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All of the Protein Interactions That Link Steroid Receptor·Hsp90·Immunophilin Heterocomplexes to Cytoplasmic Dynein Are Common to Plant and Animal Cells†

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ABSTRACT: Both plant and animal cells contain high molecular weight immunophilins that bind via tetratricopeptide repeat (TPR) domains to a TPR acceptor site on the ubiquitous and essential protein chaperone hsp90. These hsp90-binding immunophilins possess the signature peptidylprolyl isomerase (PPIase) domain, but no role for their PPIase activity in protein folding has been demonstrated. From the study of glucocorticoid receptor (GR)·hsp90·immunophilin complexes in mammalian cells, there is considerable evidence that both hsp90 and the FK506-binding immunophilin FKBP52 play a role in receptor movement from the cytoplasm to the nucleus. The role of FKBP52 is to target the GR·hsp90 complex to the nucleus by binding via its PPIase domain to cytoplasmic dynein, the motor protein responsible for retrograde movement along microtubules. Here, we use rabbit cytoplasmic dynein as a surrogate for the plant homologue to show that two hsp90-binding immunophilins of wheat, wFKBP73 and wFKBP77, bind to dynein. Binding to dynein is blocked by competition with a purified FKBP52 fragment comprising its PPIase domain but is not affected by the immunosuppressant drug FK506, suggesting that the PPIase domain but not PPIase activity is involved in dynein binding. The hsp90/hsp70-based chaperone system of wheat germ lysate assembles complexes between mouse GR and wheat hsp90. These receptor heterocomplexes contain wheat FKBPs, and they bind rabbit cytoplasmic dynein in a PPIase domain-specific manner. Retention by plants of the entire heterocomplex assembly machinery for linking the GR to dynein implies a fundamental role for this process in the biology of the eukaryotic cell.

The immunophilins are a family of protein chaperones that are distributed widely among animal and plant cells (*1*). The common feature of the immunophilins is the presence of a peptidylprolyl isomerase (PPIase)[1] domain, and they are divided into two major classes—the FKBPs, which bind immunosuppressant drugs of the FK506 and rapamycin group, and the CyPs, which bind drugs of the cyclosporin A group. The immunosuppressant drugs bind to the isomerase site and inhibit cis−trans isomerization of peptidylprolyl bonds in vitro. Despite the signature presence of a PPIase domain, the physiological functions of most of the immunophilins are still not established (*2*).

The study of mammalian heteroprotein complexes containing steroid receptors and the ubiquitous protein chaperone hsp90 led to the discovery of three high molecular weight, hsp90-binding immunophilins—FKBP52, FKBP51, and CyP-40 (reviewed in reference *3*). These high molecular weight immunophilins differ from the low molecular weight immunophilins, such as FKBP12 and CyPA, in that they contain a domain with 3 or more tetratricopeptide repeats (TPRs), which are degenerative sequences of 34 amino acids that determine their binding to a common TPR *acceptor* site located near the C-terminus of hsp90 (*4*−*6*). The major immunophilin of glucocorticoid receptor (GR)·hsp90 heterocomplexes is FKBP52, which binds directly to the receptor as well as to hsp90 (*7*).

Genes encoding plant homologues of FKBP52 (reviewed in reference *8*) have been cloned from *Arabidopsis* [62 kDa ROF1 (*9*) and 70 kDa PAS1 (*10*)], maize [mzFKBP-66 (*11*)], and wheat [wFKBP73 (*12*) and wFKBP77 (*13*)]. Like mammalian FKBP52 (*14*), some of its plant homologues are induced by heat or other forms of stress (*9*, *13*). In addition to the N-terminal PPIase domain, the plant FKBP52 homologues possess a domain with three TPRs and a calmodulin-binding domain.

Prior to the cloning of plant FKBP52 homologues, it was shown that a [^{3}H]FK506-binding protein(s) was (were) coimmunoadsorbed with hsp90 from wheat germ lysate, and coimmunoadsorption was prevented by a purified fragment of human CyP-40 comprising its TPR domain (*15*). Reddy et al. (*16*) subsequently identified the wheat hsp90-binding

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

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immunophilins as wFKBP73 and wFKBP77. Binding of both wheat FKBPs to wheat hsp90 was competed by an expressed TPR domain from rat protein phosphatase 5 (PP5) that had been shown to compete for binding of mammalian FKBP52 and CyP-40 to mammalian hsp90 (*17*). Thus, it appears that the wheat FKBPs bind to wheat hsp90 via a TPR·acceptor interaction similar to that shown for the mammalian homologues. The conservation of TPR binding to hsp90 suggests that the binding is critical to functions of the high molecular weight FKBPs as well as to functions of hsp90.

Several observations in mammalian cells suggest that both hsp90 and FKBP52 are important for the retrograde movement of the GR from the cytoplasm to the nucleus (reviewed in reference *18*). Studies utilizing a chimera of green fluorescent protein (GFP) and mouse GR have revealed two modes of steroid-dependent movement of the receptor in cells with intact cytoskeleton. Rapid movement ($t_{1/2} \sim 4.5$ min for cytoplasmic−nuclear translocation) is hsp90-dependent and occurs along cytoskeletal tracks, whereas slow movement ($t_{1/2} \sim 45$ min) is hsp90-independent and may reflect movement by diffusion (*19*). Rapid movement of the receptor is impeded by treatment with the hsp90 inhibitor geldanamycin (*19*), by microinjection of antibody against FKBP52 (*20*), and by coexpression of a fragment of FKBP52 comprising its PPIase domain (*21*).

The protein interactions of FKBP52 are consistent with the notion that it targets the receptor for retrograde movement by linking it to the retrograde movement system. For example, a 35 amino acid segment of the GR that spans the proto-signals that make up the NL1 nuclear localization signal of the receptor is sufficient for direct binding of FKBP52 (*7*). FKBP52 colocalizes with microtubules (*22, 23*), and it binds to cytoplasmic dynein (*7, 22*), a motor protein responsible for retrograde movement of vesicles along microtubules toward the nucleus (reviewed in reference *24*). Coimmunoadsorption of dynein with FKBP52 is unaffected by FK506 and is competed by the FKBP52 PPIase domain fragment, suggesting that the PPIase domain functions as a dynein interaction domain independent of its PPIase activity (*7*). GR·hsp90 heterocomplexes immunoadsorbed from cell lysates contain cytoplasmic dynein, which is present in an immunophilin-dependent manner and is competed by the FKBP52 PPIase domain (*21*).

Plant cells contain microtubule-associated motor proteins (*25, 26*), and it is likely that they also have a system for targeted movement of protein solutes (i.e., nonvesicular proteins) through the cytoplasm to the cell membrane, to the nucleus, and to various organelles. If the assembly of GR·hsp90·immunophilin·dynein heterocomplexes is important for a general system of protein movement, the protein interactions required to form these complexes may be retained in plant cells, which do not have glucocorticoid receptors. We show here that the wheat immunophilins wFKBP77 and wFKBP73 bind via their PPIase domains to mammalian cytoplasmic dynein. Incubation of immunoimmobilized mouse GR with wheat germ lysate yields GR heterocomplexes with wheat hsp90 that contain wFKBP77 and wFKBP73 and that bind rabbit cytoplasmic dynein in a manner that is specific for the wheat immunophilin PPIase domain. This conservation of FKBP PPIase domain binding to dynein along with TPR domain binding to hsp90 is consistent with the notion that similar systems may exist for moving protein solutes, such as signaling proteins, through the cytoplasm in plant and animal cells.

MATERIALS AND METHODS

Materials. Wheat germ lysate was from Promega (Madison, WI), rabbit reticulocyte lysate was from Green Hectares (Oregon, WI), and geldanamycin was obtained from the Drug Synthesis and Chemistry Branch of the Developmental Therapeutics Program, National Cancer Institute (Bethesda, MD). [6,7-^{3}H]Triamcinolone acetonide (38 Ci/mmol) and ^{125}I-conjugated goat anti-mouse and anti-rabbit IgGs were from NEN Life Sciences Products (Boston, MA). Complete-Mini protease inhibitor tablets were from Roche Molecular Biochemicals, and the proteosome inhibitor MG-132 was from Biomol (Plymouth Meeting, PA). The mouse monoclonal IgG (MAB1618) against the 74 kDa intermediate chain subunit of mammalian (bovine brain) cytoplasmic dynein was purchased from Chemicon Intl. (Temecula, CA). The BuGR2 monoclonal IgG used to immunoblot the GR and the 3G3 monoclonal IgM used to immunoadsorb mammalian hsp90 were from Affinity Bioreagents (Golden, CO). The AC88 monoclonal IgG used to immunoblot mammalian hsp90 was from StressGen (Victoria, BC, Canada). The UPJ56 antiserum against FKBP52 was a gift from Dr. Karen Leach (Pharmacia and Upjohn, Inc., Kalamazoo, MI), and the FiGR monoclonal IgG used for GR immunoadsorption was generously provided by Dr. Jack Bodwell (Dartmouth Medical School, Hanover, NH). The rabbit R2 antiserum against plant hsp90 (*27*) was a kind gift from Dr. Priti Krishna (University of Western Ontario, London, Ontario). The cDNA for rabbit FKBP52 Gly^{32}−Lys^{138} (Domain I core) encoding the PPIase domain was described previously (*28*). The baculovirus for mouse GR was obtained from Dr. Edwin Sanchez (Medical College of Ohio, Toledo, OH) and was described previously (*29*). The baculovirus for the FLAG-tagged TPR domain of rat PP5 (*30*) was kindly provided by Dr. Michael Chinkers (University of South Alabama, Mobile, AL). The cDNA used for bacterial expression of p23 and the JJ3 monoclonal IgG against p23 were gifts from Dr. David Toft (Mayo Clinic, Rochester, MN).

Conditions for Immunoadsorption and Competition. For several experiments, lysate from bacteria expressing wFKBP73 or wFKBP77 was added to 100 μL of rabbit reticulocyte lysate, and the mixture was incubated 30 min at 30 °C to allow wFKBP binding to rabbit cytoplasmic dynein. The wFKBPs were immunoadsorbed for 3 h at 4 °C to 14 μL of protein A−Sepharose with 1 μL of anti-wFKBP77 or 3 μL of anti-wFKBP73 antiserum. Immune pellets were washed 3 times by suspension in 1 mL of TEG buffer [10 mM TES, pH 7.6, 50 mM NaCl, 4 mM EDTA, 10% (w/v) glycerol] prior to gel electrophoresis and immunoblotting. Endogenous FKBP52 was immunoadsorbed from 100 μL aliquots of reticulocyte lysate in the same manner using 7 μL of UPJ56 antiserum. For competition with TPR or PPIase domain fragments, the aliquots of reticulocyte lysate were preincubated before addition of wheat FKBPs for 30 min at 30 °C with either 30 μL of Sf9 lysate expressing the TPR domain fragment of rat PP5 (*17*) or the indicated amount of purified FKBP52 PPIase domain fragment. Samples were then incubated with wheat FKBPs and immunoadsorbed as above.

To immunoadsorb hsp90·wFKBP complexes from wheat germ lysate, 30 μL aliquots of lysate were incubated for 30

min at 30 °C with a protein A−Sepharose pellet prebound with 4 μL of JJ3 monoclonal IgG against p23 and charged with 5 μg of purified human p23 to allow p23 binding to hsp90 as previously described (*15*). The resulting p23·whsp90·wFKBP heterocomplexes were then washed 2 times with 1 mL of HEG buffer (10 mM Hepes, 4 mM EDTA, 10% w/v glycerol), and incubated for 1 h at 4 °C with 300 μL of Sephacryl S-300-purified dynein. The pellets were then washed 3 times with 1 mL of HEG, and pellet-associated proteins were electrophoresed and immunoblotted.

All of the coimmunoadsorption and competition experiments were performed 2−4 times, with a representative experiment being presented in each figure. Competition by the PPIase domain fragment for wFKBP binding to dynein is repeated in various experimental formats in Figures 2, 4, and 5.

Protein Expression and Purification. pGEX-2T plasmids expressing GST-wFKBP77 or GST-wFKBP73 were used to transform *Escherichia coli* strain BL21, and pGEX λT plasmid expressing GST-rabbit FKBP52 Gly^{32}−Lys^{138} (PPIase domain fragment) was used to transform *E. coli* strain UT5600. Bacteria were lysed by sonication in PBS with one tablet of Complete-Mini protease inhibitor cocktail per 3 mL of suspension. The GST-FKBP52 PPIase domain fragment was purified by binding to GSH-agarose and incubation at 4 °C with thrombin, which cleaves at a site between the GST domain and the FKBP52. Human p23 (*31*) was purified from 10 mL of bacterial lysate by chromatography on DE52, followed by hydroxylapatite chromatography as described previously (*29*).

Rabbit cytoplasmic dynein was partially purified (~20-fold) by eluting 1 mL of reticulocyte lysate through a column (1.5 × 113 cm) of Sephacryl S-300 with HKD buffer (10 mM Hepes, pH 7.35, 100 mM KCl, 5 mM dithiothreitol). Cytoplasmic dynein was determined by immunoblotting to elute in the first five fractions comprising the void volume. These fractions were combined, Complete-Mini protease inhibitor and MG-132 were added, and this preparation is referred to hereafter as Sephacryl S-300-purified cytoplasmic dynein. Immunoadsorption of this preparation with antibody specific for the dynein intermediate chain yields coadsorption of dynein heavy chain, showing that the motor protein complex is intact.

Glucocorticoid Receptor Heterocomplex Reconstitution. Mouse GR was overexpressed in Sf9 cells as described (*29*), and cytosol was prepared by Dounce homogenization in 1.5 volumes of buffer (10 mM Hepes, pH 7.4, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1 tablet of Complete-Mini protease inhibitor mix/3 mL of buffer). The lysate was centrifuged at 100000*g* for 30 min, and the supernatant was stored at −70 °C. Receptors were immunoadsorbed from 150 μL aliquots of cytosol by rotation for 2 h at 4 °C with 14 μL of protein A−Sepharose and 7 μL of FiGR ascites, and the immune pellets were washed once with 1 mL of TEG buffer. Prior to incubation with wheat germ lysate, immunoadsorbed receptors were stripped of insect hsp90 by incubating for 2 h with 300 μL of TEG buffer containing 0.7 M NaCl and 0.2% Nonidet P-40. The immune pellets were then washed 3 times with 1 mL of TEG buffer containing 0.7 M NaCl and twice with 10 mM Hepes, pH 7.4. GR·hsp90 heterocomplexes were assembled by incubating these stripped GR immune pellets with 50 μL of wheat germ lysate, 5 μg of purified human p23, and 5 μL of an ATP-regenerating system (50 mM ATP, 250 mM creatine phosphate, 20 mM magnesium acetate, and 100 units/μL of creatine phosphokinase). The assay mixtures were incubated for 30 min at 30 °C, with suspension of the pellets by shaking the tubes every 2 min. At the end of the incubation, the pellets were washed 2 times with 1 mL of ice-cold HEG buffer. Sephacryl S-300-purified dynein (300 μL) was added to the washed pellet, which was then rotated for 30 min at 4 °C. The pellets were then washed 3 times with 1 mL of HEG and boiled in SDS sample buffer. A duplicate set of immune pellets was incubated overnight at 0−4 °C with 50 nM [^{3}H]triamcinolone acetonide and then washed and counted to determine steroid-binding activity. The steroid binding is expressed as cpm of [^{3}H]triamcinolone acetonide bound per FiGR immunopellet prepared from 100 μL of Sf9 cytosol.

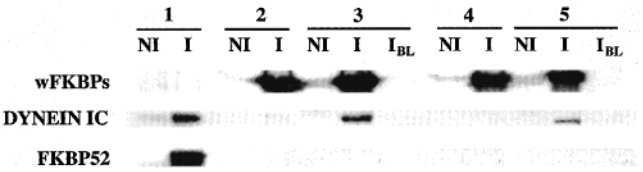

FIGURE 1: Coadsorption of cytoplasmic dynein with wheat immunophilins. Lysate from bacteria expressing equivalent amounts of wFKBP77 or wFKBP73 was mixed with 10 μL of HKD buffer or 100 μL of rabbit reticulocyte lysate, and the mixtures were incubated for 30 min at 30 °C. The incubations were immunoadsorbed with nonimmune (NI) serum or an immune (I) serum against the appropriate immunophilin. After the immune pellets were washed, proteins were resolved by SDS−PAGE and western-blotted for immunophilin and the intermediate chain of cytoplasmic dynein (Dynein IC). Condition 1, FKBP52 immunoadsorbed from reticulocyte lysate; condition 2, wFKBP77 immunoadsorbed from a mix of buffer and wFKBP77-expressing bacterial lysate; condition 3, wFKBP77 immunoadsorbed from a mix of reticulocyte lysate and wFKBP77-expressing bacterial lysate or nonexpressing bacterial lysate (I_{BL}); conditions 4 and 5, same as conditions 2 and 3 with lysate from bacteria expressing FKBP73.

Western Blotting. To assay pellet-associated proteins, immune pellets were resolved on 10% SDS−polyacrylamide gels, and proteins were transferred to Immobilon-P membranes. The membranes were probed with 0.2 μg/mL BuGR for GR, 1 μg/mL AC88 for mammalian hsp90, 0.2% R2 for wheat hsp90, 0.1% UPJ56 for FKBP52, and 0.1% anti-wFKBP77, 0.1% anti-wFKBP73, and 0.1% MAB1618 for dynein. The immunoblots were then incubated a second time with the appropriate ^{125}I-conjugated counter-antibody to visualize immunoreactive bands.

RESULTS

wFKBP77 and wFKBP73 Bind to Cytoplasmic Dynein. As shown in Figure 1 (condition 1), immunoadsorption of FKBP52 from rabbit reticulocyte lysate is accompanied by coadsorption of cytoplasmic dynein. To determine if the wheat FKBP52 homologues bind dynein, lysate from bacteria expressing either wFKBP77 (condition 3) or wFKBP73 (condition 5) was mixed with rabbit reticulocyte lysate, and the mixture was immunoadsorbed with antiserum specific for each wheat immunophilin. Rabbit dynein was coadsorbed with both of the wheat FKBPs, and it was not present in samples prepared from a mixture of reticulocyte lysate and lysate from nonexpressing bacteria.

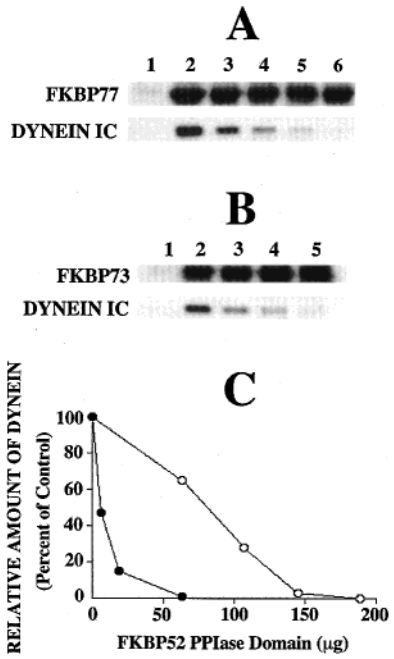

FIGURE 2: wFKBP77 binds to dynein with higher affinity than wFKBP73. Aliquots of reticulocyte lysate (100 μL) were preincubated for 30 min at 30 °C with buffer (lanes 1 and 2) or with varying amounts of purified FKBP52 PPIase fragment (lanes 3−6). At the end of the preincubation, aliquots of bacterial lysate containing equivalent amounts of either wFKBP77 (A) or wFKBP73 (B) were added, and the incubations were continued for another 30 min at 30 °C. The incubations were immunoadsorbed with nonimmune serum (lane 1) or with immune serum against the appropriate immunophilin (lanes 2−6), immune pellets were washed, and proteins were resolved by SDS−PAGE and western blotting. (C) The autoradiograms of the western blots were scanned, and the amount of dynein coadsorbed with the immunophilin is expressed as a percent of the control sample without competing PPIase domain fragment (lane 2). Open circles, wFKBP77 immunoadsorption; solid circles, WFKBP73 immunoadsorption.

To determine if the wheat FKBPs were binding to dynein via their PPIase domains, aliquots of reticulocyte lysate were preincubated with various amounts of purified FKBP52 PPIase domain (the domain I core fragment $Gly^{32}-Lys^{138}$) and then mixed with lysate from bacteria expressing wFKBP77 or wFKBP73. Each of the wheat FKBPs was immunoadsorbed, and the coadsorbed dynein was assayed (Figure 2, western blots). The western blot autoradiograms were scanned, and the amount of dynein coadsorbed in each condition is plotted in the graph as a percent of the uncompeted control sample (Figure 2C). Coimmunoadsorption of dynein with both of the wheat immunophilins was inhibited by the mammalian FKBP52 PPIase domain, suggesting that the plant immunophilins interact, either directly or indirectly, with rabbit cytoplasmic dynein via their PPIase domains. Coimmunoadsorption of dynein with the wheat immunophilins is not affected by FK506 (data not shown); thus, peptidylprolyl isomerase activity is not required for the interaction. The binding of wFKBP73 to dynein is competed by lower concentrations of FKBP52 PPIase domain fragment than the binding of wFKBP77 (Figure 2C), suggesting that the interaction of dynein with wFKBP73 is weaker than that with wFKBP77.

wFKBP77 Binds to Mammalian hsp90. FKBP52 binds independently via its TPR domain to hsp90 and via its PPIase domain to cytoplasmic dynein (*7*). This is demonstrated in

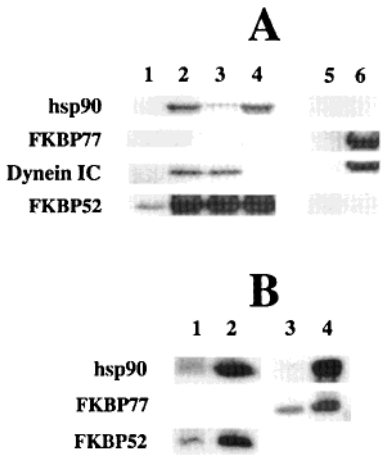

FIGURE 3: wFKBP77 binds to rabbit hsp90. (A) Rabbit hsp90 is not coadsorbed with wFKBP77. Aliquots (100 μL) of reticulocyte lysate were preincubated for 30 min at 30 °C with buffer (lanes 1 and 2) or with purified TPR domain fragment of rat PP5 (lane 3) or with purified FKBP52 PPIase domain fragment (lane 4). The mixtures were then immunoadsorbed with nonimmune serum (lane 1) or UPJ56 antiserum against FKBP52 (lanes 2−4). For lanes 5 and 6, reticulocyte lysate that was mixed with lysate from bacteria expressing wFKBP77 was immunoadsorbed with nonimmune serum (lane 5) or anti-wFKBP77 (lane 6). Washed immune pellets were then immunoblotted for the indicated proteins. (B) wFKBP77 binds to purified rabbit hsp90. A protein A−Sepharose pellet bound with the 8D3 anti-hsp90 antibody or a 3G3 pellet prebound with purified rabbit hsp90 was incubated at 4 °C with bacterial lysate expressing rabbit FKBP52 (lanes 1 and 2) or wFKBP77 (lanes 3 and 4). After washing, the pellet-bound proteins were resolved by SDS electrophoresis and immunoblotting.

Figure 3A, where immunoadsorption of FKBP52 from reticulocyte lysate was accompanied by coimmunoadsorption of hsp90 and dynein IC (lane 2). Hsp90 coadsorption was selectively competed by a TPR domain fragment (lane 3), and dynein coadsorption was selectively inhibited by a PPIase domain fragment (lane 4). When bacterial lysate containing wFKBP77 was mixed with reticulocyte lysate and wFKBP77 was immunoadsorbed, there was no coadsorption of rabbit hsp90 (lane 6). Reddy et al. (*16*) have shown that wFKBP73 and wFKBP77 bind to wheat hsp90 and that binding is competed by the same rat PP5 TPR domain fragment used in Figure 3A (lane 3) to compete for FKBP52 binding to rabbit hsp90. This supports the notion that immunophilin binding to hsp90 is a conserved protein−protein interaction, and a plant immunophilin might be expected to bind to a mammalian hsp90. The antiserum against wFKBP77 was raised against a C-terminal peptide, and this epitope may be blocked when the TPR domains, which lie in the C-terminal one-third of the immunophilin, are bound to hsp90.

Thus, in the experiment of Figure 3B, the procedure was reversed, with the immunophilin being bound to immunoimmobilized hsp90. 3G3 immune pellets or 3G3 pellets prebound with purified rabbit hsp90 were mixed with lysate from bacteria expressing FKBP52 (lanes 1 and 2) or wFKBP77 (lanes 3 and 4). In both cases, there was hsp90-specific binding of the immunophilin; thus, the plant immunophilin does bind to mammalian hsp90.

Formation of an hsp90·FKBP77·Dynein Complex in Wheat Germ Lysate. As in reticulocyte lysate, immunoadsorption of wFKBP77 from wheat germ lysate is not accompanied by coadsorption of hsp90. Thus, we wanted to

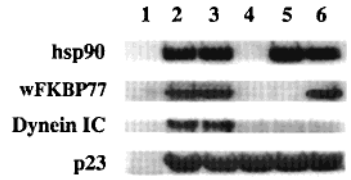

FIGURE 4: Formation of whsp90·wFKBP77·dynein complexes in wheat germ lysate. Aliquots (50 μL) of wheat germ lysate were incubated for 30 min at 30 °C with protein A−Sepharose pellets containing immunoadsorbed purified p23. Incubations contained either buffer, TPR domain fragment, 10 μM geldanamycin, or Me_2SO as indicated. The p23·whsp90·wFKBP complexes were washed and incubated with Sepharyl S-300-purified rabbit cytoplasrnic dynein with or without competing PPIase domain fragment. Proteins in the washed immune pellets were resolved by SDS electrophoresis and immunoblotting. Lane 1, immune pellet without p23 incubated with lysate then dynein; lanes 2−6, p23 pellets incubated with lysate plus buffer and then dynein (lane 2), with lysate plus 0.5 μL of Me_2SO and then dynein (lane 3), with lysate plus 10 μL of geldanamycin in Me_2SO and then dynein (lane 4), with lysate plus purified TPR domain fragment of rat PP5 and then dynein (lane 5), with lysate and then dynein plus purified FKBP52 PPIase domain fragment (lane 6).

use an approach similar to that of Figure 3B to determine if wFKBP77 would link wheat hsp90 to cytoplasmic dynein in wheat germ lysate, but two problems existed. First, there is not yet an antibody, like 3G3, that can immunoadsorb plant hsp90s in complexes with other proteins. However, in a previous study (*15*), we showed that purified human p23, an hsp90 cochaperone, added to wheat germ lysate binds to wheat hsp90 in its ATP-dependent conformation. The plant hsp90 with its associated plant immunophilins can then be immunoadsorbed with the JJ3 monoclonal IgG against p23. A second problem is that no antibody against plant dynein is available, and the available antibodies against the intermediate and heavy chains of mammalian cytoplasmic dynein do not recognize wheat cytoplasmic dynein. Thus, we purified rabbit cytoplasmic dynein ~20-fold by chromatographing reticulocyte lysate on a column of Sephacryl S-300 and pooling the fractions containing the excluded proteins. This hsp90-free and immunophilin-free rabbit dynein preparation was then added to wheat germ lysate to provide an immunodetectible source of dynein.

Accordingly, in the experiment of Figure 4, wheat germ lysate was incubated for 30 min at 30 °C with immunoadsorbed, purified human p23. The pellets were washed and incubated at 4 °C for an additional 30 min with Sephacryl S-300-purified rabbit dynein. As shown, in lane 2, wheat hsp90, wheat FKBP77 and rabbit cytoplasmic dynein are all coadsorbed with the human p23. Coadsorption of dynein was selectively inhibited by the PPIase domain fragment of FKBP52 (lane 6), and neither dynein nor wFKBP77 are present when the TPR domain fragment of rat PP5 is present to compete for binding of the plant immunophilins to plant hsp90 (lane 5). When the hsp90 inhibitor geldanamycin is present to prevent p23 binding to hsp90 (*31*), then none of the proteins are coadsorbed with p23 (lane 4). These results show that the wheat immunophilins bind independently via the TPR domain to hsp90 and via the PPIase domain to cytoplasmic dynein.

Formation of Dynein-Containing GR·hsp90 Heterocomplexes in Wheat Germ Lysate. There is now considerable evidence that the hsp90-binding immunophilin FKBP52 links GR·hsp90 heterocomplexes to cytoplasmic dynein in mammalian cells (*7*, *21*). We have shown previously that wheat germ lysate contains the machinery that forms GR·hsp90 heterocomplexes (*32*), but it has not been determined that these GR·hsp90 heterocomplexes contain plant immunophilins, and if so, whether they can link the receptor to cytoplasmic dynein. The heterocomplexes are assembled and disassembled in a highly dynamic manner in wheat germ lysate, but the complexes are quite stable to subsequent manipulation at 0−4 °C if purified mammalian p23 is present during GR·hsp90 assembly at 30 °C (*33*). In the experiment of Figure 5, mouse GR overexpressed in Sf9 cells was immunoadsorbed to protein A−Sepharose, and the associated insect chaperones were stripped off by washing with a high-salt buffer. The stripped GR (lane 2) was then incubated at 30 °C with wheat germ lysate containing purified, human p23 to stabilize the GR·hsp90 heterocomplexes during their assembly. The pellets were washed and incubated at 4 °C with Sephacryl S-300-purified rabbit cytoplasmic dynein. As shown in lane 3 of Figure 5, the GR immunopellet now contains wheat hsp90, wFKBP77, and rabbit dynein. Although not shown in this experiment, complexes containing wFKBP73 are also formed. The GR must be bound to hsp90 to have steroid-binding activity (*3*), and the wheat germ lysate has converted the receptor to the steroid-binding conformation (bar graph, lane 3). When the hsp90 inhibitor geldanamycin is present, both hsp90 binding to the GR and steroid-binding activity are abrogated (lane 4). When the TPR domain fragment is present during the 30 °C incubation with lysate, GR·hsp90 heterocomplexes with steroid-binding activity are assembled, but they do not contain wFKBP77

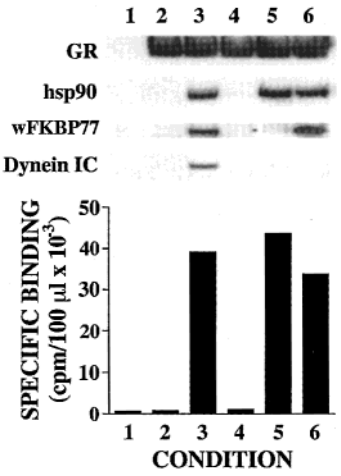

FIGURE 5: Formation of GR·hsp90 heterocomplexes containing wFKBP77 and dynein. Stripped GR immune pellets were incubated 30 min at 30 °C with 50 μL of wheat germ lysate under the conditions indicated below, and the pellets were washed. The washed pellets were then incubated for 1 h at 4 °C with Sephacryl S-300-purified rabbit cytoplasmic dynein. The pellets were washed again, and the GR-associated proteins were assayed by western blotting. A duplicate set of pellets was incubated with 50 nM [^{3}H]-triamcinolone acetonide to determine steroid binding activity. Lane 1, nonimmune pellet incubated with wheat germ lysate and then dynein; lanes 2−6, stripped GR immune pellets incubated with buffer and then dynein (lane 2), or with lysate and then dynein (lane 3), or with lysate plus 10 μM geldanamycin and then dynein (lane 4), or with lysate plus purified TPR domain fragment of rat PP5 and then dynein (lane 5), or with lysate and then with dynein plus purified FKBP52 PPIase domain fragment (lane 6). The experiment shown is representative of two similar GR·hsp90 heterocomplex assembly experiments.

or dynein (lane 5). When GR·hsp90·wFKBP77 heterocomplexes are assembled and then incubated with dynein in the presence of the competing PPIase domain fragment, dynein is not present in the final complex (lane 6).

The data of Figure 5 show that GR·hsp90 heterocomplexes formed in wheat germ lysate contain wFKBP77 and that they are linked via wheat immunophilins to cytoplasmic dynein. Although the plant immunophilin PPIase domains are responsible for the linkage to cytoplasmic dynein, the relative contribution of wFKBP77, wFKBP73, and perhaps, as yet, unidentified wheat immunophilins to that linkage is not clear.

DISCUSSION

A variety of mammalian signaling proteins (e.g., nuclear receptors, protein kinases, nitric oxide synthase, the insulin receptor, G protein $\beta\gamma$) form heterocomplexes with hsp90 (*3*). These heterocomplexes are assembled by a multiprotein machinery (hsp90, hsp70, Hop, hsp40, and p23) that, with the apparent exception of a homologue for p23, is conserved in plants (reviewed in reference *8*). Steroid receptor·hsp90 heterocomplexes formed in mammalian cells or in reticulocyte lysate contain a TPR domain protein, such as an immunophilin, and they contain cytoplasmic dynein (*21*). As we have shown here (Figure 5), GR·whsp90 heterocomplexes formed in wheat germ lysate also contain a wheat immunophilin, and they can bind mammalian cytoplasmic dynein.

Because the TPR domain proteins compete for binding to a single TPR binding site on each hsp90 dimer (*7*), any single GR·hsp90 heterocomplex is bound by only one molecule of immunophilin at any time (*34*, *35*). In addition to FKBP52, native GR·hsp90 heterocomplexes formed in mammalian cells and heterocomplexes formed in reticulocyte lysate contain other immunophilins, such as the cyclosporin A-binding protein CyP-40 and protein phosphatase 5 (PP5), an FKBP homologue (reviewed in reference *3*). In addition to GR·whsp90·wFKP77 complexes, GR·whsp90·wFKBP73 heterocomplexes are formed in wheat germ lysate (data not shown), and it is possible that other heterocomplexes are formed that contain as yet undiscovered wheat CyPs or a wheat PP5 homologue. Assembly of GR·hsp90 heterocomplexes in the presence of the TPR domain fragment blocks the binding of all of the immunophilins to hsp90 (*17*, *34*). Thus, at this time, we can only say that binding of dynein to the GR·whsp90 heterocomplex formed in wheat germ lysate requires a TPR domain immunophilin and that wFKBP77 and wFKBP73 provide part, and possibly all, of that linker function.

The function(s) of the TPR domain immunophilins in plants is (are) unknown, but the conservation of TPR domain binding to hsp90 and PPIase domain binding to cytoplasmic dynein suggests that information on immunophilin functions in mammalian signaling systems may be relevant to the functions of their plant homologues. It is important to note that glucocorticoid-dependent gene expression has been shown in tobacco cells cotransfected with cDNAs expressing the rat GR and an appropriate steroid-responsive reporter (*36*). Thus, all of the conditions for steroid regulation, including the steroid-triggered movement of the transformed GR through the cytoplasm to the nucleus, exist in plant cells.

Here, we have shown that the wheat immunophilins can link the GR·hsp90 heterocomplex to cytoplasmic dynein via conserved TPR and PPIase domain interactions (Figure 5). This conservation of the TPR and PPIase domain interactions in plant and animal immunophilins implies a fundamental role for these interactions in the biology of eukaryotic cells. We suggest that such a basic function for the hsp90-binding FKBPs may be to target the movement of signaling proteins to the nucleus and possibly other organelles (see reference *18*) by linking them to the retrograde movement system through the PPIase domain interaction with the dynein motor protein.

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