Comparative analysis of tandem C2 domains from the mammalian synaptotagmin family

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Intracellular membrane traffic is governed by a conserved set of proteins, including Syts (synaptotagmins). The mammalian Syt family includes 15 isoforms. Syts are membrane proteins that possess tandem C2 domains (C2AB) implicated in calcium-dependent phospholipid binding. We performed a pair-wise amino acid sequence comparison, together with functional studies of rat Syt C2ABs, to examine common and divergent properties within the mammalian family. Sequence analysis indicates three different C2AB classes, the members of which share a high degree of sequence similarity. All the other C2ABs are highly divergent in sequence. Nearly half of the Syt family does not exhibit calcium/phospholipid binding in comparison to Syt I, the major brain isoform. Syts do, however, possess a more conserved

function, namely calcium-independent binding to target SNARE (soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor) heterodimers. All tested isoforms, except Syt XII and Syt XIII, bound the target SNARE heterodimer comprising syntaxin 1 and SNAP-25 (25 kDa synaptosome-associated protein). Our present study suggests that many Syt isoforms can function in membrane trafficking to interact with the target SNARE heterodimer on the pathway to calcium-triggered membrane fusion.

Key words: calcium, membrane, phospholipid, soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor (SNARE), synaptotagmin.

INTRODUCTION

Synaptotagmins (Syts) are thought to regulate the fusion of secretory vesicles with the plasma membrane. In particular, brain Syt I, a calcium-binding protein, plays an important role in coupling the calcium signal to the release of neurotransmitters in neurons [1–4]. The 15 identified Syts are expressed at different levels in various tissues and may tag specific vesicles, contributing to membrane traffic properties [5–8]. All Syts share the following organization: a single transmembrane region is connected through a variable length linker to tandem C2 domains [8,9]. Structurally, a C2 domain consists of eight β -strands arranged in a barrel-like structure with a total length of approximately 125–130 amino acids [10–12]. C2 domains were initially identified in the PKC (protein kinase C) family and are found in more than 100 intracellular proteins, including membrane trafficking proteins [11,13]. In both PKC and a number of phospholipases a single C2 domain is responsible for calcium-dependent enzyme attachment to phospholipid membranes in the low micromolar calcium range [13]. Individual C2 domains from calcium-sensitive Syt isoforms bind membranes with calcium requirements similar to that of a PKC C2 domain (5–10 μ M), whereas C2 domains from calcium-insensitive Syts do not bind phospholipids, even at millimolar calcium concentrations [8,14–20]. Full-length Syt I reconstituted in liposomes drives fast calcium-triggered apposition of two membranes [21], an event that may be central for activation of the membrane-fusion machinery [22].

Membrane fusion is driven by a tight ternary complex comprising the vesicular SNARE (soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor) synaptobrevin, also known as VAMP (vesicle-associated membrane protein), and the two t-SNAREs, syntaxin and SNAP-25 (25 kDa synaptosome-associated protein), on the target membrane [23,24]. When the two

t-SNAREs meet they form a structurally distinct heterodimer, an important intermediate in the SNARE-complex assembly pathway [25,26]. The target SNAREs on the plasma membrane have been shown to form clusters of syntaxin–SNAP-25 heterodimers as a prelude to calcium-triggered exocytosis [26]. It is thought that by driving membrane apposition, Syt promotes engagement of synaptobrevin with the t-SNARE heterodimer and therefore membrane fusion [22]. However, stoichiometric isolation of Syt I in association with neuronal SNAREs, in a calcium-independent manner [27–29], points to a more direct role for Syts in the regulation of SNARE-driven membrane fusion, rather than simply calcium-triggered membrane apposition.

We recently reconstituted the high-affinity interaction of Syt I with brain-purified SNAREs in the absence of calcium and demonstrated that native Syt I specifically and stoichiometrically binds the t-SNARE heterodimer [29]. In the present study, we sought to determine the relationship between calcium/phospholipid and t-SNARE heterodimer binding properties among mammalian Syt isoforms. We show that the t-SNARE binding activity of Syt C2ABs is more conserved than calcium/phospholipid binding. Both calcium-sensitive and -insensitive C2ABs bind the t-SNARE heterodimer through a calcium-independent mechanism that is identical to native Syt I [29].

EXPERIMENTAL

C2AB sequence analysis

Full-length amino acid sequences of Syts I–XV were obtained from NCBI Protein database using the following accession numbers: I, S09595; II, AAA63502; III, P40748; IV, AAA68519; V, Q925C0; VI, Q62746; VII, AAK01451; VIII, AAK56957; IX, AAA81382; X, O08625; XI, O08835; XII, P97610; XIII,

Abbreviations used: GST, glutathione S-transferase; PKC, protein kinase C; SNAP-25, 25 kDa synaptosome-associated protein; SNARE, soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor; Syt, synaptotagmin; t-SNARE, SNARE complexes involving target proteins located on the plasma membrane.

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AAG30575; XIV, BAC76808; and XV, BAC76816. All sequences were from rat, except XIV which is from mouse. The first four residues (single letter code) for Syt tandem C2 domains (C2AB) are as defined previously [30]: I and II, LGKL; III, CGRI; IV, LGTL; V and X, CGKL; VI, CGKI; VII, LGRI; VIII, WGQL; IX and XV, LGRL; XI, LGSLL; XII, LGQV; XIII, APKL; and XIV, YGTL. Pair-wise sequence comparison of C2ABs was performed using the MacVector 6.5 program (Oxford Molecular Limited, Oxford, U.K.).

Cloning of Syts and preparation of proteins

Reverse transcriptase PCR of rat brain mRNA was used to produce GST (glutathione S-transferase)-fusion clones in pGEX-KG corresponding to the following Syt amino acid sequences: I, 95–421; II, 101–422; III, 269–588; IV, 106–425; V, 195–491; VI, 205–511; VII, 88–403; VIII, 80–395; IX, 63–386; X, 205–523; XI, 114–480; XII, 105–421; and XIII, 122–426. Recombinant Syt fragments, purified on glutathione–Sepharose beads (Amersham Biosciences), were washed with buffer A [20 mM Hepes (pH 7.0)/100 mM NaCl/2 mM EDTA] adjusted to 1 M NaCl and 1 mM dithiothreitol, before elution with 15 mM reduced glutathione in buffer A. Proteins were bound to heparin–Sepharose (Amersham Biosciences), eluted using a linear gradient (0.1–1.2 M NaCl) in buffer A, and were dialysed against buffer A. Protein concentrations were determined using Coomassie Plus 200 (Bio-Rad) and BSA as a protein standard. Syntaxin1 and SNAP-25 were purified from bovine brain cortex, as described previously [22].

Calcium/phospholipid-binding assay

Chloroform solutions of brain phosphatidylcholine, brain phosphatidylethanolamine, brain phosphatidylserine and cholesterol (all from Avanti Polar Lipids) were mixed (35:17.5:17.5:30, by mol) and dried under nitrogen. Dried lipids were resuspended in buffer B [150 mM NaCl/20 mM Hepes (pH 7.5)/4 mM EGTA]. Large multilamellar vesicles were collected by centrifugation for 10 min at 20 800 *g* and resuspended in buffer B with or without 100 μ M free calcium. Calcium concentrations were calculated using WEBMAXC program (<http://www.stanford.edu/~cpatton/maxc.html>). For lipid binding assays, approx. 3 μ g of GST–C2AB was incubated with 40 μ g of lipid in buffer B, in the presence of calcium where indicated, for 10 min at 22 °C with shaking at 500 rev./min on a platform shaker. Large multilamellar vesicles and bound protein were isolated by centrifugation for 10 min at 20 800 *g* at 4 °C. Pellets were washed once in the incubation buffer, and the bound protein was analysed by SDS/PAGE and densitometry of the Coomassie Blue-stained gel. Bound protein was expressed as a percentage of the total input.

SNARE-binding assay

All Syt–SNARE binding reactions were performed in the absence of calcium. Syt domains (approx. 3 μ g of each) attached to glutathione–Sepharose beads were incubated for 30 min at 22 °C in 100 μ l of buffer A in the presence of 1 μ M SNAREs. Beads were washed three times with 1 ml of buffer A, and the bound protein was analysed by SDS/PAGE and Coomassie Blue staining. Bound material (0.5 %) was used for Western immunoblotting. SNAREs were detected by Western blotting using monoclonal antibodies to SNAP-25 (SMI 81) and syntaxin1 (HPC-1) and enhanced chemiluminescence (WestDura reagent; Pierce, Rockford,

IL, U.S.A.) with imaging on a Bio-Rad XRS system. Binding was normalized to that of Syt I C2AB.

RESULTS

The two adjacent C2 domains, C2AB, constitute the ‘working end’ of Syts and are believed to contribute to the functional divergence of Syt isoforms [31,32]. Therefore, we limited our present study to the tandem C2 domain part of the Syt molecule. We chose to analyse the rat Syt family as the published sequences of most Syt isoforms are from rat, and three-dimensional structures are available for the C2 domains of rat Syts I and III [10,30,33], allowing precise sequence definition of the N-terminal start of the C2AB domains. The short C-terminal tail in Syt I has previously been shown to stabilize the C2B structure [33] and we therefore included C2AB tail sequences in our sequence–function analysis.

Syt C2AB amino acid sequences were analysed for pair-wise identity and similarity. The identity and similarity percentage scores are presented as a matrix in Figure 1(A), with C2ABs arranged in order of sequence identity to Syt I. Three clusters of high sequence identity and similarity were clearly identifiable, with scores being higher than 60 % and 75 % respectively. Therefore, we grouped Syts I, II and IX into class A, Syts III, V, VI and X into class B, and Syts IV and XI into class C. Other Syt C2ABs did not exhibit any detectable clustering and we classified them as unique. Interestingly, the three Syt classes are also distinguished by their genomic structures, with splice sites being virtually identical within a class, but not between the classes (Figure 1B) [34,35]. Amino acid conservation is a strong indicator of conservation of protein function, but can Syts be functionally classified on the basis of their splice site organization? We do not favour this as the unique Syt VIII has an identical splice site organization to class A [34], but nevertheless is distinct from class A on the amino acid conservation scale (Figure 1A). Functional analysis of C2ABs confirms that Syt VIII C2AB falls outside class A (see Figures 2 and 4). In addition, in class B, Syt III has an extra splice site compared with the rest of the class (Figure 1B), further illustrating that splice site organization does not reflect amino acid conservation.

Syt I, the major brain isoform, binds phospholipids as a function of micromolar calcium through a distinct region in the C2 domain structure [10,14]. Several acidic residues play a pivotal role in coordinating calcium ions in each of the two C2 domains, as determined by X-ray and NMR analysis (Figure 2A) [11,30,33]. We analysed conservation of these residues between the 15 Syt isoforms (Figure 2A). Syts from classes A and B, and the unique Syt VII have aspartate/glutamate residues in identical positions to Syt I, whereas all other isoforms either carry the acidic residues in shifted positions or are missing some of the residues altogether (Syts XIV and XV, in particular).

To test which of the C2ABs can bind the phospholipid membrane in a calcium-dependent manner, we cloned rat Syt I–XIII C2AB domains and expressed them as GST-fusion proteins in *Escherichia coli*. Poor expression of the C2ABs reported previously [36] was overcome by incubating bacteria at 22 °C for 20 h after induction of protein expression. We were able to produce 10 Syt C2ABs (I–IV, VI–IX, XII and XIII), covering the three classes and the unique isoforms, and therefore we limited our functional analysis to these isoforms. Phospholipid vesicles were prepared using the major lipid species of the cytoplasmic leaflet of the plasma membrane and secretory vesicles [37,38]: phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and cholesterol, in a molar ratio 35:17.5:17.5:30. Intracellular membrane fusion can be triggered by high nanomolar to low

Figure 1 Classification of Syt tandem C2 domains

(A) Amino acid sequence analysis of the tandem C2 domains (C2ABs) identifies three classes of Syts. C2ABs pair-wise amino acid identity and similarity percentage scores are presented in a matrix with Syt I being a reference point. The top-right and the bottom-left triangles showing the identity and similarity scores, respectively. Identity and similarity scores higher than 60% and 70% respectively are shaded in grey and represent the three classes of Syts. (B) The three Syt classes have distinctive splice site organization. Classes A and B can also be distinguished by the length of the linker region between the transmembrane regions (TMRs) and the tandem C2 domains.

micromolar calcium in a variety of intracellular trafficking pathways [39–44]. Therefore, we decided to investigate C2AB binding to the phospholipid vesicles in the presence of 100 micromolar calcium. Protein bound to the vesicles was separated from unbound material by centrifugation and quantified by densitometry following SDS/PAGE and Coomassie Blue staining. Only those C2ABs that have precise positional conservation of the Syt I calcium-binding residues exhibited calcium/phospholipid binding (Figure 2B). Four out of the 10 C2ABs tested did not support calcium/phospholipid binding, and analysis of calcium-binding regions in different C2ABs (Figure 2A) suggests that nearly half of Syt isoforms lack the calcium/phospholipid-binding ability. Although Syt IV has the closest conservation of calcium-binding residues to those of Syt I, the Syt IV C2AB does not support calcium-dependent phospholipid binding, even at 1 mM calcium, in agreement with the properties of individual calcium-insensitive C2 domains [8,15,19,20,45].

Recent studies indicate that Syt isoforms with no calcium-dependent phospholipid binding abilities are still important in

regulated secretion [46,47]. Do Syt tandem C2 domains possess a more fundamental function in exocytosis than calcium binding? Syt I C2AB binds the syntaxin1–SNAP-25 heterodimer with high affinity in a calcium-independent manner [29]. Therefore, we tested whether other C2ABs support t-SNARE binding in the absence of calcium. C2AB domains of Syt IX (class A), Syt IV (class C) and the unique Syt XIII were immobilized on beads and incubated with monomeric SNAREs or the t-SNARE heterodimer. After extensive washing, the protein attached to beads was analysed by SDS/PAGE and Coomassie Blue staining. Syt XIII C2AB did not bind syntaxin1 and SNAP-25, either alone or together. However, Syt IV and IX tandem C2 domains specifically bound the target syntaxin1–SNAP-25 heterodimer, but not the monomeric SNAREs (Figure 3), mimicking the mechanism of SNARE binding by Syt I [29].

All available C2AB isoforms were then tested for their interaction with the t-SNAREs purified from brain in triplicate experiments. For the purpose of quantitation, we chose to screen t-SNARE binding using quantitative Western blotting. Syntaxin1 and SNAP-25 immunosignals were detected using chemiluminescence and a 2¹²-bit cooled charge-coupled-device camera (Figure 4). Bound SNARE immunoreactivity was plotted with Syt I as the reference point (Figure 4). Out of 10 Syt C2ABs only the unique Syts XII and XIII did not bind the t-SNARE heterodimer, whereas the rest bound syntaxin 1 and SNAP-25 in parallel (Figure 4).

DISCUSSION

Mammalian Syts constitute a large protein family [32,34,35]. Syt family members are implicated in various types of vesicular traffic, including neuronal exocytosis, hormonal secretion, plasma membrane repair, lysosomal fusion, neurite outgrowth and the sperm acrosome reaction during fertilization (for review, see [6]). Syts are enriched in brain, but are also present in other tissues [6–8,31,35,48], and several Syt isoforms have been shown to localize to distinct subcellular organelles [5,49]. Since different Syts may account for different vesicle fusion properties throughout the cell, comprehensive functional analysis of the Syt family as a whole is required for the understanding of intracellular membrane traffic.

Amino acid sequence comparison of mammalian Syt C2AB domains demonstrates that nine Syts can be grouped into three classes, whereas the rest are unique as they currently lack close homologues (Figure 1). Syts from classes A and B and the unique Syt VII support calcium-triggered phospholipid binding at micromolar calcium concentrations. Only these members of the Syt family have full conservation of calcium-coordinating residues, matched by high amino acid conservation in the tandem C2 domain part (Figures 1 and 2). All other members, including class C Syts, do not bind phospholipid membranes at 100 μ M calcium (Figure 2B), which is reflected by the degeneration of the calcium-binding regions (Figure 2A). Importantly, a single change of an aspartate to a serine residue is sufficient to abolish phospholipid binding by C2 domains, even at 1 mM calcium, indicating that the strict conservation of aspartate and glutamate residues at defined positions is essential for the calcium/phospholipid binding ability [19]. The absence of a common motif for glutamate and aspartate residues among calcium-insensitive isoforms also indicates that these isoforms are not likely to carry a calcium-dependent function (Figure 2A).

Can the calcium-independent interaction with the t-SNARE heterodimer be a core function in the Syt family? Binding to the t-SNARE heterodimer is well conserved in the Syt family with the exception of Syt XII and Syt XIII. We detected some variation of binding to the syntaxin1–SNAP-25 heterodimer, most notably

Figure 3 Calcium-sensitive and -insensitive Syts share a common mechanism of calcium-independent binding to target SNAREs

Syntaxin1 (Syx) and SNAP-25, alone or together, were incubated with GST-C2AB beads and the level of protein bound was analysed by SDS/PAGE, followed by Coomassie Blue staining. Calcium-sensitive Syts IX and calcium-insensitive Syt IV bind the t-SNARE heterodimer, but not the monomeric SNAREs. Syt XIII C2AB did not bind syntaxin1-SNAP-25. GST-C2AB of Syt IV has a 30 kDa breakdown product.

Figure 4 Target SNARE binding by Syt family members

GST-C2ABs of Syts I–IV, VI–IX, XII and XIII were tested for binding to syntaxin1-SNAP-25 in triplicate reactions in the absence of calcium (with 2 mM EDTA). An example chemiluminescent immunoblot is shown in the upper panel. SNARE immunoreactivity bound to C2ABs was digitized using a cooled charge-coupled-device camera and plotted after normalization to the signal obtained for Syt I C2AB (lower panel). Results are expressed as the means $\pm$ S.E.M. ($n = 3$). All isoforms bind the t-SNARE heterodimer with the exception of Syt XII and XIII. Syx, syntaxin1.

functionally significant only when Syts couple to the t-SNARE heterodimers.

We have limited our study to testing a single calcium-dependent property, which is the classical function of the C2 domain protein family. Syt C2 domains have also been implicated in calcium-dependent binding, with little specificity, to various proteins, including VCP (valosin-containing protein), tubulin, syntaxin, α -latrotoxin receptor, other C2 domains and SNAP-25 [50–53]. It is not clear whether these calcium-dependent Syt–protein interactions can occur in the presence of phospholipid membranes,

to which calcium-sensitive Syts may preferentially bind. Furthermore, a recent study *in vivo* directly challenged the hypothesis that calcium-dependent Syt interaction with SNAREs has a role in the last step of exocytosis [54].

Our study provides a starting point for the full proteomic analysis of the Syt family. Nearly half of the Syt family members lack calcium/phospholipid-binding abilities, suggesting that diversification in the Syt family is not primarily linked to different calcium sensitivities. The two core properties analysed in the present study are not sufficient to explain grouping of nine Syts into the three classes. If Syts function to bridge two membranes prior to their fusion, the linker connecting C2AB to the transmembrane region could be of critical importance. Syts have variable length linkers with class A isoforms distinguished by their short linkers (55–62 amino acids) and class B isoforms carrying very long linkers (145–221 amino acids) (Figure 1B). A recent genetic study provided the first evidence for the physiological importance of the Syt linker region in neurotransmission [55]. Future functional analysis of full-length Syts, including membrane reconstitution studies, will bring additional insights into the molecular mechanisms governing a wide variety of membrane traffic in the cell and provide an answer to why the cell carries so many Syts.

We thank Joanne Chan for assistance with production of recombinant proteins.

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Received 16 September 2003/30 October 2003; accepted 12 November 2003

Published as BJ Immediate Publication 8 January 2004, DOI 10.1042/BJ20031407