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Biochim Biophys Acta. Author manuscript; available in PMC 2011 April 4.

Published in final edited form as:
Biochim Biophys Acta. 2009 June ; 1788(6): 1238–1248. doi:10.1016/j.bbamem.2009.03.018.

GENETIC EVIDENCE FOR THE REQUIREMENT OF THE ENDOCYTIC PATHWAY IN THE UPTAKE OF COENZYME Q_6 IN *SACCHAROMYCES CEREVISIAE*

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SUMMARY

Coenzyme Q is an isoprenylated benzoquinone lipid that functions in respiratory electron transport and as a lipid antioxidant. Dietary supplementation with Q is increasingly used as a therapeutic for treatment of mitochondrial and neurodegenerative diseases, yet little is known regarding the mechanism of its uptake. As opposed to other yeast backgrounds, *EG103* strains are unable to import exogenous Q_6 to the mitochondria. Furthermore, the distribution of exogenous Q_6 among endomembranes suggests an impairment of the membrane traffic at the level of the endocytic pathway. This fact was confirmed after the detection of defects in the incorporation of FM4-64 marker and CPY delivery to the vacuole. A similar effect was demonstrated in double mutant strains in Q_6 synthesis and several steps of endocytic process; those cells are unable to uptake exogenous Q_6 to the mitochondria and restore the growth on non-fermentable carbon sources. Additional data about the positive effect of peptone presence for exogenous Q_6 uptake support the hypothesis that Q_6 is transported to mitochondria through an endocytic-based system.

INTRODUCTION

Coenzyme Q (ubiquinone or Q) serves as a redox active lipid in the respiratory chain of prokaryotes and eukaryotes [1,2], and there is an increasing appreciation of its use in therapeutic, nutritional and cosmetic applications. Coenzyme Q_{10} (the subscript designates the number of isoprene units in the polyisoprene tail) has been used successfully to aid patient recovery following ischemia post reperfusion in heart transplantation [3] and cardiac surgery [4], and also shows promise as a therapeutic agent in other cardiovascular diseases including atherosclerosis [5], and chronic heart failure [6].

Dietary supplementation with Q_{10} helps slow the progression of symptoms in patients with Parkinsons disease [7,8], and may have similar benefits in Alzheimer [9] and Huntington diseases [10]. There is intense interest in mitochondrial diseases based on their apparent

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relationship with neurodegenerative diseases. A subset of mitochondrial diseases associated with decreased levels of Q_{10} such as cerebellar ataxia [11,12] Q-deficient mitochondrial diseases [13,14] can be alleviated by Q_{10} administration. Dietary supplementation with Q has been proposed to extend lifespan of rats fed diets enriched with polyunsaturated fatty acids [15]. Several studies demonstrate a decline in Q content during ageing [16], however dietary supplementation with Q_{10} did not affect life span of mice fed standard chow diets [17]. There are also studies that indicate a decrease in Q biosynthesis may actually extend lifespan of nematodes and mice [18]. Thus, there is a complex relationship between the beneficial functions of Q (as an antioxidant and in energy metabolism) and its potential to act as a source of oxidative stress (with the protein complexes of the respiratory electron transport chain).

The efficacy of Q supplementation is determined by its bioavailability to tissues and cells. Studies measuring uptake of Q_{10} have shown an extremely diverse distribution [19]. Despite the prevalent use of Q_{10} as a dietary supplement, little is known about the mechanisms responsible for its uptake. It is possible that both cellular uptake of exogenously supplied Q and intracellular transport of endogenously synthesized Q share a common pathway. Studies in human cells by cell fractionation indicate that Q_{10} is synthesized within the mitochondria and distributed to other cell membranes via the endomembrane system [20]. This system also participates in the uptake of exogenous Q_{10} suggesting that the endo-exocytic pathways play a role in distributing Q among membranes. In eukaryotes, Q is not only present in mitochondria, but is also located in a wide variety of cellular membranes and organelles, including the Golgi and endoplasmic reticulum [21]. The distribution of Q among cell membranes suggests that it may perform other functions. The reduced form of coenzyme Q, ubiquinol (QH_2), acts as an antioxidant and may prevent lipid peroxidation in membranes [22,23]. QH_2 acts directly to scavenge lipid peroxyl radicals and also acts as a co-antioxidant to quench α-tocopheryl radicals and regenerate α-tocopherol [24,25]. In addition, Q functions in the plasma membrane redox system in *Saccharomyces cerevisiae* [26] and mammalian cells [27,28], and may also participate in the electron transport chain of lysosomal membranes [29].

Despite the widespread distribution of Q, the biosynthesis of this molecule appears to be restricted. Studies in mammalian cells suggested that Q was synthesized in mitochondria, Golgi apparatus and endoplasmic reticulum [21], and it was proposed that the high level of Q synthesis in Golgi apparatus serves as a reservoir for its subsequent distribution among cell membranes. However, more recent studies in yeast, nematodes, and mammals indicate that the enzymes of Q biosynthesis are located in the matrix side of the inner mitochondrial membrane. In the yeast *Saccharomyces cerevisiae* the sub-mitochondrial localization of each of the Coq1p–Coq9p polypeptides was determined by subcellular fractionation and mitochondrial *in vitro* import experiments [30]. Furthermore, human, nematode and rat gene homologues rescue the Q biosynthetic defects of the *coq3*, *coq5* and *coq7* yeast mutants [31–35]. Recent studies have demonstrated that homologues of yeast Coq7p in *Caenorhabditis elegans* and mouse are located in mitochondria [36–38]. Further, human Coq4 is also located in mitochondria [39].

Results described above indicate that Q biosynthesis occurs within mitochondria, at least in yeast but the fact that Q is located in every cell membrane component suggests the existence of a mechanism for its distribution. Lipid transport, including phospholipids, has been extensively studied in *Saccharomyces cerevisiae* [40,41], however, very little is known about the transport of Q. Some evidence for mobilization of Q is indicated by studies with the HL-60 cell line. HL-60 cells that have lost mitochondrial DNA by ethidium bromide treatment or have been grown in a serum-deprived media contain increased amounts of Q at the plasma membrane [42]. This observation suggests a specific Q transport mechanism that

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may regulate its distribution among cell membranes in response to stress or metabolic demands.

In this study, we have tested the role of the membrane traffic pathway in cellular uptake of exogenous Q. In the first approach we used two genetic backgrounds of the yeast *Saccharomyces cerevisiae*, EG103 and CEN.PK2-1C. In Q_6 defficient mutants of both backgrounds was determined the distribution of Q_6 after it exogenous supplementation. While EG103 is known to have defects in the import of exogenous Q_6 to the mitochondria, CEN.PK2-1C is competent for such import. This behavior was associated with defects in typical traffic membrane markers. Therefore, in the second approach we analyzed the distribution of exogenous Q_6 in endocytic-deficient strains with defects in Q_6 biosynthesis. Both approaches delineate a general map of Q_6 transport that explains the bioavailability of exogenous Q in eukaryotic cells.

MATERIAL AND METHODS

Yeast strains and growth media

The yeast strains used in this study are described in Table I. Yeast were grown in rich YPD medium (1% yeast extract, 2% peptone, and 2% dextrose) and in YPG (1% yeast extract, 2% peptone, and 3% glycerol). Defined media contained 0.18% yeast nitrogen base without amino acids, 2% dextrose, 0.14% NaH_2PO_4, 0.5% $(NH_4)_2SO_4$, and either a complete amino acid supplement (SDc) or without uracil (SDc-ura). Yeast liquid cultures were grown at 30°C with shaking at 200 rpm.

In situ disruption of the coq3 gene in yeast mutants harboring defects in endocytic pathways

A *coq3::LEU2* disruption cassette was obtained from the plasmid pCleu12 [43]. In this plasmid a 0.7-kb BglII DNA segment of the yeast *COQ3* coding region has been replaced with a 2.85-kb BglII fragment of YEp13 containing the *LEU2* gene. Digestion of pCleu12 with PstI and BamHI generates a linear fragment of 4.65-kb that was used to disrupt the *COQ3* gene in each of the designated mutants of the endocytic pathway. All strains were transformed with 1 μg of the PstI/BamHI linear fragment from pCleu12 by the lithium acetate/PEG method [44]. In the strain NY431 (sec18-1) the *coq3* disruption required a *coq3::URA3* cassette. The *URA3* gene cassette was obtained by PCR from the plasmid pRS316 using specific primers containing flanking sequences complementaries with the gene *COQ3* (COQ3-URA3-F 5'-AAC GAG ATG TAA GAG CAC AGA TGC GGA ATA AGG GCG ACA C-3' and COQ3-URA3-R 5'-AAG AGT ATA CCT TTT TCG GGA TTT GAC AGC TTA TCA TCG A-3'). The amplicon was purified from the agarose gel and used to transforms the NY431 strain by the lithium acetate/PEG method [44].

The presence of the *coq3* gene deletion in the different yeast strains was ascertained by HPLC-ECD detection of Q_6 (Figure S2, Panel A) and by PCR with specific primers that amplify a *COQ3* DNA segment internal to the fragment excised by BglII digestion (Figure S2, Panel B). Primers and experimental conditions were designed with Primer Premier 5 software (Premier Biosoft International, USA). PCR was performed with Touch-Down PCR (annealing temperature 62°C to 50°C at 0.5°C/cycle) with the sense primer coq3U96 (5'-AACGAGATGTAAGAGCACAGATGC-3') and the antisense primer coq3L660 (5`-AAGAGTATACCTTTTTCGGGATTT-3'). Because this is a negative test, each PCR reaction contained a second set of primers amplifying an internal sequence of the gene *ACT1* (ACT1F 5'-TTCTGAGGTTGCTGCTTTGG-3' and ACT1R 5'-GATCCACATTTGTTGGAAGGTGGTAGTC-3'). ACT1 primers were designed to avoid non-desired interactions with *COQ3* primers and first were tested in a separate reaction.

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Purification of yeast subcellular fractions

Yeast plasma membranes were purified as described with some modifications [45]. Yeast cells were lysed by vortexing with glass beads and centrifuged (10 min at 700 × *g*) to remove debris. The supernatant was centrifuged (30 min at 20,000 × *g*) to obtain a crude membrane pellet. Crude membranes were resuspended in sucrose buffer (20% w/w sucrose, 10 mM Tris-HCl, pH 7.6, 1 mM EDTA and 1 mM dithiothreitol) and applied to a sucrose step gradient comprised of 4 ml 43% (w/w) sucrose and 2 ml 53% (w/w) sucrose in the same buffer. After centrifugation (4 h at 100,000 × *g*), plasma membranes were recovered at the 43/53 interface and reapplied to a second sucrose step gradient as before. To remove non-intrinsic plasma membrane proteins, the samples were treated with 100 mM Na_2CO_3, pH 11.5 [46], and suspended in the same buffer with 0.33 M sucrose.

Detergent-insoluble glycolipid-enriched complexes (DIGs) were isolated as described previously [47]. Cells (10–20 OD_{660} units) were broken with glass beads in TNE buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA) and centrifuged (10 min at 700 × *g*) to remove debris. The cleared lysate (1.5 ml) was incubated with TX100 (1% final concentration) for 30 min on ice. After the extraction with TX100, the lysate was adjusted to 40% Optiprep (Sigma, Spain) by adding 3 ml 60% Optiprep solution and overlaid with 7.2 ml of 30% Optiprep in TXNE (TNE, 0.1% TX100) and 1.2 ml of TXNE. The samples were subjected to centrifugation (140,000 × *g*, 10 h), and six fractions of 1.4 ml were collected from the top. The second fraction was subjected to a second incubation with TX100 and reapplied to a second Optiprep (Sigma, Spain) gradient as before to obtain a fraction of purified DIG´s by collecting the second 1.4 ml fraction from the top.

Other membrane fractions required the preparation of yeast spheroplasts, obtained by treatment with zymolyase in buffer B (1.2 M sorbitol, 20 mM potassium phosphate, pH 7.4) [48]. Spheroplasts were resuspended with C buffer (0.6 M manitol, 20 mM potassium phosphate, pH 7.4), lysed with a Dounce homogenizer and centrifuged (10 min at 700 × *g*) to remove debris. The supernatant was centrifuged 10 min at 12,000 × *g* to obtain a pellet of crude mitochondria. The resulting supernatant was centrifuged 30 min at 20,000 × *g* to remove a microsomal pellet fraction. The supernatant was centrifuged 45 min at 32,500 × *g* to obtain rough endoplasmic reticulum pellet, and then 1 hour at 100,000 × *g* to obtain smooth endoplasmic reticulum pellet. Crude mitochondria were applied to a Nycodenz step gradient of 4.5 ml 14.5% Nycodenz and 4.5 ml 20% Nycodenz in C buffer. After centrifugation (1 hour at 100,000 × *g*), mitochondria were recovered from the 14.5/20 interface. The upper fraction of the Nycodenz gradient was used to purify MAM (mitochondria associated microsomes); this fraction was applied to a sucrose step gradient of 5 ml 50% sucrose and 5 ml 22.5% sucrose in C buffer. After centrifugation (1 hour at 100,000 × *g*), the MAM fraction was recovered from the top of the gradient.

Vacuole and Golgi apparatus fractions were purified as described by Wu *et al*. [49]. Spheroplasts were obtained as above, resuspended in 0.6 M Sorbitol, 20 mM K^+-MES, pH 6.0 and lysed with a Dounce homogenizer. After cell lysis, the homogenate was centrifuged first at 700 × *g* to remove debris and 12,000 × *g* to remove an enriched mitochondria fraction. The supernatant was centrifuged 30 min at 30,000 × *g* and the pellet obtained was suspended in the same buffer and applied to a sorbitol step gradient of 5 ml 80% (w/v) sorbitol and 5 ml 25% (w/v) sorbitol in 10 mM triethanolamine, pH 7.4. The interface between the 25 and 80% sorbitol layers was collected and adjusted to 43% sorbitol and layered on a gradient prepared in 40, 43, 60, 70 and 80% increments of 2.3 ml each. Following centrifugation (120,000 × *g*, 48 h), fractions were collected from the top (0.6 ml) and monitored by immunoblot with antibodies that recognized vacuole and Golgi apparatus specific proteins.

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To check the purity of membrane fractions, SDS-PAGE and Western blotting were performed using standard methods (Figure 1). Primary antibodies that recognized specific proteins of membrane fractions were used at the following dilutions: 1:50,000 Pma1p (plasma membrane); 1:20,000 Gas1p (DIGs); 1:5000 porin (mitochondria outer membrane); 1:10,000 cytochrome c_1 (mitochondria inner membrane); 1:10,000 Sec62p (endoplasmic reticulum); 4 μg/ml Vps10p (Golgi) and 2 μg/ml Alkaline phosphatase (vacuole) from Molecular Probes. Horseradish peroxidase-linked secondary antibodies to rabbit and mouse IgG were used in 1:10,000 (rabbit) and 1:5,000 (mouse) dilutions.

Lipid extraction and determination of Q_6

Lipid extractions and quantification of Q_6 from purified membrane samples were performed as described previously [50]. Aliquots of purified membrane fractions (500 μl, 0.5–1 mg of protein) were mixed with an equal volume of 2% SDS and vortexed 1 min. Two ml of 5% isopropyl alcohol in ethanol was added, and samples were vortexed again for 1 min. To recover quinones, 5 ml of hexane were added, and the mixture was vortexed at top speed for 1 min and centrifuged at 1000 × *g* for 5 min. The upper phases recovered from three extractions were pooled and dried in a rotatory evaporator. Lipid extracts were suspended in 1 ml of ethanol dried in a speed-vac and kept at −20°C. Samples were suspended in a suitable volume of ethanol prior to HPLC injection. Q_6 and Q_9 were separated by reverse-phase high performance liquid chromatography with a C18 column and quantified with an ESA Coulochem III electrochemical detector and a 5010 analytical cell (E1, −500 mV; E2, +500 mV). A separate precolumn guard cell was set to an oxidizing mode (E, +500 mV) to convert all hydroquinones to quinones. The mobile phase was adjusted to a flow rate of 1 ml/min and was composed of methanol/ethanol/2-propanol (88/24/10) and 13.4 mM lithium perchlorate. Q_6 and Q_9 were quantified from the electrochemical detector results with Q_6 and Q_9 as external standards. The use of Q_9 as an internal standard (10 μl of a 2 mM stock) indicated a recovery of 90–100% of total Q_9 added to samples.

CPY secretion plate assay

Yeast cultures were freshly grown to logaritmic phase and washed with deionized water. 50 × 10^6 yeast cells were resuspended in 1 ml of sterile water and three ten fold dilutions were made. All dilutions were spotted on a nitrocellulose membrane which was placed on the surface of YPD plates and incubated at 30 °C for 24 h. The membranes were lifted and washed with deionized water to remove all the cells. Proteins adsorbed on the membrane were detected by inmunoblotting using monoclonal CPY antibody (Molecular Probes; 1:5,000 dilution).

Vacuolar staining with FM4-64

Yeast cells were grown in YPD media to logarithmic phase. Aliquotes of 1 ml were harvested by centrifugation and washed twice with 1 ml of PBS. Then, cells were resuspended in 1 ml of PBS and incubated with 2 μM FM4-64 at 30°C for 30 min. After incubation, yeast cells were washed twice with PBS, resuspended again in fresh PBS and observed under the fluorescence microscope (λ_{exc} = 515 nm, λ_{em} = 640 nm).

RESULTS

Exogenous coenzyme Q_6 is differentially distributed among cell membranes in CEN.PK2-1C and EG103 yeast

In order to delineate the influence of yeast genetic background on the distribution of Q_6 among yeast cellular membranes, the Q_6 content was analyzed in membranes of wild-type and *coq* mutant yeast strains derived from the CEN.PK2-1C and EG103 genetic

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backgrounds. Wild-type strains were grown in rich medium containing glucose as a fermentable carbon source (YPD). Null *coq7* mutants were cultured in the same media supplemented with 2 μM exogenous Q_6. All coq mutants are unable to synthesize Q_6 and the Q_6 detected come from the exogenous supplementation The content of Q_6 was determined in several membrane fractions including mitochondria, mitochondria-associated microsomes (MAM), smooth and rough endoplasmic reticulum (SER and RER), Golgi apparatus, vacuole, plasma membrane and detergent-insoluble glycolipid-enriched complexes (DIGs) (Figure 1 and S1). The subcellular fractionation was not significantly affected in the different mutants analyzed. Lipid extracts were prepared from each membrane fraction, and the content of Q_6 was determined by HPLC-ECD (Figure 2, Panel A and B). In wild-type cells (black bars) Q_6 content was highest in wild-type mitochondria and vacuole, being lowest in DIGs, smooth ER and plasma membrane. The MAM fraction of CEN.PK2-1C yeast was dramatically enriched in Q_6 (Figure 2, Panel A), but this enrichment was not evident in the EG103 yeast.

Addition of exogenous Q_6 restores respiration in the *coq7* null mutant derived from CEN.PK2-1C but not EG103 yeast parental strains [50]. Thus it was of interest to compare the relative amounts of exogenously added Q_6 contained in membrane fractions prepared from these *coq7* mutants. The presence of exogenously added Q_6 led to dramatic differences in the distribution of Q_6 among membrane fractions, as evidenced by the much higher Q_6 content in mitochondria, MAM and RER in the *coq7* mutant derived from CEN.PK2-1C as compared to EG103 (Figure 2, Panels A and B, white bars). The level of Q_6 in EG103coq7 mitochondria was only 22% of wild-type, while the Q_6 content of the CEN *coq7* mutant was 75% of wild-type. Q_6 levels in wild type strains are not affected significantly after the exogenous Q_6 supplementation.

Two phenotypes associated to membrane transport defects are present in EG103 genetic background

Q_6 distribution in *coq7* null mutant from EG103 genetic background suggest that membrane traffic might be involved in the uptake and transport of this lipid to the mitochondria. With the above in mind, we decided to further investigate a potential membrane trafficking defect in EG103 yeast cells.

Carboxypeptidase Y (CPY) is synthesized as a prepro form and is transported across the endoplasmic reticulum membrane. Following signal peptidase cleavage in the ER to produce a 67 kDa precursor (p1) form, it is transported through the Golgi where it acquires sugar modifications to become a 69 kDa (p2) form. Wild type cells are able to correctly deliver CPY to its final destination within the vacuolar lumen, where it is processed to a 61 kDa mature form. However, yeast mutants with defects in protein/membrane trafficking to the vacuole secrete a portion of the p2 form of CPY [51]. To investigate whether EG103 cells present this phenotype, a plate assay was performed as described in *Experimental Procedures*. We did no detect any CPY secretion associated to the parental and *coq7* null mutant strains derived from CEN.PK2-1C genetic background. Interestingly, a mild CPY secretion was detected in yeast strains from EG103 (Figure 3, Panel A), indicating a potential membrane trafficking defect associated to this genetic background.

It should be noted that whereas yeast mutants with defects in membrane traffic to the vacuole secrete CPY, there are many others mutants that secrete low levels of this protein [52]. However, it is well known that defects in trafficking to the vacuole lead to alterations of the vacuolar morphology [53]. Therefore, to further confirm the above results, we decided to analyze the vacuolar morphology of the yeast cells in both genetic backgrounds studied performing FM4-64 staining. Yeast cells grown to logarithmic phase in YPD media were harvested and incubated with the dye as described in *Experimental Procedures*. The results

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(Figure 3, Panel B) evidenced that either parental or *coq7* null cells from CEN.PK2-1C have generally one well defined vacuolar organelle. However, a number of small vacuoles were observed in yeast cells from EG103 background, which supports above CPY secretion results and strongly suggests an altered membrane trafficking pathway in EG103 cells.

The above results support the hypothesis that altered Q_6 distribution among cell membranes observed in EG103 cells may be a consequence of a partial impairment in the normal membrane trafficking processes that occur within the cell.

The endocytic pathway is involved in Q_6 uptake in yeast

To confirm whether either an impaired endocytosis or a defective membrane traffic lead to an alteration of coenzyme Q uptake and its transport to the mitochondria, we analyzed the uptake and distribution of exogenous Q_6 in mutant strains with gene defects in several endocytic transport steps such as *ERG2*, *PEP12*, *TLG2* or *VPS45* genes. Erg2p catalyzes a step in ergosterol biosynthesis and this deficiency induces the accumulation of sterol intermediates that support growth and exocytosis but fail to allow endocytic transport [54]. Pep12p is a syntaxin controlling the vesicular traffic in the prevacuolar compartment [55]. Tlg2p is a t-SNARE (target soluble N-ethyl maleimide-sensitive factor attachment protein receptor) protein required for the fusion of trans-Golgi network vesicles (TGN vesicles) with endocytic vesicles to produce early endosomes [56]. Vps45p is a SM protein (Sec1p-like/Munc-18) required for Tlg2p function [57]. These single mutant yeast strains (in the BY4741 genetic background) were obtained from the Euroscarf repository, and each retained the ability to grow on media containing glycerol as a nonfermentable carbon source (Figure 5, Panel B). Membrane traffic impairment in those mutants was confirmed by analyzing CPY secretion (Figure 4, Panel A) and performing a vacuolar staining with FM4-64 (Figure 4, Panel B). As expected, all the mutant strains showed a high level of CPY secretion and different kind of alterations in the vacuolar morphology. Using these strains and the parental BY4741 strain, mutant strains unable to synthesize Q_6 were obtained by in situ gene disruption of the *COQ3* gene. Null *coq3* mutants show the same behavior as null *coq7* mutants in terms of having defects in Q_6 biosynthesis, inability to grow on nonfermentable carbon sources, and defects in respiration. Each of the double mutants was shown to have a defect in producing Q_6 (Figure S2, Panel A) and to lack the wild-type *COQ3* gene (Figure 5, Panel B). The transformation of double mutants with the plasmid pRS12A-2.5SB (containing the *COQ3* gene) restores the growth on YPG plates (Figure S2, Panel C). Moreover, the additional *COQ3* deletion in double mutant strains did not alter either CPY secretion or FM4-64 staining phenotypes observed in BY4741 parental and mutant strains (Figure 4, panel C).

To test exogenous Q_6 uptake, each double mutant yeast strain was cultured in YPG medium supplemented with 2 μM Q_6. None of the double mutants were able to grow in this medium (Figure 5). However, the *coq3* single mutant prepared in the BY4741 genetic background showed growth rescue in YPG medium supplemented with Q_6 (Figure 6). These results suggest that endocytosis and membrane trafficking are required for Q_6 to reach mitochondria in these cells.

To determine whether the exogenously supplied Q_6 was able to reach endomembranes, the Q_6 content was determined in purified fractions of plasma membrane, Golgi apparatus, vacuole and mitochondria from the parental, the *coq3* single mutant, and each of the double mutant strains cultured in Q_6-supplemented YPD media (Figure 7, Panel A). The content of Q_6 in plasma membrane of wild-type and *coq3* mutant strains contained a similar amount of Q_6, while Golgi, mitochondria, and vacuoles isolated from the *coq3* mutant contained 59%, 57% and 32% of the wild-type Q_6 content. However, each of the *coq3*-double mutant strains lacking endocytosis related genes showed dramatically decreased Q_6 content in all

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membrane fractions analyzed. The low Q_6 content in plasma membranes of the *coq3*-double mutants is particularly intriguing, as it suggests that most of the exogenously supplied Q_6 cannot be directly incorporated into the plasma membrane. A direct insertion of Q_6 into the plasma membrane seems to be difficult due the extremely low solubility in water. Given that another lipids such as sterols are transported to the cell using protein–lipid complexes through the endocytic pathway [41], we analyzed the possibility that peptone plays the function of a vehicle for exogenous Q_6.

However, the use of constitutive deleted mutants could introduce in the analysis already affected transport pathways or putative transporters. To solve this question, has been analyzed the Q_6 exogenous transport in a double mutant NY431 *coq3* (*sec18-1/coq3*) under nonpermisive temperature (Figure 7, Panel B). Sec18p is an AAA-ATPase, the orthologue of NSF in mammalian cells. In the yeast expressing the sec18-1 mutant protein, the transport ceases after shifting the cells to the nonpermissive growth temperature [58]. NY431 *coq3* is unable to synthesize Q_6 given that harbors the *coq3* mutation. Before the addition of exogenous Q_6, this molecule is not detected in mitochodria or microsomes. After the addition of exogenous Q_6, at permisive temperature (25°C) the Q_6 is readily transported to mitochondria while at non-permisive temperature (30°C) is accumulated mainly in microsomes.

Q_6 uptake by cells requires soluble proteins

Result suggests that exogenous Q_6 could utilize the hydrophilic phase in the lumen of endocytic vesicles to be transported into the yeast cell. Since Q_6 is a hydrophobic molecule, this process would require the binding to a water soluble factor that would carry this lipid into the cell. Q_6 uptake assays were carried out with cells cultured in a rich medium containing 2% peptone, an enzymatic digest of animal protein. The digested proteins seem to be a good candidate to bind exogenous Q_6 to facilitate its transport into the cell. To test this possibility, a Q_6 uptake assay was performed with cells cultured in SD medium, which does not contain digested proteins as nitrogen source. Thus, the *coq3* null mutant strains CEN*coq3* and EG103*coq3* and the double mutant strain *coq3/erg2* (Y01812*coq3*) were cultured until stationary phase in the presence of 2 μM Q_6 with or without 2% peptone. Cells were collected after 48 hours, spheroplasts prepared, and the content of Q_6 determined (Figure 7). EG103*coq3* and CEN*coq3* strains are unable to produce Q_6 and also show a moderate amount of Q_6 when were cultured with exogenous Q_6 in synthetic media. However, the amount of Q_6 was increased after the peptone addition but only in CEN*coq3* strain, that does not show defects on membrane traffic.

DISCUSSION

Several studies performed in mammalian systems have shown that certain steps in the Q biosynthetic pathway take place in both mitochondrial and ER membranes [21,59,60]. Studies in yeast, however, suggest that Q_6 biosynthesis is carried out solely within mitochondria [30]. Here we show a higher Q_6 content in mitochondria as compared with other cell membranes in wild-type yeast strains derived from different genetic backgrounds, CEN.PK2-1C and EG103. Although Q_6 content is highest in mitochondria, it is detected in all yeast membranes indicating a Q_6 transport process from mitochondria to other cellular membranes. In this study we employed the CEN*coq7* null mutant, previously shown to be capable of taking up exogenously supplied Q_6 and respiratory electron transport is restored [50]. Exogenous Q_6 uptake by the CEN*coq7* null mutant shows a distribution profile similar in many aspects to its wild-type parent. This finding suggests that mitochondrial Q_6 uptake in the *coq7* mutant may mimic the transport of Q_6 from mitochondria to cell membranes in wild type strains.

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Several authors have indicated that the ER-Golgi system participates in the coenzyme Q secretion to the blood plasma in mammalian cells [21]. Other studies suggest that the MAM participates in the secretory pathway as a component that supplies lipids for the final assembly into very low-density lipoproteins [61]. Thus there is a precedent for the hypothesis that Q_6 transported to ER or MAM from mitochondria may use the secretory pathway to reach other cell membranes. Similarly, endocytic vesicles could also transport exogenous Q_6 to internal cell membranes. Our studies show a low but significant uptake of exogenous Q_6 by the EG103*coq7* strain, while the corresponding *coq7* mutant in the CEN.PK2-1C background exhibited nearly wild-type Q_6 content in mitochondria and plasma membrane, and higher than wild-type content of exogenous Q_6 in ER and MAM. Interestingly, the analysis of two well defined phenotypes for defective membrane trafficking (CPY secretion and anormal vacuolar morphology) showed that EG103 strains may have a mild defect in those processes, which suggest that Q_6 transport among membranes is dependent of normal endocytic and membrane transport within the cell. Furthermore, the findings reported here that yeast mutants with defects in either endocytosis (*erg2*) or several steps in membrane traffic (*pep12*, *tlg2* and *vps45*) fail to transport exogenous Q_6 to mitochondria strongly support this hypothesis. Indeed, not only there was no delivery of Q_6 to mitochondria, but none of the endocytosis mutants were able to incorporate exogenous Q_6 into other cellular membranes at significant levels, including the plasma membrane. This last observation is perhaps most surprising, as it indicates that uptake of exogenously supplied coenzyme Q_6 by the plasma membrane requires an intact endocytosis membrane trafficking system. A time course analysis of radiolabeled CoQ_{10} in human cells demonstrated that it is first incorporated in mitochondria and then delivered to other membranes including plasma membrane [20].

Even though Q_6 is a hydrophobic molecule, it is possible that when it is added exogenously in aqueous media, it is bound to soluble polypeptides, and taken up by cells via endocytic vesicles. Here we demonstrated that exogenous Q_6 uptake by cells is facilitated by peptone, indicating that Q_6 binding to the soluble proteins and peptides present in peptone partitions this lipid into the aqueous phase and allows its incorporation into the lumen of endocytic vesicles. Mutant strains defective in endocytosis (*erg2*) fail to take up exogenous Q_6 even with soluble proteins in the medium, supporting this hypothesis. These data agree with the general scheme of cholesterol trafficking in mammalian cells [62]. Uptake of CoQ_{10} by HL60 cells requires specifically lipoproteins [20]. Therefore, we propose a mechanism for uptake, in which exogenous Q_6 binds to soluble proteins, is taken up via endocytosis and travels via endocytic vesicles to the vacuole, where it must be retrieved in order to be delivered to the plasma membrane and mitochondria. The Q_6 uptake detected in synthetic media (Figure 7, Panel A) can represent a non receptor-mediated endocytic uptake (fluid-phase endocytosis) or a direct insertion of the Q_6 molecule in the plasma membrane. Also, is possible that the selected mutants do not show a total lack of endocytosis, a crucial function in the cells, and maintains a minimal activity that does not support the Q_6 uptake to mitochondria. Receptor-mediated or fluid-phase endocytosis pathways share some components. In a recent and extensive study has been found 14 genes in yeast that are related to fluid-phase endocytosis [63]. One of those genes, *TLG2*, has been used in this work and probably a *tlg2*-deleted mutant strain accumulates defects on both pathways. This interplay between both procceses may also explain the low amount of Q_6 detected in endomembranes from double mutants of endocytosis and Q_6 biosynthesis pathway. Each of the membrane traffic mutants used in this study were unable to take up exogenous Q_6 and have defects at various stages of endocytosis including the initial point of uptake (*erg2*), late endosome formation (*tlg2*, *vps45*), or vacuole maturation (*pep12*).

A point of criticism may be the use of deleted-mutants of the endocytic pathway. However, when was analyzed the Q_6 uptake in a temperature restrictive mutant such as sec18-1/coq3

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was found that at permisive temperature was produced a typical Q_6 uptake to mitochondria. This uptake was blocked at non-permisive conditions. That allow us discard that the lack of Q_6 uptake was produced by the absence of transport proteins or others factors related with the mutated genes that could not be expressed in permanent endocytic mutants. However, this experiment does not demonstrate completely that endocytic process was required for a proper Q_6 uptake. Better, it demonstrates that at least part of the process, the transport from the endomembrane system to the mitochondria is supported by the membrane traffic machinery.

Hence, this model accounts for the absence of significant amounts of exogenous Q_6 in the plasma membrane and vacuoles in each of these mutants. However, it is curious that there is very little exogenous Q_6 detected in vacuoles isolated from either the single *coq7* or *coq3* mutants (Figures 2 and 6, respectively). Perhaps the relatively high content of Q_6 in the vacuole of the wild-type strain represents the trafficking bottleneck for endogenously synthesized Q_6 originating from the mitochondria, while the trafficking bottleneck for Q_6 supplied exogenously may reside in the ER or MAM. It is interesting that other studies of exogenous Q_{10} uptake by human cells indicate that the Q_{10} accumulates mainly in a vesicular endocytic compartment [20], and only a small but significant amount of Q_{10} was assimilated by mitochondria. While the uptake of exogenous Q_6 and its delivery to mitochondria by *coq* mutant yeast is quite high, uptake into mitochondria of Q_6-replete yeast is quite low [50]. Thus, the amount of uptake of exogenous Q and its delivery to mitochondria in both human and yeast cells may reflect mechanisms that regulate mitochondrial Q content. In fact, exogenous Q_{10} uptake by human cells induces a decrease of endogenous biosynthesis maintaining Q_{10} homeostasis [20].

The vesicle traffic system for transport of exogenous Q_6 would allow transport of Q_6 to plasma membrane, and also to mitochondria. The trafficking of Q_6, whether synthesized *de novo* or supplied exogenously, is likely to share mechanisms in common with higher eukaryotes. An understanding of the processes involved in yeast uptake and assimilation of exogenously supplied Q_6 will provide insights into the process by which mammalian cells assimilate Q_{10}.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Seasson Phillips Vitellio from the School of Medecine, University of Rochester, USA for helping us with the FM4-64 staining procedure and also the Dr. Peter Novick from the Department of Cell Biology, Yale University School of Medicine, USA for gift us the strain NY431. This work was supported by the Spanish Ministerio de Ciencia y Tecnología, Grant BFU2005-03017/BMC, and by NIH GM45952 to CFC.

The abbreviations used are

DIGs	detergent-insoluble glycolipid enriched complexes
CPY	carboxypeptidase Y
MAM	mitochondria associated microsomes
Q	coenzyme Q or ubiquinone
RER	rough ER
SM	Sec1p-like/Munc-18

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SNARE	soluble N-ethylmaleimide-sensitive factor attachment protein receptor
TGN	trans-Golgi network
t-SNARE	target SNARE

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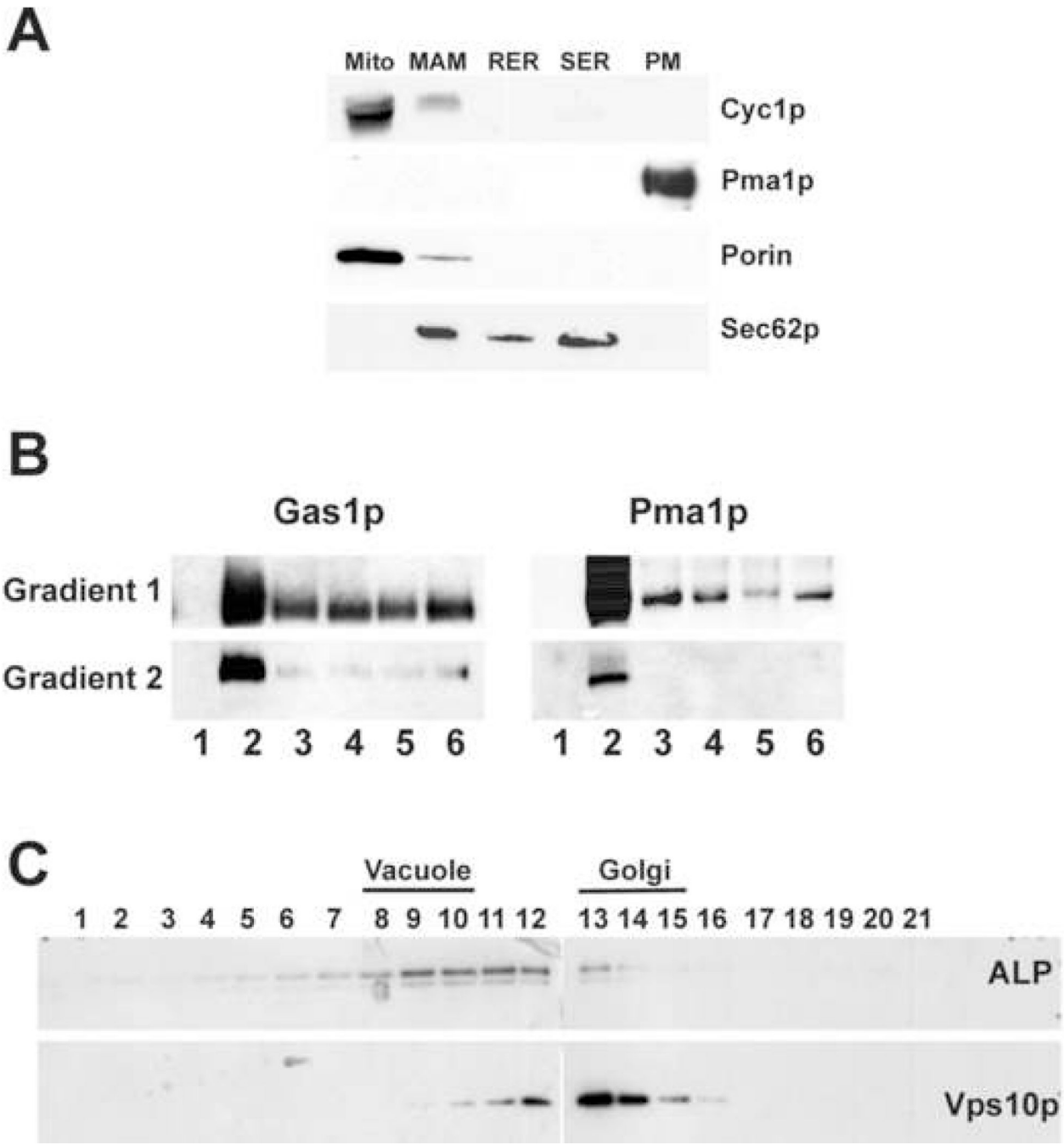

Figure 1.

Assessment of purity of yeast membrane fractions from the CEN.PK2-1C strain. In Supplementary Material was shown the corresponding analysis for the EG103 strain. Panel A: The extent of cross-contamination in endomembrane samples (30 μg of protein) was determined by SDS-PAGE and Western blot with specific antibodies to marker proteins. Panel B: DIGs were purified by an Optiprep gradient (see *Experimental Procedures*). Fraction 2 of the first gradient (enriched in both Pma1p and Gas1p marker proteins) was applied to a second gradient. Fraction 2 from the second gradient was considered as the pure DIGs fraction. Pma1p and Gas1p are considered as specific marker proteins of DIGs membranes. Panel C: Vacuole and Golgi membrane fractions were purified from a sorbitol

step gradient. Fractions most enriched with vacuole and Golgi marker proteins were identified; ALP for vacuole, and Vps10p for Golgi.

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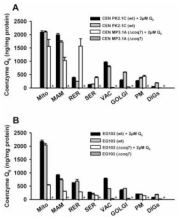

Figure 2.

Coenzyme Q_6 content in yeast membrane fractions. Lipid extracts were obtained from purified membrane fractions and the content of Q_6 was determined with HPLC-ECD as described in Experimental Procedures. Results shown are the average of three injections ± SD of two independent extractions. Panel A: CEN.PK2-1C (wt) and CEN*coq7* strains cultured in absence or presence of 2 μM. Panel B: EG103 (wt) and EG103 *coq7* strains cultured in absence or presence of 2 μM Q_6. All cells were cultured in YPD. The asterisk (*) indicates that Q_6 was not detected.

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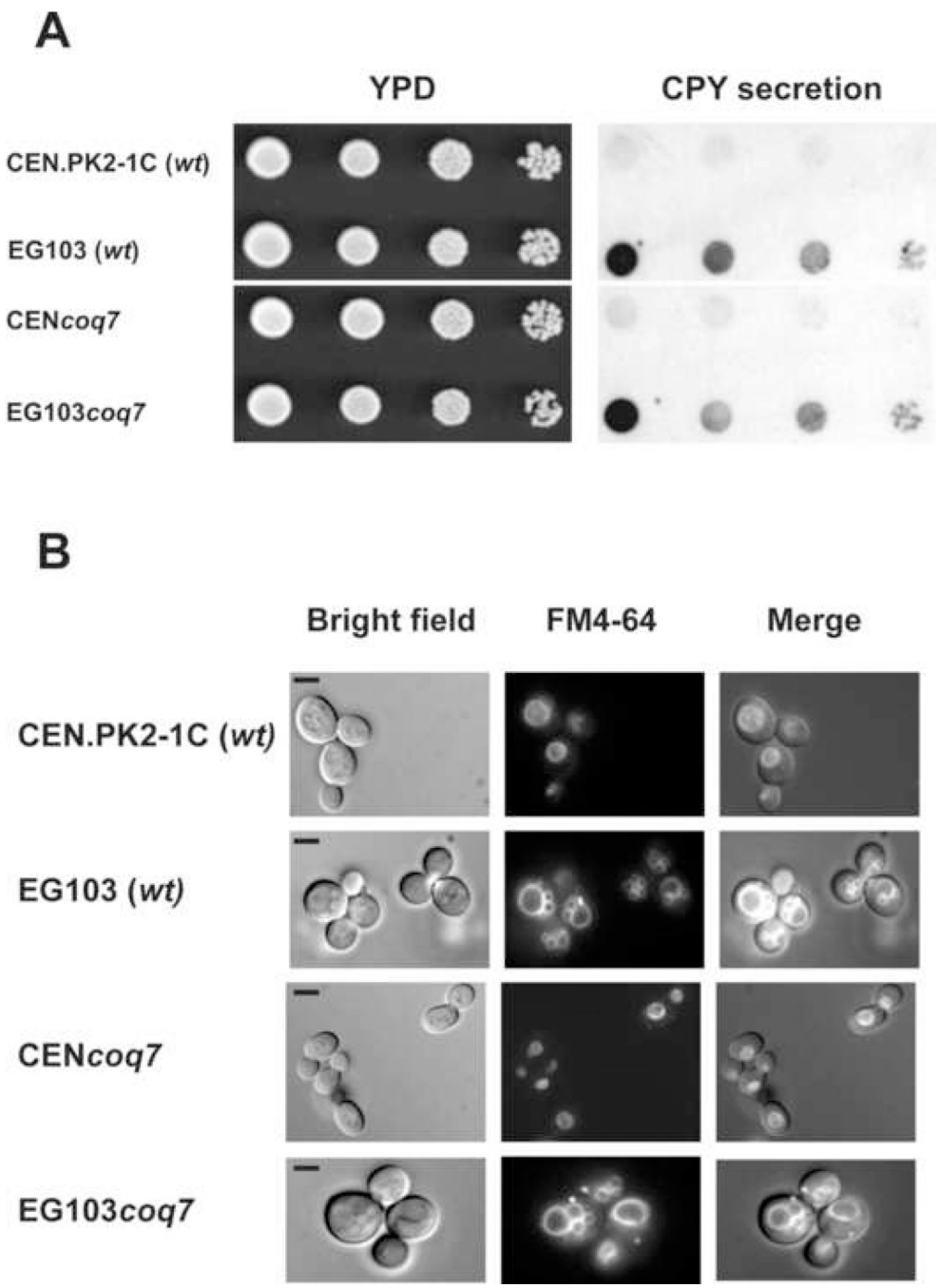

Figure 3.

Analysis of traffic membrane markers in CEN.PK2-1C and EG103 cells. Panel A: CPY secretion. Equal number of cells from freshly grown yeast cultures were deposited as undiluted, 1:10, 1:100 or 1:1000 dilutions (left to right) on a nitrocellulose filter overlaid on YPD plates. The plates were incubated for 24 hours at 30 °C and washed as described under *Experimental Procedures*. Extracellular CPY secreted from colonies was detected by inmunostaining with anti-CPY antibody (Molecular Probes). Panel B: FM4-64 vacuolar staining. Yeast cell grown to logarithmic phase in YPD were incubated with 2 μM FM4-64 at 30°C during 30 min. After incubation, cells were washed with PBS and observed under

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the fluorescence microscope. Micrographs are obtained with 1000× magnification. Bar, 5 μm. Results are representative of a set of three experiments.

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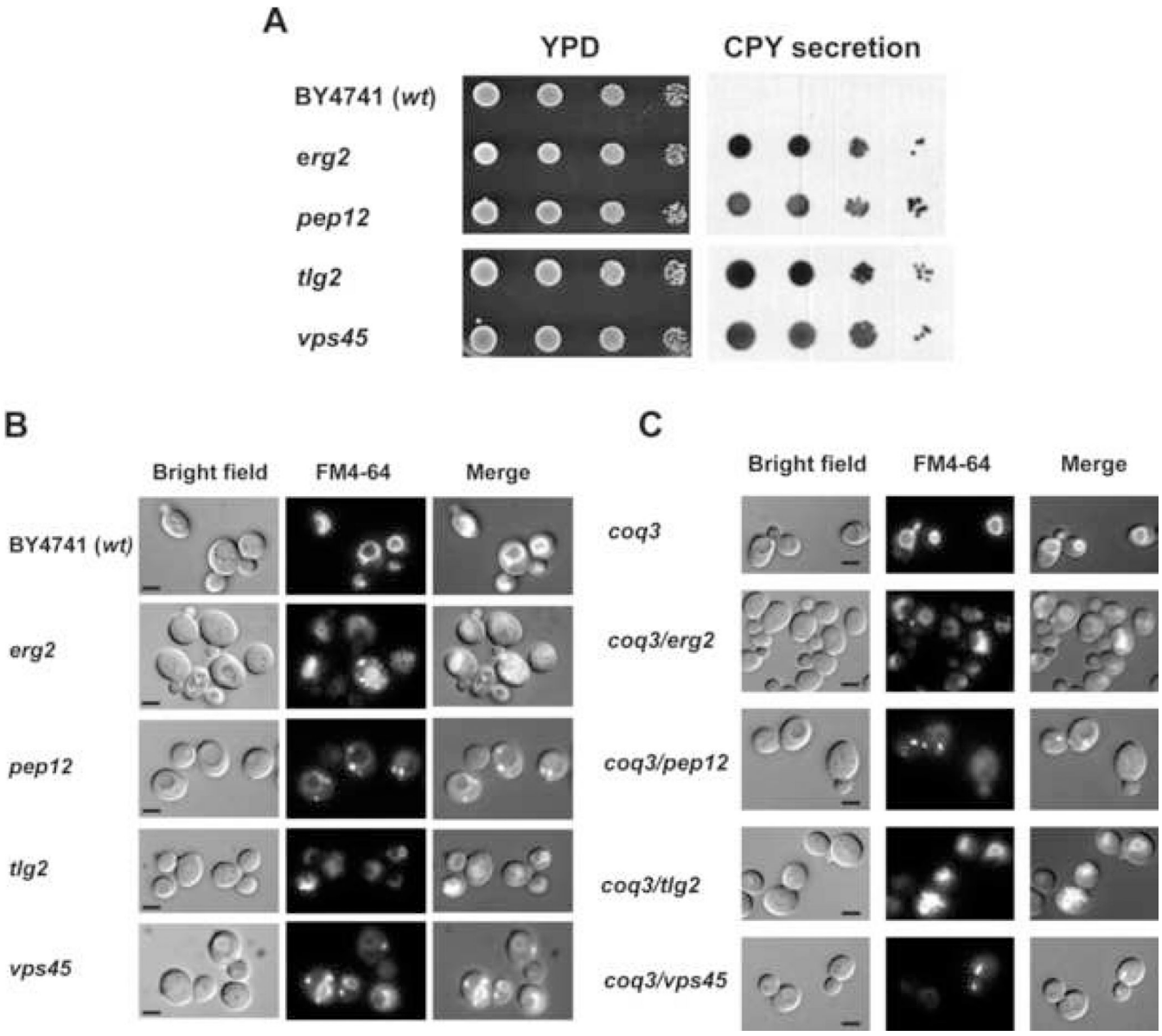

Figure 4.

Analysis of traffic membrane markers. Panel A: CPY secretion in single membrane traffic mutants. Equal number of cells from freshly grown yeast cultures were deposited as undiluted, 1:10, 1:100 or 1:1000 dilutions (left to right) on a nitrocellulose filter overlaid on YPD plates. The plates were incubated for 24 hours at 30 °C and washed as described under *Experimental Procedures*. Extracellular CPY secreted from colonies was detected by inmunostaining with anti-CPY antibody (Molecular Probes). Panel B: FM4-64 vacuolar staining. Yeast cell grown to logarithmic phase in YPD were incubated with 2 μM FM4-64 at 30°C during 30 min. After incubation, cells were washed with PBS and observed under the fluorescence microscope. Results are representative of a set of three experiments. Micrographs are obtained with 1000x magnification. Bar, 5 μm. Panel C: FM4-64 vacuolar staining in membrane traffic/coenzyme Q biosynthesis double mutants. Yeast cell grown to logarithmic phase in YPD were incubated with 2 μM FM4-64 at 30°C during 30 min. After incubation, cells were washed with PBS and observed under the fluorescence microscope.

Results are representative of a set of three experiments. Micrographs are obtained with 1000× magnification. Bar, 5 μm.

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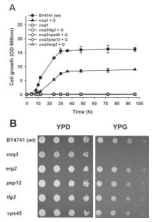

Figure 5.

Exogenous Q_6 fails to rescue yeast *coq3* mutants containing additional deletions in genes required for endocytosis. Panel A: YPG media with 2 μM Q_6 was inoculated with 0.1 OD_{660nm} units/ml of the designated yeast strains and incubated at 30°C with shaking. Data correspond to the average ± SD of five measures of the same culture. Experiment is representative of a set of two independent experiments. Panel B: Yeast strains bearing defects in membrane trafficking are able to grow in non fermentable carbon source. Equal number of cells from freshly grown yeast cultures were deposited as undiluted, 1:10, 1:100 or 1:1000 dilutions (left to right) on YPD and YPG plates. The plates were incubated for 48 hours at 30 °C.

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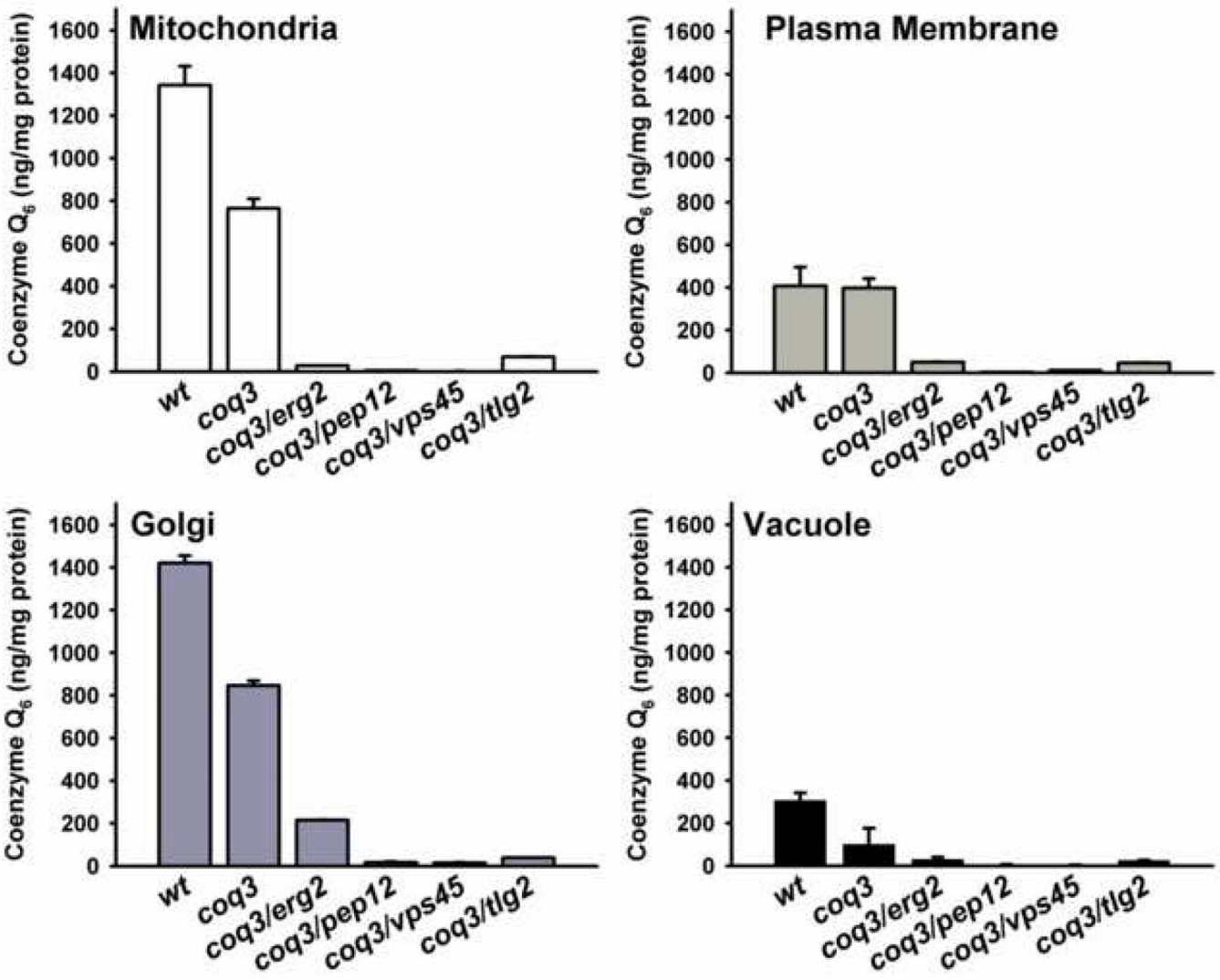

Figure 6.

Coenzyme Q_6 content in endomembranes of *coq3*/endocytic double mutants. Lipid extracts were obtained from mitochondria, plasma membrane, Golgi and vacuole fractions purified from parental (wt), *coq3* single mutant, and *coq3*/endocytic double mutants cultured in YPD with 2 μM Q_6. The content of Q_6 was determined by HPLC-ECD and results are expressed as the average ± SD of three injections. Data are representative of two independent experiments.

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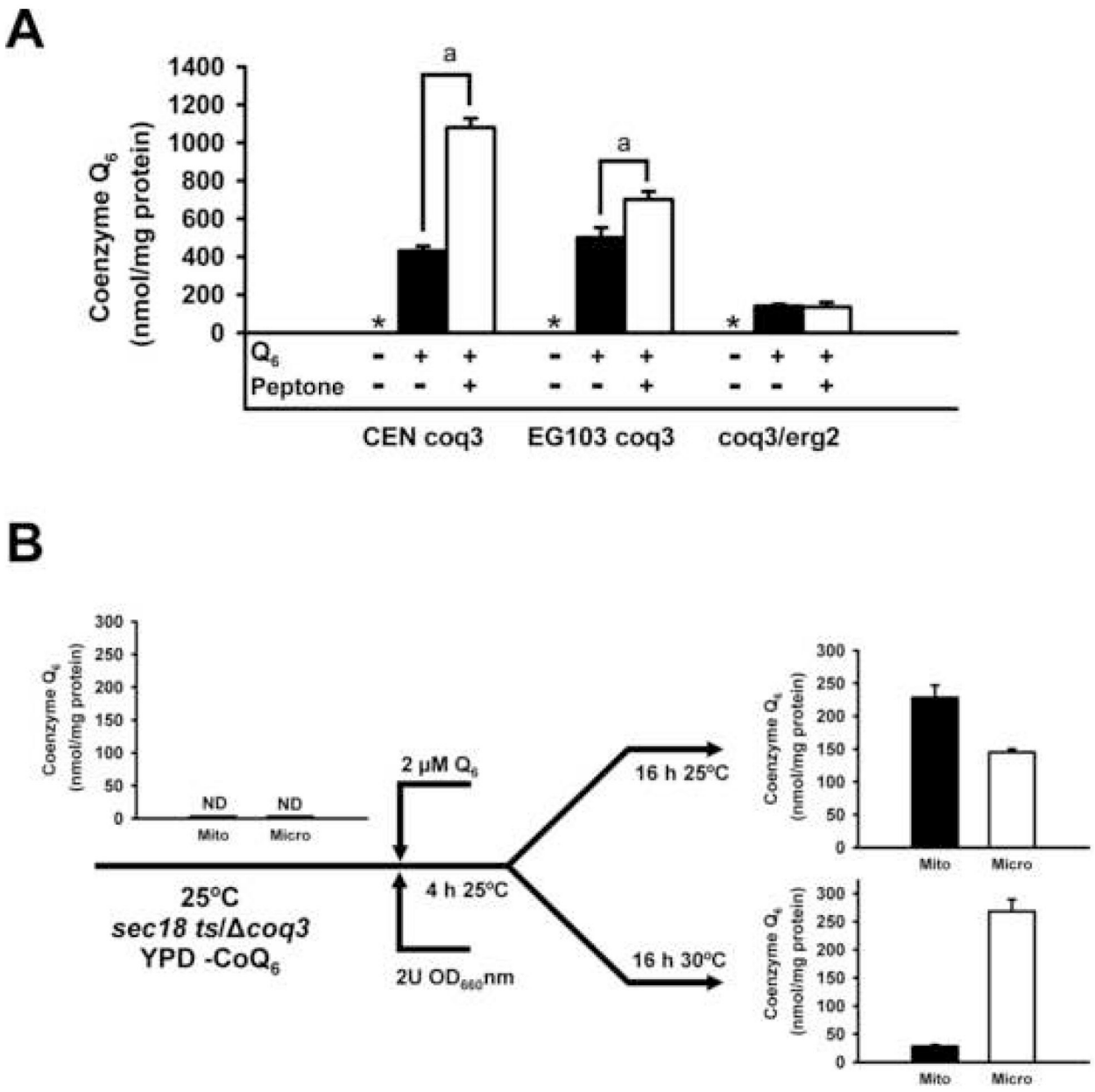

Figure 7.

Coenzyme Q_6 uptake is favored by the presence of peptone in culture media and is affected in termosensitive endocytic mutants at non-permisive temperature. Panel A: Cells from several Q-deficient strains (CEN *coq3*, EG103 *coq3* and *coq3*/*erg2*) were cultured in SD medium at 0.1 OD_{660nm}/ml for 4 hours and then exogenous Q_6 (2 μM) was added with or without 2% peptone. Cells were cultured for 48 hours, harvested, and the cell wall removed by Zymolyase digestion. Lipid extracts of protoplasts were prepared and Q_6 quantified as described in Material and Methods. Data correspond to the average ± SD of three Q_6 determinations from the same experiment. Data are representative of a set of two independent experiments. **[a]** The addition of peptone increase significantly the levels of Q_6 ($p < 0.01$). * Indicates that Q_6 was not detected in those cells and culture conditions. Panel B: Double mutants cells (sec18-ts/coq3) were cultured at permisive temperature (25°C) in YPD without the presence of Q_6 until reach 2 OD units at 660 nm. At this point exogenous Q_6 (2

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μM) was added and cells were cultured during 4 hours a the same conditions. The initial culture was splitted in two cultures; one was cultured 16 hours at permisive temperature (25°C) and the second at non-permisive temperature (30°C). Cultures were used to purify mitochondria (Mito) and microsome (Micro). Mitochondrial fractions were purified according to Material and Methods and the microsomal fractions were purified after the centrifugation of postmitochondrial supernatant at 100,000 × g 1 h at 4°C. Lipid extracts of both samples were prepared and Q_6 quantified as described in Material and Methods. Data correspond to the average ± SD of three Q_6 determinations from the same experiment. Data are representative of a set of two independent experiments.

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Table I

Genotype and sources of *S. cerevisiae* strains used in this work

Strain	Genotype	Source
EG103	*α his3Δ1, leu2-3,112 trp1-289, ura3-52, gal2*	[62]
EG103 *coq7*	*EG103Δcat5::HIS3*	[50]
EG103 *coq3*	*EG103Δcoq3::LEU2*	This study
CEN.PK2-1C	**A**, *his3-Δ1, leu2–3,112, trp1–289, ura3–52, MAL2–8c, MAL3, SUC3*	[63]
CEN *coq7*	*CEN PK2-1C Δcat5::HIS3*	[63]
CEN *coq3*	*CEN PK2-1C Δcoq3::LEU2*	This study
BY4741	**A**, *his3Δ1, leu2Δ0,met15Δ0, ura3Δ0*	Euroscarf
BY4741 *coq3*	*BY4741Δcoq3::LEU2*	This study
Y01709 (*tlg2*)	*BY4741 YOL018c::KanMX4*	Euroscarf
Y01709 *coq3*	*Y01709Δcoq3::LEU2*	This study
Y04462 (*vps45*)	*BY4741 YGL095c::KanMX4*	Euroscarf
Y04462 *coq3*	*Y04462Δcoq3::LEU2*	This study
Y00788 (*pep12*)	*BY4741 YMR202w::KanMX4*	Euroscarf
Y00788 *coq3*	*Y00788Δcoq3::LEU2*	This study
Y01812 (*erg2*)	*BY4741 YOR036w::KanMX4*	Euroscarf
Y01812 *coq3*	*Y01812Δcoq3::LEU2*	This study
NY431	*MAT a ura3-52 sec18-1*	[64]