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 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 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 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, 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.

Key Words

fibroblast growth factor, secretion, nonclassical, synaptotagmin, proteins, signal peptide, lipid binding.

Abbreviations used

FGF1 – human fibroblast growth factor-1; Syt1 – Synaptotagmin-1; ITC- isothermal calorimetry; MRC – Multiprotein Release Complex

Introduction

Fibroblast growth factors (FGFs) are β -sheet proteins (M.W ~ 16kDa) 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 a 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 40kDa 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 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 Syt1, 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 IL1 α [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 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.

Methods

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. *E. 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 hours. 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 from bovine brain, L- α -phosphatidylethanolamine (PE) from egg yolk, and L- α -phosphatidylglycerol were obtained from Avanti Polar Lipids Incorporate (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 14000 rpm, the samples were stored on ice for up to 6 hours before using.

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 μ M 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 TbCl₃ 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.

Isothermal titration calorimetry: Heat changes due to 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 $49 \times 6 \mu\text{L}$ aliquots. Small unilamellar vesicles of PS, PG or PC were used to determine the lipid-binding affinity of the proteins (in absence and in the 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 sites 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.

Proteolytic digestion assay: Limited proteolytic trypsin digestion experiments on apoC2B and C2B bound to either Ca^{2+} or Cu^{2+} in the presence and the absence of liposome (PS) were carried out at 25 ± 2 °C using trypsin (Sigma Chemical Co., St.Louis, MO). Proteolytic digestions were performed at a trypsin to substrate (C2B) molar ratio of 1:1.5. The protease activity was stopped after desired time intervals by the addition of trichloroacetic acid. The degree of proteolytic cleavage was measured from the intensity of the ~18 kDa band (on a SDS–PAGE gel) corresponding to the untreated C2B. The intensity of the C2B band ($M_w \sim 18$ kDa) intensity was measured by counting the number of pixels present in a box of a constant area using UN-SCANIT program. A constant-area box was moved horizontally across a Coomassie blue stained SDS-PAGE gel and the percentage of protein which is not degraded (at each time point), by trypsin, was measured by comparing with the intensity of the band corresponding to the untreated C2B ($M_w \sim 18$ kDa) domain

Differential scanning calorimetry: Thermal-induced unfolding was performed using a Nano III DSC (Calorimetry Sciences Corporation) fitted with a capillary cell. All samples were degassed for 2 min prior to loading, and filled cells were equilibrated for 10 min at 25 °C before the beginning of each scan. Thermograms of proteins (1.0 mg/mL) alone, and in the presence of metals/liposomes were obtained using a scan rate of 0.5 °C/min.

Before each experiment, buffer blank experiments were conducted to obtain a stable baseline. Thermograms of buffer-only scans were subtracted from each protein scan prior to analysis using software supplied by Calorimetry Sciences Corp. Reversibility of the thermal unfolding process was evaluated by repeated heating and cooling cycles in the temperature range of 10°C to 95°C. Non-superimposition of the unfolding and refolding curves/thermograms is considered as an indicator of the irreversibility of the thermal unfolding process.

Results and Discussion

The C2B domain of Syt1 binds to Ca^{2+} and is critical for triggering synchronous transmitter release in the nerve synapse [46]. Ca^{2+} binds to acidic residues located in the apical loop region of the C2B domain and facilitates intermolecular interaction(s) which are important for membrane fusion activity. Based on these data, and the vital role that copper ions play in nonclassical FGF1 export, it is important to determine the Cu^{2+} - binding affinity of the Syt1 C2B domain and examine if the lipid- binding properties are influenced by this metal ion [15].

C2B domain binds strongly to Cu^{2+} : Isothermal titration calorimetry (ITC) was used to measure the binding affinity, binding stoichiometry, and the thermodynamics of molecular interactions [40]. We examined the affinity of Cu^{2+} to C2B using ITC, and the binding isotherm characterizing the interaction of the C2B domain to Cu^{2+} shows two distinct binding modes (Fig. 1A). Unlike the typical sigmoidal isothermograms observed for most protein-ligand interactions, the binding curve characterizing Cu^{2+} - C2B domain interaction is somewhat bell-shaped. Similar complex isothermograms were observed for the binding of iron (Fe^{2+}) ions to ovotransferrin [47]. Independent fitting of the

exothermic and endothermic states of the titration curves yields a binding constant $K_{d(app)}$ value of $1.4 (\pm 0.14) \mu\text{M}$ for the endothermic phase (Fig. 1A, Table-1). The enthalpy change (ΔH) in this phase of interaction is $28 (\pm 0.3) \text{ kcal.mol}^{-1}$ (Table-1). About two Cu^{2+} - binding sites in the protein are saturated in the endothermic phase (Table-1). An exothermic phase occurs at lower molar ratios of the Cu^{2+} to protein, and is accompanied by small heat changes (ΔH , Table-1). The binding constant $K_{d(app)}$ signifying interactions in this phase is $21 (\pm 0.1) \mu\text{M}$. The exothermic phase appears to contribute to saturation of about two (~ 1.74) additional Cu^{2+} - binding sites. The ITC data show that the C2B domain binds strongly to about four Cu^{2+} ions in a biphasic manner (binding to two Cu^{2+} ions in each phase). The biphasic binding curve plausibly suggests that binding of two Cu^{2+} ions in the low affinity initial endothermic phase triggers a conformational change in the protein which further facilitates stronger binding of two additional Cu^{2+} ions at a new site(s) in the exothermic phase. Specificity of Cu^{2+} binding to C2B domain was confirmed by performing an appropriate control experiments using Mg^{2+} as negative control. (Fig. S1)

C2B has distinct binding sites for Cu^{2+} : Previous studies using sedimentation binding assays showed that the C2B domain binds weakly to membranes, with K_d values in the millimolar range [48]. Interestingly, the Ca^{2+} - binding affinity increases nearly 20-fold in the presence of phosphatidyl serine ($K_{d(app)} = 854 (\pm 5) \mu\text{M}$, [49]. To investigate further, we examined whether Ca^{2+} and Cu^{2+} share common binding sites on the C2B domain. ITC experiments were performed to examine if the binding of Ca^{2+} and Cu^{2+} to the C2B domain is mutually exclusive. An isothermogram representing the titration of the Ca^{2+} saturated - C2B domain with increasing concentrations of Cu^{2+} is almost

sigmoidal (Fig. 1B). The enthalpy change (ΔH) characterizing the binding of Cu^{2+} to the Ca^{2+} -saturated C2B domain is $\sim -34 \text{ kcal.mol}^{-1}$. Unlike the complex binding isotherm obtained for the titration of Cu^{2+} with the C2B domain, the metal interaction of the protein in the presence of Ca^{2+} occurs in a single phase with a $K_{d(\text{app})}$ value of $\sim 38 (\pm 0.5) \mu\text{M}$ (Table-1). In the presence of an excess of Ca^{2+} , there appears to be only one binding site for Cu^{2+} in the C2B domain (Table -1). The ITC results clearly show that C2B binds to Cu^{2+} and one of the two primary Cu^{2+} - binding sites on the C2B domain also binds Ca^{2+} . Therefore, there appears to be at least one site on the C2B domain that is exclusive for Cu^{2+} binding.

A lanthanide, terbium (Tb^{3+}), is a very valuable fluorescent probe that is routinely used to investigate Ca^{2+} -binding properties of proteins [47, 50]. Tb^{3+} shows an induced-fluorescence peak ($\sim$ at 565 nm) when it binds to Ca^{2+} -binding sites in proteins [16, 51]. We used Tb^{3+} fluorescence to monitor the binding of Cu^{2+} to putative Ca^{2+} - binding sites in the C2B domain. Titration of the apoC2B domain with increasing concentrations of Tb^{3+} showed a progressive increase in the fluorescence intensity at 565 nm (Fig. 2A). Interestingly, the observed increase in Tb^{3+} emission at 565 nm is accompanied by a concomitant decrease in the intrinsic tryptophan intensity at 340 nm. This spectral trend suggests that some of the tryptophan residues in the C2B domain are located in the close proximity to the $\text{Tb}^{3+}/\text{Ca}^{2+}$ - binding sites. The Tb^{3+} binding curve saturates at a $\text{Tb}^{3+}/\text{protein}$ ratio of 2:1 suggesting that two calcium-binding sites are available in the C2B domain (Fig. 2A). These results are consistent with the literature reports on the Ca^{2+} - C2B domain binding stoichiometry [41]. The competition between Ca^{2+} and Cu^{2+} to bind to the C2B domain was assessed by copper titration of the protein saturated with 500

μM Tb^{3+} (Fig. 2B). Increasing additions of Cu^{2+} to the Tb^{3+} - saturated C2B domain resulted in gradual loss of Tb^{3+} fluorescence at 565 nm. These results clearly suggest that Cu^{2+} replaces $\text{Tb}^{3+}/\text{Ca}^{2+}$ bound to the protein.

C2B specifically binds to PS vesicles: Several studies have revealed details of the interaction of the C2 (C2A and C2B) domains of Syt1 with lipid bilayers [48, 52]. The flexible Ca^{2+} -binding loops of C2 domains are thought to be buried within the lipid bilayers [46]. Although there is a broad consensus that C2 domains preferentially interact with negatively charged phospholipids, there is little or no information on how the nature of the individual phospholipids influence binding affinity of the C2 domains to the lipid bilayer. Moreover, the effect(s) of Cu^{2+} on the lipid - binding affinity of the C2B domain is still not clearly understood. In this context, we measured the binding affinity of the C2B domain to small unilamellar vesicles prepared individually from phosphatidylserine (pS), phosphatidyl glycerol (PG) and phosphatidyl choline (PC). Binding between apoC2B domain and PS vesicle is exothermic and proceeds with an enthalpy change (ΔH) of $\sim -0.12 \text{ kcal. mol}^{-1}$ (Fig. 3A). The binding constants ($K_{d(\text{app})}$) characterizing the affinity between the apoC2B domain and PS vesicles are $\sim 12(\pm 0.21) \mu\text{M}$ ($K_{d1(\text{app})}$) and $\sim 58 (\pm 0.32) \mu\text{M}$ ($K_{d2(\text{app})}$) (Table-1). Interestingly, the apoC2B domain – PS interaction is characterized by a significant increase in entropy ($\Delta S = \sim 32 \text{ cal.mol}^{-1} \cdot \text{K}^{-1}$) suggesting that the interaction between the protein and PS vesicles occurs predominantly through charge-charge interactions. In marked contrast, C2B domain showed weak binding affinity ($K_{d(\text{app})} \sim 1.1 \text{ mM}$) to unilamellar vesicles of PC, and no binding or insignificant binding to PG vesicles (Figs. 3B & 3C). These results indicate that C2B domain preferentially binds PS vesicles. ***Cu^{2+} decreases the lipid - binding affinity of the C2B***

domain: As shown earlier in figure 1, the apoC2B domain binds to Cu^{2+} with a moderate affinity. Therefore, we examined the Cu^{2+} binding affinity of the C2B domain bound to unilamellar vesicles of PS. The interaction is endothermic, and the binding isotherm indicates that the interaction proceeds in two distinct parts with binding constant ($K_{d(\text{app})}$) values of $17 (\pm 0.11) \mu\text{M}$ ($K_{d1(\text{app})}$) and $854 (\pm 5) \mu\text{M}$ ($K_{d2(\text{app})}$) for the first and second parts of the binding isotherm, respectively (Fig.4). Comparison of the binding affinity data of Cu^{2+} interaction with apoC2B alone and with C2B domain bound to unilamellar PS vesicles reveals some interesting features. Firstly, the enthalpy change (ΔH) characterizing the binding of Cu^{2+} with apoC2B ($\Delta H = \sim 28.1 \text{ kcal. mol}^{-1}$) bears an opposite sign to that observed when the metal ion interacts with C2B-PS complex. Secondly, in the presence of PS vesicles, the C2B domain appears to possess only two Cu^{2+} - binding sites as opposed to four metal binding sites in the absence of the lipid vesicles. Lastly, the K_d value for the first part ($K_{d1(\text{app})}$) of the binding isotherm in both cases does not appear to change significantly (Table-1). However, interestingly, the affinity for Cu^{2+} in the second part ($K_{d2(\text{app})}$) of the binding isotherm appears to increase nearly 40-fold (Table-1). These results suggest that the initial binding of Cu^{2+} plausibly causes a conformational change in the C2B domain which subsequently decreases its affinity for the metal ion.. ITC experiments, to examine the effects of Cu^{2+} on the lipid binding affinity of the C2B domain, could not be performed because, in the absence of the C2B domain, the PS vesicles aggregated severely when incubated with Cu^{2+} . It's obvious from the ITC data that the Cu^{2+} - binding affinity of the C2B domain drastically decreases in the presence of PS vesicles. The loss of Cu^{2+} - binding affinity of the C2B

domain may be a crucial step in the eventual translocation of FGF1 across the membrane bilayer.

Cu²⁺ - binding increases the stability of the C2B domain: Metals regulate a number of cellular processes by inducing conformational changes in proteins and/or by affecting their stability [53]. In this regard, differential scanning calorimetry (DSC) experiments and limited trypsin digestion experiments were performed to study the effects of Cu²⁺ on the stability of the C2B domain, in the presence and absence of the PS vesicles. ApoC2B domain is completely unfolded at the temperature above 60°C (Fig. 5). The unfolding process is not fully reversible and therefore detailed thermodynamic parameters associated with the unfolding of the protein could not be measured. The apparent T_{m(app)} of the apoC2B domain is ~ 46 (± 0.18)°C (Fig. 5). In the presence of Ca²⁺, the protein gets stabilized and unfolds with a T_m value of 54.6 °C (Fig. S2). Interestingly, the T_{m(app)} value of the protein shows a modest increase of ~ 2.3 (±0.09)°C in the presence of 500 µM Cu²⁺. Further increase in the concentration of Cu²⁺ to 1 mM had no significant effect on the T_{m(app)} value suggesting the saturation of the metal-induced stabilizing effect(s) on the protein. ITC experiments were performed under similar conditions with Mg²⁺ to rule out non-specific divalent metal binding effects. The results clearly show that Mg²⁺ does not bind to the C2B domain and therefore the observed modest increase observed in T_m value, in the presence of Cu²⁺ indeed stems from specific binding of the metal ion to the C2B domain (Fig. S1). The stability of the C2B domain in the presence of PS vesicles decreases by about 2 °C (T_{m(app)} ~ 44 ± 0.2°C, Fig. 5). It appears that binding of the protein in to the lipid vesicle destabilizes some of the native structural interactions at the

calcium - binding site. Interestingly, in the presence of Cu^{2+} , the $T_{m(\text{app})}$ of the C2B domain bound to the PS vesicle increases significantly ($T_{m(\text{app})} \sim 49 (\pm 0.22) ^\circ\text{C}$).

Limited trypsin digestion analysis is a valuable technique to probe ligand-induced conformational changes in proteins [54, 55]. Trypsin predominantly cleaves proteins at the C-terminal ends of basic amino acids such as lysine and arginine. The susceptibility of a protein to trypsin cleavage not only depends on the number of lysine and arginine residues in a protein but is also influenced by other factors such as, solvent accessibility of the susceptible sites, hydrogen bonds between residues at the cleavage sites and the rest of the molecule, and its propensity to local unfolding/packing [55, 56]. Therefore, limited trypsin digestion analysis was used as a technique to probe the possible conformational/structural changes induced in the C2B domain by Cu^{2+} in the presence and absence of PS vesicles. The fraction of protein cleaved by trypsin at any time point was monitored from the decrease in the intensity of the ~ 18 kDa Coomassie blue-stained band (on the polyacrylamide gel) corresponding to the intact C2B domain. The extent of cleavage of apoC2B domain increased with increase in time of incubation of the protein with trypsin (Fig. 6A). More than 85% of apoC2B domain is cleaved within 120 minutes of initiation of cleavage. In the presence of Cu^{2+} , the decrease of the C2B band intensity is only 65% suggesting that the transition metal stabilizes the protein against trypsin cleavage (Fig. 6A). These results are consistent with the ITC and the DSC data. Thus noticeably, the protein undergoes subtle conformational change(s) on binding to the metal ion. It should be mentioned that control experiments with hen egg white lysozyme showed that the observed difference(s) in the cleavage patterns are not due to non-specific effects of Cu^{2+} on the C2B domain (Fig. S3). The degree of susceptibility of C2B

domain to trypsin cleavage in the presence of the PS vesicles (after any time period of incubation of the protein with trypsin) is similar to that observed for apoC2B (Fig.6B). These observations do not completely corroborate with the DSC data wherein the $T_{m(app)}$ value of the C2B domain was found to decrease marginally in the presence of PS vesicles. The observed discrepancy between the DSC and the limited proteolytic digestion experiments may be due to the lower solvent accessibility of the trypsin cleavage sites in the protein bound to PS vesicles. The C2B domain, bound to both Cu^{2+} and PS vesicles, is significantly more resistant to trypsin cleavage (Fig. 6B). Interestingly, more than 40% of the C2B domain remains intact when it is simultaneously bound to both Cu^{2+} and the unilamellar PS vesicle (Fig. 6B). The results of the DSC and the limited trypsin digestion experiments analyzed together suggest that Cu^{2+} stabilizes the C2B domain in both its free and lipid bound forms.

Molecular events in the nonclassical secretion of FGF1: The sequence of molecular events leading to the secretion of FGF1 in to the extracellular medium is still not fully understood. Nonclassical secretion of FGF1 occurs *via* the formation of a MRC consisting of FGF1, S100A13, SphK1 and p40Syt1 (comprising of the C2A and the C2B domains) [38]. Cu^{2+} is required for the formation of the FGF1 MRC [24]. Available experimental evidence suggest that the Cu^{2+} independent binding of FGF1 to the S100A13 dimer is the first structural event in the nonclassical secretion pathway of FGF1 (Fig.7)[40]. This is supported by ITC data which shows that apo-S100A13 binds to Cu^{2+} with micromolar affinity and the S100A13 to Cu^{2+} binding stoichiometry is 1:4. Therefore, the formation of Cu^{2+} /S100A13/FGF1 ternary complex appears to be the second molecular event. The ionic form of Cu^{2+} is highly toxic to the cell and therefore it

is likely that the S100A13/FGF1 binary complex receives Cu^{2+} from cell-surface copper chaperones, such as Atx1/CCS/Cox17 [57-61] or from SphK1, which has a very high copper affinity [11]. Cu^{2+} - binding site on S100A13 and Cys 30 in FGF1 appears to be in close proximity in the Cu^{2+} /S100A13/FGF1 ternary complex (Fig.7). The formation of the homodimer of FGF1, induced by the intermolecular disulfide formed *via* Cu^{2+} induced oxidation of Cys131, appears to be the third step in the nonclassical release of FGF1 (Fig.7). Binding of Cu^{2+} to the C2 domains of p40Syt1 is likely to be the fourth step (Fig.7). ITC data suggest that Cu^{2+} binds to the Ca^{2+} binding site(s) on the C2 domains and probably increases the membrane binding affinity of the protein (C2 domain). As suggested by the ITC data, it appears that membrane binding of the FGF1 MRC decreases the Cu^{2+} - binding affinity. The C2 domains facilitate the anchoring of the FGF1 MRC to the inner side of the membrane bilayer. The critical next step is the interaction of the FGF1 MRC with annexin II [62-64]. In this context, it is worth mentioning that members of the S100 family are known to interact with annexins [63-65]. Annexin II, due to its “flip-flopping” activity between the inner and outer surfaces of the membrane could facilitate the export of FGF1 together with S100A13 bound to Cu^{2+} [54-56]. Glutathione present in the reducing environment in the extracellular medium plausibly disrupts the intermolecular disulfide bond to release the biologically active FGF1 monomer (Fig.7). Glutathione also appears to play a critical role in the recycling of Cu^{2+} ions back to the copper “chaperones” inside the intracellular compartment. It should be mentioned that in the absence of experimental evidence for occurrence of some of the molecular events, the mechanism of the nonclassical export of FGF1 proposed herein should be considered as a working hypothesis. Currently, work is in progress to

characterize the sequence of structural events that occur in the non-classical pathway of FGF1.

Conclusions

The results of this study clearly indicate that C2B domain of Syt1 binds to Cu^{2+} in a biphasic manner. The Cu^{2+} appears to bind to the Ca^{2+} binding site and causes a subtle conformational change in the protein. The conformational stability and backbone flexibility of the C2B domain significantly increases in the presence of Cu^{2+} . The C2B domain of Syt1 shows selective binding affinity to the unilamellar vesicles of phosphatidyl serine. Cu^{2+} appears to regulate the lipid binding affinity of the C2B. Our results suggest that the role of Cu^{2+} is not only in the organization of the multiprotein FGF but also is significantly involved in the final release of the growth factor in to the extracellular medium.

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References

- [1] R. Friesel, T. Maciag, Fibroblast growth factor prototype release and fibroblast growth factor receptor signaling, *Thromb Haemost* 82 (1999) 748-754.
- [2] N. Itoh, D.M. Ornitz, Fibroblast growth factors: from molecular evolution to roles in development, metabolism and disease, *J Biochem* 149 121-130.
- [3] J. Schlessinger, Common and distinct elements in cellular signaling via EGF and FGF receptors, *Science* 306 (2004) 1506-1507.
- [4] G. Szebenyi, J.F. Fallon, Fibroblast growth factors as multifunctional signaling factors, *Int Rev Cytol* 185 (1999) 45-106.
- [5] I. Prudovsky, F. Tarantini, M. Landriscina, D. Neivandt, R. Soldi, A. Kirov, D. Small, K.M. Kathir, D. Rajalingam, T.K. Kumar, Secretion without Golgi, *J Cell Biochem* 103 (2008) 1327-1343.
- [6] W. Nickel, Unconventional secretory routes: direct protein export across the plasma membrane of mammalian cells, *Traffic* 6 (2005) 607-614.
- [7] I. Prudovsky, A. Mandinova, R. Soldi, C. Bagala, I. Graziani, M. Landriscina, F. Tarantini, M. Duarte, S. Bellum, H. Doherty, T. Maciag, The non-classical export routes: FGF1 and IL-1alpha point the way, *J Cell Sci* 116 (2003) 4871-4881.
- [8] A. Jackson, S. Friedman, X. Zhan, K.A. Engleka, R. Forough, T. Maciag, Heat shock induces the release of fibroblast growth factor 1 from NIH 3T3 cells, *Proc Natl Acad Sci U S A* 89 (1992) 10691-10695.
- [9] P. Mignatti, T. Morimoto, D.B. Rifkin, Basic fibroblast growth factor, a protein devoid of secretory signal sequence, is released by cells via a pathway independent of the endoplasmic reticulum-Golgi complex, *J Cell Physiol* 151 (1992) 81-93.
- [10] I. Prudovsky, C. Bagala, F. Tarantini, A. Mandinova, R. Soldi, S. Bellum, T. Maciag, The intracellular translocation of the components of the fibroblast growth factor 1 release complex precedes their assembly prior to export, *J Cell Biol* 158 (2002) 201-208.
- [11] R. Soldi, A. Mandinova, K. Venkataraman, T. Hla, M. Vadas, S. Pitson, M. Duarte, I. Graziani, V. Kolev, D. Kacer, A. Kirov, T. Maciag, I. Prudovsky, Sphingosine kinase 1 is a critical component of the copper-dependent FGF1 export pathway, *Exp Cell Res* 313 (2007) 3308-3318.
- [12] M. Landriscina, R. Soldi, C. Bagala, I. Micucci, S. Bellum, F. Tarantini, I. Prudovsky, T. Maciag, S100A13 participates in the release of fibroblast growth factor 1 in response to heat shock in vitro, *J Biol Chem* 276 (2001) 22544-22552.
- [13] T.M. LaVallee, F. Tarantini, S. Gamble, C. Mouta Carreira, A. Jackson, T. Maciag, Synaptotagmin-1 is required for fibroblast growth factor-1 release, *J Biol Chem* 273 (1998) 22217-22223.
- [14] K.A. Engleka, T. Maciag, Inactivation of human fibroblast growth factor-1 (FGF-1) activity by interaction with copper ions involves FGF-1 dimer formation induced by copper-catalyzed oxidation, *J Biol Chem* 267 (1992) 11307-11315.
- [15] M. Landriscina, C. Bagala, A. Mandinova, R. Soldi, I. Micucci, S. Bellum, I. Prudovsky, T. Maciag, Copper induces the assembly of a multiprotein aggregate implicated in the release of fibroblast growth factor 1 in response to stress, *J Biol Chem* 276 (2001) 25549-25557.

- [16] D. Rajalingam, T.K. Kumar, C. Yu, The C2A domain of synaptotagmin exhibits a high binding affinity for copper: implications in the formation of the multiprotein FGF release complex, *Biochemistry* 44 (2005) 14431-14442.
- [17] J.D. Gaffaney, F.M. Dunning, Z. Wang, E. Hui, E.R. Chapman, Synaptotagmin C2B domain regulates Ca²⁺-triggered fusion in vitro: critical residues revealed by scanning alanine mutagenesis, *J Biol Chem* 283 (2008) 31763-31775.
- [18] E. Hui, J. Bai, P. Wang, M. Sugimori, R.R. Llinas, E.R. Chapman, Three distinct kinetic groupings of the synaptotagmin family: candidate sensors for rapid and delayed exocytosis, *Proc Natl Acad Sci U S A* 102 (2005) 5210-5214.
- [19] K.L. Fuson, L. Ma, R.B. Sutton, A.F. Oberhauser, The c2 domains of human synaptotagmin 1 have distinct mechanical properties, *Biophys J* 96 (2009) 1083-1090.
- [20] T.C. Sudhof, J. Rizo, Synaptic vesicle exocytosis, *Cold Spring Harb Perspect Biol* 3.
- [21] K. Aoyagi, T. Sugaya, M. Umeda, S. Yamamoto, S. Terakawa, M. Takahashi, The activation of exocytotic sites by the formation of phosphatidylinositol 4,5-bisphosphate microdomains at syntaxin clusters, *J Biol Chem* 280 (2005) 17346-17352.
- [22] A. Honigmann, G. van den Bogaart, E. Iraheta, H.J. Risselada, D. Milovanovic, V. Mueller, S. Mullar, U. Diederichsen, D. Fasshauer, H. Grubmuller, S.W. Hell, C. Eggeling, K. Kuhnel, R. Jahn, Phosphatidylinositol 4,5-bisphosphate clusters act as molecular beacons for vesicle recruitment, *Nat Struct Mol Biol* 20 679-686.
- [23] R. Jahn, D. Fasshauer, Molecular machines governing exocytosis of synaptic vesicles, *Nature* 490 201-207.
- [24] D.J. James, C. Khodthong, J.A. Kowalchuk, T.F. Martin, Phosphatidylinositol 4,5-bisphosphate regulates SNARE-dependent membrane fusion, *J Cell Biol* 182 (2008) 355-366.
- [25] G. van den Bogaart, S. Thutupalli, J.H. Risselada, K. Meyenberg, M. Holt, D. Riedel, U. Diederichsen, S. Herminghaus, H. Grubmuller, R. Jahn, Synaptotagmin-1 may be a distance regulator acting upstream of SNARE nucleation, *Nat Struct Mol Biol* 18 805-812.
- [26] M.E. Fealey, J.W. Gauer, S.C. Kempka, K. Miller, K. Nayak, R.B. Sutton, A. Hinderliter, Negative coupling as a mechanism for signal propagation between C2 domains of synaptotagmin I, *PLoS One* 7 e46748.
- [27] W. Kuo, D.Z. Herrick, D.S. Cafiso, Phosphatidylinositol 4,5-bisphosphate alters synaptotagmin 1 membrane docking and drives opposing bilayers closer together, *Biochemistry* 50 2633-2641.
- [28] A.L. Lai, L.K. Tamm, J.F. Ellena, D.S. Cafiso, Synaptotagmin 1 modulates lipid acyl chain order in lipid bilayers by demixing phosphatidylserine, *J Biol Chem* 286 25291-25300.
- [29] H. Takahashi, V. Shahin, R.M. Henderson, K. Takeyasu, J.M. Edwardson, Interaction of synaptotagmin with lipid bilayers, analyzed by single-molecule force spectroscopy, *Biophys J* 99 2550-2558.
- [30] J. Bai, E.R. Chapman, The C2 domains of synaptotagmin--partners in exocytosis, *Trends Biochem Sci* 29 (2004) 143-151.

- [31] V. Kiessling, S. Ahmed, M.K. Domanska, M.G. Holt, R. Jahn, L.K. Tamm, Rapid Fusion of Synaptic Vesicles with Reconstituted Target SNARE Membranes, *Biophys J* 104 1950-1958.
- [32] Y. Lai, J. Diao, Y. Liu, Y. Ishitsuka, Z. Su, K. Schulten, T. Ha, Y.K. Shin, Fusion pore formation and expansion induced by Ca^{2+} and synaptotagmin 1, *Proc Natl Acad Sci U S A* 110 1333-1338.
- [33] A. Radhakrishnan, A. Stein, R. Jahn, D. Fasshauer, The Ca^{2+} affinity of synaptotagmin 1 is markedly increased by a specific interaction of its C2B domain with phosphatidylinositol 4,5-bisphosphate, *J Biol Chem* 284 (2009) 25749-25760.
- [34] E. Rufener, A.A. Frazier, C.M. Wieser, A. Hinderliter, D.S. Cafiso, Membrane-bound orientation and position of the synaptotagmin C2B domain determined by site-directed spin labeling, *Biochemistry* 44 (2005) 18-28.
- [35] C. Bagala, V. Kolev, A. Mandinova, R. Soldi, C. Mouta, I. Graziani, I. Prudovsky, T. Maciag, The alternative translation of synaptotagmin 1 mediates the non-classical release of FGF1, *Biochem Biophys Res Commun* 310 (2003) 1041-1047.
- [36] T.M. LaVallee, I.A. Prudovsky, G.A. McMahon, X. Hu, T. Maciag, Activation of the MAP kinase pathway by FGF-1 correlates with cell proliferation induction while activation of the Src pathway correlates with migration, *J Cell Biol* 141 (1998) 1647-1658.
- [37] F. Tarantini, T. LaVallee, A. Jackson, S. Gamble, C. Mouta Carreira, S. Garfinkel, W.H. Burgess, T. Maciag, The extravesicular domain of synaptotagmin-1 is released with the latent fibroblast growth factor-1 homodimer in response to heat shock, *J Biol Chem* 273 (1998) 22209-22216.
- [38] I. Prudovsky, T.K. Kumar, S. Sterling, D. Neivandt, Protein-phospholipid interactions in nonclassical protein secretion: problem and methods of study, *Int J Mol Sci* 14 3734-3772.
- [39] C. Di Serio, L. Doria, S. Pellerito, I. Prudovsky, I. Micucci, D. Massi, M. Landriscina, N. Marchionni, G. Masotti, F. Tarantini, The release of fibroblast growth factor-1 from melanoma cells requires copper ions and is mediated by phosphatidylinositol 3-kinase/Akt intracellular signaling pathway, *Cancer Lett* 267 (2008) 67-74.
- [40] V. Sivaraja, T.K. Kumar, D. Rajalingam, I. Graziani, I. Prudovsky, C. Yu, Copper binding affinity of S100A13, a key component of the FGF-1 nonclassical copper-dependent release complex, *Biophys J* 91 (2006) 1832-1843.
- [41] I. Fernandez, D. Arac, J. Ubach, S.H. Gerber, O. Shin, Y. Gao, R.G. Anderson, T.C. Sudhof, J. Rizo, Three-dimensional structure of the synaptotagmin 1 C2B-domain: synaptotagmin 1 as a phospholipid binding machine, *Neuron* 32 (2001) 1057-1069.
- [42] R. Fernandez-Chacon, O.H. Shin, A. Konigstorfer, M.F. Matos, A.C. Meyer, J. Garcia, S.H. Gerber, J. Rizo, T.C. Sudhof, C. Rosenmund, Structure/function analysis of Ca^{2+} binding to the C2A domain of synaptotagmin 1, *J Neurosci* 22 (2002) 8438-8446.
- [43] I. Graziani, C. Bagala, M. Duarte, R. Soldi, V. Kolev, F. Tarantini, T.K. Kumar, A. Doyle, D. Neivandt, C. Yu, T. Maciag, I. Prudovsky, Release of FGF1 and p40

- synaptotagmin 1 correlates with their membrane destabilizing ability, *Biochem Biophys Res Commun* 349 (2006) 192-199.
- [44] J. Rizo, Cell biology. Staging membrane fusion, *Science* 337 1300-1301.
 - [45] K.M. Kathir, L. Gao, D. Rajalingam, A.E. Daily, S. Brixey, H. Liu, D. Davis, P. Adams, I. Prudovsky, T.K. Kumar, 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), *Biochim Biophys Acta* 1798 297-302.
 - [46] N. Coudeville, P. Montaville, A. Leonov, M. Zweckstetter, S. Becker, Structural determinants for Ca²⁺ and phosphatidylinositol 4,5-bisphosphate binding by the C2A domain of rabphilin-3A, *J Biol Chem* 283 (2008) 35918-35928.
 - [47] L.N. Lin, A.B. Mason, R.C. Woodworth, J.F. Brandts, Calorimetric studies of the binding of ferric ions to ovotransferrin and interactions between binding sites, *Biochemistry* 30 (1991) 11660-11669.
 - [48] P. Montaville, N. Coudeville, A. Radhakrishnan, A. Leonov, M. Zweckstetter, S. Becker, The PIP₂ binding mode of the C2 domains of rabphilin-3A, *Protein Sci* 17 (2008) 1025-1034.
 - [49] W. Kuo, D.Z. Herrick, J.F. Ellena, D.S. Cafiso, The calcium-dependent and calcium-independent membrane binding of synaptotagmin 1: two modes of C2B binding, *J Mol Biol* 387 (2009) 284-294.
 - [50] J. Lee, M. Blaber, The interaction between thermodynamic stability and buried free cysteines in regulating the functional half-life of fibroblast growth factor-1, *J Mol Biol* 393 (2009) 113-127.
 - [51] W.D. Horrocks, Jr., Luminescence spectroscopy, *Methods Enzymol* 226 (1993) 495-538.
 - [52] K.L. Lynch, R.R. Gerona, E.C. Larsen, R.F. Marcia, J.C. Mitchell, T.F. Martin, Synaptotagmin C2A loop 2 mediates Ca²⁺-dependent SNARE interactions essential for Ca²⁺-triggered vesicle exocytosis, *Mol Biol Cell* 18 (2007) 4957-4968.
 - [53] C.Y. Wang, S. Jenkitkasemwong, S. Duarte, B.K. Sparkman, A. Shawki, B. Mackenzie, M.D. Knutson, ZIP8 is an iron and zinc transporter whose cell-surface expression is up-regulated by cellular iron loading, *J Biol Chem* 287 34032-34043.
 - [54] C. Park, S. Zhou, J. Gilmore, S. Marqusee, Energetics-based protein profiling on a proteomic scale: identification of proteins resistant to proteolysis, *J Mol Biol* 368 (2007) 1426-1437.
 - [55] K. Weisshart, S. Friedl, P. Taneja, H.P. Nasheuer, B. Schlott, F. Grosse, E. Fanning, Partial proteolysis of simian virus 40 T antigen reveals intramolecular contacts between domains and conformation changes upon hexamer assembly, *J Biol Chem* 279 (2004) 38943-38951.
 - [56] T. Selwood, H. Smolensky, D.R. McCaslin, N.M. Schechter, The interaction of human tryptase-beta with small molecule inhibitors provides new insights into the unusual functional instability and quaternary structure of the protease, *Biochemistry* 44 (2005) 3580-3590.

- [57] L. Banci, I. Bertini, C. Cefaro, S. Ciofi-Baffoni, A. Gallo, Functional role of two interhelical disulfide bonds in human Cox17 protein from a structural perspective, *J Biol Chem* 286 34382-34390.
- [58] R. Miras, I. Morin, O. Jacquin, M. Cuillel, F. Guillain, E. Mintz, Interplay between glutathione, Atx1 and copper. 1. Copper(I) glutathionate induced dimerization of Atx1, *J Biol Inorg Chem* 13 (2008) 195-205.
- [59] J.R. Prohaska, Role of copper transporters in copper homeostasis, *Am J Clin Nutr* 88 (2008) 826S-829S.
- [60] R.A. Pufahl, C.P. Singer, K.L. Peariso, S.J. Lin, P.J. Schmidt, C.J. Fahrni, V.C. Culotta, J.E. Penner-Hahn, T.V. O'Halloran, Metal ion chaperone function of the soluble Cu(I) receptor Atx1, *Science* 278 (1997) 853-856.
- [61] L.J. Shin, J.C. Lo, K.C. Yeh, Copper chaperone antioxidant protein1 is essential for copper homeostasis, *Plant Physiol* 159 1099-1110.
- [62] A.B. Deora, G. Kreitzer, A.T. Jacovina, K.A. Hajjar, An annexin 2 phosphorylation switch mediates p11-dependent translocation of annexin 2 to the cell surface, *J Biol Chem* 279 (2004) 43411-43418.
- [63] E.C. Flood, K.A. Hajjar, The annexin A2 system and vascular homeostasis, *Vascul Pharmacol* 54 59-67.
- [64] K.A. Hajjar, A.T. Jacovina, J. Chacko, An endothelial cell receptor for plasminogen/tissue plasminogen activator. I. Identity with annexin II, *J Biol Chem* 269 (1994) 21191-21197.
- [65] L. Santamaria-Kisiel, A.C. Rintala-Dempsey, G.S. Shaw, Calcium-dependent and -independent interactions of the S100 protein family, *Biochem J* 396 (2006) 201-214.

Figure Legends

Figure. 1: Cu^{2+} binding to C2B domain.

Isothermograms representing the binding of, Cu^{2+} to C2B domain (Panel – A) and C2B domain saturated with Ca^{2+} (Panel – B). The upper panels represent the raw data and the bottom panels are the best fits of the raw data. The exothermic and endothermic parts of the binding isotherm were independently fitted using the multiple sets of sites model (Microcal Inc. Origin software). The heats of dilution (background control) was measured by titrating buffer solution(s) without metals/lipids (placed in the titrating syringe) in to buffer solution(s) containing the protein (in the reaction vessel) under identical conditions as that used in the test experiments. The resultant control isotherms were subtracted from all test binding isotherms to account for the contribution(s) due to heats of dilution.

Figure. 2: Terbium - binding to the C2B domain.

Titration of apoC2B domain with increasing concentration of TbCl_3 (Panel –A). Tb^{3+} -bound C2B domain titrated with increasing concentrations of CuCl_2 (Panel – B). *Inset* figures in each panel show the change(s) in the Tb^{3+} emission intensity (565 nm, closed circles) and intrinsic tryptophan emission intensity (340 nm, open circles). The concentration of C2B domain tested was 50 μM . All solutions were prepared in 10 mM tris buffer (pH 7.5, containing 100 mM NaCl). Appropriate blank corrections were made in all spectra. The direction of the arrows in Panels A and B indicate the increase in the Tb^{3+} fluorescence.

Figure. 3: Binding of unilamellar vesicles with vesicles containing various phospholipids

Isothermograms representing the binding of small unilamellar vesicles with small unilamellar vesicles composed of PS (Panel – A), with PC (Panel-B), and with PG (Panel – C) at pH 7.5. The upper panels show the raw data for the titration of approximately 10 mM liposome to 0.08 mM of protein. The solid line in the bottom panels represents the best-fit of the experimental data, using a sequential binding site model from Microcal Origin.

Figure. 4: Binding of Cu^{2+} to PS bound C2B domain

Isothermogram representing the binding of Cu^{2+} with the C2B domain bound to PS at pH 7.5. The upper panel shows the raw data for the titration of approximately 0.08 mM protein with 3 mM CuCl_2 . The solid line in the bottom panel represents the best-fit of the experimental data, using a sequential binding site model from Microcal Origin.

Figure. 5: DSC of C2B domain

Differential scanning calorimetry thermograms representing the unfolding of C2B domain. The protein (1.0 mg/mL) was dissolved in 10 mM tris buffer (pH 7.5) containing 100 mM NaCl. Thermograms were corrected for background noise. The experiments were performed at a scan rate of 0.5 °C/min.

Figure. 6: Limited trypsin digestion of C2B under various conditions

A- SDS-PAGE analysis of the limited trypsin digestion products of the apoC2B domain, in the presence of Cu^{2+} , PS, PS and Cu^{2+} . The intensity of the C2B domain band in lane

2, not treated with trypsin was assumed as 100%. C2B bands in the corresponding gel were normalized to this ~ 18 kDa band (indicated by an arrow). Lanes, 2-10, in each gel represents the incubation with 1.5:1 protein/enzyme concentration of trypsin. Lane 1 represents MW marker, lanes 2-10 represent the trypsin digestion products obtained after incubation of the C2B domain with trypsin for 0, 5, 15, 20, 30, 60, 90, 120, and 180 minutes respectively. Appropriate control experiments with bovine serum albumin were conducted to assess possible effects of Cu^{2+} on the activity and specificity of trypsin. The band that runs higher than that of the C2B domain corresponds to trypsin (indicated by asterisk). **B-** Depicts the percentage of digestion of the ~18 kDa C2B domain band when trypsin is incubated with, C2B domain alone (closed circles); C2B domain and Cu^{2+} (open circles); C2B domain and PS vesicles (closed squares); and C2B domain, PS and Cu^{2+} (open squares).

Figure. 7: Nonclassical release of FGF1

Cartoon representing a hypothetical scheme of events which occur in the nonclassical release of FGF1.

Table-1: Thermodynamic parameters measured from ITC experiments

Experiment number	Samples	Ligand	$K_{d(app)}$	ΔH kcal/mol	ΔS calmol ⁻¹ K ⁻¹	Number of Binding Sites
1	C2B	Cu ²⁺	$K_{d1(app)} = 1.4$ (± 0.1) μM $K_{d2(app)} = 21$ (± 0.1) μM	28.0 -0.9	121 36.7	~ 2.0 ~ 2.0
2	C2B + Ca ²⁺	Cu ²⁺	$K_{d(app)} = 38$ (± 0.5) μM	-33.5	-23.1	~ 1
3	C2B	PS	$K_{d1(app)} = 12$ (± 0.7) μM $K_{d2(app)} = 41$ (± 0.32) μM	-0.1 -0.1	32.5 19.8	~ 2 ND
4	C2B	PG	ND	ND	ND	ND
5	C2B	PC	$K_{d(app)} = 1200$ (± 0.1) μM	-0.05	19.1	1
6	C2B + PS	Cu ²⁺	$K_{d1(app)} = 17$ (± 0.11) μM $K_{d2(app)} = 854$ (± 5) μM	1.5 5.2	27.1 46.0	~ 2 ND
7	C2B	Mg ²⁺	ND	ND	ND	ND
8	C2B + Ca ²⁺	Mg ²⁺	ND	ND	ND	ND
9	C2B + PS	Mg ²⁺	ND	ND	ND	ND

ND – Not determined. The number of binding sites was round to the nearest whole number value.

Figure-1

Figure-2

Figure-3

Figure-4

Figure-5

Experiment Condition	$T_{m(app)} \text{ } ^\circ\text{C}$
C2B	46.1(± 0.18)
C2B+Cu ²⁺	48.4(± 0.01)
C2B+pS	44.1(± 0.2)
(C2B+pS)+Cu ²⁺	49.2(± 0.22)

Figure-6

Figure-7