Inferring synaptotagmin function from C2 domain stability: Analysis of structurally defined mutations in synaptotagmin 1 C2A

K L. Fuson[1], Kristofer Knutson[2], Anil Bhatta[3], Greg Gillispie[4], Candace Lange[4], Anne Hinderliter[2], **R. Bryan Sutton**[1].

[1]The University of Texas Medical Branch, Galveston, TX, USA, [2]The University of Minnesota at Duluth, Duluth, MN, USA, [3]Minnesota St. University, Moorhead, MN, USA, [4]Fluorescence Innovations, Bozeman, MT, USA.

Introduction

Synaptotagmin 1 (Syt1) triggers the release of neurotransmitter from docked synaptic vesicles through its interactions with Ca^{+2}, phospholipid and the SNARE complex. The protein is composed of two tandem C2 domains (C2A-C2B). Based on structural and biochemical evidence from the isolated domains, it is assumed that each domain is independent and binds Ca^{+2}/phospholipid with similar affinities. However, our new X-ray crystal structure of human Syt1 C2A+C2B has uncovered an extensive set of inter-C2 domain interactions, which suggest that inter-domain regulation could occur. Our structure also shows that highly conserved residues in C2A can modulate the shape of the Ca^{+2}/phospholipid binding Loop "3" of C2A in the presence of C2B. In our "closed" form of Syt1, interactions between His237 in C2A and Thr307 in C2B break a H-bond between His237 and Tyr180. This causes Loop 3 to collapse.

This locus has also been examined by genetic analysis in *Drosophila*. The "AD3" mutation in C2B (Y311N) eliminates Ca^{+2}-dependent exocytosis in *Drosophila (1)*, but a biophysical explanation for this observation has been lacking. Tyr311 in *Drosophila* Syt C2B is homologous to the Tyr180 residue in C2A. We tested whether the disruption of this single H-bond can affect the shape of the Ca^{+2} binding pocket of C2A by causing loop 3 to collapse.

Materials and methods

Materials

1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC or 16:0,18:1PC) and 1- palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine (POPS or 16:0,18:1PS) were purchased from Avanti Polar Lipids, Inc. Potassium chloride and MOPS were trace select grade from Sigma Aldrich. Chelex-100 ion-exchange resin was from Bio-Rad Labs. Chloroform, methanol and benzene were HPLC-grade.

Protein Expression and Purification.

Human synaptotagmin 1 C2A-C2B was obtained via PCR from a human hippocampal Quick-Clone cDNA library (Clontech). The cytosolic fragment of human synaptotagmin 1, containing only the cytosolic C2A and C2B, was overexpressed in *Escherichia coli* as a GST fusion protein and purified.

Preparation of LUV:

Large unilamellar vesicles (LUVs) were prepared with a POPC:POPS ratio of 3:2 by mixing the appropriate aliquots of lipids, drying the solution under a stream of argon, and vacuum desiccating the mixture overnight. The resulting film was hydrated in half the volume required with a buffer of 2mM MOPS 100mM KCL buffer, pH 7.4, and extruded through a polycarbonate filter with a 0.1 µm pore diameter using a hand-held Mini-Extruder. Lipids were extruded at a total concentration of 80 or 150 mM and then diluted for use.

Methods:

Fluorescence Lifetime analysis was conducted on a fluorescence lifetime analyzer produced by Fluorescence Innovations Inc, Bozeman MT. The wild-type C2A, C2A_Y180F, and C2A_Y180N proteins were aliquoted to a concentration of 3µM in 1.5 ml of 2mM MOPS 100mM KCL buffer. The solutions were mildly vortexed upon addition of protein, Ca^{+2} stock solution, and 60 POPC:40 POPS lipids. The denaturation curves were conducted for each protein in four different conditions: with 500µM EGTA, with 200µM 60 POPC:40 POPS, with 50µM Ca^{+2}, and with 200µM 60 POPC:40 POPS lipid in combination with 50µm Ca^{+2}. Temperatures were adjusted in two degree increments with time allowed for each setting to adjust to the temperature setpoint and scanned at 340 and 360nm for fluorescence lifetime. The lifetime waveforms were integrated to get fluorescence intensity at each wavelength.

Results

Figure 1: Stereoview of the interaction between C2A and C2B (2).

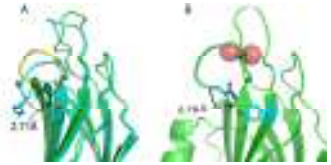

Figure 2: A) superposition of isolated C2A (green) with C2A from the C2AB structure (blue). B) Y311 in C2B

We collected structural information on mutations at the AD3 locus in C2A: Y180F and Y180N. We crystallized the Y180F mutation; however, the Y180N protein was difficult to manipulate.

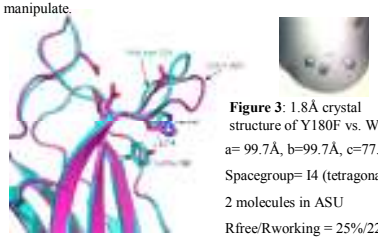

Figure 3: 1.8Å crystal structure of Y180F vs. WT

a= 99.7Å, b=99.7Å, c=77.2Å

Spacegroup= I4 (tetragonal)

2 molecules in ASU

Rfree/Rworking = 25%/22%

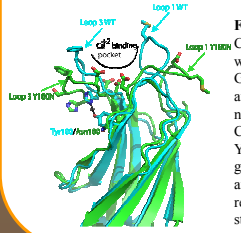

Figure 4: 1 ms simulation of C2A Y180N. Simulation was performed using GROMACS with 31,000 atoms (protein + water + neutralizing ions. Wild-type C2A is shown in blue. Y180N mutation shown in green. Ca^{+2} binding residues and lipid-associating residues are shown as green sticks in the simulation.

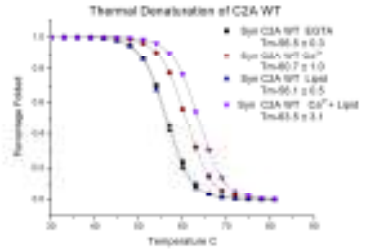

Figure 5A: Lifetime fluorescence denaturation profile of wild-type human Syt1 C2A

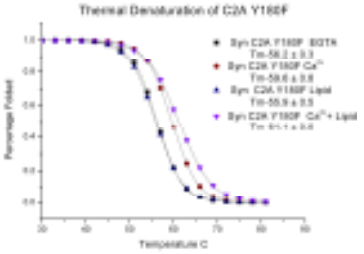

Figure 5B: Lifetime fluorescence denaturation profile of Y180F human Syt1 C2A

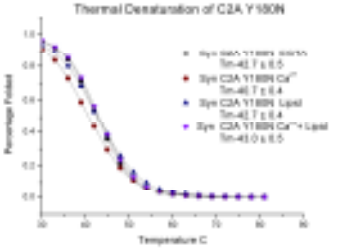

Figure 5C: Lifetime fluorescence denaturation profile of Y180N human Syt1 C2A

Conclusions

Our recent X-ray structure of Syt1 C2A+C2B demonstrated that the positioning of the C2B domain can form interactions to collapse the Ca^{+2} binding loops of C2A. Our hypothesis is that this could be a novel control feature in the C2 domain that allows one domain to bind Ca^{+2}/phospholipid, while restricting the activity of the other. To test this idea, we examined the "AD3" position in the C2A domain by mutagenesis, x-ray crystallography, molecular dynamics and lifetime fluorescence. We conclude that the "AD3" locus is a structurally important amino acid, and it can serve as a regulatory point to modulate the shape of the Ca^{+2}/phospholipid binding pocket of C2A.

We found that both the ability to form H-bonds directly with loop 3 and the steric bulk of the residue contributes to this role in the C2 domain.

While the Y180F mutation alone does not appear to adversely affect the stability of the domain, it does result a measurable change in in the position of Loop 3. On the other hand, if we eliminate both the steric bulk and the H-bonding potential at this position, the entire domain destabilizes.

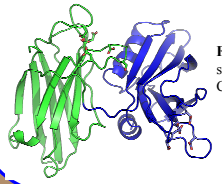

Figure 6: 2.7Å crystal structure of human Syt1 C2AB (*2*).

Literature cited

(1) Littleton, J. T., Bai, J., Vyas, B., Desai, R., Baltus, A. E., Garment, M. B., Carlson, S. D., Ganetzky, B., and Chapman, E. R. (2001) Synaptotagmin mutants reveal essential functions for the C2B domain in Ca^{+2}-triggered fusion and recycling of synaptic vesicles in vivo, *J Neurosci 21*, 1421-1433.

(2) Fuson, K. L., Montes, M., Robert, J. J., and Sutton, R. B. (2007) *Biochemistry* **46**(45), 13041-13048

(3) Kertz, J. A., Almeida, P. F., Frazier, A. A., Berg, A. K., and Hinderliter, A. (2007) *Biophys J* **92**(4), 1409-1418

For further information

Email: rbsutton@utmb.edu

PDF version of this poster is available on: http://www.ePoster.net/