The Tricalbin C2 Domains: Lipid-Binding Properties of a Novel, Synaptotagmin-Like Yeast Protein Family†

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ABSTRACT: The tricalbins are a recently discovered family of *Saccharomyces cerevisae* proteins containing a predicted N-terminal transmembrane domain and at least three C2 domains. They are thought to be yeast homologues of synaptotagmin, a hypothesis supported by structural similarities and past studies that implicated tricalbins in processes of membrane trafficking and sorting. We expressed and purified constructs consisting of single tricalbin C2 domains, and assayed their ability to bind lipids in response to calcium. Protein−lipid overlay assays indicated that the C-terminal C2 domains (C2C) of tricalbins 1 and 3 bind numerous species of acidic phospholipid, including phosphatidylserine and several phosphoinositides, and the amount of protein bound was greatly enhanced in the presence of 1 mM calcium. Sedimentation assays using mixed phosphatidylserine/phosphatidylcholine (PS/PC) vesicles confirmed that the C2C domains of tricalbin 1 and 3 bind membranes in a calcium-responsive manner and showed that they are more sensitive to calcium than the C2A domain of synaptotagmin I. Both assays revealed that all of the C2 domains of tricalbin 2 are insensitive to calcium. Fluorimetric assays exploiting the position of naturally occurring tryptophans in tricalbin 1 C2C and tricalbin 3 C2C confirmed that these domains are capable of binding calcium and that this is coupled to the binding of acidic phospholipid. Combining this with past protein−protein interaction data, we theorize that the calcium-insensitive tricalbin 2 mediates the creation of hetero-oligomeric tricalbin complexes in which tricalbin 1 or 3 or both supply a calcium-dependent membrane binding activity.

Proteins with multiple tandem C2 domains such as synaptotagmins (*1, 2*) and ferlins (*3*) have been implicated in processes involving membrane trafficking, particularly fusion-related events. This function is thought to be due to the ability of their C2 domains to bind acidic phospholipids in a calcium-dependent fashion, allowing a coordinated response to calcium signaling fluxes within the cell (*4*). There is still debate over the function of individual C2 domains within such proteins. Synaptotagmins are thought to partition certain specific functions between their two C2 domains (*2*), but these domains can work in a cooperative or synergistic manner (*5, 6*). Recent years have seen the discovery of numerous proteins with three or more C2 domains in mammals and other higher organisms, and for these relatively uncharacterized proteins, the picture is even less clear.

The tricalbins comprise a novel family of yeast proteins discovered in a search for C2-containing proteins, especially those with multiple C2 domains, in *Saccharomyces cerevisae* (*7*). They have apparent homologues in *Drosophila* (*8*) and *C. elegans* (*9*) and are hypothesized to be related to plant synaptotagmins (*9*). These very large (>130 kDa) proteins contain a predicted N-terminal transmembrane domain, three C2 domains (labeled "C2A", "C2B" and "C2C"), and a C-terminal "tail" region of variable size and sequence. The three members of the tricalbin family—tricalbin 1 (yeast open reading frame YOR086c), tricalbin 2 (YNL087w), and tricalbin 3 (YML072c)—are fairly divergent in terms of sequence identity, yet deletion studies (*7, 10, 11*) suggested that tricalbins 1 and 3 may be at least partially redundant. Δ*tcb2* and Δ*tcb1*/Δ*tcb3* deletion mutants are hypersensitive to low concentrations of cycloheximide; however, Δ*tcb1* and Δ*tcb3* single-deletion mutants failed to display any aberrant phenotype (*10, 11*). This suggested that tricalbin 1 and tricalbin 3 may be functionally redundant in this context, while tricalbin 2 maintains a unique role in cycloheximide resistance. It is interesting to note that despite the relative commonality of C2 domains in higher organisms, there are only four other known C2 proteins in yeast: protein kinase C, phospholipase C, Rsp5p (ubiquitin ligase), and Bud2p (a Ras GTPase activating protein).

The process of homotypic vacuolar fusion in *S. cerevisae* involves a soluble N-ethylmaleimide sensitive factor attachment protein receptor (SNARE) fusion complex very similar to that found in mammals, with many homologous components (*12*). Because the tricalbins are the only proteins in yeast to be structured similarly to synaptotagmin, it is possible that tricalbins may serve the same function within the SNARE machinery of yeast as synaptotagmins do in the mammalian equivalent. However, green fluorescent protein (GFP) localization studies indicate that tricalbin 2 is found primarily in punctate domains of the plasma membrane, later moving to what appear to be sorting vesicles near the vacuole (*10*). Additionally, tricalbin mutants do not display the fragmented-vacuole phenotype typically observed with mu-

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tants in the vacuole fusion process, and genetic screens have not detected a direct role of any tricalbin in vacuole fusion (*12*). Two-hybrid screens (*10*) found an interaction between the C-terminal region of tricalbin 2 (comprising the third C2 domain [C2C] and C-terminal tail region) and Pdr1p, a transcription factor that confers multidrug resistance by modulating genes responsible for drug transport, stress defense, sphingolipid biosynthesis, and endocytosis (*13*, *14*), among others. These screens also demonstrated that this region of tricalbin 2 is capable of interacting with C-terminal regions of tricalbins 1 and 3 (*10*). This suggests the existence of a heteromeric "tricalbin complex" that requires multiple members of the tricalbin family to function and may be involved in drug resistance.

To clarify the possible roles of each tricalbin within this complex, we decided to study the specific lipid interactions of C2 domains of each tricalbin isoform. To that end, we expressed and purified constructs of the C-terminal C2 domains (C2C) of tricalbins 1 and 3, as well as all C2 domains of tricalbin 2. To obtain a general profile of their lipid specificity, protein−lipid overlay assays were performed using a variety of lipid species immobilized onto nitrocellulose. We also conducted vesicle binding studies with mixed PS/PC[1] large unilamellar vesicles (LUVs). Calcium-titration curves derived from these studies indicate that increases in free calcium concentration strongly influence the ability of the C2C domains of tricalbin 1 and tricalbin 3 to bind PS-containing LUVs. Furthermore, these C2 domains appear even more sensitive to calcium than synaptotagmin I C2A, achieving half-maximal binding at calcium concentrations 2 orders of magnitude lower than that required for SytI-C2A to reach half-maximal binding. Even in the absence of calcium, all three C2 domains of tricalbin 2 bind PS vesicles to a certain extent, but increasing calcium concentrations failed to increase or decrease lipid binding.

We conclude that the C-terminal C2 domains of tricalbins 1 and 3 are calcium-dependent lipid-binding units comparable to mammalian synaptotagmin C2 domains, while tricalbin 2 likely does not bind lipids in response to calcium signaling. Implications for the possible function of the tricalbin complex, as well as for other multiple-C2 proteins, are discussed.

MATERIALS AND METHODS

Cloning of Tricalbin C2 Domains. cDNAs representing the three C2 domains of tricalbin 2 were amplified from the complete tricalbin-2 cDNA in YEP51 plasmid (*10*). The PCR primers used incorporated *Xba*I (TCTAGA) and *Sal*I (GTCGAC) restriction sites, as well as stop codons in the antisense primers. Primers used were as follows: 5′-CGC*TCTAGA*TATACCACAACTTTCCAAGACTGGC-3′ (Tcb2-C2A upstream primer, sense); 5′-GCG*GTCGAC*TTATTTTGGTTCAATAGTGGGGAAAAATCGC-3′ (Tcb2-C2A downstream primer, antisense); 5′-CGC*TCTAGA*CCTCGGATTGAAAAGTGTAGG-3′ (Tcb2-C2B primer, sense); 5′-*GTCGAC*CAGGAACAACTGGATAAAATGATA-3′ (Tcb2-C2B primer, antisense); 5′-CCC*TCTAGA*TGCCAACGGAATTGCCTCAATCAG-3′ (Tcb2-C2C primer, sense); 5′-*GTCGAC*TTATTGCTTCTCTTCGGATTTTTTGTCC-3′ (Tcb2-C2C primer, antisense). The C2C domains of tricalbins 1 and 3 were amplified directly from total chromosomal DNA of a wild-type Y500 strain of *S. cerevisae*. Primers used for these reactions were as follows: 5′-CCC*TCTAGA*TACAAAACAGTTGCCGGCAAATGACC-3′ (Tcb1-C2C primer, sense), (5′-GGC*GTCGAC*CTAAAACTTAGTTTCTTCTTCGCCCAT-3′ (Tcb1-C2C primer, antisense), 5′-CGC*GAATTC*CTAGCGTGAAACTGCCTAGCAGTG-3′ (Tcb3-C2C primer, sense; containing an *Eco*RI restriction site), and 5′-GGC*GTCGAC*TTACTGCGTGTATTCTTGAGGAACTTC-3′ (Tcb3-C2C primer, antisense). All PCR reactions were carried out with puReTaq Ready-to-Go PCR beads (Amersham Biosciences, Piscataway, NJ) in a sequence of 30 cycles consisting of 94 °C denaturation (1 min), 55 °C reannealing (2 min), and 72 °C extension (3 min). Purified reaction products were digested with the appropriate restriction enzymes (*Eco*RI and *Sal*I for Tcb3-C2C, *Xba*I and *Sal*I for all other C2 domains; all enzymes obtained from Promega, Madison, WI), ligated into bacterial pGEX-KG expression vectors (*15*), and confirmed by DNA sequencing.

Expression and Purification of C2 Domains. Cells of the *Escherichia coli* strain DH10B (Invitrogen, Carslbad, CA) were transformed with a pGEX-KG plasmid containing the C2 construct with an N-terminal glutathione S-transferase (GST) tag. A single transformant was chosen to grow overnight in a 100 mL preculture (LB with 100 μg ampicillin). The next morning, 1 L of LB (plus ampicillin) was inoculated with the preculture, and incubated for 1.5−2 h at 37 °C. Production of the protein was induced with 100 μM IPTG for 4.5−5 h at 30 °C. Cells were then pelleted and resuspended in 20 mL of PBS with 2 mM EGTA and protease inhibitors (0.5 mM PMSF and 0.5% aprotinin). Cells were lysed with a Misonix Sonicator 3000 probe sonicator (Farmingdale, NY; settings of 60% power, 50% on/off time, 2.5 min) and homogenized with 1% Triton X-100. Solid cellular debris was spun down at 12 000 × *g* for 10 min. The supernatant was added to ∼160 mg dry weight glutathione−agarose beads (preswelled in PBS) and gently mixed for 20 min at room temperature. The beads were collected by low-speed centrifugation, washed four times with 10× volumes of PBS + 1% Triton and protease inhibitors, then washed three times with 50 mM Tris, pH 8.0. Protein was eluted from the beads with four washes of 2 mL of elution buffer (10 mM free glutathione and 0.25% β-mercaptoethanol in 50 mM Tris, pH 8.0). Washes were pooled and dialyzed for 4 h against 1 L of 20 mM Hepes, pH 7.0, 50 mM KCl. Final concentration of protein was determined via Bradford assay (*16*).

Protein−Lipid Overlay Assays. Overlay assays were carried out using PIP Strips (Echelon Inc., Salt Lake City, UT), nitrocellulose strips on which various species of lipid have been immobilized. Strips were blocked for 1 h at room temperature in a solution of TBST (10 mM Tris, 150 mM NaCl, 0.1% Tween-20, pH 8.0) with 3% fatty acid free BSA (Sigma A-8806). Strips were then incubated for 1.5 h with the purified GST-tagged proteins in TBST with BSA, where protein concentration was either 0.5 μg/mL (synaptotagmin I C2A) or 0.05 μg/mL (tricalbin C2 domains). Strips were then subjected to three 5-min washes with TBST (no BSA)

[1] Abbreviations: PS, phosphatidylserine; PC, phosphatidylcholine; Rho-PE, di-18:1 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl); PI(3,5)P2, phosphatidylinositol 3,5-bisphosphate; LUV, large unilamellar vesicle; Syt, synaptotagmin; Tcb, tricalbin.

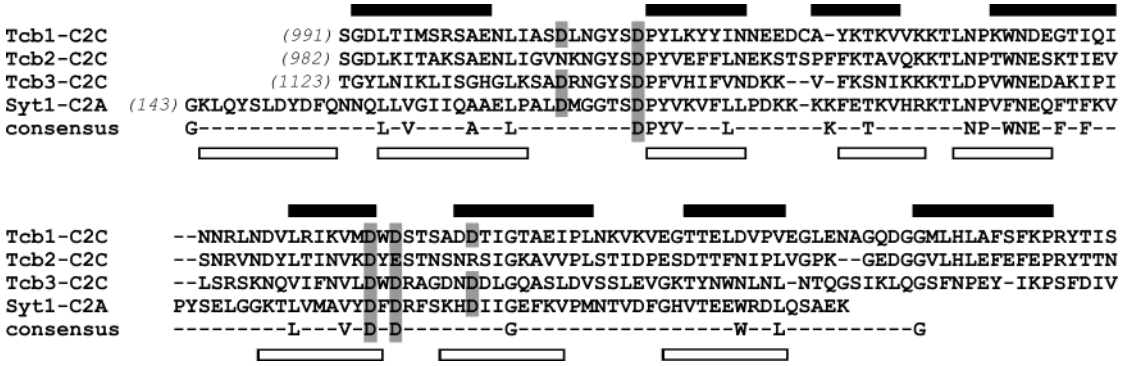

FIGURE 1: Alignment of the C-terminal C2 domains (C2C) of all three yeast tricalbins with the C2A domain of synaptotagmin I (SytI-C2A). The conserved aspartates (or glutamates) that are thought to participate in calcium binding are highlighted in gray. The "consensus" sequence is taken from Nalefski and Falke (*18*) and consists of those highly conserved residues found in over 50% of the 62 C2 domains aligned in their study. Rectangles above and below the sequences represent the predicted locations of the eight β strands; black rectangles indicate location of the strands in a topology II C2 domain (the tricalbins), and the white or hollow rectangles represent the positions of β strands in a topology I structure (synaptotagmin I).

and incubated for 2 h with TBST with BSA and rabbit anti-GST (Sigma G-7781) at 1:4000 dilution. Following another three TBST washes, the strips were incubated with horse radish peroxidase (HRP)-conjugated goat anti-rabbit IgG (American Qualex A102PN, 1:10 000 dilution) in TBST with BSA for 1.5 h. Finally, the strips were washed three to five times in dH_2O and treated with SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL) for 1−2 min. Images were then exposed on film for 30−60 s and developed. For each strip, all solutions (including washes and chemiluminescent substrate) contained either 1 mM $CaCl_2$ or 2 mM EGTA.

LUV Binding Assays. All lipids used in this portion of the study were obtained from Avanti Polar Lipids, Inc.: brain PS (840332), brain PC (840053), and di-18:1 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl) (Rho-PE; 810150). Sucrose-loaded vesicles were prepared in a procedure adapted from Rebecchi et al. (*17*). Mixtures of lipid were evaporated under an argon stream and hydrated for 30 min in a buffer containing 20 mM HEPES (pH 7.0) and 180 mM sucrose. Vesicles were then passed 10 times through a Whatman 100 nm filter using a hand-held Mini-Extruder (Avanti, Alabaster, AL). All prepared vesicles contained 0.5 mol % Rho-PE to aid in the creation of a more cohesive and easily visible pellet.

For the sedimentation assays, approximately 50 μg of LUVs were diluted into an isotonic solution of 20 mM HEPES (pH 7.0) and 100 mM NaCl. Protein (8−12 μg) and a Ca^{2+}/EGTA buffer (consisting of 2.5 mM EGTA and a quantity of $CaCl_2$ calculated to give a specific free calcium concentration at pH 7.0) were added to a final volume of 100 μL to initiate the reaction. The mixture was incubated for 15 min at room temperature, then centrifuged at 100 000 $\times$ *g* for 15 min at room temperature. The top 75 μL of the mixture was removed (so as not to disturb the pellet), leaving 25 μL of supernatant remaining with the lipid pellet. Both fractions were run on SDS−PAGE, stained with Coomassie blue stain (50% methanol, 10% acetic acid, 0.05% Coomassie blue), and then allowed to destain overnight. Gels were scanned, and densitometry analysis was performed on the bands with NIH Image 1.55. The amount of protein associated with the lipid pellet was calculated as follows: the raw scores for the "pellet" (*p*) and "supernatant" (*s*) fractions were taken and corrected for the 25 μL of supernatant in p, so the final values for pellet (*P*) and supernatant (*S*) are equal to $P = p - (s/3)$, and $S = s + (s/3)$. Thus, the percentage of protein bound (P_{bound}) to lipid is equal to $P_{bound} = 100[p - (s/3)]/[p + s]$.

Measurement of Tryptophan Fluorescence. Changes in tryptophan emission spectra were monitored with a SPEX Fluorolog 111C with excitation and emission slits set to 0.5 mm and a 300 nm cutoff filter between the sample cuvette and emission monochromator. Samples consisted of approximately 10 μg of protein (final concentration = 0.9 μM) in vesicle buffer (20 mM Hepes, pH 7.0, 100 mM KCl) in the presence or absence of 50 μg of 40:60 PS/PC LUVs (final lipid concentration = 290 μM). This mixture was added to 22 μL of 10× calcium/EGTA buffer (as used in the LUV binding assays) to a final volume of 220 μL. Excitation wavelength was set to 290 nm, and emission spectra were collected between 300 and 475 nm. Scans were repeated five times and averaged. The LUVs used in this study were prepared as above with the following changes: vesicles were comprised only of 40 mol % PS and 60 mol % PC and were suspended in a 20 mM Hepes (pH 7.0), 100 mM KCl buffer.

RESULTS

The Tricalbin C2 Domains Are a Diverse Family Possessing a Topology II Structure. Secondary structure prediction with the JPRED online program (University of Dundee, http://www.compbio.dundee.ac.uk/~www-jpred) revealed a canonical eight β-strand structure for each tricalbin C2 domain (Figure 1). The positioning of highly conserved residues within those strands allows us to deduce that the tricalbin family is of the subset of C2 isoforms called "topology II", in contrast to the synaptotagmin C2 domains, which are topology I (*18*). Topology II domains are those in which the first β strand of topology I domains has been translocated to the end of the sequence.

Apart from a few highly conserved motifs shared among all C2 domains, the primary sequences of the tricalbin C2 domains are quite diverse. Most calcium-dependent C2 domains possess five acidic residues (usually aspartate) that are involved in coordinating the calcium ions and mediating lipid interaction. Tricalbin C2 domains retain anywhere from three to all five of these residues (Table 1), and this number can vary within a single tricalbin. The first C2 domain of tricalbin 1 (Tcb1-C2A) has only three conserved acidic

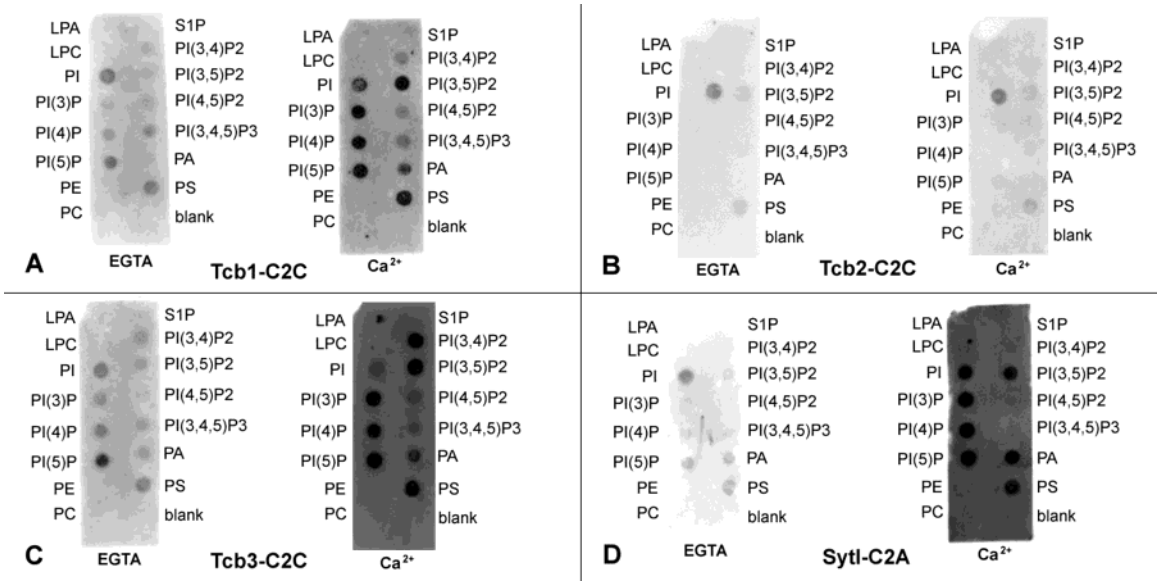

FIGURE 2: Protein−lipid overlay assays employing tricalbin C2 domains and synaptotagmin I C2A for comparison. Nitrocellulose strips spotted with a variety of lipid species ("PIP-StripsTM"; P-6001, Echelon Biosciences Inc., Salt Lake City, UT) were incubated with C2 domains as described in the text. This process was carried out in either 2.5 mM EGTA or 1 mM Ca^{2+}. Controls carried out with GST alone produced no visible spots (not pictured).

Table 1: Conserved Calcium-Coordinating Acidic Residues in Tricalbin C2 Domains[a]

Tcb1-C2A		D410		D457	D464
Tcb1-C2B	E674	D680 D727		E729	D734
Tcb1-C2C	**D1008**	**D1014**	**D1064**	**D1066**	**D1072**
Tcb2-C2A		**D403**		**D449**	**D456**
Tcb2-C2B	**D666**			**D721**	**D726**
Tcb2-C2C		**D1005**	**D1056**	**E1058**	
Tcb3-C2A	D507	D514		D566	D573
Tcb3-C2B	D812	D820	D867		D874
Tcb3-C2C	**D1150**	**D1156**	**D1204**	**D1206**	**D1212**

[a] The C2 domains used in this study are in **bold**.

residues, while its C2B and C2C retain all five. Tricalbin 3 has four each in its first two C2 domains and five in its third C2, while each C2 domain of tricalbin 2 possesses only three putative calcium-chelating residues.

Presumably, this variety in structure would allow for a diversity of function in vivo, even though initial deletion studies suggested some amount of redundancy between tricalbins (*7*, *10*). It has been proposed that tricalbins are related to plant synaptotagmins (*9*), which could also suggest properties similar to the synaptotagmin family, including unique functions for each C2 domain. Therefore we isolated and purified individual tricalbin C2 domains to study their specific biochemical properties.

Lipid-Binding Profiles of the Tricalbin C2C Domains. cDNA coding for each C2 domain of tricalbin 2, as well as the third C2 domains (C2C) of tricalbins 1 and 3 (Figure 1A, Table 1), were expressed in bacterial cultures as GST fusion proteins and purified to ≥95% purity by glutathione−agarose pull-down. Stability of these constructs in solution seemed to be enhanced by the GST tag with no noticeable effect on lipid binding. All protein−lipid overlay and LUV binding studies presented here were performed with GST-tagged protein.

Because the lipid-binding properties of tricalbin C2 domains had not yet been determined, we decided to screen a variety of lipid species to determine which lipids are preferentially bound by these domains. This was done via a protein−lipid overlay assay using nitrocellulose strips on which various phospholipid species had been immobilized, including phosphatidylserine (PS), phosphatidylinositol (PI), and various phosphoinositides, which are thought to be important in numerous signaling pathways. This technique has been successfully used with other yeast proteins to determine their lipid specificities (*19*), and an adaptation of that procedure was used here. The screen is essentially performed as a Western blot with the lipid-spotted nitrocellulose strip successively incubated with the C2 domain in question (purified as a GST fusion protein), mouse anti-GST antibody, and finally a HRP-conjugated secondary antibody directed against mouse protein. The C2C domains of tricalbins 1 (Figure 2A) and 3 (Figure 2C) showed a binding profile similar to that of the C2A domain of synaptotagmin I (Figure 2D). These C2 domains associated to some extent with PS and PI in the absence of calcium; in the presence of 1 mM Ca^{2+}, much more protein was found associated with these lipids, as well as with PI(3)P, PI(4)P, and PI(5)P. However, all three C2 domains of tricalbin 2 (Figure 2B; only C2C is shown here) bound these lipids to a similar extent both in the presence and absence of calcium.

Tcb1-C2C and Tcb3-C2C Bind PS LUVs in a Calcium-Sensitive Manner. Synaptotagmin C2 domains are well-characterized calcium-dependent lipid-binding units. To determine whether tricalbins, their putative yeast homologues, possessed similar characteristics, binding studies were carried out employing sucrose-loaded mixed PS/PC LUVs (large unilamellar vesicles). Protein binding was determined by incubating LUVs with 10 μg of protein and a calcium/EGTA buffer for 15 min, followed by centrifugation for 15 min at 100 000 × *g*. The lipid pellet (containing bound protein) and supernatant were run on separate SDS−polyacrylamide gels, from which the percentage of bound protein was calculated by gel-band densitometry.

These studies were carried out over a range of calcium concentrations from 0.1 μM to 1 mM, as well as in the

absence of free calcium (2.5 mM EGTA only). The resultant calcium titration curves (Figure 3A,B) revealed that the C2C domains of tricalbins 1 and 3 demonstrate a greater sensitivity to calcium than synaptotagmin I C2A. When 20% PS LUVs (Figure 3A) were used, half-maximal binding was achieved around 5−10 μM Ca^{2+} for the tricalbin 1 and 3 C2C domains, whereas SytI−C2A had only achieved 50% binding at 1 mM Ca^{2+}. With 40% PS vesicles (Figure 3B), Tcb1-C2C and Tcb3-C2C reached half-maximal binding at less than 1 μM Ca^{2+}, while SytI-C2A reached that point at around 30 μM Ca^{2+}.

However, tricalbin 2 C2C failed to respond to increasing concentrations of calcium. Approximately 50% of Tcb2-C2C bound to 50 μg of 40 mol % PS LUVs in the presence of EGTA only, but this number neither increased nor decreased with added calcium. This is not likely to be the result of interaction with bacterial contaminants as some have suggested (*35*), because the protein was gauged to be free of such content by A_{260}/A_{280} measurements. In fact, all three C2 domains of tricalbin 2 behaved similarly (Figure 4). When Tcb2-C2C was titrated with increasing amounts of 40 mol % PS vesicles (Figure 5), virtually all protein was found in the pellet fraction at 180−200 μg of lipid. This indicates that all of the protein is capable of binding lipid even in the absence of calcium. Lipid titrations carried out in the presence of 1 mM calcium produced identical results.

A sample of the gels from which the binding curves are calculated is shown in Figure 3C. The set displayed here comes from a single run of experiments using 40% PS vesicles (corresponding to Figure 3B); all C2 domains were studied in parallel for each experimental run, and each run was independently conducted three times. Each data point varied by less than 8% between trials, and the calculated half-maximal binding values did not significantly vary. For example, when 20% PS vesicles were used, the half-maximal binding of Tcb1-C2C and Tcb3-C2C consistently remained around 5−20 μM calcium whereas for SytI-C2A it hovered around 0.8−1.0 mM.

Fluorimetric Measurements of Calcium and Lipid Binding to Tcb1-C2C and Tcb3-C2C. The emission spectrum of tryptophan residues is known to shift on the basis of changes in local environment, such as entry into a more hydrophobic area. The C2C domains of Tcb1 and Tcb3 each contain a tryptophan residue on the third calcium-binding loop (see Figure 1), directly between two of the conserved calcium-coordinating aspartate residues (W1065 in Tcb1, W1205 in Tcb3). Since this position would presumably be brought into close proximity to the target membrane, we obtained emission spectra for Tcb1-C2C and Tcb3-C2C over a range of calcium concentrations both in the presence and absence of 40 mol % PS vesicles.

In the absence of calcium and lipid, emission spectra for both proteins reached a peak at approximately $\lambda_{em} = 352$ nm (Figure 6). Increasing concentrations of calcium reduced the intensity of this peak (Figures 6 and 7) but did not significantly shift the peak. This suggests that Tcb1-C2C and Tcb3-C2C are capable of binding calcium even in the absence of lipid. In the presence of lipid, the emission peak was seen to increase in overall intensity, and made a noticeable shift to the left (Figure 6), consistent with a change to a more hydrophobic environment. This change correlates positively with increasing calcium concentration (Figure 7).

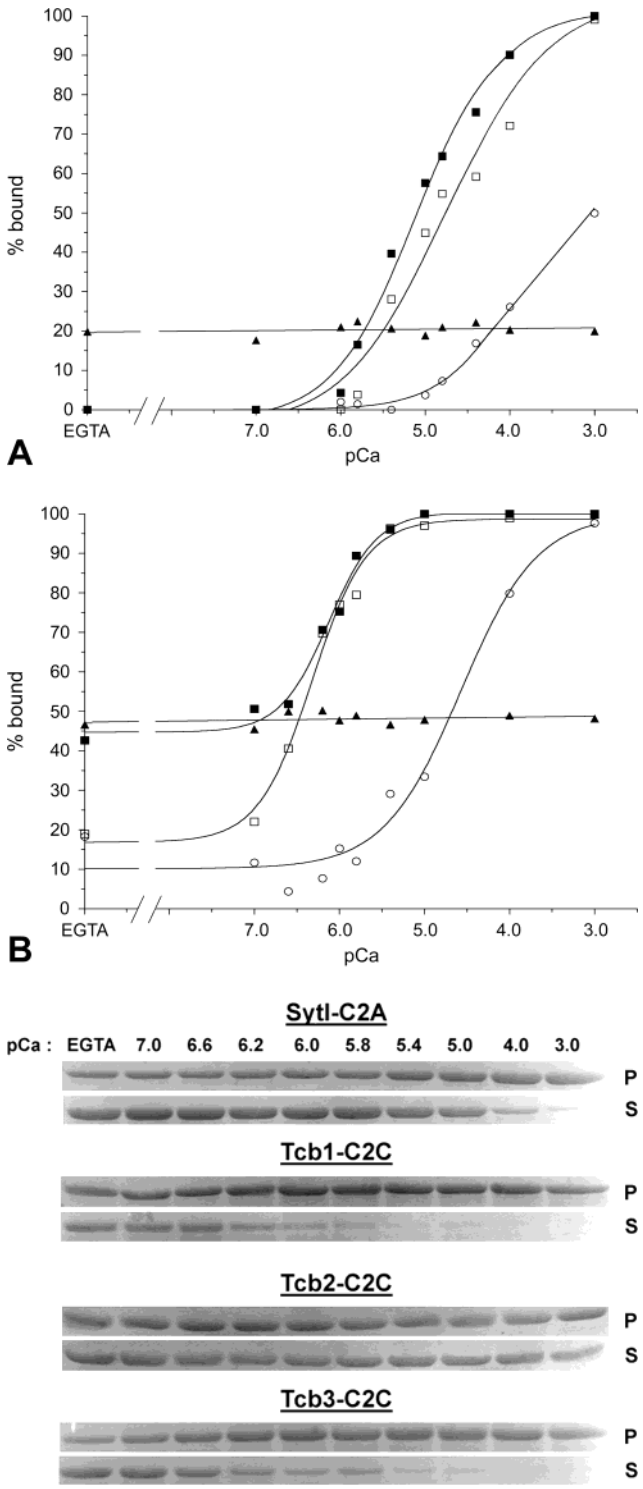

FIGURE 3: Calcium titration curves for the C2C domains of tricalbins 1 (■), 2 (▲), and 3 (□), as well as for synaptotagmin I C2A (○). These assays measured the extent of binding to large unilamellar vesicles (LUVs) containing 20 mol % PS (A) or 40 mol % PS (B, C); LUVs also contain 0.5 mol % Rho-PE (to assist in pelleting and visualization) and PC (79.5 mol % or 59.5 mol %, respectively). The percentage of protein associated with the lipid pellet (% bound) is plotted against the pCa of the reaction buffer (where pCa is equal to the negative log of free calcium concentration). Panel C shows a sample of the raw data obtained for each C2 domain in one experiment (40% PS, corresponding to panel B). The pellet fraction (*P*) contains about 25 μL of supernatant (the supernatant fraction, *S*, contains the remaining 75 μL of total supernatant; see "Materials and Methods"); final calculations in panels A and B correct for the amount of protein in *P* that actually belongs in *S*.

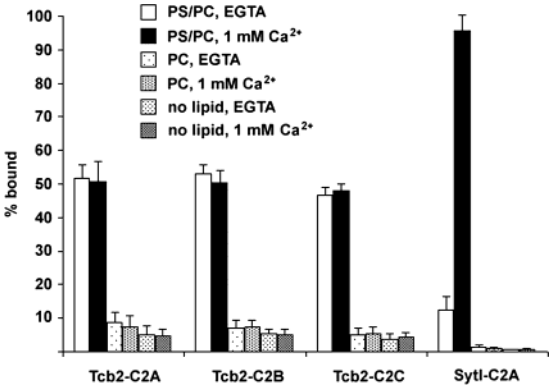

FIGURE 4: A comparison of the lipid binding activity of the C2A, C2B, and C2C domains of tricalbin 2. LUV binding assays (see text and Figure 3) were performed in the presence of either an EGTA buffer or a calcium/EGTA buffer with a free calcium concentration of 1 mM (pCa 3.0). Assays were performed using 40 mol % PS vesicles, pure PC vesicles, and in the absence of lipid (see key). Again, synaptotagmin I C2A is included for comparison. Results are averaged from three independent experiments, and error bars represent 1 SD.

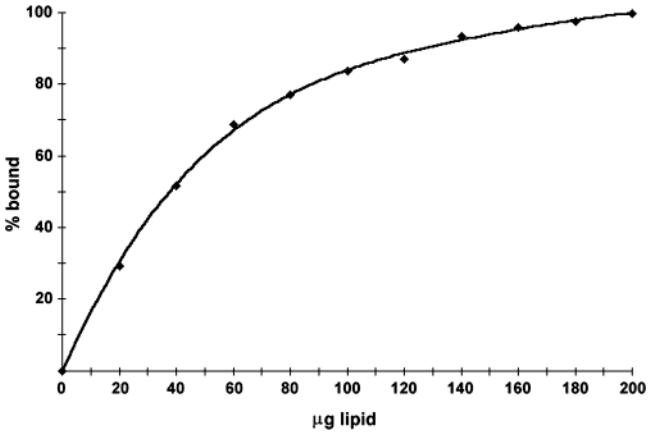

FIGURE 5: Lipid titration curve for Tcb2-C2C. LUV binding experiments were carried out as before but with a varying amount of 40 mol % PS LUVs (from 0 to 200 μg of lipid) and with calcium levels held constant. All points shown here were generated in the presence of 2.5 mM EGTA; carrying out all reactions in the presence of 1 mM calcium generated identical results (not shown).

The curves generated in the presence of lipid appear to reach maximal lipid binding at around 1.6 μM calcium (pCa 5.8), leveling off thereafter; this comes close to agreement with the LUV pelleting assay (Figure 3B), where maximal binding to 40 mol % PS LUVs is reached between pCa 5.4 and 5.0. This would appear to confirm the ability of Tcb1-C2C and Tcb3-C2C to bind phospholipid membranes and indicates that this is probably coupled to their ability to bind calcium ions.

DISCUSSION

Although initially identified as potential yeast synaptotagmin homologues, the tricalbins possess several distinct features that throw that categorization into question. In addition to the triplicate C2 domains from which they derive their name, there is a possible fourth C2 domain between the C2A and C2B of all three tricalbins. These putative fourth C2 domains retain some of the conserved motifs found in C2 domains, and secondary structure predictions indicate that they possess an eight-β-strand structure. However, they have

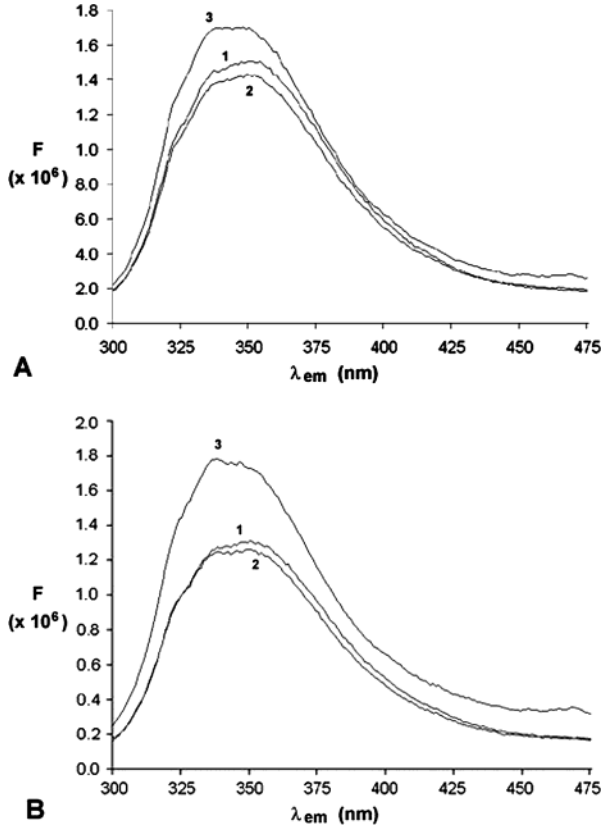

FIGURE 6: Emission spectra for Tcb1-C2C (A) and Tcb3-C2C (B), obtained as described in the text (λ_{ex} = 290 nm). The trace labeled 1 in each graph represents the spectrum obtained for the protein alone (with 2.5 mM EGTA); trace 2 corresponds to protein plus 10 μM calcium; trace 3 represents protein plus 10 μM calcium and 50 μg of 40:60 PS/PC LUVs. Addition of 2.5 mM EGTA to any sample containing calcium was sufficient to return the emission spectrum to the state seen in trace 1.

an extremely low overall sequence identity with other C2 domains (in fact, the initial high-stringency analyses used to identify yeast C2 domains failed to positively identify these as C2 domains (*10*)) and possess no more than two of the five acidic calcium-coordinating residues found in C2 domains. This suggests that these cryptic C2 domains may be vestigial remnants of the duplication event that gave rise to multiple C2 domains within the family. They may serve a structural purpose, spacing and positioning the "primary" C2 domains.

Further differentiating tricalbins from synaptotagmins is a long stretch of residues (~150 aa) of uncertain function located between the C2B and C2C domains. Secondary structure predictions indicate a mix of α helices and β strands, but BLAST and other database searches failed to turn up any known motifs or domains, nor did they show significant sequence similarity to any protein other than putative tricalbin homologues in *S. pombe* and the fungus *Erysiphe pisi*. The function of this region is unknown, though like the crypto-C2 domain, it may serve to properly position the functional C2 domains.

On the other hand, the C2 domains by themselves possess some tantalizing features that invite comparison to the synaptotagmins. BLAST sequence comparisons showed that the C2C domains of tricalbins 1 and 3 display strong sequence identities with regions of overlap of the C2A domains of synaptotagmin III and VII, respectively. In the

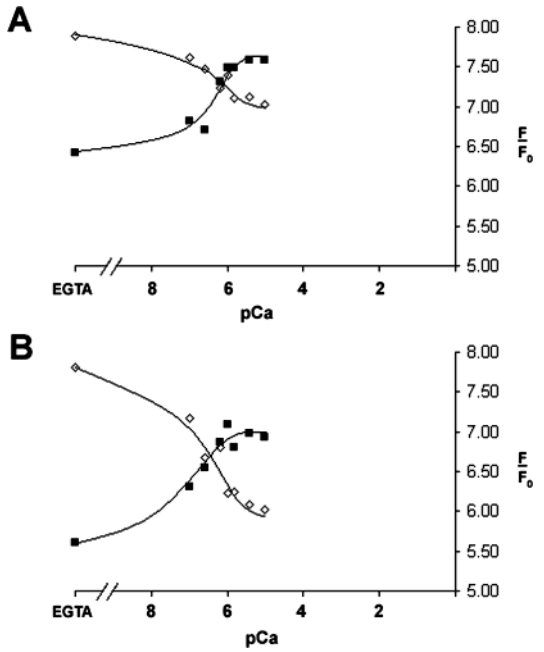

FIGURE 7: Changes in peak fluorescence as observed over a range of calcium concentrations in the presence (■) or absence (◇) of 50 μg of 40:60 PS/PC LUVs: (A) Tcb1-C2C; (B) Tcb3-C2C. Each data point was individually obtained using a fresh mixture of protein, EGTA/calcium buffer, and lipid. Possible pipetting errors and variance in protein concentration are corrected by dividing peak fluorescence (F) by baseline fluorescence at 300 nm (F_0).

region of strongest overlap (corresponding to the first six β strands of the tricalbin C2 domain, and strands two through seven of the synaptotagmin), there is a 32% identity between Tcb1-C2C and human SytVII-C2A, and 34% identity between Tcb3-C2C and human SytIII-C2A. Interestingly, the C2C domain of tricalbin 1 shares an absolutely conserved six-residue sequence (IPLNKV) with all known synaptotagmin VII C2A domains (human, rat, and mouse). This sequence is positioned at the loop between β strands 6 and 7 in tricalbin (strands 7 and 8 in synaptotagmin VII), which is located at the opposite face from the calcium- and lipid-binding loops. This sequence may serve some regulatory role that is highly conserved among the eukaryotes.

The C2C domain of tricalbin 1 shares a greater sequence identity with Tcb2-C2C (48%) than with Tcb3-C2C (30%). However, in the LUV-binding assays Tcb1-C2C and Tcb3-C2C both displayed similar calcium-response curves (Figure 3), while tricalbin 2 failed to respond to changing calcium concentrations. This may be accounted for by the fact that tricalbin 2 C2C retains only three of the five conserved putative calcium-coordinating residues (Figure 1A, Table 1), reducing the ability of Tcb2-C2C to bind calcium ions. The overall sequence identity between Tcb1-C2C and Tcb2-C2C may explain why both domains were equally capable of binding 40% PS vesicles in the absence of calcium. That is, Tcb1-C2C and Tcb2-C2C may share certain residues at or near the membrane-binding surface that confer some preference for acidic phospholipid even in the absence of calcium. Tcb3-C2C may not share these residues, resulting in its inability to bind PS in the absence of calcium.

The preference for acidic phospholipids (especially PS) suits the hypothetical role of the tricalbins in membrane trafficking at the plasma membrane or sorting bodies near the vacuole (*10*) or both. In yeast, PS is particularly abundant in the plasma membrane, comprising around 30% of the lipid found there (*20*), and in secretory vesicles, where it comprises approximately 20% (*21*). Like synaptotagmin I C2A (Figure 2D), the C2C domains of tricalbins 1 and 3 are capable of binding to phosphoinositide derivatives such as PI(3)P, PI(4)P, PI(5)P, and PI(3,5)P2 (Figure 2A,C), which suggests a possible role in PI-derived signaling pathways. PI(3,5)P2 is particularly interesting, because it has been implicated in protein sorting in the yeast multivesicular body (*22*) and vacuolar membrane sorting and recycling (*23*). Deletion of Fab1p, the yeast PI(3)P 5-kinase, results in enlarged, improperly acidified vacuoles (*23*), a phenotype similar to that observed with tricalbin mutants (*7*, *10*).

According to the LUV sedimentation assays, the C2Cs of tricalbins 1 and 3 responded to lower concentrations of calcium than synaptotagmin I C2A (Figure 3); SytI-C2A required greater than 10-fold higher calcium concentrations to achieve 50% lipid binding, compared to Tcb1-C2C and Tcb3-C2C. The level of sensitivity of the tricalbins appears comparable to that of synaptotagmins III, V, VII, and X, which also appear to have a 10- to 20-fold greater affinity for calcium than synaptotagmin I (*36*). Interestingly, these more sensitive synaptotagmins are localized to the plasma membrane, as the tricalbins appear to be in yeast (*10*), while synaptotagmin I is found in synaptic vesicles.

Budding yeast are capable of controlling calcium concentrations through the mobilization of intracellular stores (such as the vacuolar lumen, through a transient receptor potential channel, TRPC) (*24*) as well as influx of extracellular calcium through a voltage-gated calcium channel (VGCC) in the plasma membrane (*25*). Fluorescence localization studies using inducible GFP-tagged tricalbin 2 indicate that upon expression tricalbin 2 can at first be found at the plasma membrane, later moving to numerous vesicular bodies found near the vacuole (*10*). Assuming tricalbins 1 and 3 colocalize with 2 and follow a similar path, they could conceivably be subjected to calcium released into the cytoplasm by both the plasma membrane VGCC and the vacuolar TRPC. The PM VGCC seems to increase cytosolic calcium levels to around 3 μM (*26*), which falls within the range of response of the tricalbins; however, this concentration can be highly variant depending on extracellular levels of calcium. Furthermore, the PM VGCC may only act transiently to correct internal levels of calcium rather than to initiate a calcium signal (*25*, *27*). The amount of calcium released through the vacuolar TRPC is thought to be greater due to the high concentration of calcium sequestered there (approximately 1.3 mM (*28*)). Also, the vacuolar TRPC acts in response to hyperosmotic stress (*29*), which may be relevant to the apparent effect of the tricalbins on vacuole morphology (*7*, *10*), as well as the apparent ability of Tcb1-C2C and Tcb3-C2C to bind PI(3,5)-P2.

Tricalbin 2 is anomalous among the tricalbins in that all three of its C2 domains failed to bind lipid to a greater extent in response to increasing Ca^{2+} concentration. Each C2 domain lacks two of the conserved aspartates thought to participate in calcium binding, though interestingly, the missing residues differ for each C2 domain (Table 1). C2A is missing the first and third residues, C2B lacks the second and third, while C2C lacks the first and fifth. The C2C domains of tricalbin 1 and 3, on the other hand, respond to calcium with similar sensitivity; this may partially explain

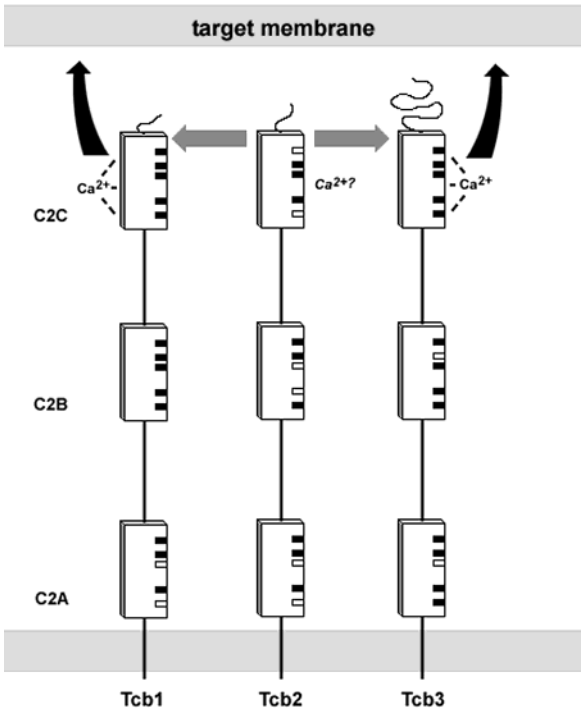

FIGURE 8: Proposed model of action of the heteromeric tricalbin complex. Tricalbin 2 (center) is able to bind tricalbin 1 (left) and tricalbin 3 (right) through its C-terminal domains (gray, straight arrows). The C2C domains of Tcb1 and Tcb3 are capable of binding to acidic phospholipid in response to calcium (black, curved arrows) through coordination of calcium ions. In this way, the entire tricalbin complex (including Tcb2 and any additional protein factors) is recruited to the target membrane in response to calcium signaling. Black rectangles within each C2 domain represent the presence of conserved aspartate residues that are thought to coordinate calcium ions; the open, or white, rectangles indicate where such residues are missing (see also Table 1).

their apparent redundancy. If tricalbins 1 and 3 are indeed calcium sensors in a membrane trafficking process, what is the purpose of tricalbin 2, which is apparently essential but does not respond to calcium? The earlier two-hybrid and mutational studies indicated that tricalbin 2 is capable of associating with tricalbins 1 and 3 (*10*). It is possible, then, that the function of Tcb2 lies in its ability to assemble the various components of the putative "tricalbin complex" (which may also include Pdr1p and other as yet unidentified proteins). Figure 8 depicts our model of how the tricalbins are organized: tricalbin 2 heterodimerizes with tricalbin 1 or tricalbin 3 (or forms multimeric complexes containing both), while tricalbins 1 and 3 supply the ability to bind the entire complex to the target membrane in response to calcium.

Another clue to the possible role of tricalbin 2 may be found by analogy to the synaptotagmin family. It is known that some synaptotagmins, such as I, II, and IV, can colocalize to the same membrane and are capable of heterodimerization (*30*, *32*). Synaptotagmin IV lacks a single aspartate in its C2A domain, which apparently abolishes the ability of SytIV to bind vesicles in a calcium-dependent fashion (*31*). Like SytII, it can form heterodimers with SytI, but this association prevents SytI from binding lipid in response to calcium (*32*). Tricalbin 2, which also seems to lack calcium sensitivity, may function in a similar fashion by binding to and negatively regulating the tricalbins, preventing them from docking with membranes until necessary. However, this seems unlikely given that the deletion studies produced identical, rather than opposing, effects for Δ*tcb2* and Δ*tcb1*/Δ*tcb3* mutants (*10*), indicating that the tricalbins work cooperatively. It is also worth noting that there are other synaptotagmin C2 domains that lack some (or even all) of the conserved calcium-binding aspartates and appear to be calcium-insensitive: SytVIII (*33*), SytIX (*34*), and SytXIV (*9*) are confirmed to be insensitive to calcium. Synaptotagmins XII and XIII are also predicted to be calcium-insensitive based on their shortage of calcium-binding residues. It has not yet been determined whether these synaptotagmins act as negative regulators as SytIV does or have some other purpose that may provide a clue toward the function of tricalbin 2. But as with the results of the present study, it would seem to demonstrate that the diversity of function to be found in C2 domains in general belies the notion that they are primarily calcium- and lipid-binding domains.

Although the C2A and C2B domains of tricalbins 1 and 3 were not studied here, it is interesting to note that in contrast to the C2C domains, not all of them retain all five of the calcium-binding aspartates. Tcb1-C2A only possesses three, while Tcb3-C2A and Tcb3-C2B have four (Tcb1-C2B appears to retain all five, assuming that the glutamates in two of the positions can adequately substitute for aspartates) (see Table 1 and Figure 8). Assuming that what was observed in Tcb2 holds up for these domains, they may not display calcium-sensitive binding to lipid. A similar situation has been observed in myoferlin, a human protein with six C2 domains and a C-terminal transmembrane domain (*3*). Only the N-terminal C2 domain (C2A)—like the tricalbin C2C domain, the one furthest from the transmembrane domain—was able to bind 50% PS vesicles in a calcium-dependent manner; the rest failed to bind membrane at all. This may be a consequence of the extremely large size of myoferlin (and by extension, the tricalbins): depending on the size and complexity of the structure, the membrane-binding face may be occluded in some way, preventing contact with the membrane. Thus lacking the evolutionary pressure to retain membrane binding, these domains may have lost that capability over time and now serve primarily to keep the "active" C2 domains properly positioned.

This study made use of vesicle sedimentation and tryptophan fluorescence assays to demonstrate that the C2C domains of tricalbins 1 and 3 bind acidic phospholipid in a calcium-sensitive fashion. They are capable of responding to much lower calcium concentrations than even synaptotagmin I C2A: concentrations that are physiologically relevant in yeast. This strongly supports the proposed role for tricalbins in calcium-dependent membrane trafficking processes.

While the tricalbins have demonstrated numerous properties that may be relevant to multiple-C2 domain structure and organization in higher organisms, little is still known of their precise function. Future work with these proteins may wish to address whether the remaining C2 domains in tricalbins 1 and 3 can bind calcium or lipid or both and whether these other C2 domains can recruit other proteins to the predicted "tricalbin complex".

The many interesting parallels between tricalbins and members of the synaptotagmin family suggest that the tricalbins may be a suitable simplified model system with

which one can identify functions of the much larger synaptotagmin family. Comparisons to the plasma-membrane-bound synaptotagmins (especially III and VII) seem particularly apt, and because the functions of these synaptotagmins are not yet clear, yeast studies may provide some valuable insights.

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