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Hsp90 binds microtubules and is involved in the reorganization of the microtubular network in angiosperms

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ABSTRACT

Microtubules (MTs) are essential for many processes in plant cells. MT-associated proteins (MAPs) influence MT polymerization dynamics and enable them to perform their functions. The molecular chaperone Hsp90 has been shown to associate with MTs in animal and plant cells. However, the role of Hsp90–MT binding in plants has not yet been investigated. Here, we show that Hsp90 associates with cortical MTs in tobacco cells and decorates MTs in the phragmoplast. Further, we show that tobacco Hsp90_MT binds directly to polymerized MTs *in vitro*. The inhibition of Hsp90 by geldanamycin (GDA) severely impairs MT re-assembly after cold-induced de-polymerization. Our results indicate that the plant Hsp90 interaction with MTs plays a key role in cellular events, where MT re-organization is needed.

Introduction

Microtubules (MTs) are highly dynamic polymers that are essential for cell growth, morphogenesis, development and signaling of plant cells (for a review, see Lloyd and Chan, 2004). During the plant cell cycle, MTs form characteristic arrays that are organized by dynamic disassembly and re-assembly of MTs in combination with movements of polymers. The microtubular dynamics are regulated by several specific microtubule-associated proteins (MAPs, for a recent review on plant MAPs see Hamada, 2007). In addition to dynamics, severing, and sliding of microtubular polymers, specific MAPs mediate the interaction of MTs with cellular compartments or organelles. The interaction of interphasic cortical MTs with the plasma membrane, for example, is mediated by phospholipase D (Gardiner et al., 2001), and the endoplasmic reticulum is co-aligned with MTs by formin FH4, suggesting a further role of FH4 at the interface of the actin and microtubular cytoskeleton (Deeks et al., 2010). In addition to a group of MT-binding proteins highly conserved in all eukaryotic cells, some binding proteins differ considerably between plant and non-plant cells, correlating with specific functions of plant MTs. Interestingly, the affinity of binding proteins is not very pronounced in many cases. It can be regulated by oligomerization, as shown for plant katanin (Stoppin-Mellet et al., 2007), or by interaction with other binding proteins, as found for the so-called +TIPs, i.e. the complex at the growing MT plus-end that regulates the microtubular elongation process (for a recent review, see Akhmanova and Steinmetz, 2008). The highly dynamic and combinatorial activity of some plant MAPs renders functional analysis difficult, because proteins that do not show pronounced affinity and/or specificity *in vitro* can act *in vivo* in concert with other proteins as specific key regulators. Such proteins that are sufficiently plastic to interact with several substrates are nevertheless expected to be very important for the control of plant MTs organization. In fact, the plant chaperone CCT (Nick et al., 2000), and heat-shock protein 90 (Freudenreich and Nick, 1998; Petrasek et al., 1998) were shown to interact with MTs in addition to other MT-independent functions.

Hsp90 is a highly conserved molecular chaperone essential for protein folding and stability in eukaryotic cells. In animals, many Hsp90 substrates, including proteins, involved in signal transduction and cell development have been described (Wegele et al., 2004). In plants, Hsp90 is essential for the stress response triggered by resistance proteins (R-proteins, Boter et al., 2007; Takahashi et al., 2003), and is involved in MAP kinase cascades (Takabatake et al., 2007). Since Hsp90 predominantly mediates the shift of regulatory and signaling proteins between active and inactive states (Rutherford and Zuker, 1994), it acts at the interface of several developmental pathways (Rutherford and Lindquist, 1998).

Among other proteins, the cytoskeletal proteins actin and tubulin have been reported as interactors of Hsp90 (Koyasu et al., 1986; Sanchez et al., 1988; Wegele et al., 2004). The interaction of Hsp90 with MTs seems to play an important role in eukaryotic cells, and its

Abbreviations: EPC, ethyl-N-phenylcarbamate; GDA, geldanamycin; MAP, microtubule-associated protein; MT, microtubule; PPB, preprophase band.

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functional importance is illustrated by a growing body of evidence in animal cells. The interaction of Hsp90 with MTs is very complex and occurs at least at four levels in animals. Hsp90 interacts with tubulin dimers in animal cells (Sanchez et al., 1988; Weis et al., 2010) as well as with assembled MTs (Fostinis et al., 1992; Williams and Nelsen, 1997). Hsp90 is also a part of the heterocomplex associating with MTs during nuclear transport of steroid hormones (Harrell et al., 2002; Pratt et al., 1999), and strikingly, Hsp90 is a core component of the centrosome (Lange et al., 2000), where it is involved in the recruitment of other centrosome-associated proteins (Basto et al., 2007; Glover, 2005) and contributes to the proper functioning of the centrosome (de Carcer et al., 2001).

Although the interaction of Hsp90 with MTs is well documented in animal cells, the functional link between these different events has remained obscure. In plants, the interaction of Hsp90 with MTs has been poorly investigated thus far. Searching for MAPs involved in plant growth regulated by light, a heat-stable protein was isolated from maize coleoptiles (Nick et al., 1995). One of these proteins was later identified as a member of the Hsp82/90 family. Hsp90 was found to co-localize with cortical MTs, the preprophase band (PPB) and phragmoplasts in tobacco VBI-0 cells (Petrasek et al., 1998) and was shown to co-assemble with MTs *in vitro* (Freudenreich and Nick, 1998).

Here, we report that plant Hsp90 is enriched in interphasic MT preparations from both non-cycling rice coleoptiles as well as from cycling tobacco BY-2 cells, and is associated with tubulin. Hsp90 binds directly to *in vitro* polymerized MTs and decorates MTs *in vivo*. Overexpression of Hsp90 in BY-2 cells has no phenotypic effect except for a mild decrease in the sensitivity to the tubulin-sequestering compound oryzalin. However, when Hsp90 activity is inhibited by geldanamycin (GDA), the recovery of cortical MTs following cold-induced elimination is impaired, suggesting that Hsp90 is required for the plastic re-organization of plant MTs.

Materials and methods

Plant material

The tobacco cell line BY-2 (*Nicotiana tabacum* L. cv. Bright Yellow 2, BY-2 (Nagata et al., 1992)) and rice seedlings were cultivated as described previously by Jovanovic et al. (2010).

Quantification of mitotic index and response to GDA

Geldanamycin (GDA, Serva) was dissolved in DMSO (stock solution 1.78 mM). GDA was added directly from the stock solution to cultivation medium to reach the final concentration. For long-term GDA treatment, cells were supplemented with 1.78 μM GDA at day 1 after subcultivation and cultivated for an additional 2 days at 26 °C.

To determine the mitotic index, cells at day 1 after subcultivation were supplemented with 178 nM or 1.78 μM GDA, collected 12, 24, 36, and 48 h after GDA addition and fixed in Carnoy fixative (3:1 (v/v) 96% (v/v) ethanol:acetic acid (Campanoni et al., 2003)). Subsequently, they were stained with 2′-(4-hydroxyphenyl)-5-(4-methyl-1-piperazinyl)-2.5′-bi(1H-benzimidazole) trihydrochloride (Hoechst 33258, Sigma–Aldrich, final concentration 1 $\mu g\,mL^{-1}$) and scored under a fluorescence microscope. At least 1200 cells were counted for each sample.

For tests of microtubule (MT) recovery, cells were incubated for 1 h at 0 °C in the presence or absence of 178 nM or 1.78 μM GDA and subsequently transferred to 26 °C for 5 min. After immunofluorescent staining of MTs, more than 100 cells in at least 6 optical fields were counted for fully, partially or no recovered MTs.

Isolation of membrane ghosts

Protoplasts from BY-2 tobacco cell suspension culture were obtained as described by Sonobe and Takahashi (1994). Briefly, the cell wall of 3-day-old BY-2 cells was removed by digestion in 1% cellulase (cellulase "Onozuka" R-10, Yakuruto Honsha Co., Ltd., Japan) and 0.1% pectolyase Y-23 (Kyowa Chemical Products Co., Ltd., Osaka, Japan), supplemented with 0.45 M mannitol, for 3–4.5 h. Protoplasts were overlaid onto the growth medium supplemented with 0.4 M sucrose and centrifuged at 200 × *g* for 10 min. Floating protoplasts were collected, filtered through a nylon mesh (mesh diameter 100 μm), re-suspended in wash buffer [10 mM PIPES (MP Biomedicals, LLC, USA), 100 mM KCl, 285 mM mannitol, pH 6.8], and allowed to attach for 3 min to poly-L-lysine (Sigma–Aldrich, St Louis, MO, USA) coated Petri dishes (diameter 19–19.5 cm) for further protein isolation, or to poly-L-lysine coated coverslips for immunostaining of MTs. Protoplasts were then lysed by incubation for 2 min in lysis buffer (7 mM PIPES, 2 mM EGTA, 10 mM $MgCl_2$, 1% DMSO, 6 mM DTT, 300 μM PMSF, pH 6.9 adjusted with KOH) and a subsequent quick-flick movement. Lysed protoplasts were briefly washed with extraction buffer (25 mM MES, 5 mM EGTA, 5 mM $MgCl_2$, 1 M glycerol, pH 6.9 adjusted with KOH) supplemented with additives (100 μM GTP, 100 μM DDT, 100 μM PMSF, 100 μM Aprotinin, 100 μM Leupeptin, 100 μM Pepstatin) diluted 1:100.

Isolation of proteins from membrane ghosts

Proteins from membrane ghosts were extracted for 1 h with 0.5% CHAPS (MP Biomedicals, Inc., USA) in extraction buffer supplemented with additives in Petri dishes swaying on ice. Proteins were collected, centrifuged at 100 000 × *g* at 4 °C for 30 min, and the supernatant was concentrated using a Vivaspin 4 filtration column (10 000 MWCO, Vivascience, USA).

MT co-sedimentation assay

10 $\mu g\,\mu L^{-1}$ bovine brain tubulin (cat. no. TL238, Cytoskeleton, Inc., USA) in PEM buffer (100 mM PIPES, 1 mM $MgCl_2$, 1 mM EGTA, pH 6.8 adjusted with KOH) was diluted 1:1 with PEMgly (20% (v/v) glycerol in PEM buffer) supplemented with 2 mM GTP and incubated at 35 °C for 20 min. 40 μM taxol and 1 mM GTP in pre-warmed (35 °C) PEM buffer was added and MTs were mixed with membrane ghost-extracted proteins or recombinant NtHsp90_MT, supplemented with 8 μM taxol and the solution was incubated at 28 °C for 1 h. To test the inhibition of its MT-binding activity, the recombinant NtHsp90_MT was supplemented by 1.78 μM GDA and incubated with polymerized tubulin at 37 °C to prevent excessive depolymerization. MTs were overlayed on the 20% sucrose cushion in extraction buffer containing protease inhibitors and sedimented by centrifugation at 100 000 × *g* at 25 °C for 1 h. MTs with bound microtubule-associated proteins (MAPs) in the sediment were precipitated with trichloracetic acid (Bensadoun and Weinstein, 1976), separated on SDS–PAGE acrylamide electrophoresis (Laemmli, 1970) and MALDI analysis was used to identify isolated proteins.

Because some amount of unpolymerized tubulin remained in the sample even after the polymerization step, an additional step, in which unpolymerized tubulin was removed by centrifugation at 100 000 × *g* at 28 °C for 30 min, was added in some cases. The polymerized tubulin found in the pellet was resuspended again in pre-warmed (37 °C) PEM buffer supplemented with 40 μM taxol and 1 mM GTP prior to the incubation with recombinant protein.