Mechanisms of Signal Transduction:

Cyclophilin-A Is Bound through Its Peptidylprolyl Isomerase Domain to the Cytoplasmic Dynein Motor Protein Complex

Mario D. Galigniana, Yoshihiro Morishima,
Philippe A. Gallay and William B. Pratt

J. Biol. Chem. 2004, 279:55754-55759.
doi: 10.1074/jbc.M406259200 originally published online October 20, 2004

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 53, Issue of December 31, pp. 55754–55759, 2004
Printed in U.S.A.

Cyclophilin-A Is Bound through Its Peptidylprolyl Isomerase Domain to the Cytoplasmic Dynein Motor Protein Complex*

Received for publication, June 4, 2004, and in revised form, October 5, 2004
Published, JBC Papers in Press, October 20, 2004, DOI 10.1074/jbc.M406259200

Mario D. Galigniana‡§, Yoshihiro Morishima‡, Philippe A. Gallay¶, and William B. Pratt‡‖

From the ‡Department of Pharmacology, The University of Michigan Medical School, Ann Arbor, Michigan 48109, the ¶Department of Immunology, the Scripps Research Institute, La Jolla, California 92037, and the §Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Programa de Regulación Hormonal y Metabólica, Consejo Nacional de Investigaciones Científicas y Técnicas de Argentina, Buenos Aires C1428EGA, Argentina

Although cyclophilin A (CyP-A) is a relatively abundant small immunophilin present in the cytoplasm of all mammalian cells, its general function(s) in the absence of the immunosuppressant drug cyclosporin A is not known. In contrast, the high molecular weight hsp90-binding immunophilins appear to play a role in protein trafficking in that they have been shown to link glucocorticoid receptor-hsp90 and p53·hsp90 complexes to the dynein motor protein for retrograde movement along microtubules. These immunophilins link to cytoplasmic dynein indirectly through the association of the immunophilin peptidylprolyl isomerase (PPIase) domain with dynamitin, a 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). Here, we show that CyP-A exists in native heterocomplexes containing cytoplasmic dynein that can be formed in cell-free systems. Prolyl isomerase activity is not required for forming the dynein complex, but the PPIase domain fragment of FKBP52 blocks complex formation and CyP-A binds to dynamitin in a PPIase domain-dependent manner. CyP-A heterocomplexes containing tubulin and dynein can be formed in cytosol prepared under microtubule-stabilizing conditions, and CyP-A colocalizes in mouse fibroblasts with microtubules. Colocalization with microtubules is disrupted by overexpression of the PPIase domain fragment. Thus, we conclude that CyP-A associates *in vitro* and *in vivo* with the dynein/dynactin motor protein complex and we suggest that CyP-A may perform a general function related to the binding of cargo for retrograde movement along microtubules.

The cyclophilins (CyPs)[1] are members of a large protein family called immunophilins because of their ability to bind immunosuppressant drugs (reviewed in Refs. 1 and 2). The cyclophilins bind the drug cyclosporin A (CsA), and a second class of immunophilins, the FK506-binding proteins (FKBPs), binds the immunosuppressant drugs FK506 and rapamycin. The immunophilins possess peptidylprolyl isomerase (PPIase) activity, and the immunosuppressant drugs occupy the PPIase site on the immunophilin, blocking its ability to direct *cis-trans*-isomerization of peptidylprolyl bonds *in vitro*. CyP-A was isolated as a 17-kDa high affinity binding protein for cyclosporin A (3), and it was later shown to be the same as a 17-kDa protein (4, 5) that was known to have PPIase activity and to accelerate refolding of urea-denatured protein *in vitro* (6). CyP-A was subsequently shown to direct the immunosuppressive effect of CsA (7), and the small FKBP, FKBP12, was shown to be the target for the immunosuppressive effects of FK506 and rapamycin (8). In both the case of CsA-bound CyP-A and that of FK506-bound FKBP12, the ultimate target of the drug-immunophilin complex is the protein phosphatase calcineurin (for review see Ref. 9). The ultimate target for rapamycin-bound FKBP12 is a phosphoinositide 3-kinase-related kinase called FRAP (FKBP-rapamycin-associated protein) (10), RAFT1 (rapamycin and FKBP12 target 1) (11), or mTOR (mammalian target of rapamycin) (12). Neither the immunosuppressant drugs nor the immunophilins CyP-A and FKBP12 bind alone to the second protein targets for immunosuppression. Only the drug-immunophilin complexes bind to calcineurin or FRAP/RAFT1/mTOR.

Although most of the work on immunophilins has focused on the small CyPs and FKBPs, like CyP-A and FKBP12, high molecular weight immunophilins have been identified that contain domains for additional protein interactions or enzymatic activity as well as a PPIase domain that binds immunosuppressant drugs (2). Several of these high molecular weight immunophilins were discovered as components of steroid receptor-hsp90 heterocomplexes. They include CyP-40, FKBP51, and FKBP52 (reviewed in Ref. 13), and they are not involved in the immunosuppressant actions of CsA or FK506. These immunophilins possess tetratricopeptide repeat (TPR) domains that bind to a TPR acceptor site located in the C terminus of the abundant and ubiquitous protein chaperone hsp90. Two roles have been proposed for these hsp90-binding immunophilins in steroid receptor action. Riggs *et al.* (14) have shown that FKBP52 selectively potentiates glucocorticoid receptor (GR)-

* This work was supported by National Institutes of Health Grants CA28010 (to W. B. P.) and AI054196 (to P. A. G.) and Agencia de Promoción Científica y Técnica de Argentina Grant FONCYT-PICT-01-14123 (to M. D. G.). Cell biology core services were supported in part by the Michigan Diabetes Research and Training Center Grant P60DK20572 from the NIDDK, National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‖ To whom correspondence should be addressed: Dept. of Pharmacology, The University of Michigan Medical School, 1301 Medical Science Research Bldg. III, Ann Arbor, Michigan 48109-0632. Tel.: 734-764-5414; Fax: 734-763-4450; E-mail: mgali@umich.edu.

[1] The abbreviations used are: CyP, cyclophilin; CyP-A, cyclophilin-A; PPIase, peptidylprolyl isomerase; FKBP, FK506-binding protein; hsp, heat shock protein; CsA, cyclosporin A; CyP-40, cyclophilin 40; TPR, tetratricopeptide repeat; GR, glucocorticoid receptor; GST, glutathione *S*-transferase; GSH, glutathione; DIC, dynein intermediate chain; TES, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethanesulfonic acid; Mes, 4-morpholineethanesulfonic acid; HISS, high ionic strength solution; HIV, human immunodeficiency virus.

dependent reporter gene activation by increasing the hormone-binding affinity of the receptor. This effect requires the prolyl isomerase activity of FKBP52 and is blocked by FK506. Another line of research has focused on the role of hsp90-binding immunophilins in targeting the movement of the GR to the nucleus (reviewed in Ref. 15). The action of these immunophilins in receptor movement is not affected by the presence of immunosuppressant drugs and thus does not require prolyl isomerase activity.

In addition to TPR domain immunophilins, GR·hsp90 heterocomplexes immunoadsorbed from cell lysates contain cytoplasmic dynein (16, 17), a molecular motor that processes along microtubule tracks toward the nucleus (18). In these complexes, the PPIase domain of the immunophilin functions as a protein-protein-binding domain to link the GR·hsp90 unit to the protein motor (19–21). The immunophilin linkage to cytoplasmic dynein is indirect by means of the dynamitin component of the dynein-associated dynactin complex (22). The binding of CyP-40 or FKBP52 to dynein is competed by a purified PPIase domain fragment of FKBP52 (21), and purified FKBP52 has been shown to bind directly via its PPIase domain to immunopurified dynamitin (22).

There is a high degree of similarity in PPIase domain structure among the various cyclophilins (2). The PPIase domain of bovine CyP-40, for example, shares 61% identity with the sequence of human CyP-A ,and with one exception, the active site residues are identical (2). Thus, we have asked whether CyP-A, like as its high molecular weight hsp90-binding homolog CyP-40, binds through its PPIase domain to the dynein/dynactin complex. Here we show that immunoadsorption of CyP-A from reticulocyte lysate or L cell cytosol yields coimmunoadsorption of cytoplasmic dynein. The linkage to dynein is indirect via the dynamitin component of the dynein-associated dynactin complex, and CyP-A binds to dynamitin in a manner that is competed by a purified PPIase domain fragment of FKBP52. Immunoprecipitation of CyP-A from cytosol containing paclitaxel and GTP to stabilize microtubules yields the coprecipitation of tubulin and dynein in a PPIase domain-dependent manner, and Cyp-A colocalizes with microtubules in NIH 3T3 cells. The observation that CyP-A is associated with the microtubule-based dynein/dynactin motor protein complex should be useful in eventually defining the housekeeping function(s) of this single domain immunophilin in mammalian cells in the absence of immunosuppressant drugs.

EXPERIMENTAL PROCEDURES

Materials—Rabbit reticulocyte lysate was from Green Hectares (Oregon, WI). Complete Mini protease inhibitor mixture was purchased from Roche Diagnostics (Mannheim, Germany). Opti-MEM I transfection medium was from Invitrogen. *Trans*-Fast transfection reagent was from Promega (Madison, WI). Enhance chemiluminescence reagents were from Amersham Biosciences. Glutathione-agarose, protein A-Sepharose, geldanamycin, hirudin, CsA, paclitaxel, rat monoclonal IgG against α-tubulin, mouse monoclonal IgG against GST, and the horseradish peroxidase-conjugated donkey anti-mouse and rabbit anti-rat antibodies were purchased from Sigma. The A-14 rabbit polyclonal IgG against c-*myc* oligopeptide was from Santa Cruz Biotechnology (Santa Cruz, CA). Fluorescent counterantibodies were from Immuno-Chemicals (West Grove, PA). Horseradish peroxidase-conjugated goat anti-rabbit IgG and BL21-competent *Escherichia coli* cells were from Pierce. The rabbit polyclonal antiserum against CyP-A was prepared in the Scripps laboratory by immunization with recombinant human CyP-A protein (23). The mouse monoclonal IgG against the 74-kDa intermediate chain of mammalian cytoplasmic dynein was purchased from Chemicon (Temecula, CA).

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 (16). The pECFP-N1 plasmid for expressing cyanine fluorescent protein was provided by Dr. Rainer Benndorf (University of Michigan Medical School, Ann Arbor, MI).

The baculovirus for the FLAG-tagged TPR domain of rat protein phosphatase 5 (24) was kindly provided by Dr. Michael Chinkers (University of South Alabama, Mobile, AL). The *myc*-tagged pCMVH50m construct encoding for p50/dynamitin (25) was a kind gift from Dr. Richard Vallee (University of Massachusetts Medical School, Worcester, MA). The pGEX-2T plasmid encoding GST-CyP-A was a kind gift from Dr. Jeremy Luban (Columbia University) (26). All of the incubation buffers used in these studies were supplemented with protease inhibitors (1 tablet of Complete Mini Plus 1000 units of hirudin/2 ml of buffer).

Coimmunoadsorption of Endogenous CyP-A and Dynein—50 μl of reticulocyte lysate were immunoadsorbed to 16 μl of protein A-Sepharose with either 5 μl of rabbit antiserum against CyP-A or 5 μl of mouse IgG against dynein intermediate chain. After 2 h at 4 °C, the pellets were washed with TEGM buffer (10 mM TES at pH 7.6, 50 mM NaCl, 4 mM EDTA, 10% v/v glycerol, and 20 mM Na_2MoO_4). Proteins were resolved by SDS-polyacrylamide gel electrophoresis, electrotransferred to an Immobilon P membrane, and Western blotted.

Formation of Complexes with GSH-Agarose-immobilized GST-CyP-A—80 μl of GSH-agarose in 80 μl of TEG buffer (TEGM buffer without molybdate) were incubated at 4 °C for 2 h with 10 μl of cytosol from BL21 *E. coli* overexpressing either human GST or human GST-CyP-A. The pellets were washed and reincubated for two additional hours in TEG buffer supplemented with 0.5 M NaCl and 0.05% Nonidet P-40 and washed three times with TEG buffer and twice with 10 mM Hepes at pH 7.5. The washed pellets containing immobilized GST-CyP-A (or GST alone) were incubated for 30 min at 30 °C with 50 μl of reticulocyte lysate supplemented with protease inhibitors, 5 μl of an ATP-regeneration system (50 mM ATP, 250 mM creatine phosphate, 20 mM magnesium acetate, and 100 units/ml creatine phosphokinase), and the indicated peptides in HKD buffer (10 mM Hepes at pH 7.5, 100 mM KCl, and 2 mM dithiothreitol).

Binding of CyP-A to Dynamitin—L929 mouse fibroblasts were grown in Dulbecco's modified Eagle's medium supplemented with 10% bovine calf serum and antibiotics. When the cells were 50% confluent, the medium was replaced by Opti-MEM I transfection medium and the incubation continued for 1 h. The medium was aspirated and replaced by a transfection mixture (at a 3-μl liposome/μg DNA ratio) preincubated for 15 min at room temperature in Opti-MEM I, which contained 3 μg of the *myc*-tagged pCMVH50m plasmid encoding for p50/dynamitin. After 1.5 h of transfection, the mixture was replaced by regular medium. After 36 h, the cells were harvested and ruptured by Dounce homogenization in HE buffer (10 mM Hepes at pH 7.5, 1 mM EDTA, and protease inhibitors) and centrifuged at 100,000 $\times$ *g* for 30 min. Cell cytosol (250 μl) was rotated for 2 h at 4 °C with 16 μl of protein A-Sepharose and 10 μl of anti-*myc* antibody. The supernatant was aspirated, and the pellet was stripped of coimmunoadsorbed proteins by preincubation with buffer supplemented with 0.5 M NaCl and 0.05% Nonidet P-40. After 2 h, the samples were washed three times with TEG buffer and twice with HE buffer. The pellets were incubated on ice with 10 μl of bacterial lysate overexpressing GST-CyP-A in the presence or absence of 90 μg of purified PPIase domain from FKBP52. The final volume was adjusted to 50 μl with HKD buffer. After 1 h, the pellets were washed with TEGM buffer and the proteins were resolved by electrophoresis followed by Western blotting.

Microtubule-stabilized Preparations—L929 fibroblasts were incubated for 15 min with 20 μM paclitaxel prior to rupture by Dounce homogenization in MES buffer (0.1 M Mes, 1 mM EGTA, and 1 mM $MgCl_2$, pH 6.9) supplemented with 20 μM paclitaxel, 100 μM GTP, and protease inhibitors. Prior to adsorption, 0.05% Nonidet P-40 was added to the cytosol and it was rotated for 30 min at 4 °C and then centrifuged at 30 p.s.i. for 15 min in an Airfuge. Aliquots (250 μl) of cytosol were incubated at 30 °C for 30 min with 25 μl of lysate from nonexpressing bacteria and 50 μl of HKD buffer or 25 μl of lysate from bacteria overexpressing GST-CyP-A and 50 μl of HKD buffer or 25 μl of lysate from bacteria overexpressing GST-CyP-A and 325 μg of purified PPIase domain of FKBP52 in HKD buffer. The HKD buffer also contained protease inhibitors. GST-CyP-A was then immobilized by rotation with GSH-agarose for 2 h at 4 °C. The pellets were washed with TEGM buffer, and the proteins were resolved by immunoblotting

Immunoblotting—Immune pellets were resolved on SDS-10% polyacrylamide gels and transferred to Immobilon P membranes. The membranes were probed with 0.1% MAB1618 for dynein intermediate chain, 0.1% A-14 for *myc*-dynamitin, 0.05% rabbit antiserum for CyP-A, and 0.05% mouse IgG for GST.

Indirect Immunofluorescence—NIH 3T3 cells were grown on 11 × 22-mm coverslips coated with fibrinogen in Dulbecco's modified Eagle's medium supplemented with 10% bovine calf serum and antibiotics. The coverslips were rinsed with phosphate-saline solution and fixed for 4 h in a freshly prepared 4% *p*-formaldehyde solution in phosphate-saline buffer. Cells were permeabilized by immersing the coverslips in acetone at −20 °C for 15 min. After washing the coverslips with phosphate-saline buffer, they were preincubated for 1 h at room temperature in HISS (high ionic strength solution) buffer (20 mM Tris at pH 8.0, 0.63 M NaCl, 0.1% Tween 20, and 3.5% bovine serum albumin) supplemented with 1% horse serum.

The cells were washed in HISS buffer and immunostained by inverting the coverslips on a 50-μl solution of HISS buffer containing 1 μl of rat anti-α-tubulin and 0.2 μl of rabbit anti-CyP-A serum. After 2 h at room temperature in a humid chamber, the cells were washed in HISS buffer for 1 h at room temperature and then incubated with a 1/100 dilution of the corresponding counter-antibodies (rhodamine-conjugated donkey anti-rabbit IgG and fluorescein isothiocyanate-conjugated donkey anti-rat IgG). The coverslips were mounted on microscope slides using a drop of Antifade mounting medium (Molecular Probes, Leiden, The Netherlands), and the cells were observed with an Olympus Fluoview-500 confocal microscope.

RESULTS

CyP-A Is Recovered in Native Heterocomplex with Dynein—To determine whether CyP-A exists in native heterocomplexes with dynein, reticulocyte lysate was immunoadsorbed with antibodies directed against the immunophilin or against the intermediate chain of dynein and proteins associated with the washed immune pellets were detected by immunoblotting. As shown in Fig. 1*A*, immunoadsorption of dynein yielded coadsorption of CyP-A (*lane 3*) and immunoadsorption of CyP-A yielded coadsorption of dynein (*lane 5*), consistent with the notion that CyP-A exists in native heterocomplexes with cytoplasmic dynein. In Fig. 1, *lanes 6–9*, the entire lanes for each immunopellet were blotted with each antibody to demonstrate the specificity of the interaction. To show that the complex does not involve only small amounts of either protein, a large amount of CyP-A was immunoadsorbed from reticulocyte lysate and the resulting immunodepletion of dynein was assessed. As shown in Fig. 1*B*, increasing immunoadsorption of CyP-A was accompanied by increasing coadsorption of dynein (*lanes 3* and *4*). At the higher amount of antibody, most of the CyP-A and a substantial amount of the dynein were adsorbed from reticulocyte lysate (*cf. lane 7* with *lane 5*). By scanning the lanes with a PhosphorImager and subtracting an adjacent background value, arbitrary units for each protein remaining in nonimmune (*lane 5*) and the highest immune (*lane 7*) supernatant were determined. A depletion of 75% CyP-A yielded 59% codepletion of dynein, indicating that a majority of each protein is in a heterocomplex containing the other protein.

To show that the binding is not a weak and possibly nonspecific interaction, reticulocyte lysate was immunoadsorbed with anti-CyP-A and the immune pellets were washed under increasingly harsh conditions. As shown in Fig. 1*C*, the complex of CyP-A with dynein persisted upon washing with TEG buffer plus 100 mM NaCl (*cf. lanes 2 and 3*) and washing with TEG plus NaCl and 0.1% Nonidet P-40 (*lane 3*). Washing under very harsh detergent conditions (NaCl, Nonidet P-40, 0.5% deoxycholate, and 1% sodium dodecyl sulfate) dissociates the CyP-A antibody from the protein A-Sepharose pellet such that most of the CyP-A and dynein are lost. Nevertheless, some complex of CyP-A and dynein can be seen under these very harsh conditions of washing (*lane 5*).

We have previously used reticulocyte lysate to achieve cell-free assembly of GR·hsp90 complexes that contain hsp90-binding immunophilins and dynein (16). In the experiment of Fig. 2, GST-CyP-A was immobilized onto glutathione beads and the beads were incubated with reticulocyte lysate under the same GR heterocomplex assembly conditions. Glutathione beads bound with GST alone did not contain dynein after incubation with reticulocyte lysate (*lane 2*), but GST-CyP-A-bound beads contained dynein (*lane 5*).

CyP-A PPIase Domain Is Required for Complex with Dynein—The formation of heterocomplexes containing the hsp90-binding immunophilins and dynein requires the PPIase domain of the immunophilin and can be blocked by competition with the PPIase domain fragment of FKBP52 (16, 19–21). In the experiment of Fig. 3, GST-CyP-A-bound beads were incubated with reticulocyte lysate in the presence of lysate from bacteria expressing the FKBP52 PPIase domain fragment and the beads were then washed and assayed for bound proteins. The presence of the PPIase domain fragment prevented CyP-A association with dynein (*cf. lanes 2* and *4*), whereas the presence of a TPR domain fragment did not affect dynein binding (*lane 5*). We have shown previously that the TPR domain fragment inhibits the binding of TPR domain immunophilins to hsp90 but does not affect their binding to dynein (16). Importantly, CsA does not affect the formation of a CyP-A complex with dynein (*lane 6*), suggesting that prolyl isomerase activity is not required.

CyP-A Interaction with Dynamitin—Recently, we have demonstrated that purified FKBP52 binds directly to purified *myc*-dynamitin, a 50-kDa component of the dynein-associated dynactin complex (22). In Fig. 4, anti-*myc* antibody was used to immunoadsorb cytosol from cells overexpressing *myc*-dynamitin and the salt-stripped immune pellet was incubated with lysate from bacteria expressing GST-CyP-A. The GST-CyP-A bound to *myc*-dynamitin (*condition 2*), and the binding was blocked by the PPIase fragment of FKBP52 (*condition 3*). Thus, similar to the hsp90-binding immunophilins, CyP-A appears to associate with dynein indirectly by binding to dynamitin.

Tubulin Is Present in the CyP-A Heterocomplexes—We have recently shown that GR heterocomplexes immunoadsorbed from L cell cytosol prepared with a microtubule-stabilizing buffer contain tubulin as well as dynein.[2] In the experiment of Fig. 5, L cells were treated briefly with paclitaxel and then ruptured in buffer containing both paclitaxel and GTP to stabilize microtubules. Cytosol was also prepared without the microtubule-stabilizing conditions. Lysate from bacteria expressing GST-CyP-A was added to each cytosol preparation, the mixture was incubated, and the GST-CyP-A was adsorbed to glutathione beads. As shown in Fig. 5, *condition 2*, dynein is present in GST-CyP-A pellets prepared under either condition but tubulin is present only when GST-CyP-A is adsorbed from cytosol prepared under microtubule-stabilizing conditions. If the PPIase domain fragment is present to compete for CyP-A binding to dynamitin, neither dynein nor tubulin is present in the GST-CyP-A pellet (*condition 3*). This finding supports a model in which CyP-A is linked to microtubules via its association with the dynein/dynactin complex.

CyP-A Is Localized to Microtubules in Vivo—In Fig. 6, 3T3 mouse fibroblasts were cotransfected with plasmids encoding for the PPIase domain fragment of FKBP52 and cyanine fluorescent protein. After 36 h, the cells were fixed and immunostained for tubulin (Fig. 6*A*, *green*) and CyP-A (Fig. 6*B*, *red*). In the untransfected cells, CyP-A is seen to colocalize with tubulin (see merge in Fig. 6*C*). The *blue image* in Fig. 6*D* shows a cotransfected cell in the middle of the field. The expression of the PPIase domain fragment did not affect the fibrillar pattern of the microtubules (Fig. 6*A*), but it disrupted the fibrillar distribution of CyP-A (Fig. 6*B*). This is consistent with the

[2] J. M. Harrell, M. D. Galigniana, P. J. M. Murphy, Y. Morishima, and W. B. Pratt, manuscript in preparation.

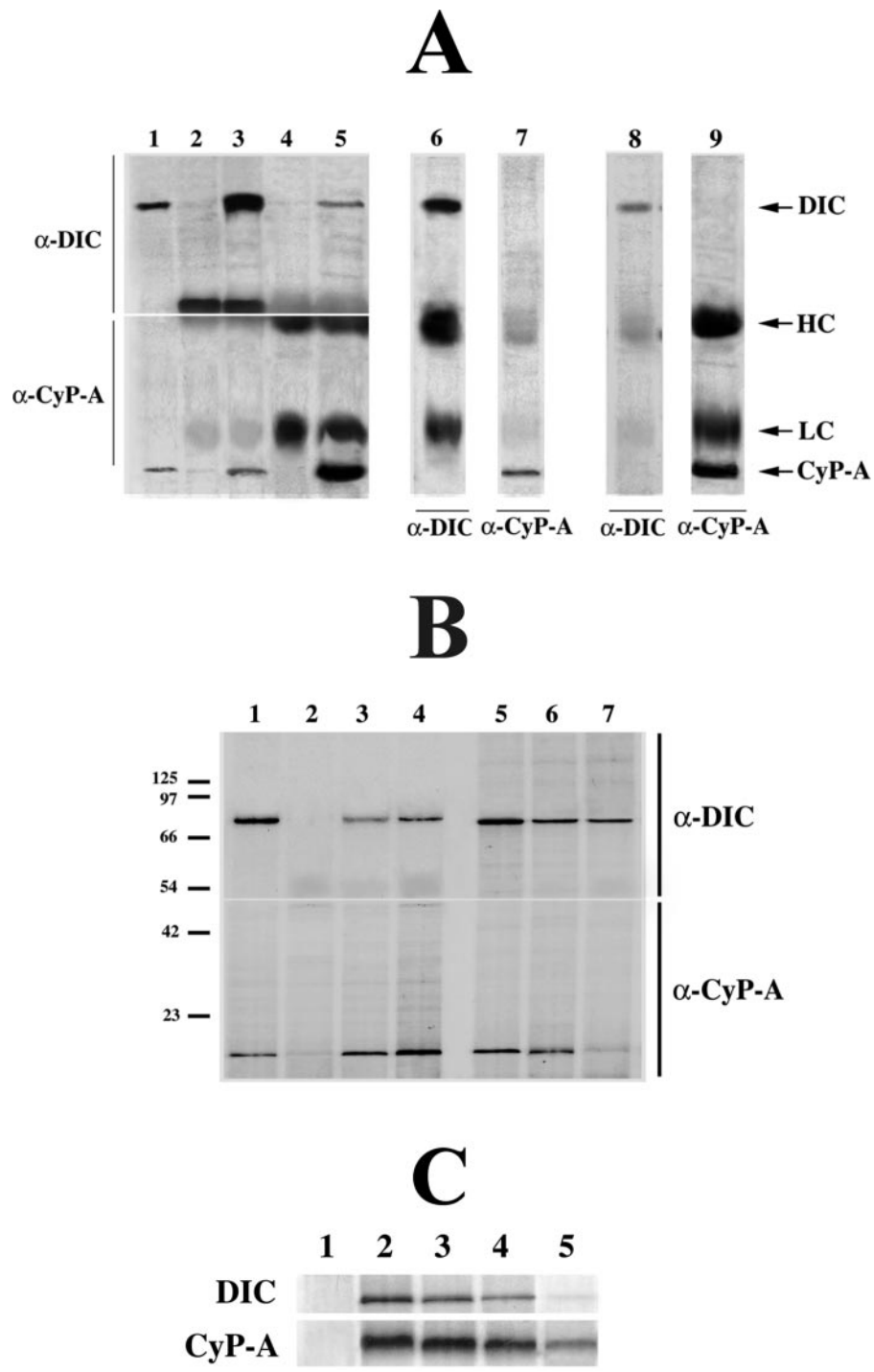

FIG. 1. **Coimmunoadsorption of CyP-A and dynein.** *A*, native complexes. Aliquots (50 μl) of reticulocyte lysate were immunoadsorbed to protein A-Sepharose with antibody against CyP-A or dynein intermediate chain (*DIC*), the immune pellets were washed, and pellet-bound proteins were resolved by SDS-polyacrylamide gel electrophoresis and immunoblotting. *Lane 1*, 4 μl of reticulocyte lysate; *lane 2*, nonimmune mouse IgG pellet; *lane 3*, anti-DIC immunopellet; *lane 4*, nonimmune rabbit serum pellet; *lane 5*, anti-CyP-A immunopellet. These lanes were cut in half, and the top was immunoblotted with anti-DIC and the bottom was immunoblotted with anti-CyP-A. *Lanes 6* and *7*, immunoadsorbed with anti-DIC and immunoblotted with anti-DIC (*lane 6*) or anti-CyP-A (*lane 7*). *Lanes 8* and *9*, immunoadsorbed with anti-CyP-A and immunoblotted with anti-DIC (*lane 8*) or anti-CyP-A (*lane 9*). For *lanes 6–9*, the full lanes that are immunoblotted show the specificity of the antibodies. *HC*, antibody heavy chain; *LC*, light chain. *B*, CyP-A is in complex with a substantial amount of dynein. Aliquots (25 μl) of reticulocyte lysate were immunoadsorbed with 4 μl of nonimmune antiserum or 2.5 or 5 μl of anti-CyP-A. The immune pellets and supernatants were resolved by SDS-polyacrylamide gel electrophoresis and immunoblotting with anti-DIC (*top*) or anti-CyP-A (*bottom*). *Lane 1*, aliquot of reticulocyte lysate; *lanes 2–4*, immune pellets from nonimmune antiserum (*lane 2*), 2.5 μl of anti-CyP-A (*lane 3*), and 5 μl of anti-CyP-A (*lane 4*); *lanes 5–7*, supernatants of the immunoadsorption with nonimmune antiserum (*lane 5*), 2.5 μl of anti-CyP-A (*lane 6*), and 5 μl of anti-CyP-A (*lane 7*). The exposure time for the immunoblot was selected after various exposure times to avoid saturation such that the signals fall in the linear range of the film, which was scanned with a PhosphorImager to yield the percentage of each protein that was immunoadsorbed as presented under "Results." *C*, the CyP-A complex with dynein withstands washing under stringent conditions. Aliquots (25 μl) of reticulocyte lysate were immunoadsorbed to protein A-Sepharose with antibody against CyP-A, and the immune pellets were washed twice with TEG buffer plus the indicated additions and once with HE buffer. *Lane 1*, nonimmune pellet washed with TEG alone; *lanes 2–5*, immune pellets washed with TEG alone (*lane 2*), TEG plus 100 mM NaCl (*lane 3*), TEG plus NaCl and 0.1% Nonidet P-40 (*lane 4*), and TEG plus NaCl, Nonidet P-40, 0.5% deoxycholate, and 1% sodium dodecyl sulfate (*lane 5*).

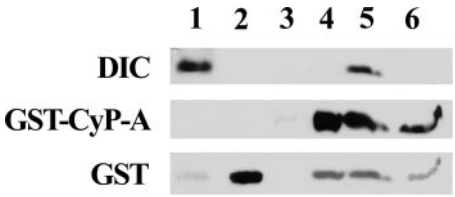

FIG. 2. **Cell-free formation of CyP-A complexes containing dynein.** Glutathione beads were mixed with GST or lysate from nonexpressing bacteria or lysate from bacteria expressing GST-CyP-A. The beads were then incubated with 0.5 M NaCl, washed, and incubated for 30 min at 30 °C with 50 μl of reticulocyte lysate or HKD buffer. The beads were then washed, and proteins were resolved by electrophoresis and immunoblotting. *Lane 1*, 6 μl of reticulocyte lysate; *lane 2*, GST-bound beads incubated with reticulocyte lysate; *lane 3*, beads bound with nonexpressing bacterial lysate incubated with reticulocyte lysate; *lane 4*, GST-CyP-A-bound beads incubated with HKD buffer; *lane 5*, GST-CyP-A-bound beads incubated with reticulocyte lysate; *lane 6*, 1 μl of lysate from bacteria expressing GST-CyP-A.

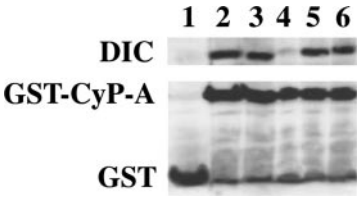

FIG. 3. **Formation of a complex containing CyP-A and dynein is inhibited by the PPIase domain fragment of FKBP52.** GST-bound beads (*lane 1*) or GST-CyP-A-bound beads (*lanes 2–7*) were incubated with 50 μl of reticulocyte lysate and HKD buffer (*lanes 1* and *2*), 10 μl of nonexpressing bacterial lysate (*lane 3*), 10 μl of lysate from bacteria expressing the PPIase domain fragment of FKBP52 (*lane 4*), 50 μg of purified TPR domain of PP5 (*lane 5*), or 1 μM CsA (*lane 6*). The final volume was adjusted to 77 μl with HKD buffer. After the incubation, the beads were washed and proteins were resolved by electrophoresis and immunoblotting.

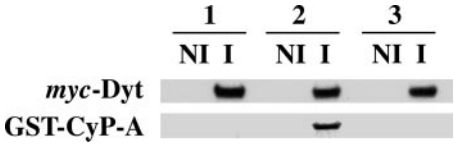

FIG. 4. **CyP-A binds to purified dynamitin.** Aliquots (250 μl) of cytosol from L cells overexpressing *myc*-dynamitin (*myc-Dyt*) were immunoadsorbed with nonimmune serum (*NI*) or with anti-*myc* serum (*I*). The immune pellets were incubated with 0.5 M NaCl, washed, and incubated for 1 h at 0 °C with 10 μl of lysate from nonexpressing bacteria (*condition 1*), 10 μl of lysate from bacteria expressing GST-CyP-A (*condition 2*), or 10 μl of lysate from bacteria expressing GST-CyP-A plus 90 μg of purified PPIase domain from FKBP52 (*condition 3*). The immune pellets were washed, and proteins were resolved by electrophoresis and immunoblotting.

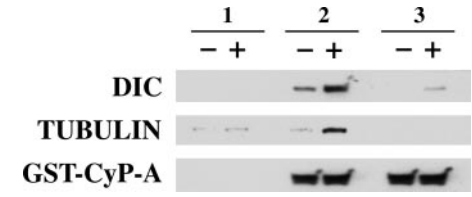

FIG. 5. **CyP-A forms a complex containing tubulin in a PPIase domain-dependent manner.** L cells were incubated with vehicle (−) or 20 μM paclitaxel (+) for 15 min prior to Dounce homogenization in tubulin-stabilizing buffer containing (+) 20 μM paclitaxel and 100 μM GTP or not (−). Aliquots (250 μl) of cytosol were incubated for 30 min at 30 °C with lysate from nonexpressing bacteria (*condition 1*) or with lysate from bacteria overexpressing GST-CyP-A plus HKD buffer (*condition 2*) or with lysate from bacteria overexpressing GST-CyP-A plus the purified PPIase domain of FKBP52 (*condition 3*). GST-CyP-A was then immobilized by rotation with GSH-agarose, the pellets were washed, and the proteins were resolved by electrophoresis and immunoblotting.

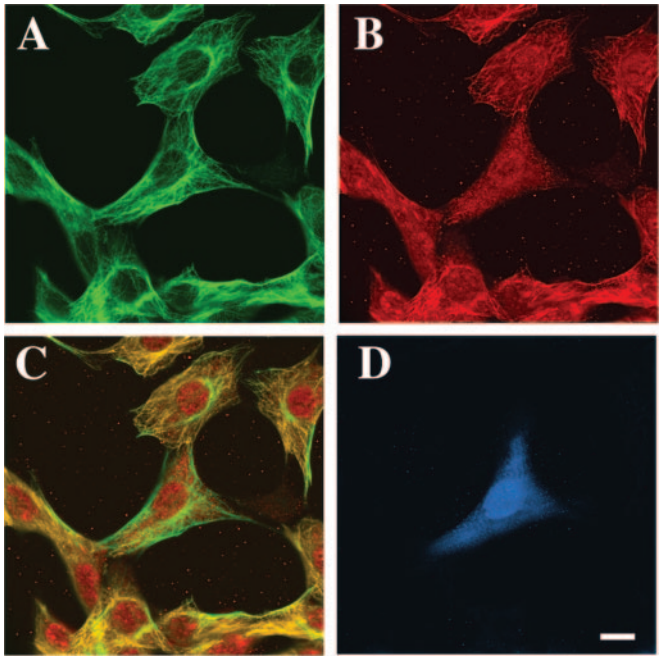

FIG. 6. **Cytoplasmic CyP-A colocalizes with microtubules.** NIH 3T3 fibroblasts were cotransfected with 1 μg of pECFP (cyanine fluorescent protein) and 8 μg of pSG5PL-FKBP52 core domain I (PPIase domain fragment of FKBP52). After 36 h, the cells were fixed and immunostained for tubulin (*A*) and CyP-A (*B*). *Panel C* is a merge of *images A* and *B*, and *panel D* identifies the transfected cell by *blue fluorescence*. *Bar C* is 10 μm.

PPIase domain fragment competing for the localization of CyP-A to microtubules.

DISCUSSION

Here, we have shown that the single domain immunophilin, CyP-A, behaves like the hsp90-binding TPR domain immunophilins in binding to the cytoplasmic dynein motor protein complex (Fig. 1). The PPIase domain fragment of FKBP52 competes for dynein binding; however, inasmuch as binding occurs in the presence or absence CsA (Fig. 3), the isomerase activity of CyP-A is not required. Thus, the binding of immunophilin PPIase domains to the motor protein complex is very different from the binding of CyP-A or FKBP12 to calcineurin where only the CsA- or FK506-bound immunophilin is a calcineurin binding partner. Again, like the hsp90-binding immunophilins, CyP-A appears to bind to dynein indirectly through an interaction of its PPIase domain with the dynamitin component of the dynein-associated dynactin complex (Fig. 4).

Cytoplasmic dynein motors process along microtubules tracks, and we show that CyP-A heterocomplexes containing tubulin and dynein can be formed in cytosol under microtubule-stabilizing conditions (Fig. 5). Importantly, we show that CyP-A colocalizes with microtubules in 3T3 mouse fibroblasts and that binding to microtubules *in vivo* is disrupted by overexpression of the PPIase domain fragment of FKBP52 (Fig. 6). Taken together, the *in vitro* and *in vivo* observations support a model in which CyP-A is associated with the retrograde movement system as a CyP-A-dynamitin-dynein-microtubule complex.

CyP-A is present as a relatively abundant protein in the cytoplasm of all mammalian cells (2). Thus, it must perform some general housekeeping function(s) important for cellular homeostasis. To date, the studies of CyP-A have largely focused on its structure and its immunosuppressive action through the association of the CyP-A·CsA complex with calcineurin (1, 2). Occasional reports have noted the CyP-A association with spe-

cific proteins, such as the YY1 suppressor of gene transcription (27) and human immunodeficiency virus, type 1 Pr55gag polyprotein precursor (26, 28). However, the associations reported have not led to a general model for CyP-A function in mammalian cells in the absence of CsA. The association of CyP-A with the dynein/dynactin motor protein complex could reveal a general housekeeping function. Dynein is thought to link to its cargo indirectly through dynactin, but it is not known how cargo is recognized (29). We summarized in the Introduction how the TPR domain immunophilins target two hsp90-regulated proteins, the GR and p53, to the dynein/dynactin complex for their retrograde movement to the nucleus (15). CyP-A could participate in such cargo recognition either directly or indirectly as part of a protein complex. Interestingly, there are several lines of evidence suggesting that HIV uses dynein and the microtubule network to transport its genome to the nucleus. Because HIV requires CyP-A to efficiently replicate in human cells (30, 31), one can envision that CyP-A acts as connector between HIV and the microtubule network in order to facilitate a secured convey of the viral genome to the nucleus.

Acknowledgments—We thank Rainer Benndorf, Michael Chinkers, Jeremy Luban, Christine Radanyi, Michel Renoir, and Richard Vallee for providing reagents used in this work.

REFERENCES

1. Galat, A. (2003) *Curr. Top. Med. Chem.* **3,** 1315–1347
2. Dornan, J., Taylor, P., and Walkinshaw, M. D. (2003) *Curr. Top. Med. Chem.* **3,** 1392–1409
3. Handschumacher, R. E., Harding, M. W., Rice, J., Drugge, R. J., and Speicher, D. W. (1984) *Science* **226,** 544–547
4. Fischer, G., Wittmann-Liebold, B., Lang, K., Kiefhaber, T., and Schmid, F. X. (1989) *Nature* **337,** 476–478
5. Takahashi, N., Hayano, T., and Suzuki, M. (1989) *Nature* **337,** 473–475
6. Fischer, G., and Bang, H. (1985) *Biochim. Biophys. Acta* **828,** 39–42
7. Liu, J. (1993) *Immunol. Today* **14,** 290–295
8. Bierer, B. E., Mattila, P. S., Standaert, R. F., Herzenberg, L. A., Burakoff, S. J., Crabtree, G., and Schreiber, S. L. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 9231–9235
9. Ivery, M. T. (2000) *Med. Res. Rev.* **20,** 452–484
10. Brown, E. J., Albers, M. W., Shin, T. B., Ichikawa, K., Keith, C. T., Lane, W. S., and Schreiber, S. L. (1994) *Nature* **369,** 756–758
11. Sabatini, D. M., Erdjument-Bromage, H., Lui, M., Tempst, P., and Snyder, S. H. (1994) *Cell* **78,** 35–43
12. Schmelzle, T., and Hall, M. N. (2000) *Cell* **103,** 253–262
13. Pratt, W. B., and Toft, D. O. (2003) *Exp. Biol. Med.* **228,** 111–133
14. Riggs, D. L., Roberts, P. J., Chirillo, S. C., Cheung-Flynn, J., Prapapanich, V., Ratajczak, T., Gaber, R., Picard, D., and Smith, D. F. (2003) *EMBO J.* **22,** 1158–1167
15. Pratt, W. B., Galigniana, M. D., Harrell, J. M., and DeFranco, D. B. (2004) *Cell. Signal* **16,** 857–872
16. Galigniana, M. D., Radanyi, C., Renoir, J. M., Housley, P. R., and Pratt, W. B. (2001) *J. Biol. Chem.* **276,** 14884–14889
17. Davies, T. H., Ning, Y. M., and Sanchez, E. R. (2002) *J. Biol. Chem.* **277,** 4597–4600
18. Vallee, R. B., and Gee, M. A. (1998) *Trends Cell Biol.* **8,** 490–494
19. Silverstein, A. M., Galigniana, M. D., Kanelakis, K. C., Radanyi, C., Renoir, J. M., and Pratt, W. B. (1999) *J. Biol. Chem.* **274,** 36980–36986
20. Harrell, J. M., Kurek, I., Breiman, A., Radanyi, C., Renoir, J. M., Pratt, W. B., and Galigniana, M. D. (2002) *Biochemistry* **41,** 5581–5587
21. Galigniana, M. D., Harrell, J. M., Murphy, P. J., Chinkers, M., Radanyi, C., Renoir, J. M., Zhang, M., and Pratt, W. B. (2002) *Biochemistry* **41,** 13602–13610
22. Galigniana, M. D., Harrell, J. M., O'Hagen, H. M., Ljungman, M., and Pratt, W. B. (2004) *J. Biol. Chem.* **279,** 22483–22489
23. Saphire, A. C., Bobardt, M. D., and Gallay, P. A. (1999) *EMBO J.* **18,** 6771–6785
24. Chen, M. S., Silverstein, A. M., Pratt, W. B., and Chinkers, M. (1996) *J. Biol. Chem.* **271,** 32315–32320
25. Burkhardt, J. K., Echeverri, C. J., Nilsson, T., and Vallee, R. B. (1997) *J. Cell Biol.* **139,** 469–484
26. Luban, J., Bossolt, K. L., Franke, E. K., Kalpana, G. V., and Goff, S. P. (1993) *Cell* **73,** 1067–1078
27. Yang, W. M., Inouye, C. J., and Seto, E. (1995) *J. Biol. Chem.* **270,** 15187–15193
28. Yoo, S., Myszka, D. G., Yeh, C.Y., McMurray, M., Hill, C. P., and Sundquist, W. I. (1997) *J. Mol. Biol.* **269,** 780–795
29. Hirokawa, N. (1998) *Science* **279,** 519–526
30. Franke, E. K., Yuan, H. E, and Luban, J. (1994) *Nature* **372,** 359–362
31. Thali, M., Bukovsky, A., Kondo, E., Rosenwirth, B., Walsh, C. T., Sodroski, J., and Gottlinger, H. G. (1994) *Nature* **372,** 363–365