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Hsp90-binding Immunophilins Link p53 to Dynein During p53 Transport to the Nucleus*

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The tumor suppressor protein p53 is known to be transported to the nucleus along microtubular tracks by cytoplasmic dynein. However, the connection between p53 and the dynein motor protein complex has not been established. Here, we show that hsp90·binding immunophilins link p53·hsp90 complexes to dynein and that prevention of that linkage *in vivo* inhibits the nuclear movement of p53. First, we show that p53·hsp90 heterocomplexes from DLD-1 human colon cancer cells contain an immunophilin (FKBP52, CyP-40, or PP5) as well as dynein. p53·hsp90·immunophilin·dynein complexes can be formed by incubating immunopurified p53 with rabbit reticulocyte lysate, and we show by peptide competition that the immunophilins link via their tetratricopeptide repeat domains to p53-bound hsp90 and by means of their PPIase domains to the dynein complex. The linkage of immunophilins to the dynein motor is indirect by means of the dynamitin component of the dynein-associated dynactin complex, and we show that purified FKBP52 binds directly by means of its PPIase domain to purified dynamitin. By using a temperature-sensitive mutant of p53 where cytoplasmic-nuclear movement occurs by shift to permissive temperature, we show that p53 movement is impeded when p53 binding to hsp90 is inhibited by the hsp90 inhibitor radicicol. Also, nuclear movement of p53 is inhibited when immunophilin binding to dynein is competed for by expression of a PPIase domain fragment in the same manner as when dynein linkage to cargo is dissociated by expression of dynamitin. This is the first demonstration of the linkage between an hsp90-chaperoned transcription factor and the system for its retrograde movement to the nucleus both *in vitro* and *in vivo*.

The tumor suppressor protein p53 is a transcription factor that can induce cell growth arrest, apoptosis, cell differentiation, and DNA repair in response to DNA strand breakage and other types of cell stress (1–3). p53 mutations occur in more than half of all human tumors (4), and inactivation of p53 is the most common alteration found in human cancer (5, 6). Although most p53 mutations in human tumors are located in the DNA-binding domain and inactivate its transcriptional activity (4), p53 inactivation may occur in other ways (2).

As has been shown for steroid receptors and some other transcription factors, p53 shuttles between the cytoplasm and nucleus (7, 8), and one mechanism of inactivation is exclusion of p53 from the nucleus (9, 10). The cytoplasmic localization of mutant p53 may have multiple mechanisms, such as increased nuclear export (11) and attachment to cytoplasmic anchor proteins (12). Like unliganded steroid receptors (13), some cytoplasmic p53 mutants retained in the cytoplasm were found to be in heterocomplex with the abundant and ubiquitous protein chaperone hsp90 (14, 15). These p53·hsp90 heterocomplexes are formed by the same hsp90/hsp70-based chaperone machinery (16) that assembles steroid receptor heterocomplexes (13).

One effect of heterocomplex assembly between hsp90 and its "client" proteins is to stabilize the client protein to degradation by the ubiquitin-proteasome pathway of proteolysis (17); this has been shown for p53 (18, 19), where its binding to hsp90 inhibits MDM2-dependent ubiquitination (20). The dynamic formation of heterocomplexes with hsp90 and its associated immunophilins is also required for rapid ligand-induced movement of aryl hydrocarbon and glucocorticoid receptors (GR)[1] to the nucleus (21, 22). Partial but complementary sets of observations regarding the mechanisms of GR·hsp90 and p53·hsp90 movement have been made that will be connected in this work to form a more complete model of the retrograde movement of p53.

GR·hsp90 heterocomplexes immunoadsorbed from cell lysates contain cytoplasmic dynein (23, 24), a molecular motor that processes along microtubular tracks toward the nucleus (25). These heterocomplexes also contain one of several immunophilins with tetratricopeptide repeat (TPR) domains that interact with a TPR acceptor site on hsp90 (13). The immunophilins are widely expressed proteins with peptidylprolyl isomerase (PPIase) domains that bind immunosuppressant drugs of the FK506 and cyclosporine A class. GR·hsp90 heterocomplexes contain one of four TPR domain immunophilins: FKBP52, FKBP51, CyP-40, or PP5, the latter being a protein phosphatase that also contains TPR and PPIase homology do-

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[1] The abbreviations used are: GR, glucocorticoid receptor; TPR, tetratricopeptide repeat; PPIase, peptidylprolyl isomerase; TES buffer, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethanesulfonic acid; CyP-40, cyclophilin 40; PP5, protein phosphatase 5; FKBP, FK506 binding protein; TPR, tetratricopeptide repeat;; DIC, dynein intermediate chain.

mains (13, 26). In these complexes, the PPIase domain functions as a protein-protein binding domain to link the GR·hsp90 unit to cytoplasmic dynein (27–29).

Although the linkage to the dynein motor has been in large part worked out for the GR, it has not been demonstrated that receptor movement is dynein-dependent. In contrast, p53 movement to the nucleus has been shown to be dynein-dependent, and it is immunoadsorbed in complexes that contain tubulin and cytoplasmic dynein (30); however, the linkage of p53 to dynein has not been defined. Here, we demonstrate that p53·hsp90 and GR·hsp90 heterocomplexes from DLD-1 human colon cancer cells exist in identical heterocomplexes containing cytoplasmic dynein and one of three immunophilins, FKBP52, CyP-40, or PP5. Competition with peptides *in vitro* shows that the PPIase domain of the immunophilin is required for dynein presence in the p53·hsp90 heterocomplexes. The immunophilin linkage to dynein is indirect by means of the dynamitin component of the dynein-associated dynactin complex, and we show that purified FKBP52 binds directly via its PPIase domain to immunopurified dynamitin. Expression of the immunophilin PPIase domain fragment in HT29-tsp53 cells inhibits nuclear translocation of p53 when cells are shifted to permissive temperature in the same manner as expression of *myc*-dynamitin. Our observations demonstrate that the hsp90-binding immunophilins are required both for p53 linkage to dynein *in vitro* and for dynein-dependent transport of p53 to the nucleus *in vivo*.

EXPERIMENTAL PROCEDURES

Materials—DLD-1 human colorectal adenocarcinoma cells were purchased from the American Type Culture Collection (Manassas, VA). HT29-tsp53 (formerly referred to as ts29-G cells) human colorectal cancer cell line overexpressing a mouse p53 temperature-sensitive mutant was described in previous works (31, 32). The Ab421 pan-specific mouse monoclonal antibody against p53 was kindly provided by Dr. Michael F. Clarke (University of Michigan Medical School). The Ab-4 mouse monoclonal IgG against mouse p53 and Ab-7 sheep antiserum against p53 were purchased from Calbiochem (La Jolla, CA). The A-14 rabbit polyclonal IgG and the 9E10 mouse monoclonal IgG against *c-myc* oligopeptide were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The MAB1618 mouse monoclonal IgG against the 74-kDa intermediate chain subunit of cytoplasmic dynein was purchased from Chemicon International (Temecula, CA). The JJ3 monoclonal IgG against p23 was provided by Dr. David Toft (The Mayo Clinic, Rochester, MN). The UPJ56 rabbit antiserum against FKBP52 was a kind gift from Dr. Karen Leach (Amersham Biosciences). The FiGR mouse monoclonal IgG used to immunoadsorb the GR was kindly provided by Dr. Jack Bodwell (Dartmouth Medical School, Lebanon, NH). The BuGR2 mouse monoclonal IgG used to immunoblot the GR and the rabbit polyclonal antibody against CyP-40 were from Affinity BioReagents (Golden, CO). The AC88 mouse monoclonal IgG against hsp90 and the N27F3–4 mouse monoclonal IgG against hsp70/hsc70 were from StressGen BioReagents (Victoria, BC, Canada). ^{125}I-Conjugated goat anti-mouse and anti-rabbit IgGs were from PerkinElmer Life Sciences. Horseradish peroxidase-conjugated goat anti-rabbit IgG was from Pierce. Horseradish peroxidase-conjugated goat anti-mouse, donkey anti-sheep antibodies, radicicol, and geldanamycin were obtained from Sigma. Rhodamine-conjugated goat anti-mouse IgG and FITC-conjugated donkey anti-rabbit IgG were obtained from Jackson ImmunoResearch (West Grove, PA). The baculovirus for the FLAG-tagged TPR domain of rat PP5 (33) and the rabbit antiserum against PP5 were kindly provided by Dr. Michael Chinkers (University of South Alabama, Mobile, AL). The pGEX1λT plasmid encoding the GST-rabbit FKBP52 Gly^{32}-Lys^{138} expression vector that comprises the PPIase core domain I (provided by Drs. Michel Renoir and Christine Radanyi, UMR8612 CNRS, Paris, France) and the purification of the PPIase core domain I protein were described previously (23, 34). The mammalian expression plasmid pSG5PL-PPIase core domain I was described in a previous work (23). The pEGFP-C3 plasmid encoding for *Aequorea victoria* green fluorescent protein was from Clontech (Palo Alto, CA). The *myc*-tagged pCMVH50m construct encoding for p50/dynamitin (35) was a kind gift from Dr. Richard B. Vallee (University of Massachusetts Medical School, Worcester, MA); Opti-MEM-I transfection medium was from Invitrogen; Trans-Fast transfection reagent was from Promega (Madison, WI); rabbit reticulocyte lysate was from Green Hectares (Oregon, WI); and Complete-Mini protease inhibitor mixture tablets were purchased from Roche Diagnostics (Mannheim, Germany).

Cell Culture—DLD-1 cells were grown at 37 °C in T-162 culture flasks containing 30 ml of Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 1.5 g/liter $NaHCO_3$, 4.5 g/liter glucose, and 1 mM pyruvate. HT29-tsp53 were grown at 39 °C (non-permissive temperature) on 22 × 22 mm coverslips placed on 35-mm plates in 2 ml of Dulbecco's modified Eagle's medium containing 10% bovine calf serum and 2 mM L-glutamine. 293-T Human fibroblasts were grown in Dulbecco's modified Eagle's medium supplemented with 10% bovine calf serum and antibiotics. All cultures were maintained under a 5% CO_2 atmosphere.

Immunoadsorptions—Confluent DLD-1 cells were harvested by scraping into ice-cold Earle's balanced saline, washed twice, and ruptured by Dounce homogenization in one volume of HE buffer (10 mM Hepes, 1 mM EDTA, pH 7.4) supplemented with 20 mM Na_2MoO_4 and one tablet of protease inhibitor mix per 3 ml of buffer. Homogenates were centrifuged at 3 °C for 30 min at 100,000 × *g*, the resultant supernatant being referred to as cytosol. For immunoadsorption of p53 or the GR, 250 μl of DLD-1 cytosol was incubated for 3 h at 4 °C with either 10 μl of Ab421 antibody or 7 μl of FiGR antibody and 16 μl of protein-A Sepharose. The pellets were washed four times with 1 ml of TEG buffer (10 mM TES, pH 7.6, 50 mM NaCl, 4 mM EDTA, 10% (v/v) glycerol, and protease inhibitors) containing 20 mM Na_2MoO_4. Immune pellets were resolved on SDS-12% polyacrylamide gels and transferred to Immobilon-P membranes. The membranes were probed with 0.1% Ab421 for p53, 0.25 μg/ml BuGR2 for GR, 1 μg/ml AC88 for hsp90, 1 μg/ml N27F3–4 for hsp70, 0.1% MAB1618 for dynein, 0.1% UPJ56 for FKBP52, 0.1% anti-CyP-40, 0.1% anti-PP5, 0.1% JJ3 mouse ascites for p23, or 0.1% A-14 for *myc*-dynamitin. The immunoblots were then incubated a second time with the appropriate ^{125}I-conjugated or horseradish peroxidase-conjugated counter-antibody to visualize immunoreactive bands. p53 was revealed by enhanced chemiluminescence. Because PP5, FKBP52, p53, and dynamitin migrate in the same region on gel electrophoresis, as do hsp70 and the dynein intermediate chain, we electrophoresed replicate samples of both non-immune and immune pellets and probed replicate immunoblots with antibody specific for each protein. Thus, the Western blots of Figs. 1, 2, and 6 are necessarily composites prepared from two or more replicate immunoblots.

Heterocomplex Reconstitution—DLD-1 cytosol was prepared in HE buffer without molybdate. After immunoadsorbing p53, coadsorbed proteins were dissociated from the immune pellet by incubating for 2 h with TEG buffer supplemented with 500 mM NaCl. The pellet was washed twice with TEG buffer and twice with 10 mM Hepes buffer at pH 7.4. p53·hsp90 heterocomplexes were then assembled by incubating the stripped immune pellets with 50 μl of rabbit reticulocyte lysate and 5 μl of an ATP-regenerating system (50 mM ATP, 250 mM creatine phosphate, 20 mM magnesium acetate, and 100 units/ml of creatine phosphokinase). The assembly mixtures were incubated for 30 min at 30 °C, with suspension of the pellets by shaking the tubes every 2–3 min. Pellets were then washed four times with 1 ml of ice-cold TEG buffer supplemented with 20 mM Na_2MoO_4 and boiled in SDS-sample buffer. In peptide competition experiments, the reticulocyte lysate was preincubated for 30 min at 30 °C with either 10 μl of lysate (8 mg of protein/ml) of Sf9 cells expressing the TPR domain fragment of rat PP5 or with 30 μg of purified PPIase domain fragment of rabbit FKBP52 (29).

Cell Transfection—When HT29-tsp53 cells were ~50% confluent, the culture medium was replaced by Opti-MEM transfection medium containing 5% bovine calf serum, and the incubation continued for 1 h as described above. The medium was aspirated and replaced by a transfection mixture (at a 3 μl liposome/1 μg of DNA ratio) preincubated for 15 min at room temperature in Opti-MEM, which contained either 0.4 μg of pCMVH50m or 1.0 μg pEGFP-C3 and 4.0 μg of pSG5PL-PPIase core domain I. Controls were transfected with pCMV or pEGFP-C3 and pSG5PL. After 1.5 h of transfection, the mixture was replaced by regular medium and the cells were incubated for an additional 24 h at the non-permissive temperature of 39 °C. The expression of dynamitin was evidenced by indirect immunofluorescence with an anti-*myc* antibody, and the co-transfection of EGFP and the PPIase domain I due to the green fluorescence exhibited by transfected cells. When *myc*-dynamitin was overexpressed in 293-T human fibroblasts, 6 μg of pCMVH50m plasmid was used. After 48 h, the cells were ruptured by Dounce homogenization in one volume of HE buffer supplemented with 20 mM Na_2MoO_4.

Indirect Immunofluorescence—Nuclear translocation of p53 was trig-

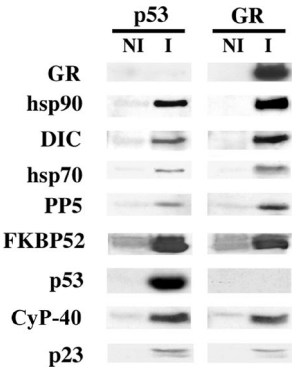

FIG. 1. **Immunoadsorption of p53 and GR heterocomplexes.** DLD-1 cell cytosol was incubated with non-immune mouse IgG (*NI*) or with antibody (*I*) against p53 or the GR. The immunopellets were washed, and proteins were resolved by SDS-polyacrylamide gel electrophoresis and immunoblotting for the indicated proteins.

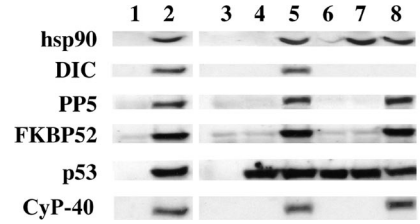

FIG. 2. **Effects of geldanamycin and competition with TPR and PPIase domain peptides on p53 heterocomplexes assembled by reticulocyte lysate.** p53 was immunoadsorbed from DLD-1 cytosol with the Ab421 antibody, and the p53-associated proteins were stripped from the immunopellet with NaCl (conditions *3-8*). The stripped p53 immunopellets were incubated with reticulocyte lysate, washed, and proteins were resolved by electrophoresis and immunoblotting. *Lane 1*, cytosol immunoadsorbed with non-immune IgG; *lane 2*, cytosol immunoadsorbed with Ab421 antibody; *lane 3*, stripped non-immune pellet incubated with reticulocyte lysate; *lane 4*, stripped immune pellet incubated with buffer; *lane 5*, stripped immune pellet incubated with reticulocyte lysate; *lane 6*, stripped immune pellet incubated with reticulocyte lysate containing 10 μM geldanamycin; *lane 7*, stripped immune pellet incubated with reticulocyte lysate preincubated with the TPR domain fragment of rat PP5; *lane 8*, stripped immune pellet incubated with reticulocyte lysate preincubated with the PPIase domain fragment of FKBP52.

gered by incubating HT29-tsp53 cells at 32 °C. Cells were fixed and permeabilized by immersion in cold (−20 °C) methanol at the indicated times, and immunostained by inverting the coverslip on 50 μl of solution of phosphate-buffered saline with 1% bovine serum albumin containing 1 μl of mouse monoclonal IgG against mouse p53 or 0.5 μl of rabbit polyclonal IgG against the *c-myc* tag of dynamitin. The coverslips were washed and reincubated with 1/100 dilution of the corresponding counter-antibody (rhodamine-conjugate goat anti-mouse IgG and fluorescein isothiocyanate-conjugate donkey anti-rabbit IgG). Cells were observed with a Leitz Aristoplan epi-illumination microscope and scored for p53 nuclear translocation as described before for the GR (21): a score of 4 was used for nuclear fluorescence much greater than cytoplasmic fluorescence, 3 for nuclear fluorescence greater than cytoplasmic fluorescence, 2 for nuclear fluorescence equal to cytoplasmic fluorescence, 1 for nuclear fluorescence less than cytoplasmic fluorescence, and 0 for nuclear fluorescence much less than cytoplasmic fluorescence. The translocation scores represent the mean ± S.E. from three experiments, in which ≥100 cells/condition/experiment were counted. Significance was analyzed by one-way analysis of variance followed by the Bonferroni *t* test.

Formation of Dynamitin·Immunophilin Complexes—Cytosol (200 μl) from 293-T cells transfected with *myc*-dynamitin was incubated with 10 μl of 9E10 mouse monoclonal IgG against *myc* and 16 μl of protein-A Sepharose. After 2 h, 0.5 M NaCl and 0.05% Nonidet P-40 were added and the incubation was continued for two more hours to strip dynamitin of associated proteins. After the pellets were washed twice with 1 ml of TEG buffer and twice with HKD buffer (10 mM Hepes, pH 7.4, 100 mM KCl, and 2 mM dithiothreitol), they were incubated for 20 min at 30 °C with 50 μl of rabbit reticulocyte lysate and 5 μl of the ATP-regenerating system. The samples were washed 4× with TEG buffer with 20 mM Na_2MoO_4 before gel electrophoresis and immunoblotting with rabbit polyclonal antibody against *c-myc* oligopeptide and specific rabbit antibodies against immunophilins. Prior to the reconstitution assay, the lysates were preincubated for 30 min at 30 °C with HKD buffer plus or minus purified PPIase core domain I of FKBP52. For direct binding of purified dynamitin to purified FKBP52, *myc*-dynamitin was immunoadsorbed from 293-T cell cytosol and stripped of associated proteins, as described above. After washing, the immunopellets were incubated for 30 min on ice with FKBP52, purified as described previously (27), in the presence or absence of purified PPIase domain I of FKBP52. The final volume was adjusted to 50 μl with HKD buffer. Pellets were washed 4 times with TEG buffer with 20 mM Na_2MoO_4, and proteins were revealed by immunoblotting with rabbit anti-*myc* antibody and the UPJ56 anti-FKBP52 antibody.

RESULTS

Composition of p53·hsp90 Heterocomplexes in DLD-1 Cells—To examine the composition of native p53·hsp90 heterocomplexes, we selected the DLD-1 human colorectal cancer cell line, which possesses a point mutation that converts Ser-241 of p53 to Phe (36). This mutant p53 is localized in the cytoplasm and exists in cytosolic heterocomplexes with hsp90 (20). In Fig. 1, mutant p53 and the GR were immunoadsorbed from DLD-1 cytosol, and proteins in the washed immunopellets were identified by immunoblotting. Hsp90 was coadsorbed with both proteins, as was some hsp70, which is an essential component of the multiprotein chaperone machinery that forms "client protein" heterocomplexes with hsp90 (13). Also present in both heterocomplexes was p23, a ubiquitous 23-kDa acidic protein that binds to the ATP-dependent conformation of hsp90 when the client protein·hsp90 complex has been assembled (13). It has been shown that p23 binds dynamically and stabilizes the GR·hsp90 heterocomplex (37).

The TPR domain immunophilins FKBP52 and CyP-40 are coimmunoadsorbed with both p53 and the GR, as is PP5 (Fig. 1). Both of these immunophilins and the immunophilin homolog PP5 have been shown to exist in cytosols in complexes that contain cytoplasmic dynein (29). Here, we use an antibody against the dynein intermediate chain (DIC) to show that dynein is coimmunoadsorbed with both the GR and p53, as previously reported (23, 24, 30). FKBP51 is another immunophilin that has been found in steroid receptor·hsp90 heterocomplexes, where it seems to counter the increase in steroid-binding affinity of the hsp90-bound GR caused by FKBP52 (38). Because we do not have an antibody to determine whether FKBP51 is in complexes that contain dynein, we have not assayed it here. Nevertheless, it seems clear from Fig. 1 that the same complex of proteins is coimmunoadsorbed with p53 as with the GR. The bands in Fig. 1 are clearly specific for the GR and p53, with non-immune pellets prepared from the same cytosol showing the background. However, the GR and p53 heterocomplexes may not be identical in that they may differ somewhat in the relative amounts of the immunophilins that are present. The specificity of the procedure is underlined by two considerations: the GR is not present in p53 immune pellets, and p53 is not present in GR immune pellets prepared from the same sample of cytosol.

Immunophilins Link the p53·hsp90 Complex to Dynein—Reticulocyte lysate contains the multiprotein hsp90/hsp70-based chaperone machinery that assembles client protein·hsp90 heterocomplexes (13), and GR·hsp90 heterocomplexes assembled in reticulocyte lysate contain both immunophilins and cytoplasmic dynein (23). Fig. 2 shows the reconstitution of p53·hsp90·immunophilin·dynein complexes by rabbit reticulocyte lysate. The native heterocomplex of mutant p53 with human chaperones and dynein is shown in *lane 2*. The immunoadsorbed p53 was then stripped of its associated proteins (*lane 4*) and incubated with reticulocyte lysate to reconstitute the heterocomplex with rabbit proteins (*lane 5*). In *lane 6*, p53 was

incubated with reticulocyte lysate that was treated with geldanamycin, an ansamycin antibiotic that binds to the unique N-terminal ATP site of hsp90 and inhibits its function (39). When hsp90 binding is blocked, there are no immunophilins or dynein associated with p53. Also, when the TPR domain fragment of PP5 is present to compete for immunophilin binding to p53-bound hsp90, p53·hsp90 complexes are formed that lack immunophilins and dynein (*lane 7*). When the PPIase domain fragment of FKBP52 is present (*lane 8*), p53·hsp90·immunophilin complexes are formed that lack dynein. Thus, *in vitro*, the immunophilins link the p53·hsp90 complex to dynein.

Radicicol Inhibits p53 Transport to the Nucleus—To examine the movement of p53 from the cytoplasm to the nucleus, we chose HT29-tsp53 cells, a stable human colon carcinoma cell line expressing a temperature-sensitive allele of murine p53 (31). This temperature-sensitive mutant of p53 is fully active and nuclear at the permissive temperature of 32 °C, but it is inactive and located in the cytoplasm at the non-permissive temperature of 39 °C (40–42). To inhibit hsp90 function, we used radicicol rather than geldanamycin. Geldanamycin has a quinone moiety that redox cycles *in vivo* to produce hydrogen peroxide (43), which induces nuclear translocation of p53 (44). Radicicol inhibits hsp90 in the same manner as geldanamycin (45), but it does not possess a quinone moiety and does not cause oxidant production (43).

Inhibition of the cytoplasmic-nuclear transport of p53 by 5 μM radicicol is shown in Fig. 3. By 60 min after the cells are switched from 39 to 32 °C, all of the p53 has progressed from the cytoplasm to the nucleus in vehicle-treated cells, whereas only partial movement has occurred in the presence of radicicol (Fig. 3*A*). From the time course of the radicicol effect shown in Fig. 3*B*, it is clear that movement is inhibited. However, the movement is not blocked, and by 90 min, most of the p53 has translocated to the nucleus in cells treated with 5 μM radicicol. In some other intact cell systems, 20 μM radicicol has been required for substantial inhibition of hsp90 (43). Unfortunately, at concentrations of 10 μM radicicol and above, the HT29-tsp53 cells detach from the plastic, and we have not been able to determine whether higher concentrations of drug produce a greater inhibition of nuclear transport.

Inhibition of p53 Transport by Expression of a PPIase Domain Fragment—To show that the overexpressed, temperature-sensitive, mutant mouse p53 in HT29-tsp53 cells undergoes dynein-dependent movement to the nucleus as reported for wild-type human p53 (30), p53 movement was examined in HT29-tsp53 cells expressing *myc*-dynamitin. Dynamitin is a 50-kDa subunit of the dynein-associated dynactin complex, and its overexpression blocks dynein function by dissociating the motor from its cargoes (35, 46). In HT29-tsp53 cells overexpressing dynamitin (Fig. 4*A*, *green*), there was very little movement of p53 (Fig. 4*A*, *red*) compared with surrounding nontransfected cells where p53 accumulated in the nucleus after the switch to permissive temperature. Overexpression of the PPIase domain fragment of FKBP52 also inhibited p53 movement (Fig. 5*A*). The extent of inhibition of p53 movement by the PPIase domain fragment (Fig. 5*B*) was similar to the inhibition produced by overexpression of dynamitin (Fig. 4*B*).

Immunophilin Interaction with Dynamitin—The observations that the PPIase domain fragment competed for the binding of p53·hsp90·immunophilin complexes to dynein (Fig. 2) and that overexpression of the PPIase domain fragment inhibited p53 translocation to the nucleus (Fig. 5) are consistent with a model in which the immunophilin PPIase domain links the p53·hsp90·immunophilin complex to the protein motor for retrograde movement. However, it is not yet clear what part of the dynein/dynactin complex interacts directly with the PPIase

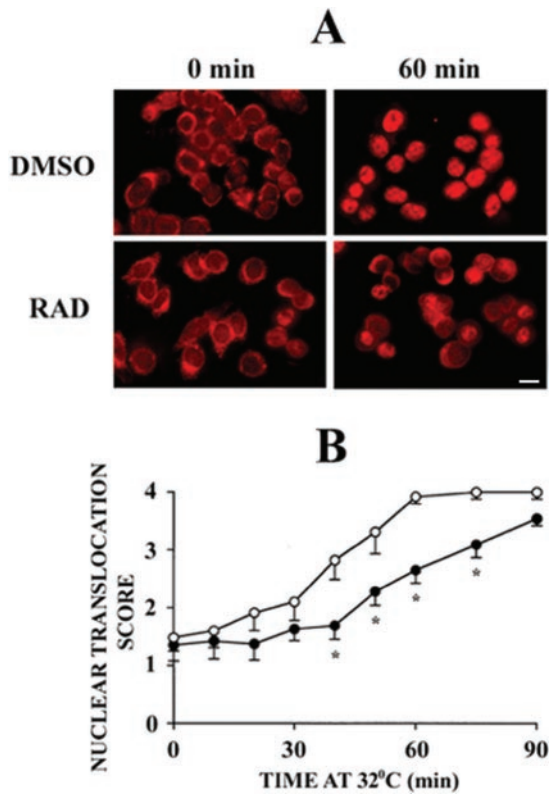

FIG. 3. **p53 translocation to the nucleus is impeded by radicicol.** *A*, HT29-tsp53 colon cancer cells were preincubated for 15 min in the presence of 5 μM radicicol (*RAD*) or 0.1% vehicle (*DMSO*), and p53 translocation to the nucleus was triggered by shifting the temperature from 39 to 32 °C. At the indicated times, the cells were fixed and permeabilized in cold methanol and then immunostained by incubating with the Ab-4 monoclonal IgG against mouse p53, followed by incubating with rhodamine-conjugated goat anti-mouse IgG. *Bar*, 10 μm. *B*, p53 nuclear translocation in cells treated with either vehicle (○) or radicicol (●) was scored as described under "Experimental Procedures" and plotted against the incubation time at the permissive temperature. Results are the mean ± S.E. of three independent experiments. *, $p \leq 0.03$, significant difference.

domain. We have demonstrated previously a very weak interaction between the PPIase domain fragment of FKBP52 and the purified expressed intermediate chain of mouse cytoplasmic dynein (29). However, PPIase domains engage in weak interactions with peptidyl prolines (47), and this binding could be nonspecific in this way. Cytoplasmic dynein is thought to link vesicles and organelles indirectly through dynactin (46), and it is possible that overexpression of dynamitin inhibits p53 nuclear translocation (Fig. 4), because it is dynamitin itself that is the component of the dynactin complex that interacts directly with the immunophilin PPIase domain.

If dynamitin binds directly to the immunophilin PPIase domain, then it should be present in immunoadsorbed p53 heterocomplexes. Because we do not have an antibody directed against dynamitin, we transfected DLD-1 cells with *myc*-dynamitin, immunoadsorbed p53 complexes, and probed Western blots of the immunoadsorbate with anti-*myc* antibody to detect the presence of dynamitin. As shown in Fig. 6*A*, dynamitin coimmunoadsorbed with p53 (*lane 2*), and its presence was eliminated by competition with the PPIase domain fragment of FKBP52 (*lanes 3 and 4*). To determine whether dynamitin binds TPR domain immunophilins, *myc*-dynamitin was immunoadsorbed to protein-A Sepharose, stripped of associated proteins by incubation with salt, and the immunopellet was incubated with reticulocyte lysate in the presence or absence of the PPIase domain fragment. It can be seen in Fig. 6*B* that the

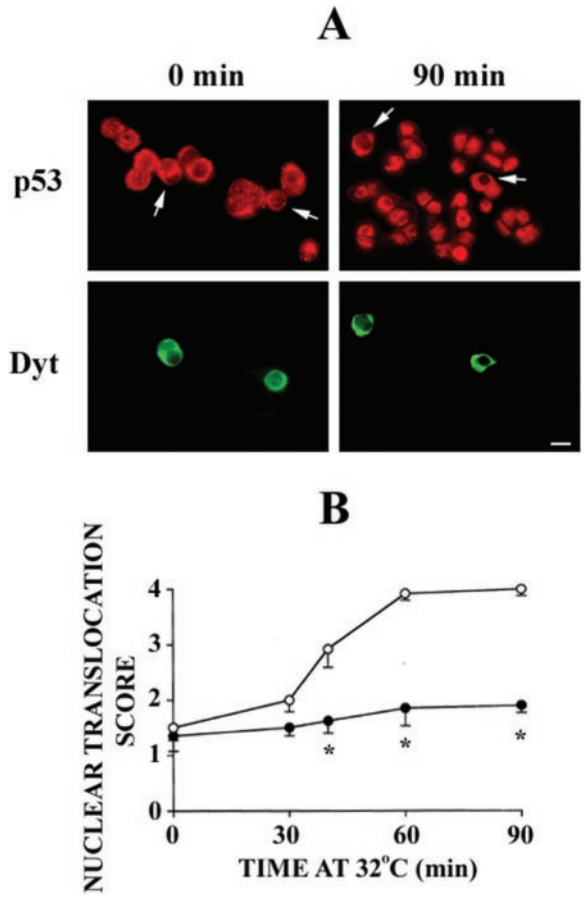

FIG. 4. **Overexpression of dynamitin inhibits p53 movement to the nucleus.** *A*, HT29-tsp53 cells were transfected with pCMVH50m plasmid encoding for *myc*-tagged dynamitin (*Dyt*). p53 nuclear translocation was triggered by shifting the temperature from 39 to 32 °C. The cells were double-stained for p53 (*red*) and dynamitin (*green*). *Arrows*, transfected cells. *Bar*, 10 μm. *B*, rate of nuclear translocation of p53 (mean ± S.E., $n = 3$) in cells transfected with empty vector (○) or with dynamitin (●). *, $p \leq 0.005$, significant difference.

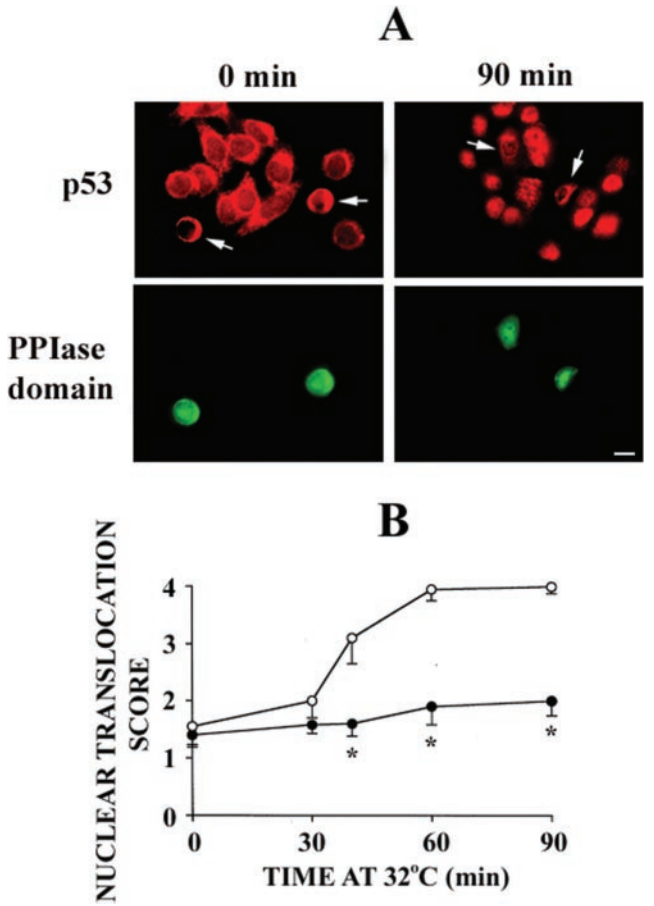

FIG. 5. **Overexpression of the PPIase domain I fragment of FKBP52 inhibits p53 movement to the nucleus.** *A*, HT29-tsp53 cells were co-transfected with pSG5PL-PPIase core domain I and pEGFP-C3 plasmids encoding for the PPIase domain of rabbit FKBP52 (Gly^{32}-Lys^{138}) and green fluorescent protein. p53 nuclear translocation was triggered by shifting the temperature from 39 to 32 °C, and cells were immunostained for p53 (*red*). *Arrows*, transfected cells. *Bar*, 10 μm. *B*, rate of nuclear translocation of p53 (mean ± S.E., $n = 3$) in cells transfected with empty vector (○) or with the PPIase domain (●). *, $p \leq 0.005$, significant difference.

stripped dynamitin (*lane 1*) bound to the three hsp90-binding immunophilins, PP5, FKBP52 and CyP-40 (*lane 2*), and the immunophilins were not bound if the PPIase domain fragment was present to compete for their binding (*lane 3*). To determine whether the immunophilin is directly interacting with dynamitin, we incubated immunoadsorbed, stripped *myc*-dynamitin with purified FKBP52. As shown in Fig. 6*C*, stripped dynamitin (*lane 3*) bindsFKBP52 (*lane 4*). This binding is competed by the purified FKBP52 PPIase domain fragment (*lanes 5 and 6*), suggesting a direct interaction between the PPIase domain of the immunophilin and the dynamitin component of the dynactin complex.

DISCUSSION

We show here that the immunophilins link the p53·hsp90 complex to cytoplasmic dynein *in vitro* (Fig. 2) and that preventing that interaction by overexpression of the PPIase domain fragment inhibits p53 movement to the nucleus *in vivo* (Fig. 5). As illustrated in the cartoon in Fig. 7, the key linkages involve immunophilin binding to hsp90 by means of the TPR domain and binding to the dynamitin component of the dynein-associated dynactin complex by means of the PPIase domain (Fig. 6). Neither binding of the immunophilins to dynein (26) nor the rate of transport of the GR (23) or p53 (data not shown) to the nucleus is affected by FK506. Thus, although the PPIase domain interacts with the dynein/dynactin complex, PPIase activity is not required for the interaction.

TPR domain immunophilins are distributed widely among animal and plant cells, and both the TPR domain binding to hsp90 and PPIase domain linkage to the dynein motor complex are conserved interactions (28, 48). This suggests that the TPR domain immunophilins perform a rather fundamental function or functions, and that there may be considerable redundancy in their action. The hsp90 binding TPR domain immunophilins were discovered as components of the steroid receptor·hsp90 heterocomplexes (13), and their functions have not been broadly studied for other transcription factors. Their presence as components that link p53 to the motor protein responsible for its retrograde movement is consistent with a broad role for these immunophilins in protein trafficking to the nucleus. The only other clear demonstration of a TPR domain immunophilin effect is that the binding of FKBP52 to the GR·hsp90 heterocomplex increases the affinity of steroid binding in a manner that requires the immunophilin PPIase activity (38). However, this effect seems to be specific to FKBP52, and it is clearly limited to the GR as opposed to other steroid receptors.

The immunophilins bind after the client protein·hsp90 heterocomplex has been assembled, and by cross-linking steroid receptor complexes, Gehring and his colleagues determined a heterotetrameric structure of 1 receptor:2 hsp90:1 immunophilin (reviewed in Ref. 49). This is the stoichiometry we suggest for the p53·hsp90·immunophilin complex in Fig. 7. The differ-

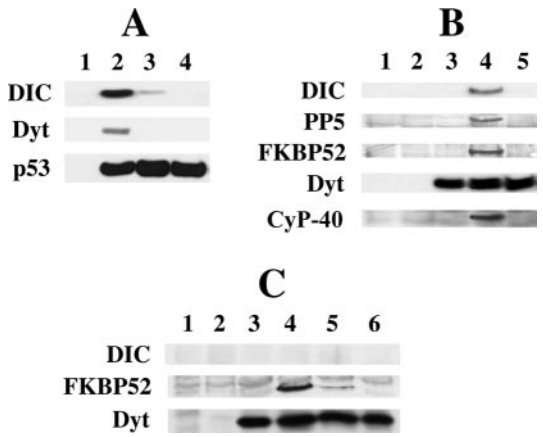

FIG. 6. **Dynamitin binding to immunophilins.** *A*, dynamitin presence in p53 heterocomplexes. Cytosol (200 μl) of DLD-1 cells transfected with *myc*-dynamitin was preincubated for 30 min at 30 °C with either buffer (*lanes 1* and *2*) or 50 μg (*lane 3*) or 100 μg (*lane 4*) of the PPIase domain of FKBP52. p53 was immunoadsorbed with the Ab421 antibody (*lanes 2-4*) or a non-immune mouse IgG (*lane 1*), and proteins were revealed by Western blotting. *B*, binding of TPR domain immunophilins to dynamitin. Dynamitin was immunoadsorbed from 293-T cell cytosol, stripped of associated proteins, and incubated with reticulocyte lysate. *Lane 1*, non-immune pellet incubated with lysate; *lane 2*, stripped immune pellet obtained from untransfected cells incubated with lysate; *lane 3*, stripped immune pellet incubated with HKD buffer; *lane 4*, stripped immune pellet incubated with lysate; *lane 5*, stripped immune pellet incubated with lysate and 100 μg of purified PPIase domain I. *C*, direct binding of FKBP52 to dynamitin. Stripped *myc*-dynamitin immunopellets were incubated with 50 μg of purified FKBP52 in the absence or presence of purified PPIase domain I fragment. *Lane 1*, non-immune pellet incubated with FKBP52; *lane 2*, immune pellet prepared from cytosol of untransfected cells that was incubated with FKBP52; *lanes 3–6*, stripped dynamitin pellets incubated with buffer alone (*lane 3*), FKBP52 alone (*lane 4*), FKBP52 plus 100 μg of PPIase domain (*lane 5*), or FKBP52 plus 200 μg of PPIase domain (*lane 6*).

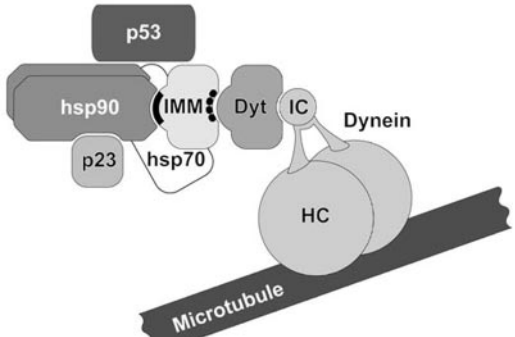

FIG. 7. **TPR domain immunophilins link the p53·hsp90 heterocomplex to dynein for retrograde movement along microtubules.** Cytoplasmic dynein is the motor protein that processes along microtubules in a retrograde movement to the nucleus. Dynein is a large multi-subunit complex (~1.2 MDa) composed of two heavy chains (*HC*) that have the processive motor activity, three intermediate chains (*IC*), and some light chains that are not shown. The immunophilin (*IMM*) links to p53-bound hsp90 by means of its TPR domain (*solid black crescent*) and it links to the dynamitin (*Dyt*) component of the dynein-associated dynactin complex by means of its PPIase domain (*dotted crescent*).

ent immunophilins compete with each other for binding to hsp90, and they exist in separate GR·hsp90 heterocomplexes (49). Nevertheless, the number of TPR acceptor sites per hsp90 dimer is somewhat controversial. Saturation binding (50) and cross-linking (27) studies are consistent with one TPR binding site per hsp90 dimer, whereas isothermal titration calorimetry studies are consistent with the binding of two molecules of TPR protein per hsp90 dimer (51, 52).

As p53 acts by regulating transcription, its translocation from the cytoplasm into the nucleus is required for the biological response. The demonstration by Giannakakou *et al.* (30) that p53 is transported to the nucleus by dynein provided an important advance in understanding the mechanism of nuclear translocation. Here, we have used p53 mutants that localize to the cytoplasm to show the similarity between p53 heterocomplexes and those of the glucocorticoid receptor, another hsp90-regulated transcription factor. This has allowed us to identify the hsp90-binding immunophilins as the linkers between p53·hsp90 and the dynein motor complex. The observations of Fig. 6 suggest that the hsp90-binding immunophilins link directly via their PPIase domains to dynamitin, which in turn connects the complex to the dynein motor. This movement model should provide an important advance in understanding how a variety of proteins that act in the nucleus are transported to that organelle.

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