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Research paper

Rapid and efficient purification of ficolin-2 using a disposable CELLine bioreactor

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ABSTRACT

The human opsonin ficolin-2 (L-ficolin) is an innate pattern-recognizing molecule that binds to acetylated moieties. Upon binding, ficolin-2 activates complement through the lectin pathway, opsonizing the target to promote phagocytic clearance. Ficolin-2 has been found to interact with a growing number of pathogenic bacteria, fungi, and viruses. Ficolin-2 also has proposed roles in host homeostasis, including the clearance of apoptotic cells. Consequently, there is an increased interest in studying ficolin-2, and access to purified ficolin-2 is necessary for these studies. Ficolin-2 purified from serum, plasma, or cell culture supernatants has been a useful tool in the characterization of ficolin-2 function; however, available protocols are laborious and inefficient, requiring additional processing of starting materials (e.g., polyethylene glycol precipitation or dialysis) and multiple steps of purification. Here, we investigated a simple solution to the problem: use of a simple, disposable bioreactor requiring only standard tissue culture equipment. Using this system, we generated cell culture supernatants containing high concentrations of recombinant ficolin-2, which permitted rapid purification of high-purity recombinant ficolin-2 without processing the supernatants. Purified recombinant ficolin-2 retained its binding capacity and supported complement activation in vitro. Bioreactor cultivation will likely be generally useful in the production of other recombinant proteins in the study of the complement system.

1. Introduction

Since its discovery in the late 1990s, ficolin-2 (also known as L-ficolin) has become a molecule of great interest. Ficolin-2 functions as an innate immune opsonin and participates in the lectin pathway of complement activation, similarly to mannose binding lectin (MBL) (Faro et al., 2008). As part of innate immunity, ficolin-2 has been described to interact with acetylated and other moieties on an ever-expanding list of pathogenic bacterial and viral targets (Fujieda et al., 2012; Kilpatrick and Chalmers, 2012; Pan et al., 2012; Luo et al., 2013; Brady et al., 2014a; Hamed et al., 2014; Vassal-Stermann et al., 2014), but it has also been shown to bind to apoptotic cells (Kuraya et al., 2005; Jensen et al., 2007) and mitochondria (Brinkmann et al., 2013), suggesting important roles in host homeostasis.

Unlike its biological function, the structure of ficolin-2 is well-understood. Ficolin-2 is comprised of an N-terminal cysteine rich domain, a long, collagen-like domain, and a C-terminal fibrinogen-like domain, which is the ligand-binding domain (Matsushita et al., 1996). Ficolin-2 trimerizes through the collagen-like domain, and the trimers form higher-order oligomers through disulfide bonds in the N-terminal domain, with the most commonly observed species existing as a 12-mer (a tetramer of trimers) arranged in a bouquet-like structure (Ohashi and Erickson, 2004). Ficolin-2 is produced in the liver and is found primarily in the serum (Matsushita et al., 1996; Ohashi and Erickson, 2004), where it associates with MBL/ficolin-associated serine proteases (MASPs) (Matsushita et al., 2000). Upon ficolin-2 binding to a target, the MASPs become activated and cleave C4 and C2 to form the classical C3 convertase C4b2a and initiate complement activation.

Studies of ficolin-2 function require purified ficolin-2, and several protocols are described to purify ficolin-2 from human serum or plasma using agarose or sepharose beads conjugated to GlcNAc or CysNAc (Krarup et al., 2004; Faro et al., 2008; Matsushita et al., 2014). However, the reported protocols are apparently inefficient, with multiple steps of purification and low reported yields. Recombinant protein expression offers flexibility, and a common approach is the translational fusion of the protein to an affinity tag such as a polyhistidine tag. However, the secreted nature of ficolin-2 requires that the tag be placed at the C-terminal end of the protein. Indeed, commercially available recombinant ficolin-2 from R&D Systems (catalog no. 2428-FC) has a polyhistidine tag at the C-terminus. Since binding of ficolin-2 occurs through the C-terminus, tagging may alter or interfere with ficolin-2 target recognition; indeed, a C-terminally his-tagged ficolin-2 produced in our laboratory failed to bind serotype 11A bacteria (unpublished

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observation), a natural ficolin-2 target (Brady et al., 2014a). Thus, recombinant ficolin-2 in its natural form is desirable.

However, purification of ficolin-2 can be laborious, and purification of recombinant ficolin-2 presents further challenges due to low concentrations of ficolin-2 in the supernatants, requiring handling and concentration of large volumes of supernatant prior to purification (Lacroix et al., 2009). To avoid these challenges, we have investigated use of bioreactors, which can permit high-concentration yields of secreted proteins. We show that a simple and inexpensive disposable bioreactor produces culture supernatants with high concentrations of ficolin-2 and that ficolin-2 can be directly purified from these supernatants without processing using GlcNAc-agarose, yielding high-purity ficolin-2.

2. Materials and methods

2.1. Culture conditions

Generation of the human ficolin-2-expressing cell line huf2E by transfection of Chinese Hamster Ovary K1 (CHO) cells with FCN2 cDNA (GenBank accession no. BC069825) in pcDNA3.1(−) was described previously (Brady et al., 2014a,b). All cell culture was performed in a humidified 37 °C incubator with 5% CO_2. For conventional cell culture, a confluent 150 cm^2 tissue culture flask of huf2E was rinsed with 5 ml Hanks' Balanced Saline Solution without magnesium or calcium (HBSS −/−) and digested with 5 ml trypsin (0.05%)-EDTA solution (Gibco 15400) in HBSS −/− for 5 min at 37 °C. After digestion, cells were thoroughly resuspended from the flask by pipetting, and 250 μl (1/20 flask, 1.6×10^6 cells) was inoculated into 100 ml fresh Dulbecco's Modified Eagle's Medium/Nutrient Mixture F12 (DMEF, Thermo Scientific SH30023) supplemented to 10% heat-inactivated fetal bovine serum (Thermo Scientific SH30080) (DMEF-FBS). Geneticin (Life Technologies 10131027) was added to a final concentration of 750 μg/ml. Supernatants were harvested at 14 days, and cells were passaged at harvest as described above.

For bioreactor culture, 1.6×10^6 huf2E cells (prepared as described above) were inoculated into 15 ml DMEF-FBS in the cell chamber of a CELLine Bioreactor Flask (Wheaton Science Products WCL1000-3); 150 ml DMEF was added to the nutrient chamber. Both chambers were supplemented to 750 μg/ml Geneticin. After 14 days of incubation, the nutrient medium was discarded. Cell chamber material (typically 20–25 ml) was removed and centrifuged at 314 ×*g* to recover cells. Cells were resuspended in 2 ml DMEF-FBS, and 1/20 cells (~5.2×10^6) were inoculated into 15 ml fresh DMEF-FBS in the cell chamber of the same bioreactor. Fresh nutrient medium and Geneticin were added as described above. It should be noted that the CELLine bioreactor is available with an adherent surface; as CHO cells are compatible with either unit, we used the suspension model and have not tested our protocols with the adherent cell model.

2.2. SDS-PAGE and immunoblotting

Ficolin-2 purity was determined through SDS-10% PAGE of a 15 μl aliquot of the indicated fractions and silver staining using Pierce Color Silver Stain Kit (Thermo Scientific 24597). Ficolin-2-containing fractions were detected through either immunoblot of SDS-10% PAGE of a 5 μl aliquot of the indicated fractions or dot blot of 100 μl serial dilutions of the indicated fractions on a 96-well dot blotting apparatus (Bio-Rad 170-6545). All immunoblots were performed on 0.45 μm nitrocellulose membranes and blocked in 5% powdered skim milk in tris-buffered saline supplemented with 0.05% tween-20. Immunodetection of ficolin-2 was achieved using a biotinylated anti-ficolin-2 antibody (R&D Systems BAF2428) and streptavidin-conjugated alkaline phosphatase (Life Technologies 43-4322), both at a dilution of 1:1000, and development using 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium chloride in 1 M tris, pH 8.8.

2.3. Buffers

The dialysis buffer (referred to hereafter as "wash buffer") reported by Lacroix et al. (2009) (145 mM NaCl, 5 mM $CaCl_2$, 20 mM Tris, pH 7.4) was the base for all other buffers, but because some solutes were acidic, solutions for purification were made from a 10× wash buffer solution and not adjusted for pH until the final solution was assembled. For elution from GlcNAc column, 500 mM N-acetyl-L-cysteine (CysNAc) was dissolved in 10× wash buffer and water and adjusted to pH 7.4 with 50% w/w NaOH before being brought to 1× (elution buffer).

2.4. Purification of ficolin-2

All steps were performed at room temperature (~20 °C) unless otherwise noted. All column applications were by gravity flow. Ten milliliters of ficolin-2-containing bioreactor supernatants were applied to a 1 ml bed of GlcNAc-agarose (Sigma-Aldrich A2278) (previously equilibrated with 20 ml wash buffer) in a 10 ml polypropylene chromatography column (Bio-Rad 731-1550). The column was washed six times with 10 ml wash buffer prior to five 1-ml elutions with elution buffer. Ficolin-2 containing fractions were identified by dot blotting, pooled, and dialyzed against ≥ 500 volumes wash buffer at 4 °C overnight.

Dialyzed ficolin-2 was supplemented with glycerol to 10% and concentrated using 3 kDa molecular weight cutoff microcentrifuge concentrator columns (Millipore UFC500396) to achieve a concentration of ~ 1 mg/ml.

2.5. Quantitation of ficolin-2

Ficolin-2 from supernatants was quantitated absolutely using a human ficolin-2 ELISA kit (Hycult Biotech HK336) or relatively by dot blot. Concentration of recovered ficolin-2 was determined by measuring absorbance at 280 nm in a 1 cm quartz cuvette (using either wash buffer or wash buffer made with 10% glycerol as a blank) assuming an extinction coefficient of 1.767 $(mg/ml)^{-1}$ cm^{-1} calculated using the Protein Calculator (http://protcalc.sourceforge.net/) based on the molecular weight and the method of Gill and von Hippel (Gill and von Hippel, 1989).

2.6. Ficolin-2 functional assays

Ficolin-2 was assayed for binding and complement activation on serotype 11A *Streptococcus pneumoniae* (strain JC03 (Calix et al., 2014)) using flow-cytometric assays as previously described (Brady et al., 2014a,b,c). C1q-depleted serum was purchased from Quidel (A509); normal human serum (NHS) was obtained from a consented, healthy adult donor in glass serum collection tubes under an IRB-approved protocol. NHS was previously determined to have 4.95 μg/ml ficolin-2 (Brady et al., 2014c); commercial C1q-depleted serum is depleted of ficolin-2 (Brady et al., 2014b). Sera were used at 5% final concentration where indicated. Briefly, JC03 was opsonized with ficolin-2 (or in buffer control) at 4 °C for 1 h, washed by centrifugation and resuspension, incubated in the presence of serum (or buffer control) for 1 h at 37 °C, and probed for ficolin-2 deposition and complement deposition with specific antibodies before analysis by flow cytometry. C3 and C4 deposition were detected by Pierce Complement C3 Antibody (Thermo Scientific LF-MA0132, used at 1:1000), detected by a phycoerythrin-conjugated goat-anti-mouse secondary antibody (Southern Biotech 1030-09, used at 1:2500), and a fluorescein isothiocyanate-conjugated anti-C4b/C4c antibody (Thermo Scientific PA1-28407, used at 1:200). Due to incompatible fluorophores, ficolin-2 binding was detected in parallel in identically prepared samples using the Pierce L-Ficolin (clone 19) monoclonal antibody (Thermo Scientific ABS005-19-02, used at 1:1000) and a phycoerythrin-conjugated goat-anti-mouse IgG secondary antibody (Southern Biotech 1030-09).