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Membrane Docking of the Synaptotagmin 7 C2A Domain: Electron Paramagnetic Resonance Measurements Show Contributions from Two Membrane Binding Loops

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Abstract

The synaptotagmin (Syt) family of proteins plays an important role in vesicle docking and fusion during Ca^{2+} -induced exocytosis in a wide variety of cell types. Its role as a Ca^{2+} sensor derives primarily from its two C2 domains, C2A and C2B, which insert into anionic lipid membranes upon binding Ca^{2+} . Syt isoforms 1 and 7 differ significantly in their Ca^{2+} sensitivity; the C2A domain from Syt7 binds Ca^{2+} and membranes much more tightly than the C2A domain from Syt1, due at least in part to greater contributions from the hydrophobic effect. While the structure and membrane activity of Syt1 have been extensively studied, the structural origins of differences between Syt7 and Syt1 are unknown. The present study used site-directed spin labeling and electron paramagnetic resonance spectroscopy to determine depth parameters for the Syt7 C2A domain, for comparison to analogous previous measurements with Syt1 C2A. In a novel approach, the membrane docking geometry of both Syt1 and Syt7 C2A was modeled by mapping depth parameters onto multiple molecular dynamics simulated structures of the Ca^{2+} -bound protein. The models reveal membrane penetration of Ca^{2+} binding loops (CBLs) 1 and 3, and membrane binding is more sensitive to mutations in CBL3. On average, Syt7 C2A inserts more deeply in the membrane than Syt1 C2A, although depths vary among the different structural models. This observation provides a partial structural explanation for the hydrophobically driven membrane docking of Syt7 C2A.

Keywords

protein-lipid interaction; membrane bound structure; calcium signaling; exocytosis; power saturation

Synaptotagmins (Syt) are a family of proteins characterized by their membrane targeting C2 domains. Syt proteins contain a transmembrane helix which anchors a cytoplasmic region consisting of two C2 domains (C2A and C2B) connected by a short linker.^{1, 2} Each C2 domain is composed of two four-stranded β -sheets forming a β -sandwich structure with three flexible Ca^{2+} binding loops, although not all Syt C2 domains bind Ca^{2+} .^{3, 4} There are 17 human isoforms of Syt, eight of which show varying degrees of Ca^{2+} binding affinity.^{5–7}

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The individual C2 domains of each Syt exhibit a variety of affinities; for example, Syt1 C2A binds anionic lipids such as phosphatidylserine (PS) nonspecifically in the presence of Ca^{2+} , while Syt1 C2B additionally binds phosphatidylinositol 4,5-bisphosphate (PIP_2) in a partially Ca^{2+} -independent fashion.^{8–12} Syt1 C2B may additionally interact with the soluble N-ethylmaleimide-sensitive fusion protein attachment receptor (SNARE) protein SNAP25 in a manner proposed to inhibit vesicle fusion prior to Ca^{2+} influx.^{13–15} Influx of Ca^{2+} into the cytoplasm releases the fusion clamp and triggers membrane insertion by the two Syt C2 domains, thereby accelerating vesicle fusion.^{1, 6, 14} The mechanism by which this happens is unknown but believed to rely on the membrane insertion and bending activity of Syt; mutations that disrupt C2 domain membrane interactions also decrease exocytosis.^{16–19} Thus, a better understanding of the structural mechanism of Syt C2 domain membrane binding is central to a fuller description of exocytosis.

Much of what is currently known about the structure of membrane-bound Syt comes from studies using electron paramagnetic resonance (EPR) spectroscopy. EPR detects the relaxation of unpaired electrons excited by incident microwaves within a magnetic field of 8–10 GHz. Incorporation of unpaired electrons into a protein of interest involves an established approach known as site-directed spin labeling,²⁰ in which unique cysteine residues are introduced at locations of interest on the target protein through site-directed mutagenesis. A nitroxide spin label such as MTSSL is then attached through formation of a disulfide bond to the engineered cysteine. EPR spectra are useful for observing local environmental interactions with the unpaired nitroxide electron, and an application termed continuous wave power saturation is capable of measuring protein-membrane docking geometry.^{21, 22} Power saturation relies on the accessibility of a spin label to various paramagnetic probes. EPR signal amplitude increases linearly with the square root of incident power up to a saturation point, which is dependent on the relaxation rate of the spin label. Heisenberg spin exchange between the spin label and extrinsic paramagnetic probes increases the relaxation rate; thus, power saturation measurements reveal the relative accessibility of the spin label to each paramagnetic probe. Paramagnetic probes with differing concentration gradients in a membrane such as O_2 (predominantly in the membrane interior) and nickel ethylenediamine diacetic acid (NiEDDA, excluded from the membrane) allow for the calculation of membrane depth parameters (Φ). The depth parameters from a library of spin-labeled protein variants can be mapped onto a known structure of the protein in order to calculate an optimized membrane docking geometry to fit the experimental results.²¹

To date the most extensively studied member of the Syt family is Syt1, which acts as a Ca^{2+} sensor for the fast synchronous release of synaptic vesicles in neuronal cells. This is the fastest known membrane fusion event, occurring in the μs to ms timescale.^{1, 23, 24} Previous EPR power saturation studies have revealed the membrane docking geometries of Syt1 C2A and C2B, both individually and in the context of the C2AB tandem.^{25–27} In agreement with qualitative studies based on fluorescence changes,^{28, 29} these EPR measurements demonstrated that both Syt1 C2A and C2B insert more deeply into membranes in the context of the C2AB tandem.³⁰ The precise mechanism by which C2A-C2B copenetration occurs is unknown. Interestingly, EPR experiments utilizing double electron-electron resonance (DEER) distance measurements of Syt1 C2AB fragments have yielded models

requiring the CBLs of C2A and C2B to be oriented in opposite directions when membrane bound, consistent with this protein's observed ability to induce negative curvature in membranes.³¹ By contrast, the isolated Syt1 C2A domain binds membranes via a combination of electrostatic and hydrophobic interactions, dominated by electrostatic contacts within the polar headgroup region.^{25, 32, 33} Its docking geometry shows membrane penetration by CBLs 1 and 3, with CBL1 located entirely within the headgroup region and CBL3 inserting somewhat more deeply.²⁵ Each of these loops contains a single hydrophobic residue at its apex: M173 on loop 1 and F234 on loop 3 of Syt1. Intriguingly, while F234 is conserved among all stimulatory Syt isoforms, M173 is somewhat variable, for example corresponding to Phe in Syt7 and Ser in Syt3.^{6, 7} It is not yet known whether the membrane binding properties of Syt1 are common to other Syt isoforms.

Presumably, the variability among Syt isoforms is indicative of the various and complex functions this family of proteins has evolved to fill.³⁴ Syt7, although not as well studied as Syt1, performs many important functions. In particular, Syt7 is involved in the regulation of insulin secretion in pancreatic β -cells. Dysregulation of insulin secretion has been implicated in the development of type 2 diabetes and in part efforts to further understand the underlying factors of this disease has lead to research into the structure and function of this protein.^{35, 36} Similar to Syt1 C2A, Syt7 C2A binds nonspecifically to anionic lipid head groups through electrostatic interactions with no particular affinity for PIP₂ beyond simple electrostatic interactions.³⁷ The Syt7 C2A domain, however, is sensitive to much lower concentrations of Ca²⁺ and also exhibits a 60-fold slower membrane dissociation rate compared to Syt1 C2A.^{37, 38} We have previously shown that the slower off rate of Syt7 C2A can be attributed in part to a stronger hydrophobic component of the Syt7 C2A docking mechanism, and we hypothesized that this is mainly due to deeper membrane penetration by F167 on CBL1.³⁷

Here, we test our previous hypothesis by measuring the docking geometry of the Syt7 C2A domain using methods analogous to those previously used for Syt1 C2A. We report depth parameters for 13 separate spin-labeled mutants, including key locations in CBLs 1 and 3. These data are interpreted by modeling docking geometry based on these depth parameters and five protein structures: three from a molecular dynamics simulation of Ca²⁺-bound Syt7 C2A, and two for comparison from a publically accessible Ca²⁺-free solution NMR structure. The results suggest a somewhat deeper membrane penetration for the isolated Syt7 C2A compared to Syt1 C2A, anchored primarily via CBL3 and secondarily by CBL1. The companion paper describes molecular dynamics simulations of Syt7 C2A binding to a lipid bilayer, revealing a balance between hydrophobic and electrostatic effects.

Experimental Methods

Materials

All materials were reagent grade unless otherwise specified. Synthetic lipids 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (phosphatidylcholine, POPC, PC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine (phosphatidylserine, POPS, PS) were obtained from Avanti Polar Lipids (Alabaster, AL) in chloroform. The spin label 1-oxyl-2,2,5,5-tetramethyl-3-pyrroline-3-methylmethanethiosulfonate (MTSSL, R1) was from Toronto Research Chemicals. Fluorescein-5-maleimide was from AnaSpec (Fremont, CA). Doxyl

lipids 1-palmitoyl-2-stearoyl-(12-doxyl)-*sn*-glycero-3-phosphocholine (12-doxyl PC), 1-palmitoyl-2-stearoyl-(10-doxyl)-*sn*-glycero-3-phosphocholine (10-doxyl PC), 1-palmitoyl-2-stearoyl-(7-doxyl)-*sn*-glycero-3-phosphocholine (7-doxyl PC), and 1-palmitoyl-2-stearoyl-(5-doxyl)-*sn*-glycero-3-phosphocholine (5-doxyl PC) were from Avanti Polar Lipids. *N*-[5-(dimethylamino)naphthalene-1-sulfonyl]-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (dansyl-PE, dPE) was from Life Technologies.

Protein Mutagenesis, Expression, Spin Labeling, and Purification

The initial goal for protein mutagenesis was to create a cysteine-free (cysless) variant of Syt7 C2A, so that unique cysteine residues could be introduced at desired positions. A GST-fused C2A expression construct encompassing residues N135-S266 was cloned from cDNA (ATCC: 11045721) as previously described.³⁷ Mutations were generated using the QuikChange II XL (Agilent) site directed mutagenesis kit using the manufacturer's protocol. The cysless mutant of Syt7 C2A, C260S, was used as the parent DNA for developing a library of single cysteine mutants. A total of 17 single cysteine mutants were created at the positions listed in Table 1. All mutations were verified by primer-extension sequencing of the full C2A coding sequence.

Each single mutant was expressed as a fusion protein with a GST tag and purified via a glutathione affinity resin (GE Healthcare) as previously described, including elution with assay buffer (140 mM KCl, 25 mM HEPES, 15 mM NaCl, 0.5 mM MgCl₂, pH 7.4) following thrombin cleavage between the GST and C2A domains.³⁷ Labeling with the methanethiosulfonate spin label (MTSSL) was achieved by incubating protein with MTSSL either 1 hr at 4 °C while the GST-C2A fusion protein was bound to the resin, or overnight at 4 °C following thrombin cleavage and elution, with approximately equivalent labeling efficiencies of >75% obtained in either case (Figure S1). Protein purity was verified by SDS-PAGE and ultraviolet absorbance spectroscopy. Due to the positive charge on Syt 7 C2A, contamination with nucleic acids is sometimes observed and can interfere with protein-lipid binding.³⁹ Thus, high-salt washes were included in the column purification protocol. We note that this removal was more critical for kinetic measurements of protein-membrane association than for EPR measurements. Only samples with an OD₂₆₀/OD₂₈₀ ratio of ~1.0, indicating only minor levels of contamination, were determined to be acceptable. Any purified proteins showing a ratio greater than 1.0 were further treated with benzonase (Sigma) for 1 h at 25 °C and a new spectrum recorded to confirm the removal of nucleic acid. EPR measurements are less sensitive to nucleic acid contamination due to the long time period over which measurements are taken, allowing proteins to be in equilibrium with the large excess of lipid vesicles. Concentrations were calculated from OD₂₈₀ measurements using an extinction coefficient of 14280 M⁻¹ cm⁻¹.

Preparation of Lipid Vesicles

Lipids in chloroform were mixed in the appropriate molar ratio, dried under N₂, and then placed under vacuum for 2 h in order to remove all traces of solvent. Dried lipids were then hydrated with assay buffer. Small unilamellar vesicles (SUV) were then generated through probe sonication, stored at 4 °C for no more than 7 days, and allowed to equilibrate overnight at 25 °C prior to EPR measurement. Vesicle stock solutions for EPR were

prepared with a molar ratio of 75:25 POPC:POPS and a total lipid concentration of 80 mM; vesicle stocks for kinetic measurements consisted of 75:20:5 POPC:POPS:dansyl-PE in order to maintain 25% anionic content, and a total lipid concentration of 3 mM.

Stopped Flow Kinetic Measurements

Membrane association and dissociation rates were measured using a BioLogic SFM-3000 stopped-flow fluorescence spectrometer essentially as outlined previously.³⁷ Briefly, protein-to-membrane fluorescence resonance energy transfer (FRET) was measured between Trp residues in the C2 domain and dansyl-PE lipids included in vesicles. Association measurements were performed by rapidly mixing protein and vesicles in the presence of 200 μ M CaCl_2 , and dissociation measurements were performed following rapid addition of 1 mM EDTA to pre-formed protein-lipid complexes. Protein-to-accessible lipid ratios were maintained at 1:100 for kinetic measurements, including 1 μ M protein and 100 μ M total accessible lipid in all association measurements (all concentrations after mixing). Observed association rates were fit to equations of the form

$$F = \Delta F_{\max} (1 - e^{-k_{\text{obs}} t}) + C, \quad (1)$$

where k_{obs} is the apparent association rate constant, $F_{\max}$ is the amplitude of the fluorescence change, and C is an offset. Dissociation profiles were subjected to nonlinear least-squares fitting to a single- or double-exponential function (Eq 2 or 3, respectively):

$$F = \Delta F_{\max} (e^{-k_{\text{off}} t}) + C \quad (2)$$

$$F = \Delta F_{\max 1} (e^{-k_{\text{off}1} t}) + \Delta F_{\max 2} (e^{-k_{\text{off}2} t}) + C \quad (3)$$

where the k_{off} are dissociation rate constants and C is an offset. Except where otherwise noted, dissociation profiles for mutants were single-exponential.

Measuring EPR Spectra

An EPR spectrum was recorded for each single cysteine variant using a Bruker ELEXSYS E500 spectrometer (9.4 GHz) with a loop gap resonator (Medical Advances). Each sample consisted of 30–150 μ M MTSSL-labeled Syt7 C2A, 1.5–2.0 mM CaCl_2 , and either no lipids present for unbound spectra or 3:1 POPC:POPS lipids for bound spectra. Although this results in Ca^{2+} concentrations higher than physiological (typically ~10–500 μ M, depending on cell type and stimulus), the C2A domain binds up to 3 Ca^{2+} ions and so at least a 3-fold molar excess is required in order to saturate membrane binding.⁴⁰ A high total lipid concentration of 30 mM (15 mM accessible), similar to previous EPR studies,^{25, 41, 42} was used in order to minimize the possibility of spin-spin interactions between the MTSSL tags of bound Syt7 C2A domains. This corresponds to a protein:accessible lipid ratio of between 1:100 and 1:500. Measurements were performed at 2.0 mW incident power with a minimum of 5 scans of 100 G. EPR spectra were normalized to their second integral representing the total number of spins. This technique has proven effective in previous studies for comparing

spectra of varying spin label concentrations particularly when comparing bound and unbound states.^{41, 42}

Continuous Wave Power Saturation Measurements

Each sample was loaded into a gas-permeable TPX capillary tube (Medical Advances). Power saturation curves were measured between 0.2 mW and 50 mW taking saturation measurements at predefined intervals, over 30 G with at least 2 scans per interval. Power saturation curves for each sample were measured under three separate conditions: (1) atmospheric oxygen (20%), (2) after equilibration under N₂ for 15 min, and (3) after addition of 10 mM NiEDDA and equilibration under N₂ for 15 min. All three accessibility measurements for a single protein were taken on the same day in order to minimize slight variations in sample preparation instrument (resonator) performance. The measurements include n-doxyl PC standards measured under the same conditions (temperature, buffer, and [Ni²⁺]) as the protein samples. Amplitudes were plotted as a function of microwave power squared and fit using *Kaleidagraph* to the equation:^{21, 22}

$$A = C P^{0.5} \left[1 + \frac{(2^{1/\varepsilon} - 1) P^{-\varepsilon}}{P_{1/2}} \right], \quad (4)$$

where A represents the peak-to-peak amplitude of the signal, C is a scaling factor, P is the microwave power, $P_{1/2}$ is the power at which half saturation occurs, and ε is the measure of homogeneity of saturation. Accessibility parameters were then calculated as follows:^{21, 22}

$$P(X) = \left[\frac{P_{1/2}(X)}{\Delta H_{pp}(X)} \right] - \left[\frac{P_{1/2}(N_2)}{\Delta H_{pp}(N_2)} \right], \quad (5)$$

where X represents either O₂ or NiEDDA paramagnetic species, $\Pi(X)$ represents the accessibility parameter for the given species, and H_{pp} is the average peak to peak line width over the linear region of the power saturation curve. Depth parameters were then calculated from the following equation:

$$\Phi = \ln \left[\frac{P(O_2)}{P(NiEDDA)} \right], \quad (6)$$

where Φ is the depth parameter. Errors on each Π and Φ measurement were calculated from the standard errors of $P_{1/2}$ and H_{pp} values. The depth parameter was measured at least twice for each mutant and three times if the first two measurements differed by more than 0.4 in value. Final values reported are weighted averages and 95% confidence interval (CI) among repeat measurements.

Modeling Docking Geometry

The experimental membrane docking geometry for Syt7 C2A was determined by first modeling the locations of MTSSL spin labels and subsequently fitting to obtain a best-fit docking geometry based on the measured depth parameters for each spin labeled mutant. Allowed configurations of MTSSL sidechains were modeled using the MTSSL Wizard plugin for PyMol (Schrödinger).⁴³ Initial estimated coordinates for each nitroxide nitrogen

(on which the free electron resides)⁴⁴ were determined by averaging the coordinates among all allowed conformations of the MTSSL tag. For the 2–5 positions at which no conformers were found using the “tight” settings of MTSSL Wizard, individual side-chain dihedral angles were defined manually to yield conformations with no structural clashes. These coordinates were then used for fitting depth parameters to a hyperbolic tangent function:

$$\Phi = A \tanh[B(d_m - C)] + D \quad (7)$$

where A and D represent the bulk values of Φ in water and hydrocarbon, C sets the inflection point of the curve, B determines the slope and, d_m represents the distance of each MTSSL spin label from the phosphate plane. As in previous studies, fits were constrained such that $(D - A) = 4.5$. For doxyl lipid standards, d_m is the published value,⁴⁵ and for MTSSL labels, d_m is given by:

$$d_m = x \sin(\theta_z) + y \cos(\theta_x) - z \cos(\theta_z) \sin(\theta_x) + y_{trans} \quad (8)$$

where (x, y, z) are the starting coordinates of the MTSSL nitrogen for each sidechain. Fitting of depth parameter data to Eqs. (7) and (8) yields an optimized docking geometry of the protein to a lipid bilayer in the xz plane, where $y = 0$ is the average phosphate position of the proximal leaflet. This optimized docking geometry can be obtained by sequentially (1) rotating the coordinates around the x axis by the best-fit angle θ_x , (2) rotating the coordinates around the z axis by the best-fit angle θ_z and (3) translating along the y axis by the best-fit distance y_{trans} . After a first round of fitting using the average MTSSL coordinates, any residues that were outliers to the hyperbolic tangent fit (more than $2 \times 95\%$ CI from the predicted Φ value) were then sequentially adjusted to a defined sidechain conformation with dihedral angles (χ) that were both (1) sterically allowed for the position and (2) low-energy based on a previous study of MTSSL conformations in solution.⁴⁶ Such adjustment was performed beginning with the farthest outliers and repeated until any further changes provided negligible improvement to the fit. The positions whose rotamer conformations were adjusted during the iterative fitting process were as follows: for Structure A, G169, N195, and L196; for Structure B, F167 and G169; for Structure C, F167 and G169; for NMR state 1, F167, G169, and L196; and for NMR state 2, G169 and S168. The dihedral angles used for each final model are given in Table S1; for the remaining majority of positions, the average coordinates from MTSSL Wizard were used. Five models were created using this method: three based on starting protein structures taken from a molecular dynamics simulation of Ca^{2+} -bound Syt7 C2A in the absence of membrane (snapshots at 6 ns, 8 ns, and 10 ns of a simulation described in the accompanying paper), and two based on starting Ca^{2+} -free structures from the protein data bank (2D8K, states 1 and 2). Each final self-consistent model is constrained by equations 7 and 8, experimental depth parameters, doxyl lipid calibration data, and the corresponding structure of Syt7 C2A.

Reanalysis of published Syt1 C2A EPR data was carried out analogously, starting with states 1–3 of structure 1BYN⁴⁷ as well as three snapshots of a molecular dynamics simulation described in the companion paper; any manually adjusted dihedral angles are listed in Table S2.

Results

Site selection, protein purification, and MTSSL labeling

The process of protein site directed spin labeling introduces an unpaired electron through the MTSSL nitroxide spin label (Figure 1). The spin label attaches through a disulfide bond to any accessible cysteine; therefore, native cysteine residues must first be removed in order to selectively label cysteine residues at the desired locations of interest. The only native cysteine in our wild-type expression construct was Cys260 near the C-terminus; this residue occurs at the beginning of the flexible C2A-C2B linker region in native human Syt7. A cysless (C260S) variant of Syt7 C2A was thus generated as a template for the creation of single cysteine mutants. The positions selected for site directed spin labeling in Syt7 C2A are analogous to positions used in a previous EPR power saturation study with Syt1 C2A.²⁵ This strategy allows for direct comparisons between Syt7 and Syt1. Spin labeled positions are summarized in Table 1 covering 16 sites on or near the Ca²⁺ binding loops 1 and 3 of Syt7 C2A, with one negative control (Q148R1) opposite the binding site.

WT, cysless, and single cysteine mutants were purified through glutathione affinity chromatography of a GST-linked protein followed by cleavage of the GST with thrombin. Nucleic acid contamination was removed to a level deemed acceptable for stopped flow kinetic measurements, and more than sufficient for equilibrium EPR measurements (see Methods). MTSSL spin labels were attached either during or after purification, with a labeling efficiency of ~75% in either case (Figure S1).

Effects of Spin Labeling on Syt7 C2A Docking Function

The methanethiosulfonate R1 spin label is a moderate polarity group that has minimal effect on membrane protein docking structure as shown in previous EPR studies.^{41, 48} Here, we used an established protein-to-membrane FRET assay to examine the kinetics of membrane binding and dissociation of each spin-labeled mutant, for comparison to the wild type and cysless Syt7 C2A. Spin-labeled mutants that produced association and dissociation rate constants within a factor of 2.7 relative to the C260S (cysless) mutant (equivalent to molar activation energies within the range $\pm RT$) were considered to have negligible effects on docking and were used for EPR studies. Summarized in Table 1 are the off and on rates for wild type, cysless, and each single-cysteine mutant.

The wild-type Syt7 C2A domain produced an observed association rate constant of 27 s⁻¹ and an EDTA-induced dissociation rate constant of 26 s⁻¹, using the liposomes here composed of 75% POPC, 20% POPS, and 5% dansyl-PE. This is a significantly slower association and somewhat faster dissociation than we reported previously for this protein domain and a more complex physiological lipid mixture.³⁷ These differences could arise from additional favorable interactions with other components of the physiological lipid mixture and are currently under investigation. However, we have also observed significant variability even with nominally identical lipid compositions, presumably due to batch differences in lipid stock concentration or homogeneity. The origins of this variability are currently under investigation. For the present study, all comparisons of on rates or off rates are between measurements made using the same preparation of liposomes. Cysless (C260S)

exhibited a ~30% slower dissociation and ~2-fold faster association relative to wild-type. Viewed in the context of activation energies, 2-fold differences in rate constants are minor; moreover, C260 resides in a flexible C-terminal tail of the protein construct used for these experiments (and in the C2A-C2B linker of the full-length protein), so the difference in on-rate is unlikely to represent a fundamental change in the C2A domain.

Nine of the mutants agreed within 2-fold of both the off and on rates for cysless. Four variants (G169R1, L196R1, L224R1, and Q148R1) had either on or off rates that were between 2- and 2.7-fold faster or slower than cysless. Two mutations, Y226R1 and A164R1, slowed association kinetics by > 2.7-fold; the reason for the differences is unknown. F229R1 showed normal association kinetics but dissociated > 2.7-fold faster than cysless, suggesting that this residue on the apex of CBL3 is essential for membrane penetration. One mutant (I235R1) had no observable kinetics, which could be due to the interior location of this residue causing the mutant protein to misfold; we note the purification yields of this mutant were also extremely low. Thus, these four mutations (F229R1, A164R1, Y226R1, and I235R1) were considered perturbing to the normal function of the Syt7 C2A protein and were excluded from further study. R228R1 and R231R1 were observed to have biphasic association profiles; however, in both of these cases the faster component was within 2-fold of wild-type and had a >3-fold greater amplitude than the slow component. The origin of the slow, low-amplitude FRET increase upon binding is unclear but could arise from either (a) a small population that binds more slowly due to alternative folding or nucleic acid contamination, which would not impact EPR measurements significantly due to the high lipid concentrations and much longer measurement timescale (minutes – hours), or (b) slow vesicle aggregation, which would not affect EPR measurements due to the much smaller protein-to-lipid ratio used in EPR samples.

EPR lineshape analysis

As a qualitative measure of changes in the local environment around a spin label during membrane docking, continuous wave EPR spectra were measured for the 13 spin-labeled mutants included in the docking model. Both bound and unbound spectra were measured for each mutant and are shown in Figure 2. As a reference, doxyl lipid EPR spectra are displayed in Figure S2. Bound protein spectra included 3:1 POPC:POPS liposomes with 30 mM total lipid concentration and excess Ca^{2+} , conditions that allow for essentially complete membrane docking of Syt7 C2A. Unbound spectra were measured prior to addition of lipid but were otherwise equivalent to the corresponding bound sample.

The spin-labeled mutants of positions in CBL1 and CBL3 display the greatest degree of signal broadening in the bound state compared to the unbound state: F167R1, S168R1, G169R1, R228R1, and R231R1 (Figure 2). Signal broadening generally indicates decreased mobility of the MTSSL tag, likely due in these cases to contact with lipids as the CBLs penetrate the membrane. The remainder of the spin-labeled mutants showed varying degrees of signal broadening upon lipid addition, indicating lipid contact and/or loss of protein rotational freedom upon docking to the vesicles.

Depth Parameters of Syt7 C2A

EPR power saturation was performed in order to measure the depth parameters for each of the 13 spin labeled positions on Syt7 C2A as well as four doxyl-PC lipids with spin labeled acyl chains at the 5, 7, 10, and 12 carbons. Doxyl-PC lipids have a known depth within the lipid membrane and are useful in calibrating the hyperbolic tangent fit used to translate depth parameters into physical depths.⁴⁵ Table 2 summarizes the depth parameters along with the O₂ and NiEDDA accessibility parameters used to calculate the depth parameters. Positive depth parameters are indicative of more deeply penetrated spin labels, while depth parameters < -1 correspond to positions in the bulk aqueous phase.

Of the 13 Syt7 C2A mutants, 7 showed depth parameters < -1 (L136R1, Q148R1, T170R1, N195R1, L196R1, L224R1, and P234R1), indicating aqueous exposed locations with little to no membrane contact. Three of the positions (F167R1, S168R1, and R228R1) had positive depth parameters, indicating membrane penetration potentially deeper than the phosphate plane of the lipid membrane. Three positions (G169R1, R231R1, and N232R1) had depth parameters between -1 and 0 suggesting close proximity to the phosphate plane. Measured depth parameters for each doxyl lipid were in good agreement with previous literature values, although these values can vary somewhat among spectrometers.⁴⁵

Several of the Syt7 C2A depth parameters are considerably deeper than those reported previously for analogous positions in Syt1. For example, F167, G169, R228, and N232 are all positions on CBL1 or CBL3 that have depth parameters in Syt7 C2A > 1.0 more positive than the corresponding positions in Syt1 C2A (Table 3).²⁵ By comparison, depth parameters at non-CBL positions were greater in Syt7 C2A by a smaller magnitude of 0.4 ± 0.2 and doxyl-PC depth parameters were greater in our study by only 0.2 ± 0.2 . Together, these data suggest that the CBLs of Syt7 C2A penetrate into membranes more deeply than those of Syt1 C2A, although determination of the docking geometry requires fitting these measured depth parameters onto a structural model.

Modeled docking geometry of Syt7 C2A

In order to translate depth parameters into physical distances from the membrane phosphate plane, a hyperbolic tangent model is used to determine a best-fit geometry based on the relationship between depth parameters and structure (Figure 3). The measured depth parameters were used to create a suite of structural models of the Syt7 C2A membrane docking geometry. Earlier EPR docking geometry studies of C2 domains have modeled geometries based on single static protein structures, and single conformations of the MTSSL side chain at each position. Both of these assumptions likely underrepresent the dynamics of membrane binding and produce relatively crude estimates of uncertainty in the final geometric model. In the case of Syt7 C2A, the available structure is an NMR ensemble of states from measurements performed in the absence of Ca²⁺. Many of these states possess CBL structures that are not relevant to the Ca²⁺-bound protein structure. Thus, we first performed molecular dynamics simulations of Syt7 C2A bound to 3 Ca²⁺ ions (in the absence of membrane), details of which are presented in the companion paper. Three representative snapshots from this simulation, which we term Structures A, B, and C, were used as bases for fitting depth parameter data, and the depths reported in Tables 3–4 are

averages among these three fits. For comparison, States 1 and 2 from the available Ca^{2+} -free NMR structure (termed NMR1 and NMR2) were also used as bases for fitting; depths from all five fits are reported in Table S3. The results provide five self-consistent docking geometries, each modeled using the same set of depth parameter data but with a different starting protein structure.

In each case, modeling consisted of three steps as in previous studies: (1) estimation of coordinates for the MTSSL tag at each position relative to the starting protein structure; (2) fitting of depth parameter data to a hyperbolic tangent function to obtain docking geometry parameters; and (3) iterative adjustment of MTSSL sidechain conformation at selected positions in order to improve self-consistent fitting.

While previous studies have estimated initial MTSSL coordinates by assigning a single rotamer conformation at each position, we instead assigned initial coordinates based on the average coordinates among allowed rotamers at each position, using the recently developed MTSSL Wizard plugin for Pymol.⁴³ Exceptions were (a) positions at which the “tight” setting of the MTSSL Wizard yielded no predicted rotamers, and (b) positions which were defined as particular rotamers during the iterative fitting process to improve fitting to the hyperbolic tangent function. In either case, rotamers were chosen which matched previously described energy minima for the MTSSL sidechain and produced no obvious steric clashes.⁴⁶ For example, T170 is located in a shallow groove that produced no rotamers in Structures 1 and 3 using MTSSL Wizard; however, it was accommodated by χ_1 and χ_2 angles of -60° (g^+ , g^+). The positions whose rotamer conformations were adjusted during the iterative fitting process are listed in the Methods and the dihedral angles used for these positions are given in Table S1.

As in previous EPR depth studies of Syt1 C2 domains, depth parameter data were fit to a hyperbolic tangent function empirically shown to model depth parameter measurements as a function of insertion depth.^{25, 26} Depth parameter varies approximately linearly with penetration depth in the membrane interior, but is independent of distance from the membrane surface for positions that are highly aqueous. Doxyl lipid standards with known penetration depth were used to calibrate the slope of the curve in the membrane interior (open circles in Figure 3). The self-consistent best-fit relationship between depth parameter and distance fit to Eq. 7–8 is shown in Figure 3A–E for all five starting structural models. The fit includes three parameters defining the curve shape (A , B , and C in Eq. 7) and three parameters defining the penetration and orientation of the C2A domain with respect to the membrane (y_{trans} , θ_x , and θ_z in Eq. 8). The protein-membrane docking geometry is then achieved by sequentially rotating the starting protein coordinates by the Euler angles θ_x and θ_z , and then translating by y_{trans} . These transformations produce a docked protein structure in which the x - z plane represents the average phosphate position of a planar membrane. The five orientations obtained from this method are shown in Figure 4.

The use of five starting structures (three containing Ca^{2+} and two without) offers a glimpse into the uncertainty associated with modeling the docking geometry by this method. Loops and external sidechains on a protein have considerably more flexibility than a single static crystal structure. Ca^{2+} -bound Structure A and NMR State 2 produced the shallowest

penetration depths, while Ca²⁺-bound Structure C and NMR State 1 produced the deepest. The distance of each spin label from the membrane phosphate plane in each model are listed in Table S3, and the averages among the three Ca²⁺-bound geometries are listed in Table 3. As expected from the hyperbolic curve fit, the more aqueous positions had a larger uncertainty associated with their position. The standard deviations were generally in the 0–3 Å range, comparable to previously estimated uncertainties associated with EPR depth parameter measurements,^{25, 42} with the exception of L136 which resides on the flexible and solvent-exposed N-terminus of the domain. For a consistent comparison of the Syt7 and Syt1 C2A docking geometries, we also re-analyzed the published Syt1 C2A depth parameters²⁵ using the same methods as our Syt7 C2A analysis. A suite of Syt1 C2A docking geometry models were created including (a) mapping MTSSL sidechain configurations using average positions from MTSSL Wizard, and (b) using six Syt1 C2A structures as bases for fitting: states 1–3 from the published NMR structure of the Ca²⁺-loaded domain,⁴⁷ and three snapshots from a molecular dynamics simulation of the Ca²⁺-loaded domain (described in the companion paper). All six of these models are shallower than the previously reported Syt1 C2A docking geometry (Table 4, Figure S3–S4, and Table S4). The discrepancy can be attributed mainly to differences in the MTSSL sidechain geometries used for fitting, particularly for two positions in CBL3, as discussed further in the legend to Figure S4. Thus, comparing docking geometries using the same modeling approach further indicates that Syt7 C2A inserts deeper than Syt1 C2A.

Table 4 also compares our modeled Syt7 C2A penetration depths to previously published geometries for Syt1 C2A.²⁵ Notably, α-carbons in CBL1 penetrate 1 to 5 Å deeper into the membrane for Syt7 C2A relative to all of the models for the isolated Syt1 C2A domain, consistent with the greater average depth parameter values of Syt7 C2A for positions in this loop. Regardless of which Syt1 C2A model is more accurate, these data are consistent with our earlier prediction that CBL1 of Syt7 C2A penetrates deeper than the corresponding region of Syt1 C2A.³⁷

Discussion

EPR depth parameter measurements are useful for measuring docking geometries of peripheral membrane proteins, with uncertainties previously reported in the 2–3 Å range.^{25, 42} Here, we note three significant findings from our EPR depth parameter study of Syt7 C2A: (i) the domain penetrates membranes somewhat more deeply than the isolated Syt1 C2A domain, although less deeply than Syt1 C2A in the C2AB tandem; (ii) uncertainty in docking geometry models arise in large part due to inherent uncertainties in mapping sidechain depth parameters onto static protein structures; and (iii) hydrophobic effects appear more critical in CBL3 than CBL1 for Syt7 C2A membrane docking, as the R1 spin label is less perturbing in place of Phe167 than at Phe229.

Comparing Syt7 and Syt1 C2A Docking Models

The extensive body of research detailing the structure and mechanism of Syt1 provides a valuable reference point for studies of other isoforms. The Syt7 C2A domain is known to bind membranes with a much greater Ca²⁺ sensitivity and with significantly slower

dissociation kinetics than its counterpart from Syt1.^{37, 49} Previously, we proposed a membrane docking mechanism for Syt7 C2A to explain this observation, in which an initial electrostatic association is followed by penetration of Phe residues in both CBL1 and CBL3 into the hydrophobic interior of the lipid membrane.³⁷ The depth measurements presented here are consistent with this hypothesis. Of the five Syt7 C2A docking geometry models shown in Figure 4, three show penetration of both Phe167 and Phe229 into the acyl chain region of the membrane. The model for Ca²⁺-bound Structure B represents the median depth among the five, and has depths closely similar to the mean of the three Ca²⁺-bound structures (Table 3, Table S3).

A comparison of previously published EPR docking geometries for Syt1 C2A to our suite of models for Syt7 C2A shows consistent and nearly equal penetration of CBL3 but variable penetration of CBL1.²⁵ Two previous depth parameter studies have been conducted for Syt1 C2A: one with the isolated domain, and one in the C2AB tandem.^{25, 27} Figures 5 and S3 show side-by-side comparisons of our Structure B docking geometry to these two previously reported docking geometries of Syt1 C2A, and Table 4 lists average α -carbon depths for our Ca²⁺-bound Syt7 C2A models compared to the four models of Syt1 C2A (two previously reported and two averages based on our re-analysis of the depth parameter data). The α -carbon at the apex of CBL1 (Phe167 of Syt7; Met173 of Syt1) increases in depth from 0.8 Å to 7.0 Å between isolated Syt1 C2A and Syt1 C2AB, and ranges from 0.2 to 4.3 Å in our Ca²⁺-bound Syt7 C2A models. By contrast, α -carbon depths for the hydrophobic apex of CBL3 (Phe229 of Syt7; Phe234 of Syt1) fall within a relatively narrow range of 4.6 to 6.0 Å in our Ca²⁺-bound models, comparable to 5.3 Å in isolated Syt1 C2A and 6.3 Å in Syt1 C2AB. Direct comparison of depth parameters along with our re-analysis of the isolated Syt1 C2A data raise the possibility that CBL3 depth may also differ between the two domains (Tables 3–4; Figure S4); thus, this comparison remains uncertain. Overall, our modeling shows a penetration depth of Syt7 C2A in which both CBL1 and CBL3 penetrate similarly or up to a few Å deeper in Syt7 C2A relative to isolated Syt1 C2A.

Uncertainty in Docking Geometry Modeling

Because EPR-based models are useful in directly visualizing docking geometries of peripheral membrane proteins, it is important to understand the origins and extent of uncertainty in the modeling process. Prior studies have reported estimated uncertainties on insertion depths, but the contributions from different stages of the measurement and modeling process have not formally been evaluated, to our knowledge. Potential sources of uncertainty include (i) experimental measurements of depth parameters of spin-labeled protein mutants, (ii) prediction of spatial coordinates for each unpaired electron with respect to the protein backbone, and (iii) fits of the measured depth parameters and coordinates to a function that describes its docking geometry. Approaches for (ii) and (iii) have varied since the development of EPR depth measurements in the 1990s, including modern methods that combine fitting with molecular simulation, e.g. via simulated annealing.^{31, 50} The present study was designed to compare results with a 2003 study of Syt1 C2A, and therefore we have used structure fitting and modeling protocols from that study where possible and adapted those methods when necessary. As a result, we have taken a somewhat novel

approach for (ii) which reveals a significant contribution to the uncertainty of the final model. Two novel components of our modeling approach are described below.

First, because the only available structure of Syt7 C2A lacks Ca^{2+} , we simulated the Ca^{2+} -bound protein domain in isolation for use as a reference structure. We used three snapshots from this simulation along with two different states of the available Ca^{2+} -free NMR structure as bases for modeling rather than a single protein structure as in most previous studies. This approach may capture effects of dynamic heterogeneity in backbone structure of the CBLs that are responsible for membrane insertion. The range of docking geometries shown in Figure 4, each generated using the same depth parameter measurements and fitting method but using different sidechain and backbone structures, illustrates the contribution of starting protein structure to the final model. Modeled penetration depths for individual residues in CBL1 varied with standard deviations of ~ 2 Å, while depths in CBL3 had smaller standard deviations of ~ 1 Å (Table 4).

Second, we modeled coordinates of each unpaired electron relative to each protein structure largely by averaging coordinates among many possible sidechain rotamers, rather than from a single rotamer at each position as has been the standard practice in previous studies. The MTSSL Wizard tool was previously developed for double electron-electron resonance (DEER) distance measurements, but to our knowledge has not previously been used for membrane depth studies.⁴³ This approach streamlines the modeling process and reflects average sidechain configurations in a protein more accurately than a single rotamer, although it is limited by not scoring free energies of the available configurations. We reverted to the historical method of defining a single rotamer at positions for which sterics of other sidechains limited the coordinate space available for the MTSSL sidechain (for which MTSSL Wizard produced no rotamers using the ‘tight’ definition of allowable contacts).

We also adopted a standard practice of defining and adjusting rotamer conformations for a few positions which did not fit well to the hyperbolic function using the average-position method.^{21, 41, 42} In particular, we manually adjusted the sidechain configurations of F167R1 and/or G169R1 in each of the structural models, in order to account for the fact that these mutants have approximately equal depth parameters despite different positions in CBL1: Phe167 is at the apex of the loop, whereas Gly169 should be much less deeply inserted. The depth parameter data can be accounted for by defining the sidechain geometries such that F167R1 sidechain is oriented parallel to the membrane surface and/or G169R1 is oriented toward the membrane interior. While these orientations might be preferred by the protein, an alternative explanation is that the adjustments compensate for other sources of error such as minor effects of mutations on penetration depth. For example, the R1 sidechain has a polarity intermediate between Gly and Phe; therefore, the F167R1 mutant has a more polar CBL1 and may penetrate less deeply, while G169R1 and S168R1 have more nonpolar CBL1 sequences and may penetrate more deeply. A similar effect may exist in Syt1 C2A with M173R1 and G174R1, both of whose sidechain positions had to be manually defined to $(\chi_1, \chi_2) = (g^+, g^+)$ in our re-analysis of these docking geometries. The fact that none of these mutations has a dramatic effect on membrane binding kinetics may reflect a secondary importance of CBL1 in membrane docking as discussed below.

Roles of CBL1 and CBL3 in membrane docking of Syt7 C2A

The differences in membrane binding and release kinetics between the F229R1 and F167R1 mutants may provide insight into the respective roles of CBL3 and CBL1 in Syt7 C2A. Phe229 in CBL3 appears to be essential to proper membrane binding, as its mutation to R1 leads to a 4-fold increased off-rate relative to the cysless form of the domain (Table 1). In contrast, kinetics were not significantly impacted by mutation of Phe167 to cysteine and addition of an MTSSL spin label. The different effects of mutating these residues may indicate a more essential role for CBL3 as compared to CBL1 in the membrane-docked structure.

Based on the relative tolerance of CBL1 to mutation and its greater uncertainty in penetration depth, it is tempting to speculate that this binding loop experiences greater structural dynamics in the membrane-bound state. However, the depth parameter is an ensemble average measurement, and does not itself provide information on the dynamics of these loops. Lineshape analysis shows that individual sidechains become more restricted upon membrane binding, but is also not informative regarding overall tilting dynamics of the protein in the membrane. In order to resolve the uncertainty in penetration depth of CBL1 and to gain information on the relative energetics of CBL1 and CBL3 insertion, alternative approaches are necessary. In the companion manuscript, we describe all-atom simulations of Syt7 C2A membrane docking that provide insight into these outstanding questions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

Syt	synaptotagmin
EPR	Electron paramagnetic resonance
PIP₂	phosphatidylinositol 4,5-bisphosphate
WT	wild type
NiEDDA	nickel ethylenediamine diacetic acid
EDTA	ethylenediamine tetraacetic acid
POPC	1-palmitoyl-2-oleoyl- <i>sn</i> -glycero-3-phosphocholine
POPS	1-palmitoyl-2-oleoyl- <i>sn</i> -glycero-3-phospho-L-serine

SNARE	soluble N-ethylmaleimide-sensitive fusion protein attachment receptor
FRET	fluorescence resonance energy transfer
PH	pleckstrin homology
GRP1	general receptor of phosphoinositides 1
PKCα	protein kinase C α
DMF	dimethylformamide
cPLA₂	cytosolic phospholipase A ₂
IPTG	isopropyl β -D-thiogalactopyranoside
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
SUV	small (sonicated) unilamellar vesicle(s)
PDB	protein data bank
MTSSL	methanethiosulfonate spin label

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Figure 1.

Spin labeling of Syt7 C2A. **A:** Solution NMR structure of Syt7 C2A (PDB ID: 2D8K).

Yellow spheres indicate residues selected for spin labeling in this study. **B:** Structure of the MTSSL tag, with dihedral angles $\chi_1 - \chi_5$ indicated.

Figure 2.

Bound and unbound EPR spectra for each spin-labeled mutant. Bound spectra (red) were measured with 30–150 μM protein and 30 mM (3:1 PC:PS) lipid membrane concentration with 1.5 – 2.0 mM Ca^{2+} . Unbound spectra (blue) were measured with similar protein concentrations but in free solution with identical Ca^{2+} concentration. Signal broadening is indicative of a less mobile spin label.

Figure 3.

Hyperbolic tangent fits of experimentally determined Syt7 C2A depth parameters. Open circles indicate doxyl lipids, solid black circles indicate single-cysteine mutants. Each fit is based on Equation 7–8. Error bars are 95% CI of depth parameter values measured in duplicate or triplicate, which are listed in Table 2. The same depth parameter data were used for each fit, and applied to (A) Ca^{2+} -bound structure A, (B) Ca^{2+} -bound structure B, (C) Ca^{2+} -bound structure C, (D) NMR structure state 1, or (E) NMR structure state 2. The relevant NMR structure is PDB ID 2D8K.

Figure 4.

Optimized best-fit docking models for Syt7 C2A based on the indicated starting structure. Black lines represent the lipid phosphate plane, blue shading represents the approximate headgroup region and yellow shading represents the approximate nonpolar acyl chain region. The phenylalanine residues at positions 167 and 229 are shown in green, and bound Ca^{2+} ions are shown in orange. Protein orientations correspond to successive Euler angle rotations of θ_x and θ_z followed by a translation along the y axis of y_{trans} , as follows: Structure A: $\theta_x = 252.8^\circ$, $\theta_z = -4.4^\circ$, $y_{\text{trans}} = -18.2 \text{ \AA}$; Structure B: $\theta_x = 268.6^\circ$, $\theta_z = 11.1^\circ$, $y_{\text{trans}} = -16.8 \text{ \AA}$; Structure C: $\theta_x = 261.8^\circ$, $\theta_z = 14.7^\circ$, $y_{\text{trans}} = -17.1 \text{ \AA}$; 2D8K State 1: $\theta_x = 17.5^\circ$, $\theta_z = 4.8^\circ$, $y_{\text{trans}} = -20.6 \text{ \AA}$; 2D8K State 2: $\theta_x = 23.1^\circ$, $\theta_z = -6.4^\circ$, $y_{\text{trans}} = -22.2 \text{ \AA}$. Starting coordinates for Structures A, B, and C are available in the Supporting Information.

Figure 5.

Comparison of (A) the median Syt7 C2A docking geometry determined here (Structure B in Figure 4) with (B) the reported Syt1 C2A docking geometry of the isolated domain²⁵ and (C) the reported Syt1 C2A docking geometry in the C2AB tandem.²⁷ Optimized docking geometries are superimposed on a simulated lipid bilayer containing 25% POPS and 75% POPC. Hydrophobic sidechains on CBL1 and CBL3 are shown in green, and Ca²⁺ ions are shown in orange. Syt1 C2A geometries are reconstructed based on the transformations reported previously.^{25, 27}

Table 1

Measured rate constants for kinetic measurements

Species	k_{off} (s ⁻¹) ^a	k_{off} relative to C260S	k_{obs} for binding (s ⁻¹) ^b	k_{obs} relative to C260S
Wild Type	26	1.5	27	0.47
C260S (cysless)	17	1	57	1
L136R1	19	1.1	35	0.61
Q148R1	20	1.2	27	0.47
A164R1 ^c	21	1.2	18	0.32
F167R1	27	1.6	52	0.91
S168R1	34	2.0	29	0.51
G169R1	19	1.1	25	0.44
T170R1	19	1.1	51	0.89
N195R1	33	1.9	65	1.14
L196R1	38	2.2	60	1.05
L224R1	35	2.1	34	0.60
Y226R1 ^c	69	4.1	5	0.09
R228R1	24	1.4	73	1.28
F229R1 ^c	69	4.1	34	0.60
R231R1	16	0.9	50	0.88
N232R1	23	1.4	34	0.60
P234R1	21	1.2	65	1.14

^a Measured by rapid addition of excess EDTA to pre-formed protein/Ca²⁺/liposome complex^b Measured by rapidly mixing protein and liposomes in the presence of excess Ca²⁺^c Species excluded from further study due to aberrant kinetics

Table 2

Probe accessibility and depth parameters for doxyl lipids and spin-labeled C2A domains

Sample Type	Π (O ₂) ^a	Π (NiEDDA) ^a	Φ ^b
L136R1	1.37 ± 0.10	7.39 ± 0.23	-1.59 ± 0.18
Q148R1	2.99 ± 0.24	12.66 ± 0.58	-1.38 ± 0.21
F167R1	3.19 ± 0.34	2.25 ± 0.31	0.27 ± 0.19
S168R1	3.32 ± 0.21	1.06 ± 0.25	1.12 ± 0.30
G169R1	2.18 ± 0.16	3.49 ± 0.26	-0.43 ± 0.17
T170R1	1.67 ± 0.49	12.01 ± 0.91	-1.85 ± 0.40
N195R1	1.86 ± 0.17	11.70 ± 0.59	-1.83 ± 0.20
L196R1	2.19 ± 0.31	14.55 ± 0.64	-1.89 ± 0.21
L224R1	1.41 ± 0.23	7.62 ± 0.42	-1.54 ± 0.24
R228R1	2.42 ± 0.25	0.92 ± 0.14	0.69 ± 0.24
R231R1	1.55 ± 0.33	2.50 ± 0.24	-0.48 ± 0.29
N232R1	1.24 ± 0.24	3.57 ± 0.35	-0.94 ± 0.46
P234R1	0.96 ± 0.14	4.18 ± 0.23	-1.42 ± 0.23
Doxyl 5	4.48 ± 0.17	0.74 ± 0.09	1.73 ± 0.21
Doxyl 7	5.97 ± 0.52	0.55 ± 0.15	2.42 ± 0.42
Doxyl 10	7.53 ± 0.36	0.70 ± 0.12	2.42 ± 0.24
Doxyl 12	9.03 ± 0.62	0.38 ± 0.16	3.13 ± 0.54

^aUncertainties reported as weighted averages from 95% confidence intervals derived from fits to power saturation curves as described in Methods (Eq. 4)

^bDepth parameter uncertainties propagated from the accessibility parameters.

Table 3

Average spin label depths from 3 docking models of Ca²⁺-bound Syt7 C2A, compared to previously published values for Syt1 C2A

Mutant	Φ^a	Average spin label depth (Å) ^b	Equivalent residue in Syt1 ^c	Syt1 C2A Φ^d
L136R1	-1.59 ± 0.18	-12.5 ± 9.3	L142	-1.9
Q148R1	-1.38 ± 0.21	-36.2 ± 2.8	Q154	N.D.
F167R1	0.27 ± 0.19	4.0 ± 0.2	M173	-1.4
S168R1	1.12 ± 0.30	6.2 ± 0.7	G174	0.4
G169R1	-0.43 ± 0.17	0.8 ± 1.3	G175	-1.8
T170R1	-1.85 ± 0.40	-6.4 ± 2.6	T176	-1.9
N195R1	-1.83 ± 0.20	-8.2 ± 0.7	T201	-1.9
L196R1	-1.89 ± 0.21	-8.8 ± 3.1	L202	-2.4
L224R1	-1.54 ± 0.24	-5.4 ± 2.8	Y229	-2.0
R228R1	0.69 ± 0.24	5.2 ± 0.5	R233	-1.4
R231R1	-0.48 ± 0.29	1.3 ± 1.0	K236	-0.6
N232R1	-0.94 ± 0.46	-2.7 ± 1.7	H237	-2.0
P234R1	-1.42 ± 0.23	-7.3 ± 2.3	I239	-2.1
Doxyl 5	1.73 ± 0.21	8.1 ^e		1.45 ^e
Doxyl 7	2.42 ± 0.42	10.5 ^e		2.05 ^e
Doxyl 10	2.42 ± 0.24	14.0 ^e		2.42 ^e
Doxyl 12	3.13 ± 0.54	16.0 ^e		2.82 ^e

^aFrom Table 2

^bMean ± S.D. of spin label nitrogen depth from three structural models based on simulations of Syt7 C2A bound to 3 Ca²⁺ ions, created as described in text. Depths from individual structural models are given in Table S3.

^cDetermined from alignment of amino acid sequences for Syt1 – Syt10 using Clustal Omega

^dFrom ref. 25

^ePreviously published values for doxyl lipids⁴⁵

Table 4Comparison of α -carbon depths between Syt7 C2A and Syt1 C2A

Residue	Syt7 C2A ($\text{\AA}$) ^a	Equivalent residue in Syt1 ^b	Syt1 C2A, reported ($\text{\AA}$) ^{a,c}	Syt1 C2AB, reported ($\text{\AA}$) ^{a,d}	Syt1 C2A, reanalysis ($\text{\AA}$) ^{a,e}	Syt1 C2A, reanalysis ($\text{\AA}$) ^{a,f}
D166	0.0 ± 1.8	D172	-1.4	4.5	-2.9 ± 1.0	-4.4 ± 0.3
F167	2.4 ± 2.1	M173	0.8	7.0	-0.3 ± 1.3	-1.7 ± 0.2
S168	1.9 ± 1.7	G174	-0.7	4.6	-1.9 ± 0.7	-2.6 ± 0.4
G169	-1.6 ± 1.9	G175	-4.5	1.8	-5.3 ± 0.8	-6.3 ± 0.4
D227	0.3 ± 0.8	D232	0.3	2.8	-4.1 ± 0.9	-4.5 ± 0.1
R228	2.5 ± 0.4	R233	2.6	2.9	-2.9 ± 0.4	-3.0 ± 0.4
F229	5.5 ± 0.8	F234	5.3	6.3	0.6 ± 0.4	0.5 ± 0.2
S230	3.8 ± 1.3	S235	4.0	7.3	0.4 ± 0.8	-0.4 ± 0.5
R231	0.4 ± 1.2	K236	0.8	5.4	-2.0 ± 0.8	-3.4 ± 0.6

^a Distance from α -carbon to phosphate plane (positive values deeper). Data are mean $\pm$ S.D. from models using three snapshots from molecular dynamics simulations of the Ca^{2+} -loaded domain as starting structures.

^b Determined from alignment of amino acid sequences for Syt1 – Syt10 using Clustal Omega

^c From ref. 25

^d From ref. 27

^e Mean $\pm$ S.D. using 1BYN states 1–3 as starting structures

^f Mean $\pm$ S.D. using simulation snapshots as starting structures