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Interaction of FAM5C with UDP-glucose:glycoprotein glucosyltransferase 1 (UGGT1): Implication of *N*-glycosylation in FAM5C secretion

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ABSTRACT

N-glycosylation of proteins is important for protein folding and function. We have recently reported that FAM5C/BRINP3 contributes to the tumor necrosis factor-α-induced expression of leukocyte adhesion molecules in vascular endothelial cells (ECs). However, regulatory mechanism of the FAM5C biosynthesis is poorly understood. Co-immunoprecipitation assay revealed the interaction of FAM5C with UDP-glucose:glycoprotein glucosyltransferase 1 (UGGT1), a glycoprotein folding-sensor enzyme. FAM5C ectopically expressed in HEK293 cells was localized to the endoplasmic reticulum and co-localized with endogenously expressed UGGT1. Molecular size of FAM5C was reduced by treatment with *N*-glycosidase F and in FAM5C-expressing cells cultured in the presence of the *N*-glycosylation inhibitor tunicamycin. FAM5C was secreted by the cells and the secretion of FAM5C was blocked by tunicamycin. Among six potential *N*-glycosylation sites, the potential site at Asn^{168} was not *N*-glycosylated, and Asn^{337}, Asn^{456}, Asn^{562}, Asn^{609}, and Asn^{641} mutants were poorly secreted by the cells. These results demonstrated that FAM5C is an *N*-glycosylated protein and *N*-glycosylation is necessary for the secretion of FAM5C.

1. Introduction

In eukaryotes, newly synthesized membrane and secretory proteins exhibit protein modifications, in particular, asparagine-linked glycosylation (*N*-glycosylation) in the endoplasmic reticulum (ER) [1]. *N*-glycosylation plays key roles in protein folding and in the regulation of protein function [2]. A consensus sequence for *N*-glycosylation is Asn–Xaa–Ser/Thr (Xaa: any amino acids except Pro) [3]. *N*-glycosylation is catalyzed by oligosaccharyl transferase, and then the outermost glucose is trimmed by glucosidase I (G-I), and further two glucose residues are trimmed by glucosidase II (G-II) [1]. Properly glycosylated proteins are correctly folded and then translocated to the Golgi apparatus, whereas severely misfolded proteins are cleared by the ER-associated degradation pathway [4]. However, misfolded glycoproteins that have been deglycosylated by G-II are reglycosylated by the ER folding-sensor enzyme UDP-glucose:glycoprotein glucosyltransferase 1 (UGGT1) that facilitates their folding [5]. Thus, UGGT1 acts as a gatekeeper for the quality control of glycosylated proteins.

Family with sequence similarity 5, member C (FAM5C, alias bone morphogenetic protein and retinoic acid-inducible neural specific protein 3 [BRINP3]) was originally identified as a protein

Abbreviations: BRINP, bone morphogenetic protein and retinoic acid-inducible neural specific protein; DMEM, Dulbecco's modified medium; ECs, endothelial cells; ER, endoplasmic reticulum; G-I, glucosidase I; G- II, glucosidase II; HEK, human embolic kidney; LC-MS/MS, liquid chromatography-mass spectrometry/mass spectrometry; mAb, monoclonal antibody; *N*-glycosylation, asparagine-linked glycosylation; pAb, polyclonal antibody; PBS, phosphate-buffered saline; TNF, tumor necrosis factor; UGGT, UDP-glucose:glycoprotein glucosyltransferase.

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homologous to BRINP1 [6]. Genetic association between polymorphisms within the *FAM5C* gene and human diseases has been reported [7,8], but biochemical properties and biological activities of FAM5C have been poorly understood. FAM5C increased the proliferation, migration, and invasion of pituitary gonadotrope cells [9] and enhanced the differentiation of cultured osteoblasts [10]. We recently reported that FAM5C contributes to the tumor necrosis factor (TNF)-α-induced expression of leukocyte adhesion molecules, such as intercellular adhesion molecule-1, vascular cell adhesion molecule-1 and E-selectin, in vascular endothelial cells (ECs), implicating the involvement of FAM5C in vascular inflammation [11].

In the present study, to gain more insight into the biochemical properties and biological activities of FAM5C, we sought to identify FAM5C-interacting proteins. We generated FAM5C stably expressing HEK293 cells and found that FAM5C interacted with UGGT1 and was *N*-glycosylated. We identified bona fide *N*-glycosylation sites and found that among six potential *N*-glycosylation sites, five potential sites were efficiently glycosylated but the other one was not. *N*-glycosylation was necessary for the secretion of FAM5C from the cells. These results provide the first evidence that FAM5C is *N*-glycosylated and that this modification is important for its secretion from the cells.

2. Materials and methods

2.1. Antibodies, plasmids and reagents

Rabbit anti-GFP polyclonal antibody (pAb) (598, Medical and Biological Laboratories, Nagoya, Japan), mouse anti-UGGT1 monoclonal antibody (mAb) (#sc-374565, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), mouse anti-calnexin mAb (AF18, #ab31290, Abcam, Cambridge, UK), mouse anti-GM130 mAb (clone 35/GM130, #610822, BD Transduction Laboratories), mouse anti-mitochondrial Hsp70 mAb (clone JG1, #MA3-028, Thermo Fischer Scientific, Waltham, MA, USA), HRP-conjugated secondary Abs (GE Healthcare Bioscience, Pittsburgh, PA, USA), fluorophore (Alexa 488 or 555)-conjugated secondary Abs (Molecular Probes, Eugene, OR, USA), and fluorophore (FITC and Cy3)-conjugated secondary Abs (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) were purchased. 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI) was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). GFP-tagged rat FAM5C (FAM5C-GFP) cDNA (pEGFP-N1-rBRINP3) was prepared as described previously [6]. Mutants of FAM5C (Asn - > Asp) were prepared using QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies Inc., Santa Clara, CA, USA). GFP cDNA (pEGFP-N1) (Life technologies, Carlsbad, CA, USA) was used as a control. Recombinant *N*-glycosidase F (PNGase F) and tunicamycin were purchased from Roche Applied Science (Penzberg, Upper Bavaria, Germany) and Wako (Osaka, Japan), respectively.

2.2. Establishment of stable cell line

Human embolic kidney (HEK) 293 cells stably expressing GFP, FAM5C-GFP or mutants of FAM5C were generated by the transfection with each cDNA using Lipofectamine 2000 (Life technologies), followed by the selection using 750 μg/ml G418 sulfate (Promega, Maddison, WI, USA), and maintained in Dulbecco's modified medium (DMEM) supplemented with 10% fetal calf serum and penicillin/streptomycin.

2.3. Immunofluorescence microscopy

Immunofluorescence microscopic analyses of 4% paraformaldehyde-fixed cells were performed as described previously [12]. In brief, the samples were incubated with 1% bovine serum albumin in phosphate-buffered saline (PBS), then incubated with Blocking One (Nacalai Tesque) in PBS. The samples were stained with the indicated Abs and then with appropriate fluorophore-conjugated secondary Abs. The fluorescent signals were visualized with a confocal laser scanning microscope (LSM700, Carl Zeiss, Oberkochen, Germany).

2.4. Immunoprecipitation and Western blotting

Immunoprecipitation and Western blotting were performed by standard methods using lysis buffer (20 mM Tris-HCl at pH 7.5, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 μg/ml leupeptin, and 1 mM PMSF) as described previously [12]. The signals were visualized by incubation with SuperSignal West Pico Chemiluminescent Substrate or SuperSignal West Femto Chemiluminescent Substrate (Thermo Fisher Scientific), and then detected using the Amersham Imager 600 (GE Healthcare Bioscience).

2.5. Mass spectrometry analysis

Mass spectrometry analysis was performed to identify proteins in the silver-stained bands in the SDS-polyacrylamide gel. The immunoprecipitates were separated by SDS- polyacrylamide gel electrophoresis and silver staining was performed using Silver Stain MS Kit (Wako Pure Chemical Industries, Ltd, Osaka, Japan) according to the manufacture's protocol. The corresponding band was excised from each sample lane, followed by in-gel digestion with 10 μg/ml sequencing grade modified trypsin (Promega) overnight at 37 °C [13]. The digested peptides were eluted with 0.1% formic acid and were subjected to liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) analysis, which was performed on a LCMS-IT-TOF (Shimadzu) interfaced with a nano reverse-phase liquid chromatography system (Shimadzu). MS/MS data were acquired in the data-dependent mode by LCMS solution software (Shimadzu) and were converted to a single text file by Mascot Distiller (Matrixscience). The file was analyzed using the Mascot (Matrixscience) MS/MS Ion Search to search, and assign the obtained peptides, to the SwissProt database.

2.6. Preparation of conditioned media and de-N-glycosylation

Conditioned media of HEK293 cells stably expressing GFP, FAM5C-GFP or mutants of FAM5C were prepared as follows. Confluent cells were cultured Opti-MEM (Life technologies) for 48 h and then culture media were centrifuged at 3000 *g* for 30 min to remove floating cells, followed by concentrated using Amicon-ultra-15 (EMD Millipore, Billerica, MA, USA). De-*N*-glycosylation was done by incubation of the conditioned media with 1 U/ml PNGase F at 37 °C overnight.

3. Results

3.1. Interaction of FAM5C with UGGT1

To gain more insight into the biochemical properties and biological activities of FAM5C, we sought to identify FAM5C-interacting proteins. For this purpose, HEK293 cells that stably express GFP or FAM5C-GFP were generated, and then proteins immunoprecipitated with anti-GFP pAb were analyzed by SDS-polyacrylamide gel electrophoresis, followed by silver staining. We found a band at ≈170 kDa in the immunoprecipitates from FAM5C-GFP-expressing cells, but not those from GFP-expressing cells (Fig. 1A). The LC-MS/MS analysis of the excised bands from