Distinct roles of the C₂A and the C₂B domain of the vesicular Ca²⁺ sensor synaptotagmin 9 in endocrine β -cells

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Synaptotagmins form a family of calcium-sensor proteins implicated in exocytosis, and these vesicular transmembrane proteins are endowed with two cytosolic calcium-binding C₂ domains, C₂A and C₂B. Whereas the isoforms syt1 and syt2 have been studied in detail, less is known about syt9, the calcium sensor involved in endocrine secretion such as insulin release from large dense core vesicles in pancreatic β -cells. Using cell-based assays to closely mimic physiological conditions, we observed SNARE (soluble *N*-ethylmaleimide-sensitive fusion protein-attachment protein receptor)-independent translocation of syt9C₂AB to the plasma membrane at calcium levels corresponding to endocrine exocytosis, followed by internalization to endosomes. The use of point mutants and truncations revealed that initial translocation

required only the C₂A domain, whereas the C₂B domain ensured partial pre-binding of syt9C₂AB to the membrane and post-stimulatory localization to endosomes. In contrast with the known properties of neuronal and neuroendocrine syt1 or syt2, the C₂B domain of syt9 did not undergo calcium-dependent membrane binding despite a high degree of structural homology as observed through molecular modelling. The present study demonstrates distinct intracellular properties of syt9 with different roles for each C₂ domain in endocrine cells.

Key words: Ca²⁺, exocytosis, membrane, secretory granule, translocation.

INTRODUCTION

Insulin secretion in pancreatic β -cells proceeds by exocytosis of LDCVs (large dense core vesicles) and Ca²⁺ constitutes the final trigger for fusion of vesicle and plasma membrane [1,2]. Synaptotagmins have been identified as a major Ca²⁺ sensor in neuronal exocytosis based on genetic, electrophysiological and biochemical evidence [3,4]. They form a family of transmembrane proteins containing two cytosolic C₂ domains, C₂A and C₂B [3,4]. The most studied synaptotagmins, syt1 and syt2, bind phospholipids and SNARE (soluble *N*-ethylmaleimide-sensitive fusion protein-attachment protein receptor) proteins in a Ca²⁺-dependent fashion via the C₂ domains and thereby attach to lipid membranes and protein complexes involved in exocytosis [3]. In reconstituted systems this mechanism seems to be instrumental for membrane fusion [5]. In addition to their interaction with exocytotic proteins, synaptotagmins may also intervene in endocytosis as suggested previously [6]. They are indeed known to interact with several components required for endocytosis [7–9]. Certain observations suggest that this may be mediated by the second C₂ domain, C₂B, although it is probably not its sole function [10–12].

The 16 synaptotagmins currently known can be classified according to their biochemical properties and by sequence alignment [13]. Ca²⁺-dependent phospholipid binding has been reported for syt1, syt2, syt3, syt5, syt7 and syt9 [14] and these isoforms have been implicated in the regulation of membrane fusion in different models. In insulin-secreting cells the use of dominant-negative mutants, recombinant proteins and knockdowns has demonstrated a role in exocytosis for syt1, syt2 and syt9, the latter being the only isoform expressed in primary β -cells [15–17]. Syt9 represents a close homologue to syt1 on sequence alignments with a consensus of 69 % (C₂A) and 72 % (C₂B), although its structure

has not yet been reported. Whereas the kinetics of membrane translocation in the case of syt9 are compatible with endocrine exocytosis, the Ca²⁺-sensitivity of the cytosolic C₂AB domains is not easily to reconcile [18–20]. In addition, divergent results have been published concerning the ability of the syt9 domains and especially the C₂B domain, to interact with phospholipids and SNARE proteins [21,22].

In view of the importance of syt9 as an endocrine Ca²⁺ sensor for the exocytosis of LDCVs, in the present study we have addressed its translocation and Ca²⁺-dependency as well as the role of the two C₂ domains. We have used cell-based assays employing transient expression and fluorescence or biochemical characterization. This also allows us to determine the site of translocation and to circumvent problems inherent to synaptotagmins expressed in bacteria [23,24]. In the present study we report Ca²⁺-dependent translocation of C₂AB with an EC₅₀ compatible with endocrine secretion and solely supported by the C₂A domain. In contrast, the C₂B domain impeded translocation but was required for subsequent localization of C₂AB to endocytotic structures.

MATERIALS AND METHODS

Antibodies

The following antibodies were employed: MAb (monoclonal antibody) anti-TfR (transferrin receptor; H684, Sigma), MAb anti-syt2, MAb anti-syt9 (BD Biosciences), anti-syntaxin (clone HPC1, Sigma), anti- β -actin (Abcam), polyclonal anti-eGFP (enhanced green fluorescent protein; SantaCruz Biotechnologies) and anti-SNAP-25 (25 kDa synaptosome-associated protein;

Abbreviations used: CCD, charged-coupled-device; eGFP, enhanced green fluorescent protein; KRB, Krebs–Ringer buffer; LDCV, large dense core vesicle; MAb, monoclonal antibody; PKC, protein kinase C; SNAP-25, 25 kDa synaptosome-associated protein; SNARE, soluble *N*-ethylmaleimide-sensitive fusion protein-attachment protein receptor; TfR, transferrin receptor; VAMP, vesicle-associated membrane protein.

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Sternberger Monoclonals, Covance Research Products). Antibodies against chromogranin A, insulin, VAMP (vesicle-associated membrane protein) 2 and synaptophysin were as previously described [25–27]. HRP (horseradish peroxidase)-linked anti-mouse or anti-rabbit secondary antibodies were purchased from Amersham Biosciences. A Cy3-labelled anti-mouse secondary antibody was obtained from Jackson ImmunoResearch.

Plasmids

The plasmid pGFP-Rab7 was provided by B. van Deurs (Department of Medical Anatomy, Panum Institute, University of Copenhagen, Copenhagen, Denmark), pGFP-CD63 by G. Griffiths (Sir William Dunn School of Pathology, University of Oxford, Oxford, U.K.), mRFP-Phogrin by W. Almers (Vollum Institute, Oregon Health Science University, Portland, OR, U.S.A.), BONT/C and BOTN/E by H. Niemann, H. Binz and T. Binz, (Institut für Biochemie, Medizinische Hochschule Hannover, Hannover, Germany) and pGFP-PKC by C. Larsson (Department of Laboratory Medicine, University of Lund, Sweden). The vector pRSET-BmStrawberry was donated by R. Tsien [28]. The plasmid pKS-Syt2 was as described previously [15].

Plasmid construction and mutagenesis

cDNA encoding mouse syt9 was generated by RT (reverse transcriptase)-PCR from total brain RNA and inserted in a pGEMT-T cloning vector. Truncated syt2 or syt9 was generated by PCR from pGEMT-syt9 or pKS-syt2 plasmids respectively, using the corresponding sense and antisense primers with an EcoRI site (underlined) inserted in the forward primer and a BamHI in the reverse primer. Primers were as follows: syt9C₂AB (amino acids 77–386), sense primer 5'-TTGGAATTCATGCTGGGCCGAGTTACATAG-3', antisense primer 5'-TCTGGATCCTCCGGGTGCAGGTATTGGCC-3'; syt9C₂A (77–233): sense primer 5'-TTGGAATTCATGCTGGGCCGAGTTACATAG-3', antisense primer 5'-TCTGGATCCAGCCACCTGCAGCTCTCTC-3'; syt9C₂B (215–386): sense primer 5'-TTGGAATTCATGAGTTTCAGTGAACCTGG-3', antisense primer 5'-TCTGGATCCTCCGGGTGCAGGTATTGGCC-3'; syt2C₂AB (101–422): sense primer 5'-TTGGAATTCATGAAGGGCAAAGGCATGAAG-3', antisense primer 5'-AGTGGATCCTTGTTCTTGCCAGAAAG-3'. The amplified coding sequences were inserted in the EcoRI/BamHI cloning sites either of pGFPN3 (Clontech Laboratories) for syt9 or in the EcoRI/BamHI cloning sites of pGFPN1 for syt2. Syt9C₂AB-mStrawberry was constructed by PCR amplification of syt9C₂AB using the primers 5'-CCCAAGCTTATGCTGGGCCGAGTTACATAG-3' and 5'-TCTGGATCCGTCCGGGTGCAGGTATTGGCC-3', HindIII and BamHI sites are underlined. After digestion with HindIII/BamHI, the amplicon was inserted in-frame into pCDNA₃-mStrawberry. The pCDNA₃-mStrawberry vector was constructed by insertion of the mStrawberry coding sequence into the restriction sites for BamHI and EcoRI of pCDNA₃. All constructs were verified by sequencing of both strands. Point mutations were introduced into the syt9 coding sequence leading to the substitution of an aspartic acid residue to an asparagine residue using the Quikchange® site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. The forward primers corresponding to each mutation were as follows (exchanged nucleotides given in bold) D145N: 5'-CCTAGGAGGTTCTCAAATCCCTATGTTAGTG-TCT-3'; D197,199N: 5'-GGTCATGGCGGTGTATAACTTTA-ATCGGTTCTCCCGCAACG-3'; D330,332N: 5'-CTGACTGT-TCTGAATTATAACAACTGGGGAAGAATGAG-3'.

Cell culture, transient transfection and biochemical membrane translocation assay

MIN6 cells (passage 21–30) and HIT-T15 cells (passage 75–85) were cultured as described previously [28,29]; after plating (72 h), MIN6 and HIT-T15 cells were transfected with plasmids using Lipofectamine™2000 (Invitrogen) or JetPEI™ (PolyPlus-Transfection) respectively according to the manufacturer's instructions. HIT-T15 cells seeded at 5×10^4 cells/ml 72 h prior transfection were grown in 24-well dishes and transfected with plasmid encoding for different cytosolic syt9 fusion proteins. After transfection (72 h), cells were washed with PBS (pH 7.4), detached by PBS containing 10 mM EDTA and centrifuged at 3000 g for 5 min at 4°C. Pelleted cells were resuspended and incubated in intracellular buffer [140 mM L-glutamic acid/monopotassium salt, 5 mM NaCl, 7 mM MgSO₄, 20 mM Hepes (pH 7), 1 µg/ml aprotinin, 1 µg/ml pepstatin, 1 µg/ml leupeptin and 0.5 mM PMSF] containing either 2 mM EGTA (non-stimulating conditions/in the absence of Ca²⁺) or 2 mM CaCl₂ with 10 µM ionomycin (stimulating conditions/in the presence of Ca²⁺) for 5 min at 37°C. Cells were then disrupted by sonication using pulses of 10 s for 1 min at 4°C [27,29]. The cell debris and the nuclei were eliminated by a centrifugation at 500 g for 10 min at 4°C. Supernatants were centrifuged at 55 000 rev./min for 1 h at 4°C (using a TLA120.1 rotor) to separate the membrane and the cytosolic fractions. The corresponding samples were suspended in Laemmli buffer, heated at 95°C for 5 min and resolved on SDS/PAGE (10% gels). Proteins were electroblotted on to PVDF, stained, blocked and incubated with the appropriate antibodies [27,29]. The signal obtained with the ECL (enhanced chemiluminescence) kit Lumi-Light^{plus} Western blotting substrate (Roche Diagnostics) was detected using a CCD (charged-coupled-device)-camera (Roperts Scientific) and quantified with FluorChem v2.00 (Alpha Innotech) [26].

In vivo membrane translocation assay

MIN6 cells (1×10^4 cell/cm²) grown on 25 mm round glass coverslips were washed twice with 1 ml of KRB (Krebs–Ringer buffer), pH 7.4, supplemented with 0.05% BSA and 3 mM glucose at 37°C [26,27]. In experiments using digitonin, cells were incubated in intracellular buffer supplemented with EGTA (0.4 mM) and then stimulated in the same buffer with a defined Ca²⁺ concentration in the presence of 30 µM digitonin. Buffered Ca²⁺ solutions were obtained as described previously [26,27] or, in the case of concentrations of free Ca²⁺ above 10 µM, calculated using Winmaxc (<http://www.stanford.edu/~cpatton/maxc.html>) employing the chelators NTA (nitrilotriacetic acid) and HEDTA (N-hydroxyethylethylenediaminetriacetic acid) [29]. When ionomycin was used, cells were first incubated in KRB (pH 7.4) supplemented with 0.05% BSA and 3 mM glucose and stimulation was performed with 1 mM Ca²⁺ and 10 µM ionomycin in KRB. Cells were kept on a heated microscope stage during acquisition as described previously [26,27]. The stimulating buffer was pressure ejected (≈ 20.7 kPa for 10 s) from a micropipette held at approximately 20 µm from the cell. Imaging of living cells was performed at 1 frame/0.5 s using an inverted microscope (Nikon TD300 equipped with a Z-drive) coupled to a monochromator (Till Photonics) and appropriate emission filters. Images were recorded by a CCD-camera (Micromax 1300Y HS, Roperts Scientific) using Metamorph software (Universal Imaging) [30]. The percentage of cells in which translocation was observed was determined. Due to leakage of unbound syt9-constructs through the pores generated by the detergent, translocation is an all-or-nothing event in digitonin-permeabilized cells.

Fluorescence microscopy of fixed cells

MIN6 cells seeded at 2.6×10^4 cells/cm² were grown on 12-mm round glass coverslips and transfected with the corresponding plasmid. After transfection (72 h), cells were washed twice with KRB and exposed to either KRB (non-stimulating conditions) or KRB containing 5 mM Ca²⁺ and 10 μ M ionomycin (stimulating conditions) for 10 min at room temperature (22°C). Cells were then fixed for 20 min at room temperature in 4% paraformaldehyde/PBS in the absence (non-stimulating conditions) or presence of 5 mM CaCl₂ (stimulating conditions). Coverslips were either directly mounted or first stained with antibodies. In the latter case, coverslips were washed once with KRB and three times with PBS containing 2% BSA. Permeabilization was performed with 0.1% saponin in PBS containing 2% BSA for 30 min at room temperature and cells were stained with corresponding antibodies. Coverslips were mounted using an antifade kit (Vectashield® mounting medium, Vector Laboratories). Imaging was performed on a LMS 510 Meta confocal laser microscope (Zeiss). Co-localization of antibody staining was quantified using Metamorph software (Universal Imaging) by analysing localization and intensities of pixels ($n = 3$) after thresholding. Values are given as means $\pm$ S.E.M. [28,30]. Co-localization masks were generated with ImageJ using the plugin 'RG2B co-localization' with a minimum ratio between channels of 0.2 and thresholds of 26 (over 255) for each channel [30].

Molecular modelling

Simulations were carried out for following systems: (i) domain C₂B of syt1 (NM001033680, amino acids 272–421 for modelling) [32] with 12323 water molecules plus 5 chloride ions, giving a total of 38,544 atoms in an initial box size of 7.37 nm³; (ii) C₂B domain of syt9 (NM016908, amino acids 239–386) with 10967 water molecules plus 12 chloride ions giving a total of 34,388 atoms in an initial box size of 7.06 nm³. These systems were solvated with SPC (simple point charge) water and energy minimized. Molecular dynamic simulations were run using Gromacs [33]. A twin-range cut-off was used for longer range interactions: 1 nm for Van der Waals interactions and 1.8 nm for electrostatic interactions. The time step was 2 fs using Shake and NPT conditions were used to constrain bond lengths (i.e. constant number of particles, pressure and temperature in the simulation). A constant pressure of 100 kPa was employed in all three directions with a coupling constant of $\theta_r = 1$ ps [34]. Water and protein were coupled separately to a temperature bath at 300 K, with a coupling constant $\theta_r = 0.1$ ps. The system was equilibrated for 2 ns and the simulation run for another ns. Results were depicted as ribbon diagrams using VMD 1.8.4 (<http://www.ks.uiuc.edu/Research/vmd/>).

Statistical analyses

For the comparison of two groups, the Student's *t* test was used.

RESULTS

Biochemical characterization of syt9C₂ domains *in situ*

In our attempt to investigate the behaviour and translocation of the cytosolic domain of syt9 we have generated constructs and mutants encompassing the C₂A and the C₂B domain (see Figure 1) and compared it with syt2C₂AB. All constructs harboured a fluorescent protein at their C-terminus, either eGFP or Strawberry

Figure 1 Expression of syt2 and syt9 constructs

(A) Schematic diagram representing the different syt9 and syt2 constructs used: syt2C₂AB (101–422), syt9C₂AB (77–386), syt9C₂A (77–233) and syt9C₂B (215–386). Mutation of the aspartic acid residue to an asparagine residue in the C₂A and/or the C₂B domains gave rise to D145N, D197N, D199N, D330N and D332N. All constructs are C-terminally tagged with a fluorescent protein (FP). (B) Expression of the constructs in HIT-T15 cells. Cells were transfected with the different variants. After transfection (72 h), cells were harvested and 20 μ g of total proteins were separated by SDS/PAGE and immunoblotted with anti-syt2, anti-syt9 or anti-eGFP antibodies. The lane number corresponds to the construct number.

[28]. Moreover, point mutations were introduced into the Ca²⁺-binding sites. Indeed, replacement of cognate aspartic acid residues by asparagine residues in the C₂A domain of syt1 has been shown to partially (D¹⁴⁵, numbering for syt9) or completely (D^{197/199} in syt9) abolish Ca²⁺-mediated effects of this domain [15,35]. Similarly, a double mutation in the Ca²⁺-binding site of the C₂B domain of syt1 (corresponding to D^{330/332} of syt9) completely abolished its function *in vivo* [11]. The encoded proteins were expressed in insulin-secreting cells (Figure 1B) as demonstrated by the use of antibodies against syt9, which also recognized the endogenous forms (right-hand panel and middle panel), except for syt9C₂B. The protein expressed by this construct no longer contained the antigenic epitope for the anti-syt9 antibody, but still reacted with anti-eGFP (Figure 1B, middle panel). Note that degradation products of fluorescent proteins were not detected.

We subsequently used these constructs to examine the translocation of syt9 using a biochemical approach to characterize the behaviour of syt9C₂AB. To this end cells were incubated in the absence or presence of Ca²⁺ (2 mM) and ionomycin (10 μ M) prior to their fractionation into supernatant (cytosol) and membrane pellets. As shown in Figure 2(A), a considerable amount of syt9C₂AB was already present at membranes in the absence of Ca²⁺ and the cation induced a complete shift of syt9C₂AB to the membrane fraction. Membrane binding of syt9C₂AB was sensitive to a high concentration of salt (Figure 2B), indicating the electrostatic nature of the interaction. Ca²⁺-sensitive synaptotagmins bind to membrane SNARE proteins such as syntaxin or SNAP-25 in a Ca²⁺-sensitive manner and this interaction is sensitive to the action of clostridial neurotoxins [36–38]. To test whether these SNARE proteins were involved in the translocation observed in the present study, we co-expressed syt9C₂AB and

Figure 2 Membrane binding of syt9C₂AB-eGFP in HIT-T15 cells

(A) After transfection (72 h) with syt9C₂AB-eGFP, HIT-T15 cells were incubated for 5 min at 37 °C in the presence of Ca²⁺ (2 mM CaCl₂ supplemented with 10 μ M ionomycin) or in absence of Ca²⁺ (2 mM EGTA) and fractionated by ultracentrifugation at 55000 rev./min. Distribution of syt9C₂AB-eGFP in supernatants (c) and membrane pellets (m) were analysed by Western blot using antibodies against syt9 and against the transmembrane protein syntaxin 1 as a control for fractionation. The distribution was quantified by densitometry as given by the histogram (on the right-hand side). Means $\pm$ S.D.s are indicated ($n = 8$). (B) Transfected cells were incubated in the absence or in the presence of Ca²⁺ supplemented with 0.5 M KCl. Fractionation and analysis were performed as described above ($n = 3$). (C) HIT-T15 cells were co-transfected with syt9C₂AB-eGFP and plasmids coding for botulinum neurotoxins BoNT/C and BoNT/E. Translocation and the effect of each toxin were analysed by immunoblotting using anti-syt9, anti-syntaxin1 or anti-SNAP-25 antibodies. Errors bars represent the S.D. ($n = 3$); * $2P < 0.05$ as compared with membranes.

botulinum neurotoxin E or C prior to analysis. Neither of the two toxins altered the Ca²⁺-sensitive distribution of syt9C₂AB despite cleavage of syntaxin 1 and SNAP-25 (Figure 2C). Note that cleavage was not complete, as transient transfection led to expression only in a fraction of cells. Taken together these data demonstrate that syt9C₂AB translocates to membranes in response to Ca²⁺ in these insulin-secreting cells. This event occurred independently of SNARE proteins and most likely implies ionic interactions with membrane phospholipids.

Next we addressed the role of the different domains of syt9C₂AB in translocation by biochemical means and compared first the C₂ domain of PKC (protein kinase C) with the C₂AB domain of syt2 and syt9 (Figure 3A). Interestingly both PKC-C₂ α and syt2C₂AB did not bind to membranes in the absence of Ca²⁺ in contrast with syt9C₂AB. As expected, a rise in Ca²⁺ resulted in a complete translocation for all three constructs. The first C₂ domain of syt9 alone, syt9C₂A, was largely cytosolic in the absence of

Ca²⁺ and fully translocated. It thus behaved as syt2C₂AB and PKC-C₂ α . Note that a double band was observed (indicated with an arrowhead) as syt9C₂A migrated just below endogenous syt9 and both were stained by the antibody used. In contrast with the C₂A domain, the C₂B domain remained mainly cytosolic despite the increase in Ca²⁺. Mutation of Asp¹⁴⁵ to an asparagine residue in syt9C₂AB did not alter translocation, whereas mutation of Asp¹⁹⁷ and Asp¹⁹⁹ rendered syt9C₂AB insensitive to Ca²⁺ (Figure 3C). We also tested the role of the aspartic acid residues in the second C₂ domain known to be required for Ca²⁺ co-ordination in syt1 and its function in neuroendocytosis [12]. Contrary to the mutations in syt9C₂A, the mutations in the second C₂ domain did not influence the Ca²⁺-induced distribution of syt9C₂AB. However, syt9C₂AB_{D330N,D332N} exhibited a considerable increase in membrane binding in the absence of Ca²⁺ similar to the reported observations on the cognate mutation in syt1C₂AB [35].

Modelling of the syt9C₂B domain

To get more insight into the differences between syt1 and syt9 we modelled the structure of syt9C₂B using the published crystal structure of syt1C₂B as a template, and performed molecular dynamic simulations following equilibration of the system (Figure 4). The syt9 sequence showed a striking overall resemblance to syt1, though some differences were apparent. The main distinction concerned the β -sheets 3 and 4 (Figure 4) which were partially or completely absent in syt9C₂B. Sheet 4 contains seven lysine residues, which are generally unfavourable for β -sheet formation and modelling has probably failed to detect the sheets documented previously by NMR and crystallography for this reason (<http://rsb.info.nih.gov/fij/> and [37]). As a control, we modelled syt1C₂B alongside syt9C₂B and again, both β -sheets were partially unfolded or were absent (results not shown). The sole additional, albeit minor, difference concerned the C-terminal acidic α -helix H2, which was shorter in syt9C₂B compared with syt1C₂B. In contrast, the main Ca²⁺ co-ordination sites were well-conserved and no difference was seen in the spatial arrangement around β -sheets 6 and 7, from which they emerge. Actually exchange of this portion in syt9C₂B by the corresponding sequence of syt1C₂B conferred 'syt1-like' biochemical properties to the mutated syt9C₂B. Thus differences in spatial arrangement do not seem to be responsible for the particular behaviour of syt9C₂B.

Translocation of syt9C₂ domains in living cells

These biochemical assays suggest that the C₂B domain does not positively influence translocation of syt9C₂AB but rather impedes it. However, this approach may be influenced by the lengthy fractionation protocol and does not allow determination of the nature of the membrane(s) syt9 is binding to. We therefore resorted to the observation of membrane translocation in living cells by the use of fluorescent videomicroscopy. Moreover, we combined this approach with the use of a detergent, digitonin, to permeabilize the membrane and the use of defined Ca²⁺ buffers to equilibrate intracellular concentrations of the cation [26,37]. A typical experiment is shown in Figure 5(A) depicting the translocation observed for syt9C₂A and syt9C₂AB. Subsequent to the stimulus and membrane attachment, we observed the disappearance of fluorescent proteins over time. This is most likely due to leakage of fluorescent proteins through the pores formed by digitonin as it was also observed for eGFP itself and only minor bleaching occurred (results not shown). Indeed, chelation of the small volume of Ca²⁺ ejected from the pipette by EGTA present

Ribbon diagrams are shown. **(A)** Syt1C₂B according to the crystal structure 1UOW. **(B)** C₂B domain of syt9 after 3 ns simulation. The loops 1 and 3 (L1 and L3), the mutation D330N/D332N (*), the unstructured part corresponding to the β -sheet 4 in syt1 and the shortened α -helix H2 are indicated. Views are given to highlight the differences between the structures.

Syt2C₂AB and syt9C₂AB exhibited distinct Ca²⁺ sensitivities EC₅₀s at around 1 μM and 10 μM free Ca²⁺ respectively (Figure 5B). In contrast, syt9C₂A behaved as syt2C₂AB in terms of Ca²⁺ sensitivity in line with our observations in the biochemical assays (see above). Again syt9C₂B remained purely cytosolic and no evidence, even on close direct inspection during the experiment or by confocal microscopy (results not shown), could be found for translocation (Figure 5B). This approach also revealed that syt9C₂AB carrying the mutation D145N exhibited some degree of translocation but only at millimolar concentrations of free Ca²⁺, whereas translocation was completely abolished by the double mutation D197N/D199N. Again, the mutation D330N/D332N in

and the endogenous protein. Syt9C₂B-eGFP was revealed with the anti-GFP antibody as it does not contain the epitope for the anti-syt9 antibody. **(C)** Syt9C₂AB-eGFP and its mutants were detected using anti-syt9.

(A) HIT-T15 cells expressing PKC-C2 α -eGFP, syt2C2 α -eGFP and syt9C2 α -eGFP were incubated for 5 min at 37 °C in the presence (2 mM CaCl₂ and 10 μ M ionomycin) or the absence (2 mM EGTA) of Ca²⁺. The subsequent fractionation was analysed by Western blot using anti-GFP, anti-syt2 or anti-syt9 antibodies for PKC-C2 α -eGFP, syt2C2 α -eGFP and syt9C2 α -eGFP respectively. Histograms on the right-hand side represent the distribution of the protein quantified by densitometry. Errors bars represent the S.D. ($n=3$); * $P < 0.05$ as compared with membranes. **(B)** The same experiments in **(A)** were performed on HIT-T15 cells transfected with syt9C2 α -eGFP and syt9C2 β -eGFP. Syt9C2 α -eGFP was revealed with the anti-syt9 antibody that detected the fluorescent protein (indicated by the arrowhead).

Figure 5 Stimulation of living cells by low micromolar Ca²⁺ translocates the C₂A, but not the C₂B domain, of syt9

(A) MIN6 cells expressing syt9C₂AB-eGFP, syt9C₂A-eGFP or syt9C₂B-eGFP were grown on coverslips and stimulated by pressure ejection of buffer containing 10 μ M free Ca²⁺ supplemented with 30 μ M digitonin. Cells were imaged at 37 °C by time-lapse microscopy. Images **a**, **c** and **e** were taken at 0 s and **b**, **d** and **f** 3 s after stimulation. (B) Membrane binding affinity of syt9 variants. Experiments were performed in MIN6 cells as described in (A). Defined concentrations of free Ca²⁺ supplemented with digitonin were used to stimulate the cells.

the C₂B domain did not alter the Ca²⁺ sensitivity of syt9C₂AB. Thus in both the biochemical and the *in vivo* approaches the C₂B domain of syt9 actually reduced the Ca²⁺-sensitivity and the extent of translocation.

Syt9C₂B is required for late translocation to endosomes

On close inspection of films it became apparent that syt9C₂AB rapidly detached from the membrane once the perfusion with buffers containing elevated Ca²⁺ was stopped, and translocated to granular intracellular structures. However, as a large amount of syt9C₂AB leaked out of the cell when using digitonin, imaging of those structures was difficult. We therefore used ionomycin and Ca²⁺ as a stimulus to improve visualization of these structures (Figures 6A–6D). At about 5 s after the beginning of membrane translocation, staining of these structures became apparent. Most interestingly, the D330N/D332N mutant of syt9C₂AB translocated to the membrane, but never subsequently moved to intracellular structures, thus indicating a role for the C₂B domain in the second step. Syt9C₂B alone never translocated and both mutants of the C₂A Ca²⁺-binding site (syt9C₂AB D145N and D197N,D199N) failed to translocate to the plasma membrane under the conditions used here (intact cells, ionomycin) and were not found on intracellular structures (results not shown). It seems therefore that prior passage to the plasma membrane is required for localization on intracellular structures.

In view of the requirement of the C₂B domain for this event we attempted to characterize the compartment(s) involved by co-expression of fluorescent fusion proteins and the use of antibodies. As the fusion proteins used as markers were tagged with eGFP, we used syt9C₂AB linked to the fluorescent protein Strawberry (syt9C₂AB-S) known to be monomeric [28]. Syt9C₂AB-S behaved as syt9C₂AB-eGFP and co-localized with eGFP-tagged syt9 upon co-transfection (results not shown). To exclude a potential interaction between fluorescent proteins, we tested the combination of syt9C₂AB-S and LPH-eGFP, a plasma-membrane marker [29,30] and SVP38-eGFP [39]. Neither of these constructs co-localized with syt9C₂AB-S suggesting that interactions between fluorescent proteins did not play any significant role (results not shown). A considerable number of compartments tested using antibodies also did not reveal any substantial degree of co-localization such as SNAP-25, syntaxin 1, VAMP2/synaptobrevin 2, insulin and chromogranin A. This excluded compartments harbouring these SNARE proteins or secretory granules as localization for syt9C₂AB-eGFP. In contrast, we observed a high degree of co-localization for the endosomal markers CD63-eGFP [40] and rab7-eGFP [41] in fixed cells (see Figures 7A and 7C). In addition, co-localization was evident for phogrin-eGFP, known to be recycled through endosomal pathways [42], and to a lesser degree for the TfR. We also determined whether co-localization can be observed in living cells by videomicroscopy (see Figure 7B). Indeed, a considerable degree of co-localization was apparent for Rab7-eGFP and syt9C₂AB-S after stimulation of cells with ionomycin. These observations are in line with an attachment of syt9C₂AB to endosomal membranes.

DISCUSSION

Synaptotagmins play a major role as Ca²⁺ sensors in exocytotic membrane fusion and in endocytosis [43,44]. Despite a considerable conservation in the Ca²⁺ and phospholipid-binding C₂ domains among several isoforms, such as syt1, syt2 and syt9, differences are apparent which may be of functional importance. Our data indicate that the C₂AB domain of syt9 is operational in living cells at Ca²⁺ levels present during exocytosis in pancreatic β -cells [20,45]. In contrast with syt1, however, the C₂B domain lowers the Ca²⁺-affinity for membrane translocation, but is required for ensuing localization on endosomal structures.

Figure 6 Syt9C₂AB-eGFP but not syt9C₂AB_{D330N/D332N}-eGFP distributes to intracellular structures after stimulation with Ca²⁺

MIN6 cells expressing syt9C₂AB-eGFP (**A**, **B** and **C**) or syt9C₂AB_{D330N/D332N}-eGFP (**E**, **F** and **G**) were stimulated by 5 mM CaCl₂ and 10 μM ionomycin. The distribution of the two fusion proteins was imaged and the three panels represent the distribution of the proteins at 0 s (**A** and **E**), 10 s (**B** and **F**) and 20 s after stimulation (**C** and **G**). GFP fluorescence was quantified in two different areas corresponding to plasma membrane (zone 1) and to the intracellular space (zone 2) (**D** and **H**). F₀, fluorescence at *t* = 0 s; F_{*t*}, fluorescence at *t*. Images are representative of at least ten independent experiments.

In contrast with previous studies, we used transient expression of synaptotagmins in mammalian cells for our biochemical and fluorescent assays. Although liposomal systems permit a good control of the variables, they do not reproduce the lipid composition or membrane protein/lipid ratio present in native preparations as glycerophospholipids are largely over-represented. It is thus important to obtain affinities at intracellular conditions. Moreover, the use of recombinant synaptotagmins expressed in bacteria has been difficult due to precipitation [46,47] or to the presence of contaminants [23,24]. Our initial biochemical characterization revealed that the interactions are sensitive to ionic forces and can occur independently from SNARE proteins. Indeed, their cleavage by clostridial neurotoxins did not alter the distribution or extent of translocation. Note that divergent results on Ca²⁺-dependent binding of syt9C₂AB to SNARE proteins had been reported previously [18,21]. Our observation does not exclude a role for the interaction with SNARE proteins in the membrane attachment of syt9, as they may direct synaptotagmins to specific sites, or in the

function of syt9 during membrane fusion, which was not tested in the present study.

Two major differences were apparent when comparing the C₂AB of syt9 and syt2. First, in both assays syt9C₂AB was already in part attached to the membrane in non-stimulated living cells or in the absence of free Ca²⁺ in biochemical assays, whereas syt2C₂AB was completely cytosolic under those conditions. The differential membrane attachment of the two isoforms in the absence of Ca²⁺ has been observed in some but not all liposome-based assays using phosphatidylcholine/phosphatidylserine vesicles and was more pronounced in the presence of PtdIns(4,5)P₂ [21,22]. Cleavage of SNARE proteins by clostridial neurotoxins did not diminish the attachment in the absence of Ca²⁺ in our assays. Interestingly, neither syt9C₂A alone nor syt9C₂B alone demonstrated any membrane binding in the absence of Ca²⁺. Pre-binding of syt9C₂AB to membranes is therefore independent of SNARE proteins and requires co-operation between the two C₂ domains. Secondly, the EC₅₀ for Ca²⁺ required in membrane

Figure 7 Syt9C₂AB redistributes to endosomes

MIN6 cells transfected with indicated constructs were incubated for 10 min at room temperature in the presence of Ca²⁺ (5 mM CaCl₂ and 10 μ M ionomycin) to allow the translocation of syt9C₂AB-FP [eGFP or S (Strawberry)] to the plasma membrane and to intracellular structures. Images corresponding to the different channels are given in the left-hand panels and middle panels; co-localization is indicated by 'MERGE' in the right-hand panels. EEA1, early endosomal antigen. (A) Cells were subsequently fixed and co-localization of the FP

translocation differed by one order of magnitude between syt2C₂AB and syt9C₂AB. Interestingly, syt9C₂A alone was indistinguishable from syt2C₂AB in this respect. The most likely explanation is provided by the increased pre-binding of syt9 in the presence of the C₂B domain which abolishes a high-affinity component. Importantly, the EC₅₀ observed here for syt9C₂AB in living cells corresponds well with the values reported for Ca²⁺-stimulated exocytosis of insulin [20,45].

Whereas the C₂A domains of syt9 behaved as expected, the C₂B domain clearly differed from syt2C₂B in respect to reported Ca²⁺-dependent membrane binding [22,48]. As to the behaviour of the C₂B domain, divergent results have been reported using either the C₂B domain alone [21] or a C₂B domain containing in addition 23 amino acids of the sequence linking C₂A and C₂B [22]. Our modelling data support the notion that amino acids 238–386 are sufficient to form a stable C₂B domain and additional N-terminal residues are not required. The observations reported in the present study clearly indicate that syt9C₂B is not capable of translocating in the cellular environment. This difference is surprising in view of the high degree of sequence conservation as well as structural arrangement between the isoforms. As membrane attachment of syt1C₂B is less resistant to salt washes than syt1C₂A, minor changes in the C₂B domain may be important [49]. Indeed, domain swapping between syt1 and syt9 around loop 3 restored Ca²⁺ sensitivity [21]. As the structure of this part of the sequence is conserved between the two isoforms in modelling, very subtle changes may be responsible for their distinct behaviour.

Although syt9C₂B was fully dispensable for Ca²⁺-dependent membrane translocation, it was clearly implicated in the subsequent attachment to intracellular structures as syt9C₂AB_{D330N,D332N} still translocated to the plasma membrane, but was not internalized. Although syt9C₂B does not bind Ca²⁺ in accordance to indirect assays as performed in the present study and by others [21], the mutation C₂AB_{D330N,D332N} is located at the base of a loop directed towards the membrane and may thus destabilize interactions [50]. Intracellular targeting of syt1C₂A has been reported but was mainly directed to the *trans*-Golgi network in MDCK (Madin–Darby canine kidney) cells in contrast with our observation on both C₂ domains of syt9 [51]. The reported localization may reflect distinct affinities for certain phosphoinositols or SNARE proteins. The co-distribution of marker proteins observed in the present study for syt9C₂AB is compatible with late endosomes as a major location and a minor location on early endosomes. Syt9 has been previously reported on endocytotic compartments in mast cells and syt9C₂B interacts with the clathrin adaptor complex AP-2, as had been shown for other synaptotagmins [7,52]. This provides a potential mechanism for internalization. As targeting to the intracellular structures required the presence of the C₂A domain, we suggest that prior contact with the plasma membrane is required. The fluorescent protein probably accompanies membrane internalization, but is not directly transferred from the cytosol to endosomes. The functional implication is yet unclear in the case of syt9. The C₂B domain of syt1 is implicated in the regulation of endocytosis and interestingly requires the Ca²⁺-co-ordinating aspartic acid residues in C₂B cognate to those which abolished internalization of syt9C₂AB [53].

What might be the functional outcome of the differences between syt1 and syt9 in terms of C₂ domains? Whereas in clonal β -cells syt1, syt2 and syt9 are expressed on LDCVs and function in

constructs analysed by confocal microscopy. (B) Living cells observed by videomicroscopy 10 min after stimulation. (C) Percentage of co-localization given as obtained from at least five experiments.

exocytosis, primary cells contain seemingly only syt9 and this isoform should therefore not be a redundant Ca²⁺-sensor [15–17,54]. The Ca²⁺ dependency of syt9C₂AB fits well with the physiological Ca²⁺ requirements for insulin exocytosis and the observed right shift may be compensated by pre-binding to the membrane via the C₂B domain. Such a mechanism may be an advantage in a cell that secretes mainly at limited time periods, i.e. in response to nutritional stimuli.

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