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NMR solution structure of C2 domain of MFG-E8 and insights into its molecular recognition with phosphatidylserine

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ABSTRACT

MFG-E8 (also known as lactadherin), which is a secreted glycoprotein from a variety of cell types, possesses two EGF domains and tandem C domains with sequence homology to that of blood coagulation proteins factor V and factor VIII. MFG-E8 binds to phosphatidylserine (PS) in membranes with high affinity. We have recently shown that the C2 domain of MFG-E8 bears more specificity toward PS when compared with phosphatidylcholine (PC), another phospholipid thought to be involved in the immune function of phagocytes. In our current study, we have determined the solution structure of the C2 domain by nuclear magnetic resonance (NMR) spectroscopy, and characterized the molecular basis of binding between the C2 domain and PS by ^{31}P-NMR spectroscopy. Furthermore, we also verified that that positively charged and aromatic residues clustered in loops 1–3 of the C2 domain play key roles in recognizing PS in apoptotic cells.

1. Introduction

Mouse milk fat globule-EGF factor 8 protein (MFG-E8), also known as lactadherin, is a soluble and heavy glycoprotein, which is expressed and secreted by various cells and tissues, including mammary epithelial cells, stimulated macrophages, stimulated endothelial cells, brain, spleen, lymph nodes, and mammary glands of human, mice, pig, and cow [1–4]. It consists of two repeated EGF-like domains on the N-terminal side, followed by two C domains (C1 and C2 domains), and has two alternative splice variants based on the presence or absence of a proline/threonine-rich domain [2]. The RGD (Arg-Gly-Asp) motif in the second EGF-like domain is recognized by some members of the integrin family of receptors, including $\alpha_v\beta_3$ and $\alpha_v\beta_5$, which facilitate cell–cell adhesion [2,5,6]. The C2 domain of MFG-E8 is the primary recognition site for PS-exposed membranes [6,7].

Macrophage clearance of apoptotic cells is a key process for preventing inflammation and the autoimmune response against intracellular antigens often released by dying cells. As cells undergo apoptosis, PS becomes exposed and is recognized by phagocytes, such as macrophages and dendritic cells as an 'eat me' signal, resulting in the engulfment of apoptotic cells [8]. MFG-E8 secreted from activated macrophages plays an important role as a linker between the PS-exposed apoptotic cells through its C2 domain and the macrophage through its RGD motif [2]. Indeed, PS-masking by a point mutation in the RGD motif induces apoptotic cell accumulation in mice [9]. Moreover, in MFG-E8-deficent mice, apoptotic lymphocytes are not efficiently engulfed by tangible body macrophages [3], with MFG-E8 deficiency leading to autoimmune diseases, such as splenomegaly, glomerulonephritis, and atherosclerosis in mice [3,10].

The C2 domain in MFG-E8 shares homology with the membrane-binding C2 domains of coagulation factors V and VIII [11,12], but is not related to the Ca^{2+}-binding C2 domains of synaptotagmin, phospholipase A2 or protein kinase C [13]. The common feature of membrane-binding C2 domains is an eight-stranded antiparallel β sandwich scaffold along with several irregular loops [14,15]. These loops exhibit considerable variability and may have potential membrane-binding amino acids that interact with the membrane and/or the hydrophilic head group of PS by stereospecific binding or electrostatic properties. Evidence from the crystal structures of the C2 domains of factor V, factor VIII and MFG-E8 suggest that a group of water-exposed hydrophobic residues and glycine residues protruding from these long loops may mediate membrane binding by insertion into the membrane [16,17]. In addition, computational docking studies provide hypothetical PS binding sites, but the sites differ between MFG-E8 and factor V or VIII [13].

In agreement with previous studies, we have recently shown that the C2 domain of MFG-E8 bears more specificity toward PS when compared with phosphatidylcholine (PC), another phospholipid thought to be involved in the immune function of phagocytes [2,18].

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Three-dimensional structural information would provide the molecular basis of the specific interaction between the C2 domain and PS. To this end, in our current study, we expressed and purified C2 domain of MFG-E8 to determine its three-dimensional structure by nuclear magnetic resonance (NMR) spectroscopy. We also performed mutagenesis studies to determine the key residues involved in the interaction with PS by analyzing the binding characteristics of mutant protein with phospholipids by ^{31}P-NMR spectroscopy. Furthermore, we confirmed that key residues, which were identified from the NMR studies, play important roles in the PS-recognition in apoptotic cells in response to death stimuli.

2. Materials and methods

2.1. Materials

^{15}N-NH_4Cl and ^{13}C-Glucose were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). Etoposide was purchased from Calbiochem (La Jolla, CA).

2.2. Construction of expression plasmids

The C2 domain (His306-Cys463) of mouse MFG-E8 (GenBank Accession No. AK171143) was cloned into a pET29b vector, as we described in detail elsewhere [18]. The enhanced green fluorescent protein (EGFP) cDNA (GenBank Accession No. U55763) was cloned into the BamHI and EcoRI sites of pGEX-4T-1 (Pharmacia Biotech, Uppsala, Sweden) to produce a new pGEX-EGFP plasmid. The DNA fragment corresponding to the C2 domain of MFG-E8 was then subcloned into the EcoRI and XhoI sites of the pGEX-EGFP. Mutations in the C2 domain (K24N, K45N, R79Q and R146Q, K24N/K45N, W26A, F81A and W26A/F81A) were generated using complementary oligonucleotides with the desired mutation via standard site-directed mutagenesis. All constructs were confirmed by DNA sequencing.

2.3. Preparation of recombinant proteins

Recombinant mouse MFG-E8 C2 domain was expressed and purified, as we previously described [18]. For NMR study, the isotopically labeled MFG-E8 C2 proteins were expressed in *Escherichia coli* (*E. coli*) BL21 (DE3) cells grown in M9 medium containing $^{15}NH_4Cl$, or $^{15}NH_4Cl$ plus [U-^{13}C]-glucose and purified by Ni^{2+}-affinity followed by Sephacryl S-200. The purified MFG-E8L C2 was concentrated to 0.8 mM in a buffer containing 20 mM sodium phosphate ($NaPO_4$), pH 6.5, 20 mM NaCl, 0.01% sodium azide (NaN_3) and 10% or 100% D_2O). For fluorescence-activated cell sorting (FACS) analysis, the plasmids were transformed into *E. coli* BL21 cells to produce GST-EGFP-C2 domain fusion proteins before being induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) for 4 h at 25 °C. Cells were lysed by sonication in a phosphate-buffered saline (PBS) containing 1 mM phenylmethanesulfonylfluoride (PMSF). The soluble fraction of the lysate was incubated with glutathione-sepharose beads (Amersham Biosciences, Little Chalfont, UK) for 1 h at 4 °C. The beads were washed with PBS and the proteins were eluted from the beads with 10 mM reduced glutathione in 50 mM Tris, pH 8.0. The concentration of the purified protein was determined by Bradford dye assay kit (Bio-Rad Laboratories).

Table 1
Structural statistics for NMR structure of MFG-E8 C2 domain.

Unambiguous NOE restraints	2067
Intra-residual (i = j)	455
Sequential (|i–j| = 1)	700
Medium range (1<|i–j|<5)	188
Long range (|i–j| ≥ 5)	724
Dihedral angle restrains	
All	110
Φ	56
Ψ	54
Hydrogen bond restraints (upper/lower)	99/99
Disulfide bond	1
Total number of restraint violations > 0.4	0
Total number of dihedral angle violations > 5°	0
Average RMSD (Å) to mean for the top 20 structures	
Residues 1–158	
Backbone atoms	1.5 ± 0.25
All heavy atoms	2.18 ± 0.23
Residues 17–20,59–96,102–104,112–156	
Backbone atoms	0.73 ± 0.10
All heavy atoms	1.40 ± 0.14
Residues 14–24,36–156	
Backbone atoms	0.92 ± 0.16
All heavy atoms	1.55 ± 0.16
Ramachandran analysis (%)	
Residues in most favored regions	59.9%
Residues in additional allowed regions	35.1%
Residues in generously allowed regions	3.7%
Residues in disallowed regions	1.4%

2.4. Nuclear magnetic resonance (NMR) spectroscopy experiments

NMR spectra were acquired on a Bruker Avance 700 MHz NMR spectrometer equipped with four RF channels and a 5 mm z-gradient TXI CryoProbe (Bruker BioSpin) at 298 K. Spectra were processed with Topspin version 1.3 (Bruker) and analyzed using Sparky (Goddard T. D. and Kneller D. G., SPARKY3, University of California, San Francisco) and NMRview [19]. Backbone assignment was made from HNCA, HNCO, HNCACB, CBCACONH and 3D 15N-HSQC-NOESY [13]. Side-chain assignments were made from 3D HC(C)H-TOCSY, H(CC)(CO)NH, (H)CC(CO)NH and ^{13}C-HSQC-NOESY. Nuclear Overhauser effects (NOE) distance constraints were provided by 3D ^{15}N- and ^{13}C-edited NOESY spectra acquired with mixing time of 120 ms and 3D aromatic ^{13}C-HMQC-NOESY spectra acquired with mixing time of 80 ms. The exchanging amide protons were monitored by dissolving the lyophilized protein in D_2O and acquiring a series of ^{15}N-HSQC spectra.

2.5. Structural calculation

NMR spatial structures of the MFG-E8 C2 were calculated using CYANA [20]. NOE distance constraints for the structure calculation were obtained from 3D ^{15}N- and ^{13}C-edited NOESY and 3D aromatic ^{13}C-HMQC-NOESY spectra. Dihedral angle restraints were calculated from chemical shifts using Torsion Angle Likelihood Obtained from Shift and sequence similarity (TALOS) and the overall secondary structure was predicted from the Chemical Shift Index (CSI) and NOE pattern [21]. The slowly exchanging amide protons were assigned as hydrogen bond donors with related hydrogen-acceptor partners on the basis of the calculated preliminary structure. The standard CYANA simulated annealing protocol was applied to 100 random structures and the resulting 20 energy-minimized structures with the lowest target function energy were selected for further analysis. Structures were analyzed using the program PROCHECK and visualized by MOLMOL, PyMOL (www.pymol.org) and Swiss-PdbViewer [21–23]. The chemical shifts and the NMR-derived restraints and atomic coordinates of the protein ensemble have been deposited at BioMagResBank (www.bmrb.wisc.edu) with accession code BMRB 17477 and the Protein Data Bank (www.pdb.org) with accession code PDB 2L9L, respectively.

2.6. 2D ^{1}H-^{15}N heteronuclear single quantum correlation (HSQC) titration with 06:0 PS

1,2-Dihexanoyl-*sn*-glycero-3-phospho-L-serine (06:0 PS) (Avanti Polar Lipids, Inc.) was dissolved into buffer containing 20 mM $NaPO_4$, pH 6.5, 50 mM NaCl, 0.01% NaN_3, to make stock solution of 3 mM concentration. The ^{15}N labeled protein sample was expressed and purified as mentioned above, and concentrated to about 0.2 mM in the buffer containing 20 mM $NaPO_4$, pH 6.5, 50 mM NaCl, 0.01% NaN_3,