Binding of hsp90-Associated Immunophilins to Cytoplasmic Dynein: Direct Binding and in Vivo Evidence that the Peptidylprolyl Isomerase Domain Is a Dynein Interaction Domain[†]

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ABSTRACT: FKBP52 is a steroid receptor-associated immunophilin that binds via a tetratricopeptide repeat (TPR) domain to hsp90. FKBP52 has also been shown to interact either directly or indirectly via its peptidylprolyl isomerase (PPIase) domain with cytoplasmic dynein, a motor protein involved in retrograde transport of vesicles toward the nucleus. The functional role for the PPIase domain in receptor movement was demonstrated by showing that expression of the PPIase domain fragment of FKBP52 in 3T3 cells inhibits dexamethasone-dependent nuclear translocation of a green fluorescent protein−glucocorticoid receptor chimera. Here, we show that cytoplasmic dynein is co-immunoadsorbed with two other TPR domain proteins that bind hsp90 (the cyclophilin CyP-40 and the protein phosphatase PP5). Both proteins possess PPIase homology domains, and co-immunoadsorption of cytoplasmic dynein with each is blocked by the PPIase domain fragment of FKBP52. Using purified proteins, we show that FKBP52, PP5, and the PPIase domain fragment bind directly to the intermediate chain of cytoplasmic dynein. PP5 colocalizes with both cytoplasmic dynein and microtubules, and expression of the PPIase domain fragment of FKBP52 in 3T3 cells disrupts its cytoskeletal localization. We conclude that the PPIase domains of the hsp90-binding immunophilins interact directly with cytoplasmic dynein and that this interaction with the motor protein is responsible for the microtubular localization of PP5 in vivo.

Steroid receptors form heterocomplexes with hsp90[1] that contain one of several immunophilins (FKBP52, FKBP51, or CyP-40) or the immunophilin homologue protein phosphatase 5 (PP5), all of which bind via tetratricopeptide repeat (TPR) domains to a TPR acceptor site on the hsp90 homodimer (reviewed in ref *1*). The steroid receptors move continuously into and out of the nucleus (reviewed in ref *2*), and there is considerable evidence that both hsp90 and the hsp90-bound immunophilin FKBP52 are involved in the cytoplasmic−nuclear trafficking of the glucocorticoid receptor (GR) (reviewed in ref *3*). The evidence for involvement of hsp90 is derived from studies with geldanamycin, an antibiotic that binds to the nucleotide binding site on hsp90 (*4*), preventing formation of normal receptor•hsp90 heterocomplexes (*5*) and impeding steroid-induced movement of the GR from the cytoplasm to the nucleus (*6*−*8*). Steroid-induced movement of the GR from the cytoplasm to the nucleus is also impeded by microinjection of antibody against FKBP52 (*9*).

FKBP52 binds directly to the GR (*10*), and several observations are consistent with the notion that FKBP52 targets the GR for movement to the nucleus by linking the receptor to the retrograde movement system. Although the majority of FKBP52 is nuclear, the fraction that is cytoplasmic colocalizes with microtubules (*11*, *12*), and immunoadsorption of FKBP52 from cytosols is accompanied by co-immunoadsorption of cytoplasmic dynein (*10*, *11*), a motor protein responsible for retrograde movement of vesicles along microtubular tracks toward the nucleus (reviewed in ref *13*). Co-immunoadsorption of dynein with FKBP52 is prevented by competition with an FKBP52 fragment comprising its PPIase domain, but dynein coadsorption is not affected by

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[1] Abbreviations: hsp, heat shock protein; GR, glucocorticoid receptor; TPR, tetratricopeptide repeat; PPIase, peptidylprolyl isomerase; FKBP, FK506 binding protein; CyP, cyclosporin A binding protein; PP5, protein phosphatase 5; AHR, aryl hydrocarbon receptor; ARA9, AHR-associated protein; dynein IC, cytoplasmic dynein intermediate chain; GST, glutathione *S*-transferase; STAT, signal transducers and activators of transcription.

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FK506 (*10*, *14*). This suggests that the PPIase domain functions as a dynein interaction domain independent of its PPIase activity. GR•hsp90•immunophilin heterocomplexes immunoadsorbed from cell lysates contain cytoplasmic dynein, which is bound via the immunophilin component and is released by competition with the FKBP52 PPIase domain fragment (*14*). Expression of the FKBP52 PPIase domain fragment in 3T3 cells markedly slows the rate of steroid-induced movement of a green fluorescent protein (GFP)−GR chimera from the cytoplasm to the nucleus (*14*). Inhibition of GFP−GR movement by both geldanamycin and the PPIase domain is abrogated in cells treated with colcemid to eliminate microtubules prior to steroid addition (*7*, *14*). Thus, rapid cytoplasmic−nuclear translocation of the receptor requires formation of GR•hsp90•immunophilin heterocomplexes and linkage to a retrograde movement system, with the immunophilin PPIase domain being critical for linkage of the receptor heterocomplex to the dynein motor.

Although it is known that FKBP12, an immunophilin without TPRs that does not bind hsp90, does not bind to cytoplasmic dynein (*10*, *14*), it is not known whether hsp90-binding immunophilins other than FKBP52 bind dynein. It is also not known whether the interaction of FKBP52 with cytoplasmic dynein is direct or indirect via a linker protein. Here, we show that cytoplasmic dynein is coadsorbed from rabbit brain cytosol with the cyclophilin CyP-40 and with the protein phosphatase PP5, a TPR protein that possesses a domain in which the amino acids are 50% homologous with those of the FK506 binding site in the PPIase domain of FKBP52 and possesses weak FK506 binding activity (*15*). In both cases, coadsorption of dynein is prevented by competition with the PPIase domain of FKBP52. Using purified bacterially expressed proteins, we show that the immunophilins bind directly to the intermediate chain of cytoplasmic dynein and that the PPIase domain is sufficient for dynein binding. By indirect immunofluorescence, we show that PP5 in the cytoplasm colocalizes with both cytoplasmic dynein and microtubules and that expression of the PPIase domain fragment of FKBP52 disrupts this localization to the cytoskeleton. These observations suggest that direct binding of the PPIase domain of PP5 to the dynein motor protein determines its microtubular localization in vivo.

MATERIALS AND METHODS

Materials. Untreated rabbit reticulocyte lysate was from Green Hectares (Oregon, WI). ^{125}I-conjugated goat anti-mouse and anti-rabbit IgGs were obtained from NEN Life Sciences Products. Sephacryl S-300 was from Amersham Pharmacia Biotech. Talon metal affinity resin was purchased from Clontech (Palo Alto, CA). The mouse monoclonal IgG (MAB1618) against the 74 kDa intermediate chain subunit of mammalian (bovine) cytoplasmic dynein was purchased from Chemicon International (Temecula, CA). The mouse monoclonal IgGs against GST and β-tubulin and the mouse monoclonal IgM against the intermediate chain of dynein were purchased from Sigma. The UPJ56 antiserum against FKBP52 was kindly provided by K. Leach (Pharmacia and Upjohn, Inc., Kalamazoo, MI), and rabbit antiserum used to immunoadsorb PP5 was prepared as described previously (*16*). The EC1 anti-FKBP52 mouse monoclonal IgG was kindly provided by L. Faber (Medical College of Ohio, Toledo, OH). The anti-FLAG M2 monoclonal IgG was from IBI (New Haven, CT), and the rabbit anti-cyclophilin 40 (C-terminal peptide) antibody was from Biomol (Plymouth Meeting, PA). The mouse monoclonal IgG against human ARA9 was from Novus Biologicals (Littleton, CO), and the rabbit antiserum against STAT5B was from Santa Cruz Biotechnology (Santa Cruz, CA). Rhodamine-conjugated donkey anti-rabbit IgG, FITC-conjugated sheep anti-mouse IgG, and FITC-conjugated goat anti-mouse IgM were from Jackson ImmunoResearch (West Grove, PA). The pGEX-2T plasmid encoding the GST−rabbit FKBP52 fusion and the pGEX1λT plasmid encoding the GST−rabbit FKBP52 Gly^{32}-Lys^{138} fusion (core domain I) comprising the PPIase domain were described previously (*17*, *18*). Rabbit FKBP52 Gly^{32}-Lys^{138} and human FKBP12 were subcloned into the pSG5PL mammalian expression vector as described previously (*14*). The pEBFB-N1 plasmid for expressing blue fluorescent protein was kindly provided by R. Benndorf (The University of Michigan Medical School). Trans-Fast transfection reagent was from Promega (Madison, WI). Purified FLAG-PP5 was prepared as described previously (*16*). The full-length mouse cytoplasmic dynein intermediate chain containing a hexahistidine tag at the N-terminus was expressed in *Escherichia coli*, solubilized from inclusion bodies, purified on a Ni^{2+}−nitrilotriacetic acid affinity column, and refolded as described previously (*19*).

Immunoadsorption of Immunophilins from Brain Cytosol. Frozen rabbit brain was thawed and homogenized in 1.5 volumes of HE buffer [10 mM Hepes and 1 mM EDTA (pH 7.4)]. The homogenates were centrifuged for 1 h at 100000*g*, and the supernatant is termed cytosol. For immunoadsorption of TPR proteins, 100 μL aliquots of rabbit brain cytosol were immunoadsorbed for 3 h at 4 °C to 14 μL of protein A−Sepharose with 7 μL of UPJ56 antiserum against FKBP52, 5 μL of anti-CyP-40, 6 μL of anti-PP5, or 10 μL of anti-ARA9. Immune pellets were washed three times with a suspension in HEG buffer [HE with 10% (w/v) glycerol] prior to gel electrophoresis and immunoblotting. Because PP5 migrates with the immunoglobulin heavy chain, 5 λ of purified FLAG-PP5 was added to samples to be immunoadsorbed with anti-PP5 serum, and the FLAG-tagged PP5 was detected on immunoblotting with anti-FLAG antibody. For competition with the PPIase domain fragment of FKBP52, 70 μL of brain cytosol was mixed with 30 μL of lysate from control bacteria or bacteria expressing the GST−FKBP52 Gly^{32}-Lys^{138} fusion and incubated for 30 min at 30 °C prior to immunoadsorption as described above.

For immunoadsorption of STAT5B, 200 μL of rabbit brain cytosol was immunoadsorbed for 3 h at 4 °C to 14 μL of protein A−Sepharose with 10 μL of antiserum against STAT5B. Immune pellets were washed, electrophoresed, and immunoblotted first for the coadsorbed dynein intermediate chain, which was developed with peroxidase. The blots were cut just above the dynein intermediate chain, and the upper portion was then probed with antiserum against STAT5B.

Western Blotting. To assay immunoadsorbed proteins, immune pellets were resolved on 10% SDS−polyacrylamide gels, and proteins were transferred to Immobilon-P membranes. The membranes were probed with 1 μg/mL EC1 for FKBP52, 0.1% anti-cyclophilin 40 antibody for CyP-40,

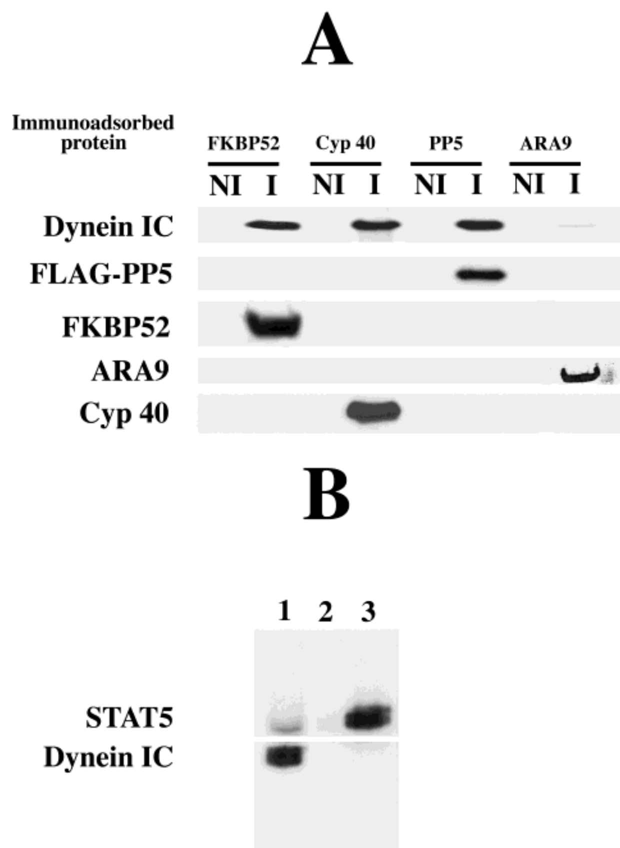

FIGURE 1: Co-immunoadsorption of cytoplasmic dynein with hsp90-binding immunophilins. (A) Immunoadsorption of immunophilins. Replicate aliquots of rabbit brain cytosol were immunoadsorbed with nonimmune antibody (NI) or antibody (I) directed against the protein indicated above each set of lanes. The proteins in the washed immune pellets were resolved by SDS−polyacrylamide gel electrophoresis and immunoblotting for the proteins indicated at the left. (B) Immunoadsorption of STAT5B. STAT5B was immunoadsorbed from 200 μL of rabbit brain cytosol with nonimmune (lane 2) or anti-STAT5B (lane 3) antiserum, and the washed immune pellets were immunoblotted for the dynein intermediate chain and STAT5B as described in Materials and Methods. A 20 μL aliquot of rabbit brain cytosol was run in lane 1.

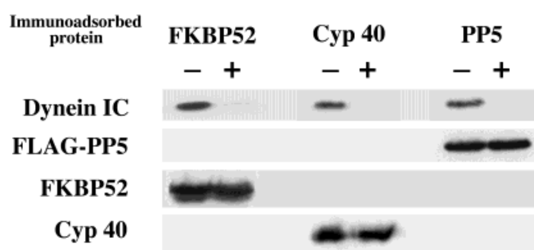

FIGURE 2: PPIase domain fragment of FKBP52 that prevents co-immunoadsorption of cytoplasmic dynein. Replicate aliquots of rabbit brain cytosol were preincubated with lysate from control bacteria (−) or lysate from bacteria expressing the PPIase domain fragment of FKBP52 (+). The proteins indicated at the top were then immunoadsorbed, and the proteins in the immune pellets were immunoblotted.

0.1% anti-FLAG for FLAG-PP5, 1 μg/mL anti-ARA9, 0.1% MAB1618 for the dynein intermediate chain, and 0.1% anti-GST. The immunoblots were then incubated a second time with the appropriate ^{125}I-conjugated counterantibody to visualize immunoreactive bands. It should be noted that the epitope for MAB1618 lies in the middle of the p150^{Glued}

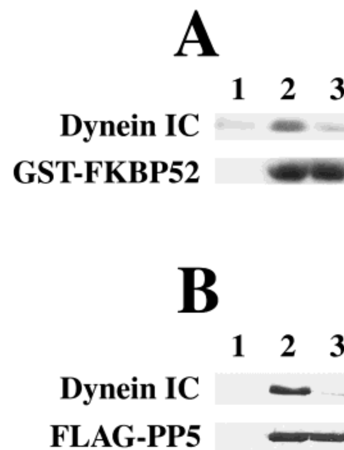

FIGURE 3: Purified GST−FKBP52 fusion and FLAG-PP5 bind partially purified cytoplasmic dynein. (A) Purified FKBP52 binds dynein. Aliquots of Sephacryl S-300-purified cytoplasmic dynein were incubated with pellets of glutathione−agarose-bound GST−FKBP52 as described in Materials and Methods, and proteins in the washed pellet were assayed by Western blotting: lane 1, glutathione−agarose bound with GST incubated with dynein; and lanes 2 and 3, glutathione−agarose bound with GST−FKBP52 incubated with dynein in the absence (lane 2) or presence (lane 3) of the purified PPIase domain fragment of FKBP52. (B) PP5 binds dynein: lane 1, protein A−Sepharose adsorbed with anti-PP5 incubated with dynein; and lanes 2 and 3, pellets containing FLAG-PP5 immunoadsorbed with anti-PP5 incubated with dynein in the absence (lane 2) or presence (lane 3) of the purified PPIase domain fragment.

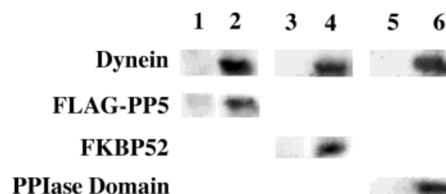

FIGURE 4: PPIase binding site on the intermediate chain of cytoplasmic dynein. Talon pellets (lanes 1, 3, and 5) and Talon pellets bound with the purified His_6-dynein intermediate chain (lanes 2, 4, and 6) were incubated with purified FLAG-PP5 (lanes 1 and 2), FKBP52 (lanes 3 and 4), or the PPIase domain fragment of FKBP52 (lanes 5 and 6), and proteins in the washed pellet were assayed by Western blotting.

binding region. Thus, it can be used for immunoblotting and immunoadsorbing free dynein but not for immunoadsorbing dynein bound to cargo.

Purification of the FKBP52 Domain I Core. pGEX λT plasmid expressing the GST−FKBP52 Gly^{32}-Lys^{138} fusion was used to transform *E. coli* strain UT5600. The fusion protein was purified by binding to GSH−agarose beads, and the core PPIase domain I of FKBP52 was released by incubation at 4 °C with thrombin.

Partial Purification of Rabbit Cytoplasmic Dynein. One milliliter of rabbit reticulocyte lysate was applied to a column (1.5 cm × 113 cm) of Sepharcryl S-300, and the column was eluted with HKD buffer [10 mM Hepes, 100 mM KCl, and 5 mM dithiothreitol (pH 7.35)]. An aliquot of each 2.5 mL fraction was Western blotted with MAB1618 for cytoplasmic dynein, which eluted in the first five fractions comprising the excluded proteins. These fractions were pooled to yield an ∼20-fold purified preparation of cytoplasmic dynein. Aliquots (300 μL) of the dynein preparation

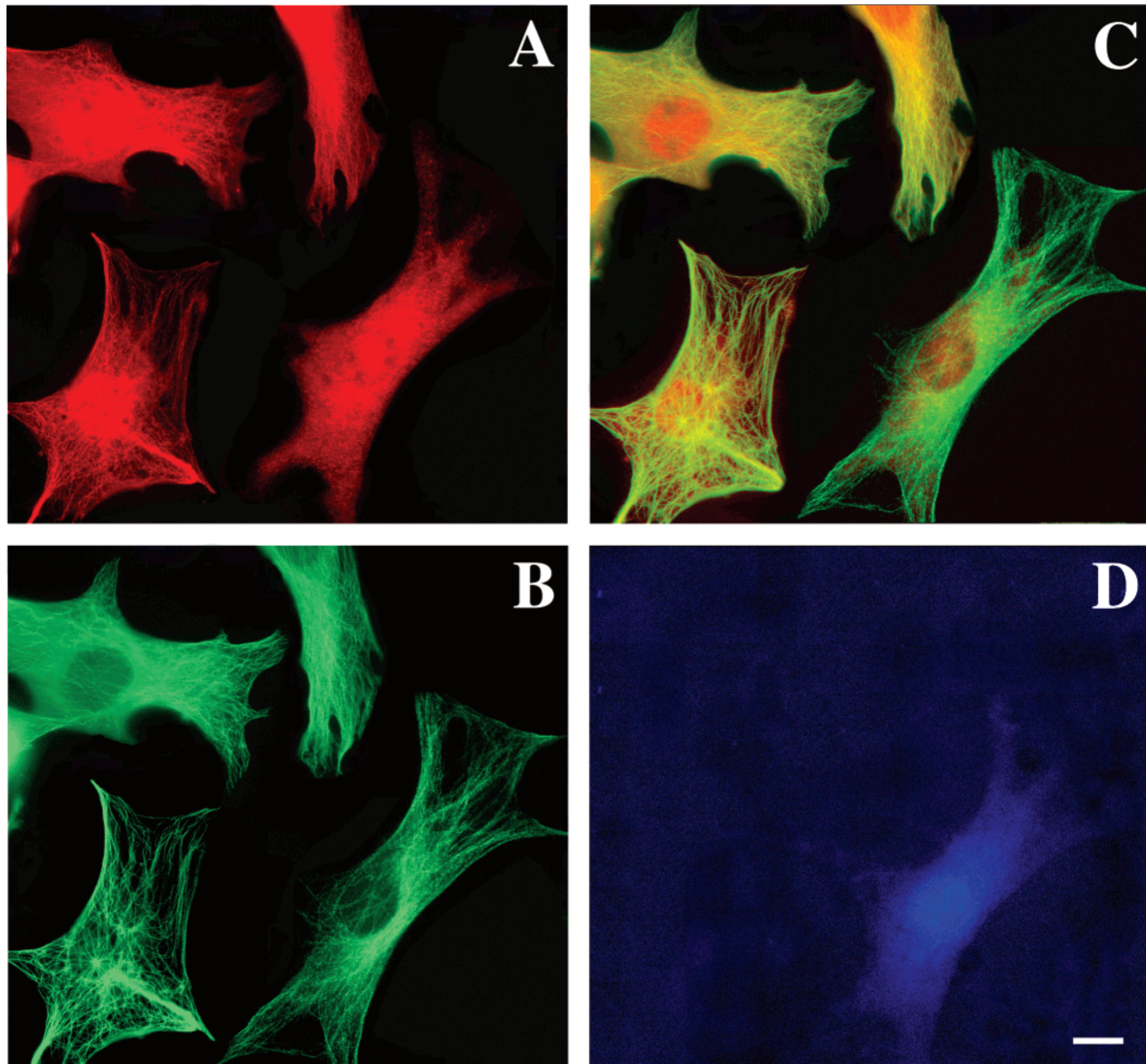

FIGURE 5: Colocalization of PP5 and cytoplasmic dynein. NIH 3T3 cells were cotransfected with pEBFP-N1 and pSG5PL-FKBP52 core domain I. The cells were immunostained for PP5 (A) and the dynein intermediate chain (B). The monoclonal IgG used to immunoprecipitate and immunoblot the cytoplasmic dynein intermediate chain cannot be used for immunofluorescence, so a monoclonal IgM antibody was used here. Panel C shows the merged image of panels A and B. The blue image in panel D identifies a transfected cell. The bar is 10 μm long.

were then incubated for 1 h at 4 °C with pellets of the glutathione−agarose conjugate bound with GST or the GST−FKBP52 fusion or with pellets of protein A−Sepharose adsorbed with anti-PP5 or anti-PP5 bound with FLAG-tagged PP5. After the pellet had been washed three times with 1 mL of HEG buffer, proteins in the pellet were detected by Western blotting.

Binding of Purified Immunophilins to the Purified Dynein Intermediate Chain. The purified mouse dynein intermediate chain (200 μg) was dissolved in 200 μL of phosphate-buffered saline, and 7 μL aliquots of this solution were added to 33 μL of 100 mM Hepes (pH 8.2) prior to adsorption to 20 μL of Talon resin for 1 h with rotation at 4 °C. The Talon pellets were washed three times with 1 mL of Hepes (pH 8.2), and the washed pellets were incubated for 1 h with rotation at 4 °C with purified FKBP52, FLAG-PP5, or the PPIase domain fragment (FKBP52 Gly^{32}-Lys^{138}) in a final volume of 50 μL adjusted with Hepes (pH 8.2). Samples were then washed three times with 1 mL of Hepes (pH 8.2), and proteins in the washed pellet were detected by Western blotting.

Cell Culture and Transfection. NIH 3T3 mouse fibroblasts were grown on 11 mm × 22 mm coverslips in 2 mL of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine calf serum, and in a 5% CO_2 in air atmosphere at 37 °C. When the cells were approximately 50% confluent, the culture medium was replaced with 0.75 mL of DMEM supplemented with 5% bovine calf serum, and the incubation was continued for 1 h. The cells were cotransfected with 4 μg/mL pEBFP-N1 expressing blue fluorescent protein and 15 μg/mL pSG5PL, pSG5PL-FKBP52 core domain I (Gly^{32}-Lys^{138}), or pSG5PL-FKBP12. The cotransfection mixture was prepared by incubating the

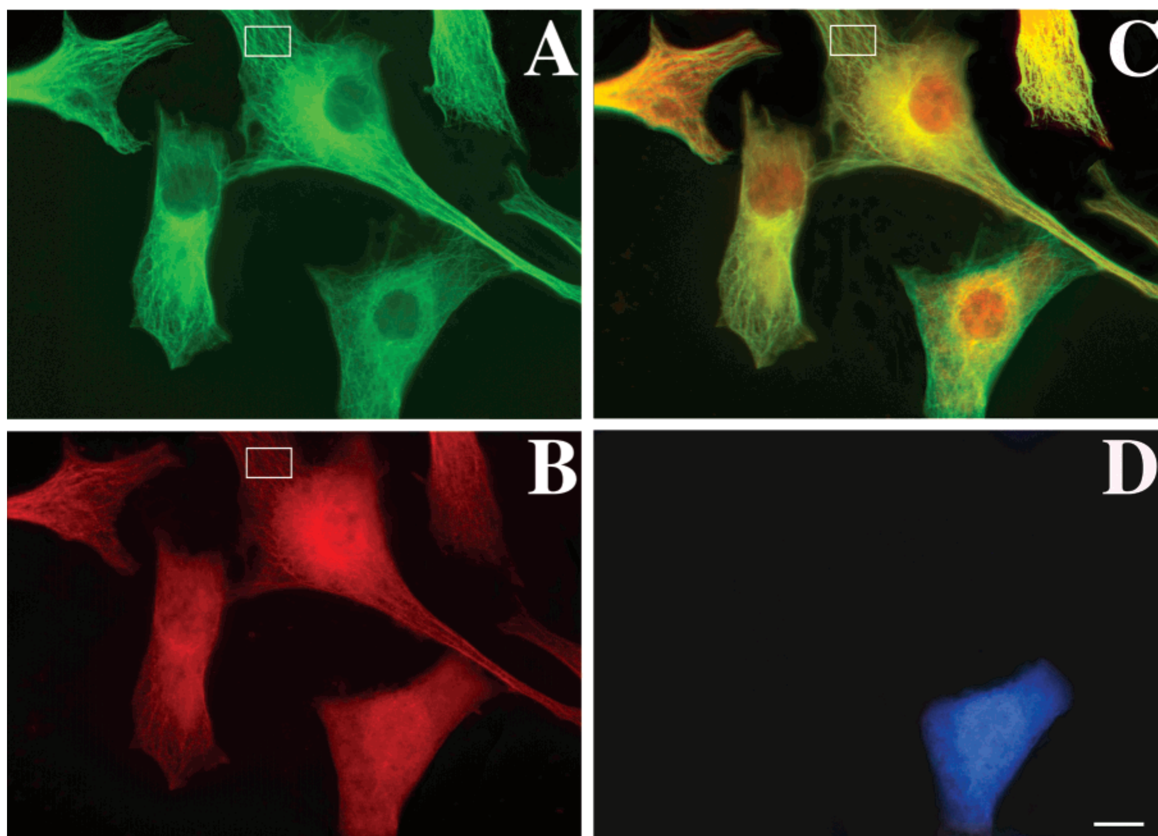

FIGURE 6: Colocalization of PP5 with microtubules. NIH 3T3 cells were cotransfected with pEBFP-N1 and pSG5PL-FKBP52 core domain I. The cells were immunostained for tubulin (A) and PP5 (B). Panel C shows the merged image of panels A and B. Panel D shows a transfected cell. The bar is 10 μm long. The region defined by the rectangles is enlarged in Figure 7.

plasmids with 4.5 μL of Trans-Fast transfection reagent per microgram of DNA in 0.25 mL of DMEM. After 10 min at room temperature, this mixture was added to the cell culture medium. One hour later, the transfection medium was replaced with 2 mL of regular medium, and the cell culture was continued for 40−48 h.

Indirect Immunofluorescence. The coverslips with transfected cells were rinsed with phosphate-buffered saline, and the cells were fixed for 8 h at room temperature in 3.0% *p*-formaldehyde in phosphate-buffered saline. Cells were permeabilized by immersing the coverslips in acetone at −20 °C for 15 min. After the coverslips had been washed with phosphate-buffered saline, the cells were immunostained by inverting the coverslilps on a 50 μL solution of phosphate-buffered saline with 1.0% bovine serum albumin containing rabbit anti-PP5 IgG (1:50), mouse anti-β-tubulin IgG (1:100), or mouse anti-dynein intermediate chain IgM (1:60). After 18 h at 8 °C in a humid chamber, the coverslips were washed with washing buffer [20 mM Tris-HCl (pH 8.0), 0.63 M NaCl, 0.05% Tween 20, and 1% bovine serum albumin] for 30 min at room temperature. The coverslips were then incubated for 2 h at room temperature with a 1:100 dilution of the corresponding counterantibody (rhodamine-conjugated donkey anti-rabbit IgG, FITC-conjugated sheep anti-mouse IgG, or FITC-conjugated goat anti-mouse IgM). The coverslips were mounted on microscope slides using a drop of mounting medium [1 mg/mL *p*-phenylenediamine in 10% phosphate-buffered saline and 90% glycerol (pH 9.0)], and the cells were observed with a Leitz Aristoplan epiillumination microscope.

RESULTS AND DISCUSSION

Co-Immunoadsorption of Dynein with hsp90-Binding Immunophilins. We have previously reported that immunoadsorption of FKBP52 from Chinese hamster ovary cell or chicken brain cytosol with UPJ56 is accompanied by coadsorption of both intermediate and heavy chains of cytoplasmic dynein (*11*). We subsequently immunoadsorbed several hsp90-binding proteins from rabbit reticulocyte lysate and found dynein co-immunoadsorbed only with FKBP52 (trace amounts were sometimes recovered with CyP-40) (*10*). Recently, we have used a broad-spectrum monoclonal IgG (MAB1618) that is very good for immunoblotting the cytoplasmic dynein intermediate chain (IC) to study the interaction of FKBP52 with dynein (*14*). We found that the association of FKBP52 with dynein is blocked by competition with a purified PPIase domain fragment without affecting its binding to hsp90, and binding of FKBP52 to hsp90 is competed with a TPR domain fragment without affecting its association with dynein (*14*). This suggests that FKBP52 can link hsp90 to the motor protein, with the PPIase domain being responsible for dynein association.

The PPIase domain defines a protein as an immunophilin, and it seems unlikely that only one of the hsp90-binding immunophilins would determine dynein association and that the other PPIase domains would be inactive in this respect. Immunoadsorbing antibodies are available for two of the three hsp90-associated immunophilins (FKBP52 and CyP-40, but not FKBP51) and for two hsp90-binding immunophilin homologues (PP5 and ARA9). In the experiment shown in Figure 1A, we have immunoadsorbed each of these

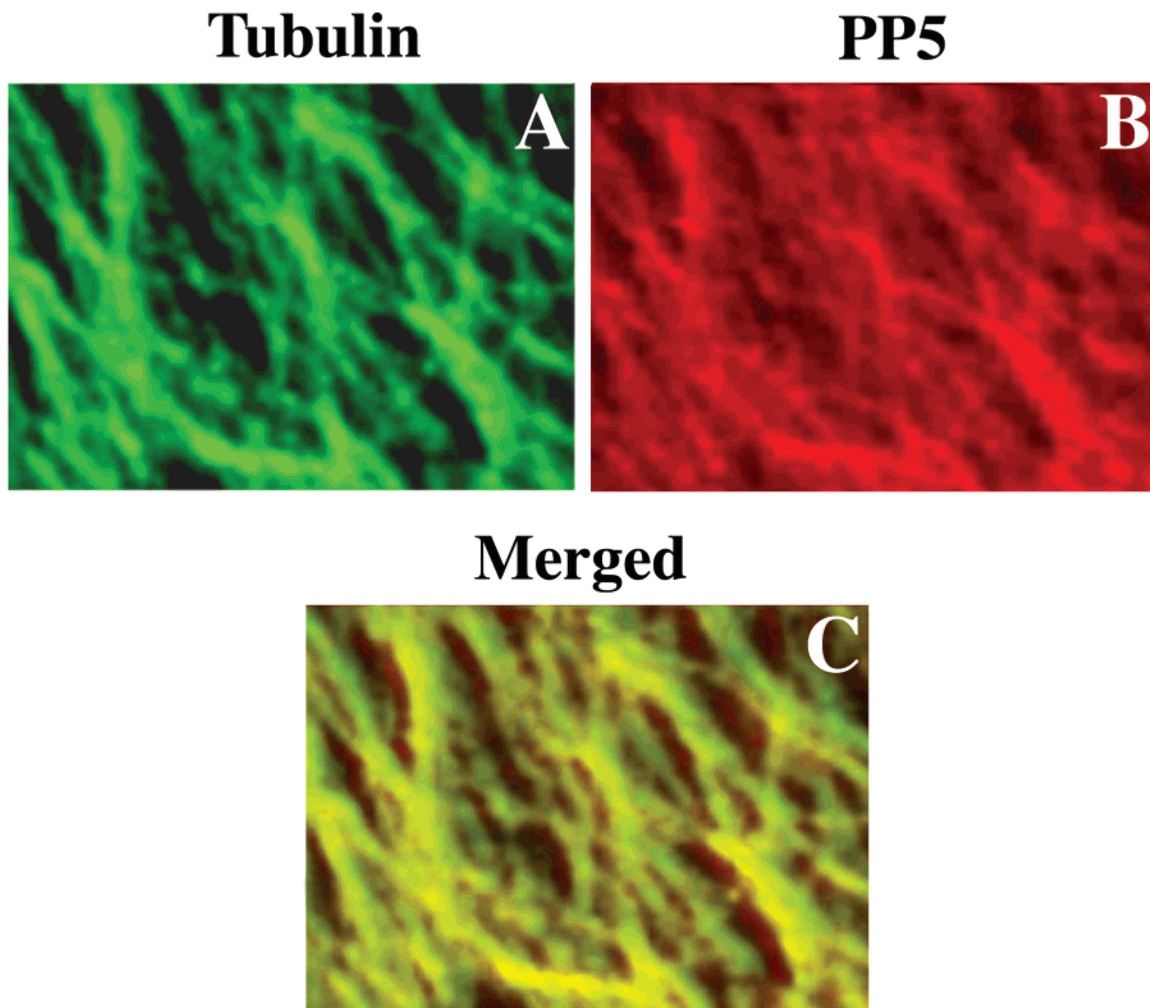

FIGURE 7: Close-up view of microtubules and PP5 in an untransfected cell. The region in the untransfected cell defined by the rectangle in Figure 6 has been enlarged to show the distribution of tubulin (A) and PP5 (B). Panel C shows the merged image of panels A and B.

proteins from rabbit brain cytosol, which contains a substantial amount of cytoplasmic dynein. Instead of washing the immune pellets with a salt-containing buffer as we have in the past, we washed the pellets with a salt-free buffer. It can be seen that under these conditions cytoplasmic dynein is co-immunoadsorbed with FKBP52, CyP-40, and PP5 (Figure 1A). As shown in Figure 2, coadsorption of dynein with each of these proteins is blocked if the brain cytosol is preincubated with lysate from bacteria expressing the PPIase domain fragment of FKBP52. Thus, there appears to be a family of hsp90-binding immunophilins possessing PPIase domains that determine the extent of association with cytoplasmic dynein. STAT (signal transducers and activators of transcription) proteins are not immunophilins and do not bind hsp90 (*7*), and in Figure 5B, STAT5B has been immunoadsorbed from brain cytosol to show that an antibody against an unrelated protein does not yield co-immunoadsorption of cytoplasmic dynein.

We know from previous work (*10*, *14*) that the small immunophilin FKBP12 does not compete for co-immunoadsorption of dynein with FKBP52, suggesting that its PPIase domain is different in that it does not bind, or binds only very weakly, with dynein or an associated protein. As shown in Figure 1A, very little, if any, dynein is co-immunoadsorbed with ARA9 (also called XAP2 and AIP), an immunophilin homologue that was isolated in yeast two-hybrid screens for proteins interacting with the aryl hydrocarbon receptor (AHR) (*20*, *21*). ARA9 is relatively abundant in brain cytosol (*22*), and it contains three TPRs in the C-terminus and a PPIase homology domain (50% similar and 27% identical with human FKBP52 domain I) in the N-terminus (*20*−*22*). ARA9 is recovered in AHR•hsp90 heterocomplexes, where it is bound both to the receptor and, via its TPR domain, to hsp90 (*20*, *21*). In co-immunoadsorption experiments, ARA9 is specifically associated with AHR•hsp90 complexes but not with GR•hsp90 complexes (*23*). The proposed role of ARA9 in AHR trafficking is the opposite of that proposed for FKBP52 in GR trafficking. Overexpression of ARA9 redistributes the AHR to the cytoplasmic compartment (*24*, *25*), and it is thought to mediate cytoplasmic retention of the receptor (*26*, *27*). To date, no protein has been identified with which ARA9 might interact to mediate such retention. Like the GR, the unliganded AHR constantly shuttles into and out of the nucleus, and it is possible that ARA9 induces cytoplasmic AHR localization by competing for the binding to hsp90 of immunophilins that associate with cytoplasmic dynein. The TPR domain proteins that bind to hsp90 compete with each other to occupy the TPR acceptor site that is present in each receptor•hsp90 heterocomplex (*10*, *15*, *28*). Thus, overexpression of a non-dynein-binding immunophilin homologue

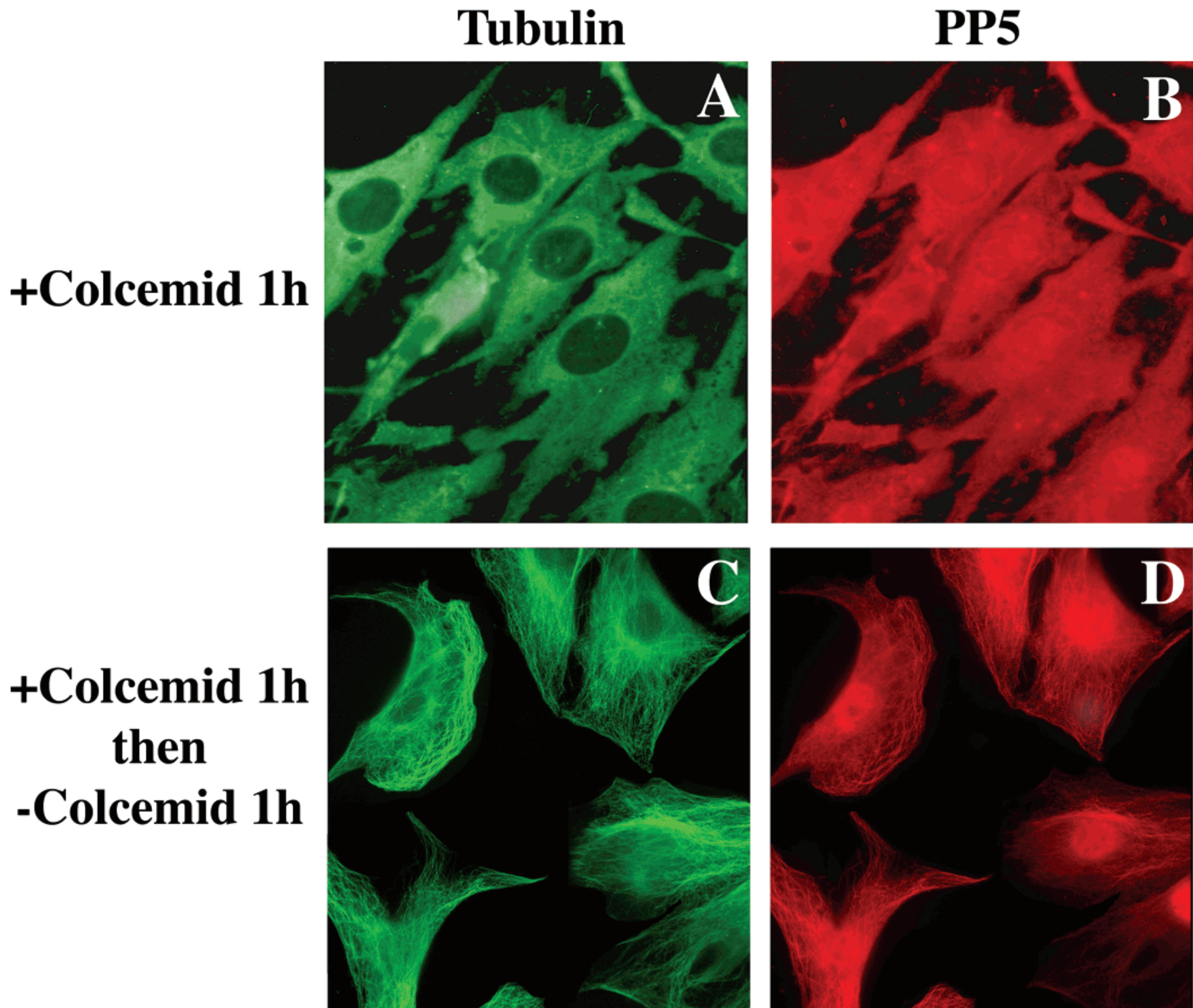

FIGURE 8: Disruption of microtubular localization of PP5 with colcemid. NIH 3T3 cells were incubated for 1 h at 37 °C with 0.6 μg/mL colcemid and immunostained for tubulin (A) and PP5 (B). Also, cells treated with colcemid for 1 h were washed and incubated for a second hour in medium without colcemid to demonstrate the return of microtubular structure (C) and fibrillar distribution of PP5 (D).

could interfere with normal cytoplasmic–nuclear shuttling via immunophilins that bind to cytoplasmic dynein, thereby promoting cytoplasmic localization of the receptor•hsp90 complex.

Purified FKBP52 and PP5 Bind to Partially Purified Cytoplasmic Dynein. All of the evidence to date for an interaction between immunophilins and cytoplasmic dynein is derived from pull-down experiments, such as those presented in Figures 1 and 2. In these experiments, both the intermediate and heavy chains of dynein are co-immunoadsorbed (*11*), consistent with binding of the immunophilin to an intact motor protein complex. The immunophilin–dynein interaction is shown in a more purified system in Figure 3. In this experiment, pellets containing purified GST–FKBP52 fusion (Figure 3A) or FLAG-PP5 (Figure 3B) were incubated with cytoplasmic dynein that had been purified ~20-fold from rabbit reticulocyte lysate by Sephacryl S-300 chromatography. Immunoadsorption of this partially purified dynein with antibody specific for the intermediate chain yields co-immunoadsorption of heavy chain (data not shown), demonstrating that the motor protein complex is intact. Figure 3 shows that both the GST–FKBP52 fusion and FLAG-PP5 bound to cytoplasmic dynein (cf. lanes 1 and 2), and the PPIase domain fragment of FKBP52 competed for the binding (lane 3).

The Immunophilins Bind Directly to the Intermediate Chain of Dynein. The intermediate chains of cytoplasmic dynein are thought to be responsible for interaction with cargo objects (*13*). In the experiment presented in Figure 4, we asked whether purified FKBP52 and FLAG-PP5 bound directly to the purified intermediate chain of mouse cytoplasmic dynein. The purified His_6-dynein intermediate chain was immobilized on Talon beads, which were then incubated with purified FKBP52 or FLAG-PP5. Both purified proteins bound to the purified intermediate chain (lanes 2 and 4), showing that the intermediate chain of the motor protein complex contains the immunophilin binding site and that the binding is direct. The immobilized dynein intermediate chain also bound the purified PPIase domain fragment of FKBP52 (lane 6), showing that the PPIase domain itself is sufficient for binding.

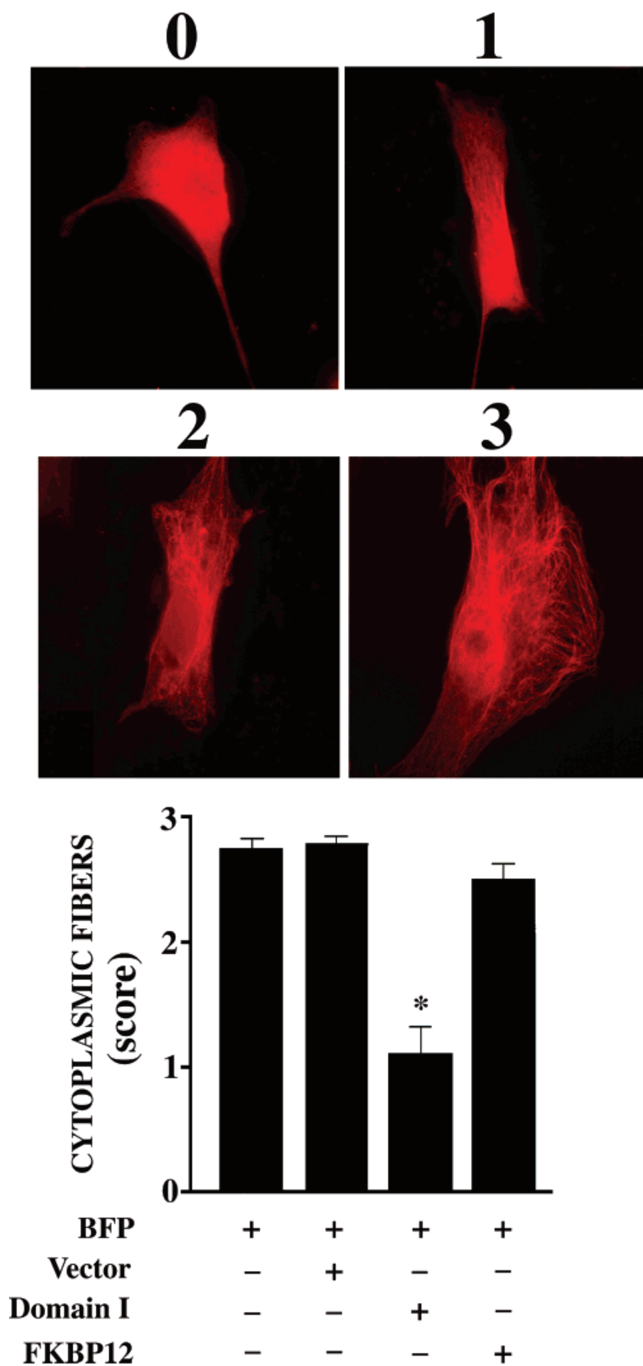

FIGURE 9: Quantitation of PPIase domain competition for PP5 colocalization with microtubules. NIH 3T3 cells were cotransfected with pEBFP-N1 (BFP) and either pSG5PL (Vector), pSG5PL-FKBP52 core domain I (Domain I), or pSG5PL-FKBP12 (FKBP12). After we had immunostained for PP5, only those cells that exhibited blue fluorescence were scored according to the presence or absence of cytoplasmic fibers, as follows: (0) no filaments, (1) filaments visible only on the cell periphery, (2) filaments observed in the cytoplasm and not over the nucleus, and (3) filaments covering the entire cell. The treatment conditions were coded and then scored by another investigator without knowledge of the code. A representative cell with its corresponding score is shown on the top. The bar graph shown below represents the score for PP5 localized on fibers for each experimental condition. Results are expressed as means ± the standard error of the mean from three experiments in which 30 cells were scored in each one. Scores were analyzed by one-way analysis of variance followed by the Bonferroni test (the asterisk denotes a difference from the other conditions at $p < 0.006$).

The PPIase Domain Fragment Disrupts PP5 Colocalization with Cytoplasmic Dynein. If FKBP52 and PP5 are bound via their PPIase domains to cytoplasmic dynein in vivo, then they should colocalize with dynein and the colocalization should be disrupted by expression of the FKBP52 PPIase domain fragment. Because the UPJ56 antiserum that we previously used to demonstrate microtubular colocalization of FKBP52 (*11*) recognizes an epitope in its PPIase domain fragment, we have examined the cellular localization of PP5. The antiserum against PP5 does not recognize the PPIase domain fragment of FKBP52, and it has previously been used to show by indirect immunofluorescence that a major portion of PP5 is located in the cytoplasm of HeLa cells (*29*). The localization of PP5 and the localization of cytoplasmic dynein in 3T3 mouse fibroblasts are shown in panels A and B of Figure 5, respectively. As can be seen in the merged image (Figure 5C), the three cells to the left show colocalization (indicated with yellow) of the two proteins to fibrillar arrays extending throughout the cytoplasm. The blue image in Figure 5D shows a cell that was transfected with plasmids expressing blue fluorescent protein and the PPIase domain fragment of FKBP52. Expression of the PPIase domain fragment did not alter the fibrillar distribution of dynein (Figure 5B), but it disrupted the fibrillar distribution of PP5 (Figure 5A). This is consistent with the PPIase domain fragment competing for PP5 binding to cytoplasmic dynein in vivo.

The PPIase Domain Fragment Disrupts PP5 Colocalization with Microtubules. The immunofluorescence localization of tubulin is shown in Figure 6A and that of PP5 in Figure 6B. As can be seen from the upper four cells in the merged image in Figure 6C, PP5 colocalized with microtubules. Again, a cell cotransfected with plasmids expressing blue fluorescent protein and the PPIase domain fragment of FKBP52 is shown in Figure 6D, and the distribution of PP5 in this cell was diffuse rather than fibrillar (Figure 6B).

The region in an untransfected cell that is defined by rectangles in Figure 6 has been enlarged in Figure 7 to show in detail the colocalization of tubulin (Figure 7A) and PP5 (Figure 7B) (see the merged image in Figure 7C). As shown in Figure 8, treatment of cells with colcemid eliminated both the microtubule network (Figure 8A) and the fibrillar pattern of cytoplasmic PP5 (Figure 8B). Once colcemid was withdrawn, microtubules reformed (Figure 8C) and PP5 returned to its fibrillar distribution (Figure 8D), confirming its microtubular localization.

To quantitate the diffuse versus microtubular localization of PP5 under various transfection conditions, the scoring system shown in Figure 9 was applied to multiple cotransfected cells. As shown by the bar graph at the bottom of Figure 9, expression of the FKBP52 PPIase domain fragment (domain I) caused a marked disruption of the microtubular localization of PP5. The non-hsp90-binding, small immunophilin FKBP12 does not bind to cytoplasmic dynein (*10*, *14*), and expression of FKBP12 does not affect the rate of steroid-induced movement from the cytoplasm to the nucleus (*14*). As shown by the bar on the right in Figure 9, transfection of 3T3 cells with human FKBP12 did not alter the microtubular localization of PP5. The level of FKBP12 in transfected cells was determined by [^{3}H]FK506 binding to be 40-fold higher than all endogenous [^{3}H]FK506 binding activity in nontransfected cells. The fact that expression of

FKBP12 does not affect the microtubular localization of PP5 is again consistent with the notion that the PPIase domains of the hsp90-binding immunophilins constitute a subclass of PPIase domains that bind to dynein.

The Model. From this work, we propose that the PPIase domains of several of the hsp90-binding, TPR domain immunophilins bind directly to a PPIase domain acceptor site located in the intermediate chain of cytoplasmic dynein. Because the co-immunoadsorption of dynein with FKBP52 is not affected by FK506 at concentrations that block peptidylprolyl isomerase activity (*10*, *14*) and because PP5 does not possess peptidylprolyl isomerase activity (*15*), PPIase domain binding to dynein IC is independent of the enzymatic activity. Disruption of PP5 colocalization with cytoplasmic dynein and microtubules by expression of the PPIase domain fragment of FKBP52 is consistent with immunophilin PPIase−cytoplasmic dynein interaction in vivo. Because these immunophilins are linking hsp90 to the motor protein, other proteins, such as steroid receptors, that are bound at any time to the chaperone site(s) on hsp90 can also be linked to the retrograde movement system.

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