

Drosophila AD3 mutation of synaptotagmin impairs calcium-dependent self-oligomerization activity

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Abstract Genetic analysis of a *Drosophila* synaptotagmin (Syt) I mutant (AD3) has revealed that Tyr-334 within the C2B domain is essential for efficient Ca^{2+} -dependent neurotransmitter release. However, little is known as to why a missense mutation (Tyr-334-Asn) disrupts the function of the C2B domain at the molecular level. Here, we present evidence that a Tyr-312 to Asn substitution in mouse Syt II, which corresponds to the *Drosophila* AD3 mutation, completely impairs Ca^{2+} -dependent self-oligomerization activity mediated by the C2B domain but allows partial interaction with wild-type proteins in a Ca^{2+} -dependent manner. This observation is consistent with the fact that the AD3 allele is homozygous lethal but complements another mutant phenotype. We also showed that the Ca^{2+} -dependent C2B self-oligomerization is inhibited by inositol 1,3,4,5-tetrakisphosphate, a potent inhibitor of neurotransmitter release. All of these findings strongly support the idea that self-oligomerization of Syt I or II is essential for neurotransmitter release in vivo. © 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Synaptotagmin; C2 domain; Self-oligomerization; Exocytosis; Synaptic vesicle

1. Introduction

Neurotransmission is now known to be achieved by a protein–protein cascade which is evolutionarily conserved from nematoda to human (reviewed in [1]). Synaptotagmin I (Syt I), an abundant synaptic vesicle protein, is one of the components of this cascade and has been shown to be essential for efficient Ca^{2+} -dependent neurotransmitter release by analysis of knock-out animals, such as mice [2], *Drosophila* [3], and *Caenorhabditis elegans* [4]. Syt I consists of a short intravesicular amino-terminus, a single transmembrane region, and two highly conserved C2 domains (C2A and C2B) in the large carboxyl-terminus (reviewed in [5–8]). Since the two C2 domains interact with various molecules necessary for synaptic vesicle exocytosis and endocytosis in vitro, including calcium [9], phospholipids [10–15], inositol polyphosphates [12,16–18], syntaxin [19], SNAP-25 [20,21], β -SNAP [22], SV2 [23], clathrin assembly protein, AP-2 [24], N- or P/Q-type calcium channels [25,26], SYNCRIIP [27], or Syt I itself [28–34], the C2

domains are thought to be the functional domains of Syt I in neurotransmitter release. Among these interactions, phospholipid or syntaxin binding to the C2A domain and self-oligomerization of the C2B domain are activated in the presence of Ca^{2+} . Although antibody or peptide injection experiments in neurons or endocrine cells have clearly demonstrated the importance of the C2 domains in synaptic vesicle trafficking [35–42], there is no direct evidence as to whether the above-mentioned protein–protein interactions, especially the C2B domain–protein interactions, are essential in vivo.

To examine the in vivo significance of C2B domain–protein interactions, we focused on the *Drosophila* AD3 mutation, in which there is a Tyr-364 to Asn substitution in the C2B domain [43,44]. This *syt* allele is homozygous lethal but complements another mutant phenotype [43,44], and thus it has been suggested that Syt I functions in an oligomerized state in neurotransmitter release. However, although it was expected that certain C2B–protein interactions would be impaired, it remains unknown why such a mutation abrogates the function of Syt. To address this, we introduced a similar mutation into mouse Syt II, a homologue of *Drosophila* Syt I, which is predominantly expressed in the caudal region of the mammalian brain, and examined the effect of the Tyr-312 to Asn (Y312N) mutation of mouse Syt II on Ca^{2+} -dependent self-oligomerization properties [28–34]. We showed that Y312N mutation impairs Ca^{2+} -dependent self-oligomerization activity, and based on this finding, we discuss the importance of Ca^{2+} -dependent oligomerization of the C2B domain in neurotransmitter release.

2. Materials and methods

2.1. Materials

AmpliQ DNA polymerase and restriction enzymes were obtained from PE Biosystems (Foster City, CA, USA) and Toyobo Biochemicals (Tokyo, Japan), respectively. Polyclonal antibody against FLAG peptide was obtained from Zymed Laboratories Inc. (San Francisco, CA, USA). Horseradish peroxidase (HRP)-conjugated anti-T7 tag antibody was from Novagen (Madison, WI, USA). D/L-myo-inositol 1,3,4,5-tetrakisphosphate (IP_4) was purchased from Dojindo Laboratories (Kumamoto, Japan). All other chemicals were commercial products of reagent grade. Solutions were made up in deionized water prepared with an Elix10 Water Purification System and Milli-Q Biocel A10 System (Millipore Corp.; Bedford, MA, USA).

2.2. Expression constructs and mutagenesis

A mutant Syt II carrying a Y312N mutation was essentially produced by means of two-step polymerase chain reaction techniques as described previously [16] by using the following oligonucleotides: 5'-CCAAACGTTAAGATCCACCTGATGCAGAACG-3' (sense; amino acid residues 311–321) and 5'-CTTAACGTTGGGGTCTGAAAG-

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Abbreviations: HRP, horseradish peroxidase; IP_4 , D/L-myo-inositol 1,3,4,5-tetrakisphosphate; Syt(s), synaptotagmin(s)

GCCCC-3' (antisense; amino acid residues 306–314) (*AcII*, underlined). The resulting Syt II cytoplasmic fragment carrying the Y312N mutation was subcloned into the *NorI* site of pEF-BOS (named pEF-T7- or FLAG-Syt II (Y312N)-cyto [12,45]. Other expression constructs (pEF-T7- or FLAG-Syt II-cyto, -Syt II (KQ)-cyto) were prepared as described previously [12,16,33].

2.3. Miscellaneous procedures

Co-transfection of pEF-T7-Syts and pEF-FLAG-Syts into COS-7 cells (5×10^5 cells, the day before transfection/10 cm dish) was carried out by the DEAE-dextran method as described previously [33,46,47] or by using the LipofectAmine Plus reagent according to the manufacturer's notes (Life Technologies, Rockville, MD, USA). Three days after transfection, cells were harvested, and the proteins were solubilized with buffer containing 1% Triton X-100, 250 mM NaCl, 50 mM HEPES-KOH, pH 7.2, 0.1 mM phenylmethylsulfonyl fluoride, 10 μ M leupeptin, and 10 μ M pepstatin A at 4°C for 1 h. Immunoprecipitation of T7-Syts in the presence or absence of 250 μ M (or 500 μ M) Ca^{2+} by anti-T7 tag antibody-conjugated agarose, SDS-polyacrylamide gel (10%) electrophoresis, and immunoblotting analyses were also performed as described previously [46]. Immunoreactive bands were captured by Gel Print 2000i/VGA (Bio Image) and quantified by Basic Quantifier Software (version 1.0) (Bio Image).

3. Results and discussion

3.1. Tyr-334 of *Drosophila* Syt I is one of the highly conserved residues among the Syt C2 domains

The *Drosophila* AD3 allele contains a Tyr to Asn substitution at amino acid position 334 within the C2B domain. This tyrosine residue is highly conserved during evolution, among other Syt isoforms [19,46], and even between the C2A and C2B domains (Fig. 1). Recent crystallographic analysis of the cytoplasmic portion of Syt III indicates that both C2 domains exhibit a similar overall structure, although they show differences in the shape of the Ca^{2+} binding pocket, the electrostatic surface potential, and the stoichiometry of bound divalent cations [48]: eight-stranded β -sandwiches with type I C2 topology and Ca^{2+} binding loops at the apex of the fold formed by the β -sandwiches. The Tyr-334 of *Drosophila* Syt I lies on the β -3 strand in the vicinity of the putative Ca^{2+} binding loop. Interestingly, the sequences surrounding Tyr-334 (SDPYVK) are also highly conserved dur-

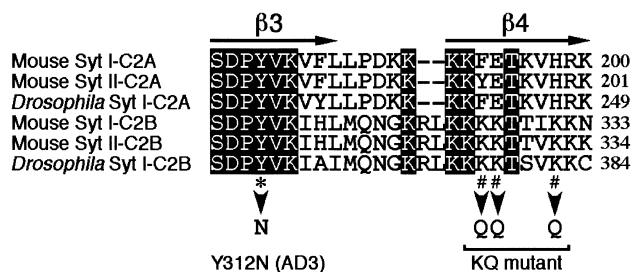


Fig. 1. Alignment of the β -3 and β -4 strands of the two C2 domains of *Drosophila* and mouse Syts. Residues that have been conserved in all sequences are shown on a black background. Note that the SDPYVK sequence is highly conserved between the two C2 domains as well as between *Drosophila* and vertebrate Syts. The asterisk indicates the position of the *Drosophila* AD3 mutation [43,44]. The number signs (#) indicate the position of the Lys residues that are only conserved in the C2B domain [16] and are essential for IP_4 binding, AP-2 binding, and self-oligomerization (so-called 'C2B effector domain') [31]. Arrows indicate the β -strands [48]. Amino acid substitutions of Syt II are indicated by arrowheads. Amino acid numbers are indicated on the right.

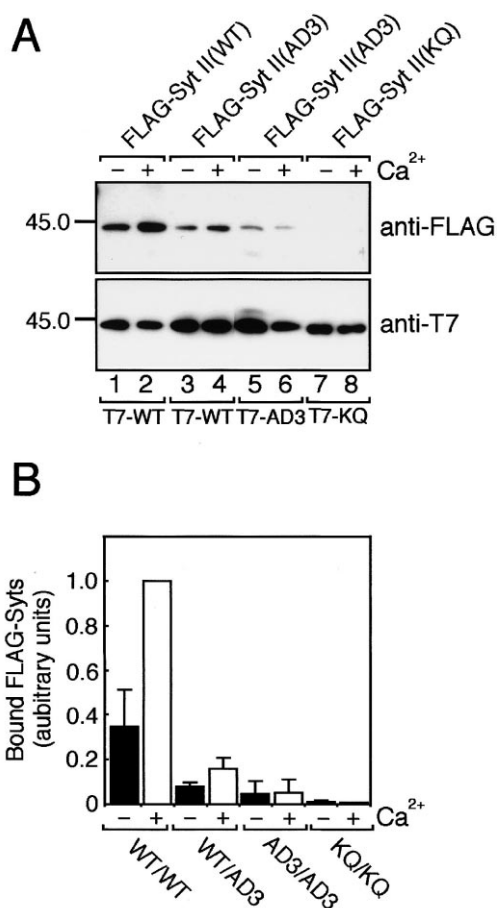


Fig. 2. Effect of Y312N mutation on self-oligomerization of mouse Syt II. pEF-T7-Syts-cyto and pEF-FLAG-Syts-cyto were co-transfected into COS-7 cells. A: Expressed proteins were solubilized with 1% Triton X-100 and immunoprecipitated by anti-T7 tag antibody-conjugated agarose as described previously [46]. Co-immunoprecipitated FLAG-Syts were first detected with anti-FLAG rabbit antibody (5 μ g/ml; upper panel). The same blot was then stripped and reprobed with HRP-conjugated anti-T7 tag antibody to ensure that the same amounts of T7-tagged proteins were loaded (1/1000 dilution; lower panel). The positions of the molecular weight markers ($\times 10^{-3}$) are shown on the left. B: Immunoreactive bands were captured by Gel Print 2000i/VGA and quantified by Basic Quantifier software. Bars indicate the S.D. of three independent experiments.

ing evolution, as well as between tandem C2 domains (Fig. 1). When the PYVK sequence of the C2A domain of Syt II was deleted, Ca^{2+} -dependent phospholipid binding activity was completely abolished [14]. Therefore, the PYVK sequence of the C2B domain is also expected of being involved in certain Ca^{2+} -dependent processes, although the C2B domain of Syt I or II itself did not show Ca^{2+} -dependent phospholipid binding activity [12]. Since most ligands bind the C2B domain in a Ca^{2+} -independent manner [12,20,24,27], we first attempted to determine whether the Y312N mutation of mouse Syt II, corresponding to the *Drosophila* AD3 mutation, affects a Ca^{2+} -independent process, i.e. Ca^{2+} -independent IP_4 binding to the mutant C2B domain. However, since the mutant Syt II proteins still bound IP_4 well (data not shown), we then investigated a possible involvement of the conserved Tyr in a Ca^{2+} -dependent process, i.e. Ca^{2+} -dependent self-oligomerization properties [28–34].

3.2. Essential role of Tyr-312 in Ca^{2+} -dependent self-oligomerization of mouse Syt II

As shown in Fig. 1 (bottom), we produced two Syt II mutants: a Y312N mutant corresponding to the *Drosophila* AD3 mutation and a KQ mutant, as a negative control, with a mutated C2B effector domain located at the β -4 strand [16,31]. This KQ mutant impairs AP-2 binding, IP_4 binding, and self-oligomerization activity [16,31,49]. The Ca^{2+} -dependent self-oligomerization activities were assayed by co-transfection of T7- and FLAG-Syts as described previously [33,46]. Briefly, T7- and FLAG-Syt II-cyto were co-expressed in COS-7 cells, and the association between FLAG-Syt and T7-Syt immunoprecipitants was evaluated by immunoblotting. The self-oligomerization activity of wild-type Syt II was activated by $500 \mu\text{M}$ Ca^{2+} (approximately a 2-fold increase; Fig. 2A,B). By contrast, both Y312N (AD3) and KQ mutants completely abolished the Ca^{2+} -activated self-oligomerization activity, and the Ca^{2+} -independent self-oligomerization activity was also dramatically reduced (Fig. 2A, lanes 5–8). The lack of Ca^{2+} -dependent self-oligomerization of these mutants was not due to the protein stability or protein expression levels, because equivalent amounts of proteins were recovered in cell lysates

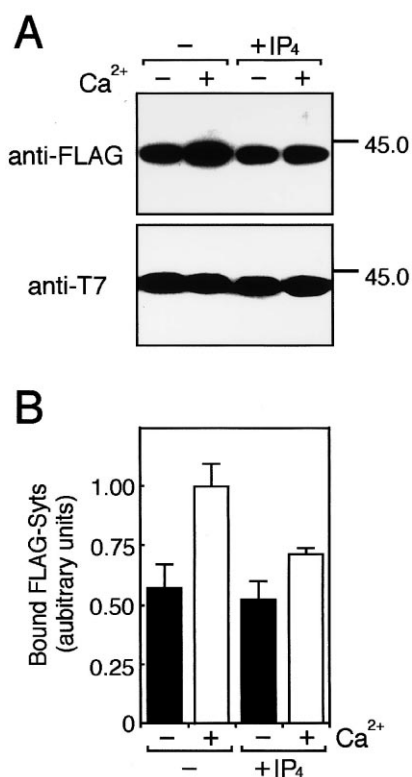


Fig. 3. Effect of IP_4 on self-oligomerization of mouse Syt II. pEF-T7-Syt II-cyto and pEF-FLAG-Syt II-cyto were co-transfected into COS-7 cells. A: Expressed proteins were solubilized with 1% Triton X-100 and immunoprecipitated with anti-T7 tag antibody-conjugated agarose in the presence and absence of $250 \mu\text{M}$ Ca^{2+} and/or $10 \mu\text{M}$ IP_4 [46]. Co-immunoprecipitated FLAG-Syts were first detected by anti-FLAG rabbit antibody ($5 \mu\text{g}/\text{ml}$; upper panel). Then, the same blot was stripped and reprobed with HRP-conjugated anti-T7 tag antibody to ensure that the same amounts of T7-tagged proteins were loaded ($1/1000$ dilution; lower panel). The positions of the molecular weight markers ($\times 10^{-3}$) are shown on the right. B: Immunoreactive bands were captured by Gel Print 2000i/VGA and quantified by Basic Quantifier software. Bars indicate the S.D. of two or three independent experiments.

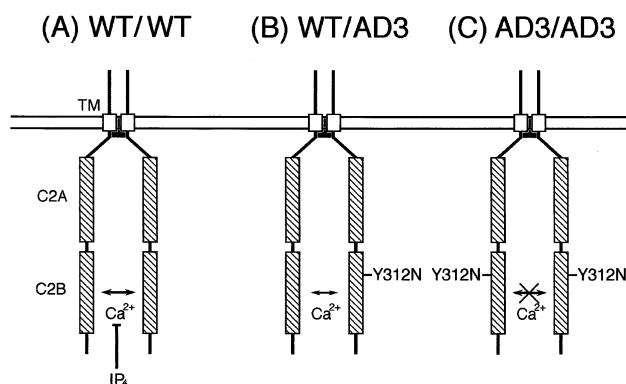


Fig. 4.

when compared to wild-type proteins (data not shown). Interestingly, however, the interaction of the AD3 mutant with wild-type proteins was significantly activated by Ca^{2+} (approximately a 2-fold increase; Fig. 2A, lanes 3 and 4). This finding indicates that the Y312N mutant can partially interact with the intact C2B domain of other mutant proteins, which is consistent with the fact that the AD3 allele can complement other mutant phenotypes [43,44]. Therefore, one attractive explanation for the ability of the AD3 allele to compensate other mutant phenotypes is that the C2B domain carrying the AD3 mutation can only act as an acceptor for Ca^{2+} -dependent oligomerization.

We recently showed that Ca^{2+} -dependent self-oligomerization mediated by the Syt II C2B domain can occur efficiently when the two molecules are tethered at the N-terminal domain (i.e. Ca^{2+} -independent oligomerization) [33]. Since the full-length Y312N mutant Syt II proteins normally associate with wild-type proteins at the N-terminal domain (data not shown), Ca^{2+} -dependent oligomerization of the AD3 mutant with the wild-type C2B domain is expected to occur more efficiently than shown in Fig. 2, where the Syt II cytoplasmic domain was used (see also Fig. 4).

3.3. Inhibition of self-oligomerization of mouse Syt II by IP_4

We previously identified Syts I and II as IP_4 binding proteins in the central nervous system [12,50] and have shown that binding of IP_4 to their C2B domains blocks transmitter secretion in squid giant synapses, superior cervical ganglion neurons, and chromaffin cells [38–40]. However, the detailed molecular mechanism remains largely unknown (i.e. disruption of specific protein–C2B domain interaction by IP_4). To examine the possible involvement of IP_4 in self-oligomerization, we measured the self-oligomerization activity of Syt II-cyto in the presence and absence of $10 \mu\text{M}$ IP_4 . As shown in Fig. 3, IP_4 significantly reduced the Ca^{2+} -dependent oligomerization activity, whereas Ca^{2+} -independent oligomerization activity was unaffected. Therefore, it is likely that the blocking of transmitter secretion by IP_4 is attributable to disruption of self-oligomerization of the C2B domain.

3.4. Conclusion

In the present study, we first found that the Y312N mutant of Syt II, which corresponds to the *Drosophila* AD3 mutation, impairs Ca^{2+} -dependent self-oligomerization activity, but that it can act as an acceptor for Ca^{2+} -dependent hetero-oligomerization with wild-type protein, indicating that the abnormal

transmitter release in the AD3 *Drosophila* mutant probably results from a lack of self-oligomerization capacity (summarized in Fig. 4). Although almost all of the C2B ligands require the C2B effector domain (Lys cluster), we speculate that the Y312 of Syt II is a specific residue necessary for Ca^{2+} -dependent self-oligomerization but not for the binding of other Ca^{2+} -independent ligands, including IP_4 . We also found that the Ca^{2+} -dependent C2B self-oligomerization was inhibited by IP_4 , a potent inhibitor of neurotransmitter release [51]. All of these findings strongly support the idea that self-oligomerization of Syts I and II is essential for neurotransmitter release in vivo.

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