Binding of Immunophilins to the 90 kDa Heat Shock Protein (hsp90) via a Tetratricopeptide Repeat Domain Is a Conserved Protein Interaction in Plants†

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ABSTRACT: In animal cell lysates, multiprotein complexes containing hsp90, hsp70, p60, p23, and several immunophilins can assemble steroid receptors and oncogenic protein kinases, such as v-Src and v-Raf, into heterocomplexes that contain hsp90 and either immunophilins or, in the case of protein kinases, p50. The complexes with hsp90 are required for the proper functioning of these signal transduction systems. Wheat germ lysate contains a similar protein folding activity that forms functional steroid receptor complexes with hsp90, but not all the components of this system have been identified. The plant chaperone system has conserved interactions with animal chaperones in that wheat hsp70 functions in the rabbit reticulocyte lysate heterocomplex assembly system and human p23 functions in the wheat germ lysate. Here, we ask if wheat germ lysate also contains immunophilins of the FK506-binding class (FKBPs) that bind to the hsp90 component of the chaperone complex *via* tetratricopeptide repeat (TPR) domains. To demonstrate the plant heterocomplex, we add purified mammalian p23, preadsorbed with the JJ3 antibody to protein A−Sepharose, to wheat germ lysate and allow ATP-dependent formation of an animal p23·plant hsp90 complex. The complex is then washed and incubated with the radiolabeled immunosuppressant drug [^{3}H]FK506, which binds in a specific manner to a coimmunoadsorbed plant FKBP. Binding of the plant FKBP to plant hsp90 is prevented by adding to wheat germ lysate a purified fragment containing the TPR domains of human cyclophilin-40. Geldanamycin, a benzoquinone ansamycin that binds to animal hsp90s and prevents their chaperone activity, binds in a temperature-dependent manner to wheat hsp90 to block formation of the p23·hsp90·FKBP heterocomplex. These data show that immunophilin binding to hsp90 *via* TPR domains is conserved in the plant kingdom as well as in the animal kingdom and that geldanamycin will be an important tool for the study of hsp90-mediated protein chaperoning in plant cells.

The immunophilins are ubiquitous and conserved proteins that bind immunosuppressant drugs such as FK506, rapamycin, and cyclosporin A (CsA)[1] [for a review, see Walsh et al. (1992)]. All members of the immunophilin protein family have peptidylprolyl isomerase (PPIase) activity, suggesting that they may play a role in protein folding in the cell (Schmid, 1993). The immunophilins may be divided into two classes: the FKBPs are binding proteins for FK506 and rapamycin, and the cyclophilins (CyPs) bind cyclosporin A. The immunosuppressant drugs occupy the prolyl-isomerase sites on the immunophilins and inhibit isomerization *in vitro*. As yet, there is only limited evidence that immunophilins play a role in protein folding *in vivo* (Heitman et al., 1992).

The low molecular weight immunophilins, such as FKBP12 and CyPA and -B, are thought to be the cellular components responsible for inhibition of T cell activation by the immunosuppressant drugs. The FKBP12−FK506 and the CyP−CsA complexes bind to and inhibit the activity of calcineurin, a Ca^{2+}/calmodulin-dependent protein phosphatase, blocking the signal pathway for T cell activation (Liu et al., 1991; Clipstone & Crabtree, 1992). Cytosolic CyPs (Gasser et al., 1990; Marivet et al., 1992) and FKBPs (Luan et al., 1993) with homology to the small immunophilins of mammals have been identified in higher plants. There is also evidence consistent with the presence of a calcineurin-like phosphatase in plants that is inhibited by FKBP−FK506 and CyP−CsA complexes (Luan et al., 1993).

Three larger immunophilins have been identified as components of steroid receptor complexes in animal cell cytosols. FKBP52 (also called p59, FKBP59, and hsp56) was isolated as a receptor-associated protein (Tai et al., 1986) and later shown to be a stress protein (Sanchez, 1990) that binds FK506 and rapamycin (Tai et al., 1992; Yem et al., 1992). Rabbit (Lebeau et al., 1986), human (Peattie et al., 1992), and mouse (Schmitt et al., 1993) cDNAs for FKBP52 have been cloned, and the protein has been shown to have PPIase activity that is inhibited by FK506 (Peattie et al.,

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[1] Abbreviations: CsA, cyclosporin A; CyP, cyclosporin A-binding protein; FKBP, FK506-binding protein; hsp, heat shock protein; TPR, tetratricopeptide repeat; PPIase, peptidylprolyl isomerase.

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1992). A 54 kDa component of the chicken progesterone receptor complex has significant sequence identity with FKBP52 (Smith et al., 1993b) and has been shown to bind FK506 (Smith et al., 1993a). In addition to these FKBPs, a cyclophilin that copurified with the bovine estrogen receptor was cloned (Ratajczak et al., 1993) and shown to be the same as a 40 kDa cyclosporin A-binding protein (CyP-40) previously purified from bovine brain (Kieffer et al., 1992) and cloned from a human library (Kieffer et al., 1993). CyP-40 has now been found in native progesterone and glucocorticoid receptor heterocomplexes as well (Milad et al., 1995; Renoir et al., 1995; Owens-Grillo et al., 1995).

A portion of the FKBP52 and a portion of the kinase-associated p50 in mammalian cell cytosols exist in a multiprotein complex with the abundant heat shock proteins hsp90 and hsp70 (Sanchez et al., 1990; Perdew & Whitelaw, 1991; Whitelaw et al., 1991; Smith et al., 1993c). Both hsp70 and hsp90 are components of a multiprotein chaperone system that "folds" the steroid receptors and protein kinases, such as v-Src and v-Raf, into a heterocomplex with hsp90 and the high molecular weight immunophilins, or hsp90 and p50 in the case of protein kinases [for review of heterocomplex assembly, see Pratt (1993) and Smith and Toft (1993)]. hsp70 is required not only for binding steroid receptors to hsp90 but also for the presence of immunophilin in the receptor heterocomplex (Hutchison et al., 1994b). Immune adsorption of hsp90 from reticulocyte lysate results in copurification of the necessary components to assemble the glucocorticoid receptor into a heterocomplex with hsp90 and fold the hormone-binding domain of the receptor into a steroid-binding conformation (Hutchison et al., 1994a). It should be noted that the receptor must be bound to hsp90 to have a steroid-binding site (Bresnick et al., 1989). A minimal receptor·hsp90 heterocomplex assembly system has now been reconstituted from purified hsp90, hsp70, p60, and p23, without the presence of immunophilins (Dittmar et al., 1996).

In direct binding studies with purified proteins, we have shown that FKBP52 (Czar et al., 1994) and CyP-40 (Owens-Grillo et al., 1995) bind to a common binding site on hsp90. The C-terminal 150 amino acids of CyP-40 share 30.7% identity with an internal region of FKBP52 (Kieffer et al., 1993) that contains 3 repetitive sequence motifs of 34 amino acids called tetratricopeptide repeat or TPR domains (Ratajeczak et al., 1993). The TPR domains of hsp56 and CyP-40 are required for their binding to hsp90 (Radanyi et al., 1994; Hoffmann & Handschumacher, 1995), and mammalian hsp90 binds a variety of TPR domain proteins to this site (Owens-Grillo et al., 1996).

In 1990, Schena et al. demonstrated that the glucocorticoid receptor expressed in plant cells is capable of activating a reporter gene linked to glucocorticoid response elements upon treatment of plant cells with glucocorticoid. Because hsp90 is required for the glucocorticoid receptor to bind steroid (Bresnick et al., 1989), we reasoned that a multicomponent hsp90/hsp70-based chaperone system must also exist in plant cells. Recently, we showed that wheat germ lysate has a chaperone system that assembles the glucocorticoid receptor into a functional heterocomplex with hsp90 (Stancato et al., 1996). We have shown that purified plant hsp70 interacts with the mammalian chaperone system (Stancato et al., 1996) and that purified human p23, a component of the mammalian foldosome, interacts with the plant chaperone system (Hutchison et al., 1995), suggesting there exists a high conservation of the protein−protein interactions and of the chaperoning mechanism between the animal and plant kingdoms.

Our goal in this work is to ask whether plant immunophilins, like the high molecular weight mammalian cell immunophilins, are associated with hsp90 in a multiprotein structure. Recently, the existence of a 55 kDa FKBP that could be a possible homolog of FKBP52 was demonstrated in leaf tissue of *Vicia faba* (Luan et al., 1994). Here, we demonstrate that a plant FKBP is bound to plant hsp90 in wheat germ lysate in a manner that is abrogated by the addition of bacterially-expressed human CyP-40 C-terminal fragment containing the TPR domains. This suggests that binding of immunophilins to hsp90 via their TPR domains is a fundamental protein−protein interaction common to both plant and animal systems. We also show that geldanamycin, a compound that binds to mammalian hsp90s and reverts v-Src-induced oncogenic transformation (Whitesell et al., 1994), interacts with plant hsp90 to block the assembly of a p23·hsp90 heterocomplex.

EXPERIMENTAL PROCEDURES

Materials. Untreated rabbit reticulocyte lysate was from Green Hectare's (Oregon, WI). Wheat germ lysate was purchased from Promega (Madison, WI). [^{3}H]Dihydro-FK506 (100 Ci/mmol) and ^{125}I-conjugated goat anti-mouse and anti-rabbit IgGs were from DuPont NEN (Boston, MA). Protein A−Sepharose and AMP-PNP were from Sigma (St. Louis, MO). Phenyl Sepharose High Performance was from Pharmacia Biotech. Cyclosporin A was provided by Sandoz Research Institute (East Hanover, NJ), and nonradioactive FK506 and rapamycin were provided by the Upjohn Co. (Kalamazoo, MI). Geldanamycin was provided by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute. The purified, bacterially-expressed human p23 and the JJ3 monoclonal IgG against p23 (Johnson et al., 1994) were kindly provided by David O. Toft (Rochester, MN). The rabbit R2 antiserum against plant hsp90 was raised against a fusion protein comprising a portion of *Pharbitis nil* (Japanese morning glory) hsp90 fused to the trpE protein of *Escherichia coli* (Krishna et al., 1995), and a detailed description of the antibody will be published elsewhere (P. Krishna, M. Sacco, J. R. H. Frappier, and R. F. Felsheim, submitted for publication). CyP-4059 is a bacterially-expressed human CyP-40 C-terminal fragment containing the FKBP52-like TPR domain, but not the CyP-18-like domain, and it was purified by Ni^{2+} affinity chromatography and thrombin cleavage (Hoffman and Handschumacher, in preparation).

Purification of p23 from Rabbit Reticulocyte Lysate. The fractionation of rabbit reticulocyte lysate on DE52 has been described previously (Hutchison et al., 1995). Briefly, rabbit reticulocyte lysate was chromatographed on DE52 equilibrated with HE buffer (10 mM HEPES, 1 mM EDTA, pH 7.35), and the proteins were eluted with a 400 mL gradient of 0−0.5 M KCl. hsp90 and p23 were detected by resolving an aliquot of each fraction by SDS−PAGE and Western blotting with appropriate antibodies. The fractions containing p23 but not hsp90 were combined, concentrated to one-tenth the original volume of lysate, and dialyzed against 10 mM

HEPES, 25 mM KCl, and 2 mM dithiothreitol. The final preparation was designated fraction C as presented in Figure 5A of Hutchison et al. (1995), and small aliquots were flash-frozen. Because p23 is highly acidic, DE52 chromatography provides a high degree of purification, and this material was used as a source of p23 for most of the experiments. If fraction C is precipitated with 1.5 M ammonium sulfate and the supernatant is fractionated on phenyl-Sepharose in a 10 mM phosphate buffer with a 1.5−0 M ammonium sulfate gradient, p23 is purified essentially to homogeneity, and this preparation was used for some experiments.

p23 Immunoadsorption. Prior to immunoadsorption, the JJ3 antibody was prebound to protein A−Sepharose (PAS) pellets by incubating 40 μL of a 20% slurry of PAS with 5 μL of ascites and 300 μL of HEG buffer (10 mM HEPES, 1 mM EDTA, and 10% glycerol, pH 7.35) for 1 h at 4 °C, followed by centrifugation and washing with HEG. p23 was immunoadsorbed from 5 μL of purified bacterially-expressed p23 (2 mg/mL) diluted in 25 μL of HE, or 30 μL of DE52 fraction C (2 mg of protein/mL) from rabbit reticulocyte lysate by rotation for 1 h at 4 °C with the JJ3−protein A−Sepharose pellet. Immunoadsorbed p23 was washed once with 1 mL of HEG prior to incubation with wheat germ lysate.

Mammalian p23·Plant Immunophilin Heterocomplex Formation and [^{3}H]FK506 Binding. JJ3 immunopellets containing p23 were incubated with 50 μL of wheat germ lysate and incubated at 0 °C for 30 min (unless otherwise noted) with resuspension of the pellets every 5 min. When additions were made to the assay mixture, volumes were kept constant by adding 5 μL of 10 mM HEPES, 100 mM KCl, pH 7.35, or 5 μL of 50 mM AMP, AMP-PNP, or ATP per 50 μL of wheat germ lysate. The immunopellets were washed by suspension and centrifugation in 1 mL of HEG buffer. To assay [^{3}H]FK506 binding, the immunopellets were suspended in 25 μL of HE buffer with 0.02% Triton X-100 containing 100 nM [^{3}H]FK506. After 2 h of incubation on ice, the immune pellets were washed twice with 1 mL of HE buffer containing 0.02% Triton X-100, and the tritium radioactivity was assayed. In experiments using geldanamycin, wheat germ lysate was preincubated for 10 min with the compound at a final concentration of 20 μg/mL or with 5% DMSO vehicle at either 0 °C or 30 °C as indicated. The geldanamycin-containing lysate was then added to the immunoadsorbed p23−protein A−Sepharose pellets, the mixture was incubated an additional 30 min, and [^{3}H]FK506 binding was assayed as described above.

For the experiment of Figure 4, wheat germ lysate (1 mL) was dialyzed (molecular mass cutoff ∼14 kDa) overnight against 100 mL of HEPES, pH 7.35, 100 mM KCl, and 2 mM DTT, followed by a second dialysis of 2 h against fresh buffer. The dialyzed lysate was used immediately for heterocomplex formation.

Gel Electrophoresis and Western Blotting. The immunoadsorbed protein A−Sepharose pellets were heated in SDS sample buffer with 10% β-mercaptoethanol, and proteins were resolved on 12% SDS−polyacrylamide gels and transferred to Immobilon-P membranes. Membranes were probed with 0.2% R2 antiserum against plant hsp90 or 0.1% JJ3 ascites against p23. The immunoblots were incubated a second time with the appropriate ^{125}I-conjugated counter-antibody to visualize the immunoreactive bands.

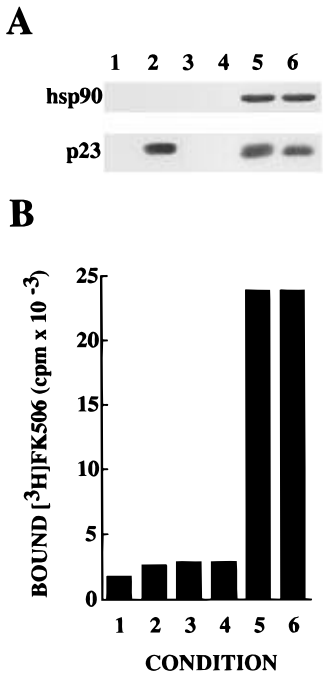

FIGURE 1: Purified, bacterially-expressed human p23 can form a heterocomplex with wheat hsp90 and a wheat FKBP. (A) Western blots. (B) [^{3}H]FK506 binding to immune pellets. For both panels A and B, conditions were as follows: lanes 1 and 2, buffer containing p23 was immunoadsorbed with nonimmune IgG-bound or JJ3-bound protein A−Sepharose, respectively; lanes 3 and 4, similar immunoadsorption of 50 μL of wheat germ lysate incubated for 30 min at 30 °C without p23; lanes 5 and 6, JJ3 immunoadsorption of 50 μL of wheat germ lysate that had been incubated with 5 μL of purified human p23 for 30 min at 30 °C (lane 5) or 0 °C (lane 6).

RESULTS

Coimmunoadsorption of hsp90 and an FKBP from Wheat Germ Lysate. Because there is no high-capacity immunoadsorbing antibody that reacts with plant hsp90s, like the IgM antibodies used to immunoadsorb mammalian hsp90 heterocomplexes (Perdew & Whitelaw, 1991), we have taken an indirect approach that utilizes a very effective immunoadsorbing antibody against mammalian p23 to coimmunoadsorb plant hsp90. An antibody to p23 has been used to isolate complexes from mammalian cell lysates that contain hsp90, the three immunophilins, FKBP52, FKBP54, and CyP-40, and minor amounts of hsp70 (Johnson & Toft, 1994). Here we add purified mammalian p23 to wheat germ lysate and allow assembly of a mammalian p23·plant hsp90 complex that can be immunoadsorbed with anti-p23. Because we have no antibodies against wheat germ FKBPs, we demonstrate the presence of immunophilin by binding [^{3}H]FK506 directly to the immunopellet, a method we have used previously to demonstrate the presence of a novel immunophilin in the complex of hsp90 with the viral transforming protein v-Raf (Stancato et al., 1994).

In the experiment of Figure 1, purified bacterially-expressed p23 was added to wheat germ lysate, and the mixture was incubated for 30 min at 30 °C (lane 5) or 0 °C (lane 6) prior to immunoadsorption of p23. It can be seen in lanes 5 and 6 of Figure 1A,B that immunoadsorption of the human p23 is accompanied by coimmunoadsorption of wheat hsp90 and that the resulting immunopellet binds [^{3}H]-FK506. Purified p23 has no FK506 binding associated with it (lane 2), and if p23 is not added to the wheat germ lysate,

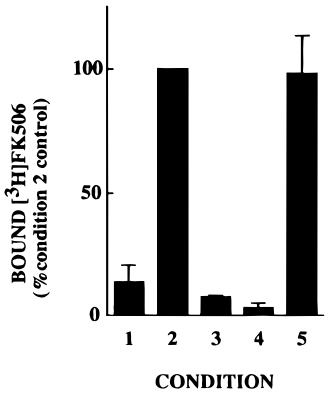

FIGURE 2: Coimmunoadsorbed wheat heterocomplex binds [^{3}H]-FK506 in an FKBP-specific manner. Wheat germ lysate was incubated with immunoadsorbed, purified rabbit p23 for 30 min at 0 °C. At the end of the incubation, the p23 immune pellets (i.e., p23 immunoadsorbed to protein A−Sepharose) were washed and incubated with 100 nM [^{3}H]FK506 in the presence of a 1000-fold excess of nonradioactive competitor. Conditions are: lane 1, lysate incubated with JJ3 prebound to protein A−Sepharose in the absence of p23, no competitor; lane 2, wheat germ lysate plus p23, no competitor; lanes 3−5, lysate plus p23 plus competing FK506 (lane 3), rapamycin (lane 4), or cyclosporin A (lane 5). The data are expressed as a percent of the [^{3}H]FK506 binding in condition 2 and represent the average of 3 experiments ± SEM.

immunoadsorption with the JJ3 antibody does not yield hsp90 or [^{3}H]FK506 binding activity in the immune pellet (lane 4).

Figure 2 shows that the FK506-binding activity in the immune pellet is specific for an FKBP. In these experiments, replicate immune pellets prepared with protein A−Sepharose-prebound p23 and wheat germ lysate were incubated with [^{3}H]FK506 in the absence of competitor (lane 2) or in the presence of a 1000-fold excess of FK506 (lane 3), rapamycin (lane 4), or cyclosporin A (lane 5). Competition by FK506 and rapamycin and the absence of competition by cyclosporin A show that the binding is specific for an immunophilin of the FK506-binding class.

Wheat FKBP Binds to Wheat hsp90 via TPR Domains. To ask if TPR domains are involved in binding of the plant FKBP (or FKBPs) to the heterocomplex, we added CyP-4059 to wheat germ lysate and incubated the mixture with protein A−Sepharose-prebound p23. The CyP-4059 fragment contains the three TPR domains of CyP-40 and its C-terminal calmodulin-binding domain (Hoffmann & Handschumacher, 1995). As shown in Figure 3B, the presence of CyP-4059 eliminates [^{3}H]FK506 binding to the immune pellet (cf. lanes 3 and 4 with lane 2). The amount of wheat hsp90 bound to human p23 in the immune pellet is not affected by CyP-4059 (Figure 3A). These data are consistent with the interpretation that the wheat FKBP binds through TPR domains to wheat hsp90.

Heterocomplex Formation Is ATP-Dependent. Formation of a complex between purified mammalian p23 and hsp90 requires ATP (Johnson & Toft, 1995). The commercial wheat germ lysate we use here has been supplemented with an ATP generation system. To determine if the formation of the p23·plant hsp90 heterocomplex was ATP-dependent, wheat germ lysate was dialyzed to eliminate endogenous ATP and then incubated with immunoadsorbed p23 in the presence of added nucleotides. As shown in Figure 4A, dialysis of lysate nearly eliminates binding of plant hsp90 to p23 (cf. lanes 2 and 3). Addition of ATP to the dialyzed

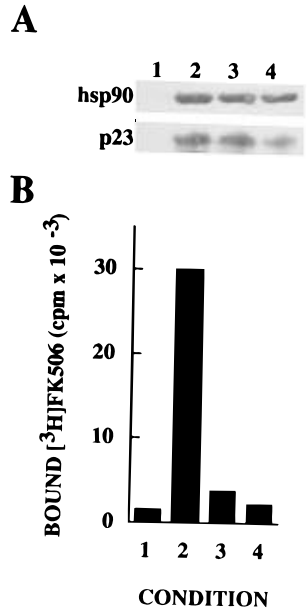

FIGURE 3: Human CyP-40 TPR domain competes for binding of wheat FKBP to wheat hsp90. (A) Western blots. (B) [^{3}H]FK506 binding to immune pellets. Wheat germ lysate was incubated with immunoadsorbed rabbit p23 in the presence or absence of purified, bacterially-expressed CyP-4059. Conditions are: lane 1, lysate incubated with JJ3 prebound to protein A−Sepharose without p23; lane 2, lysate plus preadsorbed p23; lane 3, lysate plus p23 plus 20 μg of CyP-4059; lane 4, lysate plus p23 plus 60 μg of CyP-4059.

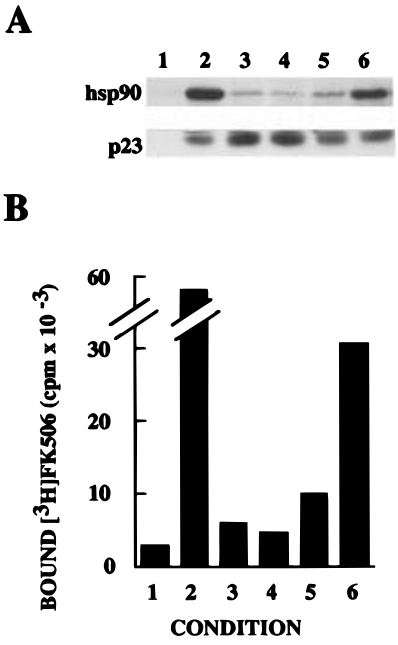

FIGURE 4: ATP is required for formation of the p23·hsp90 complex. (A) Western blots. (B) [^{3}H]FK506 binding to immune pellets. Wheat germ lysate was dialyzed as described under Experimental Procedures to eliminate ATP, and the dialyzed lysate was incubated with immunoadsorbed rabbit p23 and the indicated nucleotides for 30 min at 0 °C. Conditions are: lane 1, untreated lysate incubated with JJ3 prebound to protein A−Sepharose in the absence of p23; lane 2, untreated lysate plus preadsorbed p23; lanes 3−6, dialyzed lysate plus preadsorbed p23 and no addition (lane 3), 5 mM AMP (lane 4), 5 mM AMP-PNP (lane 5), and 5 mM ATP (lane 6).

lysate permits substantial binding of hsp90 to p23 (lane 6), but neither AMP nor AMP-PNP permits binding (lanes 4 and 5, respectively). As shown in Figure 4B, the binding of [^{3}H]FK506 to the immune pellet parallels the hsp90

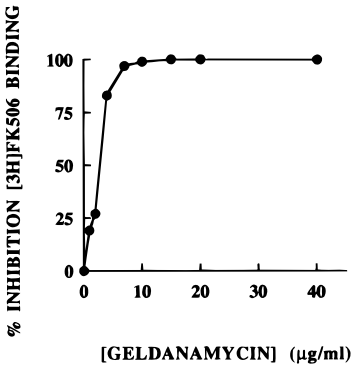

FIGURE 5: Geldanamycin inhibits heterocomplex formation in a concentration-dependent manner. Wheat germ lysate was incubated with increasing concentrations of geldanamycin for 10 min at 30 °C, and each mixture was subsequently incubated with immunoadsorbed p23 for 30 min at 0 °C. Pellets were then washed, and [^{3}H]FK506 binding was assayed.

binding, as would be expected if the wheat FKBP is bound directly to hsp90.

Geldanamycin Blocks p23·hsp90·FKBP Heterocomplex Formation. Geldanamycin is a benzoquinone ansamycin that binds specifically to mammalian hsp90s and inhibits the formation of the v-Src·hsp90 heterocomplex (Whitesell et al., 1994). Geldanamycin has also been found to disrupt mammalian p23·hsp90 complexes (Johnson & Toft, 1995). As shown in Figure 5, geldanamycin produces a concentration-dependent inhibition of heterocomplex formation as assayed by binding of [^{3}H]FK506 to the immune pellet. Half-maximal inhibition is achieved at ~2.5 μg/mL, with maximal inhibition at ~5 μg/mL. By comparison, Johnson and Toft (1995) used 10 μg/mL geldanamycin to disrupt p23·hsp90 complexes in rabbit reticulocyte lysate.

As shown in Figure 6A, geldanamycin inhibits heterocomplex formation by preventing hsp90 binding to p23 and the inhibition is temperature-dependent. In these experiments, wheat germ lysate was preincubated for 10 min with or without geldanamycin at 0 or 30 °C. Immunoadsorbed rabbit p23 was then added, and the mixtures containing the p23 immune pellet and the preincubated wheat germ lysate were incubated for 30 min at 0 or 30 °C as indicated in the chart above Figure 6A. When geldanamycin is present at 0 °C for both the preincubation and the incubation, there is little or no inhibition of hsp90 binding to p23 (cf. lanes 2 and 3), but if geldanamycin is present during either preincubation at 30 °C or incubation with p23 at 30 °C, p23·hsp90 heterocomplex formation is blocked (lanes 6 and 9, respectively). Again, the binding of [^{3}H]FK506 to the immune pellet parallels the binding of hsp90 to p23 (Figure 6B).

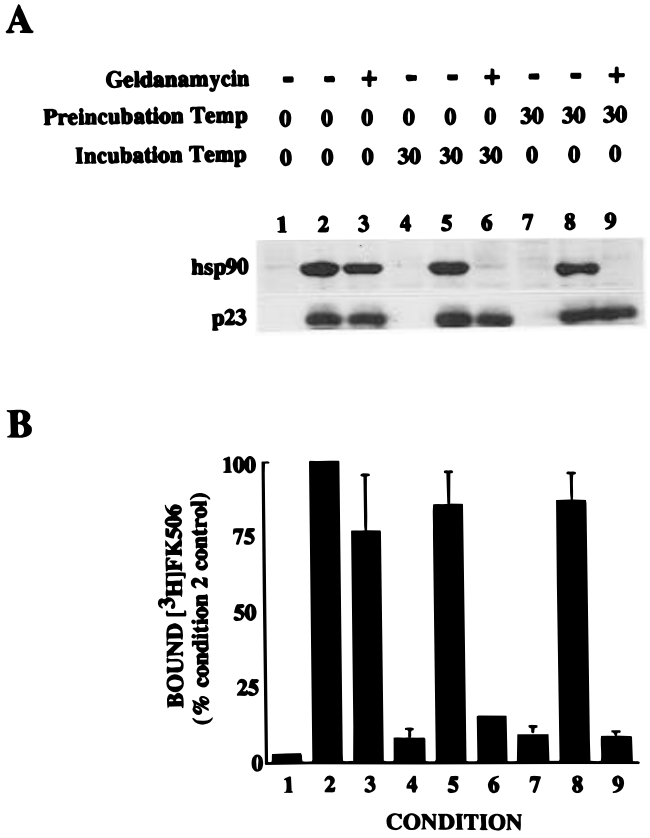

FIGURE 6: Geldanamycin inhibition of p23·plant hsp90 heterocomplex formation is temperature-dependent. (A) Western blots. (B) [^{3}H]FK506 binding to immune pellets. Wheat germ lysate was preincubated for 10 min with 5% DMSO or with geldanamycin at 0 °C (lanes 1−6) or 30 °C (lanes 7−9). The samples were then incubated with immunoadsorbed p23 for 30 min at 0 °C (lanes 1−3 and 7−9) or 30 °C (lanes 4−6). Conditions are: lanes 1, 4, and 7, lysate preincubated with 5% DMSO, and then incubated with antibody-bound protein A−Sepharose pellet without p23; lanes 2, 5, and 8, lysate preincubated with DMSO, followed by incubation with immunoadsorbed p23; lanes 3, 6, and 9, lysate preincubated with geldanamycin followed by incubation with immunoadsorbed p23. The Western blot shows a typical experiment. The [^{3}H]FK506-binding values in each of four experiments were expressed as a percent of the condition 2 control without geldanamycin, and the bar graphs present the mean ± SEM.

DISCUSSION

We have shown here that wheat germ lysate contains one, or perhaps more than one, FKBP that is present in the p23 immunopellet only under conditions that yield coimmunoadsorption of wheat hsp90. Given that studies showing direct binding of purified FKBP52 and CyP-40 to purified mammalian hsp90 have been reported (Czar et al., 1994; Owens-Grillo et al., 1995), it is reasonable to conclude from our data that the plant FKBP is binding directly to the plant hsp90. TPR domains are being identified in an expanding family of proteins (Boguski et al., 1990), and binding of FKBP52 and CyP-40 to mammalian hsp90 via their TPR domains (Radanyi et al., 1994; Hoffmann & Handschumacher, 1995) suggests that hsp90 may have a TPR domain acceptor site that allows it to interact with multiple proteins (Owens-Grillo et al., 1996). Our observation that the CyP-4059 fragment containing the TPR domains competes for plant FKBP binding to the heterocomplex suggests that the TPR domain acceptor function of hsp90 is conserved in plants.

The role of the higher molecular weight immunophilins in steroid receptor function is not defined. Although the PPIase activity might suggest a role in receptor folding (Tai et al., 1992), neither FK506 nor cyclosporin A affects glucocorticoid receptor folding by the hsp90/hsp70-based chaperone system (Hutchison et al., 1993; Owens-Grillo et al., 1995), and receptor folding has been carried out in a purified system without immunophilins (Dittmar et al., 1996). FKBP52 contains a conserved negatively charged amino acid sequence that is electrostatically complementary to the glucocorticoid receptor nuclear localization signal (NLS) (Pratt et al., 1993). Injection of antibody against this negative

region into cells impedes glucocorticoid-mediated nuclear trafficking of the receptor (Czar et al., 1995). This suggests that FKBP52 may act as an NLS recognition protein and play a role in targetted trafficking of the receptors.

In a search for yeast proteins that recognize NLSs *in vitro*, Shan et al. (1994) found a protein, NPI46, that possesses PPIase activity and is located in the nucleolus. The NH_2-terminus of NPI46 is homologous to the NH_2-terminus of Nopp140 and nucleolin, proteins that are thought to be involved in the nuclear import of proteins required for ribosome biogenesis (Meier & Blobel, 1992; Lapeyre et al., 1987). In plants, Lippuner et al. (1994) have cloned a cyclophilin from *Arabidopsis thaliana* that is expressed only in photosynthetic organs and contains an amino-terminal extension with properties of known chloroplast transit peptides. The precursor protein is imported into chloroplasts where it is processed to the predicted mature size. In considering possible functions for immunophilins in plants, it may well be important to consider a role in the targeted trafficking of proteins to organelles, as well as possible roles in protein folding.

Like hsp90 and hsp70, p23 is an ubiquitous protein in mammalian cells that is required for formation of the multiprotein steroid receptor·hsp90 heterocomplex (Johnson & Toft, 1994; Hutchison et al., 1995). It is likely that a p23 homolog exists in plants and that plant p23·hsp90·immunophilin complexes exist in the wheat germ lysate, but that has not yet been determined due to lack of antibody cross-reactivity. Because we have available an antibody that reacts with the mammalian p23, we can coimmunoadsorb plant hsp90 to protein A−Sepharose and thus demonstrate plant immunophilin binding to hsp90 by binding [^{3}H]FK506 to the immune pellet. We have shown that purified mammalian p23 is active at promoting glucocorticoid receptor folding and heterocomplex assembly by wheat germ lysate (Hutchison et al., 1995), and here we provide evidence that mammalian p23 binds directly to plant hsp90 in an ATP-dependent manner. The mechanism of p23·hsp90 complex formation is unknown, but in common with the mammalian system, formation of the hybrid system is blocked by geldanamycin.

The data of Figure 6 are unique in showing that the geldanamycin effect itself is temperature-dependent. p23·hsp90 heterocomplex formation in wheat germ lysate takes place efficiently at 0 °C (Figure 1), and if geldanamycin is present at 0 °C, complex formation is not inhibited (Figure 6). When geldanamycin is preincubated with the wheat germ lysate at 30 °C, it blocks subsequent p23·hsp90 complex formation upon addition of p23 at 0 °C. Given the direct effect of geldanamycin on mammalian hsp90 (Whitesell et al., 1994), it seems highly likely that geldanamycin directly interacts with plant hsp90 to block formation of the complex with p23.

In that hsp70 and hsp90 are ubiquitous and highly abundant proteins in plant cells, it is likely that the hsp90 chaperone system will be found to be of critical importance to a variety of plant cell functions. Genetic experiments in yeast have shown that this system is critical for proper signal transduction by both steroid receptors and v-Src (Kimura et al., 1995). By analogy, the chaperone function of hsp90 may be found critical to signal transduction systems in plants as well. Given the likely fundamental importance of hsp90 and its associated immunophilins in plant cell function, geldanamycin should prove to be a very useful tool for understanding basic mechanisms of protein folding, protein complex assembly, and protein trafficking in plant cells.

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