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Expression, purification and characterization of C2 domain of milk fat globule-EGF-factor 8-L

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Abstract

Milk fat globule-EGF-factor 8-L (MFG-E8L) is secreted by activated macrophages and functions as a linker protein or opsonin between the dying cells and phagocytes. MFG-E8L recognizes the apoptotic or dying cells by specifically binding to Phosphatidylserine (PS) exposed on the outer cell surface and enhances the engulfment of the apoptotic cells by phagocytes, thereby preventing the inflammation and autoimmune response against intracellular antigens that can be released from the dying cells. MFG-E8L contains two EGF-like domains, P/T (proline/threonine) rich domain followed by two discoidin-like domains (C1 and C2). Recent studies have shown that the C2 domain of MFG-E8L is specifically involved in interaction with PS exposed on the apoptotic cells. Towards understanding this specific molecular interaction between the MFG-E8L C2 domain and PS, we expressed, purified the C2 domain of MFG-E8L and performed the binding studies with phospholipids by ³¹P NMR experiment. We demonstrated that our recombinant construct and expression system were effective and allowed us to obtain the C2 domain and also showed that the purified C2 domain was stable and properly folded, and our ³¹P NMR studies indicated that the C2 domain had specific binding with PS.

Keywords: MFG-E8; Phosphatidylserine-binding; C2 domain; Purification; ³¹P NMR

Apoptosis is a process by which the harmful or unhealthy cells are removed and is most important step in maintaining the homeostasis of mammalian cells [1]. These apoptotic cells are rapidly engulfed by phagocytes, thereby, preventing the inflammation and autoimmune response against intracellular antigens that can be released from the dying cells [2–4]. During the apoptosis, PS present in the inner leaflet of the cell membrane is exposed on the surface, thereby, providing the recognition or eat-me signal for macrophages [5]. Milk fat globule-EGF-factor 8 (MFG-E8) secreted from activated macrophages acts as a linker protein between the apoptotic cells and phagocytes. MFG-E8 specifically binds to apoptotic cells by identifying PS exposed on the cell surface and enhances the engulfment of apoptotic cells by phagocytes [6].

Mouse MFG-E8 consists of 53- and 66-kDa soluble, heavily glycosylated proteins with two repeated EGF-like domains on the N-terminal side and of two repeated discoidin-like domains homologous to the C1 and C2 domains of the blood coagulation

factors V and VIII. MFG-E8 expressed in mouse mammary gland was shown to be composed of two isoforms: long form and short form [7]. The MFG-E8L protein has a Pro/Thr-rich domain inserted between the second EGF-like repeat and C1 domain. In contrast the short form (MFG-E8S) lacks the Pro/Thr-rich domain and is ubiquitously expressed in various tissues. From the previous studies, it was shown that MFG-E8L was involved in the identification and transportation of apoptotic cells towards the macrophages for engulfment [6,8–10] whereas the MFG-E8S is involved only in facilitation of sperm–egg interactions for fertilization [11]. In the recent studies, it was shown that the C2 domain of MFG E8L was involved in binding with PS or PS-rich membranes [12,13]. Three-dimensional structural information would provide molecular basis of the specific interaction. To this end, in our current study, we cloned, expressed, purified the C2 domain of MFG-E8L, performed preliminary 1D ^1H NMR studies, and also analyzed its binding characteristics with phospholipids by ^{31}P NMR spectroscopy.

Materials and methods

Bacterial strains, plasmids and media

Escherichia coli strain BL21 (DE3) cells were used for overproduction of MFG-E8L C2 domain. Cells were cultured in LB media (5 g/l NaCl, 5 g/l yeast extract and 10 g/l tryptone) supplemented with 30 $\mu\text{g}/\text{ml}$ kanamycin. The cDNA coding for *Mus musculus* MFG-E8L was a gift from Prof. Shigekazu Nagata.

Cloning of the M. musculus MFG-E8L C2 gene

The cDNA coding for the C2 domain (His306-Cys463) of *M. musculus* MFG-E8L sequence (GenBank Accession No. AK171143) was amplified using forward and reverse primers (f: 5'- CACTCAGCATATGAAATCGGGTCATGGTTGTTCTGAGCCCCTGGGCCTGAAG-3'; r: 5'-GCGAAGTCCTCGAGACAGCCCAGCAGCTCCAGGC G-3') primers containing *Nde*I and *Xho*I restriction sites, respectively. To enhance protein expression, the primers also contained sequences to code for MKSG at the N-terminus of the C2 domain. Polymerase Chain Reaction Polymerase Chain Reaction (PCR) was performed, and the resulting cDNA fragments were purified by gel extraction kit (Qiagen) followed by digestion with *Nde*I and *Xho*I and ligated with pET29b vector (Novagen) to produce the C2 domain containing hexahistidine at the C-terminus. The recombinant plasmid was transformed into *E. coli* DH5 α and the transformants were confirmed by DNA sequencing. The recombinant plasmid was then transformed into BL21 (DE3) cells for expression of MFG-E8L C2.

Overexpression and purification of M. musculus MFG-E8L C2

All the protein purification and centrifugation steps were performed at 4 $^{\circ}\text{C}$. The *E. coli* strain BL21 (DE3) was transformed with the recombinant plasmid construct and selected on LB agar plate containing 30 $\mu\text{g}/\text{ml}$ of kanamycin. The transformants were inoculated into 5 ml test tube culture and allowed to grow at 37 $^{\circ}\text{C}$ in a shaker at 220 rpm

for 3 h. Then the cell culture was transferred into 1 L of LB media, and the C2 was induced for 4 h at 25 °C with the addition of 1 mM isopropyl-thio- β -D-galactopyranoside (IPTG). Cells were harvested when absorbance at 600 nm reached ~0.7 by centrifugation in a Beckman JA-10 rotor at 8000 rpm for 7 min and resuspended in 10 ml/g wet cells of suspension buffer (20 mM Na-PO₄, pH 7.3, 0.5 M NaCl and 1 mM Phenylmethylsulfonyl fluoride (PMSF)). The cell suspension was then passed through microfluidizer processor (Model M-110L, Microfluidics™). Cell lysate was centrifuged at 22,000 rpm for 30 min using a Beckman JA-25.50 rotor. The supernatant containing the recombinant protein was collected carefully and was loaded on Ni²⁺-iminodiacetic acid (IDA) column (2 ml) equilibrated with the suspension buffer. The column was washed with five bed volumes of washing buffer (20 mM Na-PO₄, pH 6.5, 0.5 M NaCl, 1 mM PMSF and 25 mM Imidazole) and eluted with elution buffer (20 mM Na-PO₄, pH 6.5, 0.5 M NaCl and 0.5 M Imidazole). The eluted proteins were then analyzed by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The concentration of the purified protein was determined by Bio-Rad Laboratories protein assay kit.

MALDI-TOF/TOF analysis

Molecular weight and protein identification were confirmed by mass spectrometry. Briefly, the protein sample was desalted by using C₄ ZipTips. Impurities were then washed away with water prior to MALDI-TOF/TOF MS analysis. An aliquot of 0.3 μ l of protein (approx. 10 pmol) mixed with 0.3 μ l of Sinapinic acid matrix solution and was spotted on the MALDI sample plate and allowed to air-dry. For protein ID determination, 5 μ l of sample was digested overnight with trypsin followed by desalting with C18 ZipTips. An aliquot of 0.3 μ l of protein was mixed with 0.3 μ l of 10 mg/ml α -cyano-4-hydroxycinnamic acid (α -CHCA) matrix solution and spotted on the MALDI sample plate and allowed to air-dry. It was then subjected to MALDI-TOF/TOF analysis on Applied Bio-systems MDS SCIEX 4800 MALDI-TOF/TOF analyzer. For molecular weight determination, the MS was run in linear TOF mode. The digested peptide was run in reflection mode first for peptide mass determination followed by MS/MS mode for peptide sequencing. All spectra acquired consisted of averaged signal from 600 laser shots, and the data were processed using accompanying Bio-systems mass spectrometry software.

1D ¹H NMR spectroscopy

The purified MFG-E8L C2 was concentrated to 0.5 mM in a buffer containing 20 mM NaPO₄, pH 6.5, 20 mM NaCl and 0.01% NaN₃ and 10% D₂O. The spectral parameters used were: 128 acquisitions, 16K data points, 90 ° pulse angle, 9602 Hz sweep width, a relaxation delay of 1 s. The solvent signal suppression was achieved by ‘WATER-GATE’ [14] pulse sequence at 298K on an Avance 600 MHz spectrometer (Bruker) equipped with four-radiofrequency channels and a triple resonance cryo-probe. The 1D ¹H NMR spectrum was processed using spectrometer inbuilt Topspin (Bruker) software.

³¹P NMR spectroscopy

PS and Phosphatidylcholine (PC) were prepared separately to a final concentration of 0.25mM in a buffer containing 10mM NaPO₄, pH 6.5, 10mM NaCl, 10 mM CHAPS, 0.01% NaN₃ and 10% D₂O, and titrated with varying concentrations (0.021, 0.063, 0.087, 0.175, 0.262 and 0.350mM) of MFG-E8L C2 in same buffer conditions as that for PC and PS. The ³¹P NMR experiment were performed at 298 K on an Avance 400 MHz spectrometer Bruker, Fallanden, Switzerland) at 161.7 MHz with inverse proton gated decoupling to minimize ¹H-³¹P NOE interactions. An observation frequency range of 5000 Hz and 16K data points were used for acquisition. A pulse length of 60° and a 2s pulse cycle were utilized. Nearly 4500 accumulations were obtained before Fourier transformation of the free induction decay. Binding constant (*K_d*) for PC and PS with the C2 domain was determined by one binding site equation by plotting the chemical shift data vs the protein concentration as described [15].

Results and discussion

Cloning, expression and purification of MFG-E8L C2 domain

For the biochemical analysis and structural studies on the interaction between the C2 domain and PS, the C2 domain coding for His306-Cys463 was cloned, overexpressed, and purified by metal affinity column chromatography. To enhance protein expression, four extra residues (MKSG) were added at the N-terminus of the C2 domain. As demonstrated in Fig. 1, the construct and expression system used in this study were effective in overexpressing the C2 domain and allowed us to purify the protein for further studies. Our results showed that the purified C2 domain was nearly homogeneous on a SDS-PAGE analysis (Fig. 1, lane 7). The purified protein was stable after storage for 1 month at 4 °C.

MALDI-TOF/TOF analysis

MS was used to characterize the purified C2 domain. For molecular weight determination, the sample was prepared and subjected to MALDI-TOF/TOF experiment. The resulting spectrum (Fig. 2) showed the peak at 19407.75 Da which is in agreement with the theoretical mass (19407.7Da) of the protein primary amino acid sequence calculated from ProtParam tool [16]. For protein ID, the MS results were submitted to database search using MASCOT software which retrieved the protein ID of mouse MFG-E8S (P21956), the C2 domain of which has 100% similarity with C2 domain of MFG-E8L (Protein ID not available in SwissProt). These MALDI-TOF/TOF results, taken together, along with the results from N-terminal protein sequencing of the first 10 amino acid residues, confirms that we have cloned, expressed and purified the right protein.

1D ¹H NMR spectroscopy

The analysis of the dispersion of NMR resonance signals is a good indicator of folded globular proteins. The 1D ¹H NMR spectrum of MFG-E8L C2 (Fig. 3) showed the

characteristics of properly folded proteins featuring good dispersion of resonance lines methyl protons (-0.5–1.5ppm), α -protons (3.5–6ppm), and amide protons (6–10ppm) [17]. Our results suggest that the purified C2 domain is properly folded and ready for further biochemical and structural studies.

³¹P NMR spectroscopy

The ³¹P NMR technique was used to examine molecular interactions of proteins with charged phospholipids. In Fig. 4A, the peak marked with ‘*’ is due to the phosphorous present in the buffer binding to MFG-E8L C2 protein, and the chemical shift difference between this peak and the bound (PC) peak appearing between -1.64 and -1.39 ppm does not change at any concentrations of protein. At increasing concentrations of the protein, an uniform and significant (0.35 ppm) chemical shift changes were observed from the bound (Fig. 4A, marked B, between -1.64 and -1.39 ppm) and free-state (Fig. 4A, marked F, bottom trace, -1.74 ppm) of PC binding to the protein. The binding of phosphate in PC to the protein is in fast exchange limit as the single sharp peak is observed at all concentrations of the protein. A similar chemical shift position was observed for free PS. The stacked spectra in Fig. 4B is due to the PS binding to the protein MFG-E8L C2 in the phosphate buffer. The large central peak is (0 ppm) due to the buffer alone and the second tallest peak at -1.35 ppm is due to PS binding to the protein. This is in the slow exchange limit as the appearance of a doublet signal at -1.06 ppm (bound, marked as ‘B’ at 0.350mM C2) overlapping with the free PS signal at -1.35ppm (marked as ‘F/B’ at 0.350mM C2). The intensity and the chemical shift of the bound peak increases with respect to free PS signal and shifts towards low field uniformly with increasing concentration of the protein. In addition to it, a significant line broadening is observed at the free PS signal at -1.35 ppm without the change in chemical shift position due to the overlap of both the bound and free-state PS signal frequencies. At slightly more than 1:1 ratio of the protein–lipid concentrations, no significant chemical shift changes were observed reaching the equilibrium state indicates the higher affinity of PS binding to the protein. The dissociation constant for the PC and PS were calculated using the one-site binding titration equation, with nonlinear fit using the origin software and a dissociation constant, ‘ K_d ’ of 94 ± 0.044 and 19 ± 0.007 μ M were obtained for PC and PS, respectively (Fig. 5). From our results, it was observed that the PS had greater binding affinity towards MFG-E8L C2 compared to that of PC.

Conclusions

In conclusion, the construct and expression system allowed us to obtain the C2 domain MFG-E8L to near homogeneity as determined by SDS–PAGE, and we also confirmed the identification of the C2 domain by MALDITOF/TOF analysis. From our 1D ¹H NMR experiment, we observed that the C2 domain of MFG-E8L was well folded. Finally, from our ³¹P NMR experiments, we observed that the purified C2 domain of MFG-E8L showed selective binding with PS.

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List of Figure

- Figure 1 Expression and purification of mouse MFG-E8L C2. Protein samples at various stages were analyzed in 12% SDS-PAGE gel: lane 1, MW marker, lanes 2 and 3, before and after IPTG induction; lane 4, the supernatant of induced sample; lanes 5–7 are flow through, wash and elution from Ni^{2+} -IDA affinity column.
- Figure 2 MALDI-TOF/TOF spectrum of MFG-E8L C2. Protein sample was subjected to MALDI-TOF/TOF analysis on Applied Bio-systems MDS SCIEX 4800 MALDI-TOF/TOF analyzer as described in Materials and methods.
- Figure 3 1D ^1H NMR analysis of mouse MFG-E8L C2. Mouse MFG-E8L C2 was purified, and the protein (0.5 mM) in the buffer containing 20 mM Na-PO_4 , pH 6.5, 20 mM NaCl, 0.01% NaN_3 and 10% D_2O was used. The 1D ^1H spectrum was recorded on a Avance 600 MHz NMR spectrometer (Bruker) as described in Materials and methods.
- Figure 4 ^{31}P NMR spectrum of PC and PS. ^{31}P NMR spectra of PC (A) and PS (B) were recorded on a Avance 400 MHz NMR spectrometer (Bruker) (as described in Materials and methods) as a function of increasing concentration of MFG-E8L C2 (protein concentration given to the left of each spectrum). Peaks marked with ‘*’ are due to the interaction between the protein and phosphorus present in the buffer. Stacked plot gives the ^{31}P NMR spectra of phosphate buffer (huge central peak referenced to 0 ppm), and free PC (—1.74 ppm), PS (—1.35 ppm), respectively, as shown in the bottom traces.
- Figure 5 Titration curve for PS and PC. A fit of chemical shift data vs protein concentrations was plotted using one-site binding equation. The binding affinity is quantified by the equilibrium dissociation constant, $K_d = [E][L]/[EL]$. The equation represents a dynamic equilibrium involving free protein $[E]$, free lipid $[L]$ and the protein–lipid complex $[EL]$. A K_d value of $94 \pm 0.044 \mu\text{M}$ and $19 \pm 0.007 \mu\text{M}$ were obtained for PC and PS, respectively.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5