FEBS Letters 586 (2012) 1426–1430

journal homepage: www.FEBSLetters.org

The murine Gcap14 gene encodes a novel microtubule binding and bundling protein

Hitomi Hosono, Nao Yamaguchi, Kenzi Oshima, Tsukasa Matsuda, Daita Nadano *

Department of Applied Molecular Biosciences, Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya 464-8601, Japan

ARTICLE INFO

Article history:
Received 11 January 2012
Revised 30 March 2012
Accepted 6 April 2012
Available online 18 April 2012

Edited by Judit Ovádi

Keywords:
Microtubule
Microtubule-associated protein
Microtubule bundling
Mammal

ABSTRACT

Microtubules form flexible fibers, which are utilized in cell proliferation and differentiation. Although the flexibility of microtubules was shown to be regulated by various microtubule-associated proteins, this regulation is still far from complete understanding. Here, we report a new potential regulator of microtubules in mammals. Gcap14 colocalizes with microtubules in mammalian cells transfected with Gcap14 expression vector. Association of Gcap14 with microtubules was confirmed by biochemical subcellular fractionation. Recombinant Gcap14 protein cosedimented with pure microtubules, indicating a direct binding between the two. Furthermore, recombinant Gcap14 was shown to have the ability of inducing microtubule bundling in vitro.

Structured summary of protein interactions:
Gcap14 physically interacts with **Gcap14** by anti tag coimmunoprecipitation (View Interaction: 1, 2)

1. Introduction

Cytoskeletons, like bones in the human body, physically support lipid membrane-enclosed fragile cells in animals and determine cell morphology. In addition, cytoskeletons play roles in various processes in the cell, including cell movement and intracellular transport. To adapt to these roles promptly, cytoskeletons change their shape dynamically. For example, polymerization and depolymerization of microtubules (MTs) are intrinsically stochastic, which has been called the dynamic instability [1]. This flexibility is, however, necessary to be regulated to play the roles in the cell; dysregulation of intracellular MTs often leads to diseases including neurodegenerative diseases [2,3]. Many cytoplasmic proteins have been reported to be associated with MTs and to be essential for MT regulation [4,5]. However, the detailed mechanisms of the regulation largely remain to be clarified.

The mammary gland has a unique developmental cycle: cell proliferation in response to pregnancy, differentiation of the epithelial cells for milk secretion, and a regressive phase after weaning (involution), including extensive death of the secretory epithelial cells and tissue remodeling, for returning to a pre-pregnant state [6–8]. To know the mechanisms underlying this mammary cycle, we have screened murine genes potentially included in these events in the cycle [9–12]. During the course of this screening, we have identified a previously uncharacterized gene, Gcap14. A translated product of this gene has been found to bind specifically to MTs and bundle them in vitro.

2. Materials and methods

2.1. Cloning and expression of mouse Gcap14 cDNA

Mouse cDNA including the full-length coding region of Gcap14 (encoding a 485-aa polypeptide, GenBank ID: NM_028407) was obtained from the mouse mammary gland, according to our previous work including total RNA extraction and reverse transcription-PCR [9]. The cDNA fragment was subcloned in pCMV-Tag2B (Agilent Technologies), pcDNA3.1/Myc-His (Invitrogen), and pEGFP-c1 (BD Biosciences Clontech) to express Gcap14 protein with the N-terminal FLAG, C-terminal Myc-His, and N-terminal green fluorescent protein (GFP) tags in mammalian cells, respectively. The same cDNA was also subcloned into a pMAL-c2 vector (New England BioLabs) to express recombinant Gcap14 protein fused with maltose binding protein (MBP) in bacteria.

Culture of mammalian cell lines and vector transfection by lipofection were described previously [10,13]. For complete depolymerization of intracellular MT fibers, cells were treated with the combination of nocodazole and cold temperature [14].

2.2. Biochemical and immunochemical methods

Proteins were determined by the BCA assay (Piece). SDS-polyacrylamide gel electrophoresis (PAGE), Coomassie R-250 staining,

* Corresponding author. Address: Department of Applied Molecular Biosciences, Graduate School of Bioagricultural Sciences, Nagoya University, Furo-cho, Chikusa, Nagoya 464-8601, Japan. Fax: +81 52 789 4128.
E-mail address: nadano@agr.nagoya-u.ac.jp (D. Nadano).

0014-5793/$36.00
http://dx.doi.org/10.1016/j.febslet.2012.04.008

electroblotting, and immunodetection with primary and peroxidase-labeled secondary antibodies were performed as described previously [11,13]. The following antibodies were used as the primary antibody: rabbit antibodies against GFP and the His tag from MBL (Nagoya, Japan), mouse monoclonal antibodies against the FLAG tag (M2) and α-tubulin from Sigma, mouse monoclonal anti-glyderaldehyde-3-phosphate dehydrogenase (GAPDH) antibody from HyTest, and guinea pig anti-cytokeratins 8/18 antibody from Progen.

Cells on coverslips were fixed with glutaraldehyde, methanol, or the Cytoskelfix reagent (Cytoskeleton, Denver, CO, USA), stained with primary and fluorophore-labeled secondary antibodies, and observed under fluorescence and confocal microscopes [15,16].

After transfection of MCF-7 cells with the FLAG–Gcap14 expression vector, subcellular fractionation under MT-stabilized and MT-depolymerized conditions was performed as described previously [16,17]. Briefly, under MT-stabilized conditions, cells were pretreated with paclitaxel (taxol) and lysed with MT-stabilizing buffer plus NP-40. Under MT-depolymerized conditions, intracellular MTs were completely depolymerized as described above, and cells were then lysed with Tris buffer including NP-40 and EDTA. These cell lysates were centrifuged to obtain the supernatant and precipitate fractions. An equivalent amount of each fraction was subjected to immunoblotting with antibodies against the FLAG tag, α-tubulin, GAPDH, and cytokeratins 8/18. GAPDH and cytokeratins 8/18 were used as the markers for free cytosolic proteins and cytoskeletal fibers, respectively.

2.3. Recombinant Gcap14 protein and in vitro MT-cosedimentation assay

Recombinant Gcap14 fused with MBP was expressed in *Escherichia coli* BL21 by transformation with its expression vector described above and purified with maltose-conjugated beads (New England BioLabs). These procedures were basically according to manufacturer's instructions except for the following two points. (1) Bacteria were cultured at 20 °C in protein induction. (2) After trapping of recombinant MBP–Gcap14 on the maltose resin, two-step elution was performed with 0.1 and 3.0 mM maltose. Under these final elution conditions, the majority of the putative full-length product (calculated molecular mass, 97 kDa) was eluted with 3.0 mM maltose, as judged by SDS-PAGE. MBP was also expressed by using pMAL-c2 (no insert) and purified by the same procedure. Purified proteins were dialyzed at 4 °C against 80PEM buffer (80 mM Pipes, pH 6.9, including 2 mM $MgCl_2$ and 0.5 mM EGTA), centrifuged briefly, and stored at 4 °C until use.

For in vitro MT-cosedimentation assay, each purified recombinant protein in 80PEM buffer was mixed with Triton X-100, GTP, and paclitaxel, whose final concentrations were 0.5%, 1 mM, and 20 μM, respectively, and ultracentrifuged at 100000*g* for 60 min at 30 °C to remove a trace of insoluble material. Pure porcine brain tubulin dimer (>99% purity, Cytoskeleton) was polymerized in the presence of paclitaxel [16]. Each recombinant protein (0.2 nmol) was then mixed with porcine brain MTs (corresponding to 0.5 nmol of tubulin dimer) in 80PEM buffer, including 0.5% Triton X-100, and subjected to ultracentrifugation at 100000*g* for 15 min at 30 °C to separate the supernatant (S100) and precipitate (P100). After a brief wash of P100 with 80PEM buffer, the S100 and P100 fractions were subjected to SDS-PAGE (7.5% gel) followed by Coomassie staining.

2.4. In vitro MT-bundling assay

This assay was performed in accordance with the previous reports [18,19] with a slight modification. Each purified recombinant protein was mixed with Triton X-100 and GTP, whose final concentrations were 0.5% and 1 mM, respectively, and centrifuged at 20000*g* for 15 min at room temperature. Pure porcine brain tubulin dimer (0.1 nmol) and 0.01 nmol of HiLyte 488 dye-conjugated porcine brain tubulin dimer (Cytoskeleton) were mixed in 80PEM, including 1 mM GTP buffer and 20 μM paclitaxel, and kept for 15 min at room temperature to form fluorescent MT fibers. The purified MBP–Gcap14 or MBP (0.01 nmol) was added to the fluorescent MT fibers and incubated for 10 min at room temperature. The mixture was then diluted ten times with 1% glutaraldehyde in 80PEM (including 1 mM GTP)/100% glycerol (1:1), spotted onto a slide glass, and covered with a coverslip for observation under a fluorescence microscope (IX71, Olympus) equipped with a 40× objective. To examine the structure of thick, bundled MTs, the fixed samples were further diluted five times with the same fixation buffer to minimize accidental overlap of MT filaments. Individual MT bundles were then observed under a confocal laser microscope (FV1000-D, Olympus) with a 100× objective.

2.5. Immunoprecipitation

Cells were subjected to the complete MT depolymerization described above, lysed with 50 mM Tris–HCl, pH 7.5, containing 0.15 M NaCl, 1% NP-40, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 50 μM proteasome inhibitor I (PSI), 10 μg/ml aprotinin, 5 μg/ml leupeptin, and 5 μg/ml pepstatin A, and centrifuged twice at 20000*g* for 15 min at 4 °C. The subsequent procedures were performed using the supernatants, appropriate antibodies, and protein G-conjugated beads, essentially according to our previous report [12].

2.6. Preparation of antibodies against mouse Gcap14

cDNA encoding the C-terminal half of mouse Gcap14 protein (residues 222–485) was amplified by PCR and subcloned into the pET-32a (+) vector (Novagen) to express the Gcap14 fragment fused with thioredoxin in bacteria. The thioredoxin-fused protein was expressed in *E. coli* BL21 and purified by using the His-bind resin (Novagen) according to manufacturer's instructions. Antisera were raised in two rabbits using the purified thioredoxin-fused protein as the immunogen.

For the preparation of mouse antisera against Gcap14, the thioredoxin tag was removed from the C-terminal Gcap14 fragment by using a His-bind resin column after cleavage with enterokinase (Novagen). Four male ddY mice were immunized by injection of the Gcap14 fragment separated from the thioredoxin tag. The immunization procedures were based on our previous report [10]. Blood was collected from individual mice, and collected sera were used.

2.7. Preparation of MT-rich fraction from the mouse brain

The MT-rich fraction was prepared from the brain by MT disassembly/assembly in buffer containing glycerol, essentially according to the literature [20]. Briefly, the fresh mouse brain (about 0.4 g) was homogenized on ice in 0.2 ml 80PEM buffer and centrifuged at 22300*g* for 60 min at 4 °C. The supernatant was mixed with an equal volume of 80PEM buffer, including 8 M glycerol and 1 mM GTP, kept at 37 °C for 60 min, and ultracentrifuged at 100000*g* for 60 min at 37 °C. The clear pellet, including MTs and their associated proteins, was dissolved in the SDS-PAGE sample buffer, boiled, and subjected to SDS-PAGE.

2.8. MT stability assay

COS-1 cells plated on coverslips were transfected with the GFP–Gcap14 expression vector as described above. After 24 h of trans-