Characterization of plant p23-like proteins for their co-chaperone activities

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Abstract The small acidic protein p23 is best described as a co-chaperone of Hsp90, an essential molecular chaperone in eukaryotes. p23 binds to the ATP-bound form of Hsp90 and stabilizes the Hsp90–client protein complex by slowing down ATP turnover. The stabilizing activity of p23 was first characterized in studies of steroid receptor–Hsp90 complexes. Earlier studies of the Hsp90 chaperone complex in plants suggested that a p23-like stabilizing activity was absent in plant cell lysates. Here, we show that p23-like proteins are present in plants and are capable of binding Hsp90, but unlike human p23 and yeast ortholog Sba1, the plant p23-like proteins do not stabilize the steroid receptor–Hsp90 complexes formed in wheat germ lysate. Furthermore, these proteins do not inhibit the ATPase activity of plant Hsp90. While transcripts of *Arabidopsis thaliana* *p23-1* and *Atp23-2* were detected under normal growing conditions, those of the closely related *Brassica napus* *p23-1* were present only after moderate heat stress. These observations suggest that p23-like proteins in plants are

conserved in their binding to Hsp90 but have evolved mechanisms of action different from their yeast and animal counterparts.

Keywords Arabidopsis · Brassica · Co-chaperone · Hsp90 · Hsp70 · p23 · Sba1

Introduction

Hsp90 is required for the activation and stabilization of several signaling proteins, referred to as client proteins of Hsp90. The acidic 23-kDa protein p23 was first identified as a component of the progesterone receptor (PR)–Hsp90 complex (Johnson et al. 1994). Since then, it has come to be widely recognized as a co-chaperone of Hsp90 (Felts and Toft 2003; Pratt and Toft 2003), possessing also passive chaperoning activity itself (Bose et al. 1996) as well as a reported prostaglandin synthase activity (Tanioka et al. 2000). Thus, it would appear that p23 has both Hsp90-dependent and Hsp90-independent functions.

p23 binds to the ATP-bound state of Hsp90 and further stabilizes this conformation of Hsp90, extending the Hsp90–client protein activation time. This is achieved, in part, through interactions with several surfaces of the Hsp90 N terminus and regions beyond the charged domain (Chadli et al. 2000; Ali et al. 2006) and results in an inhibition of the ATPase activity of Hsp90 (Richter et al. 2004; Ali et al. 2006; McLaughlin et al. 2006). Though p23 is associated with many Hsp90 client proteins (Pratt and Toft 2003), its role in stabilizing Hsp90–client protein complexes is best studied in the assembly of the steroid hormone receptors with Hsp90. The minimal Hsp90 chaperone complex, consisting of five essential proteins—Hsp90, Hsp70, its co-chaperone Hsp40, Hop (Hsp70 and

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Hsp90 organizing protein), and p23 (Dittmar et al. 1996; Kossano et al. 1998)—is required for folding steroid receptors *in vitro* into their ligand-binding state (Pratt and Toft 2003).

In addition to its stabilizing role, p23 can also suppress aggregation of denatured proteins *in vitro* in an ATP-independent manner (Bose et al. 1996; Cha et al. 2009). The conserved and ordered N terminus of p23 is involved in the binding of p23 to Hsp90. However, both the N terminus and the unstructured C terminus (residues 110–160) are required for the ATP-independent chaperoning activity of p23 and for assisting in the chaperoning of steroid receptors (Weikl et al. 1999; Weaver et al. 2000). Interesting dimensions to the chaperone and co-chaperone functions of p23 are the observations that p23 can disassemble transcriptional regulatory complexes formed at the genomic response elements (Freeman and Yamamoto 2002) and that Sba1 modulates telomerase activity mainly through its own chaperone activity (Toogun et al. 2007).

From humans to yeast, the identification of p23 suggests that p23 is a ubiquitous protein. However, in earlier reconstitution studies, a p23-like stabilizing activity could not be detected in wheat germ lysate (WGL) (Hutchison et al. 1995; Dittmar et al. 1997). Notably, the addition of purified human p23 (hp23) to WGL stabilized the animal steroid receptor–plant Hsp90 complex (Hutchison et al. 1995). These observations led to the belief that the plant lysate lacked a p23-like activity. The availability of the *Arabidopsis thaliana* genome sequence allowed identification of p23-like proteins in this model plant (Krishna and Gloor 2001) and more recently in orchard grass (Cha et al. 2009). Here, we report the molecular characterization of p23-like proteins from *A. thaliana* and *Brassica napus*. Our results indicate that despite differences in the expression patterns of these proteins in the two closely related plant species, the plant p23-like proteins are conserved in their binding to plant Hsp90, but unlike animal and yeast p23/Sba1, the plant proteins neither stabilize steroid receptor–Hsp90 complexes nor inhibit the ATPase activity of plant Hsp90.

Results

Plant p23-like sequences

The human p23 and yeast Sba1 are each encoded by a single gene, though a related protein tsp23 (transcript similar to p23) of unknown function is present in humans (Castilla 1995; Weaver et al. 2000). A search for p23-like sequences in publicly available genome databases [Genbank, <http://www.ncbi.nlm.nih.gov/>; The Gene Index, <http://compbio.dfci.harvard.edu/tgi/>] identified two genes in *A. thaliana*,

three in *Oryza sativa* (rice), and ESTs representing at least one gene in numerous plant species. An alignment of a subset of plant p23-like sequences with human and yeast p23 proteins is shown in Fig. 1. These plant proteins share amino acid identities ranging from 38–60%. Bnp23-1, Atp23-1, Atp23-2, and Lep23 share 32%, 27%, 25%, and 31% amino acid identities, respectively, with the human p23. There are two notable features of plant p23-like proteins. The first is that the p23 signature sequence WPRLTKE (residues 86–92 of human p23) is fully conserved in yeast Sba1, but only partially conserved in plant p23-like proteins. A highly conserved region among plant p23-like proteins, located a few residues downstream of the signature sequence, spans residues 102–112 (KVDWDKWVDED) of Bnp23-1 and coincides with the third amino acid patch (120–125) of yeast Sba1 that is involved in making contact with Hsp90 (Ali et al. 2006). In the same context, Sba1 residues 13–16 (AQRS) are also conserved in plant p23-like proteins, while regions corresponding to Sba1 residues 31–37, 85–91, and 113–118 are less conserved when compared with Sba1 but well-conserved across plant p23-like proteins. The second notable feature is the presence of MGG repeats in some plant p23-like sequences, such as Atp23-1 (Fig. 1), Osp23-1 (GenBank accession no. NP_001061631.1), Bnp23-2 [*B. napus* Gene Index (BnGI) no. TC31271], and *Pinus* sp. p23 (TIGR Gene Index no. TC47079). A similar MG/GA rich sequence is also present in yeast Sba1, but its functional significance is not understood. Consistent with the observation that the N-terminal regions of human p23 (Weaver et al. 2000) and Sba1 (Ali et al. 2006) are involved in Hsp90 binding, the plant p23-like proteins also show a higher degree of conservation in their N-terminal regions. The small protein size is also conserved; for instance, Bnp23-1 and Atp23-1 are 178 and 241 amino acid residues long, with predicted molecular masses of 20 and 28 kDa, respectively. Nucleotide sequence analysis of *Atp23-1* and *Atp23-2* suggests the presence of six exons and five introns.

Expression of Bnp23-1

Since a recombinant *B. napus* Hsp90 was used in our earlier studies (Krishna and Kanelakis 2003), we first focused on using RT-PCR to isolate a *B. napus* cDNA encoding a p23-like protein. Interestingly, a single PCR product (534 bp) was obtained with RNA isolated from heat-stressed (HS) plants, but not with RNA isolated from plants grown at 20°C. The PCR product was cloned, and its sequence was confirmed. To corroborate and further characterize the heat-induced expression of Bnp23-1, seedlings at 48, 72, and 96 h after start of imbibition, and seedlings grown in magenta vessels for 7 and 14 days, were either maintained at 20°C or exposed to high temperatures,

Bnp23	...MSRHETVHWAQRS.....DWYITVLELFDADVVKLKLEP...EGK	37
Atp23-1	...MSRHREVEVWARTT.....EKIFLTVVLADTKDTKVNLDPE...EGV	37
Atp23-2	...MSRNPEVILWAQRS.....DKVYLTVALDFDAKDISVKCEP...QGL	37
Osp23	...MSRHREVEVWARI.....DKVYITVQLADAKDAKNVLEP...EGV	37
Lep23	...MSRHETITVWAQRS.....DKLFTITVLELFDKKNVVKLKLEP...EGK	37
Dgp23	...MSRHETVHWAQRS.....DKVYITVLELFDADVVKLNLEP...DGH	37
Sba1p	MSDKVINPQVWAAQRSTTDPERNYVLTITVSTADCAPELTIKESYIELK	50
p23	MQFASAKWYDRR.....DYVFIEFCNEDSKDYNVNFEE...KSK	35
Consensus	w d	
Bnp23	FFFSATSGASKTL.YEVDLDLDSVIVNESKASVSS..RSVFYLVRKAES	84
Atp23-1	FDPSAKVGPNHNV.YELKLELADKNVNEESKINIGE..RSIFCIIEKRAEP	84
Atp23-2	FSFSALGAQGER.FEFSLELYGKI.MTHYRKNVGL..RNIIFSICKEER	82
Osp23	FSFSATAGTGNL.YESKLELNDKNVNEESKISVGV..RSIFCIVKRAEA	84
Lep23	ELFSATAGADNVP.YEVDLDLDFDKINVDKSSSTTS..RSIVYLVRKAED	84
Dgp23	FNESAK.GSDDMG.YELDLDFDAVNVEESKAAP..STICYLVRKAES	83
Sba1p	AQSKHEHVDENVHLYLHLIDLYKEIIPKTMHKVANGQHYELKLYKRDLE	100
p23	LTFSCLGGSDFNK.HLNEIDLDFHCILPNDKHKRTD..RSILCLLRKSES	82
Consensus	g l k	
Bnp23	.KHWNSRLTKP.KGKHLFLYLVKVDNDRWVDEDED.....	115
Atp23-1	.ERWNLRLR.VKKPFHYVKVDNDRWVDEDEGSAGAADMMDMAGMEGGMG	132
Atp23-2	.SNWTRILKSEKAPYIKVDNKNKWDDEDEEVN.....	114
Osp23	.KHWKHLVDDCKAPHFVKVDNDRWVDEDDDG...ADVNVGMD.FSNF	128
Lep23	.KHWNSRLVKQGLRPVFLKVDNDRWVDEDED.....	115
Dgp23	.TWWPRLLKKGKPEVFLKVDNDRWVDEDED.....	114
Sba1p	SEWYPRITK.EKKVYPYIKTDFDKWVDEDEQDE.....	132
p23	GQSWPRITK.EKAKLNLWLSVDENNHWVDEDD.....	113
Consensus	w l d w d	
Bnp23	...KGEGGDMDFGDFD.ENGLN.....MGD.....TDE	139
Atp23-1	GGMGGMGGMGGMGGMGGMGGMGGMGMEGMDFSKLMGGMGGMGGLGGMGG	182
Atp23-2	SETASDDE..SAFVNQDSESSDDGLLYLPDLEKARKN.....	114
Osp23	GGMGGMGGMGGMGMDM..MGGMG.....GMGGMGGMMAEM..MGG	162
Lep23	...SKPEPDMDFGDMD.FSKLG.....MGEG.....PSN	140
Dgp23	...AGFGG...DFGDMD.FSKLG.....MGGDD.....GDE	138
Sba1p	VEAEGNDAAQGMDFSQMMGGAG.....GAGGAGGMDFSQMMGG	170
p23	DEDMSNDFRESEMM.....	127
Consensus		
Bnp23	IG....EEVAEEDG..DGEGETAAETKEKKIDGEKDEEGVNAKDD...	178
Atp23-1	MGMGMGGMGGMGMEFEFDSDEETAKSGDKDDDAVKEEGPATKAPAAE...	232
Atp23-2	SETASDDE..SAFVNQDSESSDDGLLYLPDLEKARKN.....	150
Osp23	MGMGMGGMGGMGMEFEFDESDEEEVSK.PQDAEKAAEAGKSQESDAKAE...	211
Lep23	FD.....VDYPEGDD..DSDFEEIEEENSEPKAAVPPSTEPGVKA....	179
Dgp23	IE.....EDEDEDNMVDSANKEVEVLVPEGSKGEEAPATTDKAP....	180
Sba1p	AGG.AGSPDMAQLQLLQAGSGGNLDMGDFKENDEEEDDEEIEPEVKA...	216
p23	NNMGGEDEDVLDPEVDGADCDSDSDDEKMPDLE.....	160
Consensus		
Bnp23		178
Atp23-1	ETTSVKEDK	241
Atp23-2		150
Osp23	TS.....	213
Lep23		179
Dgp23		180
Sba1p		216
p23		160
Consensus		

To study the heat-induced expression of Bnp23-1 in green leaves and reproductive tissues, transcript and protein levels were analyzed in plant material submerged in 40°C

buffer. Surprisingly, a 920-bp RT-PCR fragment was observed instead of the expected 534 bp fragment for the leaf sample. To understand this unexpected outcome, *B. napus* leaves were exposed to different temperatures and subsequently analyzed for Bnp23-1 transcript and protein levels. The 534 bp Bnp23-1 fragment was maximally produced from leaves exposed to 35°C, and only the 920-bp fragment was detected for leaves exposed to 40°C (Fig. 2d). The 920-bp cDNA fragment was cloned, and sequence analysis revealed that this larger fragment represents an unspliced version of the *Bnp23-1* transcript. A putative exon/intron arrangement of the *Bnp23-1* gene derived from the nucleotide sequence of the 920-bp fragment indicated the presence of five exons and four introns (not shown). These results suggest that the expression of *Bnp23-1* is temperature-

Fig. 2 Bnp23-1 transcript and protein expression in response to heat stress. **a** RT-PCR analysis of germinating *B. napus* seedlings at 48, 72, and 96 h after start of imbibition that were maintained at either 20°C (C) or given a 2-h heat-shock treatment at 40°C (HS). Seven- and 14-day-old *B. napus* seedlings were maintained at either 20°C (C) or exposed to 45°C (HS) for 2 and 4 h. Seedlings that were exposed to 45°C for 4 h were allowed to recover at 20°C (R) for 22 h. Actin was used as control (lower band); **b** immunoblot analysis of Bnp23-1 in germinating seedlings described in **a**; **c** immunoblot analysis of Bnp23-1 in 7-day-old seedlings maintained at either 20°C, exposed to 45°C for 2 and 4 h, or recovering from HS at 20°C for 24 h, 72 h, and 7 d; **d** RT-PCR analysis of first leaves from 14-day-old *B. napus* seedlings that were submerged in buffer maintained at different temperatures (20°C, 25°C, 30°C, 35°C, or 40°C) for 2 h. C no RT control; **e** immunoblot analysis of Bnp23-1 in leaf samples described in **d** (left panel) and in flower buds (FB) and flowers (F) maintained at either 20°C (C) or heat stressed at 40°C (HS) (right panel). Arrow indicates heat-induced Bnp23-1. The identity of the lower band is not known

regulated, and while splicing of its pre-mRNA in leaf tissue occurs correctly at moderately high temperatures such as 35°C, it is inhibited at 40°C.

Protein accumulation in leaves corresponded with *Bnp23-1* transcript accumulation (Fig. 2e, left panel). A heat-induced band (indicated by an arrow) was seen in leaves exposed to 35°C, but not in leaves exposed to 40°C. At present, it is unclear if the band below the HS-induced protein is a p23-related protein or if it is produced as a result of nonspecific reaction with the anti-Bnp23-1

antibody. In contrast to the leaves, only a single band representing Bnp23-1 was detected in flower buds and flowers following HS (right panel). Stresses other than HS, such as cold, dehydration, and wounding, did not induce p23 expression. Together, these results suggest that the Bnp23-1 is a heat-inducible protein.

During the course of this study, two additional *B. napus* p23-like sequences were identified in the BnGI database, referred herein as Bnp23-2 with MGG repeats (BnGI no. TC31271) and Bnp23-3 (BnGI no. TC6205). From information on the ESTs in the database, it would appear that Bnp23-2 is expressed in seeds, and Bnp23-3 is expressed in flower buds, flowers, seeds, and embryo under normal growth conditions.

Expression of Atp23-1 and Atp23-2

The heat-inducible expression of Bnp23-1 incited us to look into the expression patterns of the two *A. thaliana* genes. Primers specific to Atp23-1 and Atp23-2 were used in RT-PCR to detect transcript expression in seedlings grown at the normal growing temperature and in seedlings exposed to HS. Contrary to Bnp23-1, both Atp23-1 and Atp23-2 were expressed in the absence of stress; exposure to HS decreased transcript levels (Fig. 3a). *In silico* analysis of Atp23-1 (At4g02450) expression using AtGenExpress Visualization Tool (AVT) confirmed that environmental conditions have little or no effect on its expression (Fig. 3b) and that the gene is expressed at relatively higher levels in shoot apex and inflorescence (Fig. 3c). Since Atp23-2 (At3g03773) was not present on the ATH1 array, information on its expression could not be retrieved.

Plant p23-like proteins bind Hsp90 in an ATP-dependent manner

Bnp23-1 was cloned into pTBY2 to create a chitin binding domain (CBD)-tagged protein for further study. The Bnp23-1-CBD protein was bacterially expressed and immobilized on chitin beads (Fig. 4a). For antibody production, Bnp23-1 was cleaved from CBD (Fig. 4a) and injected into rabbits. To test for Bnp23-1 binding to plant Hsp90, Bnp23-1-CBD was immobilized and incubated with WGL to allow complex formation. Following washing, proteins retained on the chitin beads were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting with anti-Hsp90 antibody. Results shown in Fig. 4b and c indicate that wheat Hsp90 binds to Bnp23-1-CBD but not to CBD, that the binding between Hsp90 and Bnp23-1-CBD reaches equilibrium between 60 and 90 min of incubation on ice, and that the interaction between Bnp23-1-CBD and Hsp90 is salt sensitive, being disrupted almost completely by

Fig. 3 Expression of Atp23-1 and Atp23-2 transcripts. **a** RT-PCR analysis of transcripts in 14-day-old *A. thaliana* seedlings maintained at either 22°C (C) or exposed to 42°C (HS) for 2 h. **b** *In silico* analysis of Atp23-1 transcript expression in response to heat 1 h at 38°C; cold 1 h at 4°C; drought 15 min dry air stream followed by incubation in climate chamber for 1 h; osmotic 300 mM mannitol for 1 h; salt 150 mM NaCl for 1 h. **c** *In silico* analysis of Atp23-1 transcript expression in roots (21 days), hypocotyl (7 days), stem (1st node), leaves (rosette leaf #8), whole seedling (green parts; 7 days), shoot apex (before bolting; 14 days), inflorescence (after bolting; 21 days), flowers (28 days), siliques (early to late heart embryos). The data used to create **b** and **c** were obtained using AVT

250 mM NaCl. In these experiments, bands representing Hsp90 bound to Bnp23-1-CBD appear smaller than Hsp90 detected directly in WGL. This is because Bnp23-1-CBD runs at the same position as Hsp90, and its abundance in the sample distorts the Hsp90 band on the gel. Consistent with the effects of Hsp90-specific inhibitors on other Hsp90-p23 complexes (Sullivan et al. 1997; Chadli et al. 2000), preincubation of WGL with GA or radicicol (RA) at 30°C almost completely blocked the binding of Bnp23-1 to Hsp90 (Fig. 4d). Bnp23-1-Hsp90 complexes formed by incubation with WGL also contained the wheat immunophilin FKBP73 (data not shown).

Studies of Hsp90-p23 interaction in animal systems indicate that p23 binds to Hsp90 only in its ATP-bound conformation (Sullivan et al. 1997). Since the commercially available WGL contains an ATP regenerating system, the effect of nucleotides on plant p23-like proteins binding to purified plant Hsp90 was studied using purified proteins immobilized on antibody/protein A Sepharose resin; hp23 was used as a control. The results in Fig. 4e show that purified Atp23-1 and Atp23-2 can bind Hsp90 in the presence of 5 mM ATP, 5 mM ATP and 20 mM sodium molybdate or 0.5 mM ATP-γ-S. In each case, no binding was observed in the absence of ATP, indicating that the plant p23-like proteins require the ATP-bound conformation of Hsp90 for interaction. This was also observed for the interaction between Bnp23-1-CBD and purified Hsp90 (data not shown).

MGG repeats are not essential for binding of Atp23-1 to Hsp90

The presence of MGG repeats in Atp23-1, but not in Atp23-2, Bnp23-1, or Lep23, led us to examine its role, if any, in binding to Hsp90. The deleted protein, Atp23-1d (Fig. 4f), was fused to CBD and used in protein interaction studies. Neither the deletion of MGG repeats nor the fusion of CBD at the N terminus of Atp23-1d disrupted Atp23-1-Hsp90 binding (Fig. 4g, upper panel). Purified Atp23-1d expressed without any affinity tag also bound Hsp90 in an ATP-dependent manner (not shown). Thus, the MGG repeats are not essential for the binding of Atp23-1 to Hsp90.

Plant p23-like proteins bind human Hsp90

The efficient binding of human p23 with plant Hsp90 had allowed us to demonstrate the presence of plant immunophilins in this complex (Owens-Grillo et al. 1996; Reddy et al. 1998) and to stabilize the steroid receptor heterocomplex with plant Hsp90 in WGL and reconstitution studies (Hutchison et al. 1995; Dittmar et al. 1997). These studies were highly instrumental in enhancing our knowledge of

Fig. 4 Interaction of plant p23-like proteins with Hsp90. **a** SDS-PAGE analysis of recombinant Bnp23-1. Lane 1 Bnp23-1-CBD bound to chitin beads; lane 2 eluted Bnp23-1 after self-cleavage of the fusion protein. Protein size markers are shown on the left. **b** Time-dependent binding of plant Hsp90 (from WGL) to immobilized Bnp23-1-CBD. Hsp90 was detected by immunoblotting. Immobilized Bnp23-1-CBD incubated with buffer alone (extreme left) was used as a negative control. An aliquot of WGL was run on the gel to mark the position of Hsp90 (extreme right). **c** Salt stability of plant Hsp90 binding to immobilized Bnp23-1-CBD. Complexes were formed as in **a** for 60 min and then washed with buffer containing NaCl as indicated. Controls were immobilized CBD incubated with WGL for 60 min and immobilized Bnp23-1-CBD incubated with buffer alone

(first two lanes). **d** WGL was incubated with 5 μ M of GA, RA, or DMSO control for 10 min at either 30°C or 4°C prior to incubation with immobilized Bnp23-1-CBD for 60 min. **e** Immobilized plant Hsp90 was incubated with purified Atp23-1, Atp23-2, and hp23 in the absence or presence of nucleotide as indicated. After incubation, proteins were resolved on a gradient gel and detected with silver stain. The arrowheads indicate p23 proteins; arrow indicates a possible degradation product. **f** Deletion scheme of Atp23-1 to produce Atp23-1d; substitution of Gly by Ala at position 214 was a result of cloning. **g** Binding of plant Hsp90 to immobilized CBD-Atp23-1d (upper panel immunoblot; lower panel Coomassie blue stained gel). Immobilized CBD was used as negative control. The positions of CBD-Atp23-1d and CBD are indicated

the plant Hsp90 chaperone complex. To test whether plant p23-like proteins could bind human Hsp90, FLAG-tagged human p23 was immobilized and incubated with human Hsp90 in the absence or presence of competitor p23. Atp23-1 was nearly as effective as human p23 in competing with FLAG-tagged human p23 for binding to human Hsp90, while the yeast p23 and Atp23-2 were less effective (Fig. 5). The study of binding of p23 proteins, including Atp23-1d, to plant Hsp90 also indicated that human p23 and Atp23-1 are comparable in their potential to bind plant Hsp90 and that the MGG repeat region is not essential but contributes to the strength of Atp23-1 binding to Hsp90 (not shown).

Plant p23-like proteins do not stabilize steroid receptor-Hsp90 complexes

To gain insight into functions of plant p23-like proteins, we used the well-established steroid receptor assembly assay that has previously been instrumental in demonstrating functional conservation or divergence of plant Hsp90, Hsp70, Hop, and immunophilins from their animal counterparts (Dittmar et al. 1997; Krishna and Kanelakis 2003; Zhang et al. 2003). The immuno-adsorbed progesterone receptor was incubated with WGL, rabbit reticulocyte lysate (RRL), or buffer in the absence or presence of purified Atp23-1 (expressed without any affinity tag) and

Fig. 5 Competition between human p23 and plant p23-like proteins for binding to human Hsp90. FLAG-tagged C-terminal region of human p23 (*p23CFLAG*) bound to antibody/protein A Sepharose resin was incubated with 5 μ g human Hsp90 with or without competitor p23. After incubation, proteins were extracted and resolved on a 12% SDS-PAGE and stained with Coomassie blue. The heavy and light chains of the anti-FLAG antibody are the additional two bands in the gel

human p23. The hormone-binding activity of PR was used as a measure of chaperoning success. The amount of steroid bound by the receptor assembled in WGL was maximal in the presence of human p23 (Fig. 6). The addition of plant Hsp90 to WGL further boosted steroid binding. By contrast, the addition of Atp23-1 to WGL had minimal effect even in the presence of additional Hsp90. Steroid binding was best when the receptor was assembled in RRL, which naturally contains rabbit p23. Unlike in WGL, addition of human p23 to RRL did not further boost steroid binding by the receptor. Similarly, in receptor reconstitution with purified core proteins (Hsp90, Hsp70, Hsp40, Hop), a functional (steroid binding) receptor complex was formed in the presence of human p23 but not in the presence of Atp23-1 (data not shown).

Fig. 6 Reconstitution of progesterone receptor complexes. Immuno-purified PR was incubated with WGL (first nine lanes), RRL (lanes 10–12), or buffer control (lane 13) in the absence or presence of varying amounts of hp23 or Atp23-1. In two samples, 20 μ g of purified plant Hsp90 was also added. Following 30°C incubation, the samples were transferred to ice and incubated with ^3H -progesterone. After washing, bound progesterone was measured by scintillation counting

Plant p23-like proteins have no effect on the ATPase activity of Hsp90

The ATPase activity of Hsp90 is essential for its biological functions (Panaretou et al. 1998), and this activity is regulated through interaction with both co-chaperones and client proteins (McLaughlin et al. 2002, 2006; Panaretou et al. 2002). p23 has been demonstrated to inhibit the ATPase activity of human Hsp90 (McLaughlin et al. 2002, 2006), which has a stabilizing effect on Hsp90–client protein complexes. We determined the ATPase activity of purified plant Hsp90 in the absence and presence of GA as well as plant p23. GA completely inhibited the ATPase activity of plant Hsp90; the amount of GA-inhibited activity of each sample is shown in Fig. 7. Even at 1:5 molar ratio of Hsp90 to Atp23-1 or Atp23-2, there was only a small inhibitory effect of Atp23-1 protein on the ATPase activity of Hsp90. Consistent with its stimulatory effect (Panaretou et al. 2002), Aha1 increased the ATPase activity of plant Hsp90 by more than 2-fold when present in a 1:1 molar ratio. Interestingly, the addition of Atp23-1 completely inhibited the Aha1-stimulated activity of Hsp90. Since Aha1 competes with p23 for binding to Hsp90 (Harst et al. 2005), it is likely that plant p23-like proteins do not function by inhibiting the basal ATPase activity of Hsp90 but may prevent stimulation of the same by competing with Aha1 for binding to Hsp90.

Discussion

Earlier attempts using steroid receptor reconstitution and activation assays to identify a p23-like stabilizing activity in

Fig. 7 The effect of co-chaperones on the ATPase activity of plant Hsp90. Hydrolysis of ^{32}P -ATP to ^{32}P -ADP was measured using 5 μ M Hsp90, 25 μ M Atp23-1 or Atp23-2, and 5 μ M of Aha1 as indicated. Following incubations, aliquots of 1 μ L were spotted onto the PEI-cellulose and resolved by TLC. ADP/ATP ratios were quantitated using a storm phosphorimager. ATPase activity was calculated from the difference in ^{32}P -ATP hydrolysis of control and GA-inhibited samples

plant cell lysates led us to conclude that p23-like proteins are either absent in plants or they function differently from the human p23 (Krishna and Gloor 2001). The present study clarifies this issue, showing that p23-like proteins are present in plants but have some features that are distinct from animal and yeast p23 proteins.

In *A. thaliana*, two genes encode p23-like proteins; one of these, Atp23-1, contains the MGG repeats. Similarly, one of the three genes in *O. sativa*, and one of the three putatively identified genes in *B. napus*, also contains these repeats. We attribute the low expression of Atp23-1 in bacterial cells as well as its aggregation during purification to the presence of MGG repeats in this protein since deletion of these repeats in Atp23-1d resolved this problem to a great degree. Atp23-1d bound to Hsp90 but had reduced ability to compete with human p23 for binding to Hsp90 in comparison to the intact protein, suggesting that the repeats are not essential for Hsp90 binding but likely strengthen the interaction between the two proteins. The presence of two or more p23 protein members in a single plant species together with the presence of MGG repeats in at least one protein member suggests the possibility that each family member has specific cellular functions. The differences between Atp23-1 and Atp23-2 in binding to Hsp90 and in affecting Hsp90's ATPase activity also suggest that these proteins may have specific *in vivo* functions.

The various p23 sequences of plant origin (Fig. 1 and others not shown) share a higher degree of conservation in their N-terminal regions, consistent with the fact that this region is involved in Hsp90 binding. The amino acid sequence KVDWDKWVDED (residues 102–112 in Bnp23-1) is highly conserved in plant p23-like proteins. This region coincides with the third amino acid patch (120–125) of yeast Sba1 that is involved in making contacts with Hsp90 (Ali et al. 2006). In the same context, Sba1 residues 13–16 (AQRS) are also conserved in plant p23-like proteins. Thus, in all likelihood, residues conserved in all p23 proteins must be critical for binding to Hsp90, whereas other regions corresponding to Sba1's regions involved in Hsp90 binding may have evolved in plant p23 proteins for best interaction with plant Hsp90.

Sequences available in BnGI indicate that in addition to Bnp23-1, *B. napus* has at least two other genes, Bnp23-2 and Bnp23-3. An antibody raised against Bnp23-1 detected a single protein band in non-green tissues and two bands in the leaves (Fig. 2e). The HS-induced band must correspond to Bnp23-1, while the band detected under normal conditions in leaf tissue could represent either a closely related product or a nonspecific signal. According to information available in the database, Bnp23-3 is expressed under normal growing conditions in flowers, flower buds, seeds, and embryos. The lack of any signal in flowers (Fig. 2e) indicates that either the expression of Bnp23-3 is

below detectable levels or that it does not cross-react with the antibody. Since Bnp23-1 shares amino acid identities with putative Bnp23-2 and Bnp23-3 of 39% and 34%, respectively, there is a high probability that the anti-Bnp23-1 antibody does not recognize the other two proteins. A clear understanding of the *B. napus* p23 protein family will be possible when full-length cDNAs of all members of the Bnp23 protein family have been isolated and characterized.

The heat-induced expression of Bnp23-1 (present study) and of the orchard grass Dgp23 (Cha et al. 2009) suggests that such a response is common to plant p23-like proteins. Both Bnp23-1 transcripts and proteins were detected only after exposure to high temperature, with levels declining to normal after removal of the heat stress. This pattern of expression is reminiscent of heat shock proteins that protect other cellular proteins from denaturation (Parsell and Lindquist 1993). However, in contrast to HS-induced genes that are either intronless or have very few introns, Bnp23-1 gene appears to have four or more introns and is sensitive to temperature-dependent inhibition of splicing in leaf tissue (Fig. 2d). From these data, it can be extrapolated that Bnp23-1 must have functions only under moderately high temperature conditions but not under severe HS. In contrast to Bnp23-1, the two *A. thaliana* orthologs displayed constitutive expression and lack of any induction in response to heat stress. *A. thaliana* and *B. napus* are close relatives; the genes of these plant species share, on average, 87% sequence identity (Paterson et al. 2001). However, it is found that many *Brassica* phenotypes are under much more complex genetic control than are similar phenotypes in *Arabidopsis* (Lan and Paterson 2000). *Brassica* species are also expected to encompass genome changes associated with polyploidy. The consequences of polyploidy on the expression of p23-like protein gene families in the two plant species could be an interesting question to explore in the future.

A feature common to both *A. thaliana* and *B. napus* is the definite and possible higher expression of p23-like proteins in inflorescence tissue as compared to other plant tissues. This is indicated by the e-northern data for Atp23-1 (Fig. 3c) and the cDNA library sources for ESTs encoding Bnp23-2 and Bnp23-3. These data, together with the recent demonstration that mouse p23 null embryos develop but do not survive after birth (Grad et al. 2006), set the stage for plant p23-like proteins to possibly have function(s) linked to embryo development.

Despite differences in the expression and structure of Bnp23-1 and Atp23-1, both proteins as well as Atp23-2, bound Hsp90 of both plant and animal origin. Results of competition studies indicated that Atp23-1 was most efficient and comparable to human p23 in binding to plant Hsp90 (Fig. 5). These results strongly suggest that plant p23-like proteins, similar to human and yeast proteins,

function as co-chaperones of plant Hsp90. This idea is strengthened further by the observation that the wheat immunophilin FKBP73 was present in Bnp23-1–Hsp90 complexes formed in WGL (not shown). p23–Hsp90–immunophilin complexes are believed to form a functional unit, operating in the latter steps of chaperoning of client proteins (Pratt and Toft 2003).

Despite several indications that plant p23-like proteins must function as co-chaperones of Hsp90, neither of the two proteins tested stabilized the plant Hsp90–progesterone receptor complexes formed either by incubation in WGL or in the presence of purified proteins. This is in contrast to the performance of plant Hsp90, Hsp70, Hop, and FKBP73 that are either present in complexes of steroid receptors assembled in WGL or increase steroid binding by the receptor (Krishna and Kanelakis 2003; Zhang et al. 2003). Consistent with the observation that plant p23-like proteins failed to stabilize steroid receptor–Hsp90 heterocomplexes, these proteins had also little to no effect on the basal ATPase activity of plant Hsp90. We observed that Aha1, similar to its effects on yeast and human Hsp90, stimulated the ATPase activity of plant Hsp90 and that this stimulation could effectively be inhibited by Atp23-1 (Fig. 7). Based on these results, we speculate that while plant p23-like proteins do not affect the basal ATPase activity of Hsp90, they may assist in maintaining the half-lives of plant Hsp90–client protein complexes by competitively inhibiting Aha1 binding to Hsp90. Dynamic changes in the concentrations or activities of p23-like proteins and Aha1 *in vivo* would determine the rate of dissociation of a client protein from Hsp90. While our understanding of the composition and functional interrelationships of the components of the plant Hsp90 chaperone system is still in its infancy, it is clear from this study that plant p23-like proteins function via a mechanism that is somewhat different from human and yeast p23. The conclusion that plant p23-like proteins do not stabilize the animal receptor–plant Hsp90 complexes clearly does not imply that these proteins may not stabilize plant client–Hsp90 complexes in plant cells but rather points to some difference in the mode of action of plant p23 proteins compared to human and yeast p23. A more complete understanding of the co-chaperone role of plant p23-like proteins will be possible when an appropriate plant Hsp90 client protein that utilizes a p23-like protein becomes identified in the future.

Methods

cDNA cloning of plant p23-like proteins

Total RNA isolated from non-stressed and HS *B. napus* (cv. Wistar) and *A. thaliana* seedlings was reverse transcribed

into cDNA with oligo-(dT)₁₈ primer using SUPER SCRIPT™ First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad CA). A fraction of the first-strand cDNAs was used as a template for PCR. Gene-specific primers: BnF 5'AAACATATGAGTCGACATCCTACG3', BnR 5'AAGAATTCATCCTTCTTCGCATTC3'; At-1F 5'GCCATATGAGTCGTCATCCTGGAAGTG3', At-1R 5'GCGAATTCACCTGTCTTCTTAACAG3' were derived from the DNA sequences encoding *B. napus* p23-1 (Genbank accession no. AF153128) and *A. thaliana* p23-1 (Genbank accession no. AJ297951). PCR was carried out using pfu DNA polymerase (Stratagene, La Jolla Ca) with an initial denaturation step of 94°C for 2 min followed by 28 cycles of denaturation (15 s at 94°C), annealing (30 s at 60°C), and extension (1 min at 72°C). A final extension was carried out for 10 min at 72°C. The purified PCR products were cloned in pBluescript (pBS), and their sequences were verified.

Plant material and stress conditions

For germination studies, *B. napus* seeds were sterilized and left to germinate on sterile water-soaked filter paper in Petri dishes. Seedlings at 48, 72, and 96 h after start of imbibition were either maintained at 20°C (as control) or exposed to 40°C for 2 h (HS). Sterilized *B. napus* seeds were also grown on a nutrient medium in magenta vessels at 20°C (Dhaubhadel et al. 1999) for 7 and 14 days. Seedlings were maintained either at 20°C or exposed to 45°C for 2 and 4 h. Seedlings exposed to 45°C for 4 h were allowed to recover at 20°C for 22 h. For tissue collection, *B. napus* plants were grown to maturity in a growth chamber maintained at 20°C with a 16 h day length. The light intensity was 150 $\mu\text{E m}^{-2}\text{s}^{-1}$. First leaves, and flowers and flower buds, were collected from 2- and 6-week-old plants, respectively. Different plant organs were submerged in 10 mM Tris pH 8.0 and exposed to either 20°C or higher temperatures for 2 h. Sterilized *A. thaliana* seeds were exposed to 4°C for 2 days and then transferred to a growth chamber maintained at 22°C with a 16 h day length. Two-week-old seedlings grown in magenta boxes were either maintained at 22°C (control) or exposed to 42°C for 2 h (HS). All plant material was collected after treatment, quick-frozen in liquid nitrogen, and stored at –80°C.

Transcript expression by RT-PCR

RT-PCR was carried out as described above. PCR was performed for 25 and 28 cycles for analysis of Bnp23-1 and Bn-actin transcripts, respectively; 30 cycles for Atp23-1 and Atp23-2; and 26 cycles for At-actin transcripts. The following primers were used: BnF, BnR; Bn-actinF (5'ATCACCATCG GAGCTGAG3'), Bn-actinR (5'GAAGCATTCCTGTG

GACG3'); At-1F, At-1R; At-2F (5'CATTTCGGTGAAGTGT GAGCCTCA3'), At-2R (5'TGCGGTAAAGCTACTT GTTCTTG3'); At-actinF (5'TGCTCTTCCTCATGCT ATCC3'); and At-actinR (5'ATCCTCCGATCCAGA CACTG3'). PCR products were visualized on 1.2% agarose gels using a UV light transilluminator.

Data on tissue and abiotic stress specific expression of Atp23-1 (At4g02450) was generated using AVT (Schmid et al. 2005; Kilian et al. 2007) at <http://jsp.weigelworld.org/expviz/expviz.jsp>.

Construction of recombinant plant p23-like proteins

The coding regions of Bnp23-1 and Atp23-1 in pBS were excised by *NdeI* and *EcoRI* and cloned in frame with the CBD in the pTYB2 expression vector (New England Biolabs Ltd. Pickering ON) to give pBn-1 and pAt-1a, respectively. The Atp23-1 fragment was also cloned into pET-17b to produce pAt-1b. For deletion of the MGG repeats, Atp23-1 was amplified as two fragments; one was amplified using At-1F to create a *NdeI* site at the ATG start codon and a reverse primer (5'TCCCATGGCTTCC ATTCCTGCC3') to create a *NcoI* site approximately in the middle of the Atp23-1 coding region, and the second was amplified using a forward primer (5'GAGCCATGG AAGAGTTTGAAGAC3') to create a *NcoI* site and At-1R to create an *EcoRI* site at the end of the coding region. The PCR products were digested either with *NdeI* and *NcoI*, or with *NcoI* and *EcoRI*, and the fragments were cloned into the *NdeI* and *EcoRI* sites of pET-17b to produce pAt-1c. The Atp23-1d fragment from pAt-1c was also moved into pTYB12 using *NdeI* and *EcoRI* to produce pAt-1d. Atp23-2 was PCR amplified using the ABRC cDNA clone (stock no. U68171) as template and gene specific primers: 5' At-2F1 (5' TGATGAGTCGTAATCCGGAGG3'); At-2F2 (5'TATGATG AGTCGTAATCCGGAGG3'); At-2R1 (5'GATCCC TACTTGTTCCTTGCCCTTTTCC3'); At-2R2 (5' CC TACTTGTTCCTTGCCCTTTTCC3') as described (Zeng 1998). The PCR product was cloned into the *NdeI* and *BamHI* sites of pET-17b to give pAt-2. The DNA sequences of all expression constructs were verified by sequencing.

Expression and purification of plant p23-like proteins

The expression constructs pBn-1, pAt-1a, and pAt-1d were introduced into *Escherichia coli* strain ER2566 cells. Expression and purification of CBD-tagged proteins and cleavage and elution of Bnp23-1 were carried out as described before (Zhang et al. 2003).

The DNA constructs pAt-1b, pAt-1c, and pAt-2 (in pET-17b) were introduced into BL21 cells, and proteins were purified from induced bacteria as described for human p23 (Sullivan et al. 1997). Clarified bacterial lysate was

prepared by sonication of bacterial pellet, derived from 500 mL of culture, in 10 mL of TTg buffer (10 mM Tris pH 7.5, 10 mM thioglycerol) containing protease inhibitors (0.1 mM leupeptin, 0.1 mg/mL bacitracin, 0.077 mg/mL aprotinin, 1.5 μ M pepstatin, 1 mM AEBSF), followed by centrifugation at 10,000 $\times$ g for 20 min. The supernatant was applied to a 20-mL bed of DE52 resin. The fractions containing p23 were pooled and mixed with an equal volume of 3 M ammonium sulfate, pH 7.5. After centrifugation to remove precipitated proteins, the supernatant was applied to a phenyl sepharose column, and p23 was eluted using a decreasing salt gradient. The p23 containing fractions were pooled and concentrated, and the buffer was exchanged to p23 storage buffer (20 mM Tris pH 7.5, 1 mM EDTA, 50 mM KCL). This procedure was also used for purification of Bnp23-1.

Purification of Hsp90 and other proteins

Plant Hsp90, chicken Hsp90, and human Hsp70 were prepared from SF9 insect cells transfected with a baculovirus expression system as described (Park et al. 1998; Johnson et al. 2000). Human Hop and yeast hsp40 were expressed in bacteria and purified as described (Johnson et al. 2000).

Antibody generation

An aliquot of purified Bnp23-1 (200 μ g) was mixed 1:1 (v/v) with Freund's complete adjuvant (Invitrogen) and injected into the rabbit. An additional injection with 200 μ g of protein mixed with incomplete Freund's adjuvant was done after 2 weeks. Antiserum was collected 3 weeks after the second injection with the antigen. The antiserum cross-reacted with purified Bnp23-1 and specifically recognized a protein with an approximate molecular mass of 26 kDa in cell lysates of HS *B. napus*. No cross-reaction was obtained, even at a dilution of 1:2,000 (v/v), with the pre-immune serum.

Detection of plant p23-like proteins

Frozen plant material was ground using a pestle and mortar in extraction buffer (25 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0, 20 mM NaCl, 1 mM PMSF, 2 mM benzamidine, 1 μ g/mL leupeptin, and 2 μ g/mL aprotinin). The homogenate was centrifuged at 12,000 $\times$ g for 30 min at 4°C. Protein concentration was determined by the Bradford method (Bio-Rad). Proteins (60 μ g) were separated on 12.5% (w/v) SDS-PAGE and immunoblotted using anti-Bnp23-1 antibody and peroxidase-conjugated anti-rabbit IgG, each at a dilution of 1:5,000 (v/v). The antigen-antibody complexes were detected using the enhanced

chemiluminescence detection system (Amersham Biosciences, Baie d'Urfe, QC).

Protein–protein interaction assay

Total protein extract (500 μ L, $\sim$ 10 mg/mL) prepared from an induced bacterial culture was incubated with 100 μ L (bed volume) of chitin beads to immobilize the fusion protein. The beads were washed five times with 1 mL of HEG buffer (10 mM Hepes pH 8.0, 1 mM EDTA, 10% glycerol, 50 mM NaCl), and then 20 μ L aliquots of beads were each incubated with 20 μ L of WGL (Promega, Madison WI) on ice for 60 min. The beads were washed three times with 1 mL of HEG buffer, and the retained proteins were extracted in 1 $\times$ SDS sample buffer and analyzed by 7.5% SDS–PAGE, followed by immunoblotting with an anti-Hsp90 antibody (Krishna et al. 1997). For time course of Bnp23-1 interaction with Hsp90, chitin beads with immobilized protein were incubated with WGL for 15, 30, 45, 60, or 90 min before washing and analysis by immunoblotting.

For studying the effect of GA and RA on the interaction of Hsp90 with Bnp23-1, 5 μ M of GA or RA was added to WGL, and the mix was incubated for 10 min at either 30°C or 4°C. Immobilized Bnp23-1–CBD was incubated with this WGL mix on ice for 60 min, followed by washing of the beads, and analyzing of bound Hsp90. Control reactions contained WGL to which DMSO (solvent for GA and RA) had been added.

For studying interaction between Bnp23-1–CBD and purified Hsp90, Bnp23-1–CBD beads were incubated with 5 μ g of purified recombinant *B. napus* Hsp90 (Krishna and Kanelakis 2003) in the presence of 5 mM ADP, ATP, or ATP- γ -S in a buffer containing 20 mM Hepes pH 7.5, 20 mM KCl, 1 mM MgCl₂, and 0.01% NP-40 for 60 min on ice. In another reaction containing ATP- γ -S, Hsp90 was first incubated with RA for 10 min at 30°C and then ATP- γ -S was added. The samples were washed twice with the reaction buffer and analyzed for Hsp90. Immobilized CBD was used as a control in all protein–protein interaction studies. FKBP73 and Hsp70 were detected by using anti-FKBP73 and anti-Hsp70 antibodies at 1:10,000 and 1:5000 dilutions, respectively. The expression and purification of CBD–GmHop-1 has been described before (Zhang et al. 2003).

For studying interaction between plant Hsp90 and untagged p23 proteins, anti-His antibody was adsorbed to protein A Sepharose resin, and the washed resin was incubated with 7.5 μ g His-tagged plant Hsp90, 12 μ g of either Atp23-1, or Atp23-2 or human p23, without nucleotide or with 5 mM ATP, 5 mM ATP, and 20 mM sodium molybdate or 0.5 mM ATP- γ -S in BH buffer (20 mM Hepes pH 7.5, 50 mM KCl, 5 mM MgCl₂, 2 mM DTT, and 0.01% Triton X-100). The mixtures were heated

to 30°C for 30 min and then transferred to ice for 30 min. Proteins were resolved on a 10–20% gradient SDS–PAGE and detected with silver stain.

Competition assay

Assays were set up in 200 μ L of BH buffer containing 5 mM ATP, 20 mM sodium molybdate, 2.5 μ g of human p23 FLAG-tagged at the C-terminus (p23CFLAG) bound to antibody/protein A Sepharose resin, and 5 μ g human Hsp90 with or without competitor p23. The assays were incubated for 30 min at 30°C, 30 min on ice and washed 4 X with 1 mL of ice-cold BH buffer. Proteins were extracted in SDS sample buffer and resolved on a 12% SDS–PAGE and stained with Coomassie blue.

PR reconstitution assay

Salt-stripped PR adsorbed to antibody/protein A resin (Sullivan et al. 1986) was incubated for 30 min at 30°C in 200 μ L WGL, RRL, or BH buffer and indicated additions of p23 or Hsp90. The samples were transferred to ice, and labeled progesterone (³H-Progesterone, ART 663-progesterone, 50 Ci/mmol, American Radiolabeled Chemical Inc. St. Louis MO) was added to a final concentration of 1×10^{-7} M. After 90 min on ice, samples were washed 4 $\times$ with 1 mL of BH buffer. An aliquot (200 μ L) was removed for scintillation counting, and the remainder of the sample was prepared for SDS–PAGE.

ATPase assay

ATPase activity of plant Hsp90 was determined essentially as previously described (Owen et al. 2002). To determine if the measured activity was specific to Hsp90, GA was added to some samples. The reactions contained 5 μ M of purified plant Hsp90 and, where indicated, 25 μ M of purified Atp23-1 or Atp23-2. In some samples, Aha1 was also added at a concentration of 5 μ M. The samples containing protein(s), buffer, α -³²P-ATP, and ATP (50 μ M) in a final 10 μ L volume were incubated for 30–60 min at 30°C. The reactions were stopped by the addition of 10 μ L of 20 mM EDTA. Aliquots of 1 μ L were spotted onto PEI-cellulose TLC plates. ADP/ATP ratios were quantitated using a storm phosphorimager, and the ATPase activity was calculated from the difference in ³²P-ATP hydrolysis of control and GA-inhibited samples.

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