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Expression, purification, and characterization of recombinant human and murine milk fat globule-epidermal growth factor-factor 8

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ABSTRACT

Milk fat globule-epidermal growth factor-factor 8 (MFG-E8), as its name suggests, is a major glycoprotein component of milk fat globules secreted by the mammary epithelium. Although its role in milk fat production is unclear, MFG-E8 has been shown to act as a bridge linking apoptotic cells to phagocytes for removal of these dying cells. MFG-E8 is capable of bridging these two very different cell types via interactions through both its epidermal growth factor (EGF)-like domain(s) and its lectin-type C domains. The EGF-like domain interacts with $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrins on the surface of phagocytes, whereas the C domains bind phosphatidylserine found on the surface of apoptotic cells. In an attempt to purify full-length, recombinant MFG-E8 expressed in either insect cells or CHO cells, we find that it is highly aggregated. Systematic truncation of the domain architecture of MFG-E8 indicates that the C domains are mainly responsible for the aggregation propensity. Addition of Triton X-100 to the conditioned cell culture media allowed partial recovery of non-aggregated, full-length MFG-E8. A more comprehensive detergent screen identified CHAPS as a stabilizer of MFG-E8 and allowed purification of a significant portion of non-aggregated, full-length protein. The CHAPS-stabilized recombinant MFG-E8 retained its natural ability to bind both $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrins and phosphatidylserine suggesting that it is properly folded and active. Herein we describe an efficient purification method for production of non-aggregated, full-length MFG-E8.

1. Introduction

Milk fat globule-epidermal growth factor-factor 8 (MFG-E8) was originally identified as a major glycoprotein of the milk fat globule, a protein- and triglyceride-rich, membrane-bound vesicle secreted from the mammary epithelium during milk production [1]. Milk fat globules are generated when lipid droplets are formed in the cytoplasm of mammary epithelial cells and become surrounded by the plasma membrane leading to budding of a membrane-bound vesicle from the apical side of the epithelial cell (reviewed in Ref. [2]). While MFG-E8 does not contain a transmembrane domain, it peripherally associates with membrane vesicles through its interaction with anionic phospholipids. Apart from their association with milk fat globules, considerable interest in the glycoprotein components of human milk fat globules (HMFGs) stems from their differential expression in human breast tumors [3]. Antibodies have been raised against a number of these HMFG glycoproteins for use in imaging, immunotherapy, diagnostics, and histopathology as they serve as convenient tumor markers [3–7]. While murine MFG-E8 was the first to be cloned [1], the human ortholog had been studied for years as the unnamed 46 kDa antigen of the HMFG. Shortly after the cloning of murine MFG-E8, the human gene was also isolated from a breast cDNA library and was initially called BA46 (breast antigen of 46 kDa) [8]. Additional synonyms for MFG-E8 from various species include lactadherin (human), PAS-6/7 (bovine), SED1 (mouse), and rAGS (rat) [9]. In addition to production by mammary epithelial cells, MFG-E8 is also secreted by epididymal epithelial cells, vascular cells, aortic smooth muscle cells, and activated macrophages [10–13]. While the function of MFG-E8 in differentiated breast tissue remains unclear, a role for MFG-E8 in linking apoptotic cells to phagocytes during cell death has been elucidated based on the domain structure of the protein [12].

MFG-E8 is a multi-domain protein consisting of one to three

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Abbreviations used

BA46	breast antigen of 46 kDa
BSA	bovine serum albumin
CHAPS	3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate
CHO	Chinese hamster ovary
CMC	critical micelle concentration
CVs	column volumes
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
FA	formic acid
Fc	fragment crystallizable region of antibodies
FC12	Fos-choline 12
FL	full-length
FSEC	fluorescence-detection size-exclusion chromatography
HMFGs	human milk fat globules
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
Hs	*Homo sapien* or human
kDa	kilodalton
LC-MS/MS	liquid chromatography-tandem mass spectrometry
MALDI-ISD	matrix-assisted laser desorption ionization in-source decay
MALS	multi-angle light scattering
MFG-E8	milk fat globule-epidermal growth factor-factor 8
Mm	*Mus musculus* or mouse
NMR	nuclear magnetic resonance
NR	non-reduced
OG	octyl β-glucoside
PBS	phosphate-buffered saline
P/T domain	proline/threonine-rich domain
$PBST_{0.05}$	phosphate-buffered saline with 0.05% Polysorbate Tween 20
RED	reduced
RGD-motif	arginine, glycine, aspartic acid-motif
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel-electrophoresis
SEC	size-exclusion chromatography
SEC-MALS	size-exclusion chromatography coupled to multi-angle light scattering
Sf9	*Spodoptera frugiperda* cells
TBS	Tris-buffered saline
$TBST_{0.1}$	Tris-buffered saline with 0.1% Polysorbate Tween 20
TFF	tangential flow filtration
TMB	3, 3′, 5, 5′-tetramethylbenzidine
T.ni	*Trichoplusia ni*
UPLC	ultra-performance liquid chromatography.

amino-terminal epidermal growth factor (EGF)-like domains and two carboxy-terminal lectin-type C domains (C1 and C2) (reviewed in Ref. [14]). The EGF-like domains of MFG-E8 are named based on their homology to the EGF-like repeats found in the *Drosophila* Notch protein and the C domains of MFG-E8 are also known as F5/8-type C domains due to their homology to the C1 and C2 domains of the human coagulation factors V and VIII. Based on the multi-domain architecture of MFG-E8, it is not surprising that it is a bifunctional protein: the EGF-like domains bind to $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrins on the surface of cells through their arginine, glycine, aspartic acid (RGD)-motif [15–17], whereas the C domains are important for binding anionic phospholipids such as phosphatidylserine [12,15]. With this bifunctional binding activity, MFG-E8 acts as a bridge between apoptotic cells that contain a high concentration of phosphatidylserine on the outer layer of the plasma membrane and phagocytes that have integrin receptors expressed on their cell surface [12]. MFG-E8 acts to opsonize apoptotic cells and attract the body's natural clean up crew of phagocytes. While the number of C domains in MFG-E8 is constant between species at two, the number of EGF-like domains varies with a single domain found in human MFG-E8, two in bovine and murine, and three in chicken, frog, and zebrafish. Additionally, murine MFG-E8 exists as two splice variants with the long form containing a proline/threonine-rich (PT) domain between the second EGF-like domain and the C1 domain and the short form lacking the PT domain. The presence of this domain increases the affinity for anionic phospholipids and enhances phagocytosis of apoptotic cells [12].

In an attempt to produce recombinant full-length human and murine MFG-E8, we find that the protein is extremely aggregation-prone. To better understand the nature of this aggregation and how to mitigate it, we carried out systematic deletions of the individual domains of MFG-E8 and find that the C1 and C2 domains are mainly responsible for this aggregation. Furthermore, we developed a robust and simple purification method for isolation of well-behaved, recombinant MFG-E8 and describe it herein.

2. Materials and methods

2.1. Construct design and cloning

DNA constructs for full-length human (24-387) and murine (23-463) MFG-E8, missing the endogenous signal peptides, were cloned as carboxy-terminal fusions to either a human or murine Fc tag into a modified pAcGP67A vector (BD Biosciences, #554756) or modified pRK vector (Genentech) carrying a constitutive strong signal peptide for extracellular expression in insect and mammalian cells, respectively. Deletion mutants were generated with the following boundaries: human ΔC1 (Δ69-224), human ΔC2 (Δ226-387), human ΔC1,2 (Δ69-387), human ΔEGF (Δ24-67), murine ΔC1 (Δ147-303), murine ΔC2 (Δ304-463), murine ΔC1,2 (Δ147-463), murine ΔEGF ΔP/T (Δ23-145). All constructs were sequence verified.

2.2. Small-scale expression in Sf9/T.ni cells and CHO/293S cells

For small-scale expression we used either *Spodoptera frugiperda* cells (Sf9) or *Trichoplusia ni* cells (T.ni) (Expression Systems, Davis, CA) passaged in ESF 921 serum-free, protein-free media (Expression Systems, #96-001). Cultures were grown in 125 mL shake flasks in an INFORS HT Multitron Cell (Bottmingen/Basel, Switzerland) shaker incubator at 27 °C with shaking at 120 rpm. For viral production, Sf9 cells were initially placed in a 96-well plate at 1×10^6 cells/mL. Cells were then co-transfected with transfer vectors and BestBac linearized viral DNA (Expression Systems, #91-001) using TransIT (Mirus Bio, #MIR 6100). After 4 days, cultures were transferred to 24-well blocks and grown in an Innova 4230 shaker incubator at 27 °C with shaking at 250 rpm for viral amplification. The titer of recombinant baculovirus obtained after two cycles of amplification was assessed by measuring the number