hydrophilic subsegment, the pentapeptide sequence SHINP (residues 342–346), which apparently constitutes an extremely short, luminally disposed, inter-TMD loop domain. Invertase inserted into this pentapeptide sequence (Akr1-Suc2-Akr1-(D)) gets glycosylated, whereas invertase inserted at the C and E flanking sites does not (Fig. 3, A and C). Although it seems unusual for two TMDs to be so closely spaced within the sequence, we note that the yeast and human Akr1p homologues (for which homology extends across the entire protein sequence) have preserved similarly positioned hydrophobic domains (Fig. 8). At the homologous site within yeast Akr2p, there is a contiguous stretch of 43 uncharged residues, i.e. residues 298–340 (Fig. 8). The uncharged Akr2p sequence stretch is divided by a central SLVLS (residues 317–322), which is somewhat less hydrophilic than the corresponding SHINP loop segment of Akr1p. The two human Akr1p homologues, HIP14 and HIP14L, at the equivalent position, two discrete hydrophobic sequences, clearly delineated by an intervening hydrophilic sequence with multiple charged residues rather than the single long hydrophobic sequence found in Akr1p and Akr2p. It is perhaps noteworthy that for each of the four proteins, the second TMD of this pair is notably short, consisting of 15 uncharged residues in Akr1p, 18 residues for Akr2p, and 15 residues for both HIP14 and HIP14L.

In addition to the three Akr1p homologues discussed above (i.e. Akr2p, HIP14, and HIP14L), Fig. 8 also compares the Akr1p hydropathy with hydropathies for other DHHC proteins: the other five yeast DHHC proteins as well as rat GODZ, which has recently been implicated in the palmitoylation of the γ2 subunit of the ionotropic GABA_A receptor (12). As homology within this family is largely limited to the DHHC-CRD, we have used this conserved domain as a reference point for plot alignment. To predict topologies for each (Fig. 8, bars above each hydropathy plot), we made the assumption that the DHHC-CRD is oriented cytoplasmically as it is for Akr1p. Clear similarities are evident across the diverse group with regard to the spacing of TMDs that surround the DHHC-CRD. Most
notable is the fixed spacing of the DHHC-CRD to the C-terminally adjacent TMD, the spacing being constrained by the overlap of the DHHC-CRD homology with this TMD, which generally involves the 7 C-terminal DHHC-CRD residues. This invariant overlap of the putative active site (the DHHC-CRD) and the TMD suggests the possibility that essential aspects of the catalysis may occur within the bilayer plane. Extending further away from the DHHC-CRD, additional similarities are evident; the aligned plots show a roughly preserved, 220-residue core consisting of the four TMDs that surround the central DHHC-CRD (Fig. 8, gold bar extending through all of the plots). We will be interested to see whether this preserved portion represents a preserved PAT structural core for the DHHC protein family.

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