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NMR characterization of copper and lipid interactions of the C2B domain of synaptotagmin I—relevance to the non-classical secretion of the human acidic fibroblast growth factor (hFGF-1)

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ABSTRACT

Human fibroblast growth factor (hFGF-1) is a ~17 kDa heparin binding cytokine. It lacks the conventional hydrophobic N-terminal signal sequence and is secreted through non-classical secretion routes. Under stress, hFGF-1 is released as a multiprotein complex consisting of hFGF-1, S100A13 (a calcium binding protein), and p40 synaptotagmin (Syt1). Copper (Cu^{2+}) is shown to be required for the formation of the multiprotein hFGF-1 release complex (Landriscina et al. ,2001; Di Serio et al., 2008). Syt1, containing the lipid binding C2B domain, is believed to play an important role in the eventual export of the hFGF-1 across the lipid bilayer. In this study, we characterize Cu^{2+} and lipid interactions of the C2B domain of Syt1 using multidimensional NMR spectroscopy. The results highlight how Cu^{2+} appears to stabilize the protein bound to pS vesicles. Cu^{2+} and lipid binding interface mapped using 2D ^{1}H–^{15}N heteronuclear single quantum coherence experiments reveal that residues in β-strand I contributes to the unique Cu^{2+} binding site in the C2B domain. In the absence of metal ions, residues located in Loop II and β-strand IV contribute to binding to unilamelar pS vesicles. In the presence of Cu^{2+}, additional residues located in Loops I and III appear to stabilize the protein-lipid interactions. The results of this study provide valuable information towards understanding the molecular mechanism of the Cu^{2+}-induced non-classical secretion of hFGF-1.

1. Introduction

The human acidic fibroblast growth factor (hFGF-1) [1,2], a potent mitogen, is a ubiquitously expressed member of the FGF family [3–7]. hFGF-1 is a ~17-kDa all-β-sheet protein and is involved in the regulation of a wide variety of important cellular processes such as angiogenesis, morphogenesis, inflammation, tumor growth, and wound healing [8]. Interestingly, unlike most secreted proteins, hFGF-1 lacks the N-terminal signal sequence and therefore is secreted through routes that are independent of the classical endoplasmic reticulum (ER)–Golgi pathway [9].

The precise mechanism for the secretion of the signal peptide-less proteins is not completely understood. Several studies show hFGF-1 is released in response to stresses such as heat shock, hypoxia, cultivation under low serum conditions, and cell treatment with low-density lipoproteins (LDLs) [10–13]. Jackson et al. [11] demonstrated that the formation of FGF-1 homodimer is a prerequisite for its release in response to heat shock and hypoxia. Homodimer formation is facilitated by copper (Cu^{2+})-induced oxidation of a specific cysteine residue (Cys30) in hFGF-1. Cu^{2+} has been demonstrated to be required for the assembly of multiprotein hFGF-1 release complex. Prudovsky et al. [2,14], investigating the spatio-temporal characteristics of the non-classical release of hFGF-1 using real-time confocal Microscopy, showed the formation of a multiprotein hFGF-1 release complex near the inner surface of the plasma membrane [14]. The proteins assembled in the multiprotein hFGF-1 release complex include the FGF-1 homodimer, S100A13 (a calcium binding protein), and the p40 form of synaptotagmin, a protein involved in secretory vesicle docking [15,16]. More recently, the non-classically secreted enzyme sphingosine kinase 1 is also suggested to be a part of the hFGF-1 release complex [10,17–20]. However, to date, details of the molecular events leading to the formation of multiprotein FGF-1 release complex, as well as the mechanism underlying the export of this growth factor to the extracellular compartment is still an enigma.

Synaptotagmin-1 (Syt-1) is a 65 kDa synaptic vesicle protein containing a short intravesicular N-terminus, a single-pass transmembrane domain, and a C-terminal extravesicular cytosolic portion including two calcium binding C2 (C2A and C2B) domains (Fig. 1)

Abbreviations: hFGF-1, human fibroblast growth factor-1; Syt1, Synaptotagmin 1; HSQC, Heteronuclear Single Quantum Coherence; NMR, nuclear magnetic resonance

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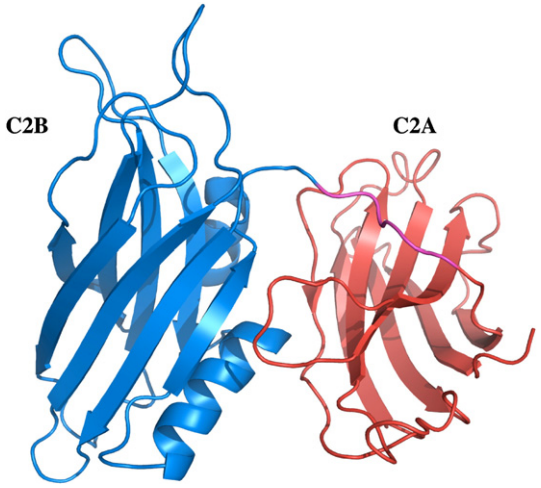

Fig. 1. Three-dimensional structure of the C2A (orange) and C2B (blue) domains of Syt1. The linker region connecting the C2 domains is shown in magenta. The structure was generated using the pdb accession code 2R83.

[21–26]. Both C2A and C2B domains bind to calcium (Ca^{2+}) and partially penetrate into the lipid bilayer, and the interactions between C2A, C2B and Ca^{2+} are believed to be critical for the membrane fusion activity of Syt-1. The 40-kDa form of synaptotagmin (p40 Syt1) represents a product of the alternative in-frame initiation of synaptotagmin mRNA translation [27–29]. It lacks the intravesicular and transmembrane domains and corresponds to the extravesicular domain of p65 Syt1. p40 Syt 1 is a member of the FGF1 export complex which is critically important for stress-induced nonclassical FGF1 release [19,30]. Although, the exact role of the C2 domains of Syt-1 in the non-classical secretion of FGF-1 is still unclear, it is believed that these lipid-binding domains are important for anchoring the multi-protein hFGF-1 release complex to the cell membrane. Indeed, mutation of lysines 326, 327 and 331 in C2B domain drastically reduced the membrane destabilizing activity of p40 Syt1 and abolished its nonclassical export [31].

The C2B domain has a β-sandwich structure formed by two layers of four antiparallel β-strands. Unlike the C2A domain, which lacks helical segments, the C2B domain contains two α-helices (Fig. 1) [32]. The helical segment located at the C-terminal end of the molecule is tightly packed against residues in β-strands III, VI, and VII [32,33]. A stretch of negatively charged residues in the C-terminal helix is in close proximity to a patch of positively charged residues that are implicated in C2B domain's interaction with other proteins. The striking dipolar distribution of charged residues on one side of the molecule is a characteristic feature of the structure of the C2B domain. Two distinct Ca^{2+} binding sites have been characterized in the C2B domain [32]. However, additional calcium binding sites could not be ruled out in the presence of interacting protein or lipid components [4,15,33–35]. Although the Ca^{2+}-dependent lipid binding affinity of the C2B domain has been well characterized, very little is known about the structural role of this domain in the Cu^{2+}-mediated non-classical secretion of hFGF-1. In this study, we investigated the influence of metal ions (Ca^{2+} and Cu^{2+}) on the structural stability and lipid binding affinity of the C2B domain. In addition, the lipid-binding interface on the C2B domain, in the absence and presence of the metal ions, has been characterized using multidimensional NMR spectroscopy. The results of this study clearly show that Cu^{2+} binds to distinct sites and significantly influences the lipid binding affinity of the C2B domain.

2. Materials and methods

Ingredients for Luria Broth were obtained from EMD Biosciences. Aprotinin, pepstatin, leupeptin, phenylmethylsulfonyl fluoride, triton X-100, terbium chloride, and β-mercaptoethanol were obtained from Sigma Co. (St. Louis). Heparin sepharose and glutathione sepharose were obtained from GE Healthcare. Labeled $^{15}NH_4Cl$ and D_2O were purchased from Cambridge Isotope Laboratories. All other chemicals used were of high quality analytical grade. All experiments were performed at 25 °C. Unless specified, all solutions were made in 10 mM Tris buffer (pH 7.5) containing 100 mM NaCl.

2.1. Expression and purification of the C2B domain

cDNA encoding the C2B domain of synaptotagmin I (residues 270 to 421) and p40 Syt1 was kindly provided by Professor Thomas Sudhof. *Escherichia coli* expressing GST-tagged C2B were induced with isopropyl-β-D-thiogalactopyranoside (IPTG) when absorbance (at 600 nm) reached 0.5–0.6, and the cells were harvested by centrifugation at 6000 rpm after 4 h of induction. The harvested cells were resuspended, and cell walls were ruptured by sonication. The cell lysate was centrifuged at 16,000 rpm for 20 min. The supernatant was then incubated with glutathione sepharose, and the resin was then extensively washed with Tris buffer saline (TBS, 20 mM Tris 150 mM NaCl) and 20 mM Tris containing 1M NaCl to remove impurities. The column was then equilibrated with cleavage buffer (20 mM Tris, 0.2 M NaCl, 2.5 mM $CaCl_2$, pH8.0), and an on-column thrombin cleavage was carried out (2 NIH units/mL) at 25 °C for 3 h. The cleaved protein (C2B) was eluted with TBS buffer as described by Ubach et al. [33]. The homogeneity of the protein was then assessed using SDS-PAGE, and the concentration of the protein was estimated on the [33] basis of the extinction coefficient ($\varepsilon = 19940\ M^{-1}\ cm^{-1}$) value calculated from the amino acid sequence of the C2B domain. Complete removal of nucleotide impurities was verified by recording UV spectrum of the protein.

2.2. Preparation of isotope-enriched C2B domain

Uniform ^{15}N labeling of C2B was achieved using M9 minimal medium containing $^{15}NH_4Cl$. To achieve maximal expression yields, the composition of the M9 medium was modified by the addition of a mixture of vitamins [36–37]. The expression host strain *E. coli* BL21 (DE3) is a vitamin B1-deficient host, and hence, the medium was supplemented with thiamine (vitamin B1) [36].

2.3. Preparation of the lipid vesicles

Small unilamellar vesicles (SUV) were prepared by dissolving the solid phospholipids (phosphatidyl serine (pS)) purchased from Avanti polar lipid, Inc.) in chloroform, followed by evaporation to dryness under nitrogen. The lipid film was suspended in 10 mM Tris buffer (pH 7.5) containing 100 mM NaCl and sonicated in an AQUASONIC-75D12 bath-type sonicator until optical clarity was obtained. The solution was finally centrifuged for 5 min at 14000 rpm in an Eppendorf microfuge before use and stored for a maximum of 6 h in ice.

2.4. NMR experiments

All NMR experiments were performed on a Bruker Avance-700 MHz NMR spectrometer equipped with a cryoprobe at 30 °C. ^{15}N decoupling during data acquisition was accomplished using the globally optimized altering phase rectangular pulse sequence, and 2048 complex data points were collected in the ^{15}N dimension. ^{1}H–^{15}N HSQC spectra were recorded at 32 scans. The concentration of the protein sample used was 0.1 mM in 90% H_2O and 10 % D_2O prepared in 50 mM MES buffer containing 150 mM NaCl and 2 mM DTT (pH 6.3). The spectra were processed on a Windows workstation using XWIN-NMR and Sparky software [38].