ACCELERATED PUBLICATION

The human SNARE protein Ykt6 mediates its own palmitoylation at C-terminal cysteine residues

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The yeast SNARE (soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor) protein Ykt6 was shown to mediate palmitoylation of the fusion factor Vac8 in a reaction essential for the fusion of vacuoles. Here I present evidence that hYkt6 (human Ykt6) has self-palmitoylating activity. Incubation of recombinant hYkt6 with [3H]Pal-CoA ([3H]palmitoyl-CoA) leads to covalent attachment of palmitate to C-terminal cysteine residues. The N-terminal domain of human Ykt6 contains a Pal-CoA binding site and is required for the reaction.

Key words: longin, palmitoylation, palmitoyl-CoA, soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor (SNARE) protein, vesicular trafficking, Ykt6.

INTRODUCTION

Palmitoylation is the attachment of fatty acids to cysteine residues of membrane proteins. The fatty acids can mediate protein–lipid interactions, thereby targeting the modified protein to membranes or membrane subdomains. In contrast with other hydrophobic modifications, i.e., myristoylation or isoprenylation, palmitoylation is often a dynamic event, with cycles of acylation and deacylation [1]. The enzymology of protein palmitoylation is poorly understood. Several enzymic activities have been characterized, but none of them was purified to homogeneity [2–4]. It has even been questioned whether enzymes were required for palmitoylation. Several proteins can be palmitoylated in vitro simply by incubating them with Pal-CoA (palmitoyl-CoA), which serves as an acyl donor in the palmitoylation reaction [5–7]. This non-enzymic palmitoylation often occurs on the same cysteine residues that are targets for palmitoylation in vivo. Recently, progress in this field was obtained in yeast with a genetic screen. 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 [8,9].

Pal-CoA is furthermore of interest because it has a stimulatory effect on vesicular transport [10,11]. This effect was attributed to protein palmitoylation, but only recently did myself and others identify the fusion factor Vac8 as the first substrate of palmitoylation by simply incubating them with Pal-CoA (palmitoyl-CoA), which serves as an acyl donor in the palmitoylation reaction [5–7]. This non-enzymic palmitoylation often occurs on the same cysteine residues that are targets for palmitoylation in vivo. Progress in this field was obtained in yeast with a genetic screen. 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 [8,9].

Ykt6 belongs to the family of SNAREs (soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptors), which are essential for vesicular trafficking in yeast and higher organisms. Fusion of vesicles with the membrane is driven by complex formation of cognate SNARE proteins [15,16]. Ykt6, the most conserved SNARE protein, is unique because it is present in the cytosol as well as on membranes. It is required for multiple transport steps, e.g., trafficking to and within the Golgi, to the yeast vacuole and for vacuole fusion. Ykt6 has a wide tissue distribution, and appears to have a specialized function in neuronal cells [17–20].

Ykt6 consists of the C-terminal SNARE motif and a regulatory N-terminal domain, which classifies Ykt6 as a member of the longin group of v (vesicular)-SNAREs [21]. The crystal structure of the N-terminal domain of Ykt6 shows the unusual hydrophobic surface that has been proposed to fold back and interact with the SNARE motif, thereby preventing its participation in SNARE complex formation [22].

In contrast with most other SNARE proteins, Ykt6 does not contain a transmembrane region, but a C-terminal CAAX box which is predicted to be farnesylated [23]. However, farnesylation alone is not sufficient to confer membrane binding of intrinsically hydrophilic proteins. In the case of the Ras protein, which is best characterized in this regard, additional sequences located in the vicinity of the CAAX-box are required for stable membrane anchorage. These are either basic amino acids or one or more cysteine residues, which are reversibly palmitoylated. Farnesylation at the CAAX box occurs co-translationally and is followed by proteolysis of AAX, carboxymethylation and palmitoylation [24,25].

Stimulated by the observation that Ykt6 also contains a conserved cysteine residue in the vicinity of the CAAX box and by the described palmitoylating activity of the protein, I have analysed whether hYkt6 (human Ykt6) might catalyse its own palmitoylation.

METHODS

Molecular biology

The hYkt6 gene was obtained from Dr James McNew (Department of Biochemistry and Cell Biology, Rice University, Houston, TX, U.S.A.). It was amplified by PCR using an N-terminal primer.

Abbreviations used: (v-)SNARE, (vesicular) soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor; Br-Pal, 2-bromopalmitate; DHHC-CRD proteins, polytopic membrane proteins with the sequence DHHC and a cysteine-rich domain; DTT, dithiothreitol; F42E, Phe42 → Glu mutation; hYkt6, human Ykt6; MEGA-8, octanoyl N-methylglucamide; NSF, N-ethylmaleimide-sensitive factor; Pal-CoA, palmitoyl-CoA.

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equipped with a SceI site and a C-terminal primer equipped with a PsiI site. The N-terminal domain of hYkt6 was created with a C-terminal primer in which a stop codon was inserted after Thr140. The cysteine mutants were constructed with PCR using C-terminal primers to convert Cys194, Cys195, or both, into serine. The PCR products were cloned into the plasmid pQE30, resulting in proteins with the amino acids MRGSHHHHHHHGSAE upstream of the start codon of hYkt6. The C-terminal domain of hYkt6 was created with an N-terminal primer equipped with a BamHI site and a C-terminal primer equipped with an Xhol site. The PCR product was cloned into plasmid pGEX-6P-1. Expression of the SNARE domain started at Ala136. The F42E (Phe42 → Glu) mutant of hYkt6 was constructed with the QuickChange® Site-Directed Mutagenesis Kit (Stratagene).

Fusion of hYkt6

PQE plasmids were transformed into M15(pREP4) cells (Qiagen) and pGEX-plasmids into BL-21 cells. Purification of hYkt6 on NNTA (Ni2+-nitrilotriacetic acid)–agarose or glutathione–Sepharose was performed as described in [7], with the following buffers: sonication buffer (50 mM Tris/HCl/300 mM NaCl/1 mM PMSF), wash buffer (50 mM Tris/HCl/300 mM NaCl/20 mM imidazole) and elution buffer (20 mM Tris/HCl/300 mM NaCl, containing 250 mM imidazole or 10 mM glutathione). Proteins were desalted using PD-10 columns (Pharmacia) with 20 mM Tris/HCl (pH 6.8)/120 mM KCl or 20 mM Pipes/KOH (pH 6.8)/120 mM KCl as eluent.

Affinity chromatography

A 100 µl portion of immobilized Cibacron Blue F3GA (Pierce) or Pal-CoA–agarose (Sigma) were equilibrated with 20 mM Pipes/KOH (pH 6.8)/120 mM KCl. The beads were then incubated with 40 µg of hYkt6 for 1 h at 4°C. Beads with bound protein were pelleted (3 min, 500 g) and the supernatant (containing unbound proteins) was removed. Beads with bound proteins were then resuspended in 20 mM Pipes/KOH (pH 6.8)/120 mM KCl, again pelleted, and the supernatant was removed (wash fractions). Washing of the beads was repeated eight times. The pelleted beads were then incubated with different CoA compounds, as indicated, for 15 min at 4°C. Beads were yet again pelleted, and the supernatant (eluate) was removed. Elution with CoA compounds was repeated once. Comparable aliquots of the input material, of the unbound proteins, of several wash fractions and of the eluates were analysed by SDS/PAGE and silver staining.

Palmitylination assay

[1H]Pal-CoA, prepared as described in [6], was dried in a Savant SpeedVac vacuum concentrator and dissolved in 20 mM Pipes (pH 6.8)/120 mM KCl/0.1 % Triton to yield a final sp. radioactivity of 100 000 c.p.m./µl. The standard palmitylation reaction mixture contained hYkt6 (20 µg, 8 µg, 3.2 µg, 10 µl of [1H]Pal-CoA (10 pmol, 100 nM), 1 mM DTT (dithiothreitol) (in a final volume of 100 µl of Tris/NaCl buffer [20 mM Tris/HCl (pH 8.5)/120 mM NaCl] and was incubated for 60 min at 30°C. Deviations from these standard conditions are indicated in the Figure legends. A 1 ml portion of chloroform/methanol (1:1, v/v) was added to the samples, and precipitated proteins were pelleted (15 min, 14000 g). Pellets were washed with 1 ml of methanol, air-dried, then resuspended in 20 µl of non-reducing SDS/PAGE sample buffer and boiled for 5 min. Samples were subjected to SDS/PAGE, and the gels were then stained with Coomassie Brilliant Blue and subjected to fluorography [7].

RESULTS AND DISCUSSION

His6-tagged hYkt6 purified from Escherichia coli (Figure 1A) was incubated with [1H]Pal-CoA. Samples were then extracted with chloroform/methanol and were subjected to SDS/PAGE and fluorography. Palmitylation of hYkt6 does not occur on ice, but requires physiological temperatures (Figure 1B). Acylation proceeds rapidly, with 50 % of total hYkt6 acylation being completed after 10 min of labelling (Figure 1C). Several compounds were tested to see whether they could stimulate the palmitylation reaction. Neither calcium (Figure 1D) nor NSF (N-ethylmaleimide-sensitive factor) (Figure 1E), regulators of many SNARE-dependent fusion events, had a significant effect on palmitylation of hYkt6. Heating of hYkt6, as well as inclusion of SDS and urea in the reaction buffer, inhibits palmitylation, which is evidence for the specificity of the reaction (Figure 1F). Liquid-scintillation counting of the radioactivity in excised bands revealed that...
approx. 1% of the Ykt6 molecules were acylated under optimal conditions. This might indicate that more efficient palmitoylation requires cofactors, e.g. lipids or other proteins, which would stimulate the reaction in vivo. Substoichiometric acylation of their substrate proteins were also reported for members of the DHHC-CRD family [8,9] and for other in vitro palmitoylation reactions [2–4].

The next set of experiments was performed to identify the palmitoylation site of hYkt6. Palmitoylation of proteins occurs on cysteine residues, which must be present in their reduced form. The N-terminal domain of hYkt6 binds Pal-CoA at a site distinct from its palmitoylation site. Myself and others have recently demonstrated, with a filter-binding assay, that the N-terminal domain of yeast Ykt6 binds Pal-CoA [14]. hYkt6 protein shows chromatographic properties that are consistent with this observation. The N-terminal domain of hYkt6 binds Pal-CoA without prior farnesylation [28].

The following experiments were performed to analyse whether acylation of hYkt6 occurs by a spontaneous (chemical) or an enzymic (autocatalytic) mechanism. Assuming an enzymic mechanism, hYkt6 should contain a binding pocket for Pal-CoA at a site distinct from its palmitoylation site. Myself and others have recently demonstrated, with a filter-binding assay, that the N-terminal domain of yeast Ykt6 binds Pal-CoA [14]. hYkt6 protein shows chromatographic properties that are consistent with this observation. The N-terminal domain of hYkt6 binds Pal-CoA without prior farnesylation [28].

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Likewise, adding Br-Pal (2-bromopalmitate), an established inhibitor of protein palmitoylation, also blocked acylation of Ykt6 at the same concentration. In contrast, palmitate had only a marginal effect on the palmitoylation reaction (Figure 4C). Inside cells, it is feasible that Br-Pal can be converted into a CoA derivative and subsequently transferred to proteins [13, 29]. However, under the experimental conditions I used, activation of Br-Pal, and thus competition with [3H]Pal-CoA in a chemical acylation reaction, can be excluded. It is more likely that Br-Pal, but not palmitate, binds to the Pal-CoA binding site of hYkt6. Indeed, Br-Pal inhibited binding of hYkt6 to immobilized Cibacron Blue (Figure 4D). Furthermore, replacing the N-terminal domain of hYkt6 with glutathione S-transferase almost completely abolished palmitoylation (Figure 4E). Thus palmitoylation of hYkt6 is dependent on its N-terminal domain, which provides further evidence for an enzymic mechanism for the reaction.

My observation with purified hYkt6 is likely to be of physiological relevance. Recent results from Rothman and co-workers [30] showed that hYkt6 is palmitoylated at Cys149 inside cells. Palmitoylation confers stable membrane binding to hYkt6, by which vesicular transport through the Golgi is stimulated. The F42E mutant of hYkt6, representing the open conformation of hYkt6, is the preferred substrate for palmitoylation in vivo. In my assay, [F42E]hYkt6 is also palmitoylated, but with slightly lower efficiency compared with the wild-type protein (Figure 4F). Thus enhanced palmitoylation of [F42E]hYkt6 in vivo is not an intrinsic property of the protein, but is rather dependent on other factors such as interacting proteins. Considering the participation of hYkt6 in many vesicular trafficking reactions it is important to identify components which regulate its autoacylation.

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REFERENCES

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