Plant Molecular Biology **33:** 457–466, 1997.

Analysis of the native forms of the 90 kDa heat shock protein (hsp90) in plant cytosolic extracts

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Received 7 June 1996; accepted in revised form 10 October 1996

Key words: hsp90, native forms, R_2 antibody

Abstract

A polyclonal antibody, R_2, was raised against a fusion protein consisting of a portion of plant hsp90 fused to the trpE protein of *Escherichia coli*. This antibody was found to be specific towards plant hsp90, showing little or no cross-reactivity with mouse and human hsp90 proteins. The R_2 antibody identified an 83 kDa protein as the hsp90 homologue in cytosolic extracts of several dicot and monocot plants. Two-dimensional gel electrophoresis indicated that at least two different isoforms of hsp90 are expressed in *Brassica napus* seedlings. An examination of the native state of hsp90 by non-denaturing gel electrophoresis showed that this protein exists as a monomer, dimer and as a high-molecular-mass complex of ca. 680 kDa in cell extracts of spinach cotyledons and leaves, *B. napus* seedlings and wheat germ. Native gel analysis and cross-linking studies of purified hsp90 showed that plant hsp90 exists predominantly as a monomer. When ^{35}S-labelled *B. napus* cytosolic extracts were immunoprecipitated with the R_2 antiserum, hsp90 and two additional proteins with approximate molecular masses of 49 and 45 kDa were detected in the immunoprecipitates. These results are consistent with the idea that hsp90:protein heterocomplexes exist in plant cells.

Introduction

The 90 kDa heat shock protein (hsp90), generally defined as a 'molecular chaperone', has the unique ability to regulate the activities of proteins with which it is normally associated [9, 21]. Proteins complexed with hsp90 in vertebrates and other organisms include the steroid hormone receptors [24, 29], oncogenic tyrosine kinases [4], casein kinase II [20], eIF-2α kinase [28], actin [19], tubulin [26], and calmodulin [18]. In mammalian cells, hsp90 has also been found to exist in a complex with two other hsps, hsp70 and hsp56, also defined as an immunophilin of the FK506 binding class [24]. The functional significance of the mammalian hsp complex has recently been reviewed [24]. A model, supported by several observations, describes the hsp complex as a self-sufficient protein folding system and as a transport particle involved in the trafficking of hundreds of proteins through the cytoplasm.

The interaction of hsp90 with steroid hormone receptors has been studied in the most detail. When complexed with hsp90, the receptor is incapable of binding DNA but has the ability to bind the steroid with a high affinity [23, 24]. In the presence of the hormone, hsp90 is released and the receptor is transformed to the DNA-binding form. The function of hsp90 is thus two-fold: (1) to stabilize the hormone-binding conformation of the steroid receptor, and (2) to mask the DNA-binding domain of the receptor in the absence of the hormone. hsp90 has been shown to inhibit the kinase activity of $pp60^{v-src}$, which enters into an association with hsp90 immediately after it is translated and remains associated with hsp90 until transported to the membrane [4]. Besides these relatively well described interactions of hsp90, it is speculated that

other as yet unidentified proteins exist in a complex with hsp90, with their biological activity being regulated through their association with hsp90. While the functions of hsp90 and the nature of its interaction with other proteins continue to be studied in detail in vertebrate and yeast cells, our knowledge of hsp90 in plant cells remains very limited. The recent finding that brassinosteroids (plant steroids) have an essential role in plant development [14], raises the possibility that an interaction between hsp90 and a yet to be identified steroid receptor may also occur in plant cells.

Hsp90 genes have been isolated from several plant species, including tomato [11], *Arabidopsis* [6, 32], maize [16], *Pharbitis nil* [8] and *Brassica napus* [12]. hsp90 transcripts were found to be strongly expressed during embryogenesis and pollen development [16] and to be present at high levels in shoot and root apices [11]. We have found hsp90 to be constitutively expressed in all *B. napus* tissues examined, albeit at different levels, and to increase upon exposure of plants to both high and low temperatures [12]. While these observations suggest a role for hsp90 in plant growth and development and during stress, studies leading to the understanding of how hsp90 may function in plant cells have not been reported.

We report here the generation and characterization of a polyclonal antibody specific for plant hsp90. Using this antibody we have investigated the isoform composition and the native forms of hsp90 in plant cells. We also show that hsp90 and two other proteins, presumably complexed with hsp90, are immunoprecipitated by the R_2 antibody from plant cell lysates.

Materials and methods

Production of TrpE-hsp90 fusion protein

The isolation and structural characterization of *Pharbitis nil hsp83A* gene has previously been described [8]. A region of this gene (residues 1923–2643, encoding 240 amino acids of the C-terminal half of the protein) was cloned into the *Eco*RI site of plasmid vector pATH1 [7] to give pTH90. Plasmid pTH90, containing a portion of the *hsp83A* coding sequence fused in frame with the *trpE* gene, was used to transform *Escherichia coli* strain M0412 (recA$^-$ LE392). Transformed cells were grown overnight in medium A (M9 medium containing 0.5% casamino acids, 0.2% glucose, 20 μg/ml tryptophan and 100 μg/ml ampicillin). Cells were collected by centrifugation, washed once with medium A minus tryptophan and then resuspended in the same medium. This culture was used to inoculate 250 ml of medium A minus tryptophan. Cells were grown to A_{600} 0.5 units at which time 250 μl of a 20 mg/ml stock solution of indole acrylic acid in alcohol was added and the cells were allowed to grow for an additional 2 h. Induced cells were collected by centrifugation, resuspended in a buffer containing 20 mM Tris-HCl pH 8.0, 50 mM NaCl and 1 mM EDTA, and lysed using a French pressure cell. The cell lysate was centrifuged at 2000 rpm for 10 min in a Beckman JA-17 rotor to remove unlysed cells, and then at 12 000 rpm for 20 min to pellet the inclusion bodies containing the fusion protein. The pellet containing the inclusion bodies was dissolved in 1$\times$ SDS sample buffer (0.0625 M Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol and 0.004% bromophenol blue). Proteins were separated on 10% preparative SDS-polyacrylamide gels [13]. Following electrophoresis, proteins were visualized by staining with 0.3 M $CuCl_2$ solution. The band representing the induced hybrid protein with an apparent molecular mass of 59 kDa, was excised and destained in 0.25 M EDTA and 0.25 M Tris-HCl pH 9.0 for 30 min, followed by incubation in elution buffer (0.025 M Tris-HCl, 0.192 M glycine pH 8.3) for 1 h. The hybrid protein was eluted from the gel overnight at 100 V using a Schleicher and Schuell Elutrap electroseparation system. The gel-purified protein was used for antibody production in rabbits and subsequently for affinity purification of the anti-hsp90 antibody.

Antibody generation

Before immunization, the rabbit was bled to collect preimmune serum. A purified preparation of the trpE-hsp90 fusion protein (250 μg) was mixed 1:1 (v/v) with Freund's complete adjuvant (BRL) and subcutaneously injected into the rabbit. An additional injection with 200 μg of protein mixed with incomplete Freund's adjuvant was done after about 3 weeks. Antiserum was collected 3 weeks after the second injection with the antigen.

Protein purification

hsp90 was purified from wheat germ [30]. A *B. napus* hsp90 cDNA, modified to encode a histidine tag at the C-terminus, was expressed in insect cells using the baculovirus expression system. The histidine-tagged protein was purified by passage over Ni^{2+}-

NTA-agarose (M. Park, C. Y. Kang and P. Krishna, manuscript submitted).

Plant material

For western blot analysis, seedlings of oilseed rape (*B. napus* L. cv. Westar), spinach (*Spinacia oleracea* L. cv. Savoy), Japanese morning glory (*Pharbitis nil* Choisy cv. Violet), soybean (*Glycine max* L. cv. Harosoy), maize (*Zea mays* L. cv. Dazzle), rye (*Secale cereale* L. cv. Musketeer), barley (*Hordeum vulgare* L. cv. Caddette), and wheat (*Triticum aestivum* L. cv. Monopol), were grown at 20 °C in growth chambers under 16 h day length (225 μE m^{-2} s^{-1}). Seedlings were subjected to heat shock at 39 °C for 2 h before harvesting. The portion of seedlings above the soil was collected, immediately frozen in liquid nitrogen and stored at −80 °C. For analysis of the native forms of hsp90, spinach cotyledons were collected before and after heat shock at 39 °C, as described above, and used immediately for making protein extracts.

For immunoprecipitation studies, *B. napus* seeds were germinated for 4 days in the dark on moist filter paper at 20 °C. Seedlings were radiolabeled with 200 μCi of Trans ^{35}S label (ICN Radiochemical Co.) in 5 ml deionized water for 6 h at 20 °C. After labelling, seedlings were washed three times with water and used immediately for preparation of protein extracts.

Preparation of cellular proteins for western blotting

Plant tissue was ground with a mortar and pestle in 1 ml extraction buffer A (20 mM Tris-HCl pH 8.0, 1 mM EDTA, 20 mM NaCl, 1 mM PMSF, 1 mM benzamidine, 1 μg/ml leupeptin, 2 μg/ml pepstatin A, 2 μg/ml pepstatin A, 2 μg/ml aprotinin) per gram tissue fresh weight. Wheat germ (ICN Biomedicals) was extracted into 10 ml extraction buffer A per gram weight. The homogenates were clarified by centrifugation at 4 °C for 45 min at 100 000 × g. Protein concentration was determined using the Bradford method (BioRad).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis

Proteins were separated by SDS-PAGE [13]. Gels were stained with Coomassie Brilliant Blue R-250, or where indicated, with silver stain (BioRad). To identify hsp90, proteins separated on SDS-polyacrylamide gels were electroblotted onto nitrocellulose membranes as described previously [12]. hsp90 was detected by sequential incubation with the R_2 antiserum and with either alkaline phosphatase-conjugated goat anti-rabbit IgG or horseradish peroxidase-conjugated anti-rabbit IgG, as described [12].

Two-dimensional gel electrophoresis

Proteins of *B. napus* seedlings were separated by two-dimensional PAGE [2, 3] and visualized by immunoblotting. The isoelectric focusing (IEF) gels and protein dilution buffer contained 2% ampholines (LKB Instruments) consisting of 40% pH 3.5–10 and 60% pH 5–8 ampholines. The SDS-separating gels were composed of a 7.5–17.5% linear gradient of polyacrylamide.

Native pore exclusion limit gel electrophoresis

Spinach cotyledons were ground in 1 ml buffer A per gram tissue. The homogenate was centrifuged at 4 °C for 45 min at 100 000 × g. A portion of the supernatant containing 30 μg of protein was mixed with an equal volume of the sample loading buffer (0.0625 M Tris-HCl pH 8.8, 20% glycerol, 0.002% bromophenol blue). Non-denaturing PAGE was performed on 4–22.5% glycerol gradient gels as described [5] but without a pH 6.8 stacking gel. The gel was run at 100 V for 48 h at 4 °C. After electrophoresis, the native gels were incubated for 30 min with transfer buffer containing 0.25% SDS and electroblotted as described above for SDS-polyacrylamide gels.

Gel filtration chromatography

Spinach leaves were homogenized in extraction buffer A, using a kitchen blender. The homogenate was filtered through several layers of cheesecloth and centrifuged at 100 000 × g for 45 min. The supernatant was adjusted to pH 8.0 and clarified by vacuum filtration before loading on an AcA44 (IBF Biotechnic, France) gel filtration column (100 cm × 2.5 cm), equilibrated with modified extraction buffer A (20 mM Tris-HCl pH 8.0, 1 mM EDTA, 20 mM NaCl, 1 mM PMSF). Fractions of 5 ml were collected and 40 μl aliquots were analyzed by both native pore exclusion limit gel electrophoresis and SDS-PAGE.

Protein cross-linking and SDS-PAGE analysis

The recombinant hsp90, expressed and purified from insect cells, was diluted in 20 mM Hepes pH 7.5 to an approximate final concentration of 0.1 mg/ml. Cross-linking was initiated at room temperature by the addition of glutaraldehyde to 0.1% in a 15 μl reaction containing 1.5 μg of the pure protein. The reaction was terminated by the addition of 5 μl of 1 M Tris-HCl pH 8.0 and prepared for SDS-PAGE. The various forms of the protein were separated by electrophoresis on 5–20% gradient SDS-polyacrylamide gels and visualized by silver staining.

Immunoprecipitation

Seedlings were ground using a mortar and pestle in 2 ml per gram tissue weight of modified RIPA extraction buffer (0.01 M sodium phosphate pH 7.2, 0.15 M NaCl, 1% NP-40, 1% sodium deoxycholate, 2 mM EGTA, 1 mM PMSF, 1 mM benzamidine, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 2 μg/ml aprotinin) at 4 °C. The homogenate was clarified by centrifugation at 100 000 $\times$ g for 45 min at 4 °C. Protein A-Sepharose beads (Sigma) were preswollen in RIPA buffer and washed three times with RIPA buffer plus 0.3% BSA. Protein A-Sepharose beads (50 μl of a 20% slurry) were incubated on ice with 25 μl preimmune or R_2 immune serum for 2 h, with periodic resuspension. Beads were washed three times with 1 mL RIPA buffer plus 0.3% BSA. ^{35}S-labelled or unlabelled cellular extracts (500 μl) were added to the washed beads and incubated for 2 h at 4 °C with agitation. Beads were washed twice with 1 ml RIPA buffer plus 0.3% BSA, and twice with 1 ml RIPA buffer. Immunoadsorbed proteins were extracted directly into 1$\times$ SDS sample buffer containing 5% 2-mercaptoethanol, and boiled for 3 min before loading on SDS-polyacrylamide gels. The gels were either silver stained, immunoblotted or prepared for fluorography by fixing, soaking in Amplify (Amersham) and then drying.

Results

Generation of an antibody against plant hsp90

To facilitate studies related to the functions of hsp90 in plants, a polyclonal antibody was raised against plant hsp90 in rabbit. The antigen consisted of a fusion protein comprising a portion of the *P. nil* hsp90 fused with the trpE protein of *E. coli*. The trpE-hsp90 fusion protein was isolated as inclusion bodies from overproducing *E. coli* cells, solubilized and separated on a SDS-polyacrylamide gel. A 59 kDa band representing the fusion protein was excised and the protein eluted from the gel. The gel purified protein, shown in Fig. 1A, was mixed with Freund's adjuvant and injected into a rabbit. The antiserum from this rabbit is referred to as R_2.

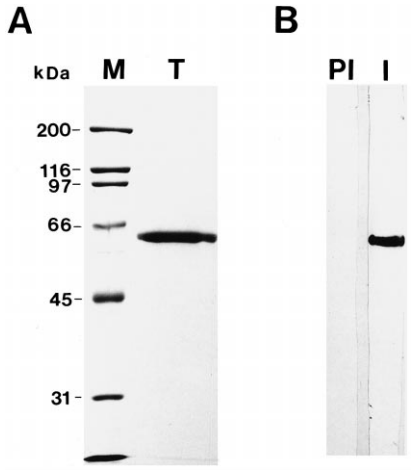

Figure 1. SDS-PAGE and immunoblot analysis of the gel purified trpE-hsp90 fusion protein. A, Purified trpE-hsp90 fusion protein was resolved on a 10% SDS-polyacrylamide gel and stained with Coomassie Blue (lane T). The molecular mass standards are shown in lane M (BioRad). B. Immunoblot of the trpE-hsp90 fusion protein reacted with the preimmune (lane PI) or immune (lane I) serum at a 1:2000 and 1:5000 dilution, respectively, followed by incubation with alkaline phosphatase-conjugated goat anti-rabbit IgG.

Specificity of the R_2 antibody

The specificity of the antibody was tested in a western blot assay using gel purified trpE-hsp90 fusion protein (Fig. 1B). A strong cross-reaction took place with the immune serum used at a 1:5000 dilution, while no cross-reaction was obtained with the preimmune serum, even at a 1:2000 dilution. The ability of the antiserum to detect hsp90 homologues in different plants was tested next in an immunoblot assay. Since the steady-state levels of constitutively expressed hsp90 could vary in different plants, the seedlings were heat-stressed prior to being collected, to ensure accumulation of sufficient amounts of hsp90. The antiserum recognized a polypeptide with an apparent molecular mass of 83 kDa in a variety of dicot and monocot plant seedlings listed in Materials and methods (data not

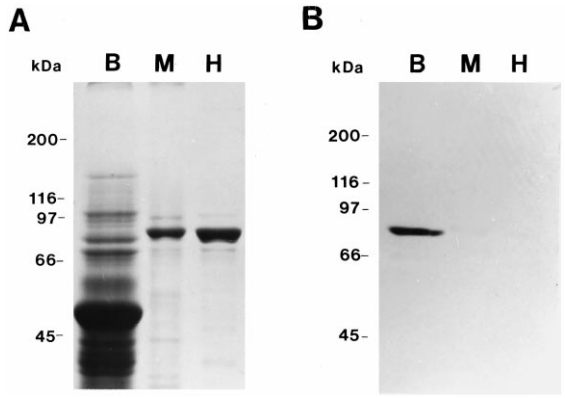

Figure 2. SDS-PAGE and immunoblot analysis of hsp90 in *B. napus* protein extracts and hsp90 purified from mouse and human. A. Total proteins (25 μg) from *B. napus* seedlings (lane B), and purified hsp90 proteins from mouse (lane M) and human (lane H) were separated on a 7.5% SDS-polyacrylamide gel and stained with Coomassie Blue. B. Western blot of *B. napus* extracts and purified mouse and human hsp90, reacted sequentially with the R_2 serum and peroxidase-conjugated anti-rabbit IgG, each at a dilution of 1:5000. The antigen-antibody complexes were detected by the chemiluminescence (ECL) system. Numbers on the left indicate molecular mass standards in kDa (BioRad).

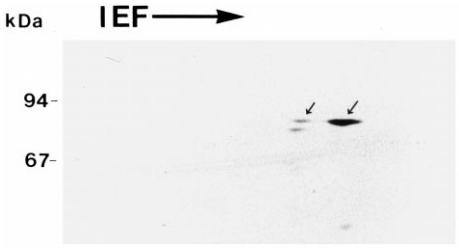

Figure 3. Analysis of *B. napus* hsp90 isoforms separated by two-dimensional gel electrophoresis. Soluble proteins of seedlings were separated by IEF-SDS-PAGE and probed with the R_2 antibody following transfer to nitrocellulose. After incubation with the secondary antibody, the antigen-antibody complexes were detected by chemiluminescence. The numbers on the left indicate molecular mass standards (Pharmacia Biotech.). hsp90 isoforms are indicated by arrows.

shown). The antibody appeared to be specific for the 83 kDa protein, identified as the plant hsp90 homolog, with almost no cross-reactivity with other proteins in cytosolic extracts (Fig. 2B, lane B).

hsp90 is highly conserved in all eukaryotes. Comparison of the amino acid sequences of plant hsp90 with human, *Drosophila* and yeast hsp90 reveals identities of 60–70% [6, 8, 16]. We were interested in knowing if the R_2 antibody cross-reacts with purified mouse and human hsp90 proteins. No cross-reaction was detected (Fig. 2B, lanes M and H), even though considerable amounts of purified mouse and human hsp90 proteins were loaded on the gel. (Fig. 2A, lanes M and H). These results suggest that the R_2 antibody is specific for plant hsp90.

Isoform composition of plant hsp90

In plants, hsp90 is encoded by a multigene family [8, 12, 16]. An approach towards understanding the functions of the various hsp90 genes, is to analyze hsp90 isoforms found in various tissues and to attempt to identify product(s) of individual genes. As a first step, we have analyzed hsp90 isoforms in 4-day old *B. napus* seedlings, using two-dimensional SDS-PAGE and western blotting. Figure 3 demonstrates the presence of at least 2 isoforms of hsp90 in unstressed *B. napus* seedlings, as detected by the R_2 antibody. Whether the R_2 antibody recognizes all hsp90 isoforms and whether all isoforms were resolved in our analysis will become clear once the hsp90 gene family in *B. napus* has been fully characterized. The spot seen below those identified by arrows represents a degradation product of hsp90. This conclusion was reached because a band of similar size was detected when the same extracts were directly subjected to SDS-PAGE.

Native forms of hsp90 in plant cell lysates

Mammalian hsp90 exists predominantly as a dimer and to a lesser extent as a monomer in its native state [17]. In order to detect the native forms of plant hsp90, purified wheat germ hsp90, the histidine-tagged hsp90, and cytosolic extracts of spinach cotyledons were subjected to pore exclusion limit electrophoresis on non-denaturing gradient gels [1]. The gel was blotted and hsp90 was detected with the R_2 antiserum. The histidine-tagged hsp90 and the wheat germ hsp90 were found to consist of monomeric, dimeric and several oligomeric forms (Fig. 4A, lanes 1 and 2). Three bands with approximate molecular masses of 83, 190 and 680 kDa were detected in spinach cell extracts (Fig. 4A, lane 3) and in *B. napus* and wheat germ extracts (not shown). Since the signal for the native forms of hsp90 was most clear in spinach cell extracts, further studies were carried out with spinach. The lower band in lane 3 represents the monomeric form of plant hsp90, the middle band represents the dimeric form, and the band close to the 669 kDa marker protein represents a high-molecular-mass species of hsp90.

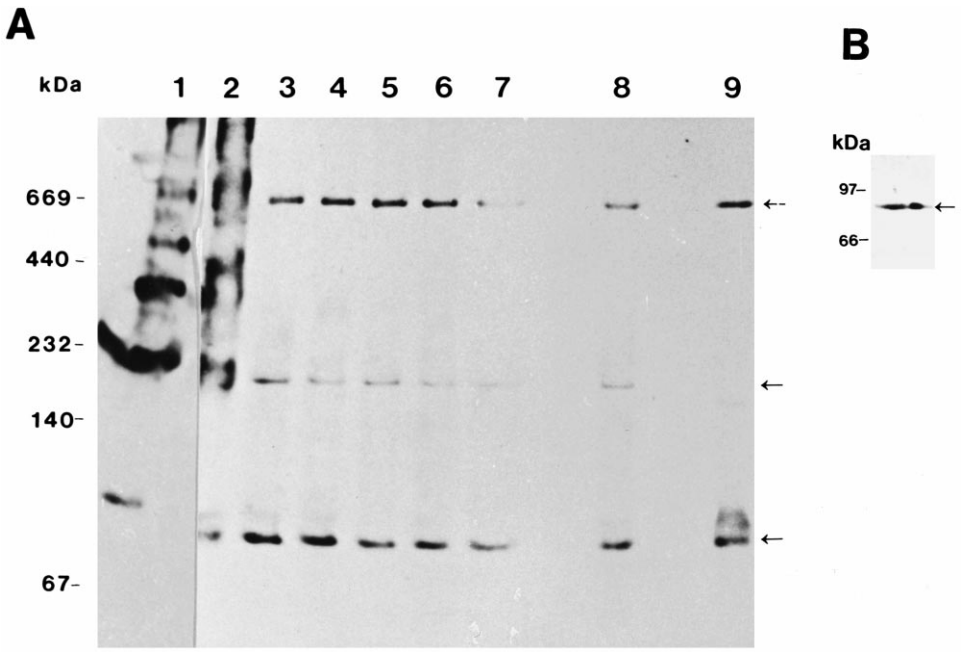

Figure 4. Analysis of the native forms of hsp90 by pore exclusion limit gel electrophoresis. A. Protein extracts of spinach cotyledons, separated on a 4-22.5% gradient non-denaturing gel, were blotted onto nitrocellulose and reacted with the R_2 antibody, followed by incubation with the secondary antibody. The antigen-antibody complexes were detected using chemiluminescence. A recombinant histidine-tagged hsp90 (lane 1), and purified wheat germ hsp90 (lane 2) were also electrophoresed on the same gel. Spinach extracts were prepared in buffer A (lane 3); with 1% NP-40 (lane 4); 2 mM $CaCl_2$ and no EDTA (lane 5); 5 mM $MgCl_2$ and no EDTA (lane 6); 1.5 M NaCl (lane 7); 5 mM ATP and no EDTA (lane 8); and 20 mM DTT (lane 9). The numbers in the left indicate native molecular mass standards in kDa (Pharmacia Biotech.). The positions of the spinach hsp90 monomer, dimer and the high molecular mass form are indicated by arrows. B. Spinach extracts were subjected to non-denaturing gel electrophoresis following which 3 mm thick gel sections were excised close to the position of the 669 kDa marker, equilibrated in SDS sample buffer for 20 min and then subjected to SDS-PAGE. hsp90 was detected by western blotting, using the chemiluminescence detection system.

Heat shock did not change the relative abundance of any of the native species detected in spinach cell extracts, although a slight increase in the amount of protein was evident as compared to the control (not shown). The high molecular mass complex was not affected by different salt conditions, 1.0% NP-40, 5 mM ATP, or 20 mM DTT (Fig. 4A, lanes 4–9), although some changes in the distribution and migration of the monomeric and dimeric forms of hsp90 were seen in the presence of DTT (lane 9).

To confirm the presence of hsp90 in the 680 kDa size band, 3 mm sections were excised from the region just above the 669 kDa marker and from two other regions of the gel below the 669 kDa marker and subjected to SDS-PAGE and immunoblotting. hsp90 was detected at the 83 kDa position following SDS-PAGE and immunoblotting in gel sections corresponding to 680 kDa (Fig. 4B), but not in the two other regions of the native gel included as controls in this analysis.

In order to fractionate the native forms of hsp90, spinach cell extracts were passed over a gel filtration column. An analysis of hsp90 by non-denaturing PAGE in various gel-filtered fractions revealed that the three forms of hsp90 were partially, but not completely, separated from one another (Fig. 5). The majority of the high-molecular-mass complex was detected in fractions 38-41, corresponding to the exclusion limit of the column. When the same fractions were analysed by SDS-PAGE and immunoblotting, an 83 kDa protein was detected (not shown), again confirming the presence of hsp90 in the high molecular mass complex.

Cross-linking of hsp90

The results of Fig. 4 indicate that hsp90 exists predominantly in the monomeric and to a lesser extent in the dimeric state in plant cell extracts. Since hsp90 was present at very high concentrations in the two

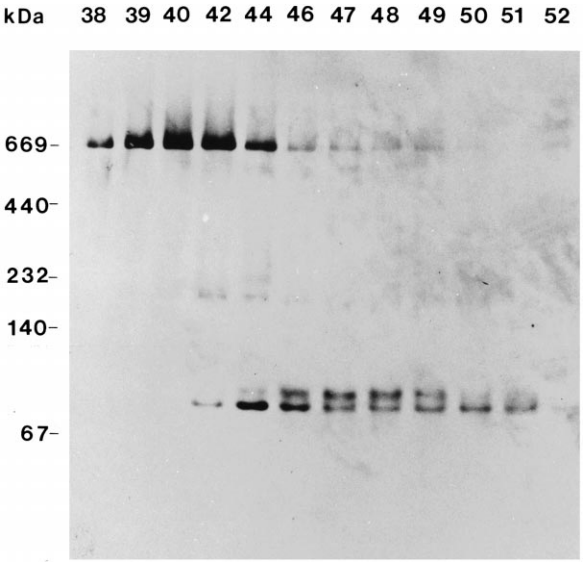

Figure 5. Analysis of hsp90 in gel-filtered spinach cytosolic extracts. An aliquot of gel-filtered fractions (see Materials and methods) were subjected to non-denaturing gel electrophoresis and immunoblotting with the R_2 antibody. The numbers on the top indicate gel filtration fraction numbers and the numbers on the left indicate molecular mass markers in kDa (Pharmacia Biotech.).

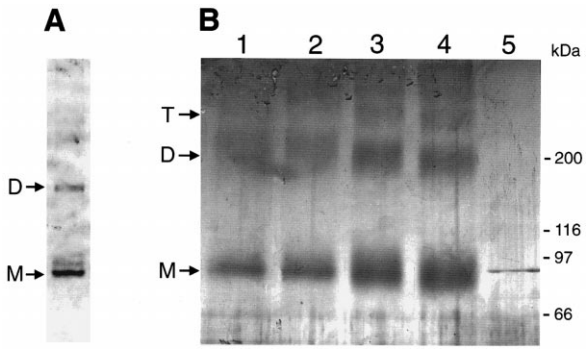

Figure 6. Oligomeric structure of purified hsp90. A. The histidine-tagged hsp90 was diluted to 0.1 mg/mL and then subjected to non-denaturing gel electrophoresis and immunoblotting with the R_2 antibody. B. The same protein was analyzed by SDS-PAGE and silver staining after crosslinking with glutaraldehyde for 1 (lane 1), 2 (lane 2), 4 (lane 3), or 10 (lane 4) min. The non-cross-linked hsp90 is shown in lane 5. The numbers on the right indicate molecular mass markers in kDa. The monomer (M), dimer (D), and tetramer (T) are indicated.

samples shown in lanes 1 and 2 of Fig. 4, it remained unclear as to what forms purified hsp90 would acquire at more dilute concentrations. The native gel analysis was therefore repeated with histidine-tagged hsp90 after dilution of the protein. The protein was also cross-linked with glutaraldehyde and subjected to SDS-PAGE. Figure 6A shows that under native conditions purified hsp90 exists both as monomers and dimers, with the monomer being in higher amounts than the dimer. A similar conclusion is reached upon analyzing the results of cross-linked hsp90 (Fig. 6B). Interestingly tetrameric and other higher oligomeric forms of hsp90 were also detected in this analysis in the absence of any divalent cations (lane 4). This is in contrast to the results obtained with animal hsp90, where higher cross-linked species were detected only in the presence of divalent cations [10]. From these results we conclude that the structural organization of plant hsp90 under native conditions is slightly different from animal hsp90.

Immunoprecipitation of hsp90 and associated proteins from plant lysates

Antibodies capable of binding free hsp90 [27] or both free and complexed hsp90 [22, 25] in vertebrate cells have been described. Antibodies with either property have served as useful tools in the investigation of vertebrate hsp90. The ability of the R_2 antibody to bind free hsp90 and hsp90:protein complexes was tested in an immunoprecipitation assay. Cytosolic extracts of ^{35}S-labelled *B. napus* seedlings were incubated with the R_2 antibody preadsorbed to protein A-sepharose, and immunoprecipitations were analyzed by SDS-PAGE, followed by silver staining or fluorography. An 83 kDa protein, indicated by an arrow, was adsorbed by the R_2 antibody (Fig. 7A and B, lane I) but not by the preimmune serum (lane PI). Fluorography revealed the presence of two other proteins with approximate molecular masses of 45 and 49 kDa, in R_2 immunoprecipitates (Fig. 7B, lane I). Immunoprecipitations were repeated several times with the same results.

The identity of the 83 kDa protein in R_2 immunoprecipitates was examined by immunoblot analysis. A *B. napus* cytosolic extract was included in this analysis as a control (Fig. 7C, lane E). The 83 kDa protein was confirmed as the hsp90 homologue (Fig. 7C, lane I). The identity of the two other proteins is currently not known.

Discussion

Hsp90 is constitutively expressed in various plant tissues and its synthesis is increased in response to both high and low temperatures, suggesting that this protein

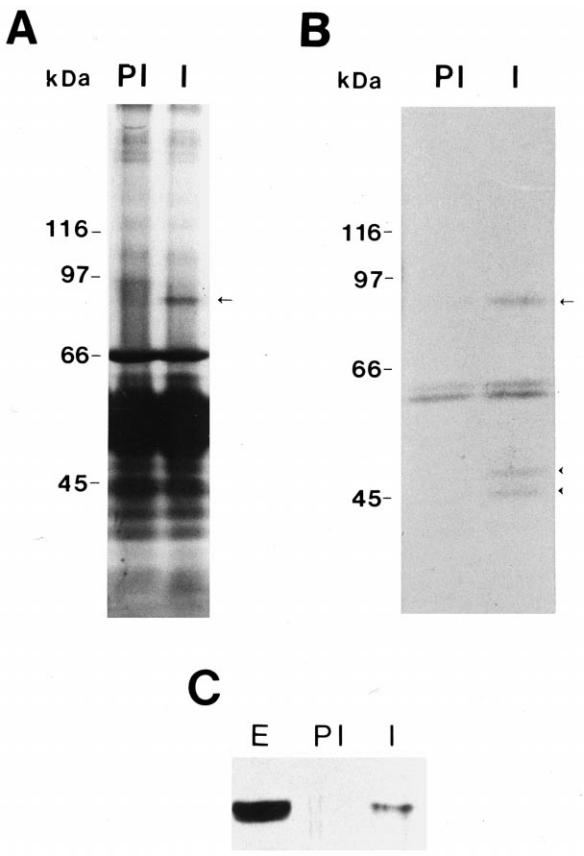

Figure 7. Immunoprecipitation of hsp90 from *B. napus* seedlings. A, Protein extracts of ^{35}S-labelled *B. napus* seedlings were incubated with protein A-Sepharose preadsorbed with preimmune serum (lane PI) or R_2 immune serum (lane I). The washed protein A-sepharose pellets were extracted in SDS-sample buffer and the proteins analyzed by SDS-PAGE and silver staining. B. Fluorogram of a gel described in panel A. hsp90 and the 45 and 49 kDa proteins are indicated by an arrow and arrowheads, respectively. Numbers on the left indicate molecular mass standards in kDa (BioRad). C. Immunoblot detection of hsp90 in preimmune (lane PI) and R_2 (lane I) immunoprecipitates of *B. napus*, using R_2 antiserum. Detection of the same protein in *B. napus* extracts is shown in lane E.

has an important role in plant growth and development as well as during stress [12]. To date, information regarding structural organization of hsp90 in plants is not available. We describe here the characteristics of a polyclonal antibody R_2 raised against a trpE-hsp90 fusion protein as the antigen. The R_2 antibody was found to be specific for plant hsp90, having the ability to interact with hsp90 homologs of both monocot and dicot plants, and to immunoprecipitate hsp90 and associated proteins from cytosolic extracts. These characteristics make R_2 antibody a useful tool in the analysis of hsp90 and of its functions in plant cells. Using this antibody we show here that hsp90 exists in monomeric and dimeric forms and a high-molecular-mass form in plant cell extracts.

The results represented in Fig. 2 clearly indicate that R_2 is specific for plant hsp90. This property of R_2 antibody has allowed us to recently show that animal and plant cell lysates share a conserved chaperone system that can fold the glucocorticoid receptor into a functional heterocomplex with hsp90 [30]. The lack of cross-reaction of R_2 with mammalian hsp90 is not unusual as it has been noted before that inter-species cross-reacting anti-hsp90 antibodies are not easily generated [25]. In the previous study, hsp90 purified from rabbit liver was used as the antigen [25], as opposed to the fusion protein used in this study. In both cases interspecies cross-reacting antibodies were not obtained. A comparison of the amino acid sequences of *P. nil* hsp90 with that of human hsp90 revealed residue identities of 69 and 80% for the first and the second exon of *P. nil* hsp90, respectively, 72% for the region of the third exon not included in the fusion protein, and 68% for the region used in constructing the fusion protein. Since vast differences in identity and similarity were not observed among different regions of hsp90, it is unlikely that the high specificity of the R_2 antibody for plant hsp90 arises solely from the region of hsp90 included in the fusion protein. Further analysis of hsp90 from different organisms in the future may allow us to understand the reason for specific immunological responses to hsp90 from different origins.

The functions of hsp90 have been demonstrated to occur through protein:protein interactions [9, 24]. Characterization of the interaction of hsp90 with proteins in other organisms has largely involved the use of antibodies capable of binding to hsp90:protein complexes [22, 25, 31]. With an antibody that has the ability to bind plant hsp90:protein complexes, it will be possible to examine the proteins that interact with hsp90 in plant cells. Using the R_2 antibody in immunoprecipitation studies, we have detected two proteins that may be in a complex with hsp90 in intact cells. Proteins that bind immunosuppressant drugs and have peptidylprolyl isomerase activity have been previously identified as components of the hsp90 heterocomplex in animal cells [24]. Due to similarities in the sizes of the two proteins detected in the R_2 immunoprecipitates and the immunophilins in the hsp90 heterocomplex [24], we propose that one or both of these proteins may be plant immunophilins. Larger-size immunophilins have been identified in plants [15]; however, since antibodies to these proteins are currently not available, our hypo-

thesis could not be tested. Our future goal is to identify these proteins by obtaining them in larger quantities and determining their N-terminus sequences.

Non-denaturing PAGE of cytosolic extracts resolved hsp90 into monomeric, dimeric and a high-molecular-mass form. The higher-molecular-mass species could either be an oligomer of hsp90 or a heterocomplex composed of hsp90 and other proteins, as discussed above. One obvious difference between the native forms of plant hsp90 and mammalian hsp90 is that the latter exists predominantly in the dimeric form where as plant hsp90 appears to be predominantly in the monomeric form. This may be due to weaker interactions between subunits of plant hsp90. We have previously noticed that plant hsp90 is less efficient than animal hsp90 in its ability to assemble the glucocorticoid receptor into a functional heterocomplex with hsp90 [30]. This may arise from differences in functional native forms or phosphorylation states of the two hsp90s, or in the chaperone complex preformed with hsp90. It will be interesting to find out in the future how plant hsp90 differs from hsp90 from other organisms both structurally and functionally, and what is the nature of the chaperone complex in plants.

Acknowledgements

We would like to thank Dr W. B. Pratt for providing purified mouse hsp90: Drs R. A. Cook and J. William for assistance with gel filtration; Stress Gen Biotechnologies Corporation for providing purified human hsp90; J. F. Cherutti for helping with amino acids comparisons; and Stefani Tichbourne and Tracey Toso for preparation of this manuscript. This work was supported by a research grant from the Natural Sciences and Engineering Research Council of Canada to P.K. Dr R. K. Reddy and M. Sacco contributed equally to the work reported in this article.

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