CMLS, Cell. Mol. Life Sci. 61 (2004) 1208–1220
1420-682X/04/101208-13
DOI 10.1007/s00018-004-4029-8

CMLS Cellular and Molecular Life Sciences

Research Article

Characterization of the yeast tricalbins: membrane-bound multi-C2-domain proteins that form complexes involved in membrane trafficking

C. E. Creutz*, S. L. Snyder and T. A. Schulz

Department of Pharmacology, University of Virginia, Charlottesville, Virginia 22908 (USA),
e-mail: Creutz@virginia.edu

Received 20 January 2004; received after revision 2 March 2004; accepted 10 March 2004

Abstract. In a survey of yeast genomic sequences encoding calcium- and phospholipid-binding C2 domains, three homologous genes were identified that encode proteins that each have three C2 domains and an apparent transmembrane domain near the N terminus. The name tricalbins is suggested for these proteins, corresponding to the open reading frames YOR086c (*TCB1)*, YNL087w (*TCB2*), and YML072c (*TCB3*). An antiserum was raised against the C-terminal portion of tricalbin 2 and used on Western blots to demonstrate that the corresponding protein is expressed in yeast and appears as a high-molecular-weight band at 130 kDa with smaller fragments at 39 kDa and 46 kDa. A fusion protein consisting of full length tricalbin 2 fused to the green fluorescent protein was expressed in cells and found to traffic from the cell surface to intracellular vesicles near the vacuole. A two-hybrid interaction screen with the C-terminal portion of tricalbin 2 indicated that tricalbin 2 binds the C-terminal portions of tricalbins 1 and 3 suggesting that the tricalbins may form heterodimers in vivo. Tricalbin 2 also interacted with the activation domain of the pleiotropic drug resistance transcription factor Pdr1p. Combinatorial disruptions of the tricalbin genes revealed that *tcb2* single mutants or *tcb1, tcb3* double mutants have an altered vacuole morphology and are hypersensitive to cycloheximide. A screen for single-copy suppressors of the cycloheximide sensitivity of tricalbin mutants yielded *RSP5*, which encodes a C2-domain-containing, ubiquitin-conjugating ligase essential for receptor-mediated and fluid phase endocytosis. The results suggest that the tricalbins function as multimers in membrane-trafficking events and may provide insights into the roles of multi-C2-domain proteins, such as the synaptotagmins, in other organisms.

Key words. *RSP5*; *PDR1*; synaptotagmin; endocytosis; vacuole; calcium-binding proteins.

The C2 domain is a calcium- and phospholipid-binding motif found in a wide range of plant and animal proteins [1]. It provides calcium control and reversible membrane localization for a number of enzymes as well as calcium regulation of membrane proteins involved in membrane-membrane interactions and fusion. Two C2 domains are present in the cytoplasmic portion of the animal protein synaptotagmin [2]. The synaptotagmin gene family has at least 14 mammalian variants, and different forms have been localized to both secretory or synaptic vesicles as well as the plasma membrane [3, 4]. Genetic studies in several model organisms indicate that the synaptotagmins play a critical role in the regulation of calcium-dependent exocytosis, and that this likely involves regulation of the interactions of the SNARE proteins which mediate membrane interactions and fusion in exocytosis [5]. The yeast *Saccharomyces cerevisiae* expresses an array of SNARE

* Corresponding author.

proteins and interacting proteins that play a role in membrane fusion at numerous locations in the cell [6]. Indeed, the initial discovery of these proteins in yeast was critical in the development of current theories of the mechanisms underlying membrane trafficking and fusion [7]. However, there is no obvious homolog of synaptotagmin in yeast, based on sequence similarity, and the secretory pathway in yeast does not appear to be regulated by calcium. Therefore, the role of C2-domain proteins in the regulation of membrane trafficking has not appeared to be amenable to analysis in the yeast system.

However, genes for C2-domain-containing proteins are present in yeast: *RSP5*, which encodes ubiquitin ligase, *PKC1*, protein kinase C, *PLC1*, a phospholipase C, and *BUD2*, a RAS GTPase-activating protein. In addition, we previously identified three yeast genes that encode proteins with a domain organization similar to that of synaptotagmin [8]. These proteins have an apparent transmembrane domain in the N-terminal region and three presumably cytoplasmic C2 domains each. We suggested the name tricalbin for these proteins [TRI (three) CA (calcium) L (lipid) BIN (binding)] and now propose that the corresponding genes be named *TCB1*, *TCB2*, and *TCB3*. In this report we provide the first description of the expression, localization, biochemical and genetic interactions, and knockout phenotype of members of the yeast tricalbin family. The results suggest a role for these proteins in membrane trafficking and indicate that the tricalbins may interact with one another as heteromeric complexes in performing their functions in the yeast cell. The observations may have relevance for the behavior and functions of multi-C2-domain proteins in other organisms.

Materials and methods

Yeast strains and general methods

The yeast strains used or constructed in this study are listed in table 1. Strain NY606 was obtained from Peter Novick (Yale University) and DBY334 from Dan Burke (University of Virginia). The tricalbin mutants were all derived from strains YPH501 and YPH499 from the Heiter laboratory. General yeast methods and molecular biology methods were as described in Rose et al. [9], and Ausubel et al. [10].

Production of anti-tricalbin 2 antibodies

Genomic DNA encoding the C-terminal 316 residues of tricalbin 2 was amplified by PCR from total yeast genomic DNA. The flanking primers contained *Sal* I and *Xba* I restriction sites allowing subcloning into pGEX-KG [11] for expression in *Escherichia coli*. This C-terminal fragment fused to glutathione transferase was expressed in *E. coli* and purified by binding to glutathione agarose beads essentially as described previously for the C2 domains of synaptotagmin [12]. The tricalbin fragment was cleaved from the GST with thrombin and purified by SDS-PAGE. The Coomassie-stained band (1–3 mg of protein) was homogenized with complete Freund's adjuvant for injection into rabbits. After one booster injection of the tricalbin fragment homogenized in incomplete Freund's adjuvant, the presence of anti-tricalbin antibodies was detected on Western blots of the recombinant antigen. Specific antibodies were purified from the serum by binding to antigen blotted onto nitrocellulose according to Olmsted [13].

Table 1. Yeast strains

YPH501 *MATa/MATalpha ura3–52/ura3–52 lys2–801amb/lys2–801amb ade2–101ochre/ade2–101ochre trp1delta63/trp1delta63 his3-delta200/his3-delta200 leu2-delta1/leu2-delta1*
YPH499 *MATa ura3–52 lys2–801amb ade2–101ochre trp1-delta63 his3-delta200 leu2-delta1*
YPH500 *MATalpha ura3–52 lys2–801amb ade2–101ochre trp1-delta63 his3-delta200 leu2-delta1*
NY606 *MATalpha leu2–3,112*
DBY334 *MATalpha leu2–3,112 ura3–52 gal1 reg1–1 pep4–3 prb1–112 can1*
The following diploid strains were derived from YPH499 and YPH501 and are in common: *MATa/MATalpha ura3–52/ura3–52 lys2–801amb/lys2–801amb ade2–101ochre/ade2–101ochre trp1delta63/trp1delta63 his3-delta200/his3-delta200 leu2-delta1/leu2-delta1*
C²Y1500 *tcb2::URA3/TCB2*
C²Y1510 *tcb1::HIS3/TCB1 tcb2::URA3/TCB2*
C²YGP *tcb1::HIS3/TCB1 tcb2::URA3/TCB2 tcb3::URA3/TCB3*
The following haploid strains are derived from the above strains and are in common: *ura3–52 lys2–801amb ade2–101ochre trp1-delta63 his3-delta200 leu2-delta1*
C²YG15 *MATalpha* (wild-type tricalbin loci)
C²YG16 *MATalpha* (wild-type tricalbin loci)
C²Y1515 *MATalpha tcb1::HIS3 tcb2::URA3*
C²Y1516 *MATa tcb1::HIS3 tcb2::URA3*
C²Y499tcb3 *MATa tcb3::URA3*
C²YG10 *MATalpha tcb1::HIS3*
C²YG3 *MATa tcb2::URA3*
C²Y1502 *MATa tcb2::URA3*
C²Y1503 ('D2' in figure 5) *MATalpha tcb2::URA3*
C²YG26 *MATa tcb3::URA3*
C²YG2 *MATa tcb1::HIS3 tcb2::URA3*
C²YG21 *MATa tcb1::HIS3 tcb3::URA3*
C²YG7 *MAT? tcb1::HIS3 tcb3::URA3*
C²YG18 *MAT? tcb2::URA3 tcb3::URA3*
C²YG6 *MATalpha tcb1::HIS3 tcb2::URA3 tcb3::URA3*
C²YG8 *MATalpha tcb1::HIS3 tcb2::URA3 tcb3::URA3*

Tricalbin 2 extraction assay

Wild-type yeast cells, strain YPH500, were homogenized in a ball mill in 150 mM NaCl, 50 mM HEPES-NaOH (pH 7.4), 5 mM EGTA, 0.2 mM phenylmethanesulfonyl fluoride, and 0.85 μg/ml aprotinin. The homogenate was centrifuged at 3000 g for 2 min to pellet unbroken cells and nuclei and the supernatant centrifuged for 10 min at 27,000 g to collect a crude membrane fraction. The membranes were resuspended in the homogenizing buffer and then diluted tenfold in phosphate-buffered saline supplemented with either 1 M NaCl, or 1% Triton X-100, or in 100 mM $NaCO_3$, pH 11.0. Fractions were incubated on ice for 20 min then centrifuged at 100,000 g at 4 °C for 20 min. Pellet and supernatant fractions were subjected to Western blotting with the anti-tricalbin 2 antibody.

Green fluorescent protein-tricalbin 2 fusion construct

DNA encoding the green fluorescent protein (GFP) was amplified from plasmid pFA6a-GFP-HIS3MX [14] by PCR and subcloned into the yeast expression vector YEp51 [15] at the *Bam*H1 and *Hind* III sites. Genomic DNA encoding the complete coding sequence for tricalbin 2 was amplified from total yeast genomic DNA and subcloned into the YEp51-GFP construct at the *Sal*I and *Bam*H1 sites. The resulting plasmid, YEp51-tcb2GFP, expresses a fusion protein of tricalbin 2 with GFP at the C terminus under control of the *GAL10* promoter. Yeast strains NY606, YPH500, and DBY334 were transformed with this plasmid and used for the localization studies. The results were equivalent with all three strains. For induction of the fusion protein, cells were grown to late log phase (approximately 1×10^7 cells/ml) in 2% glucose selective drop-out medium (Leu^-) at room temperature and then collected by centrifugation and resuspended in 2% galactose medium. Cells were examined with a ×100 oil immersion objective on a Nikon Optiphot upright microscope with fluorescent optics using a GFP filter cube from Chroma Technology Corporation. Images were captured with a Hamamatsu Orca 1 digital camera. Because the cells were grown at room temperature, and due to the lag time associated with transfer from glucose- to galactose-containing medium, the cultures did not re-enter log phase until 8–12 h after transfer to galactose medium.

Two-hybrid interaction screen

The Clontech System 3 yeast two-hybrid bait vector pGBKT7 and host strain AH109 were used for the interaction screen. Initially, DNA corresponding to the C-terminal 316 residues of tricalbin 2 were amplified from the plasmid YEp51-tcb2 described above and subcloned into pGBKT7 at the *Nco*I and *Sal*I sites. In a second screen, the 99 C-terminal residues were used as bait, using the same restriction sites. The three yeast genomic libraries prepared in vector pGAD424 as described by James et al. [16] were pooled and used for the screens (approximately 200,000 transformants in each screen). Interacting clones were selected by growing the transformed AH109 cells on Ade^-, His^- selective medium. The candidate interacting plasmids were captured from fresh liquid yeast cultures and retransformed into AH109 harboring the bait vector with the tricalbin fragment or the bait vector with no insert and retested for growth on selective media in control experiments.

Tricalbin gene disruption

The tricalbin genes were disrupted by homologous recombination using DNA cassettes containing selectable markers prepared by PCR amplification. The primers used for PCR contained 50 base pairs corresponding to portions of the tricalbin gene sequences and 20 base pairs corresponding to the selectable marker gene, following the method described by Lorenz et al. [17]. The disruptions replaced the portions of the tricalbin sequences corresponding to a point just N terminal of the transmembrane domain to the position of the second C2 domain. All disruptions were verified by PCR using primers corresponding to sequences outside the disruption sites. Initially, the gene for tricalbin 2 was disrupted in the diploid host strain YPH501using the *URA3* marker amplified from plasmid YEpDB60 [18]. The *URA3* gene replaced the coding sequence in one *TCB2* allele corresponding to amino acid residues 110–718 of tricalbin 2 to generate strain C^2Y1500. The gene for tricalbin 1 was disrupted in this diploid *tcb2* disruption strain using the *HIS3* marker amplified from plasmid pRS313 [19]. The *HIS3* gene replaced the coding sequence of one *TCB1* allele corresponding to amino acid residues 79–648 of tricalbin 1 to generate strain C^2Y1510. This diploid *tcb1, tcb2* double-mutant strain was sporulated and *tcb1* and *tcb2* single-mutant and *tcb1, tcb2* double-mutant haploid strains were selected after random spore analysis and examination of the *TCB* loci by PCR screening. The gene for tricalbin 3 was disrupted in the haploid strain YPH499 using the *URA3* marker amplified from plasmid YEpDB60. The *URA3* gene replaced the coding sequence of the *TCB3* gene corresponding to amino acid residues 223–841 of tricalbin 3 to generate strain $C^2Y499tcb3$. This *tcb3* mutant strain was crossed with the *tcb1, tcb2* double-mutant strain C^2Y1515. Diploids from this mating were picked from HIS^- plates based on diploid morphology. The resulting diploid strain, C^2YGP, was sporulated to obtain all combinations of tricalbin mutants in haploid strains after random spore analysis and screening for the disruptions by PCR (see table 1).

Visualization of vacuole morphology

Vacuoles were visualized by staining with 5- (and-6)carboxy-2′,7′ dichlorofluorescein diacetate (CDCFDA); [20] or using a carboxypeptidase S-GFP fusion protein

that was expressed from the plasmid GFP-CPS, kindly provided by G. Odorizzi and S. Emr [21].

Cycloheximide sensitivity assay

Cycloheximide was added to selective drop-out plates at concentrations of 0.05, 0.1, 0.2, 0.4, and 0.6 μg/ml. Growth of yeast strains was monitored for 24–72 h at 30 °C.

Screen for suppressors of cycloheximide sensitivity of *tcb2* mutants

A yeast genomic library in the vector pRS200 prepared by P. Hieter was obtained from the American Type Culture Collection (catalog number 77164). The library was transformed into a *tcb2* disruption strain, and transformants replica plated onto selective plates with 0.2 μg/ml cycloheximide. Approximately 200,000 transformants were monitored over 4 days. Library plasmids were isolated from liquid cultures grown from colonies growing in the presence of 0.2 μg/ml cycloheximide and retransformed into the *tcb2* mutant strain to confirm that the suppressing activity was borne by the plasmids.

Analytical methods

Protein was assayed according to Bradford [22]; SDS-polyacrylamide gels were run as described by Laemmli [23]; Western blotting was performed according to Burnette [24] using chemiluminescence for detection of peroxidase-conjugated secondary antibody. To prepare yeast samples for Western blotting, cultures were grown in YPD to a density of 5×10^7 cells/ml and 2-ml samples were collected by centrifugation and disrupted by vortexing with glass beads in SDS sample buffer and heating to 100 °C before application to the gel. DNA sequencing was performed by the University of Virginia Biomolecular Research Facility.

Results

Identification of open reading frames encoding the tricalbins

To screen for potential multi-C2-domain-containing proteins encoded by the yeast genome, the amino acid sequences of the C2 domains of mammalian protein kinase C and of synaptotagmins were used in BLAST searches of the translated yeast genomic sequences. Three open reading frames (ORFs) were found to contain three apparent C2 domains: YOR086c (tricalbin 1), YNL087w (tricalbin 2), and YML072c (tricalbin 3). Figure 1 illustrates the overall organization of the C2 domains in the encoded proteins, in comparison with mammalian synaptotagmin, which has two C2 domains. Of the three tricalbins, tricalbins 1 and 2 are smaller (1186 and 1178 residues, respectively) and are the most similar, having

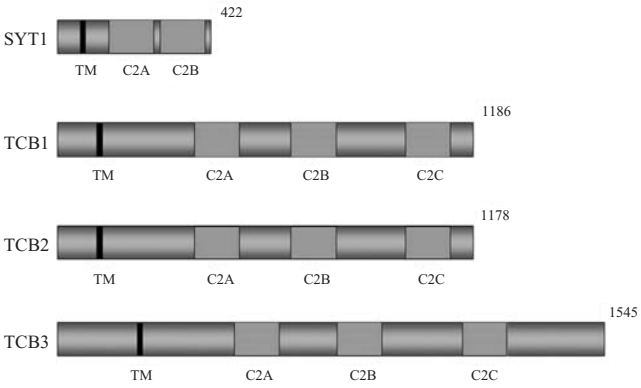

Figure 1. Schematic illustration of the domain organization of the tricalbins and synaptotagmin. SYT1, mammalian synaptotagmin 1; TCB1, TCB2, and TCB3, are the yeast tricalbins 1, 2, and 3 respectively. TM is a predicted transmembrane domain in the N-terminal portion of each protein. The C2 domains are labeled C2A, C2B, and C2C in order from the N terminus. The numbers to the right of each construct represent the number of amino acids in the full-length proteins.

55% identity in the region of overlap. Tricalbin 3 is larger at 1545 residues and is only 28–30% identical to tricalbins 1 and 2 in the regions of overlap. Tricalbin 3 also has a poorly conserved possible fourth C2 domain predicted between the first and second C2 domains (not included in fig. 1). Similar to synaptotagmin, the tricalbins each have a single region near the N terminus that is strongly predicted to form a transmembrane helix by the HMMTOP prediction program [25]. These transmembrane domains are also indicated in figure 1.

Probing current sequence databases with the tricalbin 3 sequence revealed the presence of tricalbin homologs in a number of different ascomycete genera. In addition to the important genetic and cell biological model organisms *Schizosaccharomyces pombe* and *Neurospora crassa,* tricalbin homologs were found in the fungus that causes powdery mildew in peas, *Erysiphe pisi,* and the rice blast fungus *Magnaporthe grisea.* Interestingly, these homologs all appear to correspond to tricalbin 3 in terms of overall structure in that they are of comparable size to the *S. cerevisiae* tricalbin 3 and all have a potential fourth C2 domain predicted between the first and second C2 domains indicated in figure 1. The degree of sequence identity between the tricalbin 3s of these diverse species is indicated in table 2. Of these organisms, the only other one for which there currently appear to be multiple tricalbins is *S. pombe* which has a single additional homolog predicted to have only two C2 domains (NCBI accession number NP_587865). However, sequencing of the genomes for the other ascomycetes is not yet complete.

Table 2. Amino acid sequence identities of ascomycete tricalbin 3 proteins

	S. cerevisiae	*S. pombe*	*N. crassa*	*E. pisi*	*M. grisea*
S. cerevisiae	100%	27.6%	30.7%	31.7%	30.9%
S. pombe		100%	35.1%	34.3%	35.7%
N. crassa			100%	57.5%	68.2%
E. pisi				100%	58.0%
M. grisea					100%

The tricalbin 3 sequences are available under the following NCBI protein database accession numbers: *S. cerevisiae*, NP_013639; *S. pombe*, NP_593974; *N. crassa*, XP_330699; *E. pisi*, CAB65007; *M. grisea*, EAA46726.

Analysis of the tricalbin proteins and gene promoter regions in closely related *Saccharomyces* species

The recent partial sequencing of the genomes of three additional *Saccharomyces* species permits further analysis of the conserved features of the tricalbin proteins and of the promoter regions upstream of the tricalbin genes [26, 27; sequence data available at the Saccharomyces, Genome Database: http://www.yeastgenome.org/]. In the corresponding regions of the genomes, the sequences for *TCB2* and *TCB3* are available for *Saccharomyces paradoxus, Saccharomyces mikatae* and *Saccharomyces. bayanus* to compare with the *S. cerevisiae* sequences. Sequences for *TCB1* are only available from *S. paradoxus* for comparison with *S. cerevisiae.* Alignment of the corresponding protein sequences (table 3) indicates that the tricalbins in these four species are 81–96% identical in amino acid sequence. Importantly, the pattern of the presence or absence of acidic residues involved in chelating calcium in the C2 domains [28] is 100% conserved between all species.

The degree of conservation of nucleic acid sequences in the upstream regions of the tricalbin genes is much lower, allowing an analysis of conserved intergenic sequences as potential regulatory regions. Table 4 lists all sequence motifs present in the intergenic regions upstream of *TCB2* and *TCB3* in which five or more contiguous bases are absolutely conserved among the four species. The potential promoter regions for these two tricalbin genes are apparently significantly divergent as there are no common motifs of this size upstream of both genes. Within these conserved sequences, several sites for the binding of known transcription factors are recognized: TATA-binding protein (TBP) and HSF1 for *TCB2*; and TBP and GCR1 for *TCB3*. Although the absence of data for *TCB1* from *S. mikatae* and *S. bayanus* precludes such a stringent analysis of the upstream regions of *TCB1*, potential motifs are found for ABF1, STRE, GCR1, and GCN4. However, the overall organization of this region does not significantly resemble the upstream regions of *TCB2* or *TCB3*.

Table 3. Amino acid sequence identities between the tricalbins of closely related *Saccharomyces* species

Tricalbin 1:

S. cerevisiae and *S. paradoxus*: 96.3%

Tricalbin 2:

	S. cerevisiae	*S. bayanus*	*S. mikatae*	*S. paradoxus*
S. cerevisiae	100%	83.3%	87.4%	92.7%
S. bayanus		100%	81.1%	82.6%
S. mikatae			100%	87.5%
S. paradoxus				100%

Tricalbin 3:

	S. cerevisiae	*S. bayanus*	*S. mikatae*	*S. paradoxus*
S. cerevisiae	100%	87.3%	90.6%	94.2%
S. bayanus		100%	86.7%	87.2%
S. mikatae			100%	91.1%
S. paradoxus				100%

Table 4. Conserved nucleotide sequences found upstream of *TCB2* and *TCB3* in four *Saccharomyces* species

TCB2
Upstream intergenic region: 421 bp
–375 TATCAGAA
–340 AT**TATATAA**
TBP
–284 AAAGTTTAATT
–264 GCTTTTT
–239 GAGAAAATTC**TTCGAGAA**GACAACAACA
HSF1

TCB3
Upstream intergenic region: 382 bp
–360 **TATTT**
TBP
–333 CCGAAATTTT
–313 TTTTTCA
–297 CAGCGCGAGAAGGGGGCAACA
–220 AGGGATAA
–203 A**CATCC**TTTA
GCR1
–132 TTTGC
–100 TTTCT
–74 CTAGCTTTGTTTAT
–26 AGAGCTAC
–4 GAAAA

An alternative method of analysis of the sequence motifs upstream of the tricalbin genes was applied by Kellis et al. [26] in their analysis of all of the probable genes and regulatory regions of the sequenced portions of the genomes of these four *Saccharomyces* species. By accepting imperfectly conserved motifs that are validated due to frequent occurrence on a genome-wide basis, Kellis et al. [26] recognized several motifs upstream of *TCB2* and *TCB3* (see table 5; the results of the Kellis et al. [26] analysis can be probed at the Broad Institute (M.I.T.) website: http://www.broad.mit.edu/annotation/fungi/comp_yeasts/). Included among these motifs are sites associated with HSF1 (*TCB2*, as recognized above), and MSN2/4 and PHO1 (*TCB3*).

Table 5. Motifs recognized upstream of *TCB2* and *TCB3* in a genome-wide analysis of four *Saccharomyces* species

Motif	Sequence	Associated factors
TCB2:		
10*	TYYTCGAGA	HSF1
28	TTTT-n(19)-GCKCG	
33*	TTCTTMGAAGA	HSF1
66	AATATTCTT	
TCB3:		
14	YCTATTGTT	
38	CTCCCCTTAT	MSN2/4
64	TTCTC-7n-CGC	PHO1
66	AATATTCTT	
70	CGCG-4n-GGGS	

Motif refers to the number of the motif in the listing provided in table 3 of Kellis et al. [26]. Key for degenerate nucleotides: Y=C or T; K=G or T; M=A or C; S=C or G.
* Motifs 10 and 33 in the *TCB2* gene overlap and represent the same putative promoter site.

These analyses of the upstream sequences of the tricalbin genes suggest that these genes may be subject to differential regulation. Indeed, in reviewing the data currently available from several microarray analyses of gene expresssion in *S. cerevisiae*, the expression of the tricalbin genes is apparently in general not well correlated (see the Saccharomyces Genome Database: http://www.yeastgenome.org/). In two cases, however, significant correlations were seen (Pearson coefficient >0.8): in the metabolic shift from fermentation to respiration [29], expression of *TCB2* was strongly correlated with expression of *TCB3*, while during changes in phosphate metabolism [30], expression of all three tricalbin genes was strongly correlated.

Evidence for endogenous expression of the tricalbin 2 protein

To determine if one of the tricalbin proteins is actually synthesized, a rabbit antiserum was raised against the 316 C-terminal residues of tricalbin 2, encompassing the third C2 domain, expressed in *E. coli*. Specific anti-tricalbin antibodies were affinity prepared from this serum by binding to the immobilized antigen. As illustrated in the Western blot in figure 2, these antibodies recognized a high-molecular-weight band at approximately 130 kDa as well as two distinct bands of 39 and 46 kDa. In a tricalbin 2 disruption strain, the antibody did not recognize any bands (fig. 2). We conclude that the pattern of bands represents tricalbin 2 and not cross-reacting proteins and that the disruption completely eliminated expression of tricalbin 2. The distinct bands at 39 and 46 kDa likely represent proteolytic fragments of tricalbin 2 that include the C-terminal portion of the protein that was used as the immunizing antigen. Whether the fragments of tricalbin 2 seen in the Western blot are formed in vivo or are a postmortem artifact is uncertain. However, Western blots of

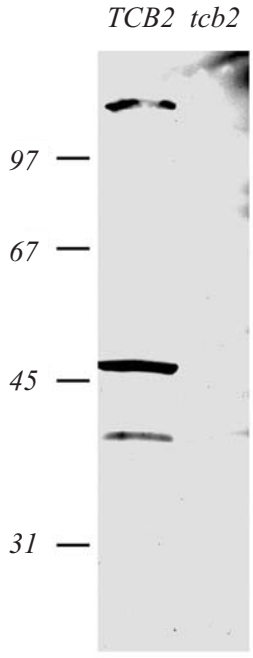

Figure 2. Western blot of total yeast homogenates probed with an antibody to tricalbin 2. '*TCB2*', a wild-type strain (YPH500), *tcb2*, a tricalbin 2 disruption strain (C^2Y1503) The numbers on the left mark the migration points of molecular-weight markers (kDa).

yeast strain DBY334, in which the gene for the *PEP4* vacuole protease is deleted, revealed a reduced intensity of the smaller tricalbin fragments (not shown), suggesting the fragments may be postmortem artifacts due to hydrolysis by proteases released from the vacuole during homogenization. Qualitative analysis of the intensity of the bands suggested a low expression level for the tricalbin 2 protein: The immunostained bands corresponding to the fragments of tricalbin 2 did not clearly correspond to identifiable bands on Ponceau-S-stained blots of the yeast homogenates.

Tricalbin 2 behaves like an integral membrane protein

Fractionation of homogenized yeast cells into crude soluble and membrane fractions, as described in Materials and methods, indicated that tricalbin 2 was associated with the membrane fraction. Furthermore, over 95% of the protein remained associated with the membrane fraction after treatment with high salt (1 M NaCl) and high pH (100 mM $NaCO_3$, pH 11.0) or with 1% Triton X-100, suggesting that tricalbin 2 may be an integral membrane protein and may be associated in larger complexes.

Localization of tricalbin 2

To visualize the localization of tricalbin 2 in vivo, a plasmid was constructed that expresses the full-length tricalbin 2 protein with GFP fused to its C terminus. The expression from this plasmid is under the control of the *GAL10* promoter so the 'life cycle' of the fusion protein

could be followed as cells were transferred from glucose to galactose medium. As illustrated in figure 3, the fusion protein first appears at the cell surface, in a patchy distribution. Subsequently, the protein enters the cytoplasm of the cell in discrete patches or on membranous vesicles. These vesicles often accumulate near the periphery of the vacuole; however, in general, the fluorescence did not appear in the vacuole membrane per se. After 15 h or more of incubation, some fluorescence in about 5–10% of the cells was found in association with structures that appeared to be microtubule asters (fig. 3). This final localization was unexpected since we anticipated that the tricalbins would be membrane bound due to the presence of the predicted transmembrane domain. However, at this late stage of expression, fragmentation of the fusion protein may have occurred, yielding a soluble fragment that can interact with the microtubules. Since expression of GFP alone in control experiments resulted in a diffuse cytoplasmic fluorescence, a fragment of tricalbin likely remains fused to the GFP and this fragment may have affinity for the microtubule system in the cell. This raised the possibility that intact tricalbin might link membranes to microtubules and that this linkage might be responsible for movement of the tricalbin to the surface membrane or back into the cell as observed. However, although incubation of the cells with 40 μg/ml nocodazole to disrupt the microtubule systems reduced or eliminated the appearance of the GFP-stained asters, this treatment did not qualitatively alter the apparent trafficking of the tricalbin-GFP fusion protein to the plasma membrane or into internal membrane systems.

Interactions of tricalbin 2

The C-terminal portions of the synaptotagmins may be important for mediating a number of interactions of synaptotagmins with proteins involved in membrane trafficking. Therefore, we sought to determine if the C-terminal portion of tricalbin 2 might also be a site of interaction with other proteins. We used the C-terminal 316 residues of tricalbin 2, including the third C2 domain, as a bait in a two-hybrid screen of a yeast genomic library. Four plasmids of interest were obtained in this screen that were validated in control experiments. Interestingly, two of these plasmids contained identical inserts encoding the 188 C-terminal residues of tricalbin 1. This suggested that tricalbins 1 and 2 may interact through their C-terminal domains.

Since we considered that the presence of the C2 domain might have caused the tricalbin 2 bait construct to associate with membranes, hindering its entry into the nucleus, the bait construct was redesigned to include only the 99 C-terminal residues of tricalbin 2. Using this construct in a two-hybrid screen, a single plasmid was obtained that was validated in control experiments. It encoded 120 C-terminal residues of tricalbin 3, suggesting that tricalbin 2 may also be able to dimerize with tricalbin 3 through interactions in the C-terminal domains of the two proteins.

The two additional plasmids validated in the initial screen with the larger tricalbin 2 bait construct were both found to encode 207 C-terminal residues of Pdr1p. Pdr1p is a transcription factor that was initially identified as playing pleiotropic functions in drug resistance and the synthesis of membrane components [31–35]. This portion of Pdr1p is in the activation domain of the transcription factor.

Tricalbin gene deletion

To assist in identifying the functions of the tricalbins, the genes for all three were disrupted by homologous recombination and yeast strains were generated that had all possible combinations of tricalbin deletions, as listed in table 1. All of the deletion strains were viable and exhibited normal growth kinetics in liquid culture and normal colony morphology at a range of temperatures (16–37 °C). Growth kinetics in high concentrations of calcium (100 mM) or in the presence of the calcium chelator EGTA were similar to those of wild-type cells under the same conditions. The cells exhibited normal sensitivity to osmotic shock and heat shock. The cells were capable of responding to mating pheromones and could mate. Diploid homozygous tricalbin mutants sporulated normally.

However, deletion of tricalbin 2 resulted in an altered vacuole morphology: instead of the multi-lobed vacuole of the wild-type cell, the mutant had an enlarged vacuole consisting of a single compartment. This was apparent by phase and Nomarski optical microscopy and when the vacuoles were stained with the carboxyfluorscein ester CDCFDA [20]. The altered morphology was also easily scored by examining the vacuoles of cells expressing a fusion construct of GFP and the vacuole protease CPS [21], as shown in figure 4. Although the morphology of the vacuole was disturbed in the mutants, the sorting of this vacuole marker to the vacuole membrane and lumen appeared normal. In addition, using a filter assay for detection of mis-sorting and secretion of the vacuole protease carboxypeptidase Y [20], no difference could be seen between the tricalbin mutants and wild-type cells.

Because the tricalbin-GFP fusion protein appeared to move from the surface membrane to the cell interior, we monitored the uptake of the fluorescent dye, lucifer yellow, by endocytosis [36] in the tricalbin deletion strains. However, the accumulation of this marker in the vacuole appeared qualitatively normal. As we previously described, the tricalbin triple mutant appeared to be slightly resistant to the growth inhibitory effects of permeant weak acids, when compared to the wild type, and the vacuoles did not appear to stain as strongly with CDCFDA, particularly in early log phase [8]. However, these effects were subtle and often difficult to score reliably.

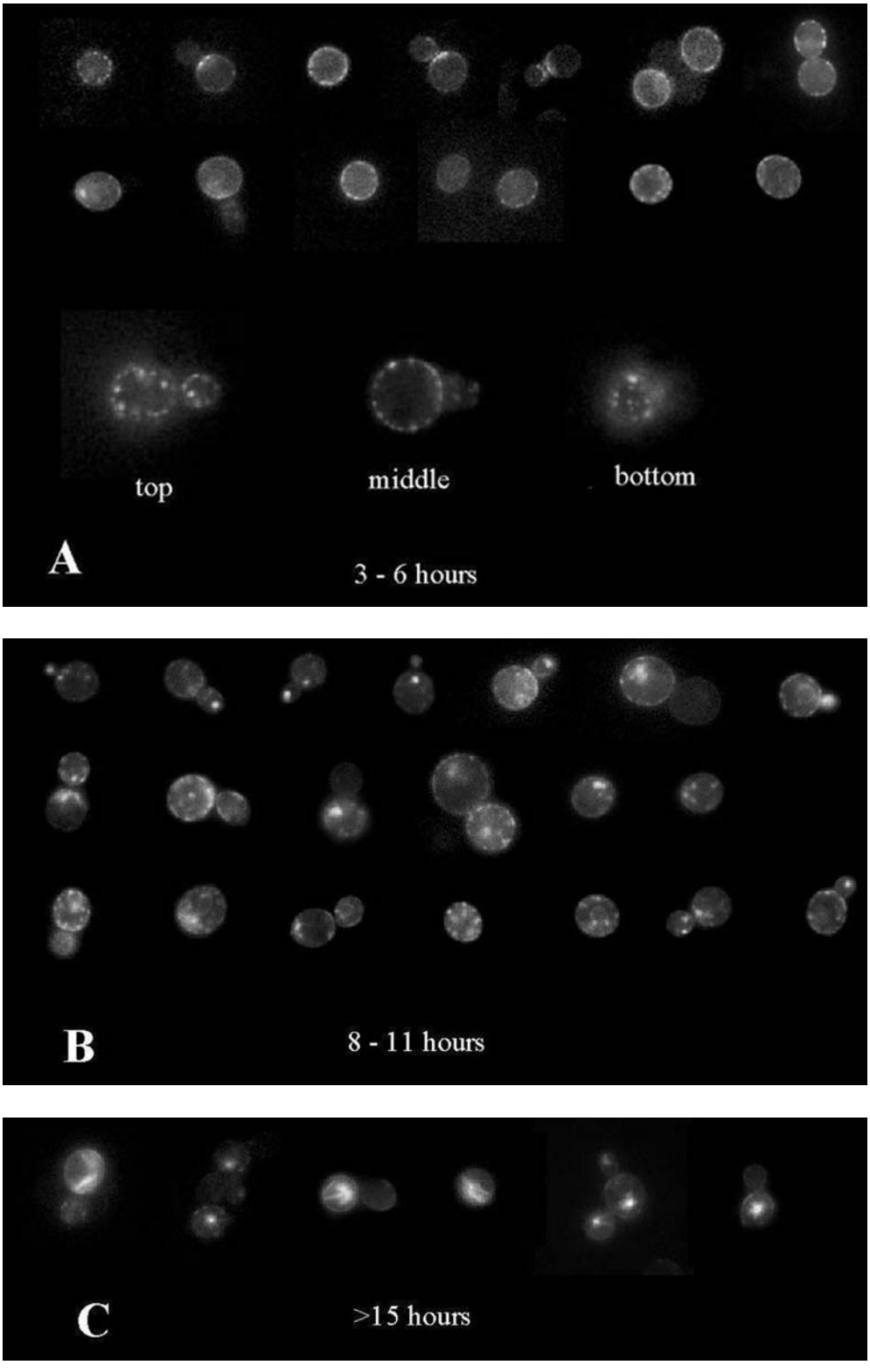

Figure 3. Time-dependent localization of a tricalbin-GFP fusion protein monitored by fluorescence microscopy. Representative yeast cells (strain YPH500) harboring plasmid YEp51-tcb2GFP are shown at various times after initiating expression of the fusion protein by shifting to galactose medium at room temperature. (A) At 3–6 h, the fusion protein is present in patches at the cell surface. At the bottom of the panel, a single budded cell is shown at higher magnification in a through-focus series representing the 'top' of the cell, the 'middle' of the cell, and the 'bottom' of the cell. The other images at lower magnification all represent a plane of focus near the middle of the cell. (B) At 8–11 h, some of the fusion protein is internalized in patches or bound to membranes throughout the cell. (C) After 15 h, some cells (about 10%) show additional localization of fluorescence on microtubule asters, as shown.

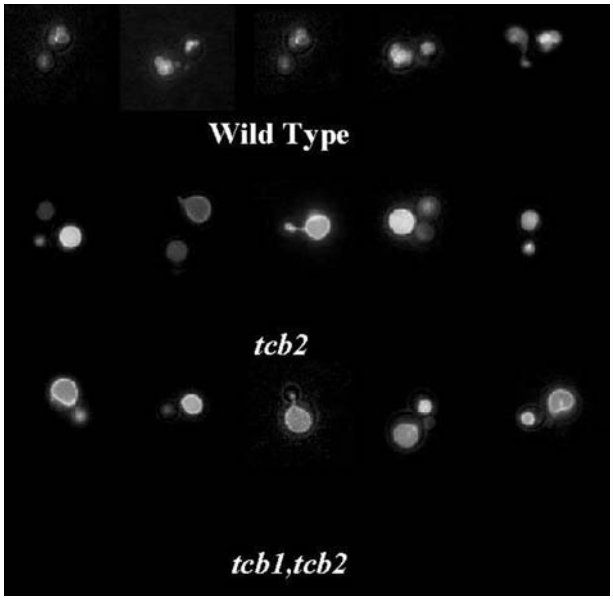

Figure 4. Altered morphology of the vacuole in tricalbin mutant strains. Vacuole membrane and lumen are visualized using a carboxypeptidase S-GFP fusion protein. In wild-type cells, the vacuole is a multi-lobed body. In *tcb2* single mutants and *tcb1,tcb2* double mutants, the vacuole is a large, single compartment.

A distinct, easily scored phenotype of the tricalbin mutants was found by following up on the implications of the two-hybrid interaction between tricalbin 2 and Pdr1p. Since disruption of *PDR1* leads to enhanced sensitivity to a number of drugs, including cycloheximide [31], we tested the sensitivity of the tricalbin mutants to a graded series of cycloheximide concentrations. *tcb2* mutants were found to be more sensitive to cycloheximide, as they would not grow on media containing 0.2 μg/ml cycloheximide, a concentration permissive for wild-type growth. Figure 5 illustrates the growth of a number of tricalbin mutants, including single, double, and triple mutants, on 0.2 μg/ml cycloheximide. Consistently, all strains with the *tcb2* deletion showed a similar hypersensitivity to cycloheximide. However, mutants with deletions in either tricalbin 1 or 3 alone were wild type in their drug sensitivity. Interestingly, the *tcb1, tcb3* double mutant was hypersensitive to cycloheximide, similar to the *tcb2* single mutant. Thus, this phenotype was produced by either deletion of tricalbin 2 alone, or simultaneous deletion of tricalbins 1 and 3.

The tricalbin mutants did not exhibit hypersensitivity to the mitochondrial poison oligomycin. Therefore, the tricalbin mutants do not appear to have the pleiotropic drug sensitivity associated with *pdr1* mutants which are hypersensitive to both cycloheximide and oligomycin.

Suppression of *tcb2* by *RSP5*

To explore additional genetic interactions of the tricalbins, a screen was conducted for single-copy suppressors of the cycloheximide hypersensitivity phenotype of the *tcb2* mutant using a yeast genomic library. Of approximately 200,000 library transformants, a total of eight plasmids were isolated that allowed the *tcb2* mutant to grow in the presence of 0.2 μg/ml cycloheximide. All eight plasmids contained genomic DNA encompassing *RSP5* and the promoter region for this gene. In addition, this gene was the only gene common to all eight plasmids. We conclude that the suppressing activity was due to *RSP5*, which encodes a ubiquitin ligase essential for ubiquitination and internalization of a number of plasma membrane proteins as well as for fluid phase endocytosis [37–38]. The colonies formed on 0.2 μg/ml cycloheximide by the *tcb2* mutants harboring the suppressing plasmids were slightly smaller than colonies formed by wild-type cells, and the vacuoles still appeared enlarged relative to wild-type. Therefore, the suppression of the *tcb2* phenotype was not complete. When the *RSP5*-containing plasmids were transformed into wild-type cells and the growth of the cells tested on higher concentrations of cycloheximide, there was no indication that the presence of an additional copy of *RSP5* increased the resistance of the wild-type cells to cycloheximide. Therefore, the suppressing effect of *RSP5* appeared to be specific for the tricalbin mutant background.

The native tricalbin 2 gene was not obtained in this suppressor screen. However, subsequent analysis of the two-hybrid library by PCR using primers flanking the gene failed to demonstrate the presence of the intact tricalbin 2 gene in this library.

The ability of *RSP5* to suppress the phenotype of the *tcb1, tcb3* double mutant, which is also hypersensitive to cycloheximide, was tested with a representative *RSP5* suppressor plasmid obtained in the *tcb2* suppression screen. This plasmid was found to suppress the double mutant to the same extent as the *tcb2* mutant, as well as the *tcb1, tcb2, tcb3* triple mutant.

Failure of mammalian synaptotagmin to complement the tricalbin mutants

Because there appeared to be a number of parallels between the structure and biology of the tricalbins and the mammalian synaptotagmins, we attempted to complement the tricalbin mutant phenotype by expressing rat brain synaptotagmin II using a previously described synaptotagmin expression vector [39]. However, synaptotagmin II was unable to suppress either the altered vacuole morphology or the cycloheximide sensitivity of the *tcb2* mutant or the *tcb1, tcb3* double mutant.

Discussion

An analysis of the presence of C2 domains encoded by the *S. cerevisiae* genome reveals that four other proteins

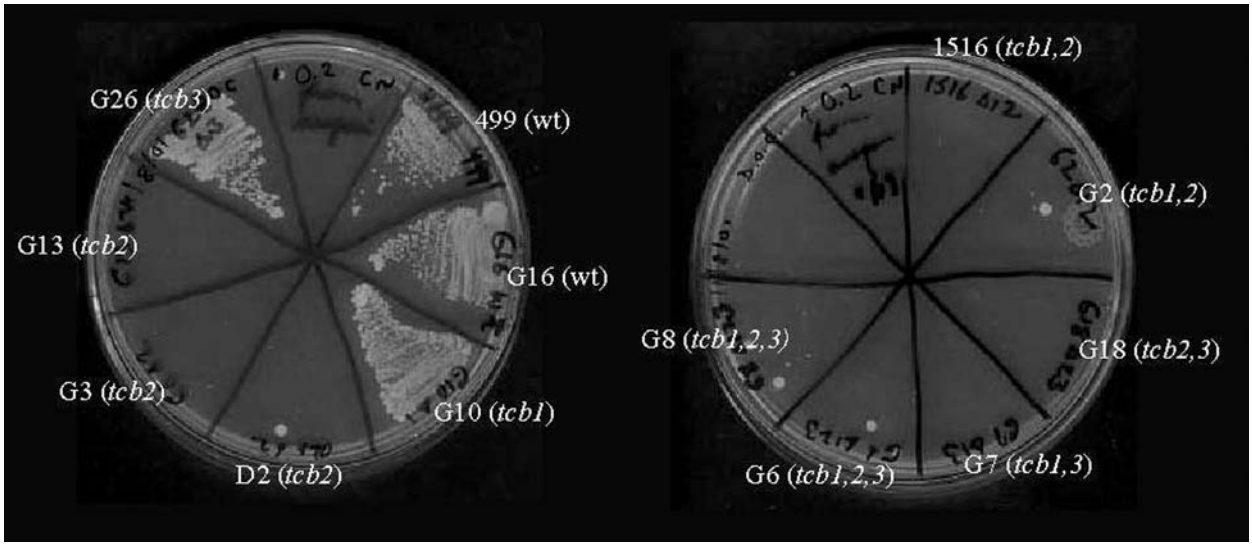

Figure 5. Hypersensitivity of tricalbin mutants to cycloheximide. Strains with tricalbin mutations as labeled (see table 1) were streaked on medium containing 0.2 µg/ml cycloheximide. Growth of wild type (wt) strains and *tcb1* or *tcb3* single mutants is similar. *Tcb2* mutants and *tcb1, tcb3* double mutants fail to grow. (No cells were streaked on the unlabeled sectors of the plates).

beside the tricalbins contain C2 domains, as summarized in the Introduction (Rsp5p, Pkc1p, Plc1p, and Bud2p). Since these proteins have only one C2 domain each, and the tricalbins each have three, in this study we have eliminated the expression of 70% of the C2 domains encoded by the yeast genome (9 out of 13). Nonetheless, the mutant cells remain viable and qualitatively similar to the wild-type under most laboratory conditions.

The localization of tricalbin 2 on the cell surface and on intracellular membranes is consistent with the presence of the predicted transmembrane domain. The route of movement of the protein after synthesis, first appearing on the cell surface and then being internalized, is reminiscent of the suspected movement of some synaptotagmins in mammalian cells. Synaptotagmin I moves from the synaptic vesicle to the plasma membrane as the vesicle membrane is incorporated in the plasma membrane during exocytosis, then it is endocytosed as the vesicle membrane is pinched off the plasma membrane by clathrin-coated pits and becomes incorporated in newly formed vesicles. In the nematode *Caenorhabolitis elegans*, a defect in synaptotagmin results in a loss of synaptic vesicle number, rather than the increase in vesicle number that might be expected if exocytosis were defective [40]. Therefore, the first step in the life cycle of synaptotagmin may also be movement from the plasma membrane to the cell interior during the formation of synaptic vesicles. However, other synaptotagmins, such as synaptotagmin VII may reside primarily on the plasma membrane [3]. We cannot rule out that tricalbin 2 may also be primarily a plasma membrane protein and that the internalization we observed represents a degradative pathway for this protein.

The final disposition of C-terminal fragments of tricalbin 2 seen in some yeast cells on microtubules suggests that the tricalbins may play a role in linking membranes, either at the cell surface or during endocytosis, to cytoplasmic microtubules. Interestingly, it has recently been found that synaptotagmin IX interacts physically with tubulin and may play a role in linking perinuclear endocytic recycling vesicles and microtubules [41]. However, in the case of the tricalbins, such a linkage may not be essential for plasma membrane insertion or endocytosis of tricalbin 2, since the movements of membrane-bound tricalbin 2 were not blocked by nocodazole treatment.

The tricalbin mutants had a defect in vacuole morphology, although trafficking of the vacuole membrane protease carboxypeptidase S appeared qualitatively normal. The type of change in vacuole morphology seen in the mutants – the formation of a single large compartment instead of a multi-lobed vacuole – can be induced by a number of unrelated stresses, including even prolonged observation under a microscope coverslip [20, 42, 43]. Therefore, the connection between the stress caused by the tricalbin mutations and vacuole morphology might be quite tenuous and indirect. Nonetheless, the apparent movement of tricalbin 2, presumably in association with membranes, from the cell surface to the perivacuolar region does suggest there may be an alteration in membrane flow to the vacuole in the mutants that could underlie the morphological changes. The tricalbin mutants were also found to be hypersensitive to cycloheximide. How can this be related to a role in membrane trafficking or to vacuole function? One speculative answer is that a defect in the vacuole might create a deficiency in one of the roles of the vacuole, that of breaking down proteins and releas-

ing amino acids for recycling in the synthesis of new proteins. If the pool of free amino acids is reduced, the efficiency of protein synthesis might be compromised. This could make the mutants particularly sensitive to inhibition in another step in the complex pathway of protein synthesis through the action of cycloheximide on the ribosome.

Of considerable interest is that *RSP5* can act as a single-copy suppressor of the cycloheximide sensitivity of the tricalbin mutants, since Rsp5p has been shown to play an essential role in the machinery of endocytosis. It is required for internalization of the plasma membrane receptor Ste2p as well as for fluid phase endocytosis [37]. This provides another indication that the tricalbins may perform a function related to membrane trafficking events. Also of interest is that Rsp5p is one of the few other yeast proteins that have a C2 domain. Mutation of the C2 domain of Rsp5p has a more profound effect on the transport of fluid phase markers to the vacuole than on receptor internalization [38], suggesting that calcium may be specifically involved in regulating the movement of the endocytic vesicles after internalization. Processes mediated by the tricalbins and Rsp5p may both be regulated in concert by changes in calcium concentration and activation of their C2 domains.

The results of the two-hybrid screen provide evidence that the tricalbins have an affinity for one another. Combined with the cycloheximide sensitivity data, the interactions appear to be of functional significance. A model that explains the importance of these interactions is illustrated schematically in figure 6. This model assumes that the tricalbins must form a heterodimer in order to carry out their biological function. One partner of the dimer must be tricalbin 2; the other can be either tricalbin 1 or 3. The tricalbin 1,2 and tricalbin 1,3 dimers are apparently functionally redundant. This model would explain why the *tcb2* mutant has the same phenotype as the *tcb1*, *tcb3* double mutant, and is consistent with the two-hybrid interactions seen between tricalbin 2 and either tricalbin 1 or 3. Although the scheme in figure 6 suggests that the tricalbin heterodimers may be anchored in the same membrane, in vivo the critical interactions could occur between tricalbins anchored in different membranes. The tricalbin interactions seen in yeast suggest an important paradigm for the function of synaptotagmins or other multi-C2-domain-containing proteins. Some synaptotagmins are known to self-associate and to associate with one another [3]. These associations may also be necessary to form functional heterodimers or larger complexes between synaptotagmins on the same or on different membranes.

The necessity for tricalbins to function as heterodimers may be a reflection of the unique properties of each of the three proteins. In detailed biochemical studies of the calcium- and lipid-binding properties of tricalbin C2 domains, we have determined that the C2 domains of tricalbin 2 interact with membranes in a calcium-independent fashion [28]. However, the lipid-binding activities of the third C2 domains of both tricalbins 1 and 3 are exquisitely sensitive to calcium in the submicromolar range [28]. Therefore, to construct a tricalbin complex that is regulated by calcium, tricalbin 2 may need to form a complex with either tricalbin 1 or 3, as was demonstrated in the two-hybrid screen and implied in the cycloheximide sensitivity study.

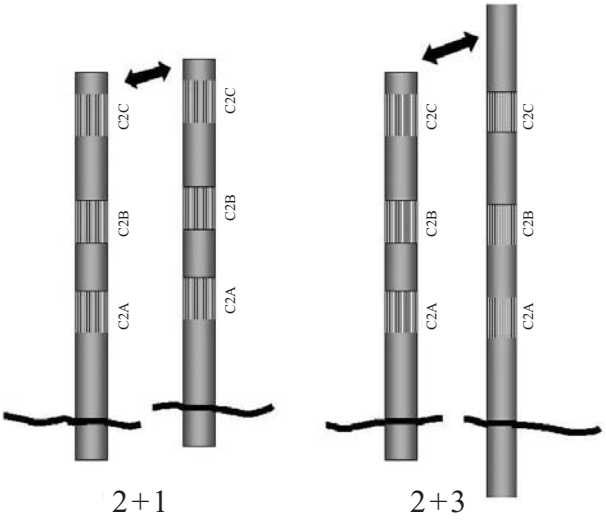

Figure 6. Hypothetical scheme for the formation of functional tricalbin heterodimers. Tricalbin 2 forms a dimer with either tricalbin 1 or tricalbin 3 through interactions at the C termini (double arrows). The dimers are functionally redundant so the tricalbin mutant phenotype emerges only if both heterodimers are disrupted either by deletion of tricalbin 2 alone, or by deleting both tricalbins 1 and 3. In the functional heterodimer, whether both tricalbins are in the same membrane or if they reside in separate membranes is not known.

In addition to interacting with the other tricalbins, tricalbin 2 was found to interact with the activation domain of the transcription factor Pdr1p in the two-hybrid assay. Defects in Pdr1p can also lead to hypersensitivity to cycloheximide, as well as other toxic compounds [31]. Pdr1p promotes expression of drug transport proteins and this may explain the sensitivity to cycloheximide of *pdr1* mutants. However, the role of Pdr1p in the yeast cell is likely to be more fundamental than one of providing resistance to toxic compounds. Pdr1p is also responsible for expression of enzymes involved in the synthesis and organization of membrane components in general, both lipid synthetic enzymes as well as lipid transport proteins [32–35]. In that sense, Pdr1p may be a type of 'master regulator' of membrane construction. We speculate that the interaction of Pdr1p with the tricalbins could play a role in regulating membrane synthesis. Although we did not detect tricalbin in the nucleus, and Pdr1p has been localized primarily to the nucleoplasm [44], Pdr1p does

have a nuclear import signal that is recognized by the Pse1/Kap12 1 nuclear importin [44]. Possibly, Pdr1p may undergo trafficking into and out of the nucleus in a fashion similar to the Pho4 transcription factor which is also a substrate of the Pse1/Kap121 importin system [45]. The binding of the activation domain of Pdr1p to tricalbin could sequester the transcription factor in the cytoplasmic compartment. If Pdr1p were then released in response to a stimulus – for example the entry of calcium into the cytoplasm which might make the C2 domain bind lipids instead of Pdr1p – the transcription factor could then enter the nucleus and initiate the expression of genes needed for the construction of new membrane. In this way, the tricalbins could play an important role in sending information to the nucleus about membrane activities and utilization.

Acknowledgement. We thank M. Smith and D. Burke of the University of Virginia for discussions, advice, and reagents during the course of this study. We also thank J. Cahill for her participation in the tricalbin localization experiments, and Phil Rock for consultations on fungal taxonomy. This study was supported by grant GM53266 from the National Institutes of Health.

1 Nalefski E. A. and Falke J. J. (1996) The C2 domain calcium-binding motif: structural and functional diversity. Protein Sci. **5:** 2375–2390
2 Perin M. S., Fried V. A., Mignery G. A., Jahn R. and Sudhof T. C. (1990) Phospholipid binding by a synaptic vesicle protein homologous to the regulatory region of protein kinase C. Nature **345:** 260–261
3 Sudhof T. C. (2002) Synaptotagmins: why so many? J. Biol. Chem. **277:** 7629–7632
4 Fukuda M. (2003) Molecular cloning, expression, and characterization of a novel class of synaptotagmin (sytXIV) conserved from *Drosophila* to humans. J. Biochem. **133:** 641–649
5 Sudhof T. C., and Rizo J. (1996) Synaptotagmins: C2-domain proteins that regulate membrane traffic. Neuron **17:** 379–388
6 Pelham H. R. (1999) SNARES and the secretory pathway – lessons from yeast. Exp. Cell Res. **247:** 1–8
7 Novick P., Field C. and Schekman R. (1980) Identification of 23 complementation groups required for post-translational events in the yeast secretory pathway. Cell **21:** 205–215
8 Creutz C. E. and Snyder S. L. (1999) The tricalbins: yeast synaptotagmin homologs? Mol. Biol. Cell **10:** 215a
9 Rose M. D., Winston F. and Hieter P. (1990) Methods in Yeast Genetics, pp. 1–198, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
10 Ausubel F. M., Brent R., Kingston R. E., Moore D.D., Seidman J. G., Smith J. A. et al. (1989) Current Protocols in Molecular Biology. Wiley, New York
11 Guan K. L. and Dixon J. E. (1991) Eukaryotic proteins expressed in *Escherichia coli*: an improved thrombin cleavage and purification procedure of fusion proteins with glutathione S-transferase. Anal. Biochem. **192:** 262–267.
12 Damer C. K. and Creutz C. E. (1994) Synergistic membrane interactions of the two C2 domains of synaptotagmin. J. Biol. Chem. **269:** 31115–31123
13 Olmsted J. B. (1981) Affinity purification of antibodies from diazotized paper blots of heterogeneous protein samples. J. Biol. Chem. **256:** 11955–11957
14 Wach A., Brachat A., Alberti-Segui C., Rebischung C. and Philippsen P. (1997) Heterologous HIS3 marker and GFP reporter modules for PCR-targetting in *Saccharomyces cerevisiae*. Yeast **13:** 1065–1075
15 Broach J. R., Li Y. Y., Wu L. C. C. and Jayaram M. (1983) Vectors for high level, inducible expression of cloned genes in yeast. In: Inovye M. (ed), Experimental Manipulation of Gene Expression, pp. 83–117, Academic Press, New York
16 James P., Halladay J. and Craig E. A. (1996) Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast. Genetics **144:** 1425–1436
17 Lorenz M. C., Muir R. S., Lim E., McElver J., Weber S. C. and Heitman J. (1995) Gene disruption with PCR products in *Saccharomyces cerevisiae*. Gene **158:** 113–117
18 Creutz C. E., Kambouris N. G., Snyder S. L., Hamman H. C., Nelson M. R., Liu W. et al. (1992) Effects of the expression of mammalian annexins in yeast secretory mutants. J. Cell Sci. **103:** 1177–1192
19 Sikorski R. S. and Hieter P. (1989) A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae.* Genetics 122: 19–27
20 Roberts C. J., Raymond C. K., Yamashiro C. T. and Stevens T. H. (1991) Methods for studying the yeast vacuole. Methods Enzymol. **194:** 644–661
21 Odorizzi G., Babst M. and Emr S. D. (1998) Fab1p PtdIns(3)P 5-kinase function essential for protein sorting in the multivesicular body. Cell **95:** 847–858
22 Bradford M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:** 248–254
23 Laemmli U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680–685
24 Burnette W. N. (1981) 'Western blotting': electrophoretic transfer of proteins from sodium dodecyl sulfate polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioactive protein A. Anal. Biochem. **112:** 195–203
25 Tusnády G. E. and Simon I. (1998) Principles governing amino acid composition of integral membrane proteins: Applications to Topology Prediction. J. Mol. Biol. **283:** 489–506
26 Kellis M., Patterson N., Endrizzi M., Birren B. and Lander E. S. (2003) Sequencing and comparison of yeast species to identify genes and regulatory elements. Nature **423:** 241–254
27 Cliften P., Sudarsanam P., Desikan A., Fulton B., Majors J., Waterston R. et al. (2003) Finding functional features in *Saccharomyces* genomes by phylogenetic footprinting. Science **301:** 71–76
28 Schulz T. A. and Creutz C. E. (2004) The tricalbin C2 domains: lipid-binding properties of a novel, synaptotagmin-like yeast protein family. Biochemistry **43:** 3987–3995
29 DeRisi J. L., Iyer V. R. and Brown P. O. (1997) Exploring the metabolic and genetic control of gene expression on a genomic scale. Science **278:** 680–6866
30 Ogawa N., DeRisi J. and Brown P. O. (2000) New components of a system for phosphate accumulation and polyphosphate metabolism in *Saccharomyces cerevisiae* revealed by genomic expression analysis. Mol. Biol. Cell **11:** 4309–4321
31 Balzi E., Chen W., Ulaszewski S., Capieaux E. and Goffeau A. (1987) The multidrug resistance gene *PDR1* from *Saccharomyces cerevisiae.* J. Biol. Chem. **262:** 16871–16879
32 Kean L. S., Grant A. M., Angeletti C., Mahe Y., Kuchler K., Fuller R. S. et al. (1997) Plasma membrane translocation of fluorescent-labeled phosphatidylethanolamine is controlled by transcription regulators *PDR1* and *PDR3*. J. Cell Biol. **138:** 255–270
33 Hazel H. B. van den, Pichler H., Valle Matta M. A. do, Leitner E., Goffeau A. and Daum G. (1999) *PDR16* and *PDR17*, two homologous genes of *Saccharomyces cerevisiae*, affect lipid biosynthesis and resistance to multiple drugs. J. Biol. Chem. **274:** 1934–1941

34 Hanson P. K. and Nichols J. W. (2001) Energy-dependent flip of fluorescence-labelled phospholipids is regulated by nutrient starvation and transcription factors, *PDR1* and *PDR3*. J. Biol. Chem. **276:** 9861–9867
35 Hallstrom T. C., Lambert L., Schorling S., Balzi E., Goffeau A. and Moye-Rowley W. S. (2001) Coordinate control of sphingolipid biosynthesis and multidrug resistance in *Saccharomyces cerevisiae*. J. Biol. Chem. **276:** 23674–23680
36 Dulic V., Egerton M., Elguindi I., Raths S., Singer B. and Riezman H. (1991) Yeast endocytosis assays. Methods Enzymol. **194:** 697–710
37 Dunn R. and Hicke L. (2001a) Multiple roles for Rsp5p-dependent ubiquitination at the internalization step of endocytosis. J. Biol. Chem. **276:** 25974–25981
38 Dunn R. and Hicke L. (2001b) Domains of the Rsp5 ubiquitin-protein ligase required for receptor-mediated and fluid-phase endocytosis. Mol. Biol. Cell **12:** 421–435
39 Damer C. K. and Creutz C. E. (1996) Synaptotagmin II expression partially rescues the growth defect of the yeast *sec15* secretory mutant. Biol. Cell **88:** 55–63
40 Jorgensen E. M., Hartweig E., Schuske K., Nonet M. L., Jin Y. and Horvitz H. R. (1995) Defective recycling of synaptic vesicles in synaptotagmin mutants of *Caenorhabditis elegans*. Nature **378:** 196–199
41 Haberman Y., Grimberg E., Fukuda M. and Sagi-Eisenberg R. (2003) Synaptotagmin IX, a possible linker between the perinuclear endocytic recycling compartment and the microtubules. J. Cell Sci. **116:** 4307–4318
42 Pringle J. R., Preston R. A., Adams A. E. M., Stearns T., Drubin D. G., Haarer B. K. et al. (1989) Fluorescence microscopy methods for yeast. Methods Cell Biol. **31:** 357–435
43 Meaden P. G., Arneborg A., Guldfeldt L. U., Siegumfeldt H. and Jakobsen M. (1999) Endocytosis and vacuolar morphology in *Saccharomyces cerevisiae* are altered in response to ethanol stress or heat shock. Yeast **15:** 1211–1222
44 Delahodde A., Pandjaitan R., Corral-Debrinski M. and Jacq C. (2001) Pse1/Kap121-dependent nuclear localization of the major yeast multidrug resistance (MDR) transcription factor Pdr1. Mol. Microbiol. **39:** 304–312
45 Kaffman A., Rank M. M., and O'Shea E. K. (1998) Phosphorylation regulates association of the transcription factor Pho4 with its import receptor Pse1/Kap121. Genes Dev. **12:** 2673–2683