Membrane stress is coupled to a rapid translational control of gene expression in chlorpromazine-treated cells

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Abstract Chlorpromazine (CPZ) is a small permeable cationic amphiphilic molecule that inserts into membrane bilayers and binds to anionic lipids such as poly-phosphoinositides (PIs). Since PIs play important roles in many cellular processes, including signaling and membrane trafficking pathways, it has been proposed that CPZ affects cellular growth functions by preventing the recruitment of proteins with specific PI-binding domains. In this study, we have investigated the biological effects of CPZ in the yeast *Saccharomyces cerevisiae*. We screened a collection of approximately 4,800 gene knockout mutants, and found that mutants defective in membrane trafficking between the late-Golgi and endosomal compartments are highly sensitive to CPZ. Microscopy and transport analyses revealed that CPZ affects membrane structure of organelles, blocks membrane transport and activates the unfolded protein response (UPR). In addition, CPZ-treatment induces phosphorylation of the translation initiation factor (eIF2α), which reduces the general rate of protein synthesis and stimulates the production of Gcn4p, a major transcription factor that is activated in response to environmental stresses. Altogether, our results reveal that membrane stress within the cells rapidly activates an important gene expression program, which is followed by a general inhibition of protein synthesis. Remarkably, the increase of phosphorylated eIF2α and protein synthesis inhibition were also detected in CPZ-treated NIH-3T3 fibroblasts, suggesting the existence of a conserved mechanism of translational regulation that operates during a membrane stress.

Abbreviations

CPZ	Chlorpromazine
Tm	Tunicamycin
3-AT	3-Aminotriazole
UPR	Unfolded protein response
ER	Endoplasmic reticulum
PI	Phosphoinositide
PS	Phosphatidylserine
PA	Phosphatidylamine
PE	Phosphatidylethanolamine

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Introduction

Control of gene expression at the level of mRNA translation is the most immediate response to environmental or cellular stresses (Dever 1999). This regulation involves the phosphorylation of the α subunit of the translation initiation factor eIF2 (Dever et al. 1992). Phosphorylated eIF2α (eIF2α-P) causes a rapid decrease of global translation initiation by reducing the activity of the eIF2 complex. eIF2 is

responsible for delivering charged methionyl initiator tRNA (Met-tRNAiMet) to the small (40S) ribosomal subunit in the first step of translation initiation. In mammalian cells, phosphorylation of eIF2α is mediated by four different protein kinases (PKR, HRI, GCN2 and PERK), which are activated in response to different cellular stresses such as a viral infection, heme deficiency in erythrocytes, nutrient starvation and accumulation of unfolded proteins in the ER [reviewed in Holcik and Sonenberg (2005)]. Gcn2p, the only eIF2α kinase in yeast, is activated upon depletion of any amino acid by binding the corresponding uncharged tRNA at its regulatory C-terminal domain, which is related to the histidyl-tRNA synthetase (HisRS) (reviewed in Hinnebusch 2005). In addition to inhibiting translation initiation of almost all mRNAs, eIF2α-P activates the expression of Gcn4p by a unique translational control mechanism (reviewed in Hinnebusch 2005). Gcn4p is the major transcriptional activator of amino acid biosynthetic genes that is activated under amino acid starvation (Natarajan et al. 2001), a cellular response named general amino acid control (GAAC).

Gcn2p activity is also induced by other environmental stresses, which all elicit the increase of Gcn4p expression (Cherkasova and Hinnebusch 2003). These findings have led to the hypothesis that Gcn4p is capable of responding to different stresses, and therefore functions in more than one transcriptional gene expression program. Notably, Patil et al. (2004) showed that Gcn4p also regulates the expression of genes that are unrelated to the activation of GAAC genes but are part of the unfolded protein response (UPR). The UPR is triggered by the accumulation of unfolded proteins in the ER and leads to the activation of the ER-resident transmembrane kinase/endoribonuclease Ire1p (Cox et al. 1993). The activated Ire1p catalyzes a nonconventional splicing reaction that governs the production of the transcription factor Hac1p. Hac1p was shown to associate with Gcn4p to bind to upstream activation sequences found in the promoters of some UPR target genes (Patil et al. 2004), thus allowing a transcriptional response to ER stress.

Recently, Chang et al. (2004) showed that the UPR is also activated after a partial membrane transport defect anywhere along the secretory pathway. Notably, the activation of the UPR alleviates the impairment of protein transport and partially suppresses the growth defect of *sec* mutants. Additionally, Leber et al. (2004) reported that a block of the secretory pathway leads to a pronounced increase of *HAC1* mRNA production, defining another mechanism of UPR control (termed S-UPR), which operates at the transcriptional level. These findings demonstrate that cells respond to a block of membrane transport to the cell surface by inducing a specific transcriptional program. In this context, we previously showed, using either *sec* mutants or chlorpromazine (CPZ), that eIF2α phosphoryla-

tion is strongly induced upon a membrane stress (Deloche et al. 2004). Thus, the expression of Hac1p and Gcn4p may be required to induce an appropriate cellular response that enables cells to cope with a membrane transport defect.

In this study, we investigated the toxic membrane effects of CPZ on actively growing *Saccharomyces cerevisiae* cells. CPZ is an antipsychotic drug historically used in the treatment of schizophrenia that blocks the activity of various neurophysiological receptors such as dopamine and serotonin receptors (Capuano et al. 2002; Trichard et al. 1998). Although CPZ was shown to directly bind to some of these receptors, recent works indicate that the diverse effects of CPZ are mostly attributable to its interaction with anionic lipids (Chen et al. 2003; Jutila et al. 2001). Here, we report that CPZ profoundly perturbs membrane properties of internal organelles and leads to a rapid translational control of gene expression.

Materials and methods

Strains, reagents and growth conditions

Yeast strains used for the study are shown in Table 1, and their construction is described below. Strains were grown in standard rich medium (yeast extract-peptone-dextrose, YPD) or in synthetic complete medium complemented with 2% dextrose (SD) and the appropriate amino acids for plasmid maintenance (Guthrie and Fink 1991). NIH-3T3 cells were maintained in DMEM supplemented with 10% fetal calf serum (GIBCO Life Science). Chlorpromazine, tunicamycin, 3-aminotriazole and thapsigargin were purchased from Sigma. Rapamycin was obtained from LC Laboratories. All these chemical products were prepared as recommended by the manufacturers.

The ODY304 (*sec7::SEC7-EGFPx3::URA3*) strain was constructed by pop-in-pop-out replacement with *SEC7-EGFP* as described in (Rossanese et al. 2001). Strains ODY326 and ODY327 were obtained by plasmid shuffling on 5-FOA plate using plasmids p[*SUI2, LEU2*; CEN] and p[*sui2S51A, LEU2*; CEN], respectively from the H1647 parental strain (25).

Chemical genomic screening analysis

The screen was performed using sublethal concentrations (10 and 20 μM) of CPZ in YPD medium buffered at pH 8.0 with 20 mM Tris–HCl pH 9.4. The EUROSCARF yeast deletion library (4,855 clones) and the parental strains BY4741 and BY4742 were replicated (in duplicate) on YPD plates at pH 8.0 containing 0, 10 and 20 μM CPZ. Cells were then incubated at 30°C and growth phenotypes were recorded after 2 days. Mutants with a no (−) or slow

Table 1 Yeast strains used in this study

Strain	Genotype	Source
BY4741	*Mata his3Δ1 leuΔ0 met15Δ0 ura3Δ0*	De Virgilio's lab
BY4742	*Matα his3Δ1 leuΔ0 met15Δ0 ura3Δ0*	De Virgilio's lab
SEY6210	*Mata leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9*	Efe et al. (2005)
LC49	*Mata leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 GLN3::myc13-kanMX6*	Cameroni et al. (2006)
ODY304	*Mata leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 sec7::SEC7-EGFPx3::URA3*	This study
RSY101	*Matα leu2-3,112 his3/4 kex2::URA3*	Schekman's lab
CY1100	*MATa leu2Δ0 lys2Δ0 ura3Δ0 sui2::Kan /* p[*SUI2, LEU2* CEN]	Cherkasova et al. (2003)
CY1101	*MATa leu2Δ0 lys2Δ0 ura3Δ0 sui2::Kan /* p[*sui2S51A, LEU2* CEN]	Cherkasova et al. (2003)
ODY197	*MATa ura3-52 leu2-3l leu2-112 trp1-Δ63::GCN4-LacZ::TRP1 sui2Δ /* pRS316[*SUI2, URA3*]	Deloche et al. (2004)
ODY198	*MATa ura3-52 leu2-3l leu2-112 trp1-Δ63::GCN4-LacZ::TRP1 sui2Δ /* pRS316[*sui2-S51A, URA3*]	Deloche et al. (2004)
H1647	*Matα ura3-52 leu2-3 leu2-112 ino2 sui2Δ HIS4-lacZ ura3-52 /* p[*SUI2, URA3*]	Dever's lab
ODY326	*Matα ura3-52 leu2-3 leu2-112 ino2 sui2Δ HIS4-lacZ ura3-52 /* p[*SUI2, LEU2* CEN]	This study
ODY327	*Matα ura3-52 leu2-3 leu2-112 ino2 sui2Δ HIS4-lacZ ura3-52 /* p[*sui2S51A, LEU2* CEN]	This study
D665-1A	*MATa* Prototrophic strain	Rose et al. (1988)

(+/−) growth phenotype relative to untreated strains were identified using the Saccharomyces Genome Database and the MIPS Comprehensive Yeast Genome Database (CYGD) and listed in Table 2.

Electron microscopy

Early log-phase cells were grown in YPD (approximately 50 OD_{600} units), harvested, and fixed in 3% glutaraldehyde, 0.1 M Na cacodylate (pH 7.4), 5 mM $CaCl_2$, 5 mM $MgCl_2$, and 2.5% sucrose for 1 h. Cells were then washed in 100 mM Tris (pH 7.5), 25 mM DTT, 5 mM EDTA, and 1.2 M sorbitol for 10 min, and resuspended in 100 mM K_2HPO_4 (pH 5.9), 100 mM citrate, and 1 M sorbitol. Following addition of oxylyticase (200 μg/ml) and Zymolyase T100 (600 μg/ml), cells were incubated for 40 min at 30°C. They were then spun down, washed, and resuspended in cold buffer containing 500 mM Na cacodylate (pH 6.8) and 25 mM $CaCl_2$, followed by osmium-thiocarbohydrazide staining. Further processing details have been described previously (Rieder et al. 1996).

Whole cell extracts and western blotting

Yeast whole cell extracts were prepared from 2 OD_{600} units of cells by a mild alkaline treatment (Kushnirov 2000). NIH-3T3 fibroblasts were lysed in RIPA buffer (150 mM NaCl, 1% NP40, 0.1% SDS, 1% deoxycholate, 50 mM Tris–HCl pH 7.4) supplemented with a cocktail of protease inhibitors (Complete, Roche) for 30 min at 4°C. Lysates were then clarified in a tabletop centrifuge. For the analysis of eIF2α phosphorylation, equal amounts of protein from different extracts were resolved by SDS-PAGE and subjected to western blotting using monospecific antibodies for phosphorylated S51 in eIF2α (Biosource/44-718G) and polyclonal antibodies that recognize total eIF2α (generous gift from T. Dever).

Polysome analysis

Sucrose gradient analyses were performed according to de la Cruz et al. (1997).

Northern blotting

Total RNA was extracted from 100 OD_{600} units of exponentially growing cells and analyzed (5 μg) by northern blot as described (De Virgilio et al. 1993). DNA probes were labeled with [α−^{32}P]CTP using the PRIME-IT II Random Primer Labeling Kit (Stratagene 300385).

[35]S pulse-chase assay and coimmunoprecipitation

Cell labeling was performed as described previously (Gaynor and Emr 1997). Briefly, mid-logarithmic phase (OD_{600} 0.6) cultures were concentrated to two OD_{600} units/ml and

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Table 2 List of gene knockout mutants sensitive to chlorpormazine

Gene name	CPZ		Functions
	10 μM	20 μM	
Endosomal Golgi proteins			
SAC1	−	−	Phosphoinositide 4 phosphatase involved in protein trafficking
VPS1	−	−	Dynamin involved in Golgi/endosomal membrane trafficking
VPS54	−	−	Subunit of VFT complex involved in Golgi membrane trafficking
VPS52	−	−	Subunit of VFT complex involved in Golgi membrane trafficking
PEP5	−	−	Subunit of the HOPS complex involved in membrane fusion
DRS2	+/−	−	P-type aminophospholipid translocase involved in secretion
PEP12	+/−	−	Endosomal t-SNARE involved in membrane fusion
APL2	+/−	−	AP-1 complex subunit required in the formation of clathrin vesicles
KRE11	+/−	−	Subunit of TRAPPII complex involved in Golgi membrane trafficking
SNF8	+/−	−	Subunit of the endosomal protein sorting complex ESCRT-II
SNF7	+/−	−	Subunit of the endosomal protein sorting complex ESCRT-III
VPS33	+/−	−	Subunit of the HOPS complex involved in membrane fusion
RCY1	+/−	−	F-box protein involved in protein recycling
VPS23	+/−	+/−	Subunit of the endosomal protein sorting complex ESCRT-I
VPS36	+	+/−	Subunit of the endosomal protein sorting complex ESCRT-II
VPS38	+	+/−	Part of the Vps34 PI3-kinase complex
MNN9	+	+/−	Alpha-1,6 mannosyltransferase of *cis*-Golgi compartment
KEX2	+	+/−	Endoproteinase of late-Golgi compartment
ER proteins			
ERG2	+/−	−	C-8 sterol isomerase
DER1	+/−	−	Involved in degradation proteins
Ribosomal components			
RPS4A	+/−	+/−	40S ribosomal protein S4
TIF3	+/−	+/−	Translational initiation factor eIF4B
FUN12	+/−	+/−	General translation factor eIF2 homolog
PTH	+/−	+/−	Peptidyl-tRNA hydrolase
MTQ2	+/−	+/−	*S*-adenosylmethionine-dependent methyl transferase
Transcription factor/nuclear proteins			
CTK3	+/−	−	Carboxy-terminal domain (CTD) kinase, gamma subunit
TAF14	+/−	−	Transcription initiation factor TFIID/cellular morphogenesis
SAC3	+/−	−	Leucine permease transcriptional regulator
THP1	+/−	−	Nuclear pore associated protein
REF2	+	+/−	RNA 3′-end formation protein
GCN5	+	+/−	Histone acetytransferase
RSC2	+	+/−	Part of the chromatin structure remodeling complex (RSC)
Metabolism—Kinase/phosphatase			
SHP1	+/−	−	Regulatory subunit for Glc7p phosphatase
CKB2	+/−	−	Beta regulatory subunit of casein kinase 2
STA1	+/−	−	Glucoamylase I (alpha−1,4 glucan glucosidase)/periplasm
YSA1	+/−	+/−	Adenosine diphosphoribose pyrophosphatase
SIT4	+/−	+/−	Serine/threonine protein phosphatase
Ions transporters/chaperone			
CCS1	+/−	+/−	Copper chaperone for superoxide dismutase Sod1p
SSQ1	+	+/−	Mitochondrial heat shock protein70
Polarized growth			
SHE4	+	+/−	Required in actin organization and intracellular mRNA localization

Table 2 continued

Gene name	CPZ		Functions
	10 μM	20 μM	
Proteins with unknown functions			
YLR169W	+	+/−	
YHR210C	+	+/−	Similarity to UDP-glucose-4-epimerase
YLR169W	+	+/−	

(+), (+/−) and (−) indicate normal, slow or no growth phenotype, respectively. All strains are isogenic to the parental BY4741, a derivative of S288C strain background

labeled with 3 μl Redivue™ PRO-MIX™ Cell Labelling Mix (Amersham Biosciences/AGQ0080) for 10 min in SD medium containing 20 μg/ml BSA. Cells were chased with 10 mM methionine, 4 mM cysteine and 0.4% yeast extract for 20 min. To separate cells from media, cell suspensions were centrifuged at 14,000g for 2 min. To assay [^{35}S] methionine/cysteine incorporation into proteins, whole cell extracts were prepared by a mild alkaline treatment (Kushnirov 2000). To determine secreted proteins, media fractions were precipitated by addition of TCA to a final concentration of 10%. After washing the pellets twice in cold acetone, proteins were solubilized in Laemmli sample buffer plus 5% β-mercaptoethanol. Labeling and immunoprecipitation of secreted pro-α-factor were performed as described (Seeger and Payne 1992). Labeled proteins and labeled immunoprecipitated pro-α-factor were resolved on SDS-PAGE gels and analyzed by fluorography.

To label NIH-3T3 fibroblasts, subconfluent dishes of cells in DMEM supplemented with 10% FCS were pretreated with the indicated drugs for 10 min prior to a pulse labeling period of 40 min with 50 μCi of Redivue™ PRO-MIX™ Cell Labelling Mix. Cells were subsequently washed two times with ice-cold PBS and lysed in RIPA buffer. The lysate was clarified in a tabletop centrifuge and 6 μg of proteins were separated by SDS-PAGE and analyzed by fluorography.

Fluorescence and indirect immunofluorescence microscopy

For CPZ detection, cells were grown in YPD buffered at pH 8.0 with 20 mM Tris–HCl pH 9.4 to mid-logarithmic phase at 30°C. For GFP images, ODY304 cells were grown in SD-URA buffered at pH 8.0 with 20 mM Tris–HCL pH 8.0 to mid-logarithmic phase at 30°C and treated or not with 100 μM CPZ for 30 min. Preparation of cells for immunofluorescence was essentially as described (Chuang and Schekman 1996). Cells were grown to 0.8 OD$_{600}$ units/ml in SD medium at 30°C. Gln3p-myc13 was detected using mouse α-Myc (9B11, Cell Signaling) at a dilution of 1:2,000. Cy3 (indocarbocyamine)-conjugated goat anti-mouse secondary antibodies (Jackson ImmunoResearch Laboratories) were used at 1:2,000 dilution. Images (100×

magnification) were obtained using a Zeiss microscope equipped with an Axiocam color digital camera and the AxioVision™ software. Figures were prepared with the use of the Adobe Photoshop 8.0 (Adobe Systems, San Jose, CA, USA) software program.

β-galactosidase assay

Cultures of cells containing the *UPRE-LacZ* plasmid (Zhou and Schekman 1999), a genomic integrated *GCN4-LacZ*, or *HIS4-LacZ* reporter were grown in synthetic complete medium to early-logarithmic phase before drugs were added for 2 h. UPR, Gcn4p and His4p expressions were measured by a standard β-galactosidase assay and the activity was normalized to the OD$_{600}$ of cells (Miller Units) used for each assay. All results are an average of at least three independent determinations.

Results

CPZ prevents yeast cell growth and accumulates on internal membrane structures in a pH-dependent manner

CPZ is a permeable amphiphilic molecule that contains a hydrophobic tricyclic ring and a hydrophilic dimethylpropylamine side chain (Fig. 1a). The hydrophobic moiety allows CPZ to intercalate and diffuse within the hydrocarbon phase of membrane bilayers. At physiological pH, the tertiary dimethylpropylamine is protonated, creating a net positive charge and leading to the accumulation of CPZ in the cytosolic membrane leaflet by the formation of electrostatic interactions with anionic lipids such as phosphatidylserine (PS), phosphatidylamine (PA) and phosphoinositides (PIs) (Fig. 1b) (Chen et al. 2003; Jutila et al. 2001; Sheetz and Singer 1974). To investigate the toxic membrane effects of CPZ on actively growing yeast cells, we spotted a wild-type culture grown to exponential phase on rich medium (YPD) plates containing increasing concentrations of CPZ, at different pH (Fig. 1c). We found that the minimal concentration of CPZ required to block growth is 30 μM at pH 8, and that lowering the pH results in

increased resistance to CPZ. At pH 3, cell growth is no longer affected by 60 μM of CPZ. These data corroborate the previously observed pH-dependent effects of CPZ on erythrocyte membranes (Ahyayauch et al. 2003), and might be explained by the fact that positively charged CPZ is unable to enter the cells and diffuse within membranes. To test this possibility further, we took advantage of the fact that CPZ fluorescence intensity increases when interacting with anionic lipids (Chen et al. 2003), which allows it to be visualized within cells. As expected, no intracellular fluorescence was detected at acidic pH (Fig. 1d), confirming that protonated CPZ cannot cross the plasma membrane bilayer. At pH 8, CPZ diffuses within the cell and partitions into visible membrane structures, which are reminiscent of the ER and vacuole compartments (data not shown). Furthermore, the presence of bright spots of fluorescence in the interior of cells (Fig. 1d) suggests that CPZ is protonated in the cytosol, allowing its binding to anionic lipids present on the cytosolic face of subcellular compartments. We therefore propose that the insertion of CPZ into the cytosolic membrane bilayer of internal organelles alters essential cellular growth activity.

A genome-wide screen reveals that mutants displaying a transport defect between late-Golgi and endosomal compartments are CPZ-hypersensitive

To gain a better insight into the biological effects of CPZ on yeast membranes, we next performed a genome-wide screen for CPZ-hypersensitivity. In analogy to the principle of the synthetic lethal screen (Huffaker et al. 1987), CPZ-hypersensitive mutants are likely to define essential biological functions that also are targeted by CPZ. For this screen, a collection of yeast deletion strains (4,857 knockout mutants) was replicated on YPD plates at pH 8.0 containing either no drug or two different sub-lethal concentrations of CPZ and grown for two days at 30°C. The genes identified as essential for normal growth in the presence of CPZ are listed in different functional categories in the Table 2. Interestingly, the most CPZ-sensitive strains bear mutations that directly perturb membrane and protein trafficking between the late-Golgi and endosomal compartments. Among the genes identified, *SAC1* encodes a phosphoinositol-4-phosphatase that regulates membrane and cytoskeleton function (Foti et al. 2001; Schorr et al. 2001). In this respect, it is noteworthy that CPZ increases the steady state level of PI4-P (phosphate at the D-4 position of the inositol ring) in human platelets and in mice fibroblasts (Frolich et al. 1992; Raucher and Sheetz 2001). PIs, but also PA and phosphatidylethanolamine (PE), which serve as a lipid platform for the recruitment of coat proteins and promote membrane curvature to initiate the formation of vesicles (Bankaitis et al. 1990; Ktistakis et al. 1996; Rothman and Wieland
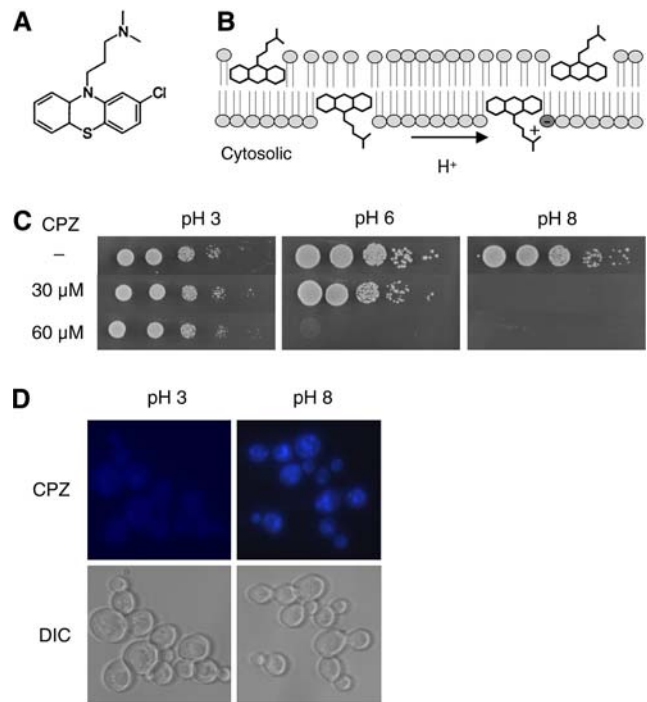

Fig. 1 The insertion of CPZ into membrane bilayers affects yeast viability. **a** Chemical structure of CPZ. **b** Schematic representation showing the interaction of CPZ with negatively charged lipids. The hydrophobic nature of CPZ promotes its association with and diffusion within the lipid bilayer membrane. When exposed to the cytosol, CPZ is protonated and accumulates in the inner leaflet of membranes by the formation of electrostatic interactions with anionic phospholipids. This model is based on data published by Chen et al. (2003). **c** The toxicity of CPZ is pH dependent. Equivalent numbers of wild-type cells (SEY6210) were serially diluted and spotted onto YPD plates containing 0, 30 and 60 μM of CPZ at pH 3, 6 or 8. **d** The accumulation of CPZ within the cells occurs only at high pH. SEY6210 (wild-type) cells were incubated with CPZ (100 μM) for 30 min at pH 3 or 8. CPZ fluorescence emission was then detected by microscopy (UV excitation DAPI/365 nm). Cells were observed by differential interference optics (*DIC*)

1996), are potential CPZ binding sites. Thus, it is likely that CPZ, by interacting with negatively charged lipids, interferes with membrane transport and produces a synergistic growth defect when combined with mutants that block membrane trafficking between the late-Golgi and endosomal compartments.

CPZ alters membrane structures of internal organelles and blocks the secretory pathways

To determine whether CPZ alters Golgi functions by interfering with the binding of proteins to Golgi membranes, we used a wild-type strain expressing a Sec7p-GFP fusion protein. Sec7p is an abundant peripheral membrane protein of the late Golgi, which is involved in vesicular membrane trafficking (Franzusoff et al. 1992). Under normal growth conditions, the Sec7p-GFP fluorescence was detected in

bright spots representing Golgi elements (Fig. 2a). In the presence of 100 μM CPZ, the fluorescence was more diffused throughout the cells, revealing a lower capacity of Sec7p to bind to Golgi membranes, and as a consequence, to regulate membrane trafficking. Since defects of membrane transport through the late-Golgi and endosomal compartments often lead to a marked distortion of organelles of the secretory pathway, we next examined CPZ-treated cells by electron microscopy analysis. Ultrastructural analysis (Fig. 2b) revealed that cells treated with CPZ accumulate enlarged ER, Berkeley bodies [membrane structures that were initially observed in mutants defective in Golgi transport (Novick et al. 1980)], as well as membrane inclusions and other undefined aberrant membrane structures. Additionally, vacuoles were also larger and less electron dense, suggesting an effect of CPZ on vacuolar membrane permeability. Another striking morphology change was the formation of small blebs on the plasma membrane, which likely result from a decrease in plasma membrane–cytoskeleton adhesion (Raucher and Sheetz 2001). Finally, high concentrations of CPZ cause formation of micelles (data not shown), which were visualized by fluorescence (Fig. 1d). Collectively, these observations further support that an important deregulation of membrane transport occurs after CPZ treatment and prompted us to investigate the effect of CPZ on the secretory pathway. To do this, we took advantage of the fact that yeast cells efficiently secrete a few proteins into the growth medium (Robinson et al. 1988). Wild-type cells were incubated with or without CPZ for 10 min at 30°C, labeled with [^{35}S]-methionine/cysteine for 10 min (pulse) and chased for 20 min. Cells and media were separated by centrifugation and proteins in the media were precipitated with TCA and resolved by SDS-PAGE. The incorporation of [^{35}S]-methionine/cysteine into proteins was reduced in cells incubated with CPZ, indicating that CPZ causes a general inhibition of protein synthesis (Fig. 3a). Protein secretion was severely affected, with only one abundant protein (migrating in the molecular weight range of the secreted Hsp150p) detected in media of cultures treated with 100 μM CPZ (Fig. 3b) (Gaynor and Emr 1997; Russo et al. 1992). In addition, under the same growth condition, the newly synthesized precursor forms of the α-factor pheromone that are normally secreted from cells lacking the endoprotease Kex2p (Fuller et al. 1988; Julius et al. 1984) were not immunoprecipitated from media using α-factor precursor antiserum (Fig. 3c), which further confirms a secretory transport defect in cells treated with CPZ.

CPZ induces the UPR pathway and *GCN4* mRNA translation

To alleviate a secretory stress, cells activate the UPR pathway that leads to the expression of specific genes, whose products promote stress adaptation thereby allowing cells
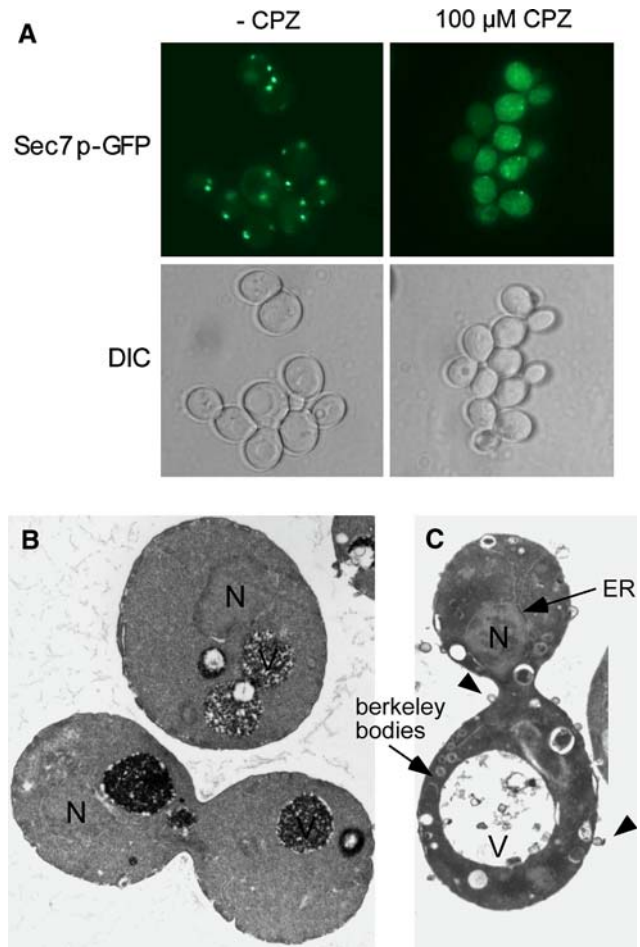

Fig. 2 CPZ perturbs membrane structures **a** CPZ reduces the binding affinity of Sec7-GFP to late-Golgi membrane. ODY304 (*SEC7*-GFP) cells were grown to mid-logarithmic phase at 30°C, treated or not with CPZ (100 μM) for 30 min and processed for fluorescence microscopy. Cells were observed by differential interference optics (DIC). Images shown are representative of more than 100 cells observed. **b**, **c** EM analysis of subcellular structure in CPZ-treated cells. SEY6210 (wild-type) cells were grown to mid-logarithmic phase at 30°C (non-treated **b**) and CPZ (100 μM) was added for 40 min **c**. Cells were subsequently processed and visualized by EM as described in Materials and methods. *Black arrowheads* indicate the presence of plasma membrane blebs. Vacuoles (*V*), nuclei (*N*), endoplasmic reticulum (*ER*), berkeley bodies are also indicated

to grow. We next tested whether the UPR is activated in CPZ-treated cells. Activation of the UPR was assayed by β-galactosidase activity (Fig. 4a) using an UPRE-*lacZ* reporter gene transformed in wild-type cells. As expected, cells treated with tunicamycin (Tm), which blocks N-protein glycosylation and elicits the UPR, exhibit elevated UPRE expression (5.8-fold increase). Treatment with 30 μM CPZ, a concentration that slightly affects protein synthesis (data not shown), results in a 2.7-fold increase of β-galactosidase activity, indicating that CPZ activates the UPR pathway at this concentration. At 100 μM CPZ, the UPR was slightly lower, a result that might be explained by
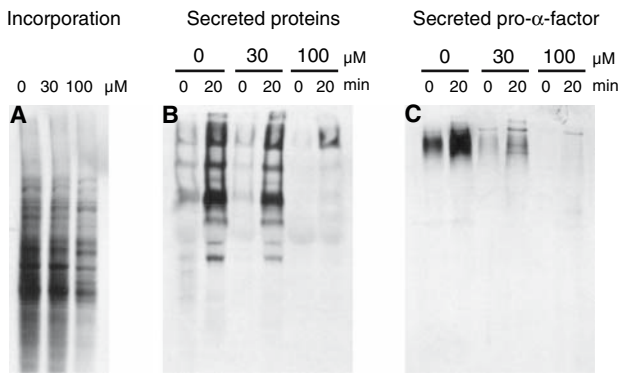
Incorporation	Secreted proteins	Secreted pro-α-factor

Fig. 3 CPZ blocks the secretory pathways. **a**, **b** SEY6210 (wild-type) cells were incubated at 30°C with the indicated concentration of CPZ (0, 30 or 100 μM) for 10 min before being metabolically [^{35}S]-labeled for 10 min and chased for 20 min. Cells and media were separated by centrifugation and protein secreted in media during the pulse-chase were precipitated with 10% TCA. The incorporation of [^{35}S] methionine/cysteine into proteins from cells after 10 min of pulse **a** and secreted [^{35}S]-proteins from media after 0 or 20 min of chase **b** were visualized by SDS-PAGE and fluorography. **c** RSY101 (*kex2Δ*) cells were metabolically [^{35}S]-labeled as described in **a** and **b**. After 0 or 20 min of chase, the pro-α-factor was immunoprecipitated from media using rabbit polyclonal pro-α-factor antibody and detected by SDS-PAGE and fluorography

the inhibitory effects of CPZ on protein synthesis and/or *GCN4* transcription. We previously showed that a CPZ treatment causes a rapid phosphorylation of eIF2α (Deloche et al. 2004). In accord with this observation and with the role of eIF2α in the activation of *GCN4*, we showed that 30 μM CPZ leads to a 3.1-fold increase of the *β*-galactosidase activity in cells containing the *GCN4-lacZ* reporter (Fig. 4b). Importantly, as in rapamycin-treated cells, CPZ-induced *GCN4* expression depends on eIF2α phosphorylation, since no increase of the *β*-galactosidase activity was observed in cells expressing the non-phosphorylated eIF2*a*^{S51A} (in a *sui2*-S51A mutant). Additionally, it is worth noting that UPRE expression is also significantly reduced in the *sui2*-S51A mutant upon an ER stress (Fig. 4a), which corroborates the previous finding that Gcn4p expression plays a major role in the UPR activation (Patil et al. 2004). Altogether, these results demonstrate that cells upregulate a subset of stress genes in response to a mild CPZ treatment. The transcription of the *HIS4* gene is known to be upregulated by Gcn4p under conditions of amino acid starvation (Drysdale et al. 1995). We thus determined whether Gcn4p induced by CPZ increases the *β*-galactosidase activity in cells containing the *HIS4-lacZ* reporter. As shown in Fig. 4c, the *HIS4-lacZ* is not derepressed under conditions where *GCN4-lacZ* activity is induced by CPZ. In contrast, the addition of 3-aminotriazole (3-AT; an inhibitor of histidine biosynthesis) increased *HIS4-lacZ* activity in an eIF2α phosphorylation-dependent manner. This finding strengthens previous results showing that the expression of Gcn4p

is not only dedicated to the control of amino acid biosynthesis (Natarajan et al. 2001). Notably Gcn4p also regulates gene products involved in degradation of autophagosomes in a process known as autophagy (Klionsky and Emr 2000). In this context, a recent study (Steffensen and Pedersen 2006) reported that induction of heterologous membrane protein production derepresses Gcn4p without increasing transcription of *HIS4*. Under these conditions, Gcn4p may stimulate expression of genes whose products are involved in autophagy, thereby contributing to the degradation of excess of useless and/or toxic proteins. It is likely that CPZ causes mis-localization of a large number of membrane proteins by affecting membrane structures and vesicular trafficking. Thus, by analogy, the increase of Gcn4p following CPZ treatment may induce transcription of both autophagy and UPR genes (see also Discussion). This transcriptional response would serve to alleviate cellular stress by increasing the degradation rate of mis-localized membrane proteins and by restoring membrane transport along the biosynthetic pathways.

Inhibition of translation initiation occurs in a prototrophic strain and does not result from TORC1 inactivation

As observed by others (Cherkasova and Hinnebusch 2003), we detected an increase of eIF2α phosphorylation in cells treated with 10 μg/ml Tm. Our data however suggest that the UPR pathway does not mediate this increase, since we observed that UPR-deficient mutants (*hac1Δ* and *ire1Δ*; data not shown) show a similar eIF2α phosphorylation level as wild-type cells when treated with Tm. This finding suggests that eIF2α phosphorylation does not result from the ER stress, but possibly indirectly from a defective membrane transport event along the secretory pathway. One hypothesis would be that CPZ inhibits translation initiation by altering the subcellular localization and/or the activity of amino acid transporters, which could then lead to a rapid depletion of the intracellular amino acid pool. For instance, the volatile anesthetic isoflurane was previously shown to induce the phosphorylation of eIF2α by inhibiting the import of some amino acids in auxotrophic strains (Palmer et al. 2002). To address this possibility, we tested the effect of CPZ on eIF2α phosphorylation and translation initiation in the prototrophic strain D665-1A (Fig. 5a). As observed in SEY6210 (auxotroph), eIF2α was also phosphorylated in D665-1A after 30 min of 30 μM CPZ treatment (Fig. 5b). Accumulation of the 80S monosome and decrease of polysome content were also evident (Fig. 5c), corroborating a translation initiation defect. Higher concentrations of CPZ led to an even greater translation defect with a higher level of eIF2α-P. At 200 μM CPZ, protein synthesis was totally blocked as judged by the lack of actively translating ribosomes (Fig. 5c). Interestingly, a significant increase of the
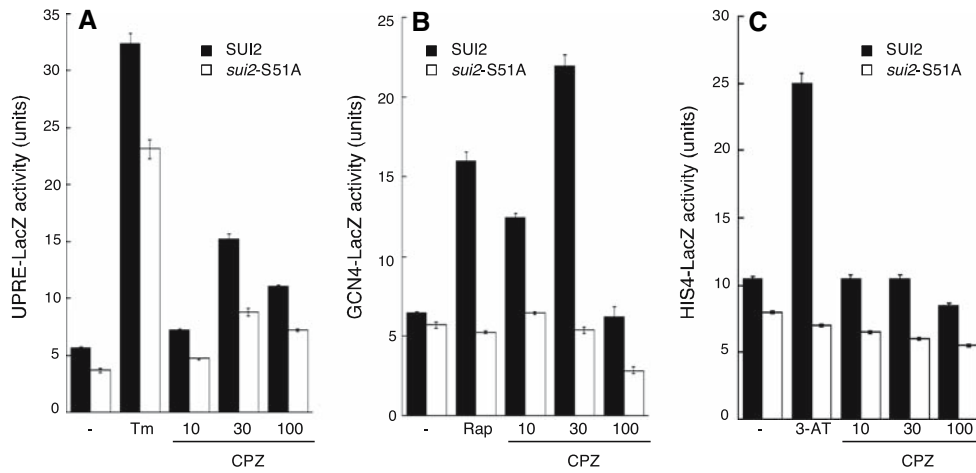
Fig. 4 CPZ activates the UPR pathway and *GCN4* expression. **a** CY1100 (*SUI2*) and CY1101 (*sui2*-S51A) strains were transformed with a reporter plasmid (*CEN*, *URA3*) carrying the *UPRE-LacZ* construct and grown in SD medium at 30°C to mid-logarithmic phase. **b** ODY197 (*SUI2*) and ODY198 (*sui2*-S51A) containing a genomic integrated *GCN4-lacZ* reporter and **c** ODY326 (*SUI2*) and ODY327 (*sui2*-S51A) containing a genomic integrated *HIS4-lacZ* reporter were grown as described in **a**. Tunicamycin (Tm 10 μg/ml), Rapamycin (Rap 1 μg/ml), 3-aminotriazole (3-AT 10 mM) or CPZ (10, 30 or 100 μM) were added for 2 h as indicated. The levels of β-galactosidase activity were measured as described in Materials and methods. The results shown are from an average of at least three independent determinations. *Black bars* are cells containing the *SUI2* allele and white bars are cells containing the *sui2*-S51A mutant allele

40S/60S ratio was observed, indicating that either the synthesis or the stability of the 60S subunit is affected. These data are reminiscent of previous studies of the group of J. R. Warner, who showed a close relationship between the secretory pathway and ribosome biogenesis (Mizuta and Warner 1994) and a transcriptional repression of ribosomal protein genes upon a CPZ treatment at 250 μM (Nierras and Warner 1999).

TORC1 (Target Of Rapamycin Complex 1) controls the activity of Gcn2p in response to cellular nutrient availability (Cherkasova and Hinnebusch 2003) and associates with membrane endocytic elements (Chen and Kaiser 2003). This raised the possibility that CPZ might inactivate TOR activity by altering the localization and/or the lipid environment of TOR proteins. To address this possibility, we examined whether CPZ changes the expression of specific genes or the levels of glycogen, which are under the control of TORC1. Gln3p is the transcription activator of nitrogen catabolic genes, which is translocated into the nucleus under low nitrogen conditions or in response to TORC1 inhibition by rapamycin (Beck and Hall 1999). A wild type strain containing a genomically integrated myc-tagged *GLN3* allele was treated with CPZ (100 μM) or rapamycin (1 μg/ml; ~1 μM) for 30 min and 60 min (data not shown) and Gln3p localization was visualized by indirect immunofluorescence. As shown in Fig. 6a, Gln3p is found throughout the cytoplasm of CPZ-treated cells, while in control experiments, rapamycin caused a strong accumulation of Gln3p in the nucleus. We next measured the mRNA levels of *HSP26* and *GLN1*, which are normally derepressed in rapamycin-treated cells

(Fig. 6b). At 30 μM, a concentration of CPZ that inhibits ribosome biosynthesis (as judged by the decrease of the mRNA levels of *RPS12* and the ribosome-associated heat shock protein *SSB1*), the expression of *GLN1* and *HSP26* genes remains still repressed. At a higher concentration of CPZ (100 μM), a slight induction of *GLN1* after 30 min and *HSP26* after 60 min was detected, suggesting that TORC1 might be partially inactivated under this condition. Finally, almost no glycogen accumulates in CPZ-treated cells as compared to cells treated with rapamycin (Fig. 6c). Taken together, our results indicate that, at least at concentrations of CPZ below 100 μM, inhibition of protein synthesis following CPZ treatment does not result from inhibition of an amino acid permease and/or TORC1 inactivation.

CPZ increases the phosphorylation of eIF2α and inhibits protein synthesis in NIH-3T3 cells

Previously, CPZ was shown to block clathrin-mediated endocytosis and the recycling of LDL receptor to the cell surface in human fibroblast cells (Wang et al. 1993). These data prompted us to determine whether a membrane transport defect induced by CPZ also results in a rapid inhibition of protein synthesis in mammalian cells. We used mice fibroblast NIH-3T3 cells that have previously been studied under conditions of CPZ treatment (Raucher and Sheetz 2001). As in yeast cells (Fig. 1a), CPZ traverses the plasma membrane and accumulates at the interior of the cell, potentially by interacting with internal membranes (Fig. 7a). To determine if CPZ inhib-
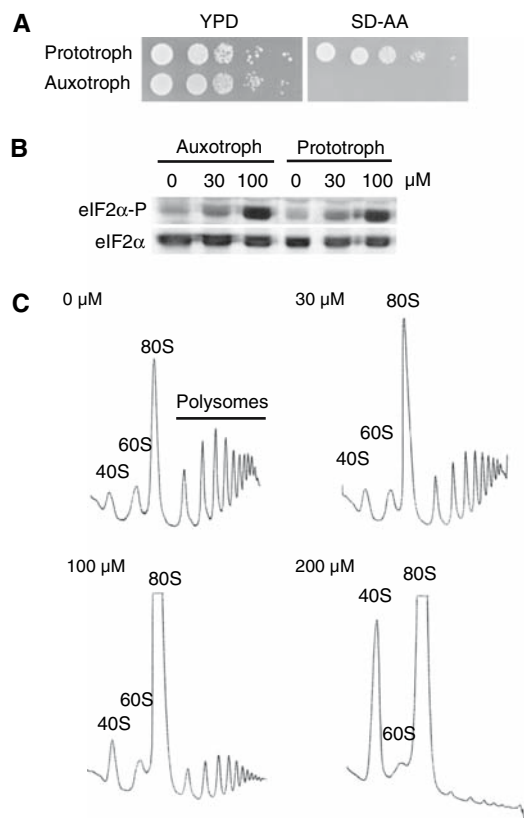
Fig. 5 Translational inhibition also occurs in a CPZ-treated prototrophic strain. **a** The prototrophic strain (D665-1A) grows in medium lacking amino acids. SEY6210 (auxotroph) and D665-1A (prototroph) cells were serially diluted and spotted onto YPD or SD-AA plates and incubated for 2 days at 30°C. **b** CPZ induces the phosphorylation of eIF2α in the prototrophic strain. SEY6210 (auxotroph) and D665-1A (prototroph) cells were grown to mid-logarithmic phase at 30°C in YPD and CPZ was added for 30 min at the indicated concentration. Whole cell extracts were prepared and phosphorylation of eIF2α (S51) was compared with the total amount of eIF2α protein determined by western analysis. **c** CPZ alters the polysome profile in the prototrophic strain. D665–1A (prototroph) cells were grown in YPD at 30°C to mid-logarithmic phase, split and the indicated concentration of CPZ was added to cultures for 45 min. Cells were then harvested and polysomes were analyzed. The positions corresponding to the 40S, 60S subunits, 80S monosomes and polysomal ribosomes are indicated

its protein synthesis, we then measured the incorporation of [^{35}S]-methionine/cysteine in CPZ-treated NIH-3T3 cells (Fig. 7b). At 30 μM concentration, almost no radiolabeled proteins were detected, indicating that CPZ rapidly blocks mRNA translation. Notably, under this condition, the uptake of radiolabeled amino acids into cells was not affected (data not shown). Importantly, this inhibition was associated with an increase in eIF2α phosphorylation, similarly as in cells exposed to either thapsigargin, dithiothreitol or Tm; all agents that are known to induce an ER stress and to inhibit protein synthesis by activating the ER-localized eIF2α kinase PERK (Fig. 7b)
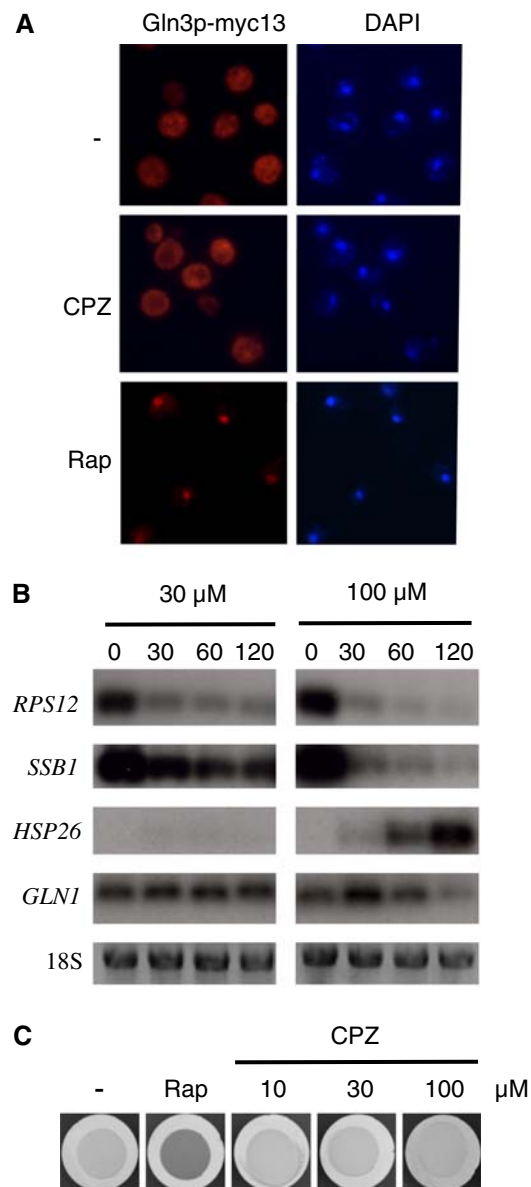

Fig. 6 TORC1-dependent signaling pathways are not affected in CPZ-treated cells. **a** LC49 (*GLN3::myc13*) strain expressing genomically tagged Gln3p-myc13 were grown in YPD and treated with CPZ (100 μM) or rapamycin (Rap 1 μg/ml). Gln3p-myc13 was visualized by immunofluorescence using monoclonal anti-Myc antibody and DNA was stained with DAPI. **b** SEY6210 (wild-type) was grown as in **a** and treated with CPZ (30 or 100 μM). At the indicated times, cells were harvested and northern analysis of indicated genes was performed as described in Materials and methods. **c** Glycogen accumulation was visualized after exposure for 1 min to iodine vapor (Dubouloz et al. 2005) on exponentially growing SEY6210 (wild-type) cells, treated or not with rapamycin (Rap 200 ng/ml) or CPZ (10, 30 or 100 μM) for 4 h

(Harding et al. 2000). Altogether these results reveal that the relationship between membrane stress and translation initiation might be conserved from yeast to mammalian cells.
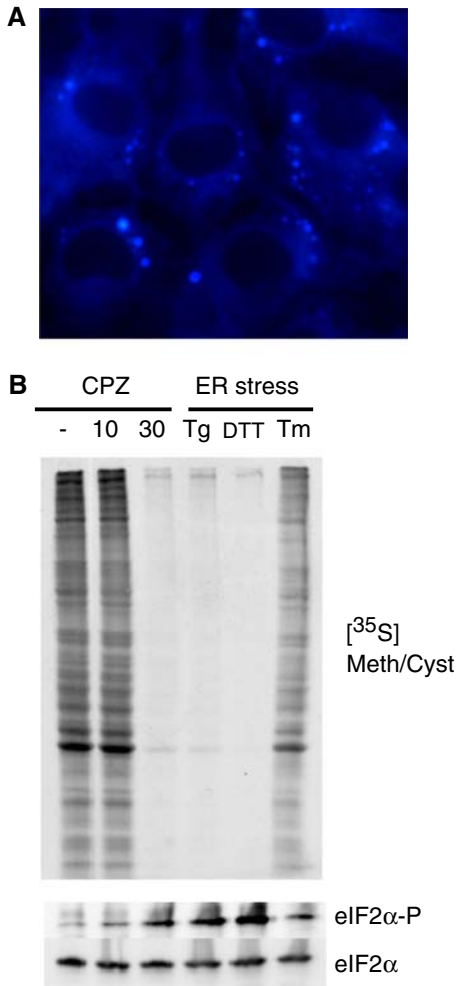
A

B

[^{35}S] Meth/Cyst

eIF2α-P

eIF2α

Fig. 7 CPZ inhibits protein synthesis and induces the phosphorylation of eIF2α in NIH-3T3 fibroblasts. **a** A fluorescent picture of CPZ-treated NIH-3T3 fibroblasts. NIH-3T3 fibroblasts were incubated with 100 μM CPZ for 30 min and fluorescence was visualized by microscopy as in Fig. 1c. **b** Protein synthesis rates were measured by the incorporation of [^{35}S] methionine/cysteine into proteins. NIH-3T3 fibroblasts were incubated with either 10 or 30 μM CPZ, 1 μM thapsingargin (*Tg*), 2 mM DTT or 10 μg/ml tunicamycin (*Tm*) for 10 min prior to a 40 min pulse labeling period. The labeled proteins in a whole cell extract were resolved by SDS-PAGE and fluorography (*upper panel*). Phosphorylation of eIF2α, from the same extracts, was detected by western analysis and compared with the total amount of eIF2α protein and shown in the *lower panel*

Discussion

CPZ perturbs internal membrane structures and protein transports

CPZ is a permeable amphiphilic molecule that partitions into the lipid cytoplasmic half of the membrane bilayer by interacting with the polar headgroups of phospholipids, resulting in the deformation of membrane structures. In the present study, we investigated the effects of CPZ on yeast membranes and demonstrate that CPZ diffuses within the cells and deforms membranes of intracellular organelles. Because negatively charged lipids such as PI, PA and PE localized on Golgi and endosomal compartments are known to induce membrane curvatures and recruit coat proteins to initiate the formation of vesicles, we argue that CPZ inhibits intracellular trafficking by modifying the structure and the net charges of membranes on secretory/endocytic compartments. Consistent with this idea, we showed that CPZ prevents the binding of Sec7p, an essential protein for secretion, onto late-Golgi membranes and rapidly blocks transport of some secreted proteins. Furthermore, our CPZ genetic screening analysis on the entire knockout gene mutant library revealed that the most CPZ-sensitive strains are those bearing mutations that alter membrane and protein trafficking between the late-Golgi and endosomal compartments.

CPZ-mediated stress leads to the expression of UPR genes and of Gcn4p, but does not inactivate TORC1 activity

Like most mutants experiencing a partial membrane transport defect (Chang et al. 2004), CPZ activates UPR gene expression. This transcriptional response is required for a rapid cellular adaptation in response to a membrane transport defect (Chang et al. 2004). The UPR integrates the Gcn4p-dependent cytosolic stress response (Patil et al. 2004). In this context, CPZ also induces *GCN4* expression. Translational induction of *GCN4* is part of the cellular response to changes in the environment such as depletion of amino acids (Natarajan et al. 2001). In CPZ-treated cells, the expression of Gcn4p depends on the Gcn2p-stimulated eIF2α phosphorylation. Since the only known way to activate Gcn2p is through binding of uncharged tRNA to its histidyl-tRNA-like domain, we first hypothesized that CPZ reduces the cytoplasmic levels of amino acids by decreasing the activity of amino acid transporters. Our data showed the phosphorylation of eIF2α still occurs in a prototrophic strain, indicating that CPZ does not block the import of amino acids. Nevertheless, we next reasoned that CPZ might reduce the activity of amino acid transporters localized on internal organelles such as the vacuoles (principal cellular amino acid storage). This might cause a decrease of the intracellular amino acid concentration, specially since the vacuolar membrane permeability appears to be affected by CPZ (Fig. 2b). However, none of the tested TORC1 readouts, which are normally induced under amino acid starvation, were significantly changed after CPZ treatments at concentration that induce Gcn4p expression. Furthermore, CPZ does not induce *HIS4* transcription, which is normally activated by Gcn4p under conditions of amino acid starvation. These results indicate that CPZ-treated cells do not seem to respond to a particular nutrient limitation

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and that Gcn4p is likely required to deal with another stress situation.

We recently showed that a decrease of phosphatidyl inositol 4-phosphate (PI4-P) levels on Golgi and ER organelles in a *pik1* (PI4-kinase) mutant result in a rapid phosphorylation of eIF2α (Cameroni et al. 2006). Like *sac1Δ* and *drs2Δ* mutants that deregulate the net charge of phospholipids on Golgi/endosomal compartments (see Table 1), the *pik1* temperature sensitive mutant is highly sensitive to CPZ at permissive temperature (data not shown). Thus, it is possible that CPZ induces eIF2α phosphorylation by interfering with the function of charged lipids such as PI4-P. PI4-Ps are essential for protein transport from the Golgi apparatus to the plasma membrane. However, because some *sec* mutants do not induce phosphorylation of eIF2α (e.g., *sec4*; Deloche et al. 2004), a block of protein transport to the cell surface does not seem to be directly linked to phosphorylation of eIF2α. Additionally, the activation of UPR does not induce eIF2α phoshorylation (data not shown) and *GCN4* mRNA translation (Steffensen and Pedersen 2006), demonstrating that Gcn2p is not stimulated by an ER stress. How eIF2α phosphorylation is induced in CPZ-treated cells remains unclear. If CPZ does not perturb the intracellular level of amino acids nor inhibit TORC1, it is unlikely to impinge on Gcn2p activity. Alternatively, CPZ may, via its interaction with specific phospholipids on intracellular organelles, disturb the activity of an eIF2α-P-targeting phosphatase(s) (Fig. 8). Notably, the type I phosphatase Glc7p that regulates homotopic vacuole fusion, ER to Golgi and endocytic transports (Peters et al. 1999), and modulates the level of eIF2α phosphorylation (Wek et al. 1992) might be (indirectly) inhibited by CPZ, thereby leading to a rapid increase of eIF2α-P. Accordingly, Gcn4p derepression in CPZ-treated cells may be a physiological response that is necessary to modulate UPR gene expression in a coordinated manner with Hac1p (Fig. 8) to alleviate a membrane transport defect. However, in contrast to Patil et al. (2004), we found that activation of the UPR does not exclusively depend on the expression of Gcn4p since UPR activation following Tm or CPZ treatment was not abolished neither in the *sui2*-S51A mutant (Fig. 4a) nor in *gcn2Δ* and *gcn4Δ* strains (data not shown). One explanation for this discrepancy is that we used a higher concentration of Tm (10 vs. 1 µg/ml), which may perturb the secretory pathway. In this regard, a block of the secretory pathway was shown to boost *HAC1* mRNA abundance (Leber et al. 2004) that, upon splicing, yields a higher production of Hac1p (S-UPR; see Introduction). It is therefore possible that an increased concentration of Hac1p, induced by a distal secretory stress from the ER, bypasses the requirement of Gcn4p to increase the UPR. Accordingly, Gcn4p may be mainly required in the early stage of the UPR, during a period where the cellular concentration of
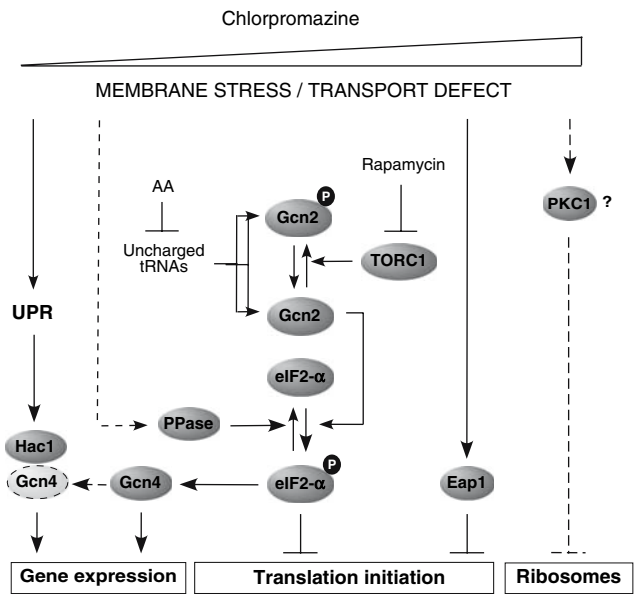

Fig. 8 Schematic representation of pathways controlling protein synthesis in response to a CPZ-stressed membrane treatment. The control of protein synthesis can be divided in two steps. The first step occurs after a mild membrane stress (secretory pathway is not blocked) and leads to the expression of specific genes under the control of the transcription factors Hac1p and Gcn4p. Crosstalk between Hac1p and Gcn4p to upregulate a subset of UPR genes may exist as described (Patil et al. 2004). The second step occurs after a more severe membrane stress and leads to the inhibition of protein synthesis. Highly phosphorylated eIF2α and the 4E-binding protein Eap1p reduce overall mRNA translation initiation. We do not exclude that other factors inhibit translation initiation (see text). Finally, a complete block of the secretory pathway leads to a drastic decrease in ribosome biogenesis, resulting in a more severe protein synthesis inhibition. This regulation might be mediated by the cell wall integrity (PKC1) pathway as described in *sec* mutants (Nierras and Warner 1999). *Arrows* and *bars* denote positive and negative interactions, respectively. *Solid arrows* and *bars* refer to direct and/or confirmed interactions, *dashed arrows* and bars refer to indirect and/or potential interactions. *Black circles* containing the letter P denote phosphorylated amino-acid residues. *UPR* Unfolded protein response; *TORC1* TOR complex 1; *AA* amino acids; *PPase* phosphatase. See text for further details

Hac1p is low (see also Fig. 4 in Patil et al. 2004). Alternatively, a secretion defect might induce additional regulatory factors that are capable of modulating the activity of Hac1p, thereby contributing to upregulation of UPR genes in the absence of Gcn4p.

CPZ rapidly inhibits protein synthesis by acting on multiple translation factors

The rapid increase of eIF2α phosphorylation in CPZ-treated cells argues that membrane stress is intimately connected to the control of translation initiation. At 100 µM, CPZ induces a much higher level of eIF2α phosporylation than at 30 µM, a concentration that was sufficient to totally derepress *GCN4*. The hyperphosphorylation of eIF2α was shown to cause a decrease in overall protein synthesis

(Fig. 8), thereby allowing cells to preserve energy and cellular resources. We have shown previously that this inhibition of translation initiation is not completely abrogated in a mutant strain where eIF2α cannot be phosphorylated (eIF2α-S51A), implying the existence of additional mechanisms of translation inhibition (Deloche et al. 2004). Notably, we reported that the eIF4E-binding protein Eap1p functions as a translation initiation inhibitor in cells treated with CPZ and in mutants that block secretory or endocytic pathways (e.g., *sec4* and *end3*). Interestingly, Eap1p was shown to interact genetically and biochemically with Scp160p (Mendelsohn et al. 2003), a ribosomal protein localized to the endoplasmic reticulum (Frey et al. 2001). It will be thus interesting to determine if Eap1p is essentially dedicated to the control of the expression of genes whose products are transported along the secretory pathway.

Finally, we cannot exclude that other translation factors such as eIF4B (i.e, Tif3p) also play a role in signaling the translation inhibition response after a membrane stress. eIF4B is an RNA-binding protein that stimulates eIF4A helicase activity to enhance the unwinding of inhibitory secondary structures in the 5′ untranslated region of mRNAs. Interestingly, the eIF4B deletion strain was found to be highly sensitive to rapamycin, an observation that corroborates the finding that the mammalian eIF4B is an indirect target of mTOR, which is responsive to amino acid starvation (Raught et al. 2004). In this regard, we previously observed that the eIF4B mutant is synthetically lethal with mutants that affect Golgi function (e.g., *vps54Δ*, *mon2Δ* (Deloche et al. 2004)) and is partially sensitive to CPZ (Table 1), indicating that the function of eIF4B is required for cell growth during a membrane (Stevens et al. 1986) stress. Finally, at 200 μM CPZ, the level of ribosomal subunits dramatically dropped, resulting in a total arrest of protein synthesis (Fig. 8). This result confirms that CPZ, at concentrations that totally block the membrane and protein trafficking processes, represses ribosomal protein (RP) gene transcription (Nierras and Warner 1999), and substantiates the existence of an intimate coordination between ribosome biosynthesis and secretion (Mizuta and Warner 1994). Altogether our results suggest that distinct regulatory pathways inhibit protein synthesis, by targeting multiple translation factors in response to a severe membrane stress.

In this study, we also showed that CPZ strongly inhibits protein synthesis in mammalian cells. Remarkably, this inhibition is also coupled to a rapid phosphorylation of eIF2α. Thus, mammals appear to possess a similar adaptive response pathway, capable of integrating membrane stress to regulate translation initiation. We believe that the control of the phosphorylation of eIF2α may directly result from UPR activation. In contrast to yeast, higher eukaryotes possess a ER-resident transmembrane kinase, PERK, that phosphorylates eIF2α upon abnormal accumulation of unfolded proteins. Accordingly, CPZ might impair ER functions by altering membranes of secretory organelles, leading to a rapid inhibition of translation via the activation of PERK. This translation inhibition is likely independent of Gcn2p and might explain why protein synthesis is more efficiently inhibited by CPZ in NIH-3T3 fibroblasts than in yeast cells (data not shown).

In conclusion, we reported that CPZ, one of the most common drugs used for people with schizophrenia worldwide alters the integrity of most membrane organelles within a cell. Our results demonstrate that membrane stress induced by CPZ leads to a rapid translational control mechanism mainly mediated by the highly conserved eIF2α translation factor.

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