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Expression, purification, and characterization of recombinant human flotillin-1 in *Escherichia coli*

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Abstract

Human flotillin-1 (reggie-2), a major hydrophobic protein of biomembrane microdomain lipid rafts, was cloned and expressed in *Escherichia coli* with four different fusion tags (hexahistidine, glutathione *S*-transferase, NusA, and thioredoxin) to increase the yield. The best expressed flotillin-1 with thioredoxin tag was solubilized from inclusion bodies, first purified by immobilized metal affinity column under denaturing condition and direct refolded on column by decreasing urea gradient method. The thioredoxin tag was cleaved by thrombin, and the flotillin-1 protein was further purified by anion exchanger and gel filtration column. The purified protein was verified by denaturing gel electrophoresis and Western blot. The typical yield was 3.4 mg with purity above 98% from 1 L culture medium. Using pull-down assay, the interaction of both the recombinant flotillin-1 and the native flotillin-1 from human erythrocyte membranes with c-Cbl-associated protein or neuroglobin was confirmed, which demonstrated that the recombinant proteins were functional active. This is the first report describing expression, purification, and characterization of active recombinant raft specific protein in large quantity and highly purity, which would facilitate further research such as X-ray crystallography.

Keywords: Flotillin-1 (reggie-2); Lipid rafts; Thioredoxin tag; c-Cbl-associated protein; Neuroglobin

Lipid rafts are newly discovered membrane microdomains enriched in sphingolipids and cholesterol. They are also called detergent-resistant membranes for their insolubility in most non-ionic detergents (reviewed in [1]). Many proteins involving in signal transduction and vesicular trafficking are abundant in lipid rafts, such as Src-family tyrosine kinases, protein kinase C, heterotrimeric and small G proteins, tyrosine kinase receptors, and G-protein-coupled receptors (reviewed in [2,3]). Besides these signaling proteins, lipid rafts also have some specific proteins, which were generally used as markers of lipid rafts, including flotillin [4,5], caveolin [6], and stomatin [7]. But the structure, function, interaction with other proteins, and linkage with disease of these proteins were not clearly defined.

Flotillins consist of two members: flotillin-1 (reggie-2) and flotillin-2 (reggie-1). They were originally discovered by their significant upregulated expression in axonal regeneration after a lesion of the goldfish optic nerve [4]. Bickel et al. [5] isolated a 'new' protein from the Triton X-100 insoluble membrane fraction of murine lung tissue in screening novel markers of lipid rafts, and named it "flotillin" by their membrane state—float like a flotilla of ships in the Triton insoluble buoyant fraction. Sequence analysis shows that flotillin-1 is identical to reggie-2 and flotillin-2 is identical to reggie-1. Flotillin-1 and flotillin-2 are homologous and evolutionary conserved [8]. Similar to stomatin, prohibitin, and bacterial membrane proteins HflK and HflC, flottillins consist of conserved SPFH domain [9].

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Human flotillin-1 gene is single copy and has 13 exons, locates at 6p21.3. It is highly expressed in brain, heart, and lung. The human flotillin-1 protein consists of 427 amino acid residues with predicted molecular weight of 47,355 Da, and predicted isoelectric point of 7.08 [10]. Flotillin-1 protein contains several potentially phosphorylated sites: residues 116 and 150 can be phosphorylated by casein kinase II, residues 160 and 238 by tyrosine kinase, and the residues 150 and 160 are evolutionary conserved. Flotillin-1 is also palmitoylated, which enables flotillin-1 associate with plasma membrane through a conserved cysteine residue, Cys-34 [11]. On cell membrane, flotillin-1, flotillin-2, and caveolins exist as hetero-oligomeric complex [12]. Flotillin-1, flotillin-2 and stomatin are the most abundant membrane proteins in erythrocyte lipid rafts. They present as independently organized high-order oligomers and act as separate scaffolding components at the cytoplasmic face of erythrocyte lipid rafts [7].

During malaria parasite *Plasmodium falciparum* infection, the association of flotillins with erythrocyte lipid rafts was disrupted, and flotillins were selectively recruited to the vacuole [13,14]. Flotillin-1 is associated with amyloid-β protein, a protein resided in lipid rafts of human brain [15]. By Western blot and immunohistochemical analysis, Kokubo et al. [16] found high flotillins expression in cortex during the development of senile plaque formation. Girardot et al. [17] also found that flotillin-1 accumulated in neuron lysosomes in Alzheimer's disease.

Flotillin-1 also has important function in the second signaling pathway required for insulin-stimulated glucose transport. Apart from the phosphatidylinositol-3-kinase-dependent pathway, c-Cbl-associated protein (CAP)[1]/Cbl complex can also first form a ternary complex with flotillin-1, then be localized to the plasma membrane lipid raft subdomain, finally stimulates glucose transport into fat and muscle cells [18]. Our research of searching the membrane proteins related to the type 2 diabetes in human erythrocyte by proteomics analysis also shows that flotillin-1 is up-regulated in type 2 diabetes patients [19]. To do further study on the reason of abnormal flotillin-1 expression in type 2 diabetes and the role of flotillin-1 in glucose uptake, we want to get large quantity of functional flotillin-1 protein.

The lipid raft proteins were normally separated from membrane insoluble fraction in 1% Triton X-100 at low temperature, with a further purification step using sucrose gradient ultracentrifuge [5]. But it is hard to isolate flotillin-1 from other lipid raft proteins. Bauer et al. [20] have isolated flotillin-1 directly from goldfish brain by two consecutive HPLC steps, the typical yield is 1 μg flotillin-1 from 30 goldfish brains. The membrane association and hydrophobic character make flotillin-1 protein purification from tissues unfeasible. Researchers tried to get flotillin-1 by express it in prokaryote [4,21], but there was no work involving purification and characterization functional full-length flotillin-1 in large quantity and high purity, so we tried to increase the expression level of flotillin-1 by using different tags, then to purify and characterize it. The biochemical activity of recombinant flotillin-1 was compared with native flotillin-1 extracted from human erythrocyte ghosts by pull-down assay. They all pulled-down by c-Cbl-associated protein or neuroglobin, both were known to interact with flotillin-1. The result showed that the recombinant and native flotillin-1 were identical.

Materials and methods

Materials

The bacterial (*Escherichia coli*) hosts DH5α, BL21 (DE3), HMS174 (DE3), the vectors pET21a, pET32a, pET43.1a, protein markers, and Western blot kits were obtained from Novagen (Madison, WI). KOD plus Pfu polymerase was purchased from Toyobo (Osaka, Japan). Nucleotides, agarose gel, DNA extraction kit, and high pure PCR purification kit were purchased from Roche Diagnostics (Indianapolis, IN). Primers were synthesized at Bioasia (Shanghai, China). DNA sequencing was performed by Bioasia (Shanghai, China). The restriction endonucleases and DNA ligation kit were purchased from Takara (Dalian, China). Nickel–nitrilotriacetic acid (Ni–NTA) Superflow column matrix was obtained from Qiagen (Chatsworth, CA). Sephacryl S-200 matrix, Sephadex G-50 matrix, Glutathione–Sepharose 4B, Thrombin, and pGEX-4T-1 vector were purchased from Amersham Biosciences (Piscataway, NJ). High Q Cartridge was from Bio-Rad (Hercules, CA). B-PER bacterial protein extraction reagent, bicinchoninic acid (BCA) protein assay reagent kit, goat anti-mouse secondary antibody labeled with horseradish peroxidase (HRP), and SuperSignal WestPico chemiluminescent substrate were from Pierce (Rockford, IL). MagneGST glutathione particles were from Promega (Madison, WI). Anti-flotillin-1 monoclonal antibody was from BD Biosciences Pharmingen (San Diego, CA). *n*-Octyl-β-D-glucoside (OG) was from Dojindo Laboratories (Kumamoto, Japan). β-Mercaptoethanol (BME), 3-[(3-cholamidopropyl)dimethylamino]-1-propanesulfonate (Chaps), dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA),

[1] *Abbreviations used:* BCA, bicinchoninic acid; BME, β-mercaptoethanol; CAP, c-Cbl-associated protein; Chaps, 3-[(3-cholamidopropyl)dimethylamino]-1-propanesulfonate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Flot1, flotillin-1; GST, glutathione *S*-transferase; His_6, hexahistidine; HRP, horseradish peroxidase; IPTG, isopropyl β-D-thiogalactopyranoside; Ngb, neuroglobin; Ni–NTA, nickel–nitrilotriacetic acid; OG, *n*-octyl-β-D-glucoside; PMSF, phenylmethylsulfonyl fluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SoHo, sorbin homology domain; TRX, thioredoxin.