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Novel Nuclear and Cytoplasmic Proteins Detected by Anti–*Zoothamnium arbuscula* (Protozoa) Spasmin 1 Antibody in Mammalian Cells Are Dependent on the Cell Cycle

Takeshi Itabashi[1,*], Takeo Terasaki[2] and Hiroshi Asai[2]

[1]Major in Integrative Bioscience and Biomedical Engineering, Graduate School of Science and Engineering, Waseda University, Tokyo, 169-8555; and [2]Advanced Research Institute for Science and Engineering, Waseda University, Tokyo, 169-8555

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Spasmin is a calcium-binding protein that is the major component of calcium-induced contractile filaments, called spasmoneme, found in vorticellid ciliates. Such filaments have not been observed in any organisms other than green algae. To determine whether calcium-induced contractile filaments resembling spasmoneme are present in higher eukaryotes, we performed immunofluorescence imaging of an anti–*Zoothamnium arbuscula* (protozoa, ciliophora) spasmin 1 polyclonal antibody in HeLa cells. In the cytoplasm, ubiquitous antigens seemed to be co-localized with microtubules at interphase, but not throughout mitosis. In the nucleus, areas linked to the nuclear envelope contained a number of hot spots. These regions were unclear during condensation of the replicated chromosomes, but became clearly visible again at cytokinesis. Immunoblotting analysis identified localized antigens during different phases of the cell cycle, including a 68/71 kDa cytoplasmic protein and a 55 kDa nuclear protein in interphase, and a 55/70 kDa protein in mitosis. The anti-spasmin 1 antibody recognized antigens in both hamster kidney BHK21 cells and Human lung cancer A-549 cells. These results suggest that novel spasmin-like proteins could be common in mammalian cells.

Key words: calcium-induced contractile filaments, cell cycle, centrin, nucleus, spasmin, tubulin.

Spasmoneme in vorticellid ciliates is an elastic organelle composed of bundles of 2–4 nm Ca^{2+}-induced contractile filaments. Hoffmann-Berling (*1*) and other investigators (*2–4*) have reported that the contraction and extension of spasmoneme *in vitro* can be cycled by changing the free Ca^{2+} concentration in the absence of ATP hydrolysis or any other organic substrates. We have focused on the structure, contractility, and constituent proteins of spasmoneme (*5–7*). The principal source of contraction is the binding of Ca^{2+} to spasmin, the major protein component of spasmoneme (*8*).

Spasmin and centrin are closely related EF-hand Ca^{2+}-binding proteins belonging to the calmodulin superfamily. A sufficient number of centrin genes have been cloned and sequenced to ascertain that centrin is a strongly conserved protein and ubiquitous among eukaryotes (*9*). In algal cells, centrin is most prominently found in Ca^{2+}-sensitive contractile fibers, the striated fiber roots that connect the basal body complex to the underlying nucleus in interphase cells (*10*). Centrin is localized to centrioles and centrosomes in cultured human cells (*11*). In addition, a Ca^{2+}-modulated contractile component around the centrosome, structurally similar and biochemically homologous to the centrin-containing fiber system of algae, has been characterized in tissue culture cells of higher eukaryotes (*12–14*).

The recent characterization of some spasmin cDNA sequences in peritrich ciliates, *Vorticella convallaria* (*15*), and *Zoothamnium arbuscula* (*7*) suggests that spasmin is a molecular species distinct from the universal centrin. Furthermore, spasmin-based structures contract and re-extend indefinitely in the absence of ATP, while centrin-based structures require ATP for cycling *in vitro* (*16*). Therefore, we investigated whether the spasmin identified in spasmoneme from vorticellid ciliates is conserved in higher eukaryotes. It is also of interest whether still unknown Ca^{2+}-dependent contractile filaments such as spasmoneme exist in a range of eukaryotic organisms.

Here we report, based on the results of confocal immunofluorescence imaging and immunoblotting with an anti–*Z. arbuscula* (protozoa, ciliophora) spasmin 1 polyclonal antibody, that HeLa cells contain several proteins immunologically related to spasmin that are dependent on location and the cell cycle.

MATERIALS AND METHODS

Cell Culture and Synchronization—Human cervical HeLa cells, hamster kidney BHK21 cells and human lung A-549 cells (American Type Culture Collection, Rockville, MD, USA) were grown in Eagle's MEM (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 10% fetal bovine serum and L-glutamine (final concentration

*To whom correspondence should be addressed. Phone: +81-3-5286-3437, Fax: +81-3-5286-3437, E-mail: t.itabashi@ruri.waseda.jp

2 mM) (Invitrogen Co., Carlsbad, CA, USA) at 37°C and 5% CO_2. To increase the yield of mitotic cells, HeLa cells were first treated with 5 μg ml^{-1} Aphidicolin (Wako Pure Chemical Industries, Ltd., Osaka, Japan) for 24 h to arrest the cell cycle at S (synthesis) phase. Following washout of the Aphidicolin, cells were cultured in MEM for an additional 8 h, and then exposed to 0.015 μg ml^{-1} Colcemid (Sigma, St. Louis, MO, USA) for 3 h. The synchronized M (mitotic) phase cells were further cultured in fresh medium before protein extraction in the next interphase or M phase.

Anti–Zoothamnium arbuscula Spasmin 1 Antibody—The *Z. arbuscula spasmin 1* cDNA (accession No. AB086262 of DDBJ/EMBL/GenBank databases) (*7*) was cloned into a pGEX-4T-1 vector (Amersham Biosciences, Tokyo, Japan), and transferred into BL21 *E. coli* for expression of the glutathione S-transferase (GST) fusion protein. The GST fusion proteins produced were recovered from the crude bacterial lysate by passage through a column of glutathione-Sepharose 4B resin (Amersham Biosciences) in accordance with the manufacturer's instructions. The GST-spasmin 1 fusion protein was cleaved at the fusion site with thrombin protease and further purified by calcium-dependent hydrophobic affinity chromatography. Fractions eluted from the glutathione-Sepharose 4B column were adjusted to a final concentration of 2 mM $CaCl_2$, and applied to a phenyl-Sepharose HP column (Amersham Biosciences) equilibrated with BP buffer (0.5 M NaCl, 2 mM $CaCl_2$, 50 mM Tris-HCl pH 7.5). The column was washed with BP buffer, and the bound proteins were eluted with EP buffer (0.5 M NaCl, 2 mM EGTA, 50 mM Tris-HCl, pH 7.5). The purified spasmin 1 protein was used to generate a mouse polyclonal anti–*Z. arbuscula* spasmin 1 antibody (Takara Bio Inc., Shiga, Japan).

For antibody purification, purified spasmin 1 was coupled to a HiTrap NHS-activated HP column (Amersham Biosciences) according to the manufacturer's instructions. From mouse antiserum, only antibodies against spasmin 1 were eluted using 100 mM glycine-HCl, pH 2.7. The Alexa Fluor 488–conjugated anti-mouse IgG antibody (Molecular Probes Inc., Eugene, OR, USA) and monoclonal Cy3–conjugated anti–β-tubulin antibody (Sigma) were purchased.

Indirect Immunofluorescence—HeLa cells, grown on 35 mm glass bottom dishes, were rinsed with PEM buffer [80 mM piperazine-*N,N′*-bis(2-ethanesulfonic acid), 5 mM EGTA, and 1mM $MgCl_2$, pH 6.9] at 37°C, and fixed with 3.7% paraformaldehyde in PEM for 20 min at 37°C. The fixed cells were permeabilized with 0.1% Triton X-100 in PEM for 5 min, followed by rehydration in phosphate-buffered saline (PBS) (three changes, 5 min each). Cells were incubated in PBS containing 10% BSA for 1 h at room temperature (approximately 25°C). After blocking, all specimens were triple-labeled with polyclonal anti–*Z. arbuscula* spasmin 1 antibody, monoclonal anti–β-tubulin antibody, and 4,6-diamidino-2-phenylindole (DAPI; Sigma). Cells on glass-bottom dishes were labeled first by incubating with anti–*Z. arbuscula* spasmin 1 antibody (10 mg ml^{-1}) for 4 h, washed five times with PBS and then incubated with Alexa Fluor 488–conjugated anti–mouse IgG antibody (1:100) for 4 h at room temperature. The specimens were then washed five times with PBS to remove excess secondary antibody, and then incubated in a mixture of Cy3-conjugated anti–β-tubulin antibody (1:50) and DAPI (0.1 μg ml^{-1}) for 4 h. Specimens were then washed five times with PBS, rinsed with deionized water, and, finally, mounted with SlowFade Light Antifade (Molecular Probes Inc.). Cells were observed under a LSM5 PASCAL microscope (Carl Zeiss Jena GmbH, Germany) equipped with epifluorescence optics using a C-Apochromat 63× water immersion objective (Carl Zeiss Jena GmbH, Germany).

Cell Extracts and Western Blot Analysis—Cells harvested in interphase or M phase were washed with PBS and centrifuged, and the cell pellet was directly treated with SDS sample buffer. Nuclear and cytoplasmic proteins in interphase cells were extracted using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotech. Inc., Rockford, IL, USA) in accordance with the manufacturer's specifications.

Proteins were analyzed by electrophoresis according to the standard methods described by Laemmli (*17*). For analysis of electrophoretic behavior, EGTA- and Ca^{2+}-containing gels were used as previously described by Huang *et al.* (*11*). After SDS-PAGE, the gels were analyzed by western blotting using an ECL plus Western Blotting Detection System (Amersham Biosciences). The gels were soaked for 15 min in blotting buffer (25 mM Tris, pH 8.3; 192 mM Glycine; 20% methanol) and transferred to Hybond ECL membranes (Amersham Biosciences). The membranes were washed with ECL blocking buffer for 1 h at room temperature. After washing with PBS-T (0.1% Tween 20 in PBS), the membranes were incubated with anti–*Z. arbuscula* spasmin 1 antibody (1 μg ml^{-1}) for 1 h at room temperature. After the membranes were rinsed with PBS-T several times, they were then incubated with HRP-conjugated anti-mouse IgG antibody (1:25,000; Amersham Biosciences) for 1 h at room temperature. The membranes were washed with PBS-T, and ECL Plus reagents and an ECL Mini-Camera (Amersham Biosciences) were used for the immunological detection of proteins.

RESULTS

Antigens Immunologically Related to Spasmin are Localized in HeLa Cells Depending on the Cell Cycle—To determine whether Ca^{2+}-dependent contractile filaments such as spasmoneme exist in cultured human cells, we performed confocal immunofluorescence microscopy using the anti–*Z. arbuscula* spasmin 1 polyclonal antibody. Triple-labeling fluorescence studies using the polyclonal anti–*Z. arbuscula* spasmin 1 antibody, a monoclonal anti–β-tubulin antibody and DAPI demonstrate that antigens immunologically related to spasmin are localized to the microtubules of HeLa cells depending on the cell cycle. Figure 1 depicts the progression of the cell cycle: interphase, prophase, metaphase, anaphase, and cytokinesis. In the cytoplasm of HeLa cells during interphase and prophase, antigens for the anti–*Z. arbuscula* spasmin 1 antibody were ubiquitous and co-localized with microtubules (Fig. 2A), but not clearly co-localized with mitotic microtubules such as those of the kinetochore and the central region of overlapping microtubules in cytokinesis (Fig. 1). However, some of the filament-like

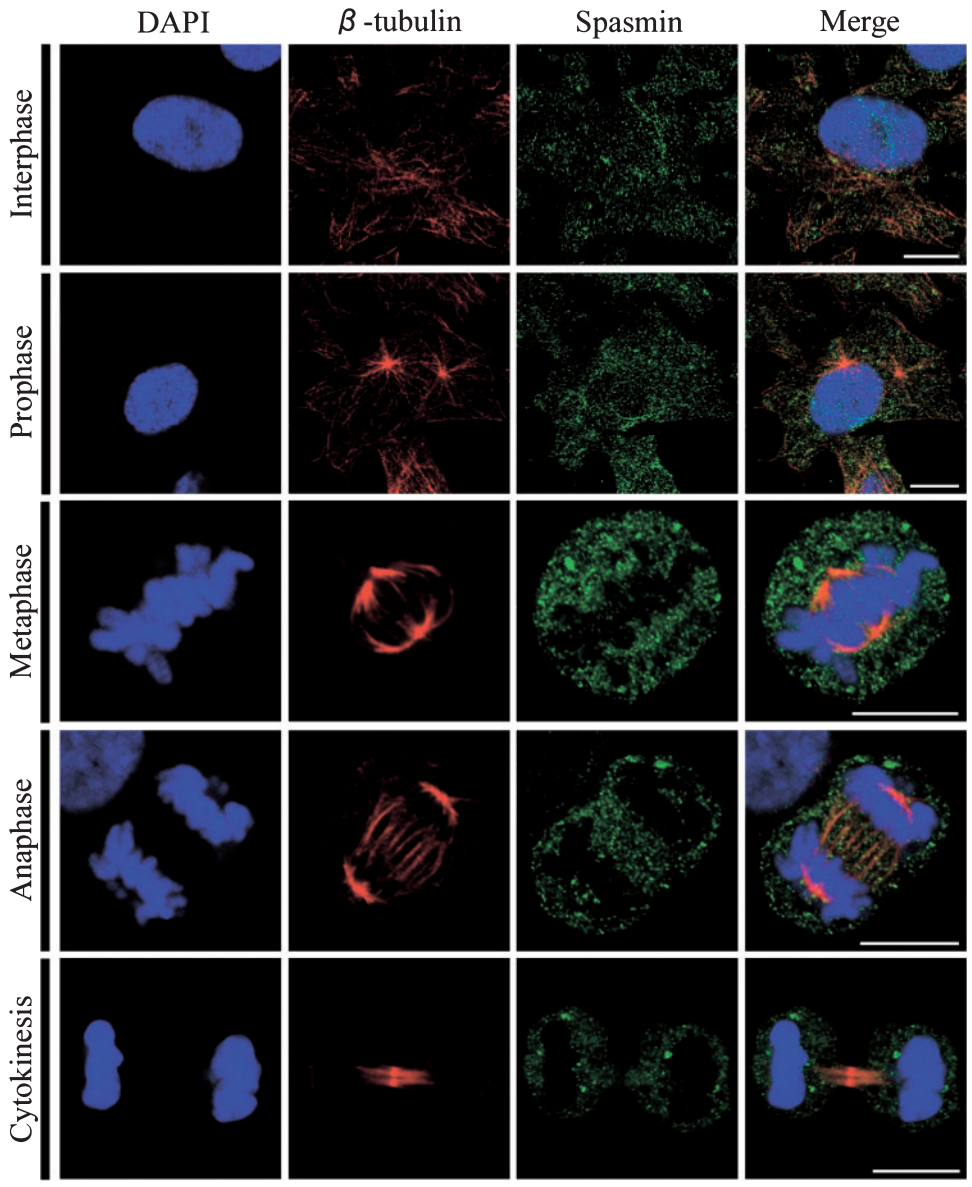

Fig. 1. **Triple-labeling of β-tubulin, spasmin and DNA in HeLa cells through mitosis.** Each row shows the same cell viewed to illustrate the different labels. Scale bar represents 10 μm.

structures recognized by the anti-spasmin antibody are not co-localized with microtubules in the cytoplasm at interphase (Fig. 2A).

To confirm the coexistence of spasmin-related protein(s) with microtubules, interphase cells treated with 10 μg/ml vinblastine for 6 h were stained with the anti–*Z. arbuscula* spasmin 1 antibody. Vinblastine generated paracrystals of tubulin within the cell after prolonged incubation. Most of the spasmin-related proteins co-localized with the tubulin (Fig. 2B); however, fragmented filaments recognized by the anti–*Z. arbuscula* spasmin 1 antibody were observed even when microtubules were depolymerized by vinblastine, and were not co-localized with the tubulin.

Interestingly, the anti–*Z. arbuscula* spasmin 1 antibody recognized antigens not only in the cytoplasm but also in the interior of the nucleus during interphase. In the DAPI image, areas with nuclear hot spots were devoid of chromatin, but associated with chromatin on their boundary with the nuclear envelope (Fig. 3). In particular, regions with a number of hot spots were found throughout the cell nucleus during interphase. At prophase, the areas with hot spots were unclear in parallel with the condensation of the replicated chromosomes. With the reassembly of a new nuclear envelope during cytokinesis, clear hot spots were newly formed (Fig. 1). In addition to the hot spots, several filamentous structures were observed despite vinblastine treatment (Figs. 2B and 3). It seems likely that the antigens detected by the anti–*Z. arbuscula* spasmin 1 antibody are components of localized structures inside the nucleus.

Anti–Z. arbuscula Spasmin 1 Polyclonal Antibody Detects Some Proteins Depending on the Cell Cycle—The human antigen recognized by the anti–*Z. arbuscula* spasmin 1 polyclonal antibody was identified by Western blotting of the total proteins of HeLa cells (Fig. 4). The anti–*Z. arbuscula* spasmin 1 antibody recognized bands in each fraction of proteins subjected to 12.5% SDS-PAGE. Both interphase and M phase cells contained two kinds of spasmin-related proteins (Fig. 4, lanes 1 and 2): a protein

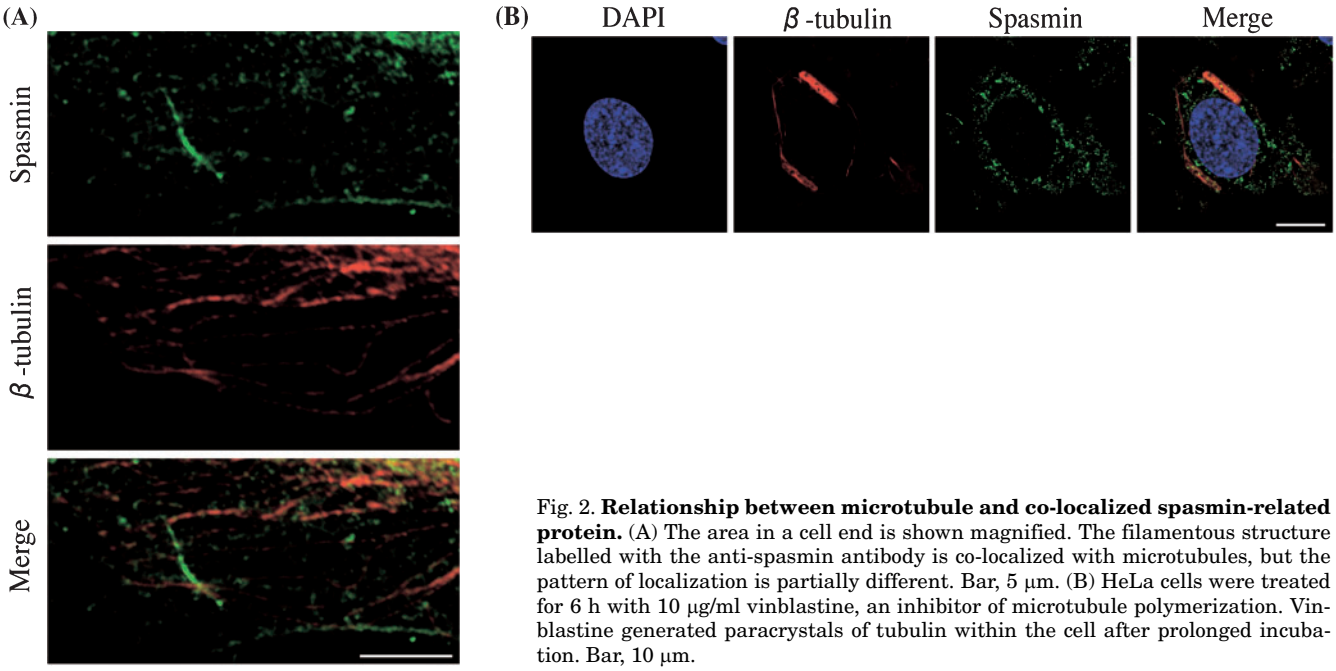

Fig. 2. **Relationship between microtubule and co-localized spasmin-related protein.** (A) The area in a cell end is shown magnified. The filamentous structure labelled with the anti-spasmin antibody is co-localized with microtubules, but the pattern of localization is partially different. Bar, 5 μm. (B) HeLa cells were treated for 6 h with 10 μg/ml vinblastine, an inhibitor of microtubule polymerization. Vinblastine generated paracrystals of tubulin within the cell after prolonged incubation. Bar, 10 μm.

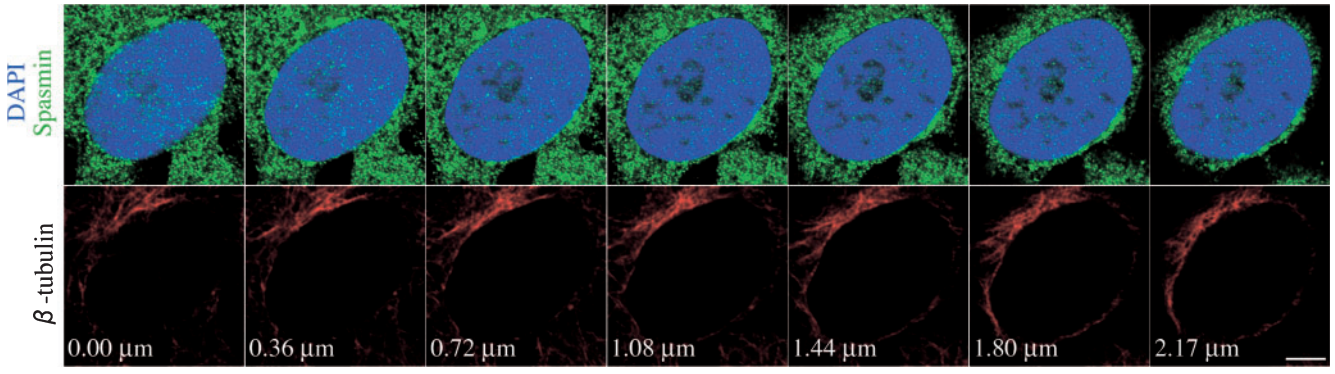

Fig. 3. **Serial optical sections starting from the base of a HeLa cell were collected at 0.36 μm intervals by confocal microscopy.** Filamentous structures and an area with hot spots labelled by the anti-spasmin antibody (shown in green) were observed. These areas were devoid of chromatin (shown in blue) and microtubules (shown in red). Bar, 5 μm.

with a calculated molecular mass of 55 kDa, and another appearing as a single band in M phase and doublet in interphase.

Interphase cells, nuclear proteins and cytoplasmic proteins were analyzed to identify antigens in the internal and external nucleus. In the nuclear fraction, interphase cells contained a nuclear 55 kDa protein (Fig. 4, lane 3) that was not present in cytoplasmic proteins (Fig. 4, lane 4). This result indicates that this protein exists solely inside the nucleus.

Cytoplasmic proteins were observed in interphase cells as two bands at approximately 68 kDa (Fig. 4, lane 4). M phase whole proteins produced a single band with a molecular mass almost identical to that of cytoplasmic proteins during interphase (Fig. 4, lane 1). Neither fraction contained a protein with the molecular mass of spasmin (20 kDa).

Immunoblot analysis with the anti–*Z. arbuscula* spasmin 1 antibody was carried out on hamster kidney BHK21 cells and human lung A-549 cells to confirm the existence of these spasmin-like proteins in other mammalian cells. Similar nuclear and cytoplasmic proteins were detected in both cell lines (Fig. 5). The nuclear spasmin-like protein in BHK21 cells had a lower molecular mass than the 55 kDa band appearing in cultured human cells, this band was not detected by the anti-mouse IgG antibody in the absence of the anti–*Z. arbuscula* spasmin 1 antibody (data not shown). These results demonstrate that mammalian cells contain antigens that are immunologically related to spasmin, dependent on the cell cycle, and located either inside or outside the nucleus.

Analysis of Electrophoretic Behavior in the Presence of Ca^{2+}—Since spasmin is a Ca^{2+}-binding protein, we tested whether the proteins detected by the anti–*Z. arbuscula* spasmin 1 antibody have Ca^{2+}-binding activity based on the fact that spasmins and centrins show a calcium-dependent mobility shift on SDS-PAGE. Proteins from the nucleus, interphase cytoplasm and M phase were subjected to SDS-PAGE in the presence of either Ca^{2+} or EGTA, and then immunoblotted with the anti–*Z. arbus-*

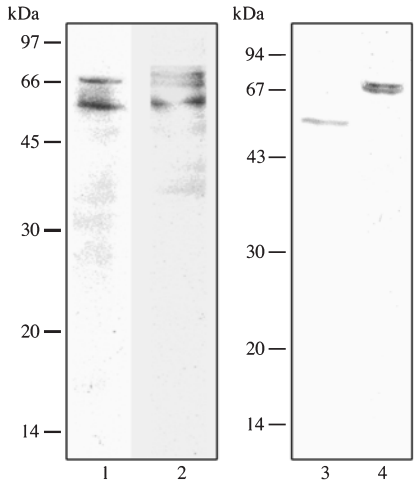

Fig. 4. **Immunodetection on nitrocellulose membranes of antigens to the anti–*Z. arbuscula* spasmin 1 antibody.** In M phase (lane 1) and interphase (lane 2), a single band or doublet around 68 kDa was detected along with a 55 kDa protein. No protein around 20 kDa was detected in any fraction. A single 55 kDa nuclear protein (lane 3) and an approximately 68 kDa cytoplasmic doublet (lane 4) were identified. Molecular weight standards are shown with their corresponding sizes indicated in kDa.

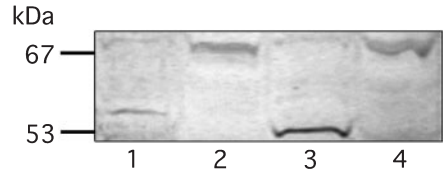

Fig. 5. **Western blot analysis of cytoplasmic and nuclear proteins in human lung A-549 and hamster kidney BHK21 cell lines.** Proteins prepared from A-549 cells (lanes 1 and 2) and BHK21 cells (lanes 3 and 4) were subjected to SDS-PAGE in 12.5% gels and transferred onto nitrocellulose membranes. A 55 kDa protein in the nuclear fraction (lanes 1 and 3) and 70 kDa proteins in the cytoplasmic fraction (lanes 2 and 4) were detected in both cell lines as in the case of HeLa cells (Fig. 4).

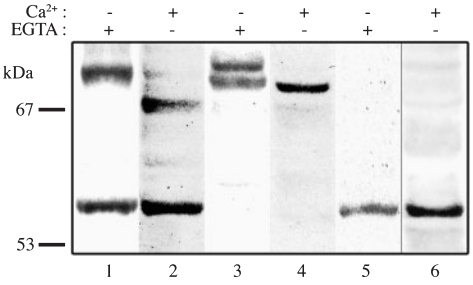

Fig. 6. **Ca^{2+}-dependent electrophoretic mobility of antigens recognized by the anti–*Z. arbuscula* spasmin 1 antibody.** Each protein was boiled in SDS sample buffer containing 5% β-mercaptoethanol plus either EGTA or $CaCl_2$ (2 mM), and then resolved on an 8% SDS–polyacrylamide gel. The corresponding proteins were detected using the anti–*Z. arbuscula* spasmin 1 antibody (Lanes 1 and 2, M phase proteins; lanes 3 and 4, cytoplasmic proteins; lanes 5 and 6, nuclear proteins). Molecular size markers are indicated in kDa on the left.

cula spasmin 1 antibody. In the cytoplasmic fraction of interphase cells, the 68/71 kDa protein formed a single band with Ca^{2+} (Fig. 6, lanes 3 and 4). In contrast, the 70 kDa protein in M phase cells formed a doublet in the presence of Ca^{2+} (Fig. 6, lanes 1 and 2). This remarkable Ca^{2+}-sensitive electrophoretic behavior was not observed for the nuclear 55 kDa protein (Fig. 6, lanes 5 and 6) or the M phase 55 kDa protein (Fig. 6, lanes 1 and 2).

DISCUSSION

In addition to the classical cytoskeleton of eukaryotic cells, nanofilaments have been identified as a fourth group of cytoskeletal elements in eukaryotes (*18*). These superfine filaments are composed of several heterogeneous components, including spasmins and centrins. In mammals, centrin proteins are ubiquitously expressed and commonly associated with centrosome-related structures such as the spindle poles of dividing cells or centrioles in centrosomes and basal bodies (*9*, *19*). In contrast, spasmins and the Ca^{2+}-induced contractile filaments composed of spasmins are thought to be specific to vorticellid ciliates. To test whether spasmin-based contractile filaments in vorticellid ciliates are found in higher eukaryotes, it is important to identify proteins that are immunologically related to spasmin. Here, we report the first detection of spasmin-related proteins in mammalian cells.

A 68/71 kDa Proteins in the Cytoplasm of Interphase Cells and a 70 kDa Protein in the Mitotic Phase Co-localize with Microtubules, Depending on the Cell Cycle—Using a polyclonal anti–*Z. arbuscula* spasmin 1 antibody with an anti–β-tubulin antibody, confocal immunofluorescence imaging of HeLa cells showed that the antibody recognized antigens localized to microtubules in interphase cells. However, these antigens were equally distributed throughout the cytoplasm as in dividing cells. Thus the antigens appear to be more selective for co-localized microtubules such as kinetochore microtubules or outer cytoskeletal microtubules during mitosis. These results suggest that the functions of these antigens are related, either directly or indirectly, to microtubules directly or indirectly. It is unclear how they discriminate between microtubules.

By Western blotting, we identified at least three kinds of antigens appearing as 68/71 kDa proteins in the cytoplasm of interphase cells and a 70 kDa protein in the mitotic phase. These novel proteins could be isoforms that exist depending on the phase of the cell cycle. Both the 68/71 kDa proteins and the 70 kDa protein have the ability to bind Ca^{2+}, and are specific to the interphase and mitotic phase, respectively. Microtubule-associated proteins, MAPs (*20*) and the tau protein (*21*) are known to associate with cytoplasmic microtubules. Similarly, the proteins related to microtubules could be the 68/71 kDa and 70 kDa proteins that are regulated by Ca^{2+}, and locate in a tubulin-dependent manner. However, some spasmin-related proteins were observed as filamentous structures that didn't localize with microtubules (Fig. 2A). These filamentous structures appeared as fragments without microtubules in the presence of vinblastine (Fig. 2B). Therefore, the cytoplasmic spasmin-related proteins could have the ability form filaments depending on microtubules.

The above studies on centrin showed that anti-centrin antiserum reacts with centrin-related proteins, the 165 kDa protein from PtK_2 cells (*12*, *13*) and 62/64 kDa proteins in a human lymphoblastic cell line (*22*). Moreover, indirect immunofluorescence imaging has demonstrated that polyclonal anti-centrin antiserum recognizes centrins and centrin-related proteins localized at the centrosomes of interphase cells, and that it redistributes to the region of the spindle poles during mitosis (*11*, *22*). Our localization results are not the same as those for centrins, indicating that the antigens immunologically related to spasmin are distinct from centrins and centrin-related proteins in mammalian cells.

The 55 kDa Protein Localized in the Nucleus—Confocal immunofluorescence microscopy studies of HeLa cells fixed in interphase revealed an area with several spots recognized by the anti–*Z. arbuscula* spasmin 1 antibody. During cytokinesis, the newly formed nucleus also showed these regions. A 55 kDa protein was found among the nuclear proteins of interphase cells by immunoblotting, and was detected among the whole proteins of mitotic phase cells. These results indicate that the 55 kDa protein is associated with the cell nucleus. These regions containing the 55 kDa proteins were devoid of chromatin materials, with chromatin associated only outside the boundary of these regions. It should be noted that this 55 kDa protein formed filamentous structures with hot spots in the nucleus (Fig. 3). It will be interesting to reveal the relationship between the 55 kDa protein–containing filaments and known components of the nuclear matrix. The nuclear matrix is composed of a profuse network of core filaments that enmesh many dense bodies of diverse size and morphology; the nuclear matrix contains many proteins found nowhere else in the cell (*23*, *24*). In HeLa cells, the cycles of nuclear expansion and contraction are regulated by Ca^{2+} alone, suggesting that nuclear contraction is caused by the nuclear matrix (*25*). Considering that spasmin is a component of the Ca^{2+}-dependent contractile filaments and spasmoneme, our results suggest that the 55 kDa protein, immunologically related to spasmin, is one of the proteins forming nuclear matrix filaments.

At the present time, we have not determined how the organization of this protein during interphase is reestablished at the end of mitosis. The function of the 55 kDa protein as a component of the nucleus of HeLa cells is also not known. Further studies utilizing immunofluorescence imaging are needed to follow these events throughout mitosis.

Taken together, the results here and elsewhere suggest that the 68/71 kDa in the cytoplasm of interphase cells, the 70 kDa protein in mitotic phase cells, and the 55 kDa protein in the nucleus are immunologically related to spasmin, and could form Ca^{2+}-dependent contractile filaments inside and/or outside the nucleus of mammalian cells. Molecular characterization of these novel proteins and ultrastructural studies using more specific antibodies will provide a greater understanding of their function, and clarify the possibility of Ca^{2+}-dependent contractile filaments.

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