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Heat-shock protein 90 is associated with microtubules in tobacco cells

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Summary. The localization of HSP90 (heat-shock protein 90) was analyzed with respect to the microtubular cytoskeleton by double-immunofluorescence and confocal laser microscopy in tobacco VBI-O cells during axial cell division and elongation. HSP90 was observed to be colocalized with cortical and radial microtubules and the nuclear envelope in premitotic cells, with the preprophase band, and with the phragmoplast. The HSP90 epitope could not be detected in mature division spindles. The association of the HSP90 epitope with radial and cortical microtubules was not continuous in space. HSP90 was organized in discrete foci that were found to be aligned with microtubules, and the distance between these foci increased, when the cells entered the elongation phase. Elimination of microtubules by drugs resulted in a loss of cell axiality and alignment of the HSP90 epitope. Together with biochemical data demonstrating binding of tobacco HSP90 to tubulin dimers these data indicate a possible role of HSP90 in the organization of microtubules.

Keywords: Chaperones; Confocal laser microscopy; Double immunofluorescence; Heat-shock protein 90; Microtubules; Tobacco cell culture VBI-O.

Abbreviations: EPC ethyl-N-phenylcarbamate; FITC fluorescein isothiocyanate; HSP90 heat-shock protein 90; MAP microtubule-associated protein; TRITC tetramethylrhodamine B isothiocyanate.

Introduction

In plants, the control of cell shape seems to be intimately linked to microtubular structures that are exclusively found in plants (for a review, see Goddard et al. 1994): Axis and symmetry of cell division are correlated with the localization of the preprophase band, a band of cortical microtubules at the site, where the new cell wall will be deposited (for a review, see Lloyd 1991). The proportionality of cell expansion is correlated with the anisotropic arrangement of cellulose microfibrils in the innermost layers of the cell wall, favouring expansion perpendicular to the preferential direction of microfibrils (for a review, see Green 1980). Directional deposition of cellulose is connected to cortical microtubules that seem to drive and guide the movement of the cellulose-synthetising enzyme complexes residing in the plasma membrane, although it is not well understood how they fulfill this task (fo a review, see Giddings and Staehelin 1991, Williamson 1991).

The control of microtubule orientation is therefore crucial for plant morphogenesis. Cortical microtubules were originally thought to be relatively inert, stable structures, irrespective of their ability to change their orientation swiftly in response to a broad range of signals such as light (Laskowski 1990, Nick et al. 1990), gravity (Nick et al. 1990, Blancaflor and Hasenstein 1993), and plant hormones (for a review, see Shibaoka 1994). This reorientation was proposed to result from sliding of polymerized microtubules along each other, such that the pitch of their helical array around the cell would change between almost transverse and almost longitudinal (Lloyd and Seagull 1985). By microinjection of fluorescence-labelled brain tubulin into living plant cells it has become possible to follow microtubule reorientation in vivo and to estimate the turnover of tubulin dimers directly (Zhang et al. 1990, Yuan et al. 1994). These

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data demonstrate that cortical microtubules are as dynamic as microtubules from animal cells. This implies a pivotal role of direction-dependent depolymerisation and repolymerisation of tubulin dimers for the reorientation of microtubules.

There are several factors that influence the dynamic behaviour of microtubules directly or indirectly: Some of them seem to be intrinsic to the microtubules themselves such as composition of different tubulin isotypes that exhibit subtle differences in charge and molecular weight (for reviews, see Cleveland 1987, Sullivan 1988) or posttranslational modifications such as tyrosination or polyglutamylation (for reviews, see Sullivan 1988, Burns and Surridge 1994). Other factors are extrinsic to tubulin such as GTP, magnesium, and temperature (Mitchison and Kirschner 1984), chaperones involved not only in the correct folding of tubulin but also the organization of the microtubular cytoskeleton (for a review, see Liang and MacRae 1997) or specific microtubule-associated proteins (for a review, see Matus 1990).

In plants, several of these factors have been reported to change in response to signals although their impact on microtubule dynamics has not been determined: Modifications of a specific tubulin isotype have been observed after application of the plant hormone gibberellin (Duckett and Lloyd 1994, Mizuno 1994). Subunits of the tubulin-folding chaperone CCT have been found recently to be regulated by the plant photoreceptor phytochrome in maize coleoptiles and to be localized along cortical microtubules and at the nuclear envelope (Himmelspach et al. 1997). In the same organ two proteins have been isolated that change their expression concomitantly with the onset of cell elongation in response to phytochrome (Nick et al. 1995). One of these proteins was expressed prior to cell elongation and disappeared in response to continuous far-red light. After purification and peptide sequencing, this microtubule-binding protein could be identified as a member of the heat-shock protein 90 (HSP90) family (Nick et al. unpubl.).

HSP90 has been known from animal cells as a protein that binds to the glucosteroid receptor as long as it is not occupied by the ligand (Kimura et al. 1995). It interacts with both actin microfilaments (Koyasu et al. 1986) and microtubules (Czar et al. 1996) and has been shown in vitro to act as a molecular chaperone (Wiech et al. 1992). In plants, an interaction of tobacco HSP90 with tubulin dimers and microtubules could be demonstrated in vitro by tubulin-affinity assays, by co-affinity chromatography on Sepharose that had been coupled to the tubulin-binding drug ethyl-N-phenyl carbamate (EPC), and by co-immune precipitation experiments (Freudenreich and Nick 1998).

To get insight into the functional significance of the binding of HSP90 to tubulin dimers and microtubules, we investigated in the present work the localization of the HSP90 epitope in cycling cells of the tobacco culture VBI-O (Smertenko et al. 1997b). This culture has the advantage of axial cell division and elongation that can be triggered and partially synchronized by addition of auxin to the culture medium. The localization of HSP90 was followed through the culture cycle in relation to the various microtubular arrays in a combination of double-immunofluorescence with confocal microscopy. The pattern is discussed with respect to a potential role of HSP90 in the control of microtubular dynamics.

Material and methods

Cultivation of cells

The tobacco cell line VBI-O (*Nicotiana tabacum* L. cv. Virginia Bright Italia) derived in 1967 from stem pith tissue (Opatrný and Opatrná 1976) has been propagated from a stock callus culture on slightly modified Heller's (1953) medium, supplemented with 1-naphthylacetic acid (5 μM) and 2,4-dichlorphenoxyacetic acid (5 μM). Batch cell suspension cultures were initiated by suspending the stationary phase inoculum in a liquid medium of the same composition. This primary cell culture was subcultivated every three weeks in 100 ml Erlenmeyer flasks with an inoculation density of $5 \cdot 10^4$ cells/ml. The cell suspensions were maintained in darkness at 25 °C on a horizontal shaker at 150 rpm. The cells were analyzed at various times after inoculation. For the experiments involving cytoskeletal drugs, the medium was complemented at the time of subcultivation either with 1 mM of colchicine or 1 mM of ethyl-N-phenyl carbamate (EPC), drugs that block tubulin polymerization (Mizuno and Suzaki 1990), or with 20 μM of taxol, a drug that inhibits microtubule depolymerisation (Morejohn 1991, Parness and Horwitz 1981). Cells were counted in a Fuchs-Rosenthal haematocytometer after staining aliquots of the cell suspension with trypan blue to check cell viability.

Double immunofluorescence

Immunofluorescence labelling of cytoskeletal proteins was performed following a protocol described in detail in Toyomasu et al. (1994). Cells were allowed to settle on glass slides that had been coated with either 0.1% (v/v) poly-L-lysine solution (Sigma, Neu-Ulm, Federal Republic of Germany) or with Meyer's fixative [1% (w/v) sodium salicylate in 1 : 1 (v/v) egg white : glycerol]. Cells were fixed for 30 min in paraformaldehyde, 3.7% (w/v), dissolved from a stock solution into warm (25 °C) microtubule-stabilizing buffer (Toyomasu et al. 1994) and washed twice for 5 min in microtubule-stabilizing buffer. Prior to antibody incubation, the cells were

incubated with a mixture of 1% macerozyme (Yakuruto, Kyoto, Japan) and 0.1% pectolyase (Yakuruto) in microtubule-stabilizing buffer for 5 min at 30 °C. Then they were blocked with 5% (v/v) goat normal serum (Sigma) in tris-buffered saline [20 mM Tris-HCl, 150 mM NaCl, 0.25% (v/v) Triton X-100, pH 7.3] at 25 °C and incubated with the primary antibodies. In cases where no HSP90 signal could be observed (this is the case for the division spindle), the usual fixation step was replaced by a fixation in cold methanol (–20 °C) for 10 min, after which the cells were washed twice for 5 min in microtubule-stabilizing buffer and then postfixed in cold acetone (–20 °C) for 6 min. This strong fixation was used to ensure that HSP90 epitopes that may remain hidden during the usual fixation would become exposed.

In a parallel set of four slides, one slide was treated with a mixture of anti-α-tubulin, anti-β-tubulin from mouse and rabbit anti-HSP90. For the second slide, the rabbit anti-HSP90 was replaced by the respective rabbit normal serum (Sigma) to test the specificity of the HSP90 signal. For the third slide, the two murine anti-tubulins were replaced by the respective normal sera to test the HSP90 signal for optical artifacts such as filter leakage. In the fourth slide, all primary antibodies were replaced by the respective normal sera to test for unspecific binding of secondary antibodies. The slides were kept in a moist chamber at 37 °C for 1 h, then washed three times with tris-buffered saline, and then incubated with a mixture of anti-rabbit-IgG conjugated to fluorescein isothiocyanate (FITC; Sigma) and anti-mouse-IgG conjugated to tetramethylrhodamine B isothiocyanate (TRITC; Sigma). To check for optical contamination of the tubulin signal by filter leakage from the HSP90 signal, control experiments were performed, where the anti-rabbit-IgG was conjugated to TRITC, whereas the anti-mouse-IgG was conjugated to FITC. These controls did not reveal any significant differences in the obtained tubulin signal (data not shown).

The cells were visualized under a confocal laser microscope (DM RBE; Leica, Bensheim, Federal Republic of Germany), using a two-channel scan with an argon-krypton laser at 488 nm and 568 nm excitation, a beam splitter at 575 nm, and 580 nm and 590 nm emission filters. To eliminate filter leakage, in some experiments, the cells were viewed by subsequent one-channel scans with identical scanning intervals. For this constellation, the FITC signal was analyzed with excitation at 488 nm, a beam splitter at 510 nm, and a 515 nm emission filter, whereas for the TRITC signal, excitation at 568 nm was followed by a beam splitter at 575 nm and a 590 nm emission filter. Each experiment was performed at least five times.

Antibodies

Mouse monoclonal anti-α-tubulin and anti-β-tubulin (Amersham, Little Chalfont, U.K.) were used at a 1 : 1000 dilution for immunofluorescence. The rabbit polyclonal anti-heat-shock protein 90 antibody had been raised against a protein from *Catharanthus roseus* (Schröder et al. 1993). In Western blots with extracts from tobacco VBI-O cells this antibody recognized a protein of 90 kDa (Freudenreich and Nick 1998) and was used at 1 : 100 for immunofluorescence. Some of the experiments were repeated with a rabbit anti-heat-shock protein 90 antibody raised against the protein from mouse (Koyasu et al. 1986) with essentially the same results (data not shown). This antibody was used in the same concentration as that against HSP90 from *C. roseus*. Anti-rabbit-IgG and anti-mouse-IgG, both conjugated to TRITC, were diluted 1 : 50, as were the anti-rabbit-IgG and anti-mouse-IgG that were conjugated to FITC.

Results

Microtubular arrays change dramatically during the cultivation cycle

The culture cycle of the VBI-O tobacco cell culture (Opatrný and Opatrná 1976) cultured in liquid medium lasts typically for two to three weeks, depending on inoculation density and culture batch. The cycle consists of two distinct phases shown in Fig. 1 A: following subcultivation, axial cell divisions produce pluricellular cell files. These files usually exhibit a clear polarity (Fig. 1 A, E) with a pointed apical pole and a rounded or sometimes flattened basal pole; during the second part of the culture cycle these pluricellular files grow by cell expansion in the long axis of the file and eventually disintegrate (Fig. 1 A, F). The complete disintegration into individual cells (Fig. 1 C) marks the end of the culture cycle. A new cycle can then be initiated by subcultivation into fresh media and addition of auxin. For the cultivation conditions used in our experiments cell divisions were most frequent around day seven after subcultivation (Fig. 1 B), resulting in long cell files that are comprised of up to ten individual cells per file (Fig. 1 B′). Between day 10 and day 20 after subcultivation the number of cells per file decreased again, due to disintegration of the files (Fig. 1 B, F).

The axial and even polar cell responses of this culture allow for studies of cell division and cell elongation with relative ease and we used this system to analyze the behaviour of HSP90 epitopes with respect to cellular development. Already by Nomarski interferential contrast microscopy (Fig. 1 C–F), massive intracellular changes can be revealed: whereas the nucleus is located near to the cell periphery in the disintegrated individual cells dominating at the time of subcultivation (Fig. 1 B, C), it moves towards the cell center at the onset of division phase (Fig. 1 D). This is accompanied by the appearance of conspicuous cytoplasmic strands that originate from the nucleus and reach the cell periphery (Fig. 1 D–F). Most of these strands are either parallel or perpendicular to the file axis producing a more or less cruciform array (Fig. 1 E, F). The strands of neighbouring cells within a file tend to meet the separating cell wall at the same site (Fig. 1 D). This coordination is lost during the disintegration of the files (Fig. 1 F).

The dynamic response of VBI-O cells becomes even more manifest, when microtubules are visualized by immunofluorescence (Fig. 2). Parallel bundles of transverse microtubules characterize the elongated

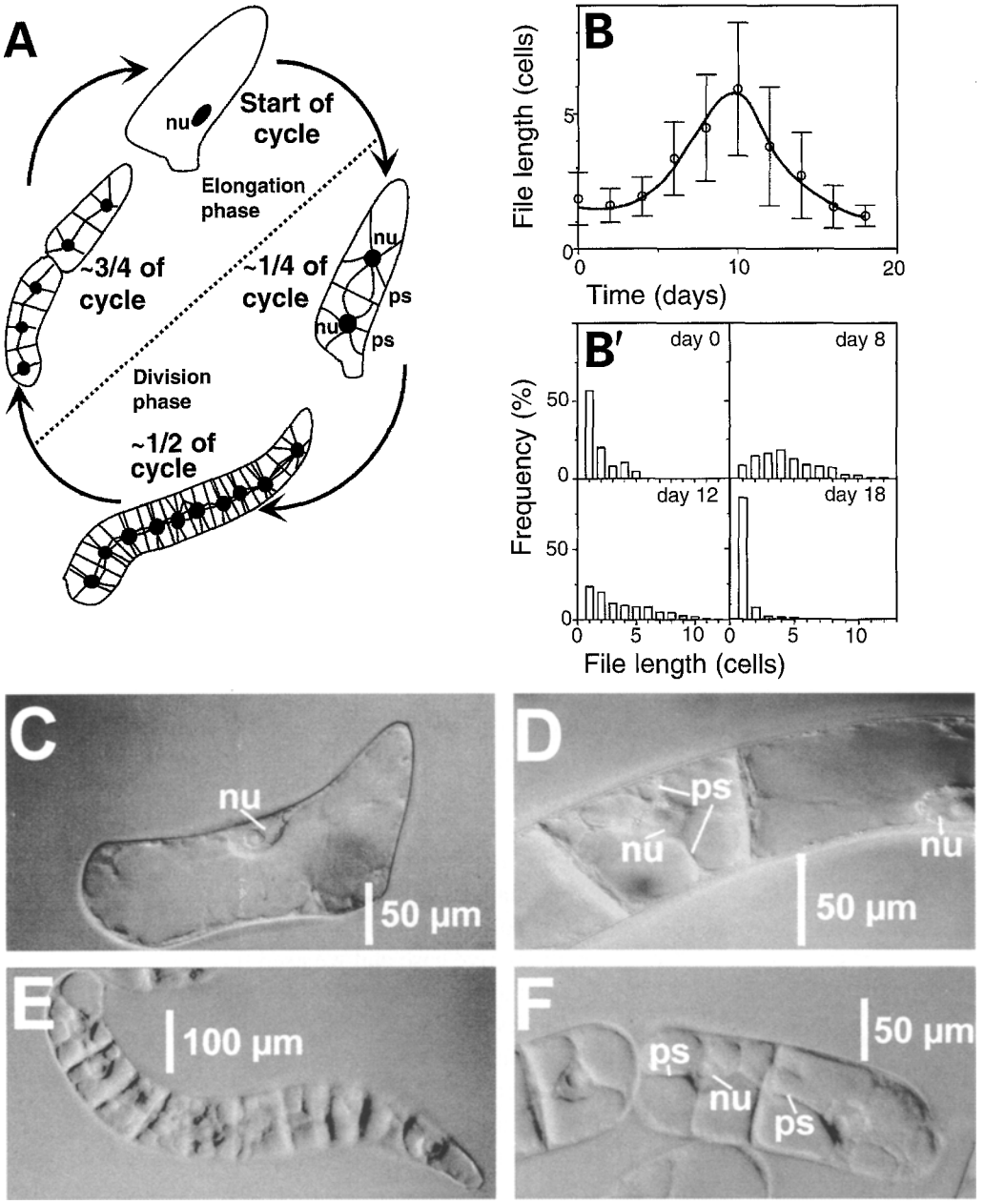

Fig. 1 A–F. Cultivation cycle of the VBI-O tobacco cell culture. **A** Schematic representation of the culture cycle; one cycle takes about 2–3 weeks. *nu* Nucleus, *ps* plasma strands connecting the nucleus and the cell wall. Time course of cell division: **B** average number of cells per file, **B′** frequency distributions over cell number per file for different stages of the culture. The maximal number of cells per file is reached at day 10 after subcultivation, cell division is most active between days 6 and 8 after subcultivation. After day 10 cell division is replaced by cell elongation and disintegration of files decreasing the number of cells per file. **C–F** Nomarski interference-contrast images of cells in different culture stages. **C** Elongated cell at the time of subcultivation, **D** cell at the beginning of division phase, the nucleus has migrated into the cell center and is attached to plasma strands, **E** pluricellular file at the end of the division phase, **F** disintegrating file in the middle of the elongation phase

cells characteristic for the stationary phase of the culture (Fig. 2 A). Cell divisions are heralded by a migration of the nucleus into the cell center and the appearance of radial microtubules that emanate from the nuclear envelope and tether the nucleus to the cell periphery (Fig. 2 B). In the next step, cortical and radial microtubules are rapidly replaced by a dense equatorial band of transverse microtubules, the preprophase band (Fig. 2 C). Then, the preprophase band disappears and the division spindle (Fig. 2 D) is formed almost simultaneously. Formation of preprophase band and division spindle seems to be an

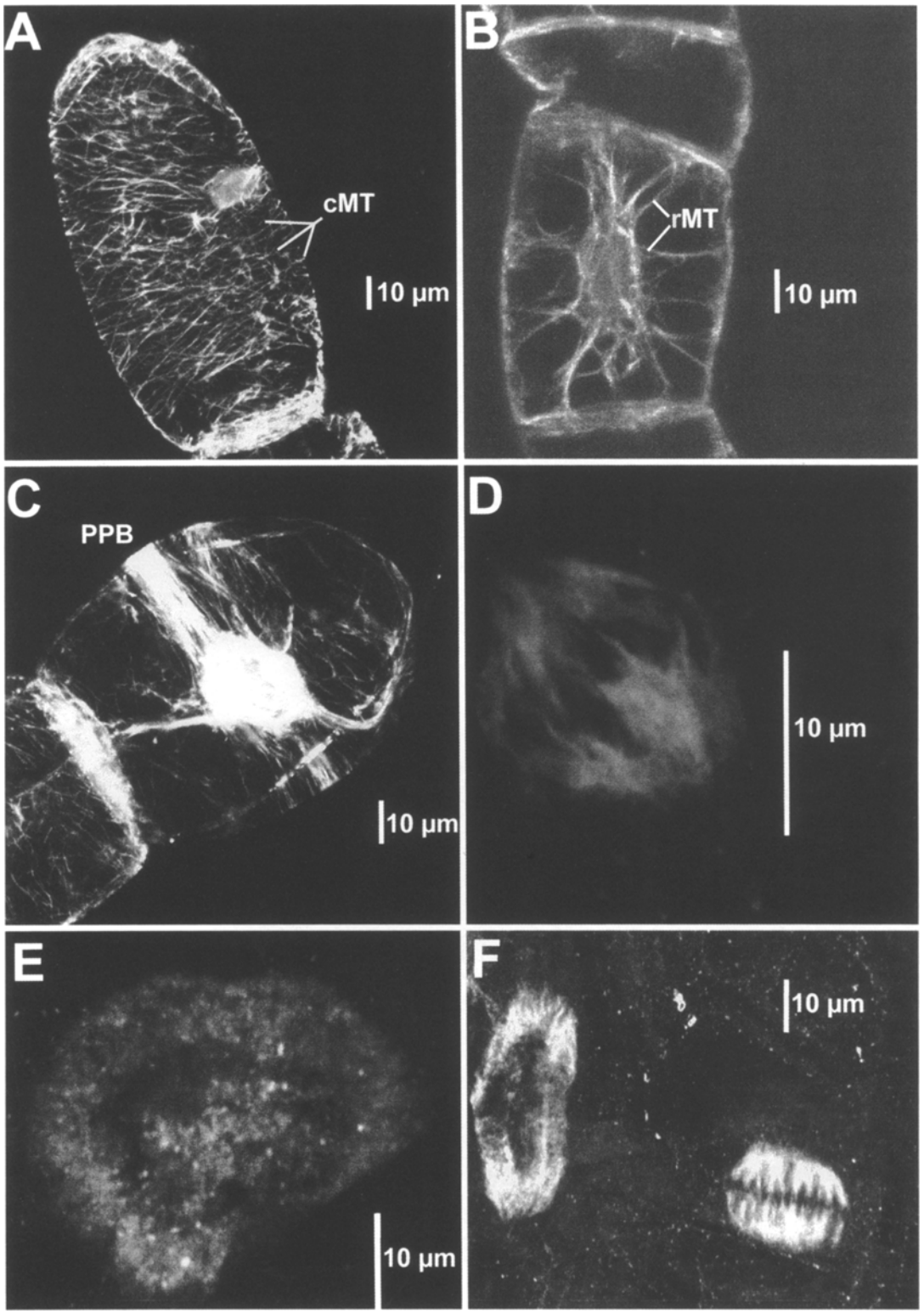

Fig. 2 A–F. Behaviour of the microtubular cytoskeleton during the cultivation cycle of VBI-O cells. **A** Elongated cell with transverse bundles of cortical microtubules (*cMT*). Note the lateral position of the nucleus. **B** Premitotic cell with radial microtubules (*rMT*) tethering the nucleus to the cell center. **C** Preprophase band (*PPB*). **D** Mature division spindle. **E** Phragmoplast. **F** Two phragmoplasts belonging to two different cell files, the left phragmoplast is oriented such that the ring-like structure becomes visible, the right phragmoplast is seen exactly from the side and reveals the interdigitating subunits

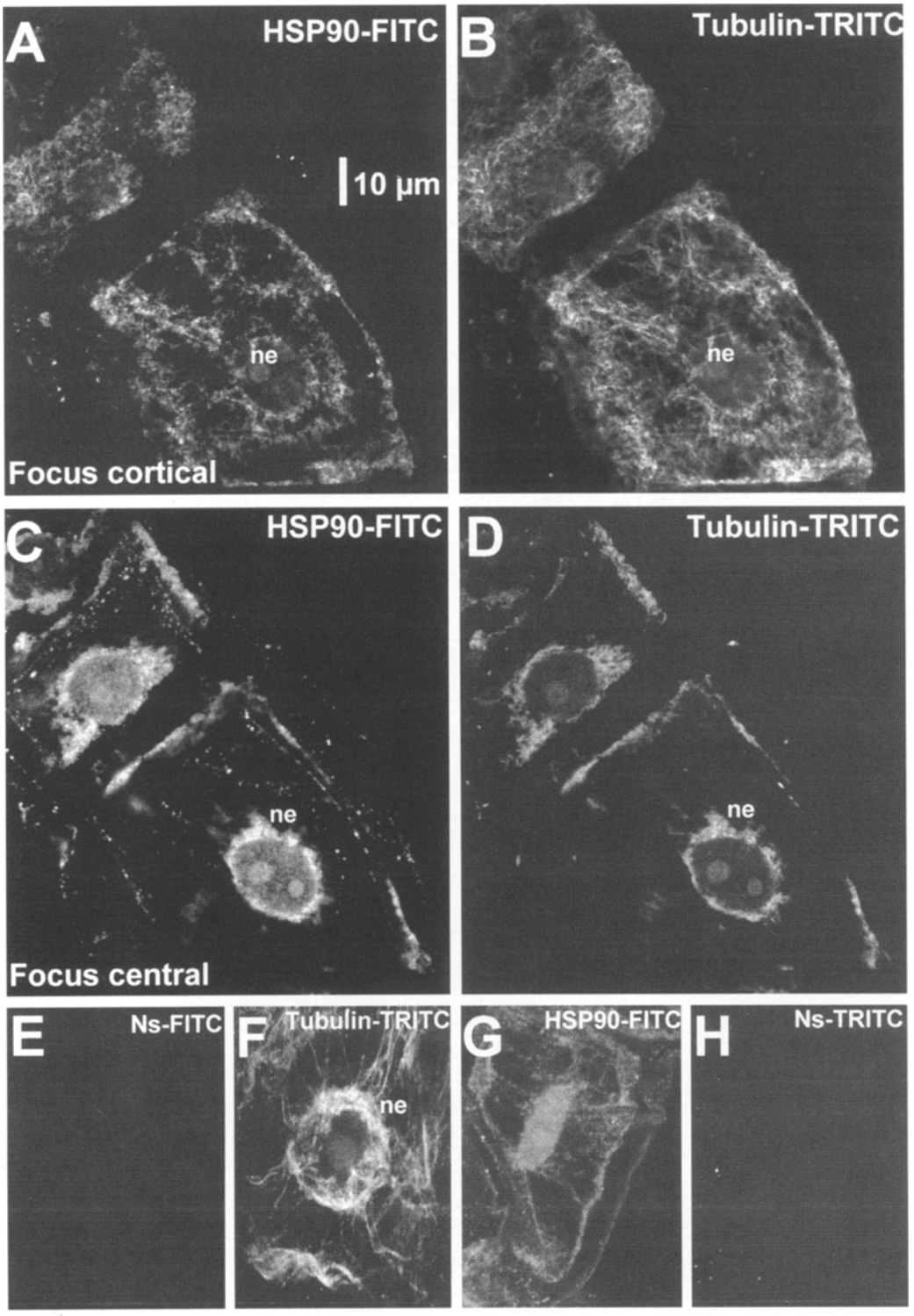

Fig. 3 A–H. Localization of HSP90 and tubulin in premitotic VBI-O cells. **A** and **B** Simultaneous staining with anti-HSP90 (**A**) and anti-tubulin (**B**) antibodies in a confocal section of the cortical region; *ne* nuclear envelope. Note the punctate staining of microtubules. **C** and **D** Confocal section of the same cell as in **A** and **B**, but 5 μm deeper in the cell stained with anti-HSP90 (**C**) and with anti-tubulin (**D**). **E** and **F** Negative control of a premitotic cell, where the anti-HSP90 antibody was replaced by a rabbit normal serum (**E**) to check for optical contamination of the HSP90 signal by the tubulin signal (**F**) and to check for unspecific binding of the FITC-conjugated secondary antibody. **G** and **H** Negative control of a premitotic cell, where the anti-tubulin antibody was replaced by a mouse normal serum (**H**) to check for unspecific binding of the TRITC-conjugated secondary antibody

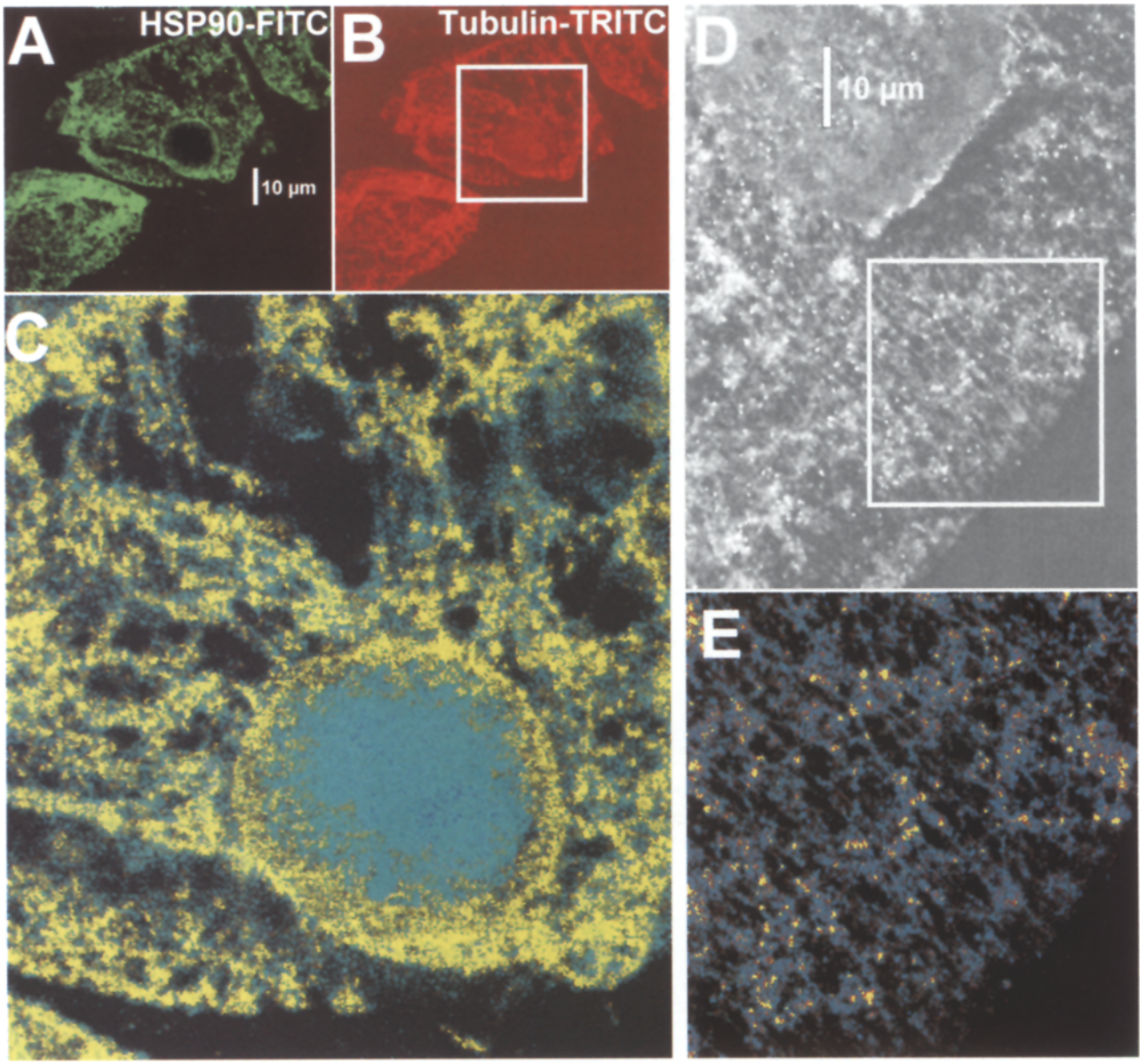

Fig. 4 A–E. Alignment of the HSP90 signal with radial and cortical microtubules and with the nuclear envelope. **A** and **B** Confocal section of a premitotic cell stained simultaneously with anti-HSP90 (**A**) and anti-tubulin (**B**) antibodies; **C** overlay of the HSP90 signal (yellow) and the tubulin signal (blue) for the cell's central region boxed in **B**. Note the alignment of the punctate HSP90 signal with radial microtubules and the nuclear envelope. **D** Confocal section of an elongating cell stained simultaneously with anti-HSP90 antibodies and anti-tubulin antibodies, the images of both channels are overlaid; **E** overlay of the HSP90 signal (yellow) and the tubulin signal (blue) for the region boxed in **D**. Note the increased spacing of HSP90 epitopes along cortical microtubules

extremely rapid process, because this transition could be observed only at a low frequency. The spindle is subsequently replaced by the phragmoplast (Fig. 2 E, F), a double ring of microtubules that is formed around the edge of the new cell plate. The phragmoplast increases in diameter during the centrifugal growth of the cell plate and eventually disintegrates. New microtubules fan out of the outer surface of the phragmoplast. In some cases, phragmoplasts are seen from the side and reveal a subdivision into two interdigitating rings of microtubules. If seen from the side, phragmoplasts superficially resemble division spindles, but they can be distinguished by their flat poles and confocal sectioning reveals a much larger diameter. Along with the disintegration of the phragmoplast, the cortical microtubules gradually reappear (Fig. 2 A).

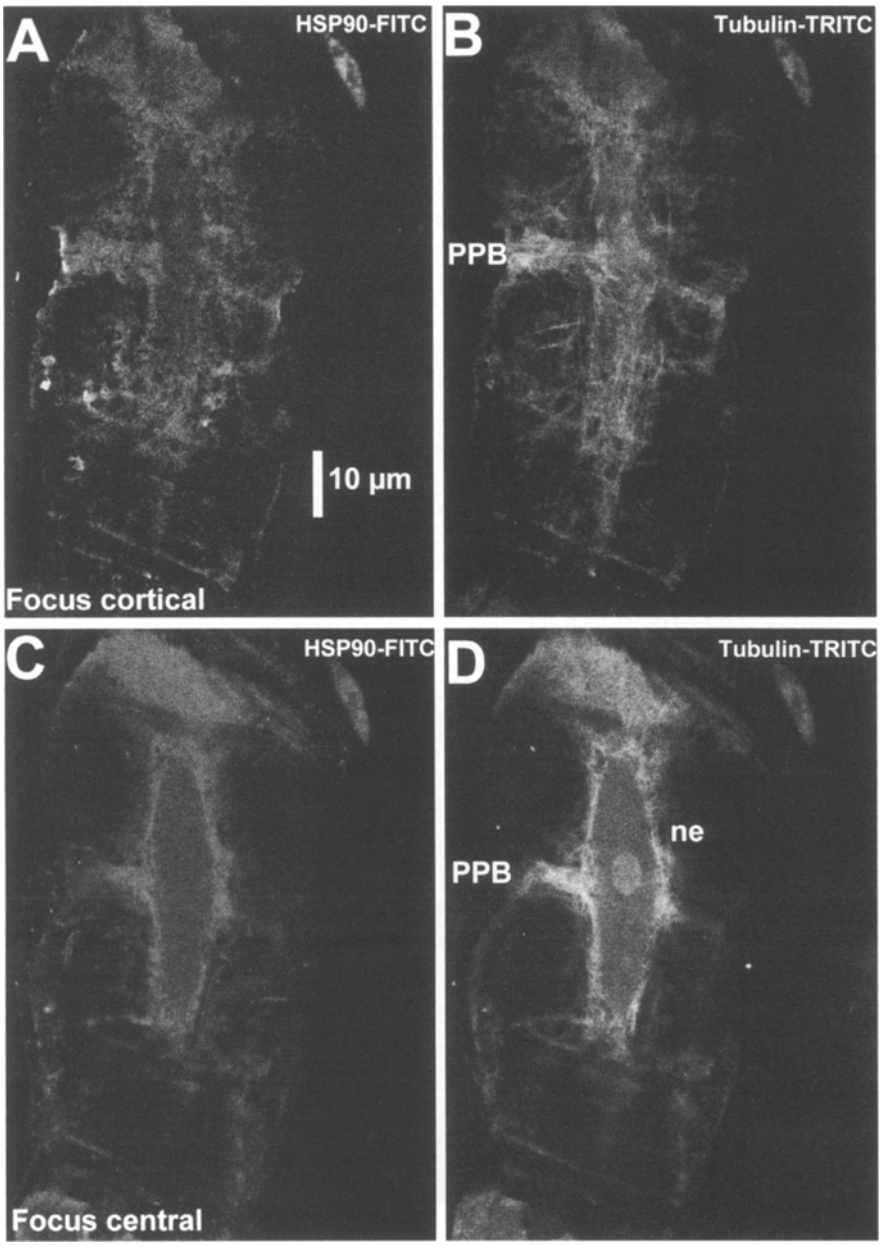

Fig. 5 A–D. Localization of HSP90 and tubulin at the onset of mitosis. **A** and **B** Confocal section of the cortical region, labeled simultaneously with anti-HSP90 (**A**) and anti-tubulin (**B**) antibodies. Note the compact concentration of the HSP90 signal in the preprophase band (*PPB*) and the punctate alignment with the longitudinal microtubules accompanying the nuclear envelope, probably representing the first steps of spindle formation. **C** and **D** Confocal section of the same cell, but 5 µm deeper to show the association of HSP90 (**C**) with the nuclear envelope (*ne*)

HSP90 is associated in discrete foci along cortical and radial microtubules in premitotic cells

The localization of HSP90 was followed through the culture cycle with respect to the microtubular cytoskeleton. The cells were double stained with monoclonal antibodies to α- and β-tubulin (visualized by secondary antibodies raised against mouse-IgG that were conjugated to TRITC), and a rabbit antibody directed against HSP90 from *Catharanthus roseus* (Schröder et al. 1993) that was visualized by secondary anti-rabbit IgG antibodies conjugated to FITC. The barrier filters were selected such that cross-contamination between the scanning channels was minimized. To exclude cross-contamination of the HSP90 signal by the tubulin signal, the secondary antibody for the detection of tubulin was chosen to be conjugated to TRITC. Negative controls with anti-HSP90 replaced by the respective normal sera checked for cross-contamination of the HSP90 signal due to the tubulin signal, and negative controls with all primary antibodies replaced by the respective normal sera tested the possibility of unspecific binding of secondary antibodies.

Premitotic cells are characterized by radial microtubules that emanate from the nuclear envelope and

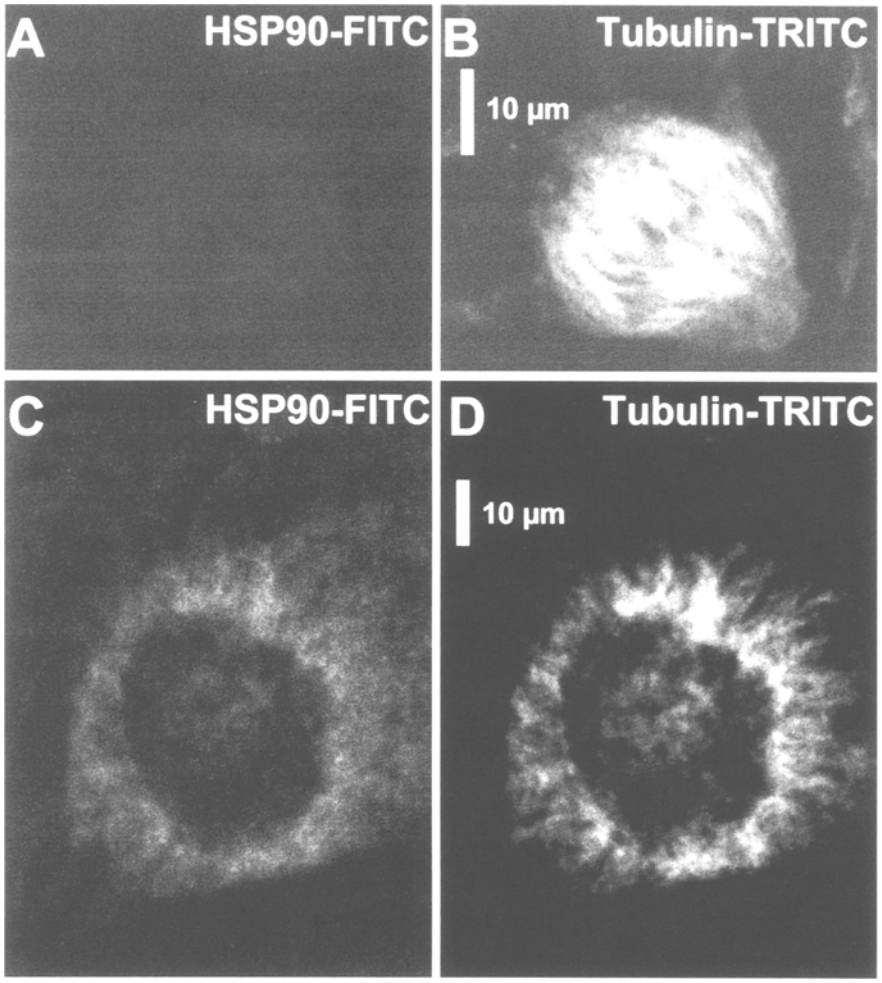

Fig. 6 A–D. Localization of tubulin and HSP90 during cell division. Simultaneous staining with anti-HSP90 and anti-tubulin antibodies of spindle (**A** and **B**) and phragmoplast (**C** and **D**). Note the absence of HSP90 epitopes from the division spindle in **B** and the decoration of phragmoplast and emerging microtubules with HSP90 in **D**

merge with cortical microtubules (Figs. 2 B and 3 B, D). They seem to tether the nucleus in a central position. The HSP90 epitope decorates these radial microtubules in form of discrete foci (Fig. 3 A, B) and is concentrated along the nuclear envelope, where radial microtubules emanate (Fig. 3 C, D). This HSP90 signal is not observed in negative controls, where the anti-HSP90 antibodies had been replaced by the respective normal serum (Fig. 3 E, F), but it is present in cells that were labelled with anti-HSP90 antibodies alone, i.e., when the anti-tubulin antisera were replaced by a mouse normal serum (Fig. 3 G, H).

The spacing between the HSP90 foci along a given microtubule was sometimes rather wide (Fig. 4), especially in the elongated cells predominant at the end of the culture cycle (Fig. 4 D, E). This made it difficult to recognize a potential association of the HSP90 signal with microtubules. Therefore, the images obtained for the HSP90-FITC signal and the tubulin-TRITC signal were combined. This overlay reveals the alignment of HSP90 foci along radial microtubules and the nuclear envelope in premitotic cells (Fig. 4 C) and the increased spacing of HSP90 foci with progressive elongation of the cell (Fig. 4 E).

HSP90 dissociates and reassociates with microtubules during cell division

Between days 6 and 12 the microtubular arrays related to cell division could be encountered frequently (Fig. 2 C–F). The HSP90 epitope was found to decorate the preprophase band in a continuous pattern (Fig. 5), but it was also concentrated in the nuclear envelope just prior to its disappearance (Fig. 5 C, D). At this time new microtubules could be observed that were arranged in a direction perpendicular to the preprophase band and probably represent the first stages of spindle formation (Fig. 5 B). These putative spindle microtubules were also labelled by the anti-HSP90 antibody, however, not continuously (Fig.

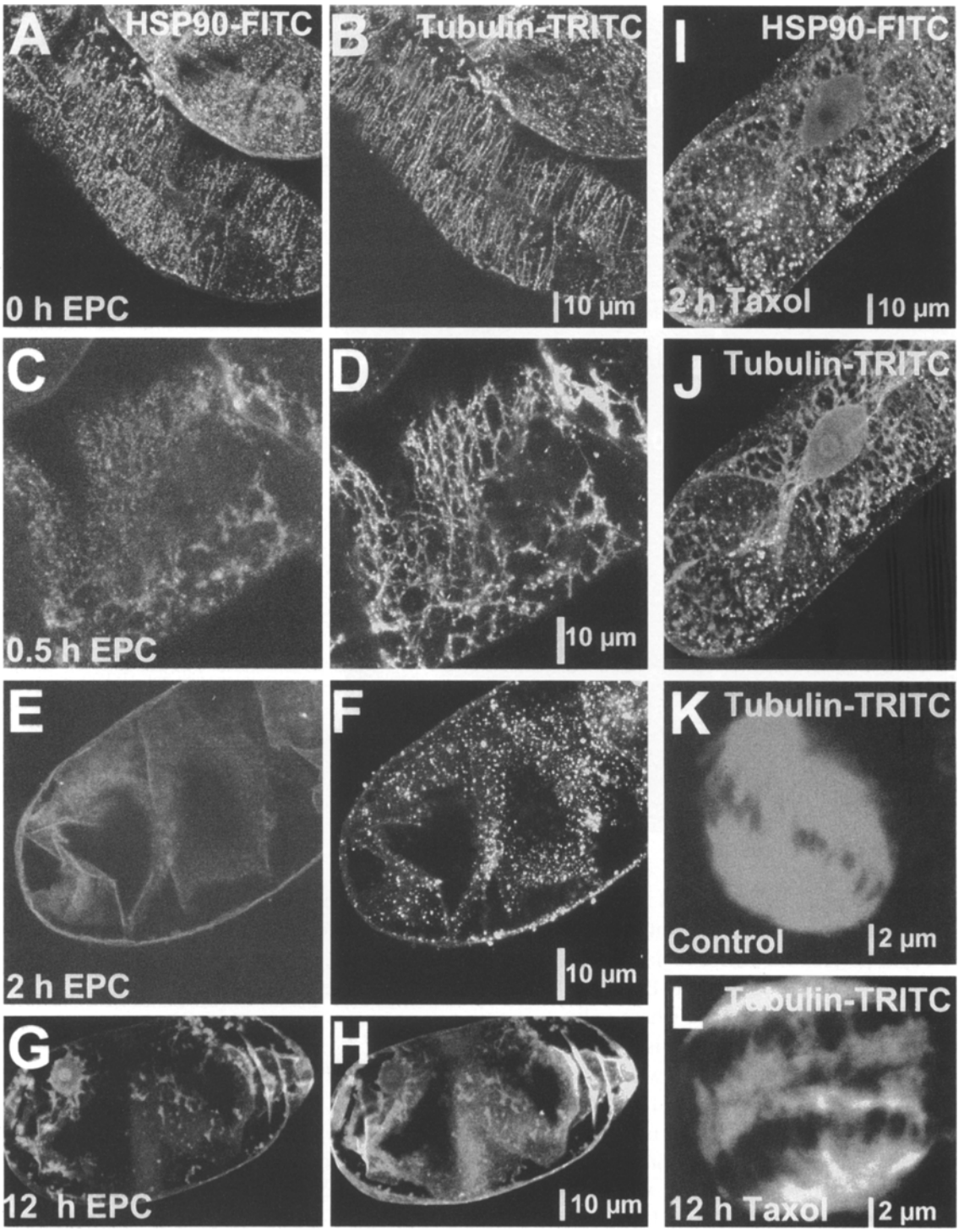

Fig. 7. A–H Time course of the response of microtubules (B, D, F, and H) and HSP90 (A, C, E, and G) to EPC, a blocker of microtubule polymerisation. Cells were stained simultaneously with anti-HSP90 and with anti-tubulin antibodies. **A** and **B** Cell prior to drug treatment with cortical microtubules that are decorated with HSP90; **C** and **D** partial disruption of microtubules mirrored in the distribution of the HSP90 signal 0.5 h after addition of 1 mM EPC to the medium; **E** and **F** complete disruption of microtubules and diffuse distribution of the HSP90 signal 2 h after addition of EPC; **G** and **H** ovoid cell shape and association of HSP90 with the nucleus 12 h after addition of EPC. **I–L** Response of microtubules and HSP90 to taxol, a blocker of microtubule depolymerisation. **I** and **J** Formation of fused cortical microtubules and simultaneous appearance of radial microtubules 2 h after addition of 20 µM of taxol. **L** Formation of abnormal spindles observed 12 h after addition of taxol. **K** Spindle of an untreated control cell

5 A, B). Interestingly, the HSP90 epitope could not be detected in mature division spindles (Fig. 6 A, B). It might be that the HSP90 protein assumes a different conformation covering the epitopes recognized by the antibody. To expose potentially hidden epitopes the fixation procedure was therefore replaced by fixation with methanol and acetone. Nevertheless, no signal of mature spindles could be observed after staining with anti-HSP90 antibodies (data not shown).

The phragmoplasts appeared as ring-like structures with new microtubules emanating from their outer edge (Fig. 2 E, F). Phragmoplast were labeled by the anti-HSP90 antibody (Fig. 6 C, D). The newly formed microtubules that fanned out from the outer edge of the phragmoplast, where decorated as well, however, again in a punctate pattern (Fig. 6 C). In negative controls, where the anti-HSP90 antibody was replaced by a rabbit normal serum only a weak background staining could be observed in the FITC channel (data not shown). Ring-shaped structures with radiating filaments could also be observed in controls, where the anti-tubulin antibodies had been replaced by a mouse normal serum (data not shown), and these structures probably represent phragmoplasts that had been decorated by the anti-HSP90 antibody.

Microtubular drugs disturb axial cell expansion and division

To assess the relation between microtubules and cell morphology, the cells were treated with the microtubule-eliminating drugs colchicine and EPC at the time of subcultivation. Both drugs eliminated microtubules within a few hours and the punctate arrays of HSP90 epitopes disappeared in parallel (Fig. 7 A–F). With increasing time, most cells lost their axiality and assumed an ovoid shape (Fig. 7 G, H). The nucleus did not migrate to the cell center as it was characteristic for the controls (Fig. 7 G, H), and the HSP90 epitope was detected mainly in the nucleus (Fig. 7 H). A similar behaviour was observed in response to colchicine (data not shown).

To assess the role of microtubule dynamics for cell morphology, the cells were treated with 20 μM taxol, a drug that blocks depolymerisation of tubulin (Morejohn 1991, Parness and Horwitz 1981). This treatment increased the number of cortical microtubules resulting in fused microtubules that were clearly decorated by the HSP90 epitope (Fig. 7 I, J). The nucleus was usually located centrally comparable to control cells and tethered by radial microtubules as in control cells (Fig. 7 I, J). The taxol-treated cells were very long, reaching almost vermiform proportions, and pluricellular files were found to be very rare (data not shown). In the later phases of the cultivation cycle, aberrant division spindles could be observed: The spindle poles seemed to have moved apart and the spindle equator was marked by a double ring of tubulin (Fig. 7 L). However, it was not possible to detect phragmoplasts.

Discussion

The HSP90 epitope is colocalized with premitotic microtubular arrays, but to varying extent

Anti-HSP90 antibodies stain premitotic radial microtubules and the nuclear envelope (Figs. 3 and 4) as well as the preprophase band (Fig. 5). The degree of association seems to be different, though: the nuclear envelope and the preprophase band are continuously labeled by the anti-HSP90 antibody (Figs. 3 C, D and 5); in contrast, HSP90 is aligned in the form of discrete foci along the radial microtubules that emanate from the nuclear envelope and seem to tether the nucleus in a central position (Fig. 2 B). This indicates that the association of HSP90 with cortical microtubules might be regulated during the culture cycle.

In addition to microtubules, anti-HSP90 antibodies labelled the nuclear envelope (Figs. 3 C and 5 C), the site where the radial microtubules characteristic for this stage emerge (Figs. 2 B and 3 D). The nuclear envelope seems to play a key role in the nucleation of new microtubules in cycling cells (Stoppin et al. 1994, Lambert 1993), and this may be related to the concentration of HSP90 at the sites where radial microtubules emerge (Figs. 3 C and 5 C).

Spindle microtubules are basically different from other microtubular arrays

There was only one microtubule array that was found to be void of HSP90 epitopes: the mature division spindle. Whereas early stages of spindle formation were characterized by a punctate decoration with HSP90 epitopes (Fig. 5 A, B), the mature spindle did not reveal any traces of HSP90 (Fig. 6 A, B). This failure to stain the spindle with anti-HSP90 was not removed by a strong fixation with acetone and methanol (data not shown). This fixation protocol should denature the protein completely and expose domains that otherwise might be hidden inside the molecule potentially interfering with the binding of the anti-HSP90 antibodies. Interestingly, HSP90

could be detected again in the phragmoplast and along the new microtubules emerging from the phragmoplast (Fig. 6 C, D). This indicates that the microtubules that establish the mature spindle are basically different from cortical microtubules and their derivatives. Recently, in tobacco cells, the spindle has been shown to be labelled specifically by antibodies that detect a specific posttranslational modification of tubulin (Smertenko et al. 1997a).

Dynamic microtubules are essential for axial cell growth and cell division

The elimination of microtubules by colchicine or EPC (Fig. 7 A–H) causes a loss of axial elongation resulting in ovoid or even spherical cells. This is consistent with earlier findings in a number of plants and can be interpreted in terms of the so called microtubule-microfibril syndrome (for reviews, see Giddings and Staehelin 1991, Williamson 1991): cortical microtubules guide the deposition of cellulose microfibrils that are laid down in parallel; transverse microtubules will thus cause transverse deposition of cellulose resulting in a lower extensibility of the cell wall in transverse direction. This anisotropy of the cell wall causes a reinforcement of cell elongation. Elimination of cortical microtubules by colchicine or EPC is therefore expected to culminate in a gradual loss of cell-wall anisotropy and thus a gradual loss of cell axiality (Fig. 7 G, H). In addition, the migration of the nucleus to the prospective site of cell-plate formation (Fig. 2 A, B) is disturbed by those drugs (Fig. 7 G, H) confirming previous results (Katsuta and Shibaoka 1988) which demonstrated a role for microtubules in this movement. Interestingly, the HSP90 epitope was found in the nucleus following treatment with EPC (Fig. 7 G).

It is possible to maintain microtubules, but to block their dynamics by addition of taxol, a drug that inhibits the depolymerization of microtubules (Parness and Horwitz 1981). Treatment with taxol produces vermiformous cells that do not divide (data not shown). This suggests that axial cell elongation can proceed independently of microtubule dynamics as long as microtubules are arranged in the right orientation. In contrast, the entry into the cell cycle seems to be inhibited by taxol, indicating that the formation of preprophase band and spindle requires the disassembly of cortical and radial microtubules consistent with results that have been obtained in wheat roots (Panteris et al. 1995). At the end of the culture cycle, when the concentration of the drug has supposedly decreased (taxol is gradually degraded at room temperature), eventually spindle formation resumes (Fig. 7 L), but cell division becomes arrested prior to phragmoplast formation consistent with observations from other groups (Yasuhara et al. 1993). The differential response of the microtubule arrays participating in cell division suggests differences in the dynamics of assembly and disassembly: radial microtubules seem to be relatively resistant to taxol (Fig. 7 I, J), whereas the transition to preprophase band and spindle formation is quite sensitive and can occur only at the end of the culture cycle, when the drug is supposedly already degraded. Even then, the transition from spindle to phragmoplast remains inhibited leading to abnormally shaped spindles (Fig. 7 L). Thus, the dynamics of disassembly and reassembly seems to be crucial for the transition between the premitotic radial microtubules towards the preprophase band and spindle arrays and, even more pronounced, for the transition between spindle and phragmoplast. In contrast, the formation of radial microtubules seems to be more independent of microtubule disassembly. The HSP90 epitope was observed along cortical and radial microtubules and in the nuclear envelope, similar to the situation in the controls (Fig. 7 I, J).

HSP90 might be involved in the nucleation of new microtubules

The HSP90 epitope has been found in those sites, that are discussed with respect to microtubule nucleation: the nuclear envelope seems to be the major microtubule-organizing center in dividing cells (Lambert 1993, Stoppin et al. 1994), the preprophase band marks the site where after completed mitosis new microtubules are nucleated (Lloyd 1991), and new microtubules are organized along the edge of the growing phragmoplast (Vantard et al. 1990). It is thus possible that HSP90 is a marker for the nucleation of new microtubules.

HSP90 has been found to bind to tubulin dimers in vitro as would be expected for a protein participating in microtubule nucleation (Freudenreich and Nick 1998). Interestingly, the capacity of HSP90 to bind to exogenous brain microtubules seems to be essentially the same, irrespective of whether the cellular extract originated from cells in division phase or from cells in elongation phase. This might indicate that the observed differences in microtubular decoration with the HSP90 epitope are not intrinsic to the HSP90 mol-

ecule (Freudenreich and Nick 1998). This interaction might therefore be regulated by developmental changes in the expression of HSP90. Alternatively, microtubules themselves might be altered in such a way that they interact to a variable extent with the HSP90 epitope. The molecular base of this difference is still unknown – one might think of different tubulin isotypes or of differences in posttranslational modifications of tubulin (for a review, see Sullivan 1988). Alternatively, the binding sites for HSP90 might be masked by microtubule-associated proteins.

Outlook

The function of HSP90 in plants is not really understood and seems to be complex in animals as well as in plants. Recently, HSP90 has been shown to be a molecular chaperone (Wiech et al. 1992) and to be associated in situ with the microtubular cytoskeleton (Czar et al. 1996). CCT, another chaperone responsible for the correct folding of tubulins and actin, has been identified as a part of the centrosomal microtubule-nucleating complex (Brown et al. 1996). Higher plants do not possess centrosomes, but they do possess functional equivalents, the microtubule-organizing centers (for a review, see Lambert 1993). The nuclear envelope seems to play a pivotal role in this respect (Stoppin et al. 1994). The observation that this major potential microtubule-organizing center is decorated with both HSP90 (Figs. 3 C and 5 C) and with CCT (Himmelspach et al. 1997) suggests a role for molecular chaperones in the nucleation of new microtubules in higher-plant cells. This would open the possibility that the components of the cytoskeleton are translated and folded close to the sites where they are subsequently assembled into the respective polymers.

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