Microtubule tracks can be detected in mouse oocytes with an antibody directed against a calcium transporter

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SUMMARY

In metaphase II-arrested mouse oocytes, most microtubules are found in the meiotic spindle, a structure that remains stable for hours despite microtubule instability. Microtubule organizing centres (MTOCs) are present at the poles of the spindle and in the cytoplasm, but the latter nucleate very few microtubules. This particular organization of the microtubule network enabled us to observe the unexpected behaviour of a protein that can associate with microtubules. We compared the distribution of a mitosis-activated calcium transport system with that of the microtubule network, by immunofluorescence, using two monoclonal antibodies, one directed against a component of the calcium transport system (7/13), and the other against the common tyrosinated form of α-tubulin (YL1/2). The 7/13 staining was associated with the spindle microtubules and with the kinetochore area. In addition, we observed many asters in the cytoplasm, around the cytoplasmic MTOCs. The majority of these asters were not stained with the anti-tubulin antibody. Moreover, these 7/13 asters either disappeared after nocodazole treatment or were enlarged after taxol treatment. Using a confocal microscope, we observed single fibres that were stained with both antibodies: the extremity furthest from the MTOC (corresponding to the + end of the microtubule) being detected by the 7/13 antibody only. All these observations suggest that the 7/13 antigen is associated with microtubule tracks that persist a few minutes after microtubule depolymerization. The possible role of these tracks in microtubule regrowth is discussed.

Key words: microtubule, calcium transport, microtubule organizing centre, mouse oocyte

INTRODUCTION

Microtubules can grow and shrink alternately as a result of their dynamic instability (Mitchison and Kirschner, 1984). Schulze and Kirschner (1988) suggested that material that promotes microtubule assembly remains localized after the depolymerization of a microtubule and facilitates the regrowth of a microtubule along the same path. Calcium plays a major role among the regulatory molecules involved in the control of microtubule dynamics. In particular, it is involved in the regulation of microtubule assembly through its interactions with microtubule-associated proteins and calmodulin (Schliwa et al., 1981; Suprenant and Rebhun, 1984; Yamamoto et al., 1985; Kumagai et al., 1986; Baudier and Cole, 1988; Padilla et al., 1990). During mitosis, calcium seems to participate in the control of spindle assembly and the movement of chromosomes (Ratan and Shelanski, 1986; Tombes and Borisy, 1989; Margolis et al., 1990). For example, proteins involved in the control of microtubule dynamics such as the calmodulin-dependent protein kinase (Ohta et al., 1990) and the calcium-dependent regulator protein of cyclic nucleotide phosphodiesterase (Marcum et al., 1978) have been localized to the mitotic apparatus. Also, the calcium and calmodulin-dependent phosphorylation of a 62 kDa protein induces microtubule depolymerization in the mitotic apparatus of the sea urchin (Dinsmore and Sloboda, 1988). Microinjection of antibodies directed against this protein arrests mitosis in dividing sea urchin embryos (Dinsmore and Sloboda, 1989).

Local concentration of intracellular calcium is achieved through the action of a calcium-transport system associated with membrane vesicles (Petzelt and Hafner, 1986). These vesicles are associated with the mitotic apparatus in sea urchin embryos (Petzelt et al., 1987) and mitosis is inhibited after the injection of antibodies directed against calcium transport enzymes in sand dollar (Silver, 1986) and sea urchin embryos (Hafner and Petzelt, 1987).

Following these observations we decided to study the localization of this calcium-transport system and its relationship with microtubule organization in a mammalian system using the mouse oocyte. We used the 7/13 monoclonal antibody specific to a membrane-bound calcium transport system (Petzelt and Hafner, 1986; Hafner and Petzelt, 1987) to stain mouse oocytes. We have observed that the antigen detected by the 7/13 antibody in mouse oocytes is associated with micro-
tubules and microtubule tracks that persist a few minutes after microtubule depolymerization. It may therefore play a role in the maintenance of the three-dimensional organization of the microtubule network, a major component in cellular morphogenesis.

MATERIALS AND METHODS

Recovery of oocytes

Swiss female mice (7-8 weeks old; Centre de Production Animale, Olivet, France) were superovulated by injection of 6.25 i.u. of pregnant mares' serum gonadotrophin (PMSG; Intervet) and 6.25 i.u. of human chorionic gonadotrophin (hCG; Intervet) 48 hours apart. Freshly ovulated oocytes were obtained by puncturing the ampullae of oviducts between 12 and 14 hours post-hCG into warm Medium 2 containing 4 mg/ml bovine serum albumin (M2 + BSA; Fulton and Whittingham, 1978). Cumulus cells were dispersed by brief exposure to 0.1 M hyaluronidase (Sigma), and the zonae pellucidae were removed by a brief treatment with acid Tyrode's, pH 2.5 (Nicolson et al., 1975), followed by a rinse in M2 + BSA.

Immunofluorescence

Oocytes were placed in specially designed stainless steel chambers as described by Maro et al. (1984), except that the chambers were coated with 0.1 mg/ml concanavalin A (Sigma). The samples were centrifuged at 450 g for 10 minutes at 37°C, then extracted for 5 minutes using 0.1% Triton X-100 in PHEM buffer (10 mM EGTA, 2 mM MgCl₂, 60 mM PIPES, 25 mM HEPES, pH 6.9; Schliwa et al., 1981), fixed with cold methanol (−20°C for 10 minutes) and processed for immunofluorescence as described previously (Maro et al., 1984).

The microtubules were stained with the rat monoclonal YL1/2 antibody directed against the tyrosinated form of α-tubulin (Kilmartin et al., 1982), (the choice of this anti-tubulin antibody will be discussed later). Different combinations of second layers were used: fluorescein-labelled anti-mouse IgM antibodies (K.P.L.) with rhodamine-labelled anti- rat antibodies (ICN), or rhodamine-labelled anti-mouse IgG+IgM antibodies (K.P.L.) with fluorescein-labelled anti-rat antibodies (Biosys). To visualize chromosomes, Hoechst dye 33258 (5 μg/ml) was added to the second layer. To prevent bleaching, the samples were mounted in Citifluor (City University, London).

Samples were observed under either a Leitz Diaplan epifluorescence microscope equipped with a ×63 objective (PlanApo; NA 1.4) or a Bio-Rad MRC-600 confocal microscope (White et al., 1987) mounted on an Optiphot II Nikon microscope equipped with a ×60 objective (PlanApo; NA 1.4) and a dual laser system (argon and helium-neon) allowing the use of three excitation wavelengths (488, 514 and 534 nm). Photographs were taken on a VM 1710 Lucius & Baer black and white high-resolution monitor using Kodak T-Max 100 film.

Electrophoresis and immunoblotting

Oocytes were lysed in SDS sample buffer, boiled and analysed using a 10% SDS-polyacrylamide gel (Laemmli, 1970), which was then stained with Coomassie Blue. Proteins were transferred onto nitrocellulose membranes, immunostained with the 7/13 antibody and visualized using alkaline phosphatase-labelled anti-mouse antibodies (Promega). Identical results were obtained when the proteins were western blotted using peroxidase-labelled anti-mouse IgM antibodies (Sigma) to perform an ECL reaction (Amersham).

Purification of meiotic spindles

A total of 200 zona-free oocytes were lysed in 20 μl of lysis buffer (0.1% β-mercaptoethanol, 0.5% Nonidet P40, 0.5 μM MgCl₂, 2 μM taxol, 1 mM PMSF, 100 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM Tris-HCl pH 8.0) for 5 minutes at 37°C. Lysates were then diluted tenfold in K-PIPES buffer (10 mM K-PIPES, pH 7.2, 1 mM EDTA, 2 μM taxol), laid on a cushion of 50% glycerol in K-PIPES buffer, centrifuged on round glass coverslips at 10,000 g for 30 minutes at 4°C, fixed with methanol for 6 minutes at −20°C (Maro et al., 1988) and prepared for immunofluorescent staining as described above.

Image analysis

Image analysis was performed in two steps, segmentation followed by quantification, using Visilog 4.1.1 (Noesis, France) software on an Iris Indigo 4000 Entry Silicon Graphics workstation (the algorithm used is available on request from C. Cibert).

Image segmentation

Segmentation of the images was performed in rectangular windows, defined to enclose the area of interest of the observed fluorescence signal (A) in each of the confocal optical sections. The segmentation procedure takes into account the general feature of the distribution of the fluorescence: ‘filaments’ emerging from central ‘spots’. The first and second steps of the segmentation process consist of the calculation of the ‘skeleton’ of filaments and the determination of the spots respectively. The binary mask corresponds to the addition of the two segmented images. The same procedure was used on the YL1/2 and 7/13 images (with necessary adjustments of the number of iterations of some functions). The main steps of the procedure were (+) indicates the steps adjusted in function of the labelling quality) as follows:

Calculation of the skeleton

Smoothing of the original images by an oriented closing and an opening

Adjustment h-equalization of the histogram (*) (the inferior threshold was coarsely defined and corresponds to the minimum of grey level of the A part of the image)

Opening (*) and subtraction (as in the classical ‘top-hat’ function) The resulting images were thresholded twice (*), the first binary image was smoothed (*) by oriented closing and was reconstructed by the second one

Separation of the contiguous blobs by calculation of the watershed lines

Calculation of the skeleton of the binary image; the different parts of the skeleton were validated after individual analysis (*) and ebarbed in a conditional way (*); the definitive skeleton was dilated once

Definition of the spots

The maximum of the labelling was defined by a threshold (*) of the smoothed original image.

Quantification

Measurements were performed in 29 concentric circular crowns, 3 pixels thick. The putative position of the MTOC was defined as the centre of the concentric circles. The area and the mean grey level of the image, which was overlapped by both the binary masks and a given crown, were measured. The areas were expressed as the percentages of the area of each crown and represented as angles (100% corresponds to 360°). The mean grey levels were expressed as the percentage of the maximum value if all the pixels of the mask had a value of 255.

RESULTS

Characterization of the 7/13 antigen in mouse oocytes

The monoclonal antibody 7/13 has been characterized in
Microtubule tracks in mouse oocytes

1901

human cells and sea urchin eggs, where it recognizes a protein involved in intracellular calcium transport (Petzelt and Hafner, 1986). In addition, injection of the 7/13 antibody into sea urchin eggs increases the intracellular calcium level and induces the depolymerization of spindle microtubules (Hafner and Petzelt, 1987). We used the 7/13 antibody to stain freshly ovulated mouse oocytes, in which most of the microtubules are found in the meiotic spindle (although many MTOCs are present in the cytoplasm; Maro et al., 1985). Immunofluorescence staining of oocytes with 7/13 antibody showed the following pattern: the meiotic spindle was labelled, with enhanced labelling at the spindle poles and at the kinetochores (Fig. 1A). In addition, many asters were detected in the cytoplasm (Fig. 1A,B). When the oocytes were double-stained with 7/13 and 5051 antibodies, an anti-serum that stains MTOCs in many species, including the mouse oocyte (Maro et al., 1985), these ‘7/13 asters’ were stained centrally by the 5051 antibodies (Fig. 1B,C). To characterize further the antigen recognized by the 7/13 antibody in mouse oocytes, we used purified mouse meiotic spindles (Maro et al., 1988). In these preparations, the 7/13 antibody did not stain the whole spindle but only the kinetochores (Fig. 2A), suggesting that the antigen is either partly extracted by 0.5% NP-40 (the detergent used during spindle purification), or removed during the centrifugation through the 50% glycerol cushion. Moreover, no associated membrane vesicles were detected when these preparations were observed with an electron microscope (Maro et al., 1988). These observations imply that the antigen detected by the 7/13 antibody is weakly associated with the spindle microtubules and may be linked to membrane vesicles, as in sea urchin eggs (Petzelt and Hafner, 1986). Finally, the 7/13 antibody was analysed by immunoblotting with mouse oocyte proteins, which permitted the detection of a major band with a relative molecular mass of around 35 kDa and a minor band at 75 kDa (Fig. 3). These two bands were still observed when similar experiments were performed after electrophoresis in reducing and non-reducing conditions and in the presence or absence of 6 M urea. This suggests that 7/13 may recognize an epitope shared by two different proteins, without excluding the possibility that the 75 kDa is a dimeric form of the 35 kDa polypeptide.

Part of the 7/13 antigen associates with microtubules in mouse oocytes

When freshly ovulated oocytes were stained with an anti-tubulin antibody, only a very few cytoplasmic asters were detected irrespective of fixation conditions (Maro et al., 1985; de Pennart et al., 1988). However, when oocytes were double-
stained with the 7/13 antibody and the YL1/2 anti-tubulin antibody (Kilmartin et al., 1982), many asters were stained with the 7/13 antibody only, and those stained with both antibodies appeared larger with the 7/13 labelling than with the YL1/2 labelling (Fig. 4A,C).

Using the confocal microscope at higher magnification, it was possible to observe clearly single fibres that were longer with the 7/13 staining than with the corresponding YL1/2 tubulin staining, and fibres that were identical with the two stainings (see Fig. 4A,B and Fig. 10 ‘control’ for schematic representation). However, we never observed fibres that appeared longer with YL1/2 than with 7/13 staining.

Since all microtubule structures were stained with the 7/13 antibody, this suggested that the 7/13 antigen associated, at least partially, with microtubules. Moreover, the existence of 7/13 fibres that were longer than the corresponding microtubules (Fig. 4) suggest that the 7/13 antigen remains in place after the depolymerization of the microtubules, at least for a short time. We tested this hypothesis by using drugs that interfere with microtubule polymerization. Taxol was used to induce the growth of stable microtubule asters in the cytoplasm (Marto et al., 1985) and nocodazole to inhibit microtubule polymerization. After a 5 minute treatment with 5 μM taxol, many asters were observed in the cytoplasm, all of which were detected by both antibodies, the 7/13 antigen being localized mostly at the (+) end of the microtubules (see Fig. 5A,B and Fig. 10 ‘taxol’ for schematic representation). This result shows that the 7/13 antigen colocalizes with microtubules when the latter are stabilized by the drug. After a 5 minute exposure to 10 μM nocodazole, the larger asters detected by the 7/13 antibody in the cytoplasm disappeared (Fig. 6A). Only small asters and dots of 7/13 positive material remained in the cytoplasm, which probably corresponded to the centre of the asters observed in control freshly ovulated oocytes (see Fig. 6 and Fig. 10 ‘nocodazole’ for schematic representation). At the same time, we were not able to detect any staining in the cytoplasm with YL1/2 (Fig. 6B). After a 15 minute treatment with 10 μM nocodazole, no dots or asters could be detected with the 7/13 antibody. These experiments suggest that the 7/13 antigen associates with growing microtubules (taxol treatment) and remains localized for a limited time in the area where microtubules were present before a shrinking phase (nocodazole treatment).

**Analysis of the interaction between the 7/13 antigen and microtubules**

To confirm our previous observations, we have examined...
carefully the asters observed under the confocal microscope using image analysis (according to the protocol described in Materials and Methods). This analysis allowed us to quantify different parameters: (i) the overlapping between the two stainings (Figs 7-9C); (ii) the surface and the length of the two stainings (Figs 7-9F,G); and (iii) the intensity of each staining according to its distance from the centre of the aster (Figs 7-9H,I).

In control oocytes, we found a good overlap of the 7/13 and the YL1/2 staining (Fig. 7C), which confirms the association of the 7/13 antigen with the microtubules. Moreover, the YL1/2-stained structures that are included in the 7/13 ones are also shorter than their 7/13 counterparts (Fig. 7F,G). Our results also revealed that the intensity of both stainings decreased as the distance from the centre of the aster to the (+) end of the microtubules increased (Fig. 7H,I). This result suggests that depolymerization of microtubules, which occurs at the (+) end of the microtubule in the aster, is followed after a short delay by the disappearance of the 7/13 staining.

Following taxol treatment, the YL1/2-stained asters were bigger (Fig. 8A,E), and structures revealed by the two staining patterns were of similar length (Fig. 8F,G), which indicates that 7/13 antigen association follows microtubule polymerization. Furthermore, the YL1/2 staining intensity decreased from the centre of the aster to the (+) end of the microtubules, whereas the 7/13 staining intensity remained constant (Fig. 8H,I). This result suggests that there is a relocation of the 7/13 antigen along stabilized microtubules.

After exposure to nocodazole, the YL1/2 staining consisted of a single spot in most cases (Fig. 9D,E) and was always shorter than the 7/13 staining (Fig. 9F,G). This result confirms our observations with control oocytes and indicates that the 7/13 antigen remains in place a few minutes after microtubules depolymerization has occurred. Moreover, the 7/13 staining intensity decreased from the centre of the aster to the (+) end of the microtubules (Fig. 9I), suggesting that disappearance of the 7/13 staining starts at the (+) end of microtubules.

**DISCUSSION**

Ovulated mouse oocytes are arrested in metaphase of the second meiotic division and it usually takes a few hours before the completion of meiosis induced by fertilization. When we used the 7/13 antibody to stain metaphase II-arrested mouse oocytes, we found that only structures closely associated with microtubules were stained with the 7/13 antibody: the meiotic spindle, the kinetochores and asters centred by cytoplasmic MTOCs. In addition, our experiments showed that the link between the 7/13-stained molecules and the spindle fibre does
Fig. 7. Image analysis of three different asters (corresponding to the top, middle and bottom panels) found in control oocytes and double-stained with the 7/13 antibody (A) and the anti-tyrosinated α-tubulin monoclonal antibody YL1/2 (E). (B) and (D) represent the binary masks of (A) and (E), respectively, and the overlap between these two masks is displayed in (C), where the 7/13 and YL1/2 stainings are represented in grey and white, respectively. Measurements were performed as described in Materials and Methods: (F) and (G) show the 7/13 or YL1/2 staining present in each crown, respectively, and in (H) and (I) the average intensity of each staining in each crown is shown.
not resist treatment with detergent and centrifugation through a 50% glycerol cushion, in good agreement with the vesicular localization of this calcium transport system observed in mammalian cells and sea urchin eggs (Petzelt and Hafner, 1986; Petzelt et al., 1987). These observations suggest that the concentration of calcium around the spindle is achieved by a specific calcium sequestering mechanism that hence controls microtubule dynamics. Such a mechanism has been proposed by Petzelt and Hafner (1986) for sea urchin embryos.

Immunoblotting with 7/13 revealed a major polypeptide at
35 kDa and a minor band at 75 kDa, both of which differ from the polypeptide found in sea urchin (46 kDa; Petzelt and Hafner, 1986). However, we believe that the polypeptide(s) recognized by the 7/13 antibody in these species are related, since they share several common properties such as association with the spindle and M-phase specific-staining. Unfortu-
Fig. 10. Schematic representation of the behaviour of microtubules (in white) and 7/13 antigen (stippled) under various experimental conditions: in control oocytes, the 7/13 staining often appears greater than the YL1/2 staining. Taxol induces a rapid polymerization of microtubules and gives similar patterns with both antibodies, sometimes greater with YL1/2, showing that the 7/13 epitope associates rapidly after microtubule polymerization. Nocodazole prevents tubulin polymerization and leads to a rapid depolymerization of the microtubules. In this case, the 7/13 staining always appears greater than the YL1/2 staining, showing that the 7/13 antigen stays in place for a few minutes after microtubule depolymerization. Hypothesis: the 7/13 antigen facilitates the growth of a new microtubule (hatched arrow) along the pre-existing track.

nately, we were not able to characterize further the localization of the 7/13 antigen with the electron microscope (EM), since the antigenicity was lost after all the EM fixation procedures that were tested.

Our studies revealed that the 7/13 antigen has a specific type of behaviour around the cytoplasmic MTOCs and is associated with microtubule tracks (see Fig. 10 for a summary of our observations). One of the most interesting peculiarities of the mouse oocyte is the presence of cytoplasmic MTOCs not associated with the spindle poles that nucleate only a very few microtubules with a very short half-life in an M-phase environment (Maro et al., 1985; de Pennart et al., 1988). The appearance of asters around the cytoplasmic MTOCs can be induced by different means, either during cold recovery (Johnson et al., 1988) or after treatment with taxol or 6-DMAP (Maro et al., 1985; de Pennart et al., 1988; Szöllösi et al., 1993). Using the 7/13 antibody, we observed many asters in the cytoplasm, around the cytoplasmic MTOCs. As expected from our previous work (Maro et al., 1985; de Pennart et al., 1988), the majority of these structures were not stained with the anti-tyrosinated α-tubulin antibody. These 7/13 asters either disappeared after nocodazole treatment or were enlarged after taxol treatment, suggesting that these structures were, at least temporarily, associated with microtubules. The use of confocal microscopy enabled us to demonstrate the presence of single fibres that were longer with the 7/13 staining than with the corresponding anti-tyrosinated α-tubulin staining, or fibres where the staining patterns were identical. In control oocytes we never observed the opposite situation, that is where the anti-tyrosinated α-tubulin-stained fibres were longer than the 7/13 stained fibres. It was always the extremity furthest from the MTOC, that is the (+) end of the microtubule, that was detected by the 7/13 antibody alone. All these observations suggest that the antigen detected by the 7/13 antibody is associated with microtubules and remains in place for a short period after microtubule depolymerization (Fig. 10). We can exclude the fact that the fixation procedure used in our experiments (extraction followed by methanol fixation) leads to the depolymerization of microtubule asters, since they were also absent when a different procedure (immediate fixation with glutaraldehyde in the presence of Triton) was used (de Pennart et al., 1988, 1993; Szöllösi et al., 1993; Verlhac et al., 1994).

The rat YL1/2 monoclonal antibody, directed against the tyrosinated form of α-tubulin, was chosen for two reasons. First, it was the only monoclonal anti-tubulin antibody that allowed us to perform double staining experiments with 7/13 antibody. Second, YL1/2 detects microtubules in the cytoplasm and in the spindle of the oocyte, while no microtubules are detected in the mouse oocyte with an anti-detyrosinated α-tubulin antibody (de Pennart et al., 1988). Moreover, the anti-detyrosinated α-tubulin cannot detect detyrosinated microtubules in the oocyte even after stabilization of the microtubules for 4 hours with taxol (de Pennart et al., 1988). Detyrosination and acetylation have been shown in many systems to take place in the more stable subpopulation of microtubules (including mouse oocytes and mouse early blastomers); de Pennart et al., 1988; Houliston and Maro, 1989). Thus, detyrosination of microtubules should take place, if at all, first at the (−) end of the microtubules that are stabilized by their association with the MTOC, rather than at the (+) end furthest from the MTOC, which is subject to growing and shrinking events (Schulze and Kirschner, 1988). Together, these previous observations permitted us to exclude the possibility that the 7/13 antigen could be distributed with detyrosinated microtubules.

The experiments reported in this paper enabled us to demonstrate the existence of microtubule tracks in mouse oocytes, first suggested in tissue culture cells by Schulze and Kirschner (1988) from their observations of microtubule behaviour in vivo. These tracks contain molecules that are able to control calcium concentration in their environment and may thus provide a molecular basis for the directed growth of microtubules along tracks occupied previously by microtubules that had subsequently depolymerized. Our observations show that these tracks persist long enough (up to 2 minutes) for the regrowth of a microtubule after a shrinking phase. They could also allow the growth of one microtubule along the path occupied shortly before by another microtubule, as observed by Schulze and Kirschner (1988). We could not demonstrate this directly by labelling in vivo the vesicles containing the 7/13 antigen, because in vivo the 7/13 antibody causes a rise in intracellular calcium leading to the depolymerization of the microtubules (Hafner and Petzelt, 1987). The localization of this calcium transport system could contribute greatly to the understanding of the maintenance of a three-dimensional microtubule array composed of unstable microtubules. Different observations provide some clues as to how an asymmetric network can be established (Bacallao et al., 1989; Houliston and Maro, 1989; Houliston et al., 1989; Jasmin et al., 1990), mainly by stabilizing microtubules under the influence of extrinsic factors (Kirschner and Mitchison, 1986). However, it is unclear how unstable microtubules are able to maintain a stable array. The behaviour, and the calcium-
transport properties of the antigen detected by the 7/13 antibody, may help to identify those mechanisms involved in the maintenance of the organization of the microtubule network.

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REFERENCES


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