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Rapid report

Targeting of proteins involved in sterol biosynthesis to lipid particles of the yeast *Saccharomyces cerevisiae*

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Abstract

In the yeast *Saccharomyces cerevisiae*, three enzymes of the sterol biosynthetic pathway, namely Erg1p, Erg6p and Erg7p, are located in lipid particles. Whereas Erg1p (squalene epoxidase) is also present in the endoplasmic reticulum (ER) to a significant amount, only traces of Erg6p (sterol C-24 methyltransferase) and Erg7p (lanosterol synthase) are found in the ER. We have chosen these three Erg-proteins as typical representatives of lipid particle proteins to study targeting to their destination. Lipid particle proteins do not contain obvious targeting motifs, but the only common structural feature is the presence of one or two hydrophobic domains near the C-termini. We constructed truncated versions of Erg1p, Erg6p and Erg7p to test the role of these hydrophobic domains in subcellular distribution. Our results demonstrate that lack of the hydrophobic domains prevents at least in part the association of the proteins with lipid particles and causes their retention to the ER. This result strongly supports the view that ER and lipid particles are related organelles.

Keywords: Lipid particle; Endoplasmic reticulum; Sterol; Protein targeting; Yeast

Nearly all organisms, including yeast, plants and mammals, as well as some prokaryotes, have the capacity to store neutral lipids in intracellular lipid droplets. These droplets consist of a highly hydrophobic core of triacylglycerols (TAGs) and/or steryl esters, surrounded by a phospholipid monolayer with only a few proteins embedded [1]. The biogenesis of lipid particles including targeting of proteins to the particles' surface, however, is still poorly understood. Common targeting motifs of the primary sequence were neither found for typical mammalian lipid droplet proteins, such as perilipins or adipose differentiation-related protein (ADRP), nor for polypeptides of plants or yeast, although plants with their oil-body-associated proteins, the so-called oleosins, have been extensively studied for targeting signals. Oleosins were postulated to play a role in preventing aggregation of storage lipids during seed maturation-desiccation [2,3] and in the biogenesis of the oil bodies themselves [4]. One model referring to the latter process suggests that newly synthesized TAGs accumulate in the hydrophobic region of the endoplasmic reticulum (ER) membrane between the two phospholipid layers. Eventually, a droplet of TAGs surrounded by a phospholipid monolayer disconnects and is released into the cytosol [5–7]. The fact that the site of TAG synthesis in plants is the ER [8,9] strongly supports the budding model and made it widely accepted. However, the crucial question remains: how do proteins reach the oil bodies? It was suggested that oleosins are synthesized on membrane-bound ribosomes [10,11] and cotranslationally inserted into microsomal membranes [12–14] in a signal-recognition-particle-dependent manner [15]. Subsequent requirement for a functional Sec61 translocon to mediate the correct insertion of the proteins into the membrane was demonstrated by Beaudoin et al. [14]. Thus, it seems likely that synthesis of oleosins and TAGs occurs within the same subcellular compartment and, moreover, that the process may be coordinated to facilitate the assembly of the oil body [3,4,16,17]. This hypothesis was supported by a study which demonstrated synthesis and occurrence of oleosins in "light" fractions of the ER enriched in TAG-synthesizing enzymes [9]. Oleosins show a characteristic three-domain structure. A

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highly conserved hydrophobic central domain of 72 amino acid residues is flanked by two amphipathic regions of more variable nature [18,19]. The importance of the hydrophobic domain, in particular of the so-called ''proline-knot'', for the correct targeting of oleosins is generally accepted.

Identification of the major proteins of lipid particles from *Saccharomyces cerevisiae* [20] was a prerequisite to study targeting of polypeptides to yeast lipid particles. We have chosen three sterol-synthesizing enzymes, Erg1p, Erg6p and Erg7p, to address this question for two reasons: (i) these three proteins, unlike other enzymes of the sterol biosynthesic pathway, which are associated with the ER [21], are either localized exclusively to lipid particles or distributed between the ER and lipid particles [22–24]; (ii) the fact that most steps of sterol biosynthesis occur in the ER, while some enzymes of this pathway are localized in lipid particles, raises the interesting question as to the collaborative interaction of these two compartments in sterol synthesis.

Following the idea that hydrophobic regions of polypeptides may be responsible for their anchoring to lipid particles, we studied the influence of such C-terminal domains of various lengths on the targeting of Erg1p, Erg6p and Erg7p to lipid particles. The subcellular distribution of the truncated constructs was determined by cell fractionation and Western blot analysis.

The haploid wild-type yeast strain *S. cerevisiae* FY1679 (*MAT**a** ura 3-52 trp1* Δ*63 leu2* Δ*1 his* Δ*200*) and the diploid wild-type strain FY1679 (*MAT**a**/*α *ura 3-52/ura 3-52 leu2* Δ*1/LEU2 his* Δ*200/HIS3 trp1* Δ*63/TRP1*) were used throughout this study. Cells were grown aerobically at 30 °C in YPD medium (1% yeast extract, 2% peptone, 2% glucose) or anaerobically in YPD supplemented with ergosterol and

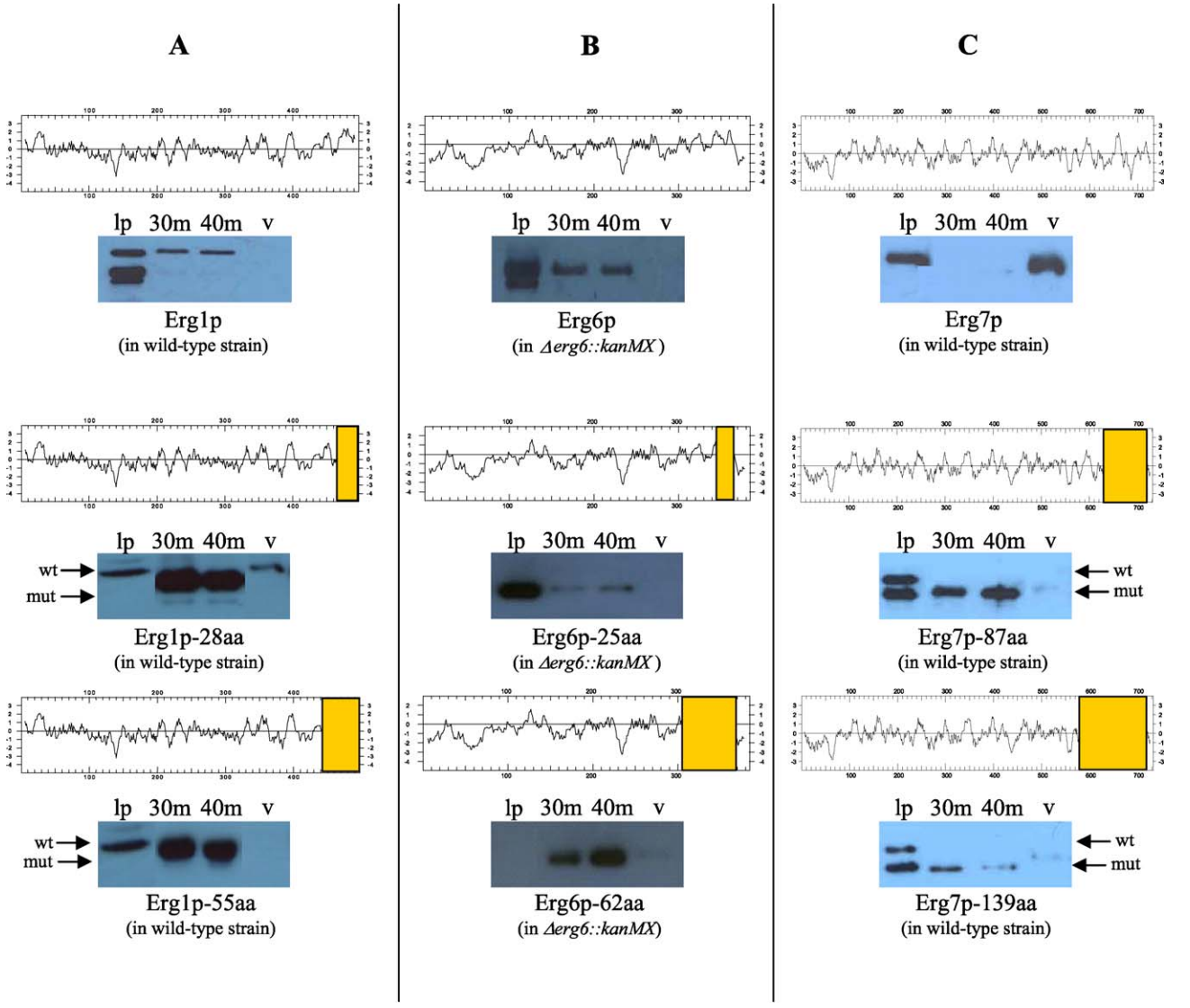

Fig. 1. Subcellular localization of overexpressed full-length proteins and truncated forms of Erg1p (A), Erg6p (B) and Erg7p (C). Positions of the hydrophobic stretches removed in the truncates are marked in gray in the Kyte–Doolittle hydropathy plots. Cell fractionation and Western blot analysis were performed as described in the text. The appearance of bands with lower molecular mass (upper lane) is probably due to degradation of full-length Erg1p and Erg6p when overexpressed. lp: lipid particles; 30m: 30.000 × *g* microsomes; 40m: 40.000 × *g* microsomes; v: vacoules.

Tween 80 [22]. Anaerobic environment was generated by using an AnaeroGen sachet [Oxoid] in a 3.5-l sealed jar.

E. coli XL1-Blue and Top10F served as hosts for plasmids used in this study. Cells were grown in LB medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl) containing 100–200 mg/l ampicillin [Carl Roth]. Yeast transformants bearing recombinant plasmids (Erg1p- and Erg7p-truncates in the FY1679 background and Erg6p-truncates in an Δ*erg6* strain with FY1679 background) were grown on leucine-free minimal medium (YNB).

Kyte–Doolittle hydropathy plots [25] of Erg1p [26], Erg6p and Erg7p (Fig. 1) were used to define the positions of the hydrophobic domains to be removed from the proteins. For the construction of *ERG1* and *ERG6* deletion alleles, PCR products with additional recognition sites introduced either at the 5′ or the 3′ end were used to replace the corresponding full-length DNA fragments of *ERG1* and *ERG6* genes in YEp351. To remove the hydrophobic sequences within Erg7p, gene Splicing by Overlap Extension (SOE) [27] was applied. Primers used for amplification of the DNA fragments for all constructs are listed in Table 1. The correct structure of all constructs was confirmed by DNA sequencing. To test the functionality of Erg1p and Erg7p truncates, *Δerg1* and *Δerg7* mutants were transformed with the respective recombinant plasmids, and transformants were assayed for ergosterol auxotrophy. In the case of Erg6p-truncates, an *Δerg6* mutant transformed with YEp351 encoding the Erg6p-fragments was tested for the occurrence of characteristic sterol intermediates [24]. None of the truncated versions of the three Erg-proteins, however, restored the original function of the enzymes (data not shown).

Microsomes, lipid particles and vacuoles were isolated by published procedures [28]. Protein quantification [29] with modifications for lipid particle proteins [22], SDS-PAGE [30] and Western blot analysis [31] were carried out by published methods. Proteins were detected by ELISA using polyclonal anti-Erg1p, anti-Erg6p and anti-Erg7p antibodies, respectively, and peroxidase-conjugated secondary antibodies.

To pinpoint the role of hydrophobic domains which might direct Erg1p, Erg6p and Erg7p to lipid particles, C-terminally truncated forms of the three Erg-proteins were constructed on the basis of Kyte-Doolittle hydropathy plots and investigated with respect to their subcellular distribution (Fig. 1). Since truncates of the three Erg-proteins were expressed from a high-copy-number vector, their localization was compared to the respective wild-type proteins expressed from the same vector. As can be seen in Fig. 1, removing the C-terminal hydrophobic domains caused at least in part a shift of the truncated proteins from lipid particles to ER fractions. In the case of Erg1p (Fig. 1A), deletion of 28 amino acids was already sufficient to retain squalene epoxidase in the microsomal fractions and to prevent association with lipid particles. Similar results were obtained when 55 amino acids were removed from the C-terminus of Erg1p.

In Erg6p, however, removing one hydrophobic domain (25aa) did not significantly change the polypeptide's subcellular distribution (Fig. 1B). In fact, Erg6p-25aa appeared to have an even stronger preference to associate with lipid particles than the overexpressed wild-type polypeptide. Erg6p-62aa, on the other hand, was completely retained in microsomes and not targeted to lipid particles any more. Thus, in contrast to Erg1p, the removal of the complete hydrophobic region at the C-terminus was required to prevent association of Erg6p with lipid particles.

C-terminal truncation of Erg7p appeared to result in the least pronounced shift in the distribution of the polypeptide

Table 1
Primers for amplification of *ERG1*, *ERG6* and *ERG7* genes and truncated alleles for cloning into the high-copy-vector YEp351

	Primer (5′ → 3′)
ERG1 (fw)	See Ref. [26]
ERG1 (rev)	See Ref. [26]
ERG1-28aa (fw)	CATTGAAAATTATATA
ERG1-28aa (rev)	CATTG*GTTAAC*CCAAGAAAC
ERG1-55aa (fw)	See *ERG1*-28aa
ERG1-55aa (rev)	TCAATT*GTTAAC*GCTTTGGC
ERG6 (fw)	TATGCA*GAGCTA*AAGGTACTGTGCTTAATC
ERG6 (rev)	GATTGA*GGATCC*TATGGAAACAGTTACGATG
ERG6-25aa (fw)	GATTTA*TCTAGA*ACCAGAAAACGCCGAAAC
ERG6-25aa (rev)	ACTATA*GCTAGC*AATGAACGTGCTATC
ERG6-62aa (fw)	GATTTA*TGGCCA*CACCAGAAAACGCCGAAACC
ERG6-62aa (rev)	See *ERG6*-25aa
ERG7 (fw)/a	TCCC*CCCGGG*GGGATGCTGCTATTCGTGATTACTGTTAC
ERG7 (rev)/a	TCCCCCC*AAGCTT*GTATTTCTCTTTTCCGTCAACTCAC
ERG7-87/139aa (fw)	ACAGGTTACCAGTCTGATCCAAAGTTTCGATCTAAGGCATTAGGTATGTACAGCAGG
ERG7-87aa (rev)	GATCGAAACTTTGGATCAGACTGGTAACCTGTATCCTTCATCTGTTTACTGACCAAG
ERG7-139aa (rev)	GATCGAAACTTTGGATCAGACTGGTAACCTGTTTGAGATTTTTTTATGAATTCGATG
ERG7 (fw)/b	AACAATTTCACACAGGAAACAGCTATGACCATGATTACGAAT TCGAGCTCGGTACCCGGGGGGATGCTGCTATTCGTGATTACT
ERG7 (rev)/b	TGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGCCAAGCTTGTATTTCTCTTTTCCGTCAA

Recognition sites for restriction enzymes are typed in italics, and complementary sequences used for SOE are underlined. Erg7 (fw/rev)/a primers were used for the construction of the truncated alleles, and Erg7 (fw/rev)/b primers for cloning into YEp351.

between lipid particles and the ER (Fig. 1C). Erg7p-87aa and Erg7p-139aa constructs could still be found in lipid particles, but a significant amount of the polypeptide was also associated with the microsomal fractions. Thus, also in Erg7p the C-terminal hydrophobic domains are required for complete targeting to lipid particles.

When the hydrophobic stretches at the C-termini of Erg1p, Erg6p and Erg7p, respectively, were fused to the green fluorescence protein (GFP), and the localization of these hybrid proteins was studied by cell fractionation and fluorescence microscopy, none of the protein fragments directed GFP to lipid particles. Hybrid proteins were rather randomly distributed within the cell (data not shown). Hydrophobic domains of lipid particle proteins thus seem to be required but not sufficient for correct targeting to lipid particles, and additional structural features appear to be important.

The study presented here is an initial attempt to understand the targeting of proteins to yeast lipid particles. Our results demonstrate that hydrophobic domains near the C-terminus of the proteins studied, Erg1p, Erg6p and Erg7p, play an important role in the subcellular distribution of these polypeptides. Most interestingly, truncation of all three proteins tested did not result in random distribution within the cell but rather in a preferential shift from lipid particles to the ER. This observation supports the idea that proteins destined to lipid particles may also have a certain affinity for the ER. The presence of hydrophobic domains and/or transmembrane spanning domains may be crucial whether or not a protein will leave the ER during a budding process by interacting preferentially with the membrane monolayer of lipid particles. However, additional structural features of the proteins appear to be equally important for the correct subcellular targeting to lipid particles. Similar observations were reported most recently for caveolins by Ostermeyer et al. [32].

Our results are in line with the idea of simultaneous budding of neutral lipids and lipid particle proteins from the ER. Major components of yeast lipid particles, steryl esters and TAGs, are synthesized to a large amount in the ER [33,34]. Proteins with hydrophobic domains, but without transmembrane spanning domains present in the ER, may accumulate in the vicinity of newly formed neutral lipids. Once a nascent lipid particle has reached its critical size, the budding process may take place.

The question as to the physiological relevance of the sterol biosynthetic pathway being distributed between two subcellular fractions remains open. The fact that three key enzymes of yeast sterol biosynthesis, Erg1p, Erg6 and Erg7p, are subject to this distributive phenomenon suggests that the presence of enzymes of a pathway in two different organelles may provide an additional regulatory possibility at the organelle level, which has not yet received sufficient attention. In the case of Erg1p, the subcellular localization appears to be even more important because the portion of this protein present in the lipid particle fraction is enzymatically inactive, whereas Erg1p of the ER is active [22]. The presence of sterol-synthesizing enzymes in two organelles also raises the question of interorganelle migration of sterol intermediates. Similar to sterols, early intermediates of phospholipid biosynthesis are also shuffled between lipid particles and the ER [35]. Surface contact of the two organelles or even specific proteins may play a role in this process. Thus, interplay of lipid particles and ER may not only be restricted to protein targeting, but may also affect the regulation of certain lipid biosynthetic pathways.

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