INTRODUCTION
Since last decade, a number of proteins have been identified as centrosome components (Andersen, 1999). Among them, $\gamma$-tubulin, a member of the tubulin superfamily (McKean et al., 2001), showing a remarkable amino acid identity among different species (Ovenchkina & Oakley, 2001; Shimamura et al., 2004) is quite extensively studied. It is a 55 kD protein, firstly localized in the spindle pole bodies of Aspergillus nidulans (Oakley & Oakley, 1989). Apart from the centrosomes, in a few studies, $\gamma$-tubulin has been localized along microtubules (MTs) in the mitotic spindle, as well as in the mid-body of animal cells (Julian et al., 1993; Lajoie-Mazenc et al., 1994). It is currently accepted that large amounts of $\gamma$-tubulin are diluted in the cytoplasm forming complexes with other proteins (Zheng et al., 1995; Jeng & Stearns, 1999; Wiese & Zheng, 1999; Schiebel, 2000; Moritz & Agard, 2001).

In angiosperm cells, $\gamma$-tubulin has been found using immunofluorescence in different regions like the nuclear envelope and the spindle poles, the prophase MT bundle, the phragmoplast, as well as along the MT bundles (Liu et al., 1993; Joshi & Palevitz, 1996; Endlé et al., 1997; Canaday et al., 2000; Panteris et al., 2000; Dibbayawan et al., 2001; Shimamura et al., 2004). It has been suggested that $\gamma$-tubulin is bound on the MT surface, or even incorporates into the lattice of MTs (Liu et al., 1993; Vaughn & Harper, 1998).

In the pteridophyte Adiantum cappilus-veneris and in the liverworts Lunularia cruciata and Marchantia paleacea, $\gamma$-tubulin has been found to extend along MT arrays during all cell division stages (Panteris et al., 2000). More recently, in the bryophyte Marchantia polymorpha it was reported that it is concentrated in the polar organizers during prophase and migrates in a cell cycle-specific manner, consistently present at all putative microtubule nucleation sites (Brown et al., 2004).

Regarding the role of $\gamma$-tubulin, it is generally accepted that it participates in MT nucleation, since it has been mainly found associated with centrosomes or microtubule organizing centers (MTOCs) (Moritz et al., 1995; Zheng et al., 1995; Moritz et al., 2000; Oakley, 2000). In a recent paper, Horio & Oakley (2003) proposed that in Arabidopsis $\gamma$-tubulin has functional MT nucleation domains and its primary
function is to nucleate MTs as is the case in animal and fungal counterparts. The different localization patterns, especially its presence along the MTs in higher plants, lead to the suggestion that it may play a different role, in relation to α- and β-tubulin (Panteris et al., 2000), such as MT stabilization (Joshi & Palevitz, 1996; Vaughn & Harper, 1998). Furthermore, it has been suggested that there may be different forms of γ-tubulin that are activated during the cell cycle (Dibbayawan et al., 2001).

Considering the above mentioned about the presence and the role(s) of γ-tubulin, brown algal cells provide an interesting model, since they are among the few algal systems bearing functionally active centrosomes in their vegetative cells during the whole cell cycle. These are the only MTOCs of this algal group, and no other MT-nucleating cytoplasmic sites have been identified. In the present study we examined the γ-tubulin localization during the vegetative cell cycle in Sphacelaria rigidula.

MATERIALS AND METHODS

Plant material and culture conditions

Male gametophytes of Sphacelaria rigidula Kützing were kindly provided by Ingo Maier (Faculty of Biology, University of Konstanz, Germany) from D. Müller’s algal culture collection. They were cultivated in Provasoli-enriched sea-water medium (Provasoli, 1968) at 16±0.2°C. The light-dark cycle was 12:12 h and the photon irradiance 30 µmol m⁻² s⁻¹ from fluorescent tubes (Sylvania, F36W/154, daylight).

γ-tubulin localization

The polyclonal anti-γ-tubulin antibody was kindly provided by Ralf Gräf (Zellbiologie, Adolph-Bute Nandt-Institut, Universität München, Germany). It has been raised in rabbits against γ-tubulin of Dictyostelium discoideum. It does not bind to α- or β-tubulin of D. discoideum (Euteneuer et al., 1998). Its specificity against γ-tubulin of higher plants has been shown by both immunofluorescence and immunoblot (Panteris et al., 2000). For the immunolocalization of γ-tubulin, the protocol described for α-tubulin immunofluorescence by Katsaros (1992) was used with a slight modification (sometimes incubation in the first antibody overnight at room temperature, and an additional extraction step, using cold methanol). The anti-γ-tubulin antibody was applied after or simultaneously with anti-a-tubulin for 90 min at 37°C. Hoechst 33258 (Sigma) was used for DNA staining.

Centrin localization

For the localization of centrin, an anti-centrin antibody (kindly provided by M. Melkonian – Botanisches Institut, Universität zu Köln, Germany) was applied together with or after anti-a-tubulin (see also Katsaros & Galatis, 1992).

RESULTS

General remarks

The distribution of γ-tubulin was examined in S. rigidula apical and subapical cells, since they are the main meristematic cells in which the cell cycle can be studied. In all cases, the signal was found always in the centrosome area, following centrosome movements. For uniformity reasons, only apical cells are shown. As centrosome area we consider the region where MTs diverge from the centrosome directing towards all the cell sites (Fig. 1c). This system changes during mitosis, when the spindle is organized (insets of Figs 2, 3, 4), while at cytokinesis two interdigitating MT systems formed from the centrosomes surround the daughter nuclei (see also Katsaros, 1992; Katsaros & Galatis, 1992).

To confirm the γ-tubulin reaction, control experiments were made without the first antibody, in which no signal was found.

Cell cycle

During interphase, γ-tubulin reaction appeared as one or two fluorescent spots close to the nuclear envelope (Figs 1a, 1b). When two γ-tubulin spots were observed, they were usually located at opposite sites of the nucleus, showing similar intensities (Fig. 1a). In some cases, the positive reaction was located in areas where the nuclear envelope shows a slight concavity. In all cell cycle stages, centrin reacting sites appear as fine dots, generally smaller than those of γ-tubulin (Fig. 1d).
During prophase, the γ-tubulin spots were always duplicated and located in opposite sites close to the nuclear envelope (Figs 2a, 2b). The intensity of the fluorescence appears slightly increased and the spots broadened compared with those at interphase. The sites of γ-tubulin always coincided with those of the MT nucleation centers, i.e. the centrosomes (Fig. 2 inset).

The fluorescence became slightly stronger during metaphase, while the diameter of the spots increased, forming a cap-like configuration (Figs 3a, 3b). This structure appears enclosing the centrosome area, where the fluorescence intensity appears brighter. By the progress of anaphase, the γ-tubulin reaction became more weak and its diameter shorter (Figs 4a, 4b). This process was continued during telophase, when the fluorescence image was similar to that of interphase. At late telophase and cytokinesis two γ-tubulin spots were observed at the polar areas of the daughter nuclei (Figs 5a, 5b).

**DISCUSSION**

From the existing literature data, two main patterns of γ-tubulin localization can be distinguished: the first occurs in cells bearing centrosomes or discrete MTOCs, like animal cells, in which γ-tubulin appears accumulated in a rather limited area (Job et al., 2003); in the second, γ-tubulin is localized along MTs and it has been described in different cell types, among which higher plant cells (Liu et al., 1993; Joshi & Palevitz, 1996; Endlé et al., 1997; Canaday et al.,

*FIGS 1a-1d. Localization of γ-tubulin in interphase cells of S. rigidula. 1a: anti-γ-tubulin immunofluorescence; 1b: Hoechst staining of DNA of the cell shown in 1a; 1c: MT organization in an interphase cell, after α-tubulin immunofluorescence; 1d: centrin localization after anti-centrin immunofluorescence.*

*FIGS 2-5. Localization of γ-tubulin in dividing cells of S. rigidula. 2: Prophase; 3: metaphase; 4: anaphase; 5: cytokinesis. 2a, 3a, 4a, 5a: anti-γ-tubulin immunofluorescence; 2b, 3b, 4b, 5b: Hoechst staining of DNA. The insets in these figures show the MT organization in the respective stages. Bars = 10 μm (magnification same in all figures).*
The role of γ-tubulin in MT nucleation is well established in different cell types bearing centrosomes (Moritz et al., 1995; Zheng et al., 1995). Discrete multi-protein ring-like complexes containing γ-tubulin have been found to exist in the centrosome site of animal cells and act as templates for MT nucleation (Moritz et al., 1995; Zheng et al., 1995; Schiebel, 2000; Moritz & Agard, 2001). Both γ-tubulin and ring complexes can be removed from centrosomes experimentally, with the coincident loss of MT nucleation potential (Schnackenberg et al., 1998). Large γ-tubulin containing complexes have also been identified in plants (Stoppin-Mellet et al., 2000; Drykova et al., 2003) as well as the plant Srp98p, a component of the small γ-tubulin containing complexes (Erhardt et al., 2002).

However, although γ-tubulin has been reported to be the kinetically dominant centrosomal MT nucleator (Hannak et al., 2002), recent studies suggest that MT nucleation may occur in the absence of γ-tubulin and that unknown mechanisms may support partial assembly of mitotic centrosomal asters (Strome et al., 2001). It seems that γ-tubulin may have other cell functions apart from being a major component of the γ-tubulin ring complex (reviewed by Job et al., 2003). Moreover, its observation in the cytoplasm as well as along MTs suggests that the role(s) of γ-tubulin may be far more complicated and diverse than initially supposed (Vaughn & Harper, 1998). Especially for plant cells, several hypotheses have been formulated about its function alternatively to MT nucleation (Joshi & Palevitz, 1996; Vaughn & Harper, 1998; Panteris et al., 2000).

In S. rigidula, the localization of γ-tubulin was limited in the centrosome area only. No signal was found in other cytoplasmic regions or along MTs. The successful localization of γ-tubulin in cells of different plant taxa, like liverworts, pteridophytes and angiosperms, by the same antibody in both immunofluorescence and immunoblot samples (Panteris et al., 2000), is a positive indication for its suitability for brown algal cells. The absence of any signal in the control specimens further supports the positive reaction.

As above mentioned, centrosomes (MTOCs) of brown algal cells are present and functionally active during the whole cell cycle. Therefore, γ-tubulin localization in S. rigidula is in accordance with similar models like those in animal cells and fungi (for reviews see Pereira & Schiebel, 1997; Oakley, 2000), supporting its MT nucleating role. The gradual increase in the fluorescence intensity, i.e. in the quantity of γ-tubulin observed by the entrance to mitosis, with maximum at metaphase, coincides with the increased number of MTs, assembled during this period (Kuriyama & Borisy, 1981). This is in accordance with electron microscope observations of Dicytota dichotoma dividing vegetative cells showing increased number of MTs assembled during mitosis in the pericentriolar area (Katsaros & Galatis, 1992).

Similar observations were reported for other algae, like the siphonous green alga Boergensenia forbesii, where γ-tubulin was localized in the centrosome area during prophase to anaphase, when MTs are organized from the centrosome, but not during telophase and interphase when MTs elongated irregularly around the nuclear envelope (Motomura et al., 2001). In Chlamydomonas, γ-tubulin was found in the basal bodies and in the flagellar transition region, consistent with a role for this protein in the nucleation of MTs of both the interphase cytoplasmic array and the mitotic spindle (Silflow et al., 1999).

It seems that a gradual accumulation of γ-tubulin takes place during the transition from interphase to metaphase, sometimes in large amounts (Vorobjev et al., 2000). The centrosomes “mature” by recruiting additional γ-tubulin ring complexes and several other proteins, resulting in an increase of the nucleation capacity of the centrosome (reviewed by Blagden & Glover, 2003). It is generally accepted that the appearance and concentration of γ-tubulin is related to the MT-nucleating capacity of the centrosomes and the MTOCs in general. The above stage-dependent activity of γ-tubulin has been confirmed in a study of centrosome dynamics in vivo, using GFP-γ-tubulin (Khodjakov & Rieder, 1999). It was shown that a rapid accumulation of γ-tubulin occurs during prophase that is again reduced by the entrance to interphase.

The differences in fluorescence intensity and dimensions of the reacting area between interphase and metaphase observed in the present study support the above mentioned hypothesis. The presence of γ-tubulin in the centrosomes and its increase during mitosis suggest a direct role in MT nucleation.

Taking into account the variety of the existing data on γ-tubulin organization in different organisms, it can be concluded that γ-tubulin is a centrosomal component and a potential MT nucleator in brown algae. The identification of multi-protein complexes
such as the γ-tubulin ring complexes in brown algae will be an interesting direction for future studies.

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