Filament Formation of MSF-A, a Mammalian Septin, in Human Mammary Epithelial Cells Depends on Interactions with Microtubules*

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Koh-ichi Nagata‡, Aie Kawajiri§, Seiya Matsui‡, Mihoko Takagishi‡, Takashi Shiromizu‡, Noriko Saitoh, Ichiro Izawa, Tohru Kiyono, Tomohiko J. Itoh, Hirokazu Hotani, and Masaki Inagaki‡**

From the Divisions of ‡Biochemistry and ‡Virology, Aichi Cancer Center Research Institute, 1-1 Kanokoden, Chikusa-ku, Nagoya 464-8681, Japan, §Department of Pathology, Nagoya University School of Medicine, Nagoya 466-8550, Japan, and the ‡Division of Biological Sciences, Graduate School of Science, Nagoya University, Nagoya 464-8602, Japan

Septins are a family of conserved proteins implicated in a variety of cellular functions such as cytokinesis and vesicle trafficking, but their properties and modes of action are largely unknown. Here we now report findings of immunocytochemical and biochemical characterization of a mammalian septin, MSF-A. Using an antibody specific for MSF subfamily proteins, MSF-A was found to be expressed predominantly in mammalian human mammary epithelial cells (HMEC). MSF-A was associated with microtubules in interphase HMEC cells as it localized with the mitotic spindle and the bundle of microtubule at midzone during mitosis. Biochemical analysis revealed direct binding of MSF-A with polymerized tubulin through its central region containing guanine nucleotide-interaction motifs. GTPase activity, however, was not required for the association. Conditions that disrupt the microtubule network also disrupted the MSF-A-containing filament structure, resulting in a punctate cytoplasmic pattern. Depletion of MSF-A using small interfering RNAs caused incomplete cell division and resulted in the accumulation of binucleated cells. Unlike Nedd5, an MSF mutant deficient in GTPase activity forms filament indistinguishable from that of the wild type in COS cells. These results strongly suggest that septin filaments may interact not only with actin filaments but also with microtubule networks and that GTPase activity of MSF-A is not indispensable to incorporation of MSF-A into septin filaments.

Septins, a family of heteropolymeric filament-forming proteins, were originally discovered in yeast to be essential for budding, and have since been identified in most eukaryotic organisms, with the exception of plants (for review, see Refs. 1–6). Although septins have 25% or greater identity over their entire length, sequence similarity is greatest in the central domain, which contains guanine nucleotide interactive motifs homologous to those of ras-related small GTPases. In addition to the conserved central domain, most septins have divergent N- and C-terminal domains, some of which contain a predicted coiled-coil region possibly involved in protein-protein interaction. Although the ras-related small GTPases function as signal transducing molecular switches through GTP/GDP-exchange and GTP-hydrolysis, little is known of the physiological significance of mammalian septins.

Although septins were initially thought to play important roles in controlling cytokinesis of budding yeast (7), it is now well established that they are also required for localized chitin deposition, bud site selection, cell cycle control, plasma membrane compartmentalization, and regulation of some kinases (for review, see Refs. 1–4). The accumulating biochemical and cell biological observations on lower eukaryotic septins suggest that either they comprise a novel cytoskeletal polymer or they function as scaffolds for assembly of signaling complexes.

Numerous mammalian homologues of yeast septins have been identified mainly based on random sequencing projects (reviewed in Ref. 6). Some of the septins probably represent alternative splicing forms. The gene mixed lineage leukemia septin-like fusion (MSF) has been identified as a fusion partner gene of mixed lineage leukemia in a case of therapy-related acute myeloid leukemia with t(11,17)(q23;q25) (8, 9). Thereafter, two alternative splicing variants, MSF-A and MSF-B, were identified (10), and another report described the complicated transcriptional pattern of MSF (11). MSF has been found to be deleted in some cases of breast and ovarian cancers; hence, it was considered to be a candidate for tumor suppressor gene (10, 12). These mutations may be associated with allelic loss of the 17q25 region (13–16). Although these findings provided insights into a possible role for MSF in leukemogenesis and oncogenesis, not only the molecular mechanism(s) regarding MSF function and tumorigenesis but also biochemical and biological properties of MSF proteins remain to be elucidated. We did a biochemical and biological characterization of MSF-A and found that MSF-A interacts with microtubules in vitro and in vivo. The biological significance of GTPase activity of MSF-A was investigated and compared with that of well characterized septin, Nedd5.

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** To whom correspondence should be addressed. Tel.: 81-52-762-6111 (ext. 7020); Fax: 81-52-763-5233; E-mail: minagaki@ichi-cc.jp.
**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—Human MSF-A, MSF, MSF-B, Nedd5, hSeptin2, and H5 were produced by PCR with Marathon-Ready cDNA (human brain) (Clontech) then subcloned into pGEX-4T3 and/or pRcRc vector containing Myc tag. The cDNA fragments of MSF-A (aa 1–586), MSF-A-N (aa 1–283), MSF-A-Cent (aa 284–561), and MSF-A-C (aa 562–586) were produced by PCR and subcloned into pGEX-4T3 or pRcRc vector harboring Myc-tag. N312MSF-A, an MSF-A mutant with a point mutation (Ser-312 to Asn) in the G1 box of the GTPase region, was prepared using the QuikChange site-directed mutagenesis kit (Stratagene). All constructs were verified by DNA sequencing.

**Preparation and Characterization of Antibodies**—The glutathione S-transferase-fused MSF fragment (aa 148–586) expressed in Escherichia coli served as the antigen. A rabbit polyclonal antibody specific for MSF proteins was produced and affinity-purified. Anti-Nedd5 antibody was kindly supplied by Dr. M. Kinoshita (Harvard University, Cambridge, MA) (17). Western blot analysis was performed, and immune-reactive bands were visualized by making use of a horseradish peroxidase-conjugated anti-rabbit antibody and the enhanced chemiluminescence Western blotting detection system (Amersham Biosciences).

**Cell Culture, Transfection, Immunofluorescence, and Microinjection**—COS-7 and HeLa cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 100 μg/ml penicillin in an air-5% CO2 atmosphere with constant humidity. HMEC cells were maintained in α-minimal essential medium containing 12.5 mM HEPES, 35 μg/ml bovine pituitary extract, 12.5 ng/ml epidermal growth factor, 1 μg/ml insulin, 10 μg/ml transferrin, 10 mM β-estradiol, 3.5 mM hydrocortisone, 0.1 μM phosphylethanolamine, 0.1 mM ethanamine, 0.1 mM sodium pyruvate, 25 μM ascorbic acid, 15 μM sodium selenite, and 1% calf serum. Transient transfection using COS-7 and HMEC cells was carried out using the LipofectAMINE method (Invitrogen). For immunofluorescence analyses, cells grown on 13-mm coverslips were fixed in 3.7% formaldehyde in phosphate-buffered saline for 15 min, then treated with 0.2% Triton X-100 for 5 min. To detect the MSF subfamily of proteins, affinity-purified anti-MSF antibody was used as the primary antibody, and Alexa 488 anti-rabbit IgG (Molecular Probes) was used as the secondary antibody. To visualize Myc-tag, actin, vimentin, or tubulin, cells were reacted with anti-Myc monoclonal antibody (9E10), rhodamine-conjugated phalloidin, anti-vimentin monoclonal antibody (I8B), or anti-tubulin monoclonal antibody (Sigma), respectively. Anti-Myc polyclonal antibody (Santa Cruz Biotechnology) was used where indicated. Alexa 488-labeled anti-mouse antibody or FluoroLink Cy3-linked anti-mouse antibody was used as a secondary antibody. When analyzing the cells, we used an Olympus LSM-GB200 confocal microscope (Figs. 2, 4 and 6) or an Olympus BH2-RFCA microscope (Fig. 5).

**Expression and Purification of Recombinant Proteins**—MSF-A, Nedd5, and their mutants were expressed in E. coli as glutathione S-transferase fusion proteins and purified on glutathione-Sepharose beads. The recombinant proteins were expressed in E. coli as glutathione S-transferase fusion proteins and purified on glutathione-Sepharose beads. The recombinant proteins were released from the beads by cleavage with human thrombin. Protein concentration was determined by the method of Bradford (36) and purity of the protein preparations was confirmed on Coomassie Blue-stained SDS-polyacrylamide gels.

**GTase Activity Assay**—GTase activity was determined as described previously (18). Briefly, recombinant septin proteins (5 μg of each) were incubated at 30 °C in 20 mM Tris/His, pH 7.5, containing 1 mM EDTA, 1 mM dithiothreitol, 0.1% Lubrol, 25 mM MgCl2, 1 μM [γ-32P]GTP (2500–3000 cpm/μmol). After incubation for various times, the reaction was terminated by addition of ice-cold 2.5% (w/v) charcoal in 50 mM NaH2PO4. The mixtures were incubated for 15 min on ice and centrifuged for 15 min at 1000 × g at 4 °C. The amount of [γ-32P]GTP, released from [γ-32P]GTP, was determined by counting the radioactivity.

**Preparation of Tubulin and Microtubule-Binding Analyses**—Purified tubulin was prepared from the bovine brain, as described previously (19). The amount of MSF-A and mutants bound to microtubules was determined by cosedimentation assay, as described previously (20). Briefly, MSF-A or the mutants (0.25 μM) and polymerized tubulin (5 μM) were incubated in 0.1 M PIPES buffer, pH 6.9, containing 0.5 μM GTP and 1 mM MgCl2. After a 10-min incubation at 37 °C, the samples were centrifuged at 100,000 × g for 30 min at 37 °C, then the amount of 32Pi released was determined by cosedimentation assay, as described previously (20).

**RNA Interference (RNAi) with Small Interfering RNAs (siRNAs)**—siRNA duplexes with the following sense and antisense sequences were made: MSF-A, 5′-GAUCUUGAGAGGUGAGGAGdTdT-3′ (sense) and 5′-CCUCUGCGUGCUAGAUCdTdT-3′ (antisense). According to the recent report (21), siRNA duplexes with the following sense and antisense sequences were also used as a positive control: MSFs, 5′-AUCAGCCGGAAUGGUCCGdTdT-3′ (sense) and 5′-CCUCUGCGUGCUAGAUCdTdT-3′ (antisense). To control for the specificity of the knockdown, we used a siRNA duplex, MSF-A-Mut, a single site-mutated version of the inhibitory sequence: 5′-GAUCUCUUCCAGUGCGGAGtTdT-3′ (sense) and 5′-CCUCUGCGUGCUAGAUCdTdT-3′ (antisense). siRNAs were supplied from Dharmaco Research Inc. (Lafayette, CO). Transfection was carried out using Oligofectamine reagent (Invitrogen). At 72 h after transfection, immunofluorescence analysis with an Olympus BH2-RFCA microscope or Western blot analysis was performed to analyze the depletion of MSF-A.

**RESULTS**

**Identification of MSF-A in HMEC Cells**—To characterize the MSF subfamily of proteins, we first prepared a rabbit polyclonal antibody against MSF then affinity-purified it on a column to which recombinant MSF had been conjugated. Specificity of the antibody was confirmed with various septin proteins overexpressed in COS-7 cells. As shown in Fig. 1A, the anti-MSF antibody specifically recognized MSF-A, MSF, and MSF-B in Western blot analyses, because the polypeptide used as an antigen contained a region common among these three splicing variants. Because other septins tested were not recognized by anti-MSF, the anti-MSF antibody is specific for the MSF subfamily of proteins. We next did a Western blot analyses to detect endogenous MSF subfamily proteins in lysates of various mammalian cell lines, including HeLa and HMEC cells.
In the cell lysates tested, three proteins with molecular masses of 76, 73, and 50 kDa, coincident with MSF-A, MSF, and MSF-B, respectively, were observed in different combinations. Preincubation of the antibody with recombinant MSF-A selectively inhibited the immunoreactivity (data not shown). RT-PCR analyses revealed the expression of MSF-A in HMEC cells as well as HeLa cells (Fig. 1C). In addition, the expression level of the 76-kDa protein significantly decreased by MSF-A-RNAi (see Fig. 5A). From these data, we concluded that the 76-, 73-, and 50-kDa proteins observed in HeLa cells are MSF-A, MSF, and MSF-B, respectively. MSF-A was predominantly expressed in HMEC cell lysates (Fig. 1B).

**MSF-A Colocalizes with Tubulin in HMEC Cells**—The mammalian septins Nedd5 and H5 have been reported to localize along with actin filaments (17, 22). We thus examined the subcellular localization of MSF-A in HMEC cells, using a confocal microscope. HMEC cells were double-stained with anti-MSF antibody and anti-tubulin antibody, rhodamine-phalloidin, or anti-vimentin antibody. As shown in Fig. 2A, MSF-A was present in filamentous structures. Double staining with an antibody to tubulin revealed a significant overlap between filamentous MSF-A and microtubules (Fig. 2, A and B). In addition, colocalization of MSF-A with microtubule networks seemed to be heterogeneous, and the extent of overlapping differed from cell to cell, which suggested dynamic features of interactions between MSF-A and microtubules. In the mitotic cells, MSF-A was localized at the mitotic spindle and the bundle of microtubules at the midzone (Fig. 2C). MSF-A was not evident at the spindle pole. We next sought to determine whether MSF-A colocalizes with other major cytoskeletons, actin filaments, and vimentin. Double staining showed no apparent codistribution of MSF-A with actin (Fig. 2D) or with vimentin filaments (Fig. 2E).

**Direct Association of MSF-A with Microtubules in Vitro**—To determine whether MSF-A directly interacts with microtubules, we examined the *in vitro* association of MSF-A with paclitaxel-stabilized microtubules, using a cosedimentation assay. We also attempted to determine the essential domain of MSF-A for binding with microtubules; for this, we used single point- and truncated MSF-A mutants (Fig. 3A). In the absence of microtubules, glutathione S-transferase-MSF-A was not sedimented, but in the presence of microtubules, a substantial portion of glutathione S-transferase-MSF-A was precipitated together with microtubules (Fig. 3B). We next determined...
whether GTPase activity of MSF-A is important for association with microtubules, because GTP hydrolysis is likely to play an important role in *in vivo* Nedd5 filament assembly (17). We tested the microtubule-binding activity of N312MSF-A, a GTPase activity-deficient mutant in which Ser312 in the G1 box is changed to Asn (see Fig. 6C). Cytochalasin B and demecolcine effectively disrupted tubulin and actin, respectively, under these conditions (A–D, G, and H). Cells treated with cytochalasin B were double stained for MSF-A (E) and actin (F). Cells treated with demecolcine were double stained for MSF-A (I) and tubulin (J). Bar, 20 μm.

Microtubule Network Is Essential for the Fibrous Distribution of MSF-A in Interphase HMEC Cells—The structural relationship between microtubules and MSF-A-containing filaments was further examined using reagents known to disrupt actin or microtubules. MSF-A sedimenting with microtubules was considered to be associated with the microtubule filaments via direct binding. The direct interaction of MSF-A with microtubules was next examined using a standard cosedimentation assay (Fig. 3). The binding of MSF-A to microtubules was saturable with an apparent *K*~*d*~ of 0.1 μM.

**Microtubule Network Is Essential for the Fibrous Distribution of MSF-A in Interphase HMEC Cells**—The structural relationship between microtubules and MSF-A-containing filaments was further examined using reagents known to disrupt actin or microtubules. MSF-A, a GTPase-deficient MSF-A mutant, MSF-A, or MSF-A-Cent (aa 284–561) had no effects on the microtubule structure (data not shown).

**Silencing Expression of MSF-A by RNA Interference Induces Incomplete Cell Division in HMEC Cells**—In the next set of experiments, we examined whether MSF-A-containing septin filaments structure is essential for microtubule network organization. As shown in Fig. 5A, protein level of MSF-A drastically decreased by RNAi using MSF-A-duplex (lane 1), whereas the control siRNA duplex, MSF-A-Mut, had little effect (lanes 2 and 6). Another duplex, tentatively termed MSFs, the effects of which on silencing of expression of MSF proteins have been reported in HeLa cells recently (21), was also used as a positive control.
control. Consequently, almost the same silencing effect was observed (Fig. 5, lane 5). The level of β-tubulin was little altered by the above siRNA treatments in Western blot analyses (Fig. 5A, lanes 3, 4, 7, and 8). However, when 500 cells were counted in which MSF-A level was drastically lowered by immunofluorescent microscopy, 14% of the cells showed reduced microtubule staining (Fig. 5B, d–f), whereas the remaining cells with decreased levels of MSF-A had almost intact microtubules (data not shown). In control experiments with MSF-A Mut, the filamentous structure of MSF-A and microtubules were little affected (Fig. 5B, a–c). Under the conditions used, the actin filament structure was not affected by the above RNAi treatments (data not shown). It is notable that 10–12% of the cells with lowered levels of MSF-A by MSF-A- or MSFs-RNAi failed to divide correctly, resulting in binucleated cells (Fig. 5, B, d–f, and C).

**GTPase Activity Is Not Required for Filament Formation of MSF-A in COS Cells**—Because GTPase activity is noted to be essential for the filamentous structure of Ned5 (17), the functional relevance of GTPase activity of MSF-A was examined and compared with that of Ned5. As depicted in Fig. 6A, N312MSF-A and N51Ned5, both of which were designed based on the well characterized ras mutant with dominant-negative activity, showed a highly reduced GTPase activity under conditions in which the wild type of each septin has GTPase activity. The wild-type and mutant septins were then individually overexpressed in COS-7 cells. As shown in Fig. 6B, N312MSF-A formed a filamentous structure indistinguishable from that of the wild type, and the filament structure was also observed when MSF-A and N312MSF-A were coexpressed (Fig. 6C, a–c). In contrast, the introduction of the GTPase-deficient mutation into Ned5 significantly abolished filament-forming activity (Fig. 6B). N51Ned5, a GTPase-deficient mutant, was not incorporated into the filament composed of wild-type Ned5, and the filament structure by wild-type Ned5 and aggregates composed of N51Ned5 are independently present in cells expressing them (Fig. 6C, d–f). Taken together, these results suggest that guanine nucleotide-binding and GTP-hydrolysis activities are not required for polymer formation of MSF-A, whereas the activities are essential for polymerization of Ned5.

Although the GTPase activity of MSF-A is not required for its binding with microtubules, it is possible that binding with microtubules may affect the GTPase activity of MSF-A. We thus measured the GTPase activity of MSF-A in the presence of various amounts of polymerized tubulin, which has undetectable GTPase activity. Consequently, tubulin had no effects on the GTPase activity (data not shown).

**DISCUSSION**

In the present study, we found that MSF-A, a member of the MSF family and predominantly expressed in mammary HMEC cells, distributes along with microtubules in the cells. The septin filament structure containing MSF-A depends on the integrity of microtubules because microtubule disruption by demecolcine induced septin filament disruption in HMEC cells. When RNAi reduced expression of MSF-A in HMEC cells, the cells displayed failed cell division more frequently than control cells; consequently, 10–12% of the MSF-A-RNAi-treated cells became binuclear. Additionally, −14% of the cells with reduced level of MSF-A showed lower levels of microtubules, although the physiological relevance of this phenotype remains to be elucidated. We noted that MSF-A directly binds with polymerized tubulin in vitro through the center domain containing guanine nucleotide-interactive motifs, although GTPase activity of MSF-A does not seem to be required for binding to microtubules.

The function of septins in cytokinesis is likely to be conserved in higher eukaryotic cells as well as in yeast (17, 23). Accumulating data on mammalian septins indicate that some, such as CDCrel-1, ARTS, and Ned5, function in vesicle fusion processes (24, 25), apoptosis (26), and neurodegeneration (27), although the precise molecular mechanisms of these processes are largely unknown. Septins also seem to play important roles in oncogenesis, because not only MSF (8, 9) but also CDCrel-1 (28) has been identified as in-frame fusions with the mixed lineage leukemia/acute lymphocytic leukemia 1 protein. The presence of a variety of septins in mammalian cells means that they are likely to be involved in various as-yet-unidentified cellular processes.

Mammalian septins are thought to be related to actin filament structures (17, 22). As for MSF-A, a recent study demonstrated that it colocalizes with actin stress fibers as well as microtubules in HeLa cells (21), suggesting interaction of MSF-A with actin filaments. On the other hand, our data showed that the filamentous structure of MSF-A in HMEC cells was conserved in cytochalasin B-treated cells. A possible explanation is that efficient interaction of MSF-A with actin filaments needs other molecule(s), such as other MSF subfamily of the proteins, because HMEC cells predominantly express MSF-A, whereas HeLa cells express MSF-A, MSF, and MSF-B equally.
It is notable that MSF-A-containing septin filaments in HMEC cells are disrupted by demecolcine treatment and MSF-A interacts with microtubules in vitro. The physiological significance of interactions between MSF-A and microtubules is an enigma; however, we speculate that MSF-A plays a role in microtubule-dependent secretory pathways because 1) microtubules play important roles within the secretory pathway in mediating the anterograde and retrograde traffic of vesicular and tubular intermediates between ER and the Golgi complex (for review, see Ref. 29), and 2) there is a large body of evidence that microtubules are involved in post-Golgi protein trafficking: microtubules are required for the efficient transport of membrane proteins to the apical surface via a direct (30) or an indirect transcytotic pathway (31, 32). It is also notable that MSF-A localizes largely along the mitotic spindle in mitotic HMEC cells, which suggests some function in mitotic processes. Although the functional relevance of MSF-A in the mitotic process remains to be elucidated, MSF-A may play a role in microtubule-dependent mitotic processes and perturbation of the function might induce cell cycle abnormalities resulting in oncogenesis.

Mammalian septins, such as Nedd5 and H5, were noted in immunofluorescent studies to be localized along with actin stress fibers (17, 22). Because H5 and Nedd5 are detectable in HMEC cells by Western blot analyses, MSF-A, Nedd5, and H5 may function, in harmony with other septin molecules, to coordinate control of actin- and tubulin-dependent cellular events. Another possibility is that these septins function as filamentous scaffolds for organization of proteins at a specific region inside the cell, because many proteins depend on septins for localization (reviewed in Refs. 1, 3, 4). Budding yeast septins were found to function as a scaffold that allows for assembly of multiprotein complexes required for activation of Gin4 and Hsl kinases, which are activated and control bud growth during mitosis (33, 34). In this context, we found that MSF-A interacts with mixed lineage kinase 2 in vivo, which is distributed together with activated c-Jun N-terminal kinase along microtubules (35), meaning that septin scaffolds probably regulate mixed lineage kinase 2-mediated c-Jun N-terminal kinase activation. The septin complex may function as scaffolds in various cellular events that also link actin and microtubule networks. Cytological investigations are under way to clarify the physiological significance of the septin complex. The results obtained by RNAi experiments are consistent with the recent observation that MSF-A and/or the splicing variants play an important role in cytokinesis in HeLa cells (21). In HMEC cells, MSF-A is possibly involved in cytokinetic process cooperatively with other septins such as Nedd5 (17) because it is a dominant MSF subtype in the cells. In addition, a weak staining pattern of microtubules was observed in ~14% of HMEC cells with reduced levels of MSF-A. Although some cells with reduced levels of MSF-A became binucleated, obvious correlation between the extent of reduction of MSF-A expression and cytokinetic defect was not observed. Whether or not MSF-A-knockdown affects the microtubule structure and how MSF-A plays a physiological role in relation to microtubule functions and cytokinesis are interesting areas for future examination. To test the role of MSF-A in microtubule organization, anti-MSF antibody was microinjected into HMEC cells. However, the antibody reacted with endogenous MSF-A and clearly colocalized with filamentous MSF-A; therefore, the antibody could not induce morphological change of microtubules (data not shown). We assume that the antibody recognized MSF-A but could not disrupt the filamentous structure.

In the present study, we report the inter-relationship of a septin with microtubules. Characterization of the MSF-A demonstrated here may represent initiation of the unraveling of a septin function linking actin and microtubule networks and various mammalian septin-dependent key events.

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