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The hsp90-FKBP52 Complex Links the Mineralocorticoid Receptor to Motor Proteins and Persists Bound to the Receptor in Early Nuclear Events[▽]

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In this study, we demonstrate that the subcellular localization of the mineralocorticoid receptor (MR) is regulated by tetratricopeptide domain (TPR) proteins. The high-molecular-weight immunophilin (IMM) FKBP52 links the MR-hsp90 complex to dynein/dynactin motors favoring the cytoplasmic transport of MR to the nucleus. Replacement of this hsp90-binding IMM by FKBP51 or the TPR peptide favored the cytoplasmic localization of MR. The complete movement machinery, including dynein and tubulin, could be recovered from paclitaxel/GTP-stabilized cytosol and was fully reassembled on stripped MR immune pellets. The whole MR-hsp90-based heterocomplex was transiently recovered in the soluble fraction of the nucleus after 10 min of incubation with aldosterone. Moreover, cross-linked MR-hsp90 heterocomplexes accumulated in the nucleus in a hormone-dependent manner, demonstrating that the heterocomplex can pass undissociated through the nuclear pore. On the other hand, a peptide that comprises the DNA-binding domain of MR impaired the nuclear export of MR, suggesting the involvement of this domain in the process. This study represents the first report describing the entire molecular system that commands MR nucleocytoplasmic trafficking and proposes that the MR-hsp90-TPR protein heterocomplex is dissociated in the nucleus rather than in the cytoplasm.

The mineralocorticoid receptor (MR) is a member of the steroid/thyroid superfamily of nuclear receptors whose transcriptional activity is triggered by aldosterone binding under normal physiologic conditions. Polarized epithelial tissues such as the distal nephron and colon are considered the classical targets of mineralocorticoids to control salt-water balance by induction of sodium reabsorption and thereby regulation of extracellular fluid volume and blood pressure. MR expression and function also extend to nonepithelial cells, such as hippocampal and hypothalamic neurons, cardiomyocytes, vascular endothelium, and adipocytes (for recent reviews, see references 65 and 52 and references therein).

MR shares considerable homology with the glucocorticoid receptor (GR), which is exemplified by the ability of some glucocorticoids to bind both receptors. It is now well established (45) that the GR (the best-studied member of the family) forms heterocomplexes with the 90-kDa and 70-kDa heat shock proteins (hsp90 and hsp70, respectively), the acidic protein p23, and proteins that possess sequences of 34 amino acids repeated in tandems, the tetratricopeptide repeat (TPR) proteins. Some of these hsp90-binding TPR proteins have peptidylprolyl-isomerase activity and are intracellular receptors for immunosuppressant drugs such as FK506, rapamycin, and cyclosporine. They belong to the relatively conserved large family of proteins known as immunophilins (IMMs) (48). Among the members of this family, some IMMs have been recovered in steroid receptor-hsp90 complexes, i.e., FKBP52, FKBP51, CyP40, and three IMM-like proteins, protein phosphatase 5 (PP5), XAP2/ARA9, and WISp39 (33, 44). Even though the biological function of these proteins in the receptor-hsp90 heterocomplex remains poorly understood, it is thought that these IMMs are not related directly to the immunosuppressant effect.

In the absence of steroid, MR oligomers reside predominantly in the cell cytoplasm (30, 35, 39, 42, 51). However, like other transcription factors, the MR is not confined to any particular compartment but continuously shuttles between the cytoplasm and the nucleus. In this sense, MR behaves like its closest partner of the superfamily, the GR.

In previous studies, we reported that the GR-hsp90-FKBP52 heterocomplex contains dynein motor proteins linked to the receptor via FKBP52 (16, 18) and suggested a potential role in GR movement for this motor protein. When the putative interaction of the GR-hsp90-based heterocomplex with structures of the nuclear pore was examined (11), we found that nucleoporins and importin β bind the GR and its chaperones, suggesting that untransformed complexes at least have the ability to interact with proteins of the nuclear pore.

Nonetheless, the observation that IMMs displayed nuclear localization of human GR expressed in dynein-deficient yeasts (50) collides with the proposed relevance of TPR proteins bound to dynein in steroid receptor signaling. More recently, analyses of GR heterocomplex composition, hormone-binding affinity, and ability to undergo hormone-induced nuclear translocation and DNA binding were performed, and no effect of

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FKBP52 loss was found for these GR properties (67). Consequently, the exact mechanistic contribution of TPR proteins to steroid receptor function remains controversial.

Here we demonstrate that the FKBP52-dynein complex, linked to the MR via hsp90, is absolutely required for the efficient transport of MR through the cytoplasm to the nucleus. We also provide strong evidence to support a model where the dissociation of the hsp90-based heterocomplex from the receptor (a process referred to as "transformation") takes place in the nuclear compartment, so the long-standing classic paradigm that supports the heuristic notion that chaperones must dissociate from the receptor upon hormone binding is called into question.

MATERIALS AND METHODS

Cell culture and plasmid transfections. L929 mouse fibroblasts, the L929 ($GR^{-/-}$)-derived cell line E82.A3, 293-T human embryonic fibroblasts, HC11 mouse mammary epithelial cells, primary rat collecting duct cells (41), and mouse embryonic fibroblasts (MEF) from FKBP52 knockout mice (FKBP52 KO cells) were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum. WCL2 cells are derivatives of CHO cells subjected to rounds of methotrexate amplification after stable cotransfection with plasmids containing the cDNAs for mouse wild-type GR and dihydrofolate reductase (54). WCL2 cells were maintained in DMEM with 10% iron-supplemented calf serum and 10 μM methotrexate. Cells were transfected according to the standard calcium phosphate precipitation method as described previously (21). Renal duct cells were isolated and cultured as described previously (41). Plasmids employed in this work were pcDNA3-flag-rMR (provided by Shigeaki Kato), pCMV6-flag-TPR and its R101A mutant (provided by Michel Chinkers), pSG5PL-PPIase domain (provided by Jack-Michel Renoir), pCMVH50m (encoding for *myc*-dynamitin; provided by Richard Vallee), pCI-Neo-flag-hFKBP51, and pCI-Neo-hFKBP52 (provided by David Smith).

Indirect immunofluorescence assays. Cells were grown on coverslips in a steroid-free medium. Nuclear accumulation was triggered by adding aldosterone (Aldo; Sigma Chemical Co., St. Louis, MO) at zero time, and the cells were fixed and immunostained by inverting the coverslip on 25 μl of a solution of primary antibody in 20 mM Tris at pH 8.8, 0.63 M NaCl, 0.05% Tween 20, 0.02% NaN_3, and 1% bovine serum albumin. After 1 h at room temperature, the cells were washed, mounted on microscope slides with an antifade solution (Fisher Scientific, Pittsburgh, PA), and observed by fluorescence microscopy in a BX-60 Olympus microscope or a Zeiss LSM5 Pa confocal microscope. To assay the effect of hsp90-disrupting agents (Sigma Chemical Co., St. Louis, MO), we followed a previously described protocol (7, 19). In those experiments where the ATPase activity of dynein was inhibited, the cells were preincubated for 30 min with 0.5 mM *erythro*-9-[3-(2-hydroxynonyl)]-adenine (EHNA; BioMol International, Plymouth Meeting, PA), with the drug being left in the culture medium during the course of the experiment (5, 9, 62). When the quantification of fluorescence was required, cells were analyzed with Media Cybernetics Image-Pro Plus software, and both the nuclear and cytoplasmic fluorescence was quantified as the median intensity for each compartment. The nuclear fraction was calculated as the ratio of nuclear pixels to total pixels.

Immunoprecipitation. Cells were harvested by being scraped into ice-cold Earle's balanced saline, washed twice, and ruptured by Dounce homogenization in 1 volume of HEM buffer at pH 7.4 (10 mM HEPES, 1 mM EDTA, 20 mM Na_2MoO_4). Homogenates were centrifuged at 3°C for 30 min at 66,000 × *g*, and the resultant supernatant is referred to as the cytosol. For immunoadsorption of Flag-MR, 200 μl of cytosol was incubated for 2 h at 4°C with 2 μl M2 antibody (Sigma) or nonimmune IgG and 18 μl of protein A-Sepharose. Endogenous MR was immunoprecipitated from renal duct cells as described in previous studies (41), using a rabbit antiserum raised against human MR (gifted by Gerald Litwack). The pellets were washed four times with 1 ml of TEGM buffer at pH 7.6 [10 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES), 50 mM NaCl, 4 mM EDTA, 10% (vol/vol) glycerol, and 20 mM Na_2MoO_4]. Proteins in the immune pellets were resolved by Western blotting with the following antibodies: AC88 clone for hsp90 and N27F3-4 clone for hsp70 (StressGen, Ann Arbor, MI); MAB1618 clone for dynein (Chemicon, Temecula, CA); BuGR2 anti-GR clone, anti-CyP-40, JJ3 clone for p23, and anti-FKBP51 (Affinity BioReagents, Golden, CO); anti-PP5 serum (gifted by Michel Chinkers); UP30 antiserum for FKBP52 and 8D3 anti-hsp90 clone (gifted by William Pratt); F5 mouse monoclonal IgG for p60/Hop (gifted by David Smith); A-14 clone for *myc*-dynactin2 and anti-$p150^{Glued}$ (BD Transduction Laboratories, San Diego, CA); and TUB2.1 clone for tubulin (Sigma). The immunoblots were then incubated with the appropriate counterantibodies conjugated with horseradish peroxidase and revealed by enhanced chemiluminescence. Because PP5, FKBP52, p53, and dynactin2 migrate in the same region upon gel electrophoresis, as do hsp70 and the dynein intermediate chain, we electrophoresed replicate samples of both nonimmune and immune pellets and probed replicate immunoblots with antibody specific for each protein. Thus, the Western blots in some figures are necessarily composites prepared from two or more replicate immunoblots.

Heterocomplex reconstitution. We followed a previously described protocol for heterocomplex reconstitution (20, 26). Flag-MR was immunoadsorbed from cell cytosol overexpressing the receptor by use of the M2 antibody coupled to protein A-Sepharose (Sigma), whereas hsp90 was immunoadsorbed from reticulocyte lysate with the 8D3 antibody cross-linked to Actigel-ALD (Sterogene, Carlsbad, CA). The immune pellets were stripped of coadsorbed proteins by incubation for 2 h with TEG buffer supplemented with 0.5 M KCl. The pellet was washed twice with 1 ml TEG buffer and twice with 1 ml 10 mM HEPES buffer at pH 7.4, and the heterocomplexes were then assembled by incubating the stripped 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 to 3 min. Pellets were then washed four times with 1 ml of ice-cold TEGM buffer and boiled in SDS sample buffer. In peptide competition experiments, the reticulocyte lysate was supplemented with purified peptides as described in previous works (18, 26, 59). The association of partially purified dynein (15) with FKBP52 was assayed using bacterially expressed glutathione *S*-transferase (GST)-FKBP52 immobilized to glutathione (GSH)-agarose.

Sucrose gradient ultracentrifugation. Steroid binding assays and continuous sucrose gradient ultracentrifugation were performed as described in previous studies (13, 39), by prelabeling MR with [1,2,6,7-^{3}H]Aldo (NEN Life Sciences, Cambridge, MA). The transformed and untransformed peaks (i.e., bound to and dissociated from the hsp90-based complex, respectively) were calibrated with samples treated with high ionic strength (transformed peak) or protected by the addition of molybdate to the medium (untransformed peak). The soluble fraction of lysed nuclei (nucleoplasm) was resolved in its native form or after preincubation of each fraction with an anti-hsp90 IgM (8D3 clone) able to recognize the chaperone in a complex.

Digitonin-permeabilized cells. E82.A3 cells were permeabilized with 25 μg/ml digitonin and incubated with Flag-MR–hsp90 heterocomplexes released from M2 anti-Flag immune pellets with Flag peptide (Sigma) as described in previous studies (42). For Fig. 7D, the permeabilized cells were incubated with dithiosuccinimidyl propionate (Pierce, Rockford, IL)-cross-linked Flag-MR (11), with purified Flag-TPR peptide from rat PP5 (58), or with a DNA-binding peptide (DBP) of MR (see sequence in Fig. 7D) synthesized by the University of Michigan Protein Core Facility. The incubation mixture contained 40 μl of Adam's buffer supplemented with an ATP-regenerating system and 10 μl of E82.A3 cytosol (~40 μg protein). After 20 min at 30°C (with and without 100 nM Aldo in the medium), the coverslips were rapidly washed with Adam's buffer and fixed with cold methanol, and the localization of MR was visualized by indirect immunofluorescence. Alexa Fluor 488-labeled albumin (Molecular Probes, Eugene, OR) was used as a control to test the nuclear envelope integrity after treatment with digitonin.

RESULTS

hsp90 is required for MR movement to the nucleus and for MR stability. To analyze the influence of hsp90 on MR cytoplasmic transport, E82.A3 fibroblasts expressing Flag-MR were preincubated with 1 μM Aldo for 1 h on ice to form steroid-receptor complexes, followed by a short incubation of 15 min with 2 μM geldanamycin (GA) to inhibit hsp90. When the temperature was raised to 37°C, MR underwent a rapid aldosterone-dependent shift to the nucleus (Fig. 1A, panel b versus panel a). While GA alone did not affect the primary subcellular localization of MR (Fig. 1A, panel c), the hsp90-disrupting agent impaired the steroid-dependent MR accumulation in the nucleus (panel d). Figure 1B shows a kinetic analysis of MR movement to the nucleus and shows that GA

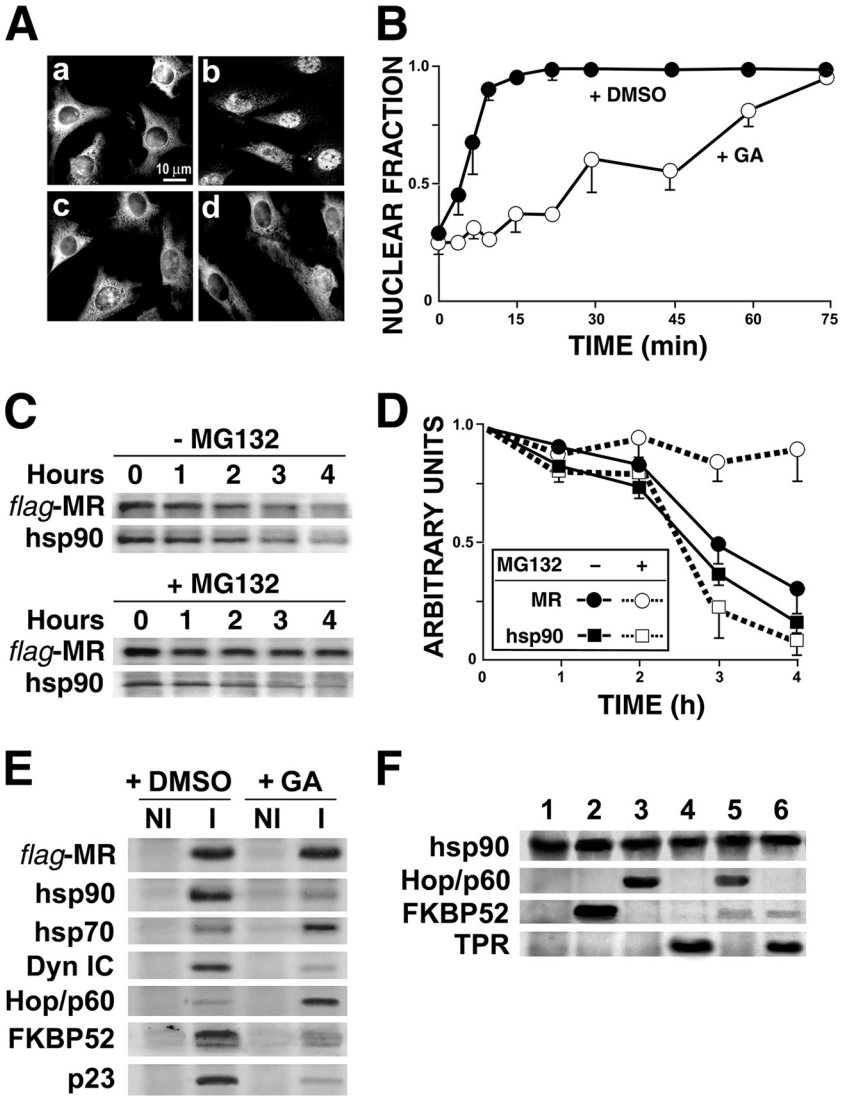

FIG. 1. MR retrotransport requires a functional hsp90 complex. (A) Inhibitory effect of GA on MR nuclear accumulation. E82.A3 cells expressing Flag-MR were incubated on ice for 1 h with 0.1% ethanol (a and c) or 1 μM Aldo (b and d). Next, 2 μM GA (c and d) or 0.1% dimethyl sulfoxide (DMSO) (a and b) was added to the medium, and the incubation was continued on ice for 15 min. The temperature was shifted to 37°C (zero time), and MR was visualized by indirect immunofluorescence after 15 min. (B) The nuclear accumulation rate of MR was measured for the indicated incubation times at 37°C. Data are means ± standard errors of the means (SEM) ($n = 3$). (C) GA treatment of intact cells destabilizes MR by proteasome degradation. Flag-MR was immunoprecipitated from the cytosol of cells treated with 2 μM GA for the indicated times, in the presence or absence of 10 μM MG132. Coadsorbed hsp90 is also shown. (D) The relative amounts of MR and hsp90 shown in panel C were semiquantified by band density scanning (mean ± SEM; $n = 3$). (E) 293-T cells were treated with 0.1% DMSO (vehicle) or 2 μM GA for 3 h in the presence of 10 μM MG132 to prevent MR degradation. The receptor was immunoprecipitated with anti-Flag antibody (I; immune) or a nonimmune IgG (NI), and the associated proteins were resolved by Western blotting. (F) hsp90 was immunopurified from reticulocyte lysate, stripped of associated proteins by high ionic strength, and incubated with pure recombinant proteins. Stripped hsp90 was incubated with buffer (lane 1), 50 μg FKBP52 (lane 2), 50 μg Hop/p60 (lane 3), 50 μg Flag-TPR peptide (lane 4), 50 μg FKBP52 and 100 μg Hop/p60 (lane 5), or 50 μg FKBP52 and 100 μg Flag-TPR peptide (lane 6).

does not block MR translocation but impairs MR cytoplasmic trafficking. This experiment implies that under normal conditions, hsp90 is required for rapid and efficient cytoplasmic transport of MR to the nucleus.

Cytosol from GA-treated cells was immunoprecipitated with anti-Flag antibody and Western blotted for MR and hsp90 (Fig. 1C, upper panel). A marked loss of receptor was evidenced, without any change in the fraction of receptor bound to hsp90 (densitometric analyses are shown in Fig. 1D). The protective effect of MG132 (Fig. 1C, lower panel) demonstrates that the loss of MR is due to proteasome-dependent digestion, suggesting that the disruption of the chaperone function makes the receptor unstable and more sensitive to degradation. Inasmuch as the half-life for MR disappearance from GA-treated cells is about 2.5 h, this effect cannot account for the failure of Aldo to promote nuclear accumulation after GA treatment under the conditions used for Fig. 1A. These results strongly suggest that the inhibitory effect of GA on MR nuclear

accumulation is due to the effect of this drug on the functional properties of hsp90.

The immunoprecipitation of MR yielded the specific coimmunoprecipitation of chaperones and cochaperones normally associated with steroid receptor complexes, i.e., hsp90, hsp70, p23, and FKBP52 (Fig. 1E). It was reported in previous works that immunoadsorption of FKBP52 is accompanied by coimmunoadsorption of dynein (18, 27). Consistent with the presence of FKBP52 in MR complexes, the intermediate chain of cytoplasmic dynein was also revealed in the Western blot. This result confirms our previous observation (21) showing that dynein binds to the MR-hsp90 complex. As described for the progesterone receptor (PR) expressed in COS cells (61) and endogenous GR in L cells (7), Fig. 1E also shows that the MR recovered from GA-treated cells was arrested in an intermediate maturation state of the heterocomplex assembly which was depleted of hsp90 and the hsp90-interacting cochaperone p23 (for a recent review, see reference 60). As a consequence of the loss of the hsp90-FKBP52 complex, the association of dynein with the MR heterocomplex was also lost. This observation is consistent with the impairment of MR nuclear accumulation observed in Fig. 1A for cells treated with GA.

Figure 1E also shows that in GA-treated cells, the MR complex has greater amounts of hsp70 and the TPR protein Hop/p60. Because GA favors the persistence of Hop/p60 in the complex, we analyzed whether this TPR protein impairs FKBP52 binding to hsp90. Immunopurified hsp90 was incubated with recombinant FKBP52 in the presence of recombinant Hop/p60. Figure 1F shows that FKBP52 was displaced from hsp90 (lane 5 versus lane 2) due to competition of Hop/p60 for the only TPR acceptor site of hsp90 (59). A similar effect was observed in the presence of an excess of recombinant TPR peptide (lane 6).

TPR protein swapping. We next asked whether the swapping between Hop/p60 and FKBP52 impairs dynein binding to the hsp90-based complex. Reticulocyte lysate was depleted of hsp90 by three passages through a column of the 8D3 anti-hsp90 antibody covalently bound to Sepharose, and the resultant hsp90-free lysate was used as a source of TPR proteins and dynein. The hsp90-FKBP52-dynein complex could be reconstituted on 8D3 immune pellets of hsp90 that were first stripped of associated proteins and incubated with hsp90-free lysate in the presence of ATP (Fig. 2A, lane 1). The addition of 50 μg (lane 2) or 100 μg (lane 3) of Hop/p60 to the lysate inhibited the reconstitution of the complex with FKBP52 and dynein, whereas more Hop was recovered bound to hsp90. This observation is in line with the inhibitory effect of GA on MR transport to the nucleus (Fig. 1A) and with the loss of dynein bound to the MR complex recovered from GA-treated cells (Fig. 1E).

Steroid receptors are able to form complexes with several TPR domain proteins, in particular FKBPs (44, 60). Among the members of this subfamily, FKBP52 and FKBP51 seem to compete not only for their binding to hsp90 but also for the functional consequences of that interaction. Thus, FKBP51 represses GR, PR, MR, and Aldo receptor (AR) steroid-binding capacity and transcriptional activity, whereas FKBP52 shows no significant effect on steroid receptor action or a slight activation effect if the amount of endogenous FKBP52 is sufficient (6, 21, 24, 49, 66, 67). The experiment shown in Fig. 2B, using purified FKBPs or TPR peptide bound to immobilized

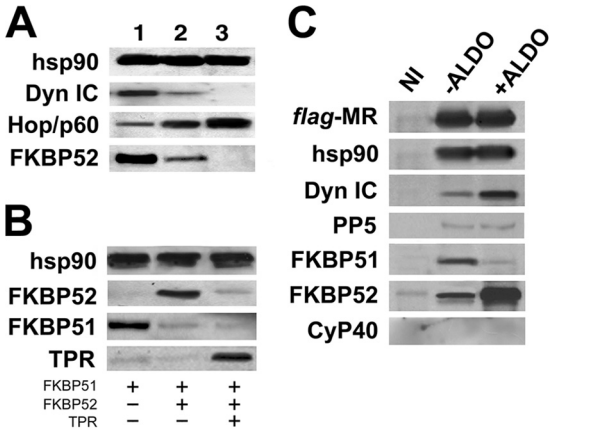

FIG. 2. Swapping of TPR proteins. (A) Stripped hsp90 was incubated with hsp90-free reticulocyte lysate (source of endogenous TPR proteins and dynein) supplemented with buffer (lane 1), 50 μg Hop/p60 (lane 2), or 100 μg Hop/p60 (lane 3). (B) FKBPs compete for the TPR acceptor site of hsp90. Stripped hsp90 pellets were incubated with 50 μg FKBP51 in the presence of 100 μg FKBP52 or 100 μg FKBP52 and 100 μg Flag-TPR peptide. (C) Effect of hormone binding to MR on IMM recruitment to the complex. E82.A3 cells transfected with Flag-MR were incubated on ice for 1 h with vehicle (−Aldo) or 1 μM aldosterone (+Aldo) to allow steroid binding but not receptor translocation to the nucleus. MR was then immunoadsorbed, and the coadsorbed proteins were resolved by Western blotting. NI, nonimmune pellet.

hsp90, demonstrates that an excess of FKBP52 displaces FKBP51 prebound to its binding site on hsp90 and that such competition is TPR domain dependent.

Under physiological conditions, ligand binding to the receptor should be the natural switch that promotes FKBP swapping. In a pioneer study by the E. Sanchez laboratory, it was reported that the hormone causes exchange of FKBP51 for FKBP52 in GR complexes (8). Figure 2C shows that when the cells were incubated on ice with Aldo to permit ligand binding without triggering MR nuclear translocation, larger amounts of FKBP52 were recruited to the MR complex, whereas FKBP51 was dissociated. Because dynein is bound to FKBP52, the motor protein was also recruited. In the context of the biological action proposed here for the hsp90-FKBP52-dynein complex, this observation is reasonable, since the MR heterocomplex is modified upon ligand binding to be translocated more efficiently to the nuclear compartment. Moreover, it is also biologically convenient that FKBP51 is released from the complex because this IMM does not bind dynein (66) and shows negative regulatory effects on MR action (21).

On the other hand, it should be pointed out that in all the assays performed with different cell types or in heterocomplex reconstitution assays with the reticulocyte lysate system, the IMM CyP40 was never recovered associated with the MR-hsp90 complex, suggesting that it is unlikely that this TPR domain protein plays a key role in MR signaling.

MR heterocomplexes bind to microtubules. If dynein motor proteins are responsible for the hsp90-dependent transport of MR, it would be expected that the receptor is linked to the cytoskeleton via the hsp90-IMM complex. The disruption of microtubules and/or microfilaments showed that MR was capable of moving toward the nucleus (data not shown), but the

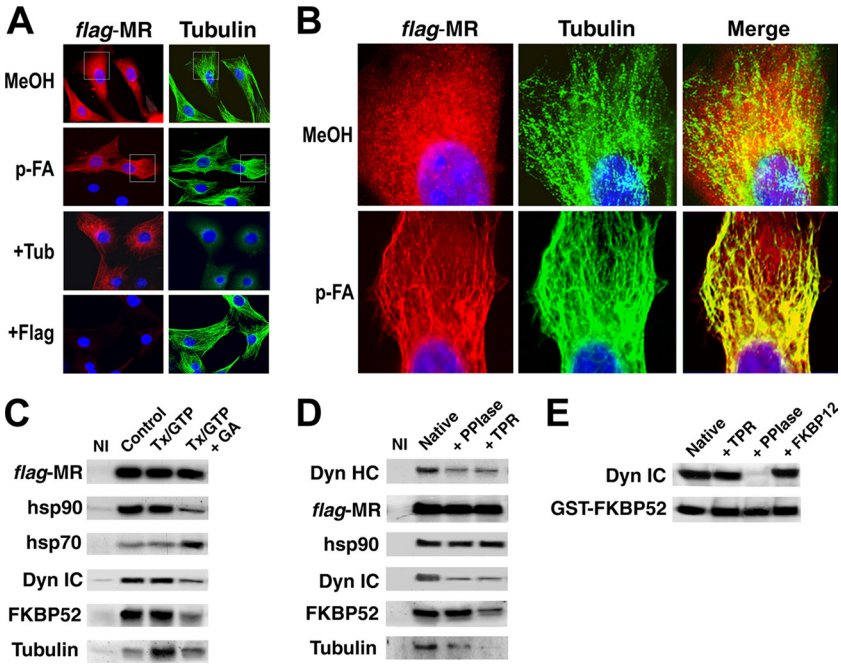

FIG. 3. MR heterocomplexes bind microtubules. (A) NIH 3T3 fibroblasts were transfected with Flag-MR and grown in a steroid-free medium. After 36 h, the cells were fixed and permeabilized in cold (−20°C) methanol (MeOH) for 10 min or in 4% *p*-formaldehyde (p-FA) for 1 h at room temperature and further permeabilized by immersing the coverslips in acetone at −20°C for 5 min. The cells were stained for MR with anti-Flag M2 antibody (red) and for tubulin with the TUB2.1 antibody (green). +Tub and +Flag, indirect immunofluorescence after preincubation of the primary antibody with an excess of pure tubulin or Flag peptide. (B) Close-up view of microtubules and MR. The areas defined by the white rectangles in panel A were enlarged to show the strong colocalization of MR with microtubules in *p*-formaldehyde-fixed cells (p-FA) compared with the diffuse distribution of MR observed in cells fixed with methanol (MeOH). (C) Taxol increases tubulin association with MR heterocomplexes. Flag-MR was immunoadsorbed with a nonimmune IgG antibody (NI) or the anti-Flag M2 IgG from E82.A3 standard cytosol (control) or supplemented with 20 μM Taxol and 100 μM GTP to stabilize microtubules (Tx/GTP). Tx/GTP+GA represents a Taxol/GTP-stabilized cytosol obtained from cells pretreated with 2 μM GA for 1 h. (D) FKBP52 domains prevent the assembly of the MR complex with tubulin. Flag-MR immune pellets were stripped of endogenous chaperones by high ionic strength and reincubated with 50 μl of E82.A3 cytosol containing an ATP-regenerating system and either 20 μl of buffer (native), 200 μg/20 μl PPIase domain (PPIase), or 200 μg/20 μl Flag-TPR domain. NI, nonimmune pellet. (E) Interaction of dynein with the PPIase domain of FKBP52. Bacterially expressed GST-FKBP52 was immobilized on GSH-agarose gel, stripped of associated proteins, and incubated for 20 min at 30°C with 60 μl of partially purified dynein from reticulocyte lysate in a medium containing 10 mM ATP and either 20 μl buffer (native), 200 μg Flag-TPR peptide/20 μl, 200 μg PPIase peptide/20 μl, or 200 μg FKBP12/20 μl.

inhibitory effect of GA on MR movement observed in Fig. 1A was lost. This observation indicates that when the integrity of the cytoskeleton is disrupted, the regulatory mechanism of the hsp90-dependent transport is also lost and MR can move freely in that environment. This observation agrees with the previously reported effect of GA on GR movement, which requires an intact cytoskeleton net (17, 19). Nonetheless, if the GA-sensitive MR retrotransport does require intact microtubules, then the putative association of MR with cytoskeletal structures remains to be demonstrated.

Figure 3A shows that MR colocalizes with tubulin in cells fixed with *p*-formaldehyde for 1 h. This association could not be seen when the cells were fixed with cold methanol, suggesting that the interaction is weak and requires a cross-linker. The regions defined by white boxes in Fig. 3A are enlarged in Fig. 3B to show with more detail the colocalization of MR and tubulin in *p*-formaldehyde-fixed cells compared to those fixed with methanol. The specificities of the signals generated by both anti-Flag and anti-tubulin antibodies were tested by blocking the primary antibody with an excess of Flag peptide and recombinant tubulin, respectively. As a result, the signal of the blocked antigen was totally lost (Fig. 3A, +Tub and +Flag panels). This control ruled out eventual cross-reactions of the antibodies and strongly supports the interpretation that MR is associated with microtubules in intact cells.

Figure 3C shows that the immunoprecipitation of MR yielded tubulin coimmunoprecipitated with the heterocomplex (control). When cells were ruptured in a buffer supplemented with paclitaxel and GTP to stabilize microtubules (22, 27), greater amounts of tubulin were recovered in the immune pellet. This interaction faded when the cells were treated with the hsp90 inhibitor GA. This suggests that the binding of MR to microtubules is only partially dependent on the hsp90-FKBP52 complex. To test this interpretation, cytosol samples were incubated with an excess of recombinant peptidyl-prolyl isomerase (PPIase) peptide or recombinant TPR peptide prior to the immunoprecipitation assay. Figure 3D shows that dynein was released from the heterocomplex in both cases. This indicates that FKBP52 is a key component for such association with the motor protein complex. Note that tubulin was fully released from the complex when FKBP52 was competed by an excess of the TPR peptide, whereas the PPIase peptide was

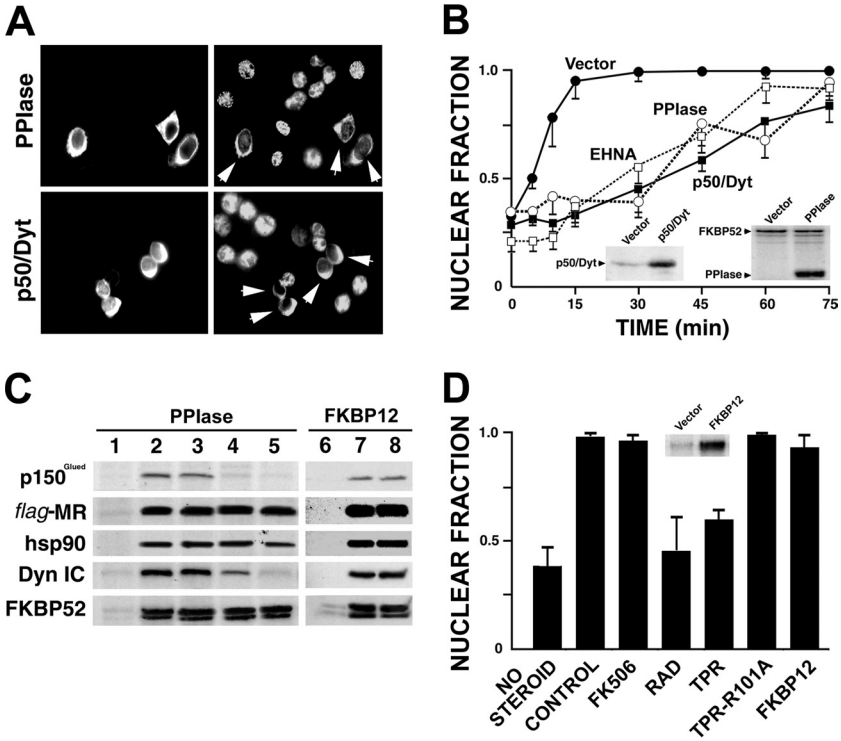

FIG. 4. Disruption of the dynein/dynactin complex impairs MR nuclear accumulation. (A) Renal duct cells were transfected with myc-p50/dynactin2 or the PPIase domain of FKBP52. Endogenous MR nuclear accumulation was achieved after 15 min of incubation with 1 μM Aldo. Cells were stained for MR (right) and either p50/dynactin2 or the PPIase domain (left). Arrowheads show the cytoplasmic localization of MR in transfected cells. (B) Nuclear accumulation rate of MR for cells transfected with PPIase domain or p50/Dyt. Note that MR nuclear accumulation was equally impaired in cells treated with the dynein inhibitor EHNA. The inset shows the expression of p50/Dyt or the PPIase domain compared to the endogenous level of FKBP52. (C) The PPIase domain, but not FKBP12, prevents dynein binding to the MR heterocomplex. Flag-MR was immunoprecipitated from 293-T cells cotransfected with vector, the PPIase domain, or FKBP12. Conditions were as follows (μg of plasmid are given in parentheses): lanes 1 and 6, nonimmune pellets; lanes 2 (2 μg), 4 (5 μg), and 7 (5 μg), immune pellets from cells cotransfected with empty vector; lanes 3 and 5 (2 μg and 5 μg), immune pellets from cells cotransfected with the PPIase domain of FKBP52; lane 8, immune pellet from cells cotransfected with FKBP12 (5 μg). (D) Nuclear accumulation was quantified in renal duct cells grown in steroid-free medium (no steroid) or incubated with Aldo for 20 min under the following conditions: 0.1% DMSO (control), 1 μM FK506, 2 μM radicicol (RAD), TPR domain, TPR mutant (R101A) unable to bind hsp90, or FKBP12. The inset shows the level of overexpression in FKBP12-transfected cells. Results in panels B and D are means ± SEM ($n = 3$).

able to promote the partial dissociation of tubulin. This suggests that the PPIase domain is also involved in linking the IMM to the cytoskeleton, although it is less efficient than the TPR domain. In conclusion, it is highly reasonable to conclude that the IMM plays an essential role in the association of MR with microtubules.

Figure 3D shows that the presence of dynein in the MR complex is IMM dependent. In Fig. 3E, it is shown that the complex could be reconstituted after an incubation of pure GST-FKBP52 with partially purified dynein. When the incubation mixture was supplemented with an excess of either TPR domain, PPIase domain, or FKBP12, only the PPIase domain of FKBP52 was able to prevent dynein binding. This confirmed that the association of dynein with FKBP52 occurs through the PPIase domain of the IMM.

MR nuclear accumulation is impaired by disruption of the hsp90-FKBP52-dynein molecular machinery. Dynactin is a multiprotein complex required for most, if not all, forms of dynein-based movement. It works as an adapter that allows dynein to bind a variety of cargoes. Recently, it was shown that the overexpression of a dynactin subunit, p50/dynactin2 (p50/Dyt), mediates the disruption of the entire oligomer by binding to endogenous subunits (34), which in turn leads to the disassembly of the dynactin complex, resulting in dynein inactivation. If our model for MR transport is correct, it could be predicted that the overexpression of p50/Dyt should prevent (or at least impair) MR nuclear translocation. Because Fig. 3E shows that dynein (and consequently dynactin) is bound to the complex via the PPIase domain, a similar prediction for the movement of MR would be valid for the overexpression of the PPIase peptide. To test this hypothesis, renal duct cells were transfected with the PPIase domain or p50/Dyt, and the subcellular localization of endogenous MR was observed after exposing the cells to Aldo for 15 min. Figure 4A shows that MR was cytoplasmic in transfected cells only (arrowheads). The nuclear translocation of endogenous MR was equally delayed when the molecular machinery of movement was disrupted by overexpression of the PPIase peptide or p50/Dyt (Fig. 4B) or by inhibition of the ATPase activity of dynein with EHNA (5, 9, 62). Also, note that the nuclear accumulation rate is the same as that measured when hsp90 was disrupted by GA (Fig. 1B).

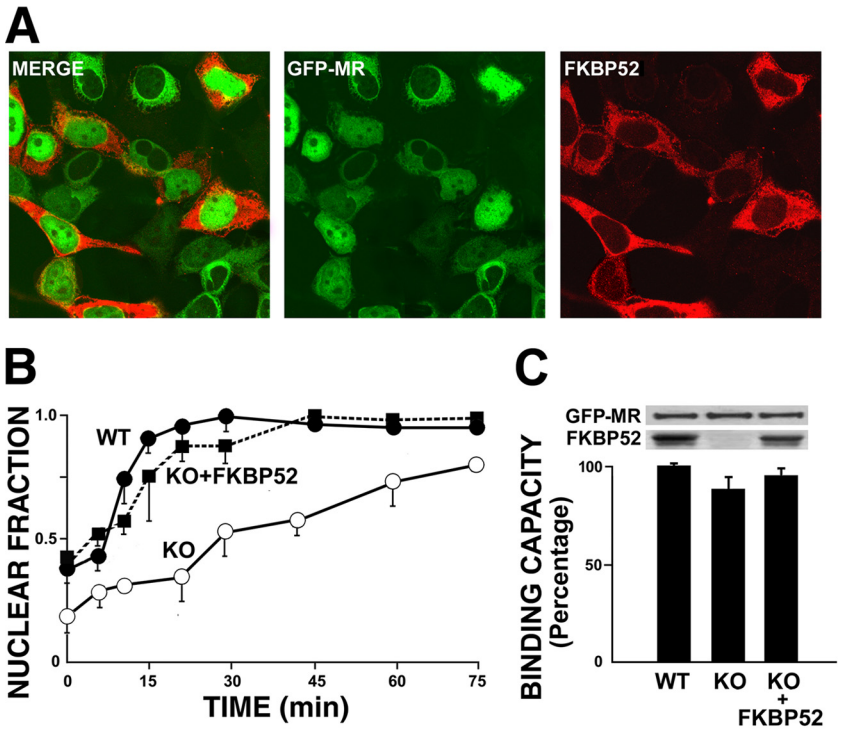

FIG. 5. Nuclear accumulation rate of MR in FKBP52 KO MEF cells. (A) MEF cells obtained from FKBP52 KO mice were cotransfected with GFP-MR and FKBP52. After 15 min of incubation with 1 μM Aldo, the cells were fixed and FKBP52 was visualized by indirect immunofluorescence (rhodamine-labeled cells). (B) The nuclear accumulation rate of GFP-MR was measured as described in the legend for Fig. 1. (C) GFP-MR was immunoprecipitated with an anti-MR rabbit serum, and the immune pellets were incubated with 5 nM [^{3}H]Aldo (±1 μM radioinert Aldo). Bars show the specific binding for wild-type cells (WT), FKBP52 KO cells (KO), and FKBP52 KO cells transfected with FKBP52 (KO+FKBP52). Results are means ± SEM (n = 5). The Western blots show the coadsorption of FKBP52 with GFP-MR.

Recently, it was reported (34) that the excess of p50/Dyt causes the complete release of a dynactin subunit, p150^{Glued}, which is a key component of the dynactin complex that functions as a linker with dynein and is directly bound to microtubules (63) regulating dynein/dynactin function (10). For Fig. 4C, we analyzed the presence of p150^{Glued} in the FKBP52-dynein complex. p150^{Glued} was coimmunoprecipitated with MR, whereas the overexpression of the PPIase peptide of FKBP52 was accompanied by the loss of both p150^{Glued} and cytoplasmic dynein. This effect was specific, since the overexpression of FKBP12, a highly homologous protein with similar enzymatic activity and size to those of the PPIase peptide, was ineffective to dissociate both motor proteins.

Figure 4D shows that in parallel with the effect of radicicol (used here as an intra-experiment control), the overexpression of the TPR peptide impaired the nuclear accumulation of MR in a similar manner to that of the hsp90 inhibitor. The lack of effect of a TPR mutant (R101A) unable to interact with hsp90 (53) proves that the inhibitory effect observed by overexpression of the TPR peptide is specific for the association of hsp90 with TPR factors, most likely FKBP52, but also with other potential dynein-interacting IMMs, such as PP5 (21), FKBP-L (33), and any other still unknown TPR factor that could replace FKBP52. The inhibitory effect observed by overexpression of the TPR domain strengthens the conceptual importance of the biological role of TPR proteins in the movement mechanism of the receptor.

Inasmuch as the PPIase domain of FKBP52 is essential for the transport machinery of MR, the effect of the drug FK506 was assayed. The PPIase inhibitor showed no effect on MR transport, which suggests that the enzymatic activity of FKBP52 is not required. In agreement with the lack of effect on dynein binding to the MR heterocomplex (Fig. 4C), the overexpression of FKBP12 did not affect the nuclear translocation of MR (Fig. 4D).

Inefficient MR transport to the nucleus in FKBP52$^{-/-}$ fibroblasts. MR nuclear translocation was studied in MEF obtained from FKBP52 knockout mice. Figure 5A shows that GFP-MR was cytoplasmic in cells treated with Aldo for 15 min, unless FKBP52 was reintroduced by transfection (see rhodamine-labeled cells). MR showed a lower nuclear translocation rate in FKBP52 KO cells than in wild-type MEF cells or KO cells where the IMM was reintroduced by transfection (Fig. 5B). Note that the nuclear localization rate of MR at time zero, i.e., in cells not exposed to steroid, was significantly lower ($P <$ 0.002) than the translocation rate measured for KO cells or KO cells transfected with FKBP52. This suggests that the IMM may be related to the constitutive subcellular localization of the receptor even in the absence of ligand. Figure 5C demonstrates that the less efficient nuclear accumulation of MR in KO cells cannot be assigned to a lower steroid-binding capacity of MR or a low level of receptor expression.

MR is recovered bound to hsp90 immediately after its nuclear internalization. Because the MR utilizes the hsp90 het-

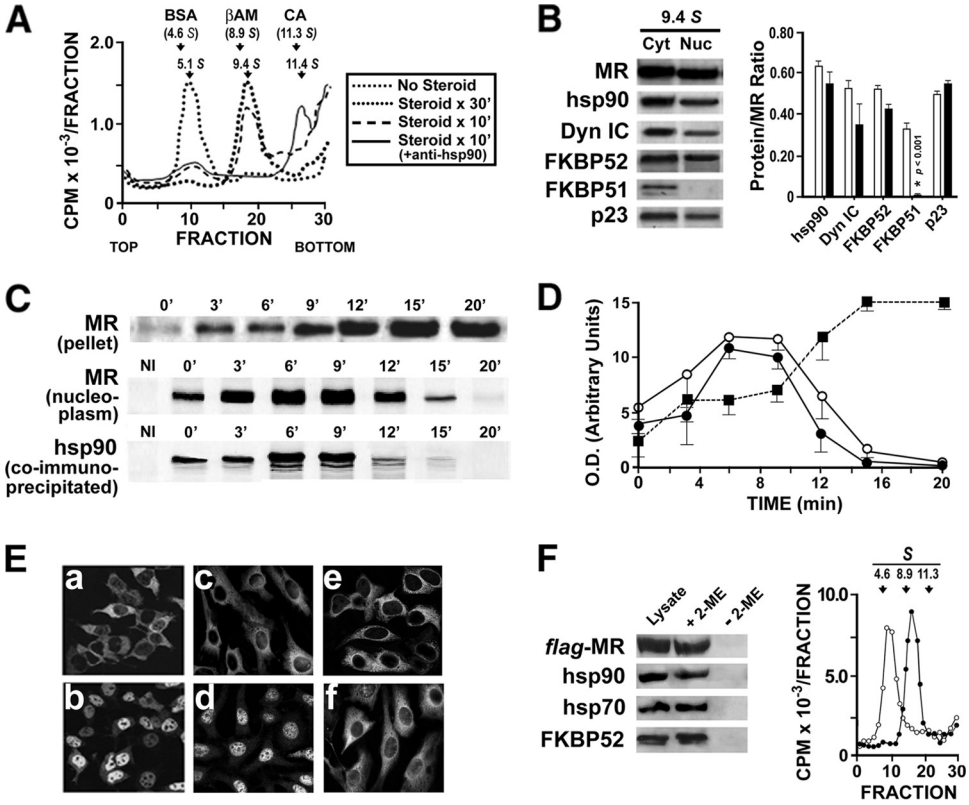

FIG. 6. MR transformation is a nuclear event. (A) Primary renal duct cells grown in suspension were incubated with [^{3}H]Aldo for 10 min, and nuclei were immediately isolated by a quick centrifugation at 2°C and lysed by three freeze-thaw cycles. Soluble nucleoplasmic MR isoforms were resolved in a continuous sucrose gradient before (gray dashed line) and after (gray continuous line) preincubation with anti-MR IgM. In the latter case, note the switch of the untransformed peak of MR from 9.4S to 11.4S. The column was calibrated with bovine serum albumin (BSA) (4.5S), β-amylase (β-AM) (8.9S), catalase (CA) (11.3S), untransformed MR (9.4S) obtained in a buffer supplemented with molybdate, and transformed MR (5.1S) obtained from cells incubated for 30 min with steroid and lysed in a buffer supplemented with 0.5 M KCl. (B) Untransformed (9.4S) peaks were pooled, and MR was immunoprecipitated. Coadsorbed proteins were resolved by Western blotting. Cyt, control of MR heterocomplex obtained from cell cytosol; Nuc, MR heterocomplexes from nucleoplasmic fractions. The bar graph shows a densitometric analysis of proteins bound to MR for the cytosolic (white bars) and nuclear (black bars) fractions. Results are means ± SEM for four independent assays. (C) Nucleoplasmic MR was immunoprecipitated after different periods of incubation with Aldo. Coadsorbed hsp90 is shown at the bottom, and MR associated with the insoluble pellet of chromatin is shown at the top. NI, nonimmune. (D) The optical densities of the bands shown in panel C were plotted against the times of incubation with hormone. ■, MR in the insoluble pellet; ○, MR in the soluble nucleoplasm; ●, hsp90 coimmunoadsorbed with soluble MR. Results are means ± SEM for six assays. (E) Cross-linked MR heterocomplexes were incubated with digitonin-permeabilized E82.A3 cells (a and b) or FKBP52 KO MEF cells (c to f). MR subcellular localization was visualized by indirect immunofluorescence in untreated cells (a and c) or cells incubated with 1 μM Aldo (b, d, and f). Conditions were as follows: a, E82 cells without steroid; b, E82 cells with steroid; c, MEF cells without steroid; d, MEF cells with steroid; e, permeabilization control of MEF cells incubated with Alexa Fluor 488-labeled albumin; f, control of MEF cells treated with steroid in a buffer not supplemented with cytosol and ATP. (F) Cross-linking controls. The Western blot shows the coimmunoprecipitation of hsp90, hsp70, and FKBP52 with Flag-MR from untreated cytosol (lysate) or from dithiosuccinimidyl propionate-cross-linked complexes boiled in sample buffer supplemented (+2-ME) or not (−2-ME) with β-mercaptoethanol. The plot on the right shows a sucrose gradient supplemented with 0.5 M KCl for cross-linked MR cytosol preincubated with [^{3}H]aldosterone followed by treatment with (○) or without (●) β-mercaptoethanol.

erocomplex for its cytoplasmic transport, an obvious corollary for this model is that the heterocomplex should not dissociate immediately upon steroid binding as the classic model posits. We used a continuous sucrose gradient to analyze the transformed-to-untransformed MR ratio in nuclei isolated from cells exposed to steroid for 10 min. The gradient was calibrated with untransformed MR stabilized with molybdate (9.4S peak), with transformed MR obtained from cells treated with steroid for 30 min and extracted with high ionic strength (5.1S peak), and with commercial standard proteins (Fig. 6A). Nucleoplasmic extracts obtained from cells treated with hormone for 10 min (a time when most MR molecules are nuclear) showed that ~75% of nuclear MR was still untransformed (i.e., bound to an hsp90 complex) and was recovered in the 9.4S peak. To test if hsp90 belongs to this complex, the nuclear samples were preincubated with an anti-hsp90 IgM (clone 8D3) able to recognize the chaperone in complexes with other factors, and then the [^{3}H]Aldo-labeled MR was analyzed in a sucrose gradient. Interestingly, the untransformed 9.4S peak switched to 11.4S after preincubation with the IgM antibody, indicating that hsp90 was still bound to the nuclear form of MR.

To analyze the composition of the putative untransformed nuclear MR, several 9.4S peaks were pooled and the MR was immunoprecipitated. hsp90, p23, FKBP52, and the dynein intermediate chain were recovered in the immune pellet in similar fashion to that for the immune pellets obtained from cy-

toplasmic 9.4S peaks (Fig. 6B), whereas FKBP51 was not present in the nuclear 9.4S fraction. This agrees with the observation that FKBP52 replaces FKBP51 upon steroid binding (Fig. 2C). The bar graph in Fig. 6B shows a densitometric analysis of each protein normalized to the amount of immunoprecipitated MR. Only FKBP51 showed a significant difference ($P < 0.001$).

Figure 6C shows the rate of appearance of the MR-hsp90 complex in the soluble fractions of nuclei isolated from cells incubated with Aldo. In agreement with the results shown in Fig. 6A, the Western blots show that hsp90 was recovered in the MR immune pellet during the first 10 min of incubation with steroid. From 12 min onwards, the signal of soluble MR faded, as well as the amount of coimmunoprecipitated hsp90. Western blots were scanned and plotted (Fig. 6D). The disappearance of soluble MR and coimmunoadsorbed hsp90 paralleled the binding of the receptor to the insoluble fraction of chromatin and nuclear matrix.

To provide more conclusive evidence that MR is able to translocate through the nuclear pore in its untransformed state, Flag-MR was overexpressed in 293-T cells, immunopurified with anti-Flag antibody, released from the immune pellet with Flag peptide, and cross-linked to the hsp90-based heterocomplex with dithiosuccinimidyl propionate (11). The cross-linked receptor was cleared of free MR, excess reagents, and other free factors by a quick centrifugation for 30 s at 12,000 rpm in a minicolumn packed with Sephacryl S200 equilibrated in Adam's buffer (1). The cross-linked MR heterocomplex then was incubated with digitonin-permeabilized E82.A3 cells in Adam's buffer supplemented with an ATP-regenerating system, with or without Aldo. Figure 6E shows that MR was cytoplasmic in the absence of steroid in E82.A3 cells (panel a) and MEF KO cells (panel c) and totally nuclear with Aldo in both cell types (panels b and d, respectively). Such nuclear translocation occurred even though MR was bound to the hsp90 heterocomplex and clearly demonstrates that the receptor can translocate through the nuclear pore in its untransformed state. Two controls were performed to test the integrity of the nuclear envelope after the treatment of MEF cells with digitonin. Figure 6E, panel e, shows that the fluorescence of Alexa Fluor 488-labeled albumin was entirely cytoplasmic, and panel f shows that the cross-linked MR complex remained cytoplasmic in the presence of Aldo in a medium lacking ATP and not supplemented with cytosolic factors (1). Figure 6F shows two controls that demonstrate the efficiency of the cross-linking reaction, namely, a sucrose gradient profile and a Western blot for cross-linked complexes. Note that in the latter case, the large MR heterocomplex could not enter the gel unless the cross-linking was previously reversed with β-mercaptoethanol.

Taken together, the experiments shown in Fig. 6 demonstrate that MR transformation may take place in the nucleus within a time frame of 10 to 15 min after steroid binding. Therefore, the dissociation of the hsp90 complex is not an early event triggered immediately after hormone binding, as has always been believed to date.

TPR protein balance affects the subcellular distribution of MR. In contrast to wild-type cells, FKBP52 KO cells showed significantly lower amounts of nuclear MR in the absence of steroid (Fig. 5B). This may indicate that FKBP52 affects the basal subcellular distribution of MR, for example, because FKBP52 favors the nuclear retention of the receptor. Moreover, Fig. 4D shows that the overexpression of TPR peptide reduced the nuclear fraction of MR, a property already observed even in the absence of ligand (not shown). According to the above hypothesis, the effect of the TPR peptide may be related to a "dominant-negative" effect on MR anchorage to nuclear structures. To analyze whether the subcellular distribution of unliganded MR is affected by the level of expression of physiological TPR factors, we studied its subcellular distribution in 293-T cells and COS-7 cells transfected or not with FKBP51 or FKBP52.

In the absence of hormone, MR showed stronger nuclear localization in COS-7 cells than in 293-T cells (Fig. 7A, vector or Vec). This distribution parallels the higher FKBP52/FKBP51 ratio shown for MR immune pellets from COS-7 cells than for those from 293-T cells. On the other hand, the overexpression of FKBP51 (+FKBP51 or 51) promoted MR exclusion from nuclei in both cell lines, whereas the overexpression of FKBP52 (+FKBP52 or 52) did not seem to affect MR subcellular distribution, perhaps due to the fact that the endogenous levels of FKBP52 are already sufficient to retain more receptors in the nuclear compartment. This speculation is in agreement with the nuclear score measured for FKBP52 KO cells (Fig. 5) versus the same cells where FKBP52 was reintroduced by transfection.

To analyze whether the effect of FKBP52 on receptor distribution is also observed with GR, we extended the analysis to the endogenous levels of expression of this receptor in several cell types (Fig. 7B). L929 fibroblasts and HC11 mammary epithelial cells showed equivalent FKBP52/FKBP51 expression ratios and, consequently, similar nucleus-to-cytoplasm distributions of unliganded GR. However, WCL2 cells, a CHO-derived cell line that overexpresses mouse GR in a constitutive manner (54), showed a greater FKBP52/FKBP51 ratio due to higher expression of endogenous FKBP52 and a lower level of expression of FKBP51. In this specific cell line, the GR was localized in the nucleus even in the absence of steroid. In summary, all of these results emphasize the fact that FKBP52 is important not only for the molecular mechanism of steroid-dependent transport of steroid receptors to the nucleus but also for their basal subcellular redistribution in the absence of ligand.

The nucleocytoplasmic balance of a given protein depends on the balance of two processes, nuclear import versus nuclear export. Figures 4D, 5B, and 7 strongly argue in favor of a role for TPR domain proteins in the distribution balance of the MR. On the other hand, the DNA-binding domain (DBD) of steroid receptors possesses a highly charged cluster of basic amino acids that apparently complements a negative cluster of amino acids in the structure of FKBP52 (46), and there is a link between the binding site of this IMM and the hinge region of GR, where its NL1 region is located (59). It has also been reported that the DNA-binding domains of multiple nuclear receptors are related to a still unknown nuclear export mechanism that seems to be independent of Crm1 (3). Therefore, we analyzed the possible involvement of these two pathways in the nuclear localization of MR.

Cells transfected with GFP-MR were preincubated with Aldo to allow GFP-MR accumulation in the nucleus. The

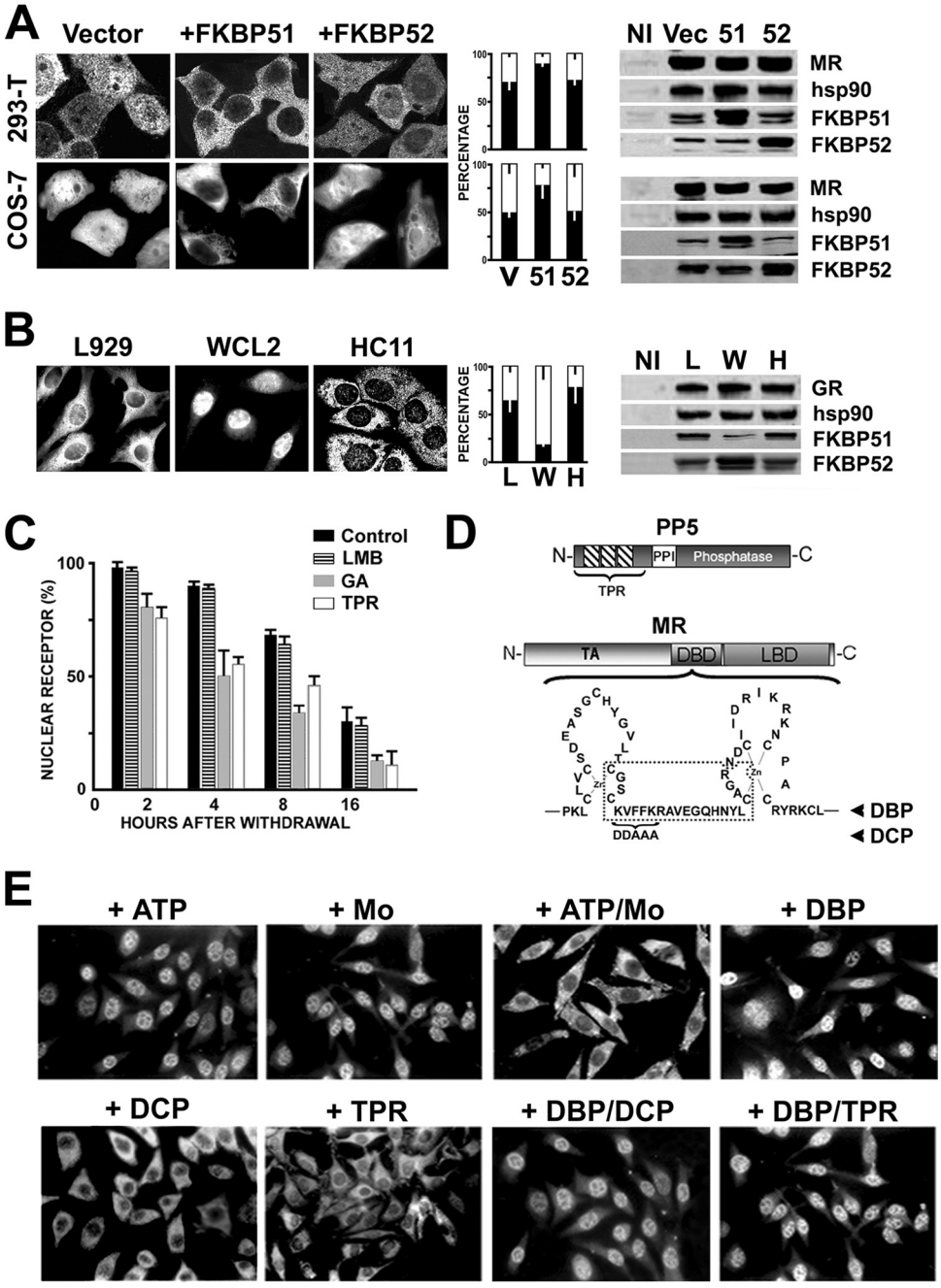

FIG. 7. FKBP52 expression favors steroid receptor nuclear retention. (A) 293-T cells and COS-7 cells were cotransfected with GFP-MR and either FKBP51 or FKBP52 and then cultured in a steroid-free medium. The primary subcellular localization of the receptor was visualized by indirect immunofluorescence without the addition of steroid. (B) Primary subcellular localization of endogenous GR, visualized in L929 (L), WCL2 (W), and HC11 (H) cells. The bar graphs shown in panels A and B represent the percentages of nuclear (white bars) and cytoplasmic (black bars) receptors for more than 100 cells counted in each experiment (mean ± SEM; $n = 3$). The Western blots on the right show the coadsorbed hsp90 and FKBPs for MR (A) and GR (B). Vec or V, cells transfected with vector only; NI, nonimmune pellet. (C) Nuclear export of GFP-MR was measured in E82.A3 cells preincubated with 10 nM Aldo, washed, and reincubated in a steroid-free medium with 0.1% DMSO (control), 10 ng/ml leptomycin B (LMB), or 2 μM geldanamycin (GA). TPR, cells cotransfected with the TPR domain of rat PP5 reincubated without additions after steroid withdrawal. The nuclear fraction of MR was measured for more than 100 cells per experiment (mean ± SEM; $n = 4$). (D) Schematic representation of recombinant TPR peptide from rat PP5, the DNA-binding peptide (DBP) (dotted box), and a control peptide (DCP) where the five amino acids next to the first loop were replaced by the DDAAA sequence. (E) GFP-MR was translocated to the nuclei of E82.A3 cells with Aldo. The coverslips were washed, permeabilized with digitonin, and incubated in Adam's buffer supplemented with 10 mM ATP, 20 mM molybdate (Mo), or 10 mM ATP plus 20 mM molybdate (all the other incubations), without further additions (ATP/Mo) or in the presence of a 1 mM concentration of each peptide or mixture of peptides (DBP, DCP, TPR, DBP and DCP, or DBP and TPR).

concentration of steroid was reduced to the minimal amount (10 nM) required to promote rapid and efficient nuclear translocation and to avoid further interference of steroid nonspecifically bound to the plastic or the glass, which could be released into the medium during subsequent incubations. After being washed with medium five times, the coverslips were changed to a new dish containing culture medium without steroid (time zero). Figure 7C shows the nuclear export rate of GFP-MR, which was similar to that reported for the GR in other studies (14, 17, 55). While the Crm1 inhibitor leptomycin B showed no effect on the nuclear export rate of MR, both the overexpression of TPR peptide (see the scheme in Fig. 7D) and the hsp90 inhibitor GA accelerated MR export, to similar extents. This result suggests that the hsp90-FKBP complex may be related to the process.

To explore the possible involvement of TPR proteins in nuclear export, E82.A3 fibroblasts transfected with GFP-MR were incubated with 10 nM Aldo for 60 min, washed, and permeabilized with digitonin. These permeabilized cells were incubated without steroid for 20 min at 30°C in the presence of 10 mM ATP or 20 mM molybdate. None of these individual treatments *per se* affected the nuclear localization of MR (Fig. 7E), but both components together facilitated MR nuclear export. These results agree with those previously reported for the GR under the same experimental conditions (64, 68). Interestingly, a DNA-binding peptide (DBP) including the central region between the two zinc fingers of the DBD (see scheme in Fig. 7D) prevented the nuclear export of MR, whereas a control peptide (DCP) where the key sequence KVFFK (3) was replaced by DDAAA showed no effect. The TPR peptide did not affect the nuclear export mechanism mediated by the DBP. These results suggest that the nuclear export mechanism of MR depends on its DBD and appears to be independent of the classical export system based on Crm1.

DISCUSSION

This work shows that the complex of hsp90 and the TPR domain immunophilin FKBP52 links the MR with the dynein/dynactin complex that powers the receptor's movement toward the nucleus. Inasmuch as this molecular bridge provides the traction chain for the receptor to be transported throughout the cytoplasm, presumably via microtubule tracks, its dissociation from the MR should not be an early event that follows ligand binding. It is clear that the chaperone-based heterocomplex is required for the normal mechanism of transport. Actually, the experimental evidence supports a model where the dissociation of the hsp90-based complex from the receptor should take place in the nucleus after 10 or 15 min of steroid binding. This conclusion is reasonable if the model for MR movement is understood from the perspective that hsp90-FKBP52 is required for cytoplasmic transport.

MR nuclear accumulation was equally impaired by disruption of the movement machinery at any level, i.e., with hsp90 inhibitors, the replacement of FKBP52 by another TPR protein, disruption of FKBP52-dependent interactions with the PPIase peptide, inhibition of the ATPase activity of dynein with EHNA, or the disassembly of the dynactin complex. In all cases, MR still moved toward the nucleus, but in a very ineffective manner, such that the half-life for the nuclear translocation rate was increased by an order of magnitude (i.e., from 4 to 5 min to 50 to 60 min). This suggests the existence of an alternative, hsp90-FKBP52-independent mechanism of movement.

The interchange of IMMs is one of the earliest events described for receptor activation upon hormone binding and was first reported for the GR (8). More recently, it was shown that this is a highly versatile process, since the nature of the factors involved in this swapping depends on the type of steroid bound to the receptor (21). Thus, aldosterone binding to the MR favors the replacement of FKBP51 by FKBP52, which is relevant from the functional point of view, since FKBP51 inhibits the mineralocorticoid response (21) and does not bind dynein (66), whereas FKBP52 is required to link the receptor to the dynein motor protein. At variance with the case for aldosterone, the binding of the synthetic agonist 11,19-oxidoprogesterone (40) to MR also favors the recruitment of the IMM-like Ser/Thr phosphatase PP5, a factor that also shows the ability to bind the motor protein (15). In order to detect the highly dynamic process of protein interchange, the temperature was lowered to 0°C to decrease the kinetics. Such a low temperature reduces the possibility that protein swapping is energy dependent. Because the MR interchanged factors even at low temperature, the most reasonable explanation is that steroid binding induces a conformational change of the MR whose consequence is the release of FKBP51 and the recruitment of FKBP52 or PP5. The differential pattern of proteolytic fragments observed by limited proteolysis of MR bound to Aldo or 11,19-oxidoprogesterone suggests that this is the case (21). It should be pointed out that in cells incubated on ice, Aldo-MR complexes keep moving toward the nucleus, although at a very low rate, so MR becomes nuclear after 2.5 to 3 h of incubation on ice. Even though movement at physiological temperatures occurs by active transport, MR movement at the low temperature should occur by passive diffusion only. This led us to speculate that when the movement machinery is disrupted, the alternative mechanism for cytoplasmic transport may be simple diffusion. This speculation is also supported by the observation (2, 17, 19) that after the disruption of all cytoskeletal filaments, a condition where active transport on cytoskeletal tracks is not possible, the GR equally concentrates in the nucleus, in a steroid-dependent manner. In this sense, Perrot-Applanat et al. (38) shifted the PR to the nucleus whether the cytoskeleton was intact or disrupted, and similar to our experiments with MR, Nishi et al. (36) found no effect on GR nuclear accumulation in cells treated with colchicine and nocodazole.

A question to be analyzed is whether or not it is possible that MR transformation occurs at the nuclear pore and that the receptor moves into the nucleus and is reassembled on the nucleoplasmic side. It is now clear that hormone-free (25) and hormone-bound (32) receptors can freely shuttle between the nuclear and cytoplasmic compartments and that the equilibrium of these movements determines whether any given receptor is predominantly in the cytoplasm or the nucleus. Yet almost nothing is known about the factors that control this equilibrium. The requirement of receptor-hsp90 complexes for efficient movement throughout the cytoplasm and the detection of these complexes in the nuclear compartment immediately after the MR becomes nuclear raise the concept that the heterocomplex passes intact through the nuclear pore. This

possibility is strongly supported by the experiments shown in Fig. 6, in particular due to the ability of cross-linked complexes to translocate to the nucleus in an aldosterone-dependent manner. Whether the receptor is primarily cytoplasmic or nuclear, how transformation takes place is uncertain to date. However, nuclear transformation also takes place in those receptors that are constitutively nuclear in the absence of hormone. It is possible that posttransductional modifications modify the structure of the receptor-hsp90 complex in the nucleus, promoting the dissociation of the chaperone, which allows the consequent recognition of the DBD for the promoter sequences.

If the heterocomplex translocates intact through the nuclear pore, then the chaperone system should interact with structures of the pore. In a very recent study (11), we found that hsp90, hsp70, p23, and FKBP52 interact with the integral nuclear pore glycoprotein Nup62. GR also binds to Nup62, and this association is more efficient when both proteins, GR and Nup62, are chaperoned by the hsp90-based heterocomplex and seems to be regulated dynamically by cytosolic factors. It is interesting that TPR proteins such as FKBP52 and PP5 are able to interact with Nup62. Moreover, importin β1 was also recovered associated with GR and hsp90. In agreement with the results shown in Fig. 6 for MR, we have also shown that cross-linked GR complexes accumulate in the nuclei of $GR^{-/-}$ cells permeabilized with digitonin (11), strengthening the interpretation that the entire steroid receptor heterocomplex can pass intact through the nuclear pore and that transformation is not the first mandatory event required for receptor nuclear translocation upon hormone binding, as always believed.

A priori, the predicted large size of the complex may make its passage through the nuclear pore relatively unlikely. However, there is a compelling number of examples in the literature supporting the active passage through the nuclear pore of macromolecular proteins (including entire viral particles), dextrans, and gold-coated structures of high molecular mass (12, 23, 31, 37, 43, 47, 57). In this sense, the nuclear pore is a highly flexible and plastic structure (28, 29). Interestingly, recent studies (4, 56) have shown that the nuclear envelope permeability and its electrical conductivity increased sharply after 10 min of stimulation with Aldo, and cell nuclei were found to be swollen by about one-third of their original volumes. All of these effects were prevented by spironolactone. It was postulated that the increased permeability was linked to the electrical conductivity of the nuclear pore channels, which are transiently more open, and that macromolecules can readily travel through the central channel pathway. If this model is correct, in further studies it would be important to determine whether the chaperone system associated with the nuclear pore undergoes any qualitative or quantitative modification that favors the import mechanism of steroid receptor heterocomplexes.

Because the subcellular localization of a given protein is not dependent on its nuclear import only, we also analyzed some aspects of the MR nuclear export mechanism. Again, TPR domain proteins such as FKBP52 seem to play a key role, although for different reasons from those discussed for the nuclear import mechanism. Figures 4D, 5B, and 7 suggest that TPR proteins, most likely FKBP52, favor receptor anchorage to the nucleus. Thus, MR nuclear localization is disfavored by competence of the hsp90 acceptor site with the TPR peptide (Fig. 1F, 2B, 3D, and 4D), higher FKBP51/FKBP52 ratios (Fig. 7) or the absence of FKBP52 (Fig. 5). Whether or not hsp90 is also involved in this process requires further studies, in particular due to the effect observed in cells treated with GA (Fig. 7C). However, our results cannot rule out the possibility that such accelerated nuclear export of MR with GA is the result of the indirect effect of the drug on nuclear hsp90-FKBP52 complexes that may anchor the receptor to nuclear structures. This speculation agrees with the equivalent nuclear export rates measured for MR in both GA-treated cells and TPR-overexpressing cells (Fig. 7C).

MR does not possess a typical nuclear export signal able to be recognized by Crm1. This is in agreement with the lack of effect of leptomycin B on nuclear export. The experiment in Fig. 7E suggests that the DBD is implicated in the nuclear export mechanism of MR. This observation parallels results already reported for the GR (3). It is possible that the DBD of MR is involved in some kind of association with an export factor that recognizes this domain, such that the saturation of this system with the DBP simply blocks nuclear export. It is interesting to speculate that the signal involved in the nuclear export mechanism is the same one that binds the receptor to the DNA. Thus, the putative association with a nuclear export factor should also release the receptor from its nuclear sites of action.

Many signaling pathways regulate the activity of transcription factors by controlling their subcellular localization. The cytoplasmic retention of transcriptionally inactive factors such as the steroid receptors by hsp90 has been a heuristic model posited for many years. The evidence provided here clearly demonstrates that the hsp90-FKBP52 complex is required for MR trafficking and that the dissociation of that complex is not an event required for nuclear translocation. On the contrary, the complex is necessary for an efficient nuclear translocation rate for the receptor. Moreover, studies performed with neurons demonstrated that when rapid movement is abrogated by GA, the GR is rapidly targeted for proteasome degradation (14). Figure 1C also shows that this may be the case for the MR, an observation that strengthens the importance of having an efficient transport system like that provided by the hsp90-FKBP52 molecular machinery bound to the receptor.

It is possible that the movement system described here may also apply to several other factors, different from steroid receptors, that are also associated with the hsp90-FKBP52 complex. Actually, the movement of soluble factors in the cell is a poorly understood field that requires more studies to unravel still unknown regulatory mechanisms that affect their subcellular distribution. In particular for the case of transcription factors, controlling their localization is a complex process that may be specific to each signaling pathway. Unmasking these processes could ultimately allow us to modulate these pathways with potential therapeutic purposes.

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