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Copper binding affinity of the C2B domain of synaptotagmin-1 and its potential role in the nonclassical secretion of acidic fibroblast growth factor

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ABSTRACT

Fibroblast growth factor 1 (FGF1) is a heparin-binding proangiogenic protein. FGF1 lacks the conventional N-terminal signal peptide required for secretion through the endoplasmic reticulum (ER)–Golgi secretory pathway. FGF1 is released through a Cu^{2+}-mediated nonclassical secretion pathway. The secretion of FGF1 involves the formation of a Cu^{2+}-mediated multiprotein release complex (MRC) including FGF1, S100A13 (a calcium-binding protein) and p40 synaptotagmin (Syt1). It is believed that the binding of Cu^{2+} to the C2B domain is important for the release of FGF1 into the extracellular medium. In this study, using a variety of biophysical studies, Cu^{2+} and lipid interactions of the C2B domain of Syt1 were characterized. Isothermal titration calorimetry (ITC) experiments reveal that the C2B domain binds to Cu^{2+} in a biphasic manner involving an initial endothermic and a subsequent exothermic phase. Fluorescence energy transfer experiments using Tb^{3+} show that there are two Cu^{2+}-binding pockets on the C2B domain, and one of these is also a Ca^{2+}-binding site. Lipid-binding studies using ITC demonstrate that the C2B domain preferentially binds to small unilamellar vesicles of phosphatidyl serine (PS). Results of the differential scanning calorimetry and limited trypsin digestion experiments suggest that the C2B domain is marginally destabilized upon binding to PS vesicles. These results, for the first time, suggest that the main role of the C2B domain of Syt1 is to serve as an anchor for the FGF1 MRC on the membrane bilayer. In addition, the binding of the C2B domain to the lipid bilayer is shown to significantly decrease the binding affinity of the protein to Cu^{2+}. The study provides valuable insights on the sequence of structural events that occur in the nonclassical secretion of FGF1.

1. Introduction

Fibroblast growth factors (FGFs) are β-sheet proteins (MW ~ 16 kDa) which participate in the regulation of key biological processes such as, cell proliferation, cell differentiation, angiogenesis, and wound healing [1–4]. FGFs exhibit their cellular functions by binding to specific cell surface tyrosine kinase receptors [1–4]. Therefore, they need to be released from the cells into the extracellular compartment [5]. Interestingly, two of the most ubiquitous members of the FGF family, FGF1 and FGF2, lack an N-terminal signal peptide that is required for the secretion of proteins through the classical endoplasmic reticulum (ER)–Golgi secretory pathway [6,7]. The exact mechanisms by which these FGFs are exported to the extracellular medium are still not clear. Previous studies have demonstrated that cells release FGF1 in response to stress conditions such as heat shock, hypoxia, growth factor starvation, and upon treatment with low-density lipoproteins. The ER–Golgi secretory pathway inhibitor Brefeldin A does not block the export of FGF1 and FGF2 [8,9]. However, studies using specific inhibitors have shown that the secretion of FGF1 and FGF2 is dependent of ATP and is not mediated by exocytosis [8,9]. A multiprotein release complex (MRC) involving FGF1, S100A13 and the 40 kDa form of synaptotagmin (Syt1) is formed at the inner leaflet of the cell membrane [10]. Sphingosine kinase (SphK1) was demonstrated to be an additional component of the FGF1

Abbreviations: FGF1, human fibroblast growth factor-1; Syt1, synaptotagmin-1; ITC, isothermal calorimetry; MRC, multiprotein release complex; PS, phosphatidyl serine; PC, phosphatidyl choline; PG, phosphatidyl glycerol; PE, phosphatidyl ethanolamine; ER, endoplasmic reticulum; SphK1, sphingosine kinase; TTM, tetrathiomolybdate; IPTG, isopropyl-β-d-thiogalactopyranoside; SUV, small unilamellar vesicles; MW, molecular weight

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MRC [11]. Similar to FGF1, S100A13, p40 Syt1 and SphK1 are released *via* the nonclassical protein secretion route, and all these proteins are critical for FGF1 export [11–13]. All members of FGF1 MRC including FGF1, bind copper with high affinity [11,14,15]. Studies using NIH3T3 cells have indicated that the stress-induced release of FGF1 is inhibited by the copper (Cu^{2+}) chelator, tetrathiomolybdate (TTM), suggesting that the Cu^{2+} plays a crucial role in the assembly of the multiprotein FGF release complex [11,15]. *In vitro* studies indicate that Cu^{2+} induces the formation of an FGF-dimer through formation of intermolecular disulfide bond formation involving Cys30 [14]. Rajalingam et al. showed that copper-induced FGF1 dimer formation is inhibited by amlexanox, an anti-inflammatory drug which inhibits FGF1 export [16]. However, the precise role of copper (Cu^{2+}) in the assembly of MRC is still unclear.

The 65 kDa form of Syt1 participates in the docking of the exocytotic vesicles to the cell membrane [17,18]. The extravesicular cytosolic portion of the Syt1 is located at its C-terminal end and contains two calcium-binding modules called the C2A and C2B domains [19,20]. Several elegant studies have characterized the lipid binding interactions of the C2A and C2B domains [21–29]. The C2A and C2B domains facilitate the penetration of FGF1 into the lipid bilayer and therefore play a critical role in the Syt1-mediated docking of exocytotic vesicles [23, 30–34]. Syt1 also exists as a 40 kDa form (p40 Syt1), in the cytosol [13,35]. This form represents a product of the alternative in-frame initiation of Syt1 mRNA translation [35]. Interestingly, unlike p65 Syt1, p40 Syt1 only contains the extravesicular portion and conspicuously lacks the intravesicular and transmembrane domains [13,35]. p40 Syt 1, but not p65 Syt1, is a constituent of the FGF1 MRC whose formation is critical for the release of FGF1 through the nonclassical pathway [35–37]. Although the precise role of the individual protein constituents of the FGF1 MRC is uncertain, it is believed that the C2A and C2B domains of p40 Syt1, due to their high lipid-binding affinity, play an important role in anchoring the FGF MRC to the cell membrane [38].

There has been increased interest in understanding the role of Cu^{2+} in the nonclassical export of signal peptide-less proteins such as, FGF1 and IL1α [5,10,11,16,38–40]. Competitive metal binding studies with S100A13 revealed that the protein binds to Cu^{2+} with micromolar affinity [40]. However, site-directed mutagenesis studies of the Cu^{2+}-binding residues in S100A13 showed that the mutation of the putative Cu^{2+}-binding residues did not significantly affect its interaction with FGF1. On the other hand, the C2 domains of p40 Syt1 have been shown to bind to Ca^{2+} with reasonably high affinity [41,42]. Interestingly, combined mutations of lysines 326, 327 and 331 significantly lowered the membrane destabilizing activity of p40 Syt1 and inhibited its nonclassical secretion [43]. It is believed that Cu^{2+} could potentially bind to the C2B domain of p40 Syt1 and influence the anchoring of FGF1 MRC to the lipid membrane. In addition, the three-dimensional structure of C2B shows a cluster of negatively charged residues in the C-terminal helix, which is in spatial proximity to a dense patch of positively charged residues. This arrangement of charged residues is shown to provide an interaction surface for ligand/protein binding [41,44]. In this context, herein, we examine the binding affinity of the C2B domain to Cu^{2+}. In addition, we also investigate the binding affinity of the C2B to lipid vesicles in the presence and absence of metals. The results of this study clearly show that the lipid-binding affinity of the C2B domain is significantly influenced by Cu^{2+}. They also suggest an important role of the C2B domain of p40 Syt1 in the nonclassical secretion of FGF1.

2. Methods

2.1. Protein expression, purification, and lipid vesicle generation

Bacterial expression and purification of the C2B domain of Syt1 were carried out as described in detail [45]. Briefly, cDNA encoding the C2B domain of synaptotagmin I (residues 270 to 421) and p40 Syt1 was kindly provided by Professor Thomas Sudhof. *Escherichia coli* transformed with plasmid coding for GST-C2B were induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and the cells were harvested by centrifugation after 4 h. After purification, the purity of the protein was assessed using SDS-PAGE, and the samples were subsequently concentrated.

Small unilamellar vesicles (SUV) were prepared as described previously [20]. Briefly, L-α-phosphatidylcholine (PC) from egg yolk, L-α-phosphatidylserine (PS) from bovine brain, L-α-phosphatidylethanolamine (PE) from egg yolk, and L-α-phosphatidyl glycerol were obtained from Avanti Polar Lipids Incorporated (Alabaster, AL). All samples were dissolved in chloroform, evaporated to dryness, and the lipid film was suspended in 10 mM tris buffer (pH 7.5) containing 100 mM NaCl and sonicated until optical clarity was obtained. After centrifugation for 5 min at 14,000 rpm, the samples were stored on ice for up to 6 h before using.

2.2. Steady-state fluorescence measurements

All fluorescence spectra were collected on a Hitachi F-2500 spectrofluorometer at 2.5 nm resolution, using an excitation wavelength of 280 nm at 25 °C. Fluorescence measurements were conducted at a protein concentration of 50 μM in 10 mM tris buffer (pH 7.5) containing 100 mM NaCl. Appropriate corrections were made for background noise. For terbium titrations, a stock solution of 50 mM $TbCl_3$ in 10 mM tris (pH 7.5), containing 100 mM NaCl, was prepared. The excitation wavelength was set at 280 nm, and bandwidths for excitation and emission were set at 2.5 and 10 nm, respectively. Appropriate background corrections were performed to correct for dilution and concentration-dependent inner filter effects. The final increase in volume of the solution, after Tb^{3+} titration, was less than 5% of the initial volume.

2.3. Isothermal titration calorimetry

Heat changes due to the binding of various ligands [Ca^{2+}, Cu^{2+}, or small unilamellar vesicles of PS, PG, or PC] to C2B were analyzed using a VP-ITC titration microcalorimeter (MicroCal Inc., Northampton, MA). Copper concentrations were estimated using a GBC 932 plus atomic absorption spectrometer. All protein and ligand solutions were degassed under vacuum and equilibrated prior to titration. The sample cell (1.4 mL) contained 0.08 mM C2B in 10 mM tris buffer (pH 7.5) containing 100 mM NaCl. All titrations were carried out in 10 mM tris buffer to avoid precipitation of the metal ions. ApoC2B was prepared by dialyzing against 10 mM tris (pH 7.5) buffer, containing 100 mM NaCl and EDTA, to chelate all bound metal ions. C2B protein was then extensively dialyzed against 10 mM tris (pH 7.5) buffer, containing 100 mM NaCl. Upon equilibration, 3 mM Ca^{2+} or Cu^{2+} was injected in 49 × 6 μL aliquots. Small unilamellar vesicles of PS, PG or PC were used to determine the lipid-binding affinity of the proteins (in the absence and presence of the Ca^{2+} or Cu^{2+}). The reaction cell contained the protein. PS, PG or PC vesicle suspensions containing the same buffer were added in serial injections of 6 μL. The concentration of the protein(s) in the cell was about 0.08 mM, whereas the total concentration of the lipids in the syringe was about 10 mM. The resulting titration curves were corrected for the protein-free buffer [10 mM tris (pH 7.5) containing 100 mM NaCl] and analyzed using the *Origin* software supplied by MicroCal Inc. The raw ITC data were individually fitted with different binding models (One Set of Sites, Two Sets of Sites, Multiple Sets of Sites and Sequential binding site models) using MicroCal Origin software provided by the vendor (GE Healthcare, Inc.). The appropriateness of the fitting model(s) was judged based on the χ^2 values of the fits. The binding model with the least χ^2 value was chosen as the best fit to represent the interaction.

2.4. Proteolytic digestion assay

Limited proteolytic trypsin digestion experiments on apoC2B and C2B bound to either Ca^{2+} or Cu^{2+} in the presence and absence of