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Cloning, expression, purification, crystallization and preliminary X-ray diffraction crystallographic study of human synaptotagmin 5 C2A domain

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Synaptotagmin acts as the Ca^{2+} sensor for neural and endocrine exocytosis. Synaptotagmin 5 has been demonstrated to play a key role in the acquisition of cathepsin D and the vesicular proton ATPase and in Ca^{2+}-dependent insulin exocytosis. The C2 domains modulate the interaction of synaptotagmin with the phospholipid bilayer of the presynaptic terminus and effector proteins such as the SNARE complex. This study reports the cloning, expression in *Escherichia coli*, purification, crystallization and preliminary X-ray analysis of the C2A domain of human synaptotagmin 5 with an N-terminal His_6 tag. The crystals diffracted to 1.90 Å resolution and belonged to the hexagonal space group $P6_5$, with unit-cell parameters $a = b = 93.97$, $c = 28.05$ Å. A preliminary model of the protein structure has been built and refinement of the model is ongoing.

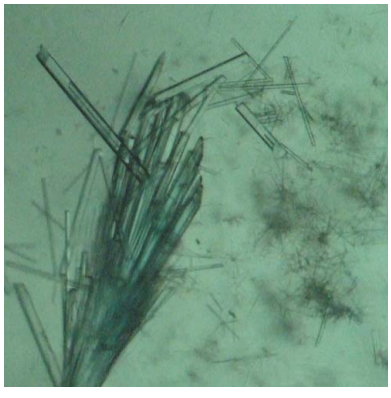
1. Introduction

Early studies of synaptic exocytosis demonstrated that Ca^{2+} floods into the synapse before the propagation of an action potential, implying that there is a Ca^{2+} sensor on the presynaptic terminus. Calmodulin, protein kinase C (PKC) and annexins have all been suggested as possible candidates for the Ca^{2+} trigger of exocytosis (Pollard *et al.*, 1980; Hutton, 1986; DeLorenzo, 1982). More recently, however, strong evidence has accumulated that points to the synaptic vesicle protein synaptotagmin (syt) as the receiver for the Ca^{2+} signal in the neuron (Südhof, 2004; Chapman, 2002). This protein belongs to a family of integral membrane proteins that are predominantly found on vesicles in neural and endocrine tissues (Südhof, 2002) and could potentially transmit the Ca^{2+} signal through its interactions with the SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor; SNAP receptor) complex. Vesicles could then fuse with the plasma membrane and release their contents.

Syt is composed of a short intravesicular NH_2-terminal region, a single membrane-spanning domain, a lysine- and arginine-rich region and two homologous C2 domains which are associated by a short flexible linker. The C2 domain was first characterized in conventional PKC isoforms and has been implicated in Ca^{2+}-dependent phospholipid binding (Parker *et al.*, 1986). The C2 domain of syt has been shown to bind Ca^{2+} and regulate phospholipid membrane fusion (Hui *et al.*, 2005; Bowen *et al.*, 2005).

17 isoforms of the syt family have been identified in mammals. These isoforms are distributed in various tissues and regulate different vesicle–membrane fusion processes. Syt 5 has been demonstrated to function as an exocytosis regulator which is recruited to the nascent phagosome and remains associated throughout the maturation process to control the acquisition of cathepsin D and the vesicular proton ATPase (Vinet *et al.*, 2009). It is restricted to glucagon-producing islet α-cells and controls Ca^{2+}-dependent insulin exocytosis (Saegusa *et al.*, 2002; Iezzi *et al.*, 2004).

The structures of the isolated and tandem C2 domains of several isoforms of syt have been revealed and the Ca^{2+}-binding sites within the Ca^{2+}-binding pockets have been identified in some of these (Sutton *et al.*, 1995, 1999; Shao *et al.*, 1997; Cheng *et al.*, 2004; Fuson *et*



al., 2007; Vrljic *et al.*, 2010; Xue *et al.*, 2010). Although syt isoforms are highly similar in sequence and structure, the structural basis of their different regulatory functions has not been elucidated. With the view of understanding differences in the regulation of vesicle–membrane fusion among syts and the acquisition of phagosome, microbicidal and Ca^{2+}-dependent insulin exocytosis, we have carried out preliminary X-ray diffraction analysis of the first C2 domain of syt 5 (syt 5 C2A domain) in preparation for comparative structural analysis.

2. Materials and methods

2.1. Cloning, expression and purification

The cDNA of human syt 5 C2A domain (residues 102–242), which was obtained *via* PCR from a human brain tissue cDNA library, was cloned into p28 (derived from pET28a, Novagen) excised using *Nde*I and *Xho*I to create a recombinant syt 5 C2A domain with an N-terminal hexahistidine tag (MGHHHHHHM). The sequence of the cDNA of the human syt 5 C2A domain was verified by DNA sequencing. A single colony of *Escherichia coli* Rosetta (DE3) (Novagen) cells harbouring the expression vector was cultured in 16 ml Luria–Bertani broth overnight and was then used to inoculate 1.6 l medium containing 50 µg ml^{-1} kanamycin. Cells were grown at 310 K for 2.5 h until an $OD_{600\,nm}$ of 0.5 was reached; protein expression was then induced for 3 h with 0.2 m*M* isopropyl β-D-1-thiogalactopyranoside (IPTG) at the same temperature. The cells were collected and resuspended in 50 ml binding buffer (20 m*M* Tris–HCl pH 8.0, 500 m*M* NaCl). After disrupting the cells by sonication, they were centrifuged at 15 200*g* for 0.5 h. The recovered clean lysate supernatant was loaded onto Ni–NTA agarose (GE Healthcare) resin pre-equilibrated with binding buffer. The tagged protein was eluted with 30 ml binding buffer including 500 m*M* imidazole. The protein was then concentrated to 1 ml for further purification using Superdex 75 (GE Healthcare) gel-filtration chromatography. The retention volume corresponding to the target protein indicated that it is a monomer in solution. Fractions corresponding to the peak were pooled and concentrated to 34 mg ml^{-1} using a 3 kDa cutoff Amicon centrifugal ultrafilter concentrator (Millipore). Examination of the purified protein by SDS–PAGE revealed a single band that corresponded to the expected molecular weight. Protein concentration was measured with the BCA Protein Assay Kit (Pierce).

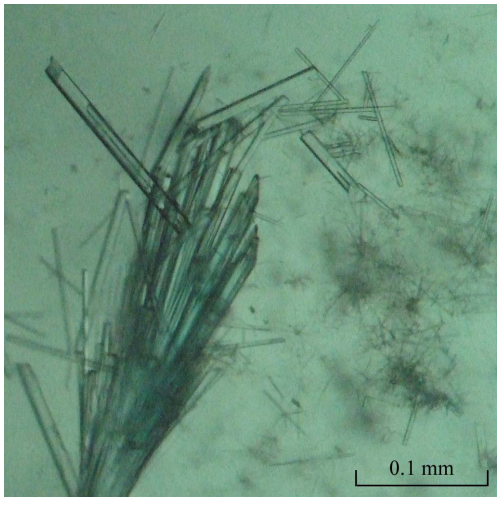


Figure 1
Crystals of the syt 5 C2A domain as grown in 0.1 *M* calcium acetate, 0.1 *M* sodium acetate pH 5.6, 12.5%(*w*/*v*) PEG 4000 and used in the diffraction experiment.

Table 1
Data-collection statistics for the syt 5 C2A domain crystal.

Values in parentheses are for the highest resolution shell.

Space group	$P6_5$
Wavelength (Å)	1.5418
Unit-cell parameters (Å)	$a = b = 93.97$, $c = 28.05$
Resolution (Å)	35.00–1.90 (1.93–1.90)
Total No. of observations	138485
No. of unique reflections	11445
Completeness (%)	99.9 (99.1)
Average $I/\sigma(I)$	33.9 (5.7)
R_{merge}†	0.079 (0.425)

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the observed intensity and $\langle I(hkl)\rangle$ is the mean intensity of reflection *hkl*.

2.2. Crystallization

Preliminary screening for initial crystallization conditions was performed by the hanging-drop vapour-diffusion method using ProPlex (Molecular Dimensions) at 287 K by mixing 1 µl 34 mg ml^{-1} protein solution with an equal volume of reservoir solution in 24-well plates. Small needle-shaped crystals were obtained from the condition 0.1 *M* calcium acetate, 0.1 *M* sodium acetate pH 4.5, 10%(*w*/*v*) PEG 4000. Conditions were further optimized using various concentrations of PEG 4000 *versus* a pH range of 4.0–5.6 in the presence or absence of 0.1 *M* calcium acetate. Crystals of good diffraction quality (Fig. 1) appeared in about 20 d from 0.1 *M* calcium acetate, 0.1 *M* sodium acetate pH 5.6, 12.5%(*w*/*v*) PEG 4000.

2.3. X-ray diffraction data collection and processing

Before data collection, the crystals were soaked for a short time in a cryoprotectant solution consisting of 25%(*v*/*v*) PEG 300, 0.1 *M* calcium acetate, 0.1 *M* sodium acetate pH 5.6, 12.5%(*w*/*v*) PEG 4000 and flash-cooled in a nitrogen stream at 100 K. X-ray diffraction data were collected using Cu $K\alpha$ X-rays generated by a Rigaku MicroMax-007 rotating-anode generator (Rigaku) equipped with a MAR345dtb 34.5 cm diameter image-plate detector (MAR Research). The crystal-to-detector distance was kept at 150 mm and the crystal was rotated through a total of 229° with 1° rotation per frame over 15 min. The crystal diffracted to a resolution of 1.90 Å. All data were indexed, integrated and scaled with *HKL*-2000 (Otwinowski & Minor, 1997). Analysis of the systematic absences in the diffraction data narrowed the possible choice of space group to either $P6_5$ or $P6_1$. Inspection of the molecular-replacement solution obtained using *MOLREP* (Vagin & Teplyakov, 2010) from the *CCP*4 suite (Winn *et al.*, 2011) confirmed the space group to be $P6_5$. The final statistics of data collection and processing are tabulated in Table 1.

3. Results and discussion

The C2A domain of syt 5, an exocytosis regulator from *Homo sapiens*, was successfully cloned in *E. coli* and purified to homogeneity with an N-terminal His_6 tag. The molecular weight of 17.15 kDa for monomeric His_6-tagged syt 5 C2A domain predicted from the sequence was confirmed by 12% SDS–PAGE. The protein was crystallized using 0.1 *M* calcium acetate, 0.1 *M* sodium acetate pH 5.6, 12.5%(*w*/*v*) PEG 4000.

Crystals of syt 5 C2A diffracted to a maximum resolution of 1.90 Å (Fig. 2) and belonged to the hexagonal space group $P6_5$, with unit-cell parameters $a = b = 93.97$, $c = 28.05$ Å. The resulting R_{merge} was 7.9% overall and the mosaicity of the crystal was 0.56–0.70°. The calculated Matthews coefficient and solvent content (Matthews, 1968) were

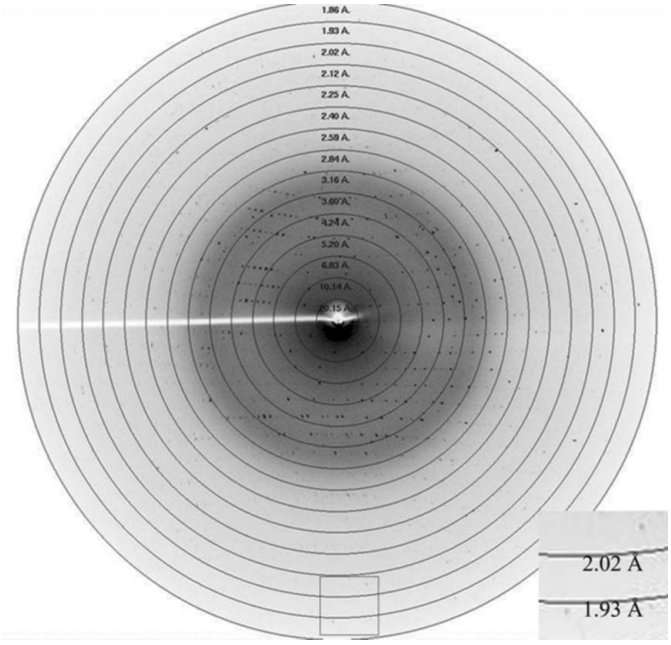


Figure 2
Diffraction image of a typical crystal of the syt 5 C2A domain.

2.08 Å^3 Da^{-1} and 41.01%, respectively, assuming the presence of one molecule per crystal asymmetric unit. An initial model for the structure was obtained using the crystal structure of the syt 1 C2A domain (PDB entry 3f04; F. Guo, R. Dakshinamurthy, S. K. K. Thallapuranam & J. Sakon, unpublished work; 64.7% amino-acid sequence identity) *via MOLREP* (Vagin & Teplyakov, 2010). The resulting R factor decreased to 25.6% and the $R_{\rm free}$ factor was improved to 33.5% after refinement in the resolution range 30.76–1.90 Å. Final model building by restrained refinement using *REFMAC*5 (Murshudov *et al.*, 2011) and manual refinement using *Coot* (Emsley & Cowtan, 2004) is currently in progress.

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