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Evidence for Glucocorticoid Receptor Transport on Microtubules by Dynein*

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Rapid, ligand-dependent movement of glucocorticoid receptors (GR) from cytoplasm to the nucleus is hsp90-dependent, and much of the movement system has been defined. GR·hsp90 heterocomplexes isolated from cells contain one of several hsp90-binding immunophilins that link the complex to cytoplasmic dynein, a molecular motor that processes along microtubular tracks to the nucleus. The immunophilins link to dynein indirectly via the dynamitin component of the dynein-associated dynactin complex (Galigniana, M. D., Harrell, J. M., O'Hagen, H. M., Ljungman, M., and Pratt, W. B. (2004) *J. Biol. Chem.* 279, 22483–22489). Although it is known that rapid, hsp90-dependent GR movement requires intact microtubules, it has not been shown that the movement is dynein-dependent. Here, we show that overexpression of dynamitin, which blocks movement by dissociating the dynein motor from its cargo, inhibits ligand-dependent movement of the GR to the nucleus. We show that native GR·hsp90·immnunophilin complexes contain dynamitin as well as dynein and that GR heterocomplexes isolated from cytosol containing paclitaxel and GTP to stabilize microtubules also contain tubulin. The complete movement system, including the dynein motor complex and tubulin, can be assembled under cell-free conditions by incubating GR immune pellets with paclitaxel/GTP-stabilized cytosol prepared from GR$^-$ L cells. This is the first evidence that the movement of a steroid receptor is dynein-dependent, and it is the first isolation of a steroid receptor bound to the entire system that determines its retrograde movement.

As the initial step in their action, transcription factors, such as steroid receptors, p53, and HSF1, must move in a targeted manner through the cytoplasm to the nucleus. Until recently, there has been little mechanistic understanding of how protein solutes (*i.e.* non-vesicle-associated proteins) undergo such retrograde trafficking. Because the glucocorticoid receptor (GR)[1] moves rapidly and quantitatively from the cytoplasm to the nucleus in a ligand-dependent manner, it has been a useful model for studying the movement process (reviewed in Refs. 1 and 2). Like other steroid receptors, the GR forms heterocomplexes with hsp90, and experiments testing the effects of molybdate and geldanamycin on GR trafficking have led to the concept that a dynamic process of receptor·hsp90 complex assembly-disassembly is required for rapid nuclear translocation (3, 4). Geldanamycin is a quite specific inhibitor of hsp90 heterocomplex assembly (5) that has been shown to inhibit the translocation of several hsp90-regulated transcription factors, including glucocorticoid, androgen, and aryl hydrocarbon receptors, as well as the tumor suppressor protein p53 (6–11). Recently, it has been shown that GR mobility within the nucleus is also hsp90-dependent (12).

Retrograde hsp90-dependent movement of the GR in the cytoplasm occurs along cytoskeletal tracts (6), and a number of biochemical observations have led to a model of the movement system. GR·hsp90 heterocomplexes immunoadsorbed from cell lysates contain cytoplasmic dynein (13, 14), a molecular motor that processes along microtubules toward the nucleus (15). The receptor·hsp90 heterocomplexes contain one of several immunophilins possessing tetratricopeptide repeat (TPR) domains that bind to a TPR acceptor site on hsp90 (1). The signature domain of the immunophilins is the peptidylprolyl isomerase (PPIase) domain, which is the binding site for immunosuppressant drugs of the FK506 or cyclosporine A class. The immunophilins link the GR·hsp90 heterocomplex to the dynein·dynactin movement machinery via their PPIase domains (16–18).

GR·hsp90 heterocomplexes contain one of three TPR domain immunophilins (FKBP52, FKBP51, and cyclophilin 40) or protein phosphatase 5 (PP5), a protein phosphatase that contains TPR and PPIase homology domains (reviewed in Ref. 1). Immunoadsorption of FKBP52, cyclophilin 40, or PP5 is accompanied by coimmunoadsorption of dynein, and coimmunoadsorption of dynein is competed by a purified PPIase domain fragment of FKBP52 (16, 18). The PPIase domain fragment also competes for the presence of dynein in GR·hsp90·immunophilin complexes (13, 17), and expression of the fragment in mouse fibroblasts impedes ligand-dependent GR nuclear translocation to the same extent as treatment of cells with geldanamycin (13). Thus, there is both *in*

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[1] The abbreviations used are: GR, glucocorticoid receptor; hsp, heat shock protein; PP5, protein phosphatase 5; FKBP, FK506 binding protein; TPR, tetratricopeptide repeat; PPIase, peptidylprolyl isomerase; TES, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethanesulfonic acid.

vitro and *in vivo* evidence that GR·hsp90 complexes are linked via TPR domain immunophilins to cytoplasmic dynein. However, it has not been demonstrated that receptor movement is dynein-dependent.

The tumor suppressor protein p53, which also forms heterocomplexes with hsp90 (19, 20), is immunoadsorbed in complexes that contain dynein and tubulin, and its movement to the nucleus has been shown to be dynein-dependent (21). Dynein dependence was determined by showing that overexpression of dynamitin abrogates p53 nuclear accumulation (21). Dynamitin is a 50-kDa subunit of the dynein-associated dynactin complex, and its overexpression blocks movement by dissociating the motor protein from cargo (22, 23). Here, we show that dynamitin overexpression inhibits ligand-dependent GR nuclear translocation in 3T3 mouse fibroblasts to the same extent as treatment with the hsp90 inhibitor geldanamycin or overexpression of the PPIase domain fragment. We show that immunoprecipitation of GR from cytosol containing paclitaxel and GTP to stabilize microtubules yields coprecipitation of tubulin as well as dynein. Both tubulin and dynein are uncoupled from the GR·hsp90·immunophilin complex by competition with a purified PPIase domain fragment of FKBP52, consistent with the immunophilin forming the bridge linking the receptor to the movement system. This work represents the first isolation of a steroid receptor bound to the entire system determining its retrograde movement.

EXPERIMENTAL PROCEDURES

Materials

Untreated rabbit reticulocyte lysate was from Green Hectares (Oregon, WI). ^{125}I-conjugated goat anti-mouse and anti-rabbit IgGs were obtained from PerkinElmer Life Sciences. Peroxidase-conjugated rabbit anti-rat IgG was from Sigma. The A-14 rabbit polyclonal IgG against c-*myc* oligopeptide was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The MAB1618 mouse monoclonal IgG against the 74-kDa intermediate chain subunit of dynein and the rabbit antiserum against dynamitin were purchased from Chemicon Intl. (Temecula, CA). The UPJ56 rabbit antiserum against FKBP52 was provided by Dr. Karen Leach (Pfizer, Inc., Ann Arbor, MI). Purified rat IgG against α-tubulin was purchased from Accurate Chemical and Scientific Corp. (Westbury, NY). The FiGR mouse monoclonal IgG used to immunoadsorb the GR was provided by Dr. Jack Bodwell (Dartmouth Medical School, Lebanon, NH). Mouse monoclonal IgGs against p150^{Glued} and dynactin p50 (dynamitin) were from BD Transduction Laboratories (San Diego, CA). The BuGR2 monoclonal IgG used to immunoblot the GR was from Affinity BioReagents (Golden, CO). The AC88 and EC1 mouse monoclonal IgGs against hsp90 and FKBP52, respectively, were from StressGen Bioreagents (Victoria, Canada). The rabbit antiserum against PP5 and the baculovirus for the FLAG-tagged TPR domain of rat PP5 were kindly provided by Dr. Michael Chinkers (University of South Alabama, Mobile, AL). Rhodamine-conjugated goat anti-mouse IgG and fluorescein isothiocyanate-conjugated donkey anti-rabbit IgG were from Jackson ImmunoResearch (West Grove, PA). The pGEX1λT plasmid encoding the glutathione *S*-transferase 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 peptide fragment were described previously (13, 24). The *myc*-tagged pCMVH50m construct encoding for p50/dynamitin (22) was a kind gift from Dr. Richard Vallee (University of Massachusetts Medical School, Worchester, MA). Opti-MEM-1 transfection medium was from Promega (Madison, WI). The E82.A3 subline of mouse L cells was kindly provided by Dr. Paul Housley (University of South Carolina School of Medicine, Columbia, SC).

Methods

Cell Culture and Transfection—For immunoadsorption experiments, L929 mouse fibroblasts were grown in a monolayer in Dulbecco's modified Eagle's medium with 10% calf serum. The cells were harvested by scraping into Earle's balanced saline, suspended in HE buffer (10 mM Hepes, pH 7.4, and 1 mM EDTA) with 20 mM Na_2MoO_4 supplemented with one tablet protease inhibitor mix/3 ml of buffer and ruptured by Dounce homogenization. The cell homogenates were centrifuged for 1 h at 100,000 $\times$ *g*, and the supernatant from this centrifugation is referred to as the cytosol.

For visualization by indirect immunoflourescence, 3T3 fibroblasts were grown on 11 $\times$ 22-mm coverslips in 2 ml of Dulbecco's modified Eagle's medium supplemented with bovine calf serum. When cells were ~50% confluent, the culture medium was replaced by Opti-MEM transfection medium containing 5% bovine calf serum, and the incubation was continued for 1 h. The medium was aspirated and replaced by a transfection mixture (at 3 μl of liposome/μg of DNA) preincubated for 15 min at room temperature in Opti-MEM, which contained 0.4 μg of pCMVH50m or pCMV. After 1.5 h of transfection, the mixture was replaced by regular medium, and the cells were incubated for an additional 48 h, prior to the addition of 1 μM dexamethasone to initiate nuclear translocation of the GR.

Indirect Immunoflourescence—At various times after dexamethasone addition, the cells were fixed, permeabilized by immersion in cold (−20 °C) methanol, and immunostained by inverting the coverslip on 50 μl of a solution of phosphate-buffered saline with 1% bovine serum albumin containing 1 μl of FiGR mouse monoclonal IgG against the GR or 0.5 μl of rabbit polyclonal IgG against the *myc* tag of dynamitin. The coverslips were washed and reincubated with a 1:100 dilution of the corresponding counter antibody (rhodamine-conjugated goat anti-mouse IgG and fluorescein isothiocyanate-conjugated donkey anti-rabbit IgG). The cells were observed with a Leitz Aristoplan epi-illumination microscope and scored for GR translocation as described before (6), using a score of 4 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 means ± S.E. from three experiments, in which ≥30 cells/data point/experiment were counted. Significance was analyzed by one-way analysis of variance followed by the Bonferroni *t* test.

Immunoadsorption—For immunoadsorption of tubulin-containing GR complexes, L cells were incubated for 20 min with 10 μM paclitaxel prior to homogenization in HE buffer containing 20 mM Na_2MoO_4, 20 μM paclitaxel, and 100 μM GTP to stabilize the microtubules. Prior to GR immunoadsorption, 0.04% Nonidet P40 was added to the cytosol, and it was centrifuged for 15 min in an Airfuge. GR was immunoadsorbed from air centrifuged cytosol by incubating 250-μl aliquots for 3 h with rotation at 4 °C with 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 12% SDS-polyacrylamide gels and transferred to Immobilon-P membranes. The membranes were probed with 0.25 μg/ml BuGR2 for GR, 1 μg/ml AC88 for hsp90, 0.1% MAB1618 for dynein, 0.1% A-14 for *myc*-dynamitin, 0.2% anti-dynamitin serum for endogenous dynamitin, 0.1% UPJ56 for FKBP52, 0.1% anti-PP5, or 0.1% anti-tubulin. The immunoblots were then incubated a second time with the appropriate ^{125}I-conjugated or horseradish peroxidase-conjugated counter antibody to visualize immunoreactive bands. For immunoadsorption of FKBP52, 300 μl of L cells were immunoadsorbed to protein A-Sepharose with 10 μl of UPJ56, and the immunoblot was probed with 1 μg/ml EC1 or 2 μg/ml anti-dynactin p50 for dynamitin or 2 μg/ml anti-p150^{Glued}.

Reconstitution of GR Heterocomplexes Containing Tubulin—Mouse GR was overexpressed in Sf9 cells as described previously (25), cytosol was prepared, and receptors were immunoadsorbed from 60-μl aliquots as described above. The immune pellets were washed once with 1 ml of TEG buffer and then 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 once 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 cytosol from the GR$^-$ subline (E82.A3) of L929 cells containing 20 mM paclitaxel, 100 μM GTP, and 5 μl of an ATP-generating system (50 mM ATP, 250 mM creatine phosphate, 20 mM magnesium acetate, and 100 units/ml of creatine phosphokinase). The assay mixture was incubated for 30 min at 30 °C, with suspension of the pellets by shaking of the tubes every 2 min. At the end of the incubation, the pellets were washed three times with 1 ml of ice-cold TEG buffer with 20 mM Na_2MoO_4 and boiled in SDS sample buffer.

Atomic Force Microscopy—To prepare samples for microscopy, FiGR-bound protein A-Sepharose pellets were incubated with 200 μl of L cell cytosol, and the immunoadsorbed GR was stripped of associated chaperones by incubating the immunopellet for 2 h at 4 °C with 350 μl of 0.5

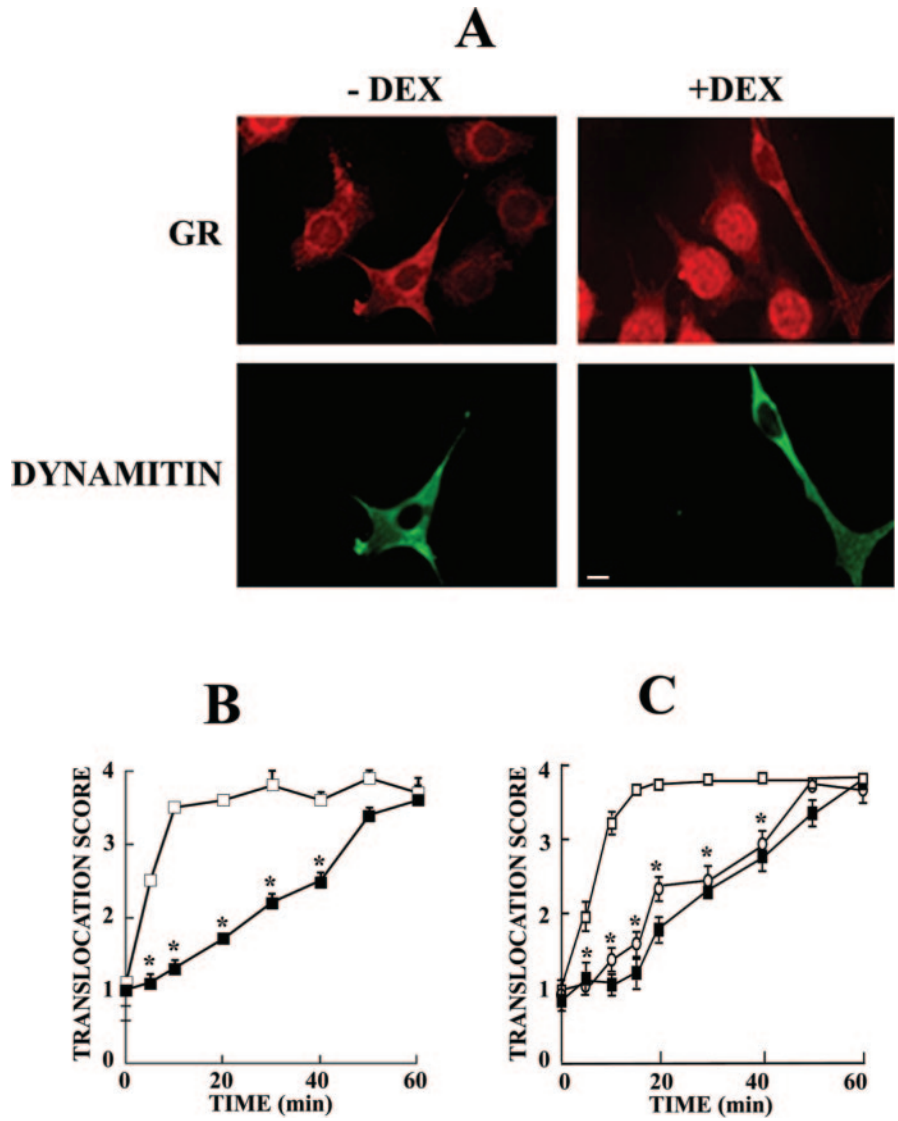

FIG. 1. **Overexpression of dynamitin inhibits GR movement to the nucleus.** *A*, NIH 3T3 fibroblasts were transfected with pCMVH50m plasmid encoding *myc*-tagged dynamitin. After 2 days, GR nuclear translocation was initiated by the addition of 1 μM dexamethasone (DEX), and after 10 min, the cells were double-stained for GR (*red*) and dynamitin (*green*). The *bar* in the *lower right panel* represents 10 μm. *B*, rate of GR nuclear translocation (means ± S.E.) in cells transfected with empty vector □ or with dynamitin (■). *, differences are significant at $p \leq 0.01$. *C*, historical data showing inhibition of green fluorescent protein-GR nuclear translocation by geldanamycin (■) or by overexpression of the PPIase domain fragment of FKBP52 (○). These data are from Galigniana *et al.* (13).

M NaCl in TEG buffer. The pellets were then washed three times with 1 ml of TEG buffer followed by a wash with 1 ml of Hepes buffer (10 mM Hepes, pH 7.4). The immunopellets were then incubated for 30 min at 30 °C with 50 μl of rabbit reticulocyte lysate and 5 μl of an ATP-regenerating system and washed once with TEG buffer and once with Hepes buffer. The GR and GR-bound proteins were released into the supernatant by incubating the pellets with 1 mM of epitope peptide in 50 μl of HE buffer with 20 mM Na_2MoO_4 for 5 min at 30 °C as described by Murphy *et al.* (26). At the end of the incubation, 16.7 μl of buffer was added, the tubes were vortexed and centrifuged, and 50 μl of the supernatant was collected for analysis by atomic force microscopy.

All of the atomic force images were made with a Nanoscope IIIa Extended Multimode Scanning Probe Microscope from Digital Instruments (Santa Barbara, CA) using a 120 × 120 μm "JV" scanner and were conducted under fluid in tapping mode. NP-S oxide-sharpened silicon nitride probes with a spring constant of 0.32 N/m and a cantilever length of 100 μm were used for imaging. Nominal radius of curvature for tips was reported to be ≤40 nm by the manufacturer. Fresh mica substrates were prepared by cleaving the mica surface immediately preceding sample deposition. Ten μl of GR and associated proteins released from the FiGR-protein A-Sepharose pellet by peptide competition was deposited on the mica substrate and allowed to absorb for 2 min, and 120 μl of buffer was added to the fluid cell to ensure tip immersion. Scan parameters were optimized for each sample, with a typical tapping drive frequency of 9 kHz and a scan rate of 1 Hz. Images for each sample were obtained with a scan size of 1 × 1 μm. Because the theoretical width of the proteins is significantly smaller than the nominal tip radius, the height of each identified particle is used as a measure of its relative size. This makes it possible to limit the error associated with tip convolution artifacts. The heights of all identified particles were determined with Digital Instruments off-line section analysis routines. Previous studies have identified the relative height distortion caused by mica substrate surface interactions (27), and molecular mass was determined by comparing calculated particle size to known dendrimer and protein complex standards (27, 28).

RESULTS

Inhibition of GR Transport by Expression of Dynamitin—To determine whether cytoplasmic dynein is required for rapid translocation to the nucleus, dexamethasone-dependent movement of the endogenous GR was examined in 3T3 mouse fibroblasts expressing *myc*-dynamitin. As shown in Fig. 1*A*, GRs (*red*) in cells not expressing *myc*-dynamitin have translocated to the nucleus within 10 min of exposure to dexamethasone. However, in the cell expressing *myc*-dynamitin (*green*), the liganded GR remains cytoplasmic. The time course in Fig. 1*B* shows that the rate of GR translocation is slowed from a $t_{1/2}$ of ~4 min in control cells (*open squares*) to a $t_{1/2}$ of ~40 min in cells overexpressing *myc*-dynamitin (*solid squares*). For purpose of comparison, Fig. 1*C* presents data from Galigniana *et al.* (13) showing inhibition of receptor translocation in 3T3 cells by treatment with the hsp90 inhibitor geldanamycin (*solid squares*) and by overexpression of the PPIase domain fragment of FKBP52 (*open circles*). It is clear that dynamitin overexpression, geldanamycin treatment, and PPIase domain overexpression inhibit ligand-dependent GR translocation to the same extent.

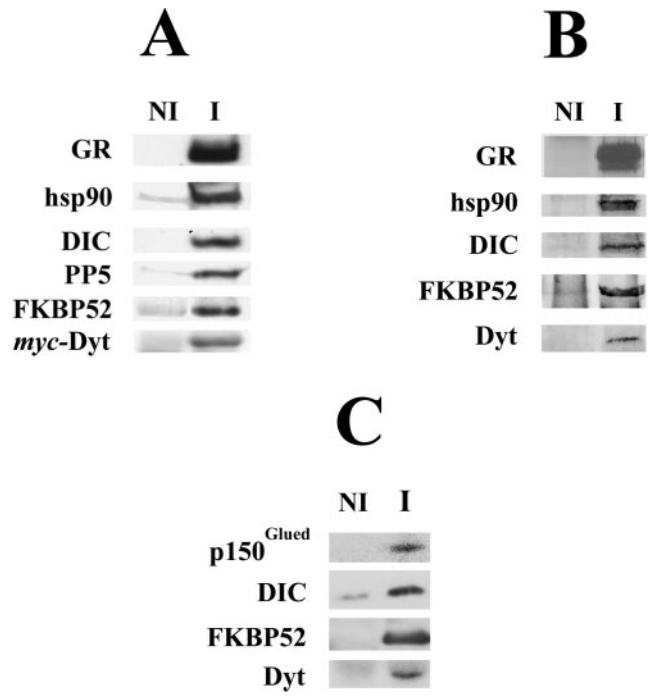

FIG. 2. **Dynamitin is present in GR heterocomplexes.** *A*, immunoadsorption of GR from cells expressing *myc*-dynamitin yields coadsorption of *myc*-dynamitin (*myc-Dyt*). Cytosol (250 μl) of L cells overexpressing *myc*-dynamitin was immunoadsorbed with nonimmune IgG (*NI*) or with FiGR antibody against the GR (*I*). After washing, proteins in the immune pellets were resolved by SDS-polyacrylamide gel electrophoresis and immunoblotting. *DIC*, dynein intermediate chain. *B*, coimmunoadsorption of endogenous dynactin proteins with GR. Cytosol from untransfected L cells was immunoadsorbed with nonimmune IgG or with FiGR antibody, and immunopellets were immunoblotted for the dynamitin (*Dyt*) component of the endogenous dynactin complex. *C*, FKBP52 exists in a cytosolic complex with dynactin and dynein. FKBP52 was immunoadsorbed from L cell cytosol, and the immune pellet was blotted for dynein, dynamitin and $p150^{Glued}$.

Dynamitin Is a Component of GR Heterocomplexes—Cytoplasmic dynein links to vesicles and organelles indirectly through dynactin (23), and it may be that nonvesicular proteins, such as the GR, link to dynein via dynactin as well. Dynamitin is a component of the dynactin complex, and we have shown previously that it binds directly to the PPIase domain of FKBP52, an hsp90-binding immunophilin component of GR·hsp90 heterocomplexes (11). Our interpretation of the dynamitin inhibition of GR movement shown in Fig. 1 is that the overexpressed *myc*-dynamitin is binding the immunophilin PPIase domain, and because *myc*-dynamitin is in great excess of dynein·dynactin, the great majority of the GR·hsp90·immunophilin complexes will bind to free *myc*-dynamitin that is not associated with the dynein·dynactin motor system. If this is the case, then *myc*-dynamitin should be present in GR heterocomplexes.

We tested this proposal by immunoadsorbing GR from cytosol of L cells expressing *myc*-dynamitin and probed Western blots of the immunoadsorbate with anti-*myc* antibody to detect the presence of *myc*-dynamitin. L cells were chosen because they have a much higher level of GR (~3-fold) than 3T3 fibroblasts. As shown in Fig. 2*A*, *myc*-dynamitin is present in GR·hsp90·immunophilin heterocomplexes. There are also GR heterocomplexes containing dynein, as shown by the presence of the dynein intermediate chain. It should be noted that *myc*-tagged dynamitin is present only in the low percentage of cells that are transfected, and the dynein shown in Fig. 2*A* is in GR heterocomplexes present in the great majority of cells that are not expressing *myc*-dynamitin.

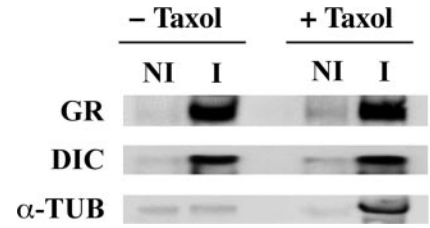

FIG. 3. **GR heterocomplexes are bound to microtubules.** GR was immunoadsorbed from 250-μl aliquots of L cell cytosol prepared with (+ *Taxol*) or without (– *Taxol*) 20 μM paclitaxel and 100 μM GTP to stabilize microtubules. Proteins in washed nonimmune (*NI*) and immune (*I*) pellets were revealed by Western blotting with antibodies specific for GR, dynein intermediate chain (*DIC*), and α-tubulin.

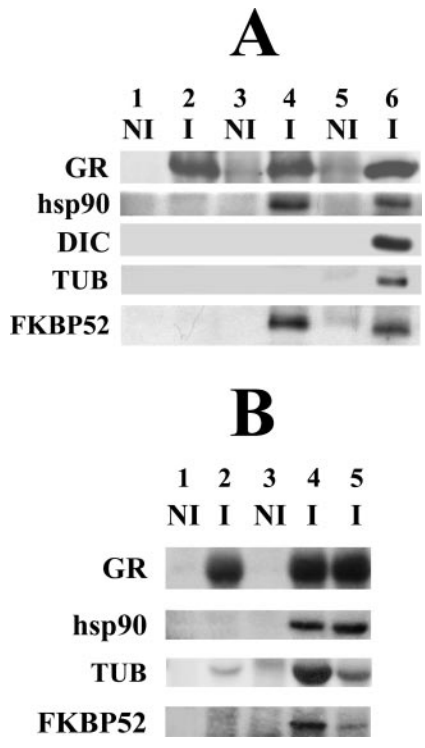

FIG. 4. **Assembly of GR heterocomplexes bound to microtubules in E82 cytosol.** *A*, the PPIase fragment competes for assembly of GR complex with tubulin. Stripped GR immune pellets (*I*) or nonimmune IgG pellets (*NI*) were incubated for 30 min at 30 °C with E82 cell cytosol prepared in buffer containing 20 μM paclitaxel and 100 μM GTP that was preincubated for 30 min at 30 °C under conditions indicated below. The pellets were then washed, and the GR and associated proteins were assayed by Western blotting as described under "Methods." *Lanes 1* and *2*, pellets incubated with buffer alone (no cytosol); *lanes 3* and *4*, pellets incubated with cytosol preincubated with 90 μg of purified core domain I of rabbit FKBP52; *lanes 5* and *6*, pellets incubated with cytosol preincubated with buffer. *B*, the TPR domain fragment competes for assembly of GR complex with tubulin (*TUB*). *Lanes 1* and *2*, pellets incubated with buffer alone; *lanes 3* and *4*, pellets incubated with E82 cytosol preincubated with 5 μl of lysate from control Sf9 cells; *lane 5*, GR immunopellet incubated with E82 cytosol preincubated with 5 μl of lysate from Sf9 cells expressing the TPR domain fragment of rat PP5. A trace amount of insect (Sf9) tubulin is present in the GR immune pellet (*lane 2*). *DIC*, dynein intermediate chain.

If the GR is linking to dynein via dynactin, then GR heterocomplexes immunoadsorbed from cytosol of untransfected L cells should contain endogenous dynamitin. Fig. 2*B* shows that immunoadsorption of the GR is accompanied by coadsorption of endogenous dynamitin as well as dynein. If proteins link to the dynein·dynactin motor system via hsp90-binding immunophilins, then immunoadsorption of an immunophilin should be accompanied by coadsorption of both dynein and dynactin proteins. Fig. 2*C* shows that immunoadsorption of FKBP52 from L

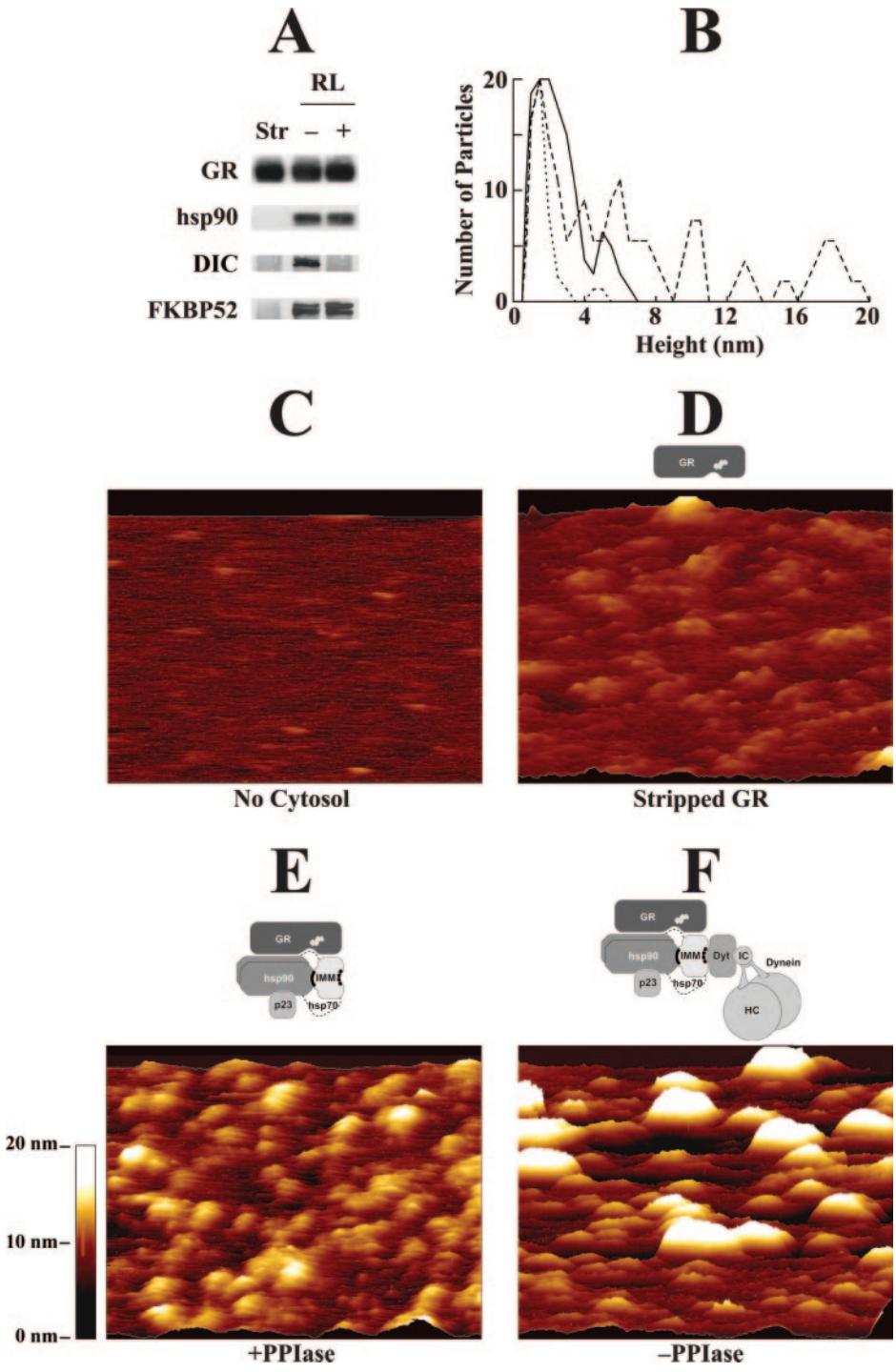

FIG. 5. **Visualization of GR·hsp90·immunophilin·dynein complexes by atomic force microscopy.** *A*, samples to be imaged. FiGR-bound pellets were incubated with L cell cytosol and stripped of endogenous chaperones. The immunopellets were incubated with buffer alone (*Str*) or reticulocyte lysate (*RL*) and an ATP-regenerating system preincubated with (+) or without (−) the purified PPIase domain fragment of FKBP52. After washing, the immunopellets were incubated with epitope peptide, and proteins in the supernatants were resolved by Western blotting. *B*, heights of protein complexes from peptide-released, stripped GR (*dotted line*), GR incubated with reticulocyte lysate pretreated with PPIase domain fragment (*solid line*), or GR incubated with reticulocyte lysate without PPIase domain fragment (*dashed line*) were measured by sectional analysis. Over 100 particles were measured for each condition, but for purposes of comparing relative distributions, the data are normalized such that the highest peak is set at 20 particles, which was the actual peak value for the dashed line. *C–F*, images of immunopellets prepared as in *A* and competed with epitope peptide to release complexes from the antibody. Supernatants from no cytosol control (*C*), stripped GR (*D*), GR incubated with reticulocyte lysate and PPIase domain fragment (*E*), and GR incubated with reticulocyte lysate without PPIase domain fragment (*F*) were overlaid on a mica substrate, and adherent protein complexes were visualized by atomic force microscopy. The images are of 1 × 1 μm scans tilted 30° toward the observer to aid definition, and the height scale is from 0 nm (*black*) to 20 nm (*white*). *DIC*, dynein intermediate chain.

cell cytosol yields coadsorption of the dynactin components dynamitin and p150^{Glued} as well as dynein.

GR Heterocomplexes Bind to Microtubules—Giannakakou *et al.* (21) ruptured cells in buffer containing paclitaxel and GTP to stabilize microtubules, and under those conditions, p53 immunoadsorption was accompanied by coimmunoadsorption of tubulin. In Fig. 3, GR was immunoadsorbed from L cell cytosol prepared without and with these microtubule-stabilizing agents. Although dynein is present in GR immune pellets prepared under either condition, tubulin is present only in GR heterocomplexes immunoadsorbed from cytosol prepared under microtubule stabilizing conditions.

Inasmuch as the immunophilins link the GR·hsp90 complex to the dynein·dynactin motor complex via the immunophilin PPIase domain and dynein binds to microtubules, the presence of microtubules in the GR·hsp90 heterocomplexes should be PPIase domain-dependent. To determine whether this was the case, GR immune pellets were incubated with concentrated cytosol from E82.A3 cells. The E82 subline of L929 fibroblasts was selected for glucocorticoid resistance, and E82 cells do not contain any GR mRNA or GR protein (29). As shown in Fig. 4*A*, incubation of GR immune pellets with E82 cytosol containing paclitaxel and GTP to stabilize microtubules yielded assembly of GR·hsp90 complexes containing dynein and tubulin (*lane 6*). When the E82 cytosol was preincubated with the purified PPIase domain fragment of FKBP52, neither dynein nor tubulin were present in the heterocomplex (*lane 4*). This supports a model in which GR heterocomplexes are bound to microtubules

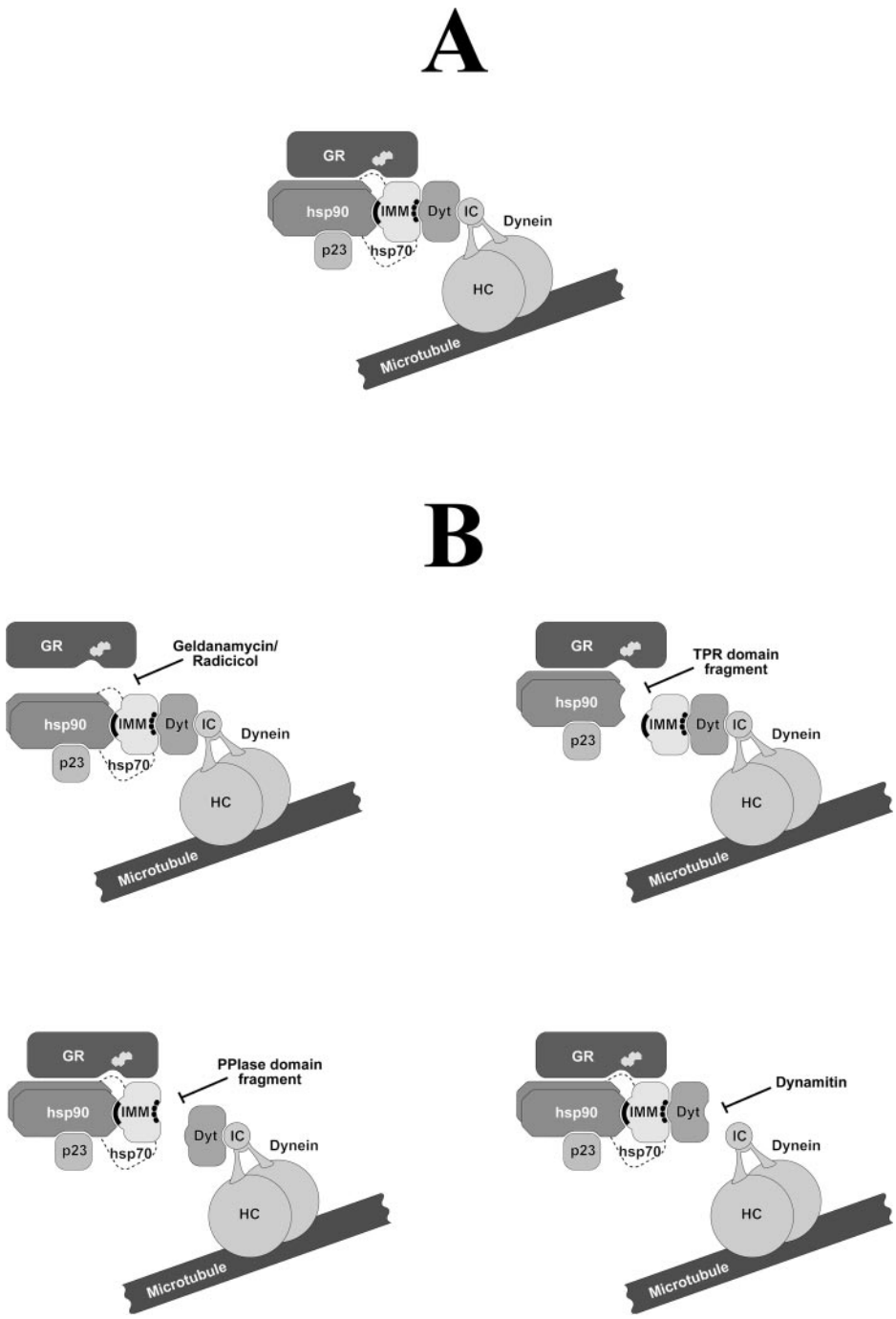

FIG. 6. **The complete GR movement system complex and agents for selective uncoupling of the system.** *A*, illustration of the complete movement system. *B*, sites of uncoupling by the hsp90 inhibitors geldanamycin and radicicol, by the TPR domain fragment of PP5, by the PPIase domain fragment of FKBP52, and by dynamitin (*Dyt*). Immunophilin (IMM) TPR domain (*solid crescent*), PPIase domain (*dotted crescent*), Dynein heavy chains (*HC*), intermediate chains (*IC*).

through dynein. In Fig. 4*B*, GR heterocomplexes were assembled in microtubule-stabilized E82 cytosol in the presence or absence of the TPR domain fragment of PP5. As shown in *lane 5*, the TPR domain fragment competes for the presence of both FKBP52 and tubulin in the assembled GR heterocomplexes.

Visualization of GR·hsp90·Immunophilin·Dynein Complexes by Atomic Force Microscopy—We have previously imaged both the GR and GR·hsp70 complexes by atomic force microscopy (26). In the event that at least some of the very large receptor complexes with the movement machinery remain intact through the sample preparation procedure, we wanted to use this technique to visualize GR associated with the dynein motor protein complex. As we have previously reported, GR·hsp90·immunophilin·dynein complexes can be assembled by incubating stripped GR immune pellets with rabbit reticulocyte lysate (13). The complexes can be released from the immune pellet by competition with epitope peptide (26), and Fig. 5*A* shows an immunoblot of three states of the GR released from the immunopellets: the stripped GR (*Str*) and the stripped GR that was incubated with rabbit reticulocyte lysate in the absence (−) or presence (+) of the PPIase domain fragment to compete for immunophilin association with the dynein·dynactin complex. Fig. 5 (*C–F*) shows the atomic force microscopy images of the no GR control (*C*), the stripped GR (*D*), the GR·hsp90·immunophilin complexes (*E*), and GR·hsp90·immunophilin complexes containing dynein (*F*).

The heights of over 100 individual particles in each sample were determined, and a summary is presented in Fig. 5*B*, where the *solid line* shows complexes formed in the presence of the PPIase domain fragment to prevent GR heterocomplex association with dynein, and the *dashed line* represents the dynein-containing GR complexes distributing in multiple

peaks. The largest complexes shown in *white* in Fig. 5*F* distribute between 16 and 20 nm in height (Fig. 5*B*, *dashed line*) and have a calculated molecular mass of ~2.5 MDa. A GR·hsp90·immunophilin complex of 1:2:1 stoichiometry has a molecular mass of ~350 kDa, which is the calculated mass of the smaller of the two peaks defined by the *solid line* in Fig. 5*B*. Dynein has a mass of ~1.2 MDa (23). The molecular composition of an associated dynactin complex in this case is not known, but assuming a minimum complex of only $p150^{Glued}$, $p135^{Glued}$, and dynamitin with a stoichiometry of 1:1:4 (30), the molecular mass would be ~500 kDa. Thus, a predicted minimal size for GR·hsp90·immunophilin complexes containing dynein would be at least 2.05 MDa, which approaches the calculated molecular mass of the large white particles (Fig. 5*F*) of 16–20 nm in height (Fig. 5*B*, *dashed line*).

DISCUSSION

Fig. 6 illustrates the GR movement system and the tools we have used to uncouple the movement system *in vitro* and to inhibit GR movement *in vivo*. As shown in Fig. 1, preventing the receptor from binding to hsp90 with geldanamycin, preventing the GR·hsp90·immunophilin complex from binding to dynamitin by overexpression of a PPIase domain fragment, and preventing the whole cargo complex from binding to dynein by overexpressing dynamitin all inhibit the rate of GR translocation to the nucleus to the same extent. When rapid, hsp90-dependent movement is inhibited by any of these methods, slow movement can occur. This slow movement apparently reflects diffusion, and in neurites where proteins cannot move by diffusion, geldanamycin blocks retrograde movement of the GR (31), suggesting that hsp90 is required for rapid GR movement.

We have previously reported a very weak interaction between the PPIase domain fragment of FKBP52 and the purified expressed intermediate chain of cytoplasmic dynein (18). PPIase domains engage in weak interactions with peptidyl prolines, and this binding could be nonspecific in this way. Subsequently, we showed that purified FKBP52 binds purified *myc*-dynamitin to form a complex that withstands rigorous washing conditions (11). FKBP52 binding to dynamitin is blocked by competition with a purified PPIase domain fragment, suggesting that the PPIase domain directly interacts with dynamitin (11). Fig. 2 shows that dynamitin is present in GR heterocomplexes containing immunophilins. Thus, in the model of Fig. 6, we have suggested that dynamitin is the component of the dynein·dynactin complex with which the immunophilin in the GR heterocomplex interacts. Consistent with this model, immunoadsorption of FKBP52 is accompanied by coadsorption of the dynactin proteins dynamitin and $p150^{Glued}$ as well as dynein (Fig. 2C)

Although immunolocalization studies with most antireceptor antibodies in most cells have found the GR to be diffusely located throughout the cytoplasm, in several reports, the Gustafsson laboratory was able to demonstrate colocalization of the GR with microtubules (reviewed in Ref. 32). The vitamin D receptor has also been reported to colocalize with microtubules (33). But the data of Fig. 3 present the first biochemical evidence of steroid receptor linkage to microtubules. In 1993, we reported that GRs in L cell cytosol were converted to particulate form under conditions that promote microtubule polymerization (34). However, the particulate contained large amounts of actin and vimentin as well as tubulin, and the system did not permit analysis of how the GR was linked to the cytoskeleton. Thus, for the last decade we have focused on defining the proteins that are present in GR·hsp90 heterocomplexes and the linkages involved (reviewed in Ref. 2). In Fig. 3, we see that immunoadsorption of GR from L cell cytosol prepared under conditions that stabilize microtubules yields coimmunoadsorption of tubulin. When GR heterocomplexes containing tubulin are assembled in stabilized cytosol, the presence of both tubulin and dynein in the heterocomplex is competed by the PPIase domain of FKBP52 (Fig. 4*A*), showing that the presence of tubulin is immunophilin-dependent. Consistent with this model, when GR heterocomplexes are assembled in the presence of a TPR domain fragment to compete for immunophilin binding to hsp90, the presence of both tubulin and FKBP52 in the complex is reduced. In that both dynein and tubulin are present in an immunophilin-dependent manner, it is reasonable to indicate in Fig. 6 that the dynein motor links the receptor heterocomplex to the microtubules.

At this time, we are clearly at an early stage of understanding the role of hsp90 in signaling protein trafficking through the cytoplasm to the nucleus. Whether hsp90 plays a general role in the targeted movement of proteins or a more specific role in the role in the movement of a limited number of transcription factors is not known. What we propose is that one function of the hsp90/hsp70-based chaperone machinery in forming client protein·hsp90 complexes is to "capture " proteins into multichaperone complexes that, through the hsp90-bound immunophilins, can link them to motor systems for their movement along cytoskeleton. An important concept is that the chaperone machinery can interact with proteins in their native, least energy state without regard to the size, shape, amino acid sequence, or function of a protein (1). This ability to interact with a wide variety of client proteins combined with the diversity that arises from the various TPR domain proteins that associate with the client protein and hsp90 may provide an integrated system for targeted movement of proteins to diverse sites of action within the cell.

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