European Journal of Cell Biology 96 (2017) 119–130

Contents lists available at ScienceDirect

European Journal of Cell Biology

journal homepage: www.elsevier.com/locate/ejcb

Research paper

CP39, CP75 and CP91 are major structural components of the *Dictyostelium* centrosome's core structure

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ARTICLE INFO

Article history:
Received 24 October 2016
Received in revised form
13 December 2016
Accepted 9 January 2017

Keywords:
Dictyostelium
Mitosis
Microtubules
Centrosome
Nucleus

ABSTRACT

The acentriolar *Dictyostelium* centrosome is a nucleus-associated body consisting of a core structure with three plaque-like layers, which are surrounded by a microtubule-nucleating corona. The core duplicates once per cell cycle at the G2/M transition, whereby its central layer disappears and the two outer layers form the mitotic spindle poles. Through proteomic analysis of isolated centrosomes, we have identified CP39 and CP75, two essential components of the core structure. Both proteins can be assigned to the central core layer as their centrosomal presence is correlated to the disappearance and reappearance of the central core layer in the course of centrosome duplication. Both proteins contain domains with centrosome-binding activity in their N- and C-terminal halves, whereby the respective N-terminal half is required for cell cycle-dependent regulation. CP39 is capable of self-interaction and GFP-CP39 overexpression elicited supernumerary microtubule-organizing centers and pre-centrosomal cytosolic clusters. Underexpression stopped cell growth and reversed the MTOC amplification phenotype. In contrast, in case of CP75 underexpression of the protein by RNAi treatment elicited supernumerary MTOCs. In addition, CP75RNAi affects correct chromosome segregation and causes co-depletion of CP39 and CP91, another central core layer component. CP39 and CP75 interact with each other directly in a yeast two-hybrid assay. Furthermore, CP39, CP75 and CP91 mutually interact in a proximity-dependent biotin identification (BioID) assay. Our data indicate that these three proteins are all required for proper centrosome biogenesis and make up the major structural components of core structure's central layer.

1. Introduction

The centrosome serves as the major microtubule-organizing center and is therefore of crucial importance for organelle positioning, intracellular transport and mitotic spindle organization. Centrosomes in organisms capable of cilia formation contain centrioles, hollow cylinders mainly consisting of microtubules, which are surrounded by pericentriolar material containing microtubule nucleation complexes. In G1, there are two centrioles, an older mother centriole and a daughter centriole that was born in the previous cell cycle. During G1 and G0 the mother centriole serves also as the basal body for a primary cilium. This centrosome type is opposed by acentriolar centrosomes found in organisms that have lost the capability to build cilia such as most fungi and many Amoebozoa (Gräf et al., 2015). Thus, centrosomes in the amoebozoan *Dictyostelium discoideum* consist of a cylindrical, three-layered core structure surrounded by a so-called corona (Moens, 1976). The corona is the functional equivalent of the pericentriolar matrix and contains γ-Tubulin nucleation complexes that are represented by dense nodules on the electron microscopic level (Daunderer et al., 1999; Euteneuer et al., 1998). At the first glance, the tri-laminar core structure of the *Dictyostelium* centrosome resembles a yeast spindle pole body, which mainly consists of a stack of three plaques. Yet, closer analysis has shown that these structures resemble each other mainly from a morphological point of view, while there are only little similarities with regard to the molecular composition of the individual layers and their behavior during centrosome duplication (Ueda et al., 1999). Centrosomes duplicate once and only once per cell cycle, whereby the actual duplication process is synchronized with S-phase in most organisms. However, in *Dictyostelium* centrosome duplication starts in prophase, when the central, layered core

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http://dx.doi.org/10.1016/j.ejcb.2017.01.004
0171-9335/

structure expands and the corona with its microtubule nucleation complexes disappears, together with the interphase microtubules (Kitanishi-Yumura and Fukui, 1987). In late prophase, the central layer of the core structure disappears, which allows separation of the two outer layers and coincides with their insertion into the nuclear envelope. Instantly microtubules are nucleated from the nuclear surface of the now nuclear envelope-embedded two layers that have now become the mitotic spindle poles organizing a central spindle. In metaphase, the two poles are opposed to each other and astral microtubules extending into the cytoplasm appear at their edges that are bent towards the cytosolic face of the nucleus. Pole separation appears to employ both astral and spindle microtubules (Tikhonenko et al., 2016). As the poles separate, the folding process of the poles continues and in telophase each pole has folded back onto itself in a way that the former cytosolic surface becomes buried inside a new, layered structure. The microtubule-nucleating, former inner surface organizes a new corona with a radial microtubule cytoskeleton and the central layer reappears (Kuhnert et al., 2012b). Compared to our detailed knowledge about the morphological changes during the centrosome cycle in *Dictyostelium* our insights in dynamic assembly and regulation of centrosomal proteins are still limited.

In a previous study nine of 34 candidate proteins identified in a proteomic screening assay for new centrosomal components were confirmed as genuine centrosomal components (Reinders et al., 2006; Schulz et al., 2009). According to the distribution of the respective green fluorescent protein (GFP) fusion proteins in deconvolved confocal images of isolated centrosomes, four of these proteins, named CP39, CP55, CP75 and CP91, were designated to the so far poorly characterized three-layered core complex. Among these proteins CP55 and CP91 have been characterized more thoroughly. While CP55 is a structural component of the outer core layers with a role in centrosome duplication and requirement for integrity and stability of the corona (Kuhnert et al., 2012a), CP91 was characterized as the first component of the central layer. It is required for centrosome integrity, proper chromosome segregation and it appears to mediate in cohesion of the two outer layers (Putzler et al., 2016). Here we characterize CP39 and CP75, two further essential components of the core structure. Our data indicate that these two proteins together with CP91 are the major structural components of the central layer of the core structure.

2. Results

2.1. Endogenous CP39 localizes to the centrosomal core structure

The *cepA* gene encodes a 346 aa (amino acids) protein with a calculated molecular mass of 39.9 kDa and an unusual amino acid composition with several poly-asparagine and poly-glutamine stretches. The protein was hence named CP39. Its only homologue identified by BlastP is a 39.0 kDa protein of *Dictyostelium purpureum* sharing about 30% of its amino acids with CP39. As this is a quite low degree of identity for orthologues of two species belonging to the same genus, it is not surprising that sequence comparisons disclosed no CP39 orthologue in more distantly related organisms. Sequence analysis with the COILS program (window size 28; Lupas et al., 1991) predicted a single coiled coil region from amino acid 198 to 235.

An overexpressed GFP-CP39 fusion protein produced in the course of our analysis of the centrosomal proteome was already assigned to the centrosomal core structure (Schulz et al., 2009). Yet, as overexpression produced clear, phenotypic abnormalities (see below) we preferred to perform further analyses on its cell cycle-dependent localization with the endogenous protein. Thus, we raised a rabbit polyclonal antibody against recombinant CP39. In immunoblots endogenous CP39 migrated with an apparent molecular mass of ~45 kDa. Its total cellular protein level seems to be quite low, since it can only be detected in fractions enriched with centrosomes, whereas it was completely absent from the cytosolic fraction (Fig. 1A). This high apparent molecular mass for a 39-kDa protein is in agreement with the behavior of the GFP fusion protein, which also migrated slower than calculated in SDS electrophoresis gels (Schulz et al., 2009).

To elucidate the subcentrosomal localization of CP39, the antibody was used to stain the protein in wild type AX2 cells in comparison with the corona protein DdCP224 (Fig. 1B–D). While DdCP224 showed a donut-shaped distribution, CP39 was concentrated at a small dot in the middle of the centrosome.

2.2. CP39 is regulated during mitosis

Our earlier study had shown that overexpressed GFP-CP39 is absent from the spindle poles of metaphase spindles, however, it was not clear at which mitotic stage CP39 dissociates from the centrosome and when it re-appears (Schulz et al., 2009). Thus, the polyclonal antibody was used to stain endogenous CP39 through all mitotic stages (Fig. 1E). In early prophase, where most but not all microtubules have been severed from the centrosome, the protein could still be observed at the centrosome, co-localizing with the brightest spot in the tubulin staining. Later, in prometaphase the signal disappeared and remained undetectable until early telophase. To follow localization of CP39 through mitosis in living cells, a strain expressing red fluorescent marsRFP-CP39 in addition to GFP-TubA was generated.

Movie S1 shows three mitotic cells, two of which (cell A and B) are captured from G2 till next S-phase including all stages of mitosis, the third is already in prophase at time 0 s (cell C) (Fig. 1F, supplementary Movie S1). In G2 the red signal of the fluorescently labeled CP39 is present at the centrosome. At the G2/M transition (140 s for cell A, 560 s for cell B) the green-labeled microtubule network has collapsed, while the red signal remained, indicating that CP39 stays at the centrosome during prophase. 60 s. later, when the cell is in prometaphase, marsRFP-CP39 is no longer detectable at the centrosome. The signal stays absent throughout metaphase and anaphase. A weak marsRFP-CP39 signal re-appears in early telophase (A: 920 s., B: 1080 s.; Fig. 1F arrowheads) and strengthens in intensity as the poles separate further. The behavior of both endogenous CP39 and marsRFP-CP39 during mitosis coincides with the known presence and absence of the central layer of the core structure. Thus, we conclude that CP39 is a component of this central layer.

2.3. CP39 is essential and overexpression causes supernumerary centrosomes

Since repeated efforts to knock out *cepA* via homologous recombination failed, we concluded that CP39 is essential. To circumvent this problem, the CP39 knockout construct, which mediates blasticidin resistance, was transformed into a newly generated G418-resistant GFP-CP39 strain. The resulting GFP-CP39ko strain carries a knockout of the endogenous protein and the expression level of GFP-CP39 can be regulated by the growth conditions. In axenic medium expression is high, since both the actin-6 promoter driving GFP-CP39 expression is active and G418 resistant strains usually contain multiple chromosomal tandem insertions of the transgenic protein expression vector (Barth et al., 1998). Yet, when grown on *Klebsiella aerogenes* (Ka) bacteria, the actin-6-promoter is repressed and protein expression is low (Liu et al., 2002).

When grown in HL5c culture medium high expression of the GFP fusion protein led to a distinct phenotypic abnormality: in shaking culture, 66% of all GFP-CP39ko cells contained supernu-