Biochemical characterization of the vacuolar palmitoyl acyltransferase

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Abstract Vacuole fusion requires Sec18p-dependent acylation of the armadillo-repeat protein Vac8p that has been isolated with cis-SNARE complexes. To gain more insight into the mechanism of acylation, we analyzed the palmitoylation reaction on isolated vacuoles or in vacuum detergent extracts. Re-combinant Vac8p is palmitoylated when added to vacuoles and is anchored to membranes after modification. The palmitoyl acyltransferase (PAT) extracted from vacuum membranes is functional in detergent extracts and shows all characteristics of an enzymatic activity: It modifies exogenous Vac8p in a temperature-, dose- and time-dependent manner, and is sensitive to bromo-palmitate, a known inhibitor of protein palmitoylation in vivo. Importantly, PAT is specific for palmitoyl-CoA, since myristoyl- and stearyl-CoA can compete with labeled Pal-CoA only at 10-fold higher amounts.

1. Introduction

Palmitoylation is the covalent attachment of fatty acids to cysteine residues of membrane proteins. The fatty acids can mediate protein–lipid and protein–protein interactions, thereby targeting the modified protein to membranes or membrane subdomains [1]. In general, four families of proteins exist that are targets to palmitoylation: (1) proteins containing an N-terminal myristic acid flanked by cysteines, (2) proteins with a C-terminal prenylated Caax box, (3) proteins with a cluster of cysteine residues in the middle of the molecule, and (4) proteins that are modified proximal to their transmembrane domain. For most proteins palmitoylation is essential for their function and occurs in the vicinity of a membrane [1]. It has been questioned whether enzymes were required for acylation since some studies suggested that palmitoylation might be a non-enzymatic event [2–4]. Only very recently, the family of DHHC-CRD proteins (polytopic membrane proteins with the sequence DHHC and a cysteine-rich domain) has been presented as potential acyltransferases for proteins with a C-terminal Caax-box in yeast. Erf2p (in a complex with Erf4p) appears to be necessary to mediate acylation of Ras, whereas Akr1p palmitoylates the C-terminal CC sequence of the casein-kinase Yck2p [5–8]. Surprisingly, none of the members of this family is essential for yeast growth. In Drosophila, acylation of the homeobox protein hedgehog requires the putative acyltransferase skinny hedgehog [9,10]. However, acylation of hedgehog occurs via an amide bond, which makes it unlikely that skinny hedgehog is also responsible for the more common mode of palmitoylation by thioester bonding [11].

Activated fatty acids, the lipid substrates for protein palmitoylation, are involved in vesicular trafficking. Pal-CoA stimulates both the budding of transport vesicles from the Golgi as well as the Golgi- and the yeast vacuole fusion assay [12–15]. Using a non-hydrolyzable analog of Pal-CoA, it was suggested that this stimulatory effect might be due to palmitoylation of proteins, which thereby get activated to function in the fusion reaction [13]. Interestingly, Pal-CoA is most effective at limiting NSF/Sec18p concentrations [12] indicating that Pal-CoA and NSF/Sec18p act together in a step required for protein transport and palmitoylation.

The yeast vacuole fusion assay is a model system to analyze membrane trafficking [16]. Vacuole fusion depends on a cascade of events that can be divided into a priming, docking and fusion step. During priming, ATP hydrolysis by Sec18p triggers disassembly of a multisubunit SNARE complex into its subunits [17], which is a prerequisite for docking and fusion [18]. Using the yeast vacuole fusion assay we have recently identified Vac8p as the first protein substrate of palmitoylation required for a vesicular trafficking reaction [19,24]. Vac8p was originally isolated as a protein involved in vacuole inheritance [20–23]. Vac8p is myristoylated cotranslationally at its N-terminal glycine residue, and subsequently becomes palmitoylated at three neighboring cysteine residues [21,23]. Palmitoylation, but not myristoylation, is essential for vacuole inheritance, although both hydrophobic modifications are required for complete localization of Vac8p to the vacuole [21]. During the yeast vacuole assay Vac8p becomes palmitoylated only at the beginning of the reaction and palmitoylated Vac8p is essential for vacuole fusion, probably acting at a stage following trans-SNARE pairing [9,24]. So far, only Sec18p has been identified to have a role in the palmitoylation reaction, and might be involved in releasing cis-SNARE-bound Vac8p, thus supplying the substrate for palmitoylation.

We have now established an in vitro assay to analyze the acyltransferase in detail. PAT activity can be extracted from vacuole membranes and can specifically palmitoylate recombinant Vac8p in detergent extracts. Specificity of Pal-CoA demonstrates that substrate specificity appears to be one of its important characteristics.

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2. Materials and methods

2.1. Reagents and strain

[9,10-3H]Palmitic acid (50 Ci/mmol) was obtained from Hartmann Analytik (Romansteg, Germany). [14C]-Pal-CoA was synthesized with [14C]-palmitate and acyl-CoA-synthetase as described in Section 2.4. 2-Bromo-palmitate was from ACROS, Belgium. All other reagents were purchased from Sigma. The yeast strains used in this study were as described in [38,39]. Antibodies to Sec18p were made to a recombinant bacterial-expressed protein [15]. When we added recombinant Sec18p to vacuole fusion assays, antibodies to Sec18p do not block fusion, nor palmitoylation, indicating that the antibody has been quenched by the additional pure protein (our unpublished observations).

2.2. Purification of Vac8-GST and His6-Vac8p from Escherichia coli

His8-Vac8p cloned into the pQE30 plasmid (N-terminal His8-tag on Vac8p), was expressed in the absence of the human N-myristoyl-transfase (see below) and was purified as described in [19]. A PCR fragment encoding Vac8p or Vac8C457A was cloned as a Ncol/NcoI fragment into a pETGEX vector, thus generating a C-terminally tagged NMTco-expression in E. coli (kindly provided by Roger Clegg, Hannah Research Institute, Ayr, UK). NMTco-expression in E. coli leads to efficient-myristoylation [40], although we have not analyzed the efficiency of myristoylation of Vac8p in our system. Vac8-GST and Vac8C457A-GST were purified with glutathione-agarose, eluted with 50 mM Tris-HCl, pH 7.4, 300 mM NaCl, 10 mM glutathione, and dialyzed into 10 mM PIPES/KOH, pH 6.8 ± 150 mM KCl, 5 mM MgCl2.

2.3. Labeling of the vacuole fusion assay

Palmitoylation of vacuoles with [14C]-Pal-CoA or [14C]-Pal-CoA was done as described except that recombinant His8-Vac8p (5 µg) or His6-Vac8p were added [19]. Extraction of vacuoles with high salt or TX-100 (Fig. 1d) was done as follows. Vacular pellets from an identical incubation were resuspended in 300 µl high salt buffer (1 M KCl, 20 mM HEPES/KOH, pH 7.4, 1, 1x PIC) and incubated for 15 min on ice. Non-extracted material was pelleted (30 min, 20,000 × g) and the supernatant was precipitated with trichloroacetic acid (TCA). The pellet from the salt extraction was resuspended in Triton buffer (0.1% Triton X-100, 50 mM KCl, 20 mM HEPES/KOH, pH 7.4) and incubated for 15 min. Non-extracted material was again pelleted and the pellet and the TCA-precipitated supernatant were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography.

2.4. Effect of 2-bromo-palmitate on acyl-CoA synthesis

Acyl-CoA synthetase (2 µl, 10 mg/ml in phosphorin buffer, from Pseudomonas fragi; [41]) was added to 50 µg units in 25 µl of protein (in 1x PIC) was incubated for 10 min at 28°C in glass vessels with coenzyme A (3 µl, 10 mM in distilled water), [14C]-palmitic acid (25 µCi, 50-60 Ci/mmol, dissolved in 5 µl ethanol), 1 mM ATP, and 4 mM MgSO4 in a final volume of 50 µl phosphorin buffer (pH 7.4). Br-Pal (1 µl) was added from a 90°C-cooked stock solution in ethanol. The reaction was stopped by adding 500 µl acetonitrile/1 M phosphoric acid (9:1) and precipitated proteins were pelleted (10 min, 5000 × g) and the supernatant was extracted three times with 1 ml of toluol to remove the remaining [14C]palmitic acid. Aliquots of the lower phase, which contains [14C]-Pal-CoA, were counted in a liquid scintillation counter.

2.5. Palmitoylation assay

Unless mentioned otherwise, BJ3505 vacuoles were preincubated under fusion conditions for 15 min at 30°C. Vaculars were pelleted (10 min, 12,000 × g), resuspended in PS buffer (10 mM PIPES/KOH, pH 6.8, 200 mM sorbitol) containing 0.5 × PIC (protease inhibitor cocktail: 7.5 µM pefabloc SC, 7.5 ng/ml leupeptin, 3.75 µM o-phenanthroline and 37.5 µg/ml pepstatin) and again pelleted. Membranes from a 200 µl reaction were solubilized in 100 µl Triton buffer (0.25% Triton X-100, 50 mM KCl, 20 mM HEPES/KOH, pH 7.4 and 1 × PIC) and incubated for one h at 4°C with agitation. Insoluble material was pelleted (15 min, 20,000 × g) and the supernatant was used immediately in the PAT assay or stored at −80°C. Where indicated, vacuoles were extracted with 1 M KCl, 20 mM HEPES/KOH, pH 7.4. Vacular extract (10 µl, about 5 µg) was incubated with Vac8-GST and [3H]Pal-CoA in 20 mM PIPES, pH 6.8, 120 mM KCl in a final volume of 100 µl for 15 min at 30°C. 1 ml of chloroform/methanol (1:2) was added and precipitated proteins were pelleted at 10,000 × g for 5 min. The pellet was washed with 1 ml of methanol and again centrifuged. Proteins were resuspended in non-reducing sample buffer and subjected to SDS-PAGE and fluorography.

3. Results

3.1. Specific palmitoylation of recombinant Vac8p

We sought to bypass the association of endogenous Vac8p with the SNARE complex by using soluble Vac8p as substrate for palmitoylation. When added to the vacuole fusion reaction, recombinant Vac8p with an N-terminal His-tag (His6-Vac8p) was palmitoylated in a concentration-dependent manner (Fig. 1a). Palmitoylation requires ATP and CoA, but not cytosoł, demonstrating that the PAT activity is likely to reside on the vacuole. ATP and CoA are required for the synthesis of Pal-CoA from [14C]-Palmitate. The palmitoyl-transfer reaction analyzed with the vacular extract and [14C]-Pal-CoA as

Fig. 1. Yeast vacuoles palmitoylate exogenous Vac8p. a: Efficient palmitoylation of exogenous Vac8p. Vacuole fusion reactions (60 µg vacuoles in 300 µl) containing cytosoł (0.5 µg/ml), coenzyme A (10 µM) and ATP (100 µM) as indicated were supplemented with recombinant His6-Vac8p and [14C]-Pal-CoA (150 µCi) and incubated for 20 min at 30°C. Vacuoles were then pelleted, washed and subjected to non-reducing SDS-PAGE and fluorography. b: Labeling of exogenous Vac8p occurs early. Vacuole fusion reactions containing cytosoł, ATP and CoA were started and 5, 10, 20 or 40 min later 5 µg His6-Vac8p and [14C]-Pal-CoA were added and incubations were continued for 15 min. Analysis was done as in panel a. c: A fraction of exogenous Vac8p is found on vacuoles after palmitoylation. Vacuole fusion reactions containing cytosoł, ATP, CoA and 5 µg His6-Vac8p were incubated for 15 min at 30°C. Vacuoles were pelleted and washed twice with PS buffer. The supernatant was precipitated with 13% (v/v) TCA, washed with ethanol and resuspended in reducing sample buffer. Supernatants (20%, lane 2) and the complete vacuolar pellet (lane 1) were applied to SDS-PAGE. In a parallel experiment, vacuoles were salt-extracted (see Section 2) and the initial amount of vacuole (vac, lane 3), the complete supernatant (S, lane 4) and the vacuolar pellet (P, lane 5) were analyzed by Western blotting with anti-His antibodies (Qiagen). d: Exogenous Vac8p associated with vacuoles is palmitoylated. Vacuole fusion reactions supplemented with 5 µg His6-Vac8p and [14C]-Palmitate were incubated for 15 min. Vacuoles were then pelleted, washed and directly solubilized in SDS-PAGE buffer (vac), extracted with high salt or Triton X-100. Supernatants (S) and pellets (P) were then subjected to SDS-PAGE and fluorography. e: Antibodies to Sec18p block acylation of exogenous Vac8p. Vacuole fusion reactions containing 5 µg His6-Vac8p were incubated as before. Where indicated, antibodies of Sec18p were added. Analysis was as in panel a.
indicating that enzymatic events are not required for binding. Membrane also occurs during incubation on ice (not shown), resulting extracts were incubated with [3H]Pal-CoA and with disassembly. Vacuoles were then collected by centrifugation, activity from vacuole membranes, we preincubated vacuoles to the vacuole[21]. To prepare extracts with palmitoylating vitrolabeling, possibly due to the excess amount of the added different times to the fusion reaction, [3H]-labeling was ob-

3.2. Extraction of PAT from vacuolar membranes
To establish an assay that measures PAT activity we generated a C-terminally tagged Vac8-GST fusion protein, which, due to its higher molecular weight, separates during SDS-PAGE from endogenous Vac8p. We prepared Vac8-GST in the presence of the human N-myristoyltransferase from E. coli (see Section 2). Vacuoles efficiently palmitoylate Vac8-GST using [3H]Pal-CoA as a lipid donor, whereas a similar construct with alanine substitutions in the N-terminal cysteines did not get modified (Fig. 2a). Since non-myristoylated His6-Vac8p and myristoylated Vac8-GST are equally labeled, we assume that the myristoyl anchor may be dispensable for in vitro labeling, possibly due to the excess amount of the added protein. The anchor is, however, essential for in vivo targeting to the vacuole[21]. To prepare extracts with palmitoylating activity from vacuole membranes, we preincubated vacuoles in the presence of ATP at 26°C to allow SNARE-complex disassembly. Vacuoles were then collected by centrifugation, solubilized in Triton X-100 or extracted with high salt. The resulting extracts were incubated with [3H]Pal-CoA and with or without Vac8-GST. Proteins were then precipitated with chloroform/methanol to remove non-covalently bound fatty acids and subjected to SDS-PAGE and fluorography. In the presence of detergent alone we were able to extract PAT activity, indicating that the activity is membrane-associated (Fig. 2b). We then conducted several control experiments to characterize the activity. Palmitoylation increases with increasing concentrations of substrate and membrane extract (Fig. 2c). Preincubation of vacuoles with anti-Sec18p prior to extraction abolished recovery of PAT, indicating that Sec18p is proximal to PAT on the vacuole. No labeling of Vac8p was seen in the absence of the extract, indicating that non-enzymatic palmitoylation does not occur under these conditions. Labeling of Vac8p was complete after 10 min and was best at 30°C, similar to our observations with endogenous Vac8p (Fig. 2d,e; [19]). In contrast, non-enzymatic palmitoylation usually requires several hours before completion [3]. In addition, heating of Vac8p or the extract, as well as trypsin addition to the extract prior to addition to the in vitro assay abolished acylation, indicating that the PAT is a protein (Fig. 2f). Furthermore, a mutant of Vac8p with alanine substitutions in the three N-terminal cysteine residues is not labeled, indicating that palmitoylation occurs at the same sites that are acylated in vivo (Fig. 2f).

3.3. Inhibitors and specificity of the acylation reaction
The fatty acid analog 2-bromo-palmitate (Br-Pal) is known to inhibit protein palmitoylation in vitro and in vivo when labeling is done with [3H]palmitate. Br-Pal could therefore inhibit either the synthesis of activated fatty acids by acyl-CoA synthetase or the transfer of the activated fatty acid by PAT, or both. To understand its precise mode of inhibition, we analyzed the effect of Br-Pal on acyl-CoA synthesis. Purified acyl-CoA synthetase (from P. fragi) was incubated with [3H]palmitate, CoA, ATP and increasing concentrations of Br-Pal, and synthesized [3H]Pal-CoA was extracted and counted. Br-Pal blocked Pal-CoA synthesis completely at 100 µM (Fig. 3a). Similarly, the same concentration of Br-Pal was sufficient to block acyl-CoA synthesis on isolated
vacuoles (Fig. 3b), demonstrating that the synthesis of Pal-CoA is blocked by Br-Pal. In addition, Br-Pal also interferes with the transfer of fatty acids. When Vac8-GST was incubated with the detergent extract and [3H]Pal-CoA, Br-Pal inhibited acylation of Vac8p completely (Fig. 3c). Thus, Br-Pal inhibits both Pal-CoA synthesis and fatty acid transfer. If Br-Pal would block the transfer by possibly binding to a Pal-CoA binding pocket within the acyltransferase, we would expect that the PAT should be able to discriminate between Pal-CoAc binding pocket within the acyltransferase, we would expect that the PAT should be able to discriminate between Pal-CoAbindingpocketwithintheacyltransferase,wewould 

The mean of four experiments including the standard error is shown. b: TLC analysis. Vacuole fusion reactions were labeled with [3H]palmitate for 15 min in the absence or presence of Br-Pal. Lipids were extracted and analyzed by TLC and fluorography. PL = phospholipids, FA = free fatty acids. c: Inhibition of acyltransferase activity by Br-Pal. The Triton extract (7.5 µg) was incubated with 5 µg Vac8-GST and increasing concentrations of Br-Pal (1 µl of a 100× stock solution in ethanol) for 30 min at 30°C.

Fig. 3. Br-Pal and cold acyl-CoAs inhibit fatty acid transfer. a: Inhibition of acyl-CoA synthetase by Br-Pal. Purified acyl-CoA synthetase was incubated with [3H]palmitate and increasing concentrations of Br-Pal. Non-converted [3H]palmitate was then extracted with Triton X-100 and synthesized [3H]Pal-CoA was counted (see Section 2 for details). The vacuolar PAT shows 10-fold higher specificity for Pal-CoA compared to other activated fatty acids, e.g. Myr-CoA and Stear-CoA. The combination of these data excludes that we are dealing with an uncatalyzed, non-enzymatic reaction, which has been described for some proteins in vitro [3,29].

Identification of palmitoyltransferases has been unsuccessful due to the instability of the enzyme. Recently, proteins of the DHHC-cysteine-rich domain proteins (DHHC-CRD) have been implicated as acyltransferases for palmitoylation at the C-terminus of target proteins. Erf2p (in a complex with Erf4p) has been implicated as acyltransferase for palmitoylation at the C-terminus of target proteins. Erf2p (in a complex with Erf4p) [7,8]. In vitro reconstitution and mutations of the histidine or cysteine residues of Erf2p and Akr1p suggest a direct role of these proteins in acylation. Erf4p may act as a chaperone for Erf2p [7]. Similarly, Sec18p might cooperate with the vacuolar PAT to promote palmitoylation. The identification of the vacuolar PAT may provide details of this interaction.

Interestingly, the Erf2/4 complex is specific for Ras2p and only little acylation is observed with N-myristoylated Gpa1p, the α-subunit of the trimeric GTPase of the yeast plasma membrane. Likewise, acylation of Ras2p is impaired in cells lacking Akr1p. Furthermore, Erf2/4 localize to the ER and Akr1p to the Golgi and endosomes. In yeast, seven DHHC proteins have been identified by sequence comparison. All are non-essential, though it is possible that they have overlapping function. No DHHC-CRD protein seems to be required for maintaining vacuole morphology [30]. In contrast, yeast cells carrying a mutant of Vac8p with alanine substitutions for the palmitoylated cysteines have the same multilobed vacuoles observed for cells lacking Vac8p altogether. We therefore consider it unlikely that a DHHC family member is identical to the vacuolar PAT.

Vac8p is a myristoylated protein required for vacuole fusion and inheritance [20–23,31]. We have previously shown that acylation of Vac8p is an early event in vacuole fusion and inhibited by antibodies to Sec18p. A portion of endogenous Vac8p is associated with the vacuolar SNARE complex, and Vac8p is separated from the SNAREs during Sec18p/ATP-dependent priming [19]. The actual function of Sec18p is, however, unclear in this reaction, since palmitoylation is not ATP-dependent (Dietrich et al., submitted). Only recently, a novel function of the Sec18p homolog NSF has been described. NSF activates fusion of post-mitotic Golgi membranes in an ATP hydrolysis-independent manner [32], by associating with the SNARE GOS-28 together with GATE-16 and α-SNAP prior to fusion [33]. Additional functions of NSF in the activation of the glutamate receptor at the post-

Fig. 4. Lipid specificity of the vacuolar acyltransferase. Triton extracts were incubated with [3H]Pal-CoA (20 nM) and cold Myr-CoA, Pal-CoA or Stear-CoA (added from a 100× stock solution in water) as indicated.

4. Discussion

Our study shows that the yeast vacuolar membrane contains a palmitoylating activity (PAT), which can be extracted with detergent, but not with high salt. The latter finding is consistent with all published studies describing a PAT activity [25–28] and indicates that PAT closely associates with the membrane, either via a transmembrane region or by a hydrophobic modification. Our data reveal that the vacuolar PAT fulfills all criteria of an enzyme: (i) it is protease-sensitive, (ii) has a temperature optimum, (iii) modifies its natural substrate Vac8p in a time- and dose-dependent manner and at its previously identified palmitoylation sites, and (iv) the activity is blocked by the palmitoylation inhibitor Br-Pal. The vacuolar PAT shows 10-fold higher specificity for Pal-CoA compared to other activated fatty acids, e.g. Myr-CoA and Stear-CoA. The combination of these data excludes that we are dealing with an uncatalyzed, non-enzymatic reaction, which has been described for some proteins in vitro [3,29].

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synapse are also known [34–37]. We are presently characterizing the acyltransferase to unravel the precise role of Sec18p in this process.

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**References**