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The actin-capping protein CapG localizes to microtubule-dependent organelles during the cell cycle

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ABSTRACT

Extensive cross-talk between the actin and the microtubule cytoskeletons has been reported. Especially in mitosis, processes dependent on actin- and microtubule-based structures alternate and regulate each other in a complex cascade leading to division into two daughter cells. Here, we have studied the subcellular localization of the filamentous actin-capping protein CapG. Fluorescence microscopy of endogenous CapG and EGFP-tagged CapG revealed CapG localization at the mother centriole in interphase, the mitotic spindle in mitosis and the midbody ring in abscission. Surprisingly, nucleoporin Nup62, an interaction partner of CapG, also localized to the midbody ring at the end of abscission and colocalized with CapG. We propose a role for the actin-binding protein CapG as a mediator of cross-talk between the actin cytoskeleton and microtubule-based organelles that regulate cell division.

Processes that require cellular asymmetry such as cell motility, neuronal pathfinding, wound healing and cell division involve cross-talk between the actin and microtubule cytoskeletons [1]. Interaction between the two cytoskeletons is structural or regulatory. Interactions are classified as structural when actin and microtubules are physically linked. Regulatory interactions are those that involve proteins such as Rho family GTPases which transmit signals between actin and microtubules. Various organelles, such as the centrosome, the mitotic spindle and the midbody, use microtubules as a structural constituent. Interactions between the actin cytoskeleton and microtubule-dependent organelles can also be classified structurally or regulatory. Mitotic spindle mediated regulation of the actomyosin contractile ring and F-actin mediated transport of the centrosomes prior to mitosis are examples of regulatory and structural interactions, respectively.

The microtubule cytoskeleton is required to maintain the polarized distribution of actin-dependent protrusions at the leading edge of migrating fibroblasts [2]. It has been suggested that microtubule growth at the leading edge could promote local activity of Rac in the cell front to drive lamellipodial protrusion [3]. Conversely, microtubule depolymerization behind the lamellium could activate RhoA to drive actomyosin contraction [4]. Thus, the actin cytoskeleton provides force-generating structures to extend the cell front, whereas the microtubule cytoskeleton coordinates these actin-based structures in migrating cells. In addition, microtubules coordinate focal adhesion disassembly in migrating cells [5].

Centrosome reorientation in migrating cells is coupled to the capture of microtubule + ends to the actin cortex by Cdc42 and cytoplasmic Dynein [6]. At the onset of mitosis, filamentous actin and myosin II have been shown to provide the force required to separate centrosomes and drive them to opposite ends of the nucleus [7]. The attachment of F-actin to the centrosome is mediated by cortactin [7]. Correct positioning of centrosomes is necessary to initiate and correctly form the spindle. Proper spindle orientation is also mediated by interaction between microtubules and cortical actin [8]. Thereafter, the mitotic spindle controls the precise localization of the contractile actomyosin ring to form the cleavage furrow in the plasma membrane [1]. Disruption of the spindle before formation of the actomyosin ring inhibits formation of this organelle and prevents furrow ingression in cytokinesis [9]. Following cytokinesis, the midbody, a microtubule-dependent organelle, performs abscission, the process by which daughter cells become physically separated. Separation of daughter cells occurs when exocytotic vesicles fuse with the plasma membrane in the midbody at a special structure called the midbody ring [10]. It has been suggested that the contractile ring and the midbody ring, which are distinct organelles with markedly different characteristics, are nonetheless templated at the same time [11].

CapG is an actin filament capping protein of the Gelsolin family [12]. Its capping activity is activated by calcium and inhibited by

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membrane polyphosphoinositides but unlike Gelsolin, CapG does not sever actin filaments [12]. CapG also localizes in the nucleus and we recently showed that CapG is imported in the nucleus by the transport receptor NTF2, Ran GTPase and nucleoporin Nup62 [13,14]. In the nucleus, CapG is actively transported to transcriptionally active nucleoli [15]. Here, we present evidence that CapG might mediate cross-talk between the actin cytoskeleton and microtubule-based organelles involved in mitosis.

Materials and methods

Reagents. Thymidine, saponin, nocodazole and 4,6-diamidino-2-phenylindole (DAPI) were obtained from Sigma–Aldrich (St. Louis, USA). Digitonin was from Calbiochem (Darmstadt, Germany).

Plasmids, recombinant proteins and antibodies. CapG cDNA was subcloned in the pEGFP-N1 vector (Clontech-Takara Bio Europe, St-Germain-en-Laye, France). CapG was also subcloned in the pcDNA6-myc-His vector (Invitrogen, Merelbeke, Belgium). Purification of Myc-CapG-V5-His_6 is described by Van Impe et al. [13]. Rabbit polyclonal anti-CapG antibody was affinity purified [16]. Mouse monoclonal anti-V5 antibody was obtained from Invitrogen. Alexa 488-conjugated goat anti-rabbit antibody and Alexa 594-conjugated goat anti-mouse antibody were from Molecular Probes (Invitrogen, Merelbeke, Belgium). Mouse monoclonal anti-Acetylated tubulin antibody, mouse monoclonal anti ε-tubulin antibody, mouse monoclonal anti γ-tubulin antibody and mouse monoclonal anti α-tubulin antibody were from Sigma–Aldrich. Mouse anti-Ran antibody and mouse anti-Nup62 antibody were from BD Biosciences (Erembodegem, Belgium).

Cell culture and cell processing. HeLa, MDCK-AZ and HEK293T cells were maintained at 37 °C in a humidified 10% CO_2 incubator and grown in DMEM (Gibco) supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin and 100 IU/ml penicillin. HeLa cells were transiently transfected using lipofectamine reagent (Invitrogen) according to the manufacturer's instructions. HEK293T cells, seeded on rat tail collagen-coated coverslips, were transfected using calcium phosphate. For thymidine treatment, HEK293T cells were plated on collagen-coated coverslips, allowed to recover for 24 h and stimulated with 2 mM thymidine in DMEM for 13 h, followed by 11 h incubation in fresh medium before processing for immunofluorescence microscopy. In the microtubule regrowth assay, HEK293T cells were plated on collagen-coated coverslips, allowed to recover for 24 h and treated with 10 μg/ml nocodazole for 2 h at 37 °C to depolymerize microtubules. Cells were then allowed to recover in fresh medium for 15 min to polymerize new microtubules. For saponin extraction, HEK293T cells were plated on collagen-coated coverslips, allowed to recover for 24 h and treated with 0.02% saponin in PHEM buffer (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 2 mM MgCl, 0.02% BSA, pH 6.9) for 5 min at 37 °C. Cells were then fixed in 100% methanol at −20 °C for 6 min. For cell permeabilization with digitonin, MDCK-AZ cells were grown on glass coverslips for 48 h. Cells were washed in binding buffer (20 mM Hepes pH 7.3, 110 mM potassium acetate, 5 mM sodium acetate, 2 mM magnesium acetate, 2 mM DTT, 0.5 mM EGTA and protease inhibitors) and then treated with digitonin (20 μg/ml) for 4 min on ice. After permeabilization, the cells were washed twice with binding buffer and inverted on top of 50 μl import reaction mix containing 4 μM myc-CapG-V5-His and an energy-regenerating mixture (1 mM ATP, 0.1 mM GTP, 5 mM creatine phosphate and 20 U/ml creatine phosphokinase) and incubated at 30 °C for 30 min. Cells were washed twice in binding buffer and further processed for immunofluorescence microscopy.

Immunostaining and immunofluorescence microscopy. Cells were washed with PBS, fixed with 3% paraformaldehyde for 20 min at room temperature and permeabilized with 0.2% Triton X-100 in PBS for 5 min. Paraformaldehyde was neutralized with 0.75% glycine for 20 min. Alternatively, cells were fixed and permeabilized by a 6 min incubation in 100% methanol at −20 °C. Cells were then blocked in 1% BSA in PBS for 30 min and incubated with primary antibody for 1 h at 37 °C. Cells were washed in PBS, then incubated with secondary antibody and 4,6-diamidino-2-phenylindole (DAPI) for 30 min at room temperature. Cells were first stained with rabbit primary antibody, thereafter with mouse primary antibody. Following immunostaining, samples were analyzed using a Carl Zeiss Axiovert 200 M Apotome epifluorescence microscope (63× 1.4NA oil objective) equipped with an Axiocam cooled CCD camera and processed using Axiovision software (Zeiss, Göttingen, Germany).

Results and discussion

CapG localizes to the centrosome during interphase

In a previous study, we reported that CapG interacts with Ran, a major regulator of nucleo-cytoplasmic protein transport, and this interaction is necessary for nuclear import of CapG [14]. We also demonstrated that Ran can accompany CapG in the nucleolus [15]. In addition, Ran localizes to the centrosome [17]. Therefore, we hypothesized that CapG possibly acts in concert with Ran at the centrosome. Reminiscent of many centrosomal proteins, Ran localization at the centrosome can only be visualized in methanol-fixed cultured cells [17]. Analysis of the subcellular localization of endogenous CapG revealed that CapG localized to the centrosome of methanol- but not paraformaldehyde-fixed HEK293T cells (Fig. 1A, second panels). γ-Tubulin is a marker for both centriole bodies. CapG only localized to one of both centrioles, in contrast to Ran which localizes to both centriole bodies (Fig. 1A, left panels). To determine to which centriole CapG localizes, we stained HEK293T cells for ε-tubulin, a marker for the appendages of the mother centriole, and acetylated tubulin, which stains the primary cilium that only grows on the appendages of the mother centriole (Fig. 1A, right panels) [18,19]. These stainings revealed that CapG specifically localized to the appendages of the mother centriole. To further confirm that CapG localized to the appendages, we performed a microtubule regrowth assay (Fig. 1B). The newly polymerized microtubules seemed attached to a ring-shaped CapG staining, indicating that CapG indeed localized at the appendages of the mother centriole (Fig. 1B). We also analyzed the localization of EGFP-tagged CapG in γ-tubulin-stained HEK293T cells (Fig. 1C). CapG-EGFP localized to the centrosome, supporting the previous results. Strikingly, CapG-EGFP localized to both centriole bodies and completely overlapped with γ-tubulin (Fig. 1C). Possibly, addition of the EGFP tag perturbs the exact position of CapG at the centrosome. Alternatively, excess CapG accumulates at a transient location during transit to the appendages of the mother centriole. Thus, increased expression of CapG could shift the balance between a transient and a more stable location at the centrosome. The reported interaction between CapG and Ran could account for this observation since Ran also localizes to both centriole bodies. Ran has already been shown to assist CapG import into the nucleus and CapG transport to the nucleolus [14,15]. In this perspective, we can hypothesize that Ran also recruits CapG to the centriole bodies prior to further transport to the appendages of the mother centriole.

The localization of endogenous CapG at the centrosome persisted until the onset of mitosis in prophase. Subsequently, CapG relocalized to the mitotic cytoplasm (supplementary data, Figs. S1 and S2). CapG reappeared at the centrosome in abscission at the end of cytokinesis, simultaneously at both connected daughter cells (Fig. 1D and supplementary data, Fig. S2). Interestingly, CapG