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A carboxyl-terminal cysteine residue is required for palmitic acid binding and biological activity of the *ras*-related yeast *YPT1* protein

Constance M.T.Molenaar, Reinhild Prange and Dieter Gallwitz

Max Planck Institute for Biophysical Chemistry, Department of Molecular Genetics, PO Box 2841, D-3400 Göttingen, FRG

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The *Saccharomyces cerevisiae YPT1* gene codes for a *ras*-like, guanine nucleotide-binding protein which is essential for cell viability. The functional significance of two consecutive cysteines at the very carboxyl-terminal end of this protein and in *ypt* homologues of other eukaryotic species was examined. *YPT1* gene mutations were generated that either led to substitutions by serine or the deletion of one or both C-terminal cysteines. The consequences of the mutations were checked in cells after replacing the wild type with the mutant genes. It was found that as long as one of the cysteines was retained, the protein was fully functional. The *YPT1* protein could be labelled with [^{3}H]palmitic acid that appeared to be bound in an ester linkage. The wild-type protein was evenly distributed between soluble and membrane-associated proteins, the palmitoylated form was predominantly in the crude membrane fraction. The mutant protein lacking the C-terminal cysteines was not palmitoylated and was exclusively found in the soluble fraction. The extension by three residues, −Val−Leu−Ser, generating a *ras*-typical C-terminal end, did not interfere with the mutant *YPT1* protein's function although it resulted in a reduced labelling with palmitic acid.

Key words: palmitoylation/*ras*-related/*Saccharomyces cerevisiae*/yeast/*ypt1* protein

Introduction

The oncoproteins of the mammalian *ras* gene family are thought to be involved in signal transduction and cell proliferation. Prerequisite for proper functioning is their ability to bind and hydrolyze GTP and to attach to the inner surface of the plasma membrane (Barbacid, 1987 for review). Stable membrane association seems to be the result of a post-translational modification whereby palmitic acid becomes bound via a thioester linkage to a conserved cysteine located four residues from the carboxyl-terminal end in all *ras* proteins (Sefton *et al.*, 1982; Willumsen *et al.*, 1984; Chen *et al.*, 1985; Buss and Sefton, 1986).

In the yeast *Saccharomyces cerevisiae*, RAS1 and RAS2 proteins, which are highly analogous to the mammalian *ras* proteins, are likewise palmitoylated at their similarly positioned carboxyl-terminal cysteine residues (Fujiyama and Tamanoi, 1986; Deschenes and Broach, 1987). If this modification is prevented, either by inactivation of an enzyme involved in fatty acid acylation (Powers *et al.*, 1986) or by mutation of the relevant cysteine residue (Deschenes and Broach, 1987), yeast *RAS* proteins are no longer bound to membranes and lose biological activity.

The *ras*-related *YPT1* protein, which was first discovered in the yeast *S.cerevisiae* (Gallwitz *et al.*, 1983) but has recently also been found in mammalian cells (Haubruck *et al.*, 1987; Touchot *et al.*, 1987), the slime mold *Dictyostelium discoideum* (A.Kimmel, personal communication) and the fission yeast *Schizosaccharomyces pombe* (our unpublished data), is characterized by two consecutive cysteine residues residing at the very carboxyl-terminal end. The ubiquity of *ypt* proteins in eukaryotic cells and the remarkable conservation of the two C-terminally located cysteines prompted us to investigate the possible role of this structural feature for the functioning of these guanine nucleotide-binding proteins.

The yeast *YPT1* protein is essential for cell viability (Schmitt *et al.*, 1986). A detailed mutational analysis of the single yeast *YPT1* gene was therefore undertaken which allowed to easily test for an impairment of protein function *in vivo* after replacing the wild-type with the different mutant genes that were altered in the region coding for the carboxyl-terminal end. Our studies show that at least one of the two C-terminal cysteine residues is required for palmitic acid binding, membrane attachment and biological function of the protein.

Results

At least one of the two C-terminal cysteine residues is required for YPT1 protein function

To investigate a possible functional significance of the two consecutive cysteines at the C-terminal end of the yeast *YPT1* protein, one or both residues were either deleted or exchanged for serine (Table I). Codon changes were created by site-directed mutagenesis using synthetic oligonucleotides. The wild-type *YPT1* gene on chromosome VI was then exchanged with the mutant genes by homologous recombination in diploid leucine-auxotrophic yeast cells. As described previously (Schmitt *et al.*, 1986), cells were transformed with linear DNA fragments carrying a particularly mutated *YPT1* gene, the yeast *LEU2* gene as selectable marker and genomically-derived sequences flanking the *YPT1* gene. Transformants heterozygous for wild-type and mutant *YPT1* gene, verified by Southern analysis using mutant oligonucleotides, were finally sporulated to search for the viability of mutant cells.

As outlined in Table I, the deletion of the two cysteines or their substitution for serine residues resulted in a complete loss of *YPT1* protein function: similar to a *YPT1* gene disruption (Schmitt *et al.*, 1986) spores carrying the respective mutant genes could often germinate but were unable to go through more than one division. In contrast, the substitution for serine of either one of the two cysteine residues or the deletion of only one cysteine led to perfectly functional

Table I. Mutations at the carboxyl-terminal end of the *YPT1* protein and their effects on cell viability

Protein	C-terminal sequences and mutations[a]												Effect of mutations on cell growth
	200												
YPT1^{WT}	– Asn	Thr	Gly	Gly	Gly	**Cys**	**Cys**	*					
	– AAC	ACC	GGT	GGG	GGC	TGC	TGT	TGA	–				viable
YPT1$^{\Delta 202-206}$	– Asn	Thr	*										
	– AAC	ACC	TGA	GGG	GGC	TGC	TGT	TGA	–				lethal
YPT1$^{\Delta 205.206}$	– Asn	Thr	Gly	Gly	Gly	*							
	– AAC	ACC	GGT	GGG	GGC	TAA	TGT	TGA	–				lethal
YPT1$^{\Delta 206}$	– Asn	Thr	Gly	Gly	Gly	**Cys**	*						
	– AAC	ACC	GGT	GGG	GGC	TGC	TGA	TGA	–				viable
YPT1^{Ser205}	– Asn	Thr	Gly	Gly	Gly	Ser	**Cys**	*					
	– AAC	ACC	GGT	GGG	GGC	TCA	TGT	TGA	–				viable
YPT1^{Ser206}	– Asn	Thr	Gly	Gly	Gly	**Cys**	Ser	*					
	– AAC	ACC	GGT	GGG	GGC	TGC	AGT	TGA	–				viable
YPT1$^{Ser205.206}$	– Asn	Thr	Gly	Gly	Gly	Ser	Ser	*					
	– AAC	ACC	GGT	GGG	GGC	AGC	AGT	TGA	–				lethal
YPT1^{Ext}	– Asn	Thr	Gly	Gly	Gly	**Cys**	**Cys**	Val	Leu	Ser	*		
	– AAC	ACC	GGT	GGG	GGC	TGC	TGT	GTC	CTG	AGT	TGA	–	viable

[a]Transitions and transversions generated by site-directed mutagenesis to result in stop or serine codons are underlined. Translation termination codons are indicated by asterisks.

proteins. Cells expressing these mutant proteins had growth properties identical to wild-type cells.

Deletion or substitution of the C-terminal cysteine residues did not affect the guanine nucleotide-binding capacity of the mutated proteins (data not shown).

Deletion of both C-terminal cysteines interferes with membrane attachment of the YPT1 protein

In analogy to other GTP/GDP binding regulatory proteins, like G and *ras* proteins, the *YPT1* gene product might serve its function in a membrane-bound state.

We examined the distribution of wild-type *YPT1* protein and the mutant form lacking the C-terminal five residues in cellular extracts that were separated by centrifugation into soluble and crude membrane fractions. Soluble proteins and proteins extracted with 1% Triton X-100 and 0.5% sodium desoxycholate (DOC) from a washed membrane fraction were separated by SDS–PAGE on a 25 cm long gel, transferred to a nitrocellulose filter and reacted with a polyclonal antibody directed against an MS2 polymerase/*YPT1* fusion protein (Schmitt *et al.*, 1986; Wagner *et al.*, 1987). As shown in Figure 1, the 23.5 kd *YPT1* protein (b, in lanes 1–4) seems to be evenly distributed between soluble and membrane-attached proteins whereas a cross-reacting protein with an apparent mobility of ~24.5 kd, presumably the *YPT1*-related *SEC4* gene product (Salminen and Novick, 1987), was only identified in the crude membrane fraction (a, lanes 2 and 4). Most importantly, the unfunctional mutant *YPT1* protein lacking the C-terminal residues (c, lane 3) was exclusively in the fraction of soluble proteins.

This result clearly indicates that the C-terminal cysteine residues or the last five amino acids are required for membrane attachment and further suggests that the intimate contact of the *YPT1* protein with some cellular membrane is a necessity for the protein's activity.

The YPT1 protein is palmitoylated

Mutation or deletion of the cysteine residue four amino acids

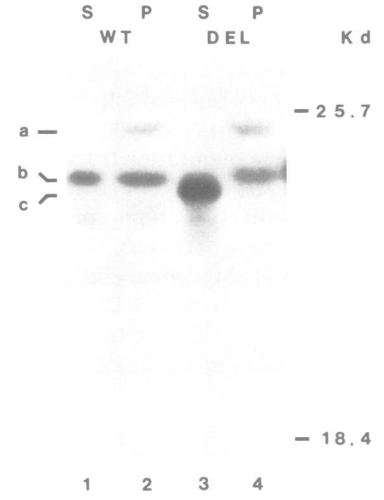

Fig. 1. Distribution of wild-type and mutant *YPT1* proteins in yeast cells. Extracts from exponentially growing yeast cells were separated into crude membrane (P, **lanes 2** and **4**) and soluble (S, **lanes 1** and **3**) fractions and subjected to SDS–PAGE. Proteins were transferred to a nitrocellulose filter and reacted with affinity-purified antibody against an MS2 polymerase/*YPT1* fusion protein, followed by ^{125}I-labelled anti-rabbit IgG. Diploid strains were either homozygous for the wild-type *YPT1* gene (WT, **lanes 1** and **2**) or carried the wild-type *YPT1* gene on one chromosome VI and the mutant *YPT1*$^{\Delta 202-206}$ gene (see Table I) on the other (DEL, **lanes 3** and **4**); both genes were under the transcriptional control of the *YPT1* promoter. ^{14}C-labelled protein markers were used as molecular weight standards. Bands a, b and c are referred to in the text.

from the carboxyl-terminus of mammalian *ras* proteins and the yeast *RAS2* protein prevents fatty acid acylation and membrane association of these proteins (Willumsen *et al.*, 1984; Deschenes and Broach, 1987). From the results in the foregoing section it seemed likely that modification by fatty acid binding to a C-terminally located cysteine could play an important role for the attachment of the *YPT1* protein to membranes.

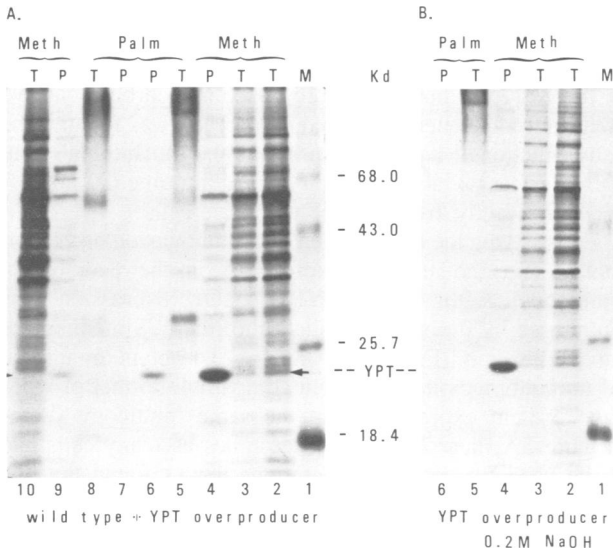

Fig. 2. Fatty acid acylation of wild-type *YPT1* protein. A haploid *pep4/leu2* strain (**lanes 7–10**) and the same strain transformed with the multicopy vector pAAH5-YPT (**lanes 2–6**) were labelled with [^{35}S]methionine (**lanes 2, 3, 4, 9** and **10**) or [^{3}H]palmitic acid (**lanes 5–8**). Extracts were prepared (2–6, in duplicate) and either used directly (**lanes 2, 3, 5, 8** and **10**) or treated with *YPT1*-specific immune sera. Extracts from [^{35}S]methionine-labelled cells were diluted 1 to 100 with identically prepared extracts of non-labelled cells of the appropriate yeast strain in order to obtain comparable amounts of protein. Thereby, total extracts and immunoprecipitates originate from 0.1 ml (**lanes 3, 5** and **8**), 0.2 ml (**lanes 2** and **10**), 5 ml (**lanes 4** and **9**) or 10 ml (**lanes 6** and **7**) of yeast culture. In **lane 1**, ^{14}C-labelled marker proteins with indicated molecular weights were separated. Samples 1 to 6 were loaded twice on the gel. After electrophoresis, part of the gel was soaked in 20% methanol (**A**), the other part in 0.2 M NaOH/20% methanol (**B**). The fluorograms shown are the result of film exposure for one week (**A**) or three weeks (**B**).

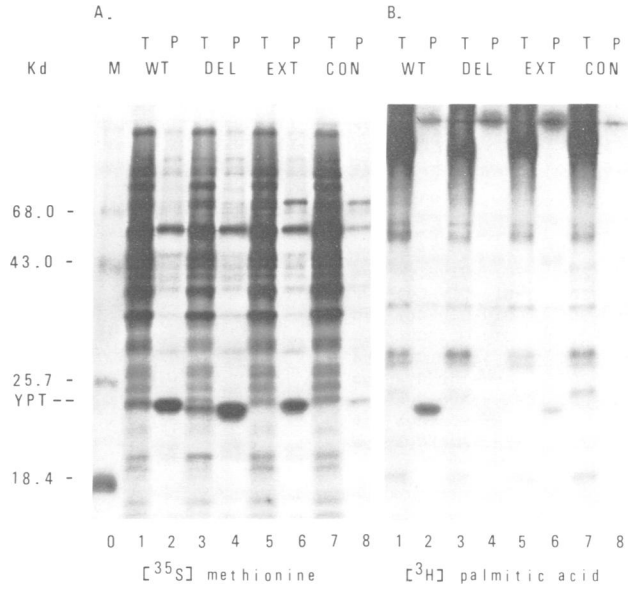

Fig. 3. Fatty acid acylation of wild-type and mutant *YPT1* protein. A haploid *pep4/leu2* strain (**lanes 7** and **8**) and the same strain transformed with either the plasmids pAAH5-*YPT1*^{Ext} (**lanes 5** and **6**), pAAH5-*YPT1*$^{\Delta 202-206}$ (**lanes 3** and **4**) or pAAH5-YPT (**lanes 1** and **2**) were labelled with [^{35}S]methionine (**A**) or [^{3}H]palmitic acid (**B**). Extracts were prepared and either used directly (**lanes 1, 3, 5** and **7**) or treated with *YPT1*-specific immune sera (**lanes 2, 4, 6** and **8**). [^{35}S]methionine-labelled proteins were diluted 1 to 100 (see legend to Figure 1). Total cell extracts and immunoprecipitates originated from 0.1 ml [**lanes 1, 3, 5** and **7**, in (**B**)], 0.2 ml [**lanes 1, 3, 5** and **7**, in (**A**)], 5 ml [**lanes 2, 4** and **8**, in (**A**)], 10 ml [**lanes 2, 4** and **8**, in (**B**)], 15 ml [**lane 6**, in (**A**)] and 30 ml [**lane 6**, in (**B**)] of yeast culture. Molecular weights of ^{14}C-labelled marker proteins (**lane 0**) are indicated. The polyacrylamide gel was soaked in 20% methanol before treatment with DMSO and DMSO/PPO. The fluorograms in (**A**) and (**B**) were obtained by film exposure for 10 days and 5 weeks, respectively.

To test this, a protease-deficient *pep4* strain harbouring the 2 μ plasmid-based recombinant vector pAAH5-YPT (Schmitt *et al.*, 1986) to overproduce the yeast *YPT1* protein, was labelled with [^{3}H]palmitic acid and, in parallel, with [^{35}S]methionine. The strain without plasmid was radioactively labelled likewise. Total protein isolated in the presence of detergents after a 2-h labelling period was used to precipitate the *YPT1* protein with the affinity-purified *YPT1* antibody mentioned above. Immunoprecipitates (P) and total protein (T), either labelled with palmitic acid or methionine, were separated by SDS–PAGE and subjected to autoradiography (Figure 2A). In comparing lanes 2–4 and 9,10 it is evident that already among the total protein of overproducing cells the methionine-labelled *YPT1* protein became easily visible on the autoradiogram. Immunoprecipitation with saturating amounts of antibody revealed the difference of ^{35}S-labelled *YPT1* protein in normal and overproducing cells (lanes 4 and 9).

The pattern of palmitic acid-labelled proteins (lanes 5 and 8) resembled that described previously by Wen and Schlesinger (1984) and Powers *et al.* (1986): only a limited number of proteins was labelled. Immunoprecipitation with *YPT1* antibody, however, allowed to clearly identify radioactively labelled *YPT1* protein in overproducing cells (lane 6). No labelled band was seen in normal cells (lane 7). We concluded from this result that the *YPT1* protein was indeed palmitoylated and sought to examine whether the fatty acid was bound to the protein via an amide or an ester linkage. Since ester bonds are alkali-labile, the methionine- and palmitic acid-labelled proteins, separated by SDS–PAGE, were treated with 0.2 N NaOH/20% methanol. Part of the same acrylamide gel with identical samples seen in Figure 2A (lanes 1 to 6) was directly soaked in alkali and, after drying, was subjected to autoradiography (Figure 2B). The swelling of the gel in water after treatment in the alkaline solution led to a broadening of protein bands which required a longer film exposure time to obtain the autoradiogram in Figure 2B, which has to be compared to that seen in part A of this figure. Although the intensity of all radioactive bands in Figure 2B is weaker, it is evident that the signals from the palmitic acid-labelled proteins (lanes 5 and 6) almost vanished. It seems likely therefore that the fatty acid is bound to the *YPT1* protein via an ester linkage.

By fractionating a cellular extract into a soluble and a crude membrane fraction as described in the previous section, we found that the palmitic acid-labelled *YPT1* protein was nearly exclusively associated with the membrane fraction (data not shown).

Fatty acid is bound to a C-terminal residue

The loss of *YPT1* protein function and its inability for membrane attachment caused by deletion of the C-terminal cysteine residues suggested that palmitic acid is linked to one or both of these cysteines and mediates the association of the protein with a cellular membrane, similar to the situation with *ras* proteins.

	RAS		
183-189	-Ser-Cys-Lys-Cys-Val-Leu-Ser	Human	$c\text{-}ras^H$
183-189	-Ile-Lys-Lys-Cys-Ile-Ile-Met	Human	$c\text{-}ras^K$
180-186	-Lys-Lys-Gln-Cys-Leu-Ile-Leu	*Dictyost.*	*ras1*
216-219	-Thr-Lys-Cys-Cys-Val-Ile-Cys	*S.pombe*	*ras1*
303-309	-Gly-Gly-Cys-Cys-Ile-Ile-Cys	*S.cerev.*	*RAS1*
316-322	-Gly-Gly-Cys-Cys-Ile-Ile-Ser	*S.cerev.*	*RAS2*
	YPT		
203-206	-Gly-Gly-Cys-Cys	*S.cerev.*	*YPT1*
202-205	-Gly-Gly-Cys-Cys	Mouse	*ypt1*
202-205	-Gly-Gly-Cys-Cys	Rat	*rab1*
209-212	-Gly-Gly-Cys-Cys	Rat	*rab2*
205-208	-Lys-Ala-Cys-Cys	*Dictyost.*	*ypt1*
200-203	-Asn-Thr-Cys-Cys	*Dictyost.*	*ypt2*
197-200	-Lys-Arg-Cys-Cys	*S.pombe*	*ypt1*
211-214	-Ser-Gln-Cys-Cys	*S.pombe*	*yho1*
212-215	-Ser-Asn-Cys-Cys	*S. cerev.*	*SEC4*

Fig. 4. Comparison of carboxyl-terminal sequences of *ras* and *ypt* proteins from different eukaryotes. Sequence data are from Capon *et al.* (1983; *c-H-ras*), McGrath *et al.* (1983; *c-K-ras*), Reymond *et al.* (1984; *Dictyostelium discoideum ras1*), Fukui and Kaziro (1985; *S.pombe ras1*), Powers *et al.* (1984; *S.cerevisiae RAS1,2*), Gallwitz *et al.* (1983; *S.cerevisiae YPT1*), Haubruck *et al.* (1987; mouse *ypt1*), Touchot *et al.* (1987; rat *rab1,2*) and Salminen and Novick (1986; *S.cerevisiae SEC4*). Sequences of *Dictyostelium discoideum ypt1* and *ypt2*, *S.pombe yho1* and *S.pombe ypt1* are from A.Kimmel and M.Yamamoto (personal communications) and our own unpublished data. Mouse *ypt1* and rat *rab1* proteins have identical sequences.

Yeast cells transformed with the vector pAAH5 carrying either the wild-type *YPT1* gene or the mutant gene with an opal mutation in codon 202 (thereby deleting the C-terminal five residues of the protein) were labelled with [^{3}H]palmitic acid or with [^{35}S]methionine. *YPT1* protein was immunoprecipitated and subjected to electrophoresis to identify the palmitoylated protein. In Figure 3, an autoradiogram is presented which shows the label of total (T) and immunoprecipitated proteins (P) derived from cells overproducing the wild-type (lanes 1 and 2) or the mutant *YPT1* protein (lanes 3 and 4). The mutant protein which runs slightly faster can clearly be seen to lack the palmitic acid label (Figure 3B, lane 4). The faint and somewhat diffuse radioactive band visible in the position of the mutant protein might result from the incorporation of amino acids generated from metabolized palmitic acid. We conclude from this finding that in the wild-type *YPT1* protein the fatty acid is bound via an ester linkage to one or both of the cysteine residues at the carboxyl-terminal end of the protein.

The YPT1 protein with a C-terminus typical for ras proteins is functional and becomes palmitoylated

Because of the conspicious conservation of two consecutive cysteines at the C-terminus of *ypt* proteins from different species on the one hand and of the structure Cys-A-A-X (A = aliphatic, X = any residue) found at the carboxyl-terminal end of *ras* proteins on the other (Figure 4), we considered the possibility that these rather typical structural features might play a role for substrate specificity of the two groups of proteins regarding acylating enzymes or for the association of the proteins with membranes of different cellular compartments.

In a first attempt to clarify the significance of the conserved C-terminal sequence of the *YPT1* protein, the gene was mutated such that the protein product was extended by three residues, Val−Leu−Ser, thereby resembling the mammalian *c-ras*^{H} protein with respect to the carboxyl-terminus. This mutant gene, either under the control of the *GAL10* or the *YPT1* promoter, was introduced into chromosome VI by gene replacement and, to our surprise, was found to be fully functional: haploids expressing the mutant gene only were perfectly viable and their generation time did not differ significantly from that of wild-type cells.

For studying the modification by palmitoylation of the mutant protein, the gene was expressed from the recombinant multicopy vector pAAH5-YPT^{Ext}. Overexpression was monitored by immunoprecipitation of the [^{35}S]methionine-labelled protein (Figure 3A, lane 6). To obtain an amount of immunoprecipitated protein comparable to that of wild-type protein expressed from the same multicopy vector (Figure 3A, lane 2), about three times as many cells had to be used for extract preparation. It was found that the *YPT1*^{Ext} mutant protein, despite its extended C-terminus, had a very similar electrophoretic mobility to the wild-type protein. Most importantly, it was clearly labelled with palmitic acid but to a lesser extent than the wild-type protein (compare lanes 2 and 6 of Figure 3B).

Whether the mutant protein, in order to be functionally active, has to be processed by deletion of its three additional residues is still an open question.

Discussion

It has been realized in the past few years that modification by lipids of eukaryotic and viral proteins is rather common and that these modifications might be a means by which proteins attach to cellular membranes (for recent reviews see Sefton and Buss, 1987; Cross, 1987). Fatty acids can be linked to the polypeptide chain either indirectly via ethanolamine bound to a glycosylated phosphatidylinositol moiety or directly by an amide, oxyester or thioester bond. Palmitic acid, generally found in a thioester linkage to a cysteine residue, has been identified to modify *ras* proteins in mammalian cells (Sefton *et al.*, 1982; Buss and Sefton, 1986; Chen *et al.*, 1985) and in yeast (Fujiyama and Tamanoi, 1986; Powers *et al.*, 1986; Deschenes and Broach, 1987).

With the work presented in this report we have demonstrated that the yeast *YPT1* protein, a member of a family of *ras*-like, guanine nucleotide-binding proteins with a highly conserved C-terminus consisting of two consecutive cysteines, is likewise modified by palmitic acid binding. As the binding to the protein of a significant part of the fatty acid was labile to alkaline methanolysis, we conclude that palmitic acid is linked to the *YPT1* protein by an ester bond. Two lines of evidence point to one or both of the C-terminal cysteine residues being the site for palmitoylation. Firstly, the mutant protein lacking the last five amino acids (Gly−Gly−Gly−Cys−Cys) was not labelled with palmitic acid. Secondly, this as well as all other mutant *YPT1* proteins from which both C-terminal cysteines were either deleted or substituted for serine proved to be biologically inactive. This was in contrast to those mutants that had retained either one of the cysteine residues and which were perfectly functional within the cell. As the GTP-binding capacity of the mutant proteins was not altered the conclusion is justified that fatty acid acylation of the *YPT1* protein is a modification essential for the functioning of this protein. The presence of palmitic acid-labelled wild-type pro-

tein in the membrane fraction only and the absence of the nonpalmitoylatable mutant protein from cellular membranes suggest in addition a connection between fatty acid acylation and membrane association. This is reminiscent of yeast and mammalian mutant *ras* proteins with a serine substitution for the conserved cysteine four residues from the carboxyl-terminal end which are no longer substrates for palmitoylation and do not associate with the plasma membrane. (Willumsen *et al.*, 1984; Deschenes and Broach, 1987).

From structural comparisons it seems appropriate to look at *ras* and *ras*-like proteins as different groups or families of guanine nucleotide-binding proteins present in all eukaryotes from yeast to mammals. Besides other structural features, *ras* (Barbacid, 1987, for review) and *rho* proteins (Madaule *et al.*, 1987) on the one hand and *ypt* proteins (Haubruck *et al.*, 1987) on the other have distinct and highly conserved carboxyl-termini (see Figure 4) with cysteine residues required for palmitoylation. This suggested to us that separate enzymes or protein sorting mechanisms might be responsible for acylating members of these families or directing them to different cellular membranes. As shown in this report, however, the yeast *YPT1* protein, when mutated to become *ras*-typical with respect to its C-terminus, i.e. Cys-A-A-X, could fully complement the wild-type *YPT1* protein terminating with a cysteine residue. There still appears to be a notable difference between the yeast *RAS* and *YPT1* proteins with respect to the functional significance of their C-terminally located cysteine residues. As can be seen in Figure 4, like the *YPT1* protein which has two glycine residues preceding the terminal cysteines, the *S.cerevisiae RAS* proteins exhibit an identical structure, two glycines followed by two cysteines in front of the last three residues. However, whereas our studies on the *YPT1* protein show that either one of the two cysteines is able to retain the protein's function, the substitution of cysteine-319 with serine of *RAS2* rendered this protein unfunctional despite the cysteine in position 318 (Deschenes and Broach, 1987). In this context it is worth mentioning that this mutant *RAS2* protein with a serine replacing cysteine-319 allows cellular growth when overexpressed from a plasmid-born *RAS2* gene (Deschenes and Broach, 1987).

There has been an extensive debate regarding the possible processing of mammalian and yeast *ras* proteins. Precursors of these proteins seem to undergo processing on their way to or at the plasma membrane independently of fatty acid modification (Shih *et al.*, 1982; Ulsh and Shih, 1984; Fujiyama and Tamanoi, 1986). Although the acylated *ras* protein appears to have a slightly higher electrophoretic mobility than the fatty acid-free protein, the latter form of the protein still seems to run faster than the precursor on SDS-containing gels (Chen *et al.*, 1985; Buss and Sefton, 1986; Magee *et al.*, 1987). It therefore seems possible, and it has not been excluded yet, that the acylated form of the *ras* proteins is proteolytically processed resulting in the loss of the last three amino acids. The mature form would then resemble the *ypt* proteins with respect to the C-terminus in having a cysteine in the ultimate position. Such a processing step could also be envisaged for the *YPT1*^{Ext} protein carrying the additional tail of three residues. Indeed, a slower migrating form of this mutant protein but not of the wild-type *YPT1* protein was observed in 5-min pulse labelling experiments with [^{35}S]methionine (our unpublished data).

Materials and methods

Oligonucleotide-directed mutagenesis, cloning of mutant genes and transformation of yeast cells

Point mutations of the *YPT1* gene were created in a recombinant M13mp8 phage carrying the *YPT1* gene by a previously described procedure (Schmitt *et al.*, 1986). The following oligonucleotides were used: (i) 5′-CAGCCCCTCAGGTGTTGG-3′; (ii) 5′-CTTGTTCAACATTAGCCCCCACCG-3′; (iii) 5′-GCTTGTTCATCAGCAGCCC-3′ to convert Gly 202, Cys 205 and Cys 206 to a nonsense codon, respectively; (iv) 5′-CTTGTTCAACATGAGCCCCCACCG-3′; (v) 5′-GCTTGTTCAACTGCAGCCCC-3′ to substitute serine for cysteine at positions 205 and 206, respectively, and (vi) 5′-CTTGTTCAACTGCTGCCCCCACCG-3′ to change the cysteines to serine residues at both positions 205 and 206. The insertion of nine base pairs encoding the sequence Val−Leu−Ser was achieved by direct cloning of a chemically synthesized *Hin*dII−*Bss*HII fragment having the desired sequence into the M13mp8 recombinant. The *Bss*HII restriction site five nucleotides 3′ of the translation stop codon, in combination with restriction sites within the protein-coding region, was used to generate fragments from the different phages that were then inserted into the plasmids pYA102-LEU2^{Bam} and pAAH5-YPT (Schmitt *et al.*, 1986) in place of the corresponding 'wild-type' sequences.

The diploid yeast strain DAH2215 (*MATa/MATαYPT1/YPT1 leu2/leu2 his3/+ his4/+*) was transformed with 6.9 kb *Hin*dIII fragments isolated from the different pYA102-LEU2^{Bam} derivatives for insertion of mutant *YPT1* genes into chromosome VI. A haploid *pep4/leu2* strain was transformed with the plasmids pAAH5-YPT carrying the wild-type or mutated *YPT1* genes.

Procedures for cloning, yeast transformation, tetrad analysis, verification of mutants and ascertainment of successful gene replacement were as described previously (Schmitt *et al.*, 1986; Wagner *et al.*, 1987).

Isotopic labelling and preparation of cell extracts

Cells were grown at 30°C to a density of 0.7 at OD_{600} in synthetic medium containing 5% galactose. After adjustment of the medium to pH 6.8, proteins were labelled with [^{35}S]methionine (Amersham; 1065 Ci/mmol; 15 μCi/ml culture) or 9,10[^{3}H]palmitic acid (New England Nuclear; 28.5 Ci/mmol in ethanol; 100 μCi/ml culture) for 2 h. Labelled cells were washed once in ice-cold extraction buffer A (10 mM Tris−HCl, pH 7.4, 100 mM NaCl, 0.1 mM EDTA, 0.1 mM DTT, 1 mM PMSF, 1% Triton X-100, and 1% sodium deoxycholate, DOC). The pellet was frozen at −80°C and lysed in 300 μl of ice-cold buffer A (per 10 ml of cell culture) with 300 μl glass beads by vortexing 12 times in 30 s-bursts followed by chilling on ice. After incubation on ice for 1 h to extract the proteins, the lysate was transferred into an Eppendorf cap by pipetting, avoiding the glass beads that were washed twice with 100 μl buffer B (10 mM Tris−HCl, pH 7.4, 100 mM NaCl). The extract was clarified by centifugation at 14 000 *g* for 10 min.

Cell fractionation and immunoblot analysis

Crude membrane (P100) and soluble (S100) fractions from exponentially growing yeast cells were prepared essentially as described by Fujiyama and Tamanoi (1986). Briefly, cells were lysed in sorbitol buffer without detergents and centrifuged for 30 min at 100 000 *g*. Proteins were extracted from the washed membranes by incubation in phosphate buffer, pH 7.0, containing 1% Triton X-100 and 0.5% DOC. S100 and P100 extracts from ~2 × 10^7 cells were subjected to SDS−PAGE. Immunoblot analysis of the separated proteins was performed as described (Schmitt *et al.*, 1986), using affinity-purified antibodies against the MS2 polymerase/YPT fusion protein.

Immunoprecipitation of proteins and SDS−PAGE

The [^{35}S]methionine-labelled cell extracts were diluted 1 to 100 with identically prepared extracts of nonlabelled cells. Extracts were incubated with affinity-purified antibodies against the MS2 polymerase/*YPT1* fusion protein (Schmitt *et al.*, 1986) for 4 h at 0°C in the presence of 0.5% NP40. Immune complexes were isolated with Pansorbin cells (Calbiochem), washed four times with buffer C (20 mM Tris−HCl, pH 7.4; 150 mM NaCl, 0.5% NP40, 0.5% DOC and 0.1% SDS) and once with 20 mM Tris−HCl, pH 6.8. Total yeast extracts and immunoprecipitates were taken up in SDS- and 2-mercaptoethanol-containing buffer according to Laemmli (1970), boiled for 3 min and subjected to SDS−PAGE.

Treatment of the gels and fluorography

The polyacrylamide gels were soaked in 20% methanol or in 0.2 M NaOH/20% methanol for 2 h at 23°C immediately following electrophoresis. After three 20 min washes with DMSO, the gels were soaked in

DMSO–PPO for 4 h and washed in water for 1 h. The dried gels were exposed to presensitized Kodak XAR-5 films at −70°C.

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