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ARTICLE *in* BIOCHEMISTRY · JANUARY 2008

Impact Factor: 3.02 · DOI: 10.1021/bi701372c · Source: PubMed

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Differential Recruitment of Tetratricorpeptide Repeat Domain Immunophilins to the Mineralocorticoid Receptor Influences both Heat-Shock Protein 90-Dependent Retrotransport and Hormone-Dependent Transcriptional Activity†

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Received July 11, 2007; Revised Manuscript Received September 19, 2007

ABSTRACT: The mineralocorticoid receptor (MR) forms oligomers with the heat-shock protein 90 (Hsp90) -based heterocomplex, which contains tetratricopeptide repeat (TPR) domain immunophilins (IMMs). Here we investigated the unknown biological role of IMMs in the MR·Hsp90 complex. Upon hormone binding, FKBP52 was greatly recruited to MR·Hsp90 complexes along with dynein motors, whereas FKBP51 was dissociated. Importantly, the Hsp90 inhibitor geldanamycin impaired the retrograde transport of MR, suggesting that the Hsp90·IMM·dynein molecular machinery is required for MR movement. To elucidate the mechanism of action of MR, the synthetic ligand 11,19-oxidoprogesterone was used as a tool. This steroid showed equivalent agonistic potency to natural agonists and was able to potentiate their mineralocorticoid action. Importantly, aldosterone binding recruited greater amounts of FKBP52 and dynein than 11,19-oxidoprogesterone binding to MR. Interestingly, 11,19-oxidoprogesterone binding also favored the selective recruitment of the IMM-like Ser/Thr phosphatase PP5. Each hormone/MR complex yielded different proteolytic peptide patterns, suggesting that MR acquires different conformations upon steroid binding. Also, hormone/MR complexes showed different nuclear translocation rates and subnuclear redistribution. All these observations may be related to the selective swapping of associated factors. We conclude that (a) the Hsp90·FKBP52·dyenin complex may be responsible for the retrotransport of MR; (b) a differential recruitment of TPR proteins such as FKBP51, FKBP52, and PP5 takes place during the early steps of hormone-dependent activation of the receptor; (c) importantly, this swapping of TPR proteins depends on the nature of the ligand; and (d) inasmuch as FKBP51 also showed an inhibitory effect on MR-dependent transcription, it should be dissociated from the MR·Hsp90 complex to positively regulate the mineralocorticoid effect.

One of the most abundant classes of transcriptional regulators in metazoans is the nuclear receptor superfamily, which includes receptors for a great variety of ligands such as retinoic acids, thyroid hormones, dioxin, sterols, fatty acids, leukotrienes, and prostaglandins. Among these transcription factors, there is a cluster of phylogenetically related proteins that function as transducers of steroid ligands, the steroid receptor subfamily (*1*).

Evolutionary studies suggest that the mineralocorticoid receptor (MR)[1] seems to be the youngest nuclear receptor among the members of the subfamily (*2*). In mammals, this receptor is expressed at the greatest quantity in sodium-transporting epithelia such as the distal part of the nephron and colon, sweat and salivary glands, and the cardiovascular system. It is also abundant in the central nervous system (particularly in hippocampus) and brown adipose tissue, whereas it shows very low expression in other tissues. Nevertheless, MR does not show the abundance of the other members of the steroid receptor subfamily, which has made characterization of its molecular properties very difficult to achieve.

It is now well established (*3*, *4*) that steroid receptors are capable of forming heterocomplexes with the 90-kDa and 70-kDa heat shock proteins (Hsp90 and Hsp70), the acidic protein p23, and proteins that possess sequences of 34 amino acids repeated in tandems, the TPR proteins. Some of these Hsp90-binding TPR proteins have peptidylprolyl isomerase activity and are intracellular receptors for immunosuppressant

† This work was supported by grants from ANPCyT (01-14123 and 26495), CONICET (PIP-6167), and the NIH Fogarty International Center (Grant R03-TW007162). All authors are members of the Argentine Research Council.

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[1] Abbreviations: MR, mineralocorticoid receptor; GR, glucocorticoid receptor; Hsp90, 90-kDa heat-shock protein; IMM, high molecular weight immunophilin; TPR, tetratricopeptide repeats; GA, geldanamycin; 11-OP, 11,19-oxidoprogesterone; DOC, 11-deoxycorticosterone; EC_{50}, effective concentration of ligand required to produce 50% of the maximum effect.

10.1021/bi701372c CCC: $37.00
Published on Web 11/15/2007

drugs such as FK506, rapamycin, and cyclosporine A. Therefore, they are clustered into the relatively conserved and large family of proteins known as immunophilins (IMMs) (*5*). Among the members of this family, only five IMMs have been recovered to date in steroid receptor·Hsp90 complexes: FKBP52, FKBP51, CyP40, and two IMM-like proteins, protein phosphatase 5 (PP5) and XAP2/ARA9. Even though the biological function of these proteins in the receptor·Hsp90 heterocomplex remains poorly understood, it is accepted that those IMMs are not related to the immunosuppressant effect.

In the absence of steroid, MR oligomers reside predominantly in the cell cytoplasm (*6*−*10*). Like other transcription factors, MR does not remain confined to any particular cell compartment but continuously shuttles between cytoplasm and nucleus. The mechanism by which MR movement takes place is unknown. It has always been assumed that simple diffusion is the driving force for moving soluble proteins, which become "trapped" in their sites of action by protein−protein or nucleic acid−protein interactions. Alternatively, soluble proteins may utilize molecular machinery, in which case, movement would be likely to involve cytoskeletal tracks, similar to vesicle transport. It has been recently shown that the intermediate chain of the motor protein dynein (Dyn IC) coimmunoprecipitates with the Hsp90·FKBP52 complex of the glucocorticoid receptor (GR) (*11*). When the complex is disrupted by the Hsp90 inhibitor geldanamycin, the nuclear translocation rate of GR is dramatically delayed and the receptor is targeted to proteasomal degradation (*12*).

On the other hand, it has been demonstrated that the proapoptotic factor p53 requires intact microtubules for moving toward the nucleus, dynein being the motor protein that powers its retrotransport (*13*). Interestingly, the cytoplasmic forms of p53 are also bound to IMMs via Hsp90, suggesting that the Hsp90·IMM complex is related to the molecular machinery of movement (*14*). Preliminary studies have shown that dynein motor proteins are also recovered with MR, so we hypothesized that, like GR and p53, MR retrotransport may be dependent on the same heterocomplex. Inasmuch as our knowledge about the biological role of IMMs is poorly understood, and because the molecular mechanism of action of MR has remained elusive for decades, this study was focused on (a) whether or not the cytoplasmic retrotransport of this receptor is Hsp90·IMM- and dynein-dependent, (b) if the nature of the agonist influences the association of TPR proteins with MR, and (c) whether IMMs influence the MR-dependent biological response.

EXPERIMENTAL PROCEDURES

Materials. Proteases, aldosterone, 11-deoxycorticosterone (DOC), and SC9420 [7α-(acetylthio)-17α-hydroxy-3-oxo-pregn-4-ene-21-carboxylic acid γ-lactone] were purchased from Sigma Chemical Co. (St. Louis, MO). [1,2-3H]-Aldosterone, [^{35}S]cysteine, and [^{35}S]methionine were from NEN Life Science Products (Boston, MA). 11,19-Oxidoprogesterone (11-OP) and its bent isomer 6,19-oxidoprogesterone (6-OP) were synthesized as described (*15*). pCI-Neo-hFKBP51 and pCI-Neo-hFKBP52 plasmids encoding for FKBP51 and FKBP52 were kindly provided by Dr. David F. Smith (Mayo Clinic, Scottsdale, AZ) and Dr. Jack-Michel Renoir (Faculté de Pharmacie, Châtenay-Malabry Cedex, France) respectively. FLAG-tagged pcDNA3-rat MR was a kind gift from Dr. Shigeaki Kato (University of Tokyo). The rabbit pAbhMR polyclonal antibody against MR was a kind gift from Dr. Gerald Litwack (Jefferson Cancer Institute, Philadelphia, PA).

Cell Culture and Transfections. Three different cell lines were used in this work: 293-T human fibroblasts, NIH-3T3 mouse fibroblasts, and the L929-derived cell line E82.A3, all of them being grown in Dulbecco's modified Eagle's medium supplemented with 10% bovine calf serum. 293-T cells were used for transactivation assays by cotransfection of 100 ng of FLAG-MR, 2 μg of mouse mammary tumor virus−luciferase (MMTV-Luc), and 100 ng of Rous sarcoma virus (RSV)−β-galactosidase according to the calcium phosphate precipitation standard method. After 36 h in a medium containing charcoal-stripped serum, cells were stimulated with steroid for 12 h. Both luciferase and β-galactosidase activities were measured, and the luciferase activity was normalized to the β-galactosidase expression. E82.A3 fibroblasts were used for coimmunoprecipitation assays. Cells were transfected for 2 h with FLAG-tagged MR by use of Trans-Fast reagent (Promega, Madison, WI) in Opti-MEM medium (Gibco). The transfection medium was replaced by Opti-MEM medium supplemented with 3% (v/v) charcoal-stripped serum, and the cells were grown for an additional 48 h. Cells were placed on ice for 15 min, and 1 μM steroid was added to the incubation medium to allow steroid binding to MR without nuclear translocation (*16*−*18*). After 1.5 h, the cells were homogenized in HEM buffer [10 mM *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid (Hepes) at pH 7.4, 1 mM ethylenediaminetetraacetic acid (EDTA), and 20 mM Na_2MoO_4) and centrifuged at 120000*g* for 30 min at 0 °C. MR complexes were immunoprecipitated from 300 μL of cytosol with 2 μL M2 anti-FLAG antibody from IBI (New Haven, CT) prebound to protein A−Sepharose. After the pellets were washed five times with 1 mL of TEGM buffer [10 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (Tes), pH 7.6, 50 mM NaCl, 4 mM EDTA, 10% (v/v) glycerol, and 20 mM Na_2MoO_4], proteins were resolved by Western blotting (*19*−*21*).

Indirect Immunofluorescence. Cells grown on coverslips were fixed and permeabilized in methanol for 15 min at −20 °C. Coverslips were inverted onto 25 μL of blocking buffer [20 mM Tris at pH 8.8, 0.63 M NaCl, 0.05% Tween-20, 0.02% NaN_3, and 1% bovine serum albumin (BSA)] containing 0.25 μL of anti-FLAG M2 antibody, incubated for 2 h at room temperature, washed with blocking buffer, and inverted onto 25 μL of blocking buffer containing 0.25 μL of rhodamine-conjugated counterantibody. 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 Pascal confocal microscope. To assay the effect of the Hsp90-disrupting agent geldanamycin (GA) on FLAG-MR movement, we followed a previously described protocol for GR (*22*). Transfected E82.A3 cells were placed on ice for 15 min and then treated for 2 h with 1 μM steroid to allow steroid binding to MR but not nuclear translocation. Then, 2.5 μM geldanamycin or vehicle (0.05% dimethyl sulfoxide, DMSO) was added to the medium and the incubation was continued on ice for

an additional 15 min. Cells were shifted to 37 °C to allow steroid-bound MR movement to the nucleus. Coverslips were rinsed with phosphate-buffer saline and fixed in cold (−20 °C) methanol for 10 min. MR was visualized by indirect immunofluorescence with the M2 anti-FLAG antibody, and the nuclear fraction of MR was scored by quantifying cytoplasmic and nuclear fluorescence of 100 cells with the Zeiss LSM 5 Image Examiner software. The term nuclear fraction of MR represents the nuclear/total pixels ratio.

Limited Proteolysis of MR. MR was translated in vitro in a reticulocyte lysate system using the TNT-coupled transcription/translation kit from Promega (Madison, WI) in the presence of [^{35}S]Met and [^{35}S]Cys. The ^{35}S-radiolabeled MR was immunoprecipitated with the rabbit pAbhMR antibody, and the pellet was washed and preincubated for 15 min at 20 °C with 1 μM steroid. Pellets were then cooled on ice for 30 min and treated with chymotrypsin from bovine pancreas (10 or 20 units/mL) for 5 min at 0 °C or with trypsin from porcine pancreas (20−120 μg/mL) for 10 min at 0 °C. The reaction was stopped by boiling the reaction mixture in SDS sample buffer, and proteins were resolved by SDS−PAGE. Gels were fixed for 30 min in methanol/acetic acid/water (30:10:60), treated with Intensify Enhancer (Kodak), and dried, and proteins were visualized by autoradiography.

Mineralocorticoid Properties. Mineralocorticoid in vivo effect was measured as the Na^+/K^+ urinary ratio per milligram of excreted creatinine in 48 h adrenalectomized Sprague-Dawley male rats as described (*23*). Steroid-binding assays were measured in rat kidney cytosol by competition of [^{3}H]aldosterone with increasing concentrations of unlabeled steroids (*24*). Nonspecific binding (~25% of total binding) was measured with 1000-fold excess unlabeled aldosterone and subtracted from the total binding. The dissociation rate constant of [^{3}H]aldosterone-labeled MR complexes was measured by isotopic dilution with 100-fold excess of unlabeled aldosterone added to the incubation medium in the equilibrium when the free tracer was precleared by charcoal/dextran adsorption. In all binding assays performed in this work, buffers contained 0.1 μM RU28362 to prevent possible cross-reaction with GR. Data were analyzed by one-way nonparametric analysis of variance followed by the Kruskal−Wallis test.

RESULTS

Biological Properties of MR Ligands. It is known that ligand binding to steroid receptors promotes a conformational change in the receptor that may vary according to the nature of the hormone. Consequently, the biological properties of each steroid/receptor complex may be unique (for a recent review, see ref *25* and references therein). While glucocorticoids show a highly bent conformation (typically from −27° for cortisol to −36° for dexamethasone), aldosterone possesses a minimally angled steroid nucleus at the A/B ring junction (−8.5°) that is rigidly preserved due to the presence of a hemiketalic additional ring. Recently we have characterized a novel synthetic MR agonist, 11,19-oxidoprogesterone (11-OP) (*26*), that also shows a minimally bent conformation (+4.4°) and, like aldosterone, it also shows an extra ring due to the presence of a $C_{11}-C_{19}$ ether bridge that confers on the steroid a relatively rigid structure. Thus, 11-OP shows biochemical and biological properties similar to those shown by endogenous mineralocorticoids, but not glucocorticoid actions or GR binding capacity ($K_d \gg 3$ μM). Nevertheless, preliminary results have shown some atypical biological properties of 11-OP that make this ligand a useful tool to elucidate some aspects of the molecular mechanism of action of MR, in particular, the role of high molecular weight IMMs in the MR molecular mechanism of action.

Figure 1A depicts a dose−response curve for electrolyte elimination, showing that 11-OP is as potent as DOC for the entire range of the dose−response curve and is as potent as aldosterone at doses equal to and higher than 1 μg/100 g body weight. In contrast, the highly bent (−57.8°) conformer 6,19-oxidoprogesterone (6-OP) is devoid of mineralocorticoid effect. This is in agreement with the relative binding affinities for MR shown by both synthetic ligands (20−25 nM for 11-OP and 2−3 μM for 6-OP) (*26*). Interestingly, a potentiation of the in vivo mineralocorticoid effect was measured when a suboptimal dose of 60 ng of aldosterone (~50% of maximum effect) was co-injected with 0.6 ng 11-OP, a dose that is ineffective per se (Figure 1B). Such potentiation on aldosterone action was specifically dependent on 11-OP since it was not observed when 0.6 or 10 ng of DOC was co-injected with aldosterone. 11-OP was also able to fully potentiate the effect of 100 ng of DOC (~50% of maximum effect) in similar fashion as described for aldosterone, whereas 6-OP showed no effect when it was co-injected with any steroid (data not shown). Importantly for the ends of this study, the biological response was totally prevented by the MR antagonist SC9420, which indicates that the effect in vivo is entirely MR-dependent.

The potentiation effect observed with 11-OP led us to ask whether this effect could also be reproduced in vitro. Figure 1C depicts the concentration−response curves for aldosterone and 11-OP (from 10^{-12} to 10^{-7} M) in cells cotransfected with the MMTV-Luc gene reporter. Mimicking the in vivo dose−response curve, 11-OP showed lower potency than aldosterone to induce luciferase activity, although both steroids reached the same maximum effect (~20−25-fold induction). Importantly, 0.05 nM 11-OP, a very low active concentration of steroid (2-fold induction only), was able to potentiate MR-dependent transcriptional activity of 5 pM aldosterone from 4-fold to 12-fold induction, and at higher concentrations of aldosterone, an approximately 80-fold induction was observed (■). Control experiments (not shown) demonstrated that cells transfected with the reporter gene alone did not respond to cortisol, progesterone, 17β-estradiol, or R1881. Moreover, the biological response in MR-transfected cells was totally abolished by SC9420 but not by RU486. This clearly demonstrates that the induction of luciferase activity was entirely dependent on the expression of exogenous MR.

Inasmuch as the potentiation effect of 11-OP on aldosterone/MR-dependent biological response also occurred in cultured cells, it was inferred that this property should involve a basic molecular mechanism rather than a more complex in vivo regulation (e.g., longer plasma half-life, different distribution volume, more active metabolites, enzymatic activity in other organs, steroid bioavailability, influence of plasma carriers, etc.). One possible mechanism for the potentiation effect may take place at transcriptional level, for example, due to a more efficient interaction of MR with

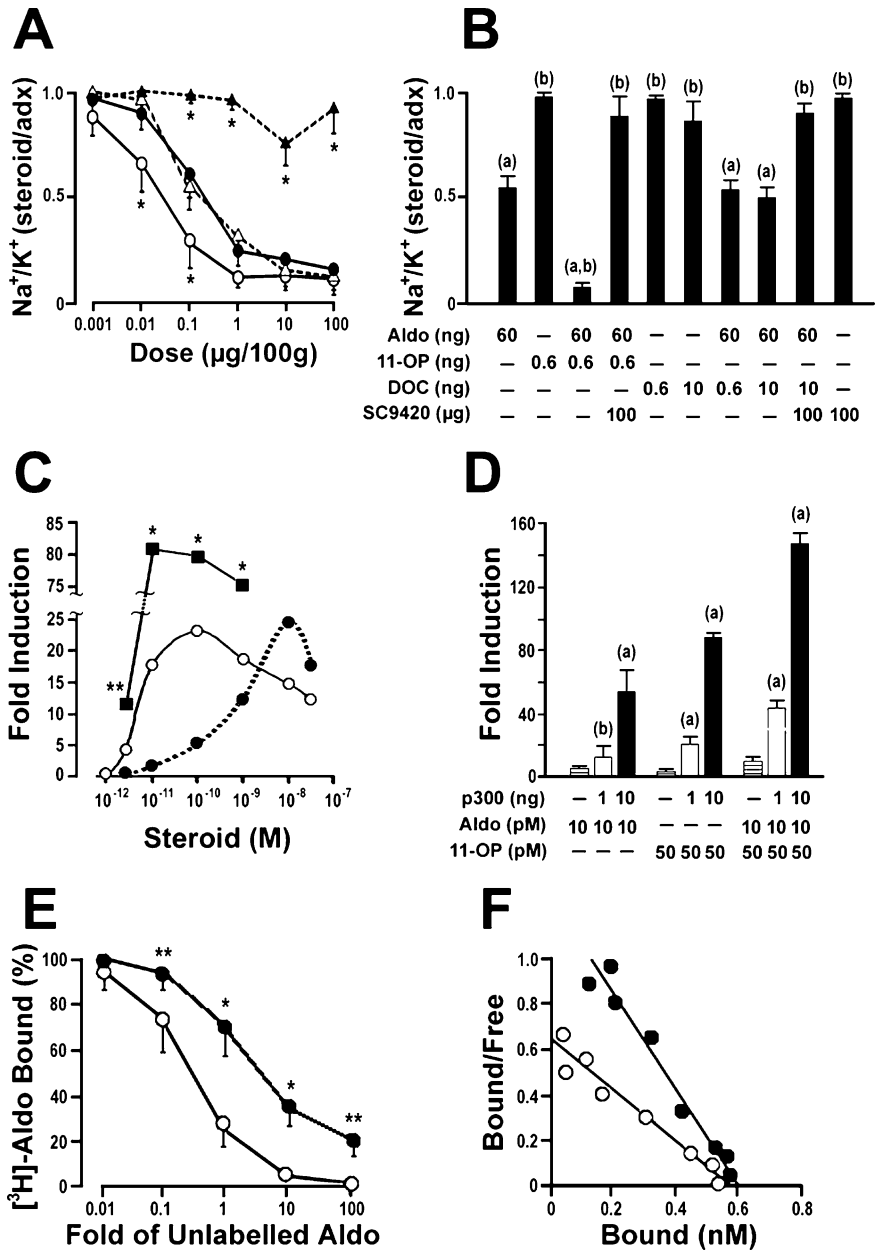

FIGURE 1: Biological and biochemical properties of the ligands. (A) Dose−response curves. The anti-natriuretic/kaliuretic ratio (mean ± SEM) was plotted against the dose of steroid injected in adrenalectomized (adx) male rats (8 rats/dose). (○) Aldosterone; (●) DOC; (△) 11-OP; (▲) 6-OP. *Different from 11-OP at $p \leq 0.002$. (B) Potentiation effect in vivo. The mineralocorticoid response was measured after co-injection of the indicated doses of steroids. Note that the 11-OP dose used in this assay is ineffective per se. Results are the mean ± SEM of 12 rats per condition. Letters indicate difference ($p \leq 0.002$) from (a) the adx rat group or (b) rats injected with 60 ng of aldosterone. (C) Potentiation of transcriptional activity. Luciferase activity was measured in 293-T cells as a function of the steroid concentration and normalized to β-galactosidase activity. (○) Aldosterone; (●) 11-OP; (■) aldosterone concentration shown on the *x*-axis in the presence of constant 0.05 nM 11-OP. Results are the mean ± SEM of three independent experiments performed in triplicate. Asterisks indicate difference from aldosterone at *$p \leq 0.001$ and **$p \leq 0.01$. (D) Coactivator-enhanced potentiation. 293-T cells were cotransfected with pCMV-p300 and the potentiation effect on the induction of the luciferase reporter gene activity was measured. Hatched bars are controls where the coactivator was not transfected; open and solid bars show induction of luciferase activity in cells transfected with 1 ng and 10 ng of pCMV-p300, respectively. Results are the mean ± SEM of three experiments each performed in triplicate. Letters indicate difference from basal levels at (a) $p \leq 0.001$ or (b) $p \leq 0.01$. (E) 11-OP binding increases the affinity of MR for aldosterone. Kidney cytosol was prelabeled with 4 nM [^{3}H]aldosterone for 5 h at 0 °C, and then the tracer was competed for 12 h at 0 °C with increasing concentrations of unlabeled aldosterone, in the presence (●) or absence (○) of a fixed concentration (30 nM) of 11-OP. Specific binding was normalized as a percentage of the maximum. Results are the mean ± SEM of three experiments, each one performed in quadruplicate. Asterisks indicate difference from aldosterone alone at *$p \leq 0.001$ or **$p \leq 0.01$ (F) Scatchard plot. K_d for aldosterone was measured ($n = 4$) in the absence (○) or presence (●) of 1 nM 11-OP.

coactivators. In this sense, it is known that p300 functions as a coactivator for many members of the steroid receptor subfamily, and because it has always been assumed that MR behaves like GR, the notion that MR must be activated by p300 has been posited in the literature. However, only two studies have actually addressed this issue. While a recent report by Hultman et al. (*27*) suggests that p300 does not interact with MR, Fuse et al. (*28*) have shown a weak enhancement (2−3-fold induction) of steroid-dependent transactivation of full-length rat MR. Therefore, we tested

Table 1: Dissociation Rate of Ligand/MR Complexes[a]

steroid	$k_{-1} \times 10^{-3}$ (min^{-1})	$k_{+1} \times 10^{-3}$ (min^{-1})	K_d (nM)	n_H (r)
aldosterone	1.87 ± 0.12	2.18 ± 0.14	0.85 ± 0.11	0.91 (0.88)
aldosterone + 11-OP	0.58 ± 0.12	1.91 ± 0.22	0.31 ± 0.13	0.88 (0.94)

[a] [^{3}H]Aldosterone/MR complexes in the equilibrium plateau (6 h on ice) were cleared of free [^{3}H]aldosterone with charcoal−dextran and reincubated at 4 °C with 100-fold excess unlabeled aldosterone with respect to the tracer, in the presence (B_s) or absence (B_0) of 2 nM 11-OP. The dissociated radioactivity was adsorbed with charcoal−dextran every 5 min for 30 min, and the specific binding was measured in the supernatant. Ln (B_s/B_0) was plotted versus incubation time, and the dissociation constant rate, k_{-1}, was measured from the slope; k_{+1} was calculated from this value and the relative K_d measured by Scatchard analysis. Results are the mean ± SD of four experiments performed in triplicate. n_H, Hill coefficient; r, linear regression coefficient for the Hill plot.

our hypothesis by cotransfection of very low amounts of p300 (1 and 10 ng) to analyze whether the maximum potentiation effect reached with both steroids was able to surpass the addition of the transcriptional activities induced by each individual steroid when the cells offer more coactivator to MR. Figure 1D shows that the transfection of pCMV-p300 greatly increased the maximum potentiation effect in a p300-dependent manner (up to ~140-fold induction when 10 ng of DNA was transfected), suggesting that one possible mechanism by which the amplification effect may occur may be more efficient recruitment of coactivators to the promoter. This hypothesis is compatible with the significant increase of the biological response, which greatly surpasses the maximum measured in the concentration−response curve described in Figure 1C (from 25- to 80-fold).

Regardless of whether or not p300 is more efficiently recruited to the complex and is responsible for the potentiation effect, it is clear that the simultaneous binding of both steroids is related to the stabilization of a more active conformation of the receptor, so MR becomes more efficient to interact with other proteins in a ligand-dependent fashion. If this interpretation were correct, the particular conformation of MR-induced 11-OP binding may be of great utility for revealing many aspects of the molecular mechanism of action of MR.

Inasmuch as 11-OP shows no binding properties to steroid receptors other than MR, and because the cells used in these assays did not respond unless MR was transfected, we wondered if 11-OP exerts any effect of addition or potentiation on aldosterone binding to MR. Figure 1E shows a competition curve of increasing concentrations of unlabeled aldosterone incubated for 12 h with pre-existing [^{3}H]-aldosterone/MR complexes formed by incubation of kidney cytosol with the tracer for 5 h at 0 °C. It should be pointed out that, under these experimental conditions, the plateau is reached in ~1 h.

As expected, [^{3}H]aldosterone was displaced with an $EC_{50} = 3.2 \pm 0.9$ nM (○). If 11-OP binds to the same binding site as aldosterone, this ligand would be expected to add its competition effect to that of unlabeled aldosterone, so a shift of the competition curve to the left may be predicted. On the other hand, if 11-OP were ineffective in modifying the competitive profile of aldosterone, the competition curve should be the same. Surprisingly, the simultaneous presence of a constant concentration of 11-OP shifted the curve to the right (●), increasing the EC_{50} to 16.5 ± 1.8 nM. In other words, 5-fold more unlabeled aldosterone is required to displace the tracer from the binding site. Taken together, this experiment and those shown in the other panels suggest that the 11-OP-dependent amplification of the mineralocorticoid response may be due to the stabilization of an active, aldosterone-like conformation of MR. That this is the case is shown by the Scatchard plot depicted in Figure 1F. [^{3}H]-Aldosterone binding to MR was measured in the presence or absence of 1 nM 11-OP, a concentration that is unable to compete with the natural ligand. 11-OP significantly increases MR affinity for aldosterone by approximately 3 times (K_d decreases from 0.92 ± 0.11 to 0.30 ± 0.06 nM, $p \leq 0.005$). Scatchard plots performed with higher concentrations of 11-OP that are able to displace aldosterone from MR yielded a typical competitive inhibitory pattern on aldosterone binding to MR (data not shown), so the apparent K_d of MR for aldosterone increases 2-fold (2.15 ± 0.35 nM) and more than 3-fold (3.91 ± 0.50 nM) in the presence of 30 and 100 nM 11-OP, respectively. The number of total binding sites remained constant in all cases.

One possible explanation for this effect is that 11-OP may bind with high affinity to an alternative binding site in MR, this site being a potential regulator or stabilizer of the active (aldosterone-bound-like) form of MR. Aldosterone should not be recognized by this site because the Scatchard plots show only one slope (as shown in Figure 1F). On the other hand, because competition curves with 11-OP demonstrates that this pregnane steroid (at higher concentrations) displaces aldosterone from MR, it may be postulated that 11-OP plays a dual role: at low concentrations, unable per se to efficiently compete with aldosterone, 11-OP occupies this alternative binding site, whereas at higher concentrations, 11-OP also binds to the aldosterone-binding pocket and consequently competes with the tracer.

Next we investigated whether the reason 11-OP increases the K_d for aldosterone is because aldosterone binding to MR is faster or because aldosterone dissociates at a slower rate. Table 1 shows that the association rate constant (k_{+1}) for [^{3}H]aldosterone binding was not affected, whereas the simultaneous presence of 11-OP clearly decreased the dissociation rate constant (k_{-1}) of aldosterone by 3-fold. Because the Hill coefficients (n_H) measured for all conditions are approximately equal to unity, this imply that the occurrence of allosteric effects is unlikely.

In summary, the results summarized in Figure 1 suggest that the binding of 11-OP to MR enhances the affinity of the receptor for aldosterone. This effect may involve the stabilization of a more active conformation of MR, which retains aldosterone in its binding pocket for longer periods of time. If true, this implies that 11-OP should induce a differential conformational change of MR favoring its more active conformation in a nonsequential manner (because of the n_H measured in Table 1), which in turn may affect certain protein−protein interactions that were less efficient or directly absent before stabilizing such conformation.

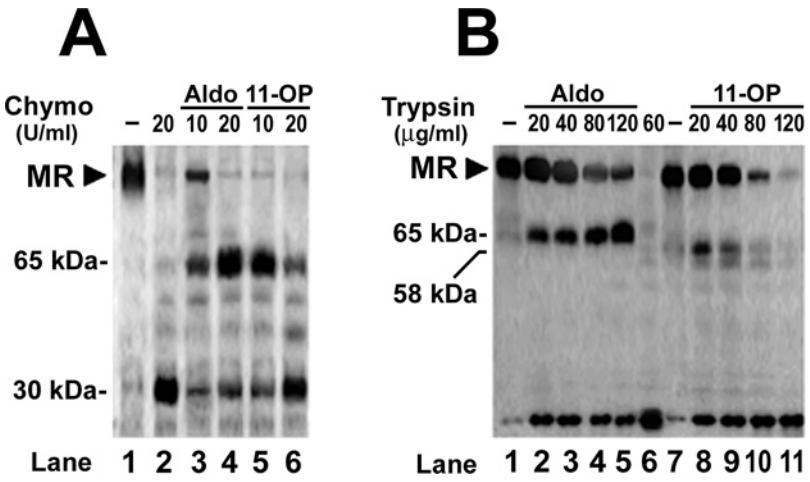

FIGURE 2: Controlled proteolysis of steroid/MR complexes. MR was translated in a reticulocyte lysate system ($n = 3$), and the ^{35}S-labeled MR was immunoprecipitated, washed, and incubated with 1 μM steroid or vehicle. Ligand/[^{35}S]MR complexes were incubated on ice with (A) chymotrypsin (10 or 20 units/mL) for 5 min at 0 °C in duplicate or with (B) trypsin (20−120 μg/mL) for 10 min at 0 °C in duplicate. Peptides were resolved by SDS−PAGE and visualized by autoradiography. The figure shows representative protein profiles for each treatment. (A) Lane 1, unliganded MR; lane 2, unliganded MR treated with 20 units/mL chymotrypsin; lanes 3−6, MR preincubated with aldosterone (lanes 3 and 4) or 11-OP (lanes 5 and 6) followed by digestion with 10 units/mL (lanes 3 and 5) or 20 units/mL chymotrypsin (lanes 4 and 6). (B) Unliganded MR (lanes 1 and 7), aldosterone/MR complexes (lanes 2−5), or 11-OP/MR complexes (lanes 8−11) were treated with 20 μg/mL (lanes 2 and 8), 40 μg/mL (lanes 3 and 9), 80 μg/mL (lanes 4 and 10), or 120 μg/mL trypsin (lanes 5 and 11). Lane 6, unliganded MR incubated with 60 μg/mL trypsin.

Ligand-Induced MR Conformation. It would be possible that MR undergoes differential conformational changes upon binding of each steroid or both steroids together and, consequently, the recruitment of factors required for the molecular mechanism of action of MR may be accordingly affected.

We tried to demonstrate putative conformational changes of MR upon steroid binding by analyzing its peptide fingerprint upon controlled proteolysis. MR was translated in a reticulocyte lysate system in the presence of radioactive Cys and Met. The resultant [^{35}S]MR was immunoprecipitated, washed, and treated with α-chymotrypsin (Figure 2A) or trypsin (Figure 2B). Figure 2A shows that unliganded MR is greatly digested by chymotrypsin compared to the 110-kDa band observed in controls incubated without protease (lane 2 vs lane 1). Two smaller fragments (∼65 and ∼30 kDa) were generated by limited chymotrypsinization of both steroid/MR complexes. Interestingly, aldosterone/MR and 11-OP/MR complexes show different proteolytic profiles. Some full-length MR that has resisted the controlled degradation can still be seen for aldosterone/MR complexes (lane 3) but not for 11-OP/MR complexes (lane 5). Inverted proteolytic peptide ratios can also be seen (compare lanes 4 vs 6 and lanes 3 vs 5). The 65-kDa fragment is more resistant for aldosterone/MR complexes, and the 30-kDa fragment appears to be derived from the former. This 30-kDa peptide was fully degraded under all conditions when longer incubation times (≥10 min) were tested (data not shown).

Tryptic digestion of MR for 10 min at 0 °C also showed different stability for each steroid/MR complex (Figure 2B). Both full-length MR and its partially digested intermediates were more resistant to degradation when aldosterone was bound to the receptor. Trypsinization of aldosterone/MR complexes also yielded the 65-kDa peptide observed by chymotrypsinization, whereas 11-OP/MR-derived fragments yielded a distinctive 58-kDa peptide. When MR was preincubated with the naturally occurring agonist DOC, the peptide pattern of MR obtained by treatment with both proteases was identical to that observed with aldosterone, whereas neither progesterone nor 6-OP was able to protect MR against degradation (data not shown).

Unfortunately, when proteolysis was performed with MR bound to both steroids, the proteolytic pattern was not different from that observed with aldosterone alone for several aldosterone/11-OP concentration ratios (i.e., 10 nM/1 nM, 10 nM/10 nM, 10 nM/50 nM, 10 nM/100 nM, and 1 μM/1 μM) (data not shown). These results imply that 11-OP may stabilize an aldosterone-like active conformation of MR or that the conformational change generated by binding of both ligands is slender and the method is consequently not sensitive enough to detect such slight differences. We favor the latter speculation because each individual steroid actually generates different conformational changes, due to the potentiation effect observed in vivo and in vitro, and because of some of the results shown later in this work.

Ligand-Dependent Recruitment of IMMs to the MR·Hsp90 Heterocomplex. If 11-OP binding to MR generates a different receptor conformation than aldosterone binding, the nature and/or amount of soluble factors recruited by the steroid/MR complex may be accordingly influenced. To determine whether the ligand may be selective in this regard, E82.A3 cells overexpressing FLAG-MR were incubated on ice with aldosterone, 11-OP, or vehicle. Cells were homogenized and MR was immunoprecipitated from cytosol. Figure 3A shows a representative experiment where the coimmunoprecipitated Hsp90-binding IMMs and dynein were detected by Western blotting.

Hsp90, the high molecular weight IMMs FKBP51 and FKBP52, and the IMM-like protein phosphatase PP5 coimmunoprecipitated with unliganded MR. Dynein intermediate chain (Dyn IC) was also specifically recovered in the MR immune pellet, most likely bound to the Hsp90·IMM complex (*29*). Upon steroid binding, more FKBP52 and Dyn

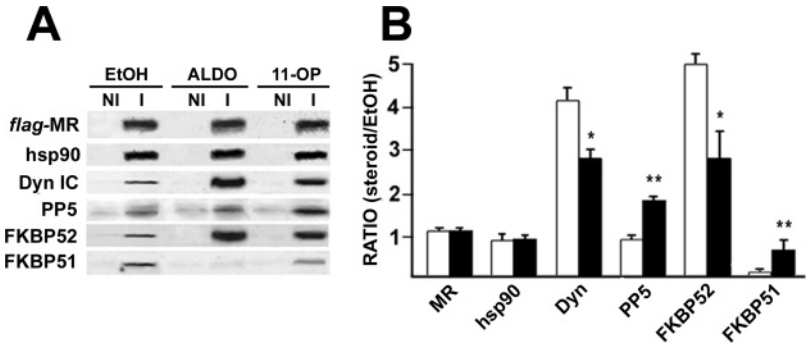

FIGURE 3: Differential recruitment of immunophilins. E82.A3 cells transfected with FLAG-MR were incubated on ice with 1 μM steroid. MR was immunoadsorbed and proteins were resolved by Western blotting. (A) Representative experiment. Bands were scanned and quantified; the bar graph shown in panel B depicts the steroid-treated/vehicle-treated ratio for each protein from three independent experiments. Open bars, aldosterone/MR complexes; solid bars, 11-OP/MR complexes. Asterisks indicate difference from Aldo/MR complexes at $*p \leq 0.001$ and $**p \leq 0.01$. NI, nonimmune antibody; I, anti-FLAG antibody.

IC were recovered with MR, whereas the amount of Hsp90 remained constant. This indicates that the FKBP52·motor protein complex is recruited to the MR·Hsp90 complex upon ligand-dependent activation of the receptor. On the other hand, the signal of FKBP51 decreased, perhaps due to the competition of FKBP52 that is recruited to the MR·Hsp90 complex and shares the same TPR acceptor site of Hsp90 (*30*).

Even though 11-OP binding also caused recruitment of FKBP52 and Dyn IC to MR, it occurs to a much lower extent compared to MR/aldosterone complexes. This is more clearly seen in the bar graph depicted in Figure 3B, where the steroid/vehicle signal ratios are plotted for each protein after the bands of three independent experiments are scanned. Interestingly, PP5 is recruited to the complex only by 11-OP binding, whereas some residual amount of FKBP51 still persists with the 11-OP/MR complex. However, when the cells were exposed to both steroids, the profile of coimmunoprecipitated proteins was the one shown with aldosterone alone (data not shown). Consequently, even though 11-OP was present, no larger amounts of PP5 nor residual amounts of FKBP51 were recovered with the MR·Hsp90 complex.

The differential recruitment of soluble factors such as IMMs and Dyn IC by MR upon binding of one steroid or the other is in agreement with the interpretation derived from Figure 2; that is, a different conformational change is induced in MR upon binding of aldosterone or 11-OP, which in turn influences the nature and/or quantity of factors associated with the complex. Importantly, this experiment demonstrates for the first time that the recruitment of TPR proteins by a given receptor·Hsp90 complex is dependent on the nature of the ligand. This observation may have strong consequences in a physiologic milieu where MR is exposed to several simultaneous stimuli in different cell or tissue contexts.

Nuclear Translocation Rate of MR. FKBP52 has been implicated in the retrotransport mechanism of p53 by connecting the nuclear factor-associated chaperone Hsp90 with dynein motors (*14*). Therefore, if the recruitment of IMMs and dynein to MR is selectively affected by the ligand, it may be possible that this affects the retrotransport rate of MR. First, we studied the nuclear accumulation rate of MR in E82.A3 cells upon ligand binding. This L929 fibroblast-derived cell line is a subclone where GR was knocked out (*31*) and shows the property of not expressing other steroid receptors. Therefore, E82.A3 cells are optimal to study the properties of transfected MR without the interference of other endogenous steroid receptors.

Figure 4A shows that MR is predominantly cytoplasmic under hormone-free conditions, whereas it becomes entirely nuclear after 20 min of incubation with steroid. To study whether the Hsp90 complex is involved in MR movement, cells were first incubated with steroid on ice to allow hormone binding. Then the Hsp90 inhibitor GA was added to the medium and the incubation continued on ice for an additional 15 min. MR movement to the nucleus was triggered by shifting the temperature to 37 °C. While GA alone did not affect the subcellular localization of MR, the nuclear relocalization of MR was inhibited. This implies that MR retrotransport is functionally linked to the Hsp90 heterocomplex.

Figure 4B demonstrates that the nuclear translocation rates for each steroid/MR complex are not the same. Clearly, 11-OP (●) moves at a slower rate than aldosterone (▲) or DOC (■), such that the average translocation half-time for 11-OP/MR complexes is twice as long (8.4 ± 0.6 min) as that measured with each natural agonist (4.0 ± 1.0 min). The Hsp90-disrupting agent GA was equally effective for impairing the nuclear accumulation with all three steroid/MR complexes (open symbols). Incubation times longer than 50−60 min showed that the entire population of MR still reaches the nucleus in the presence of GA (asterisk). Therefore, GA slows down but does not abolish MR retrotransport, suggesting the existence of an alternative, Hsp90-independent mechanism of nuclear translocation for MR.

MR recovered from cells treated with GA showed that the proteins associated with the heterocomplex underwent qualitative and quantitative changes (Figure 4C). A significantly lower amount of the Hsp90·FKBP52·dynein molecular machinery remained associated with MR after treatment with the inhibitor, whereas larger amounts of Hsp70 and the TPR protein Hop/p60 (which competes with FKBPs for Hsp90-binding) were recovered in the complex. This is in agreement with the functional consequence evidenced in Figure 4B for cells treated with GA, which strengthens the interpretation that MR is retrotransported via the Hsp90·FKBP52·dynein molecular machinery.

Subnuclear Distribution of MR. Data with 11-OP support a model in which MR undergoes a differential conformational

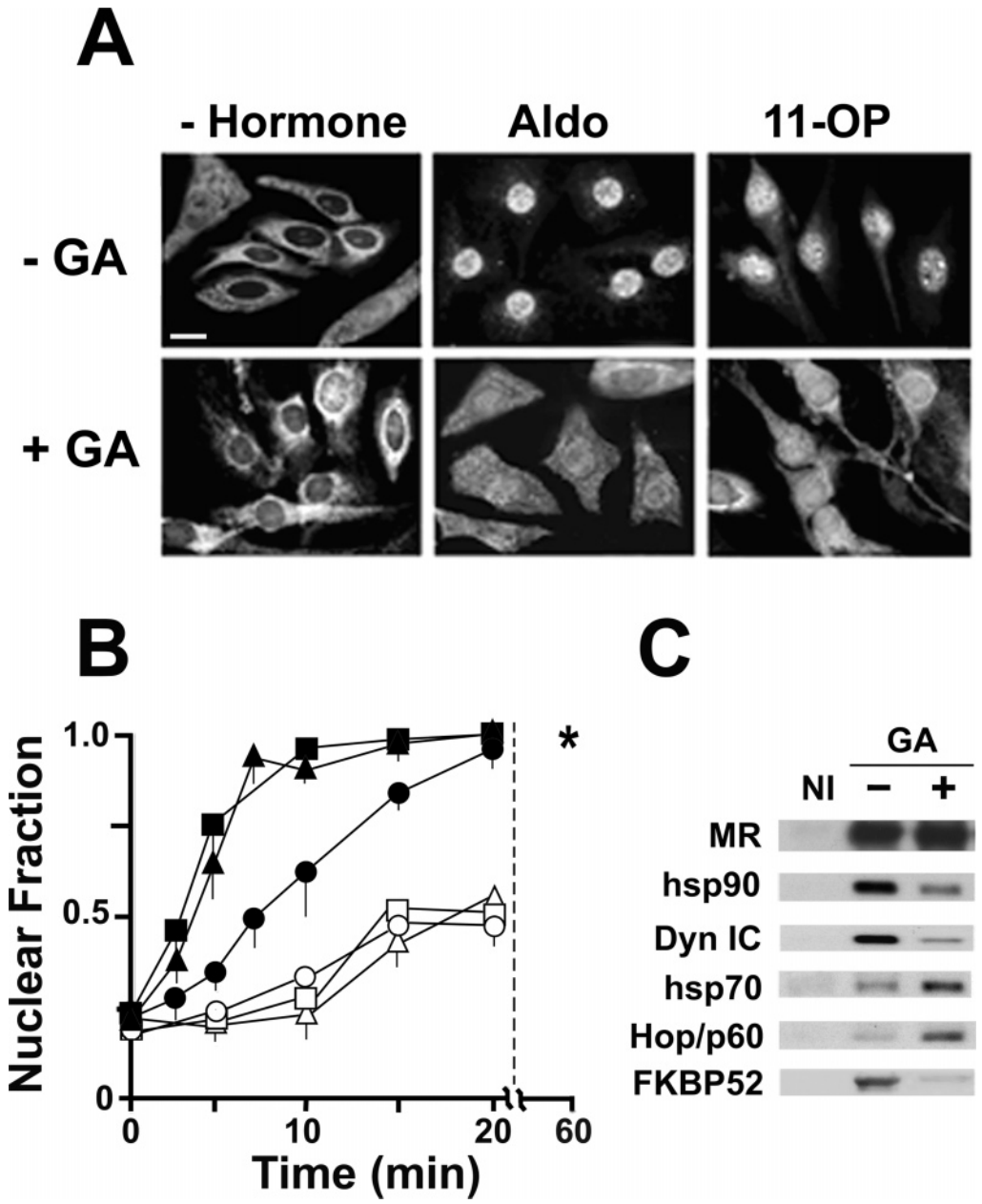

FIGURE 4: Geldanamycin impairs the nuclear translocation of MR. E82.A3 fibroblasts transfected with MR were placed on ice and incubated with 1 μM steroid for 1.5 h. Subsequently, 2.5 μM geldanamycin (GA) or 0.1% (v/v) DMSO was added to the medium, and the incubation was continued for 15 min. The temperature was shifted to 37 °C (zero time) to trigger MR movement toward the nucleus. (A) MR localization. Cells were fixed in cold methanol after 20 min with steroid, and MR was visualized by indirect immunofluorescence with an epi-illumination fluorescence microscope. White bar, 10 μm. (B) MR retrotransport rate. Fluorescence in the cytoplasm and nucleus was quantified for more than 100 cells after various incubation times at 37 °C. The plot depicts the nuclear translocation rates for aldosterone (▲, △), DOC (■, □), and 11-OP (●, ○) in the absence (solid symbols) or presence (open symbols) of GA. The asterisk represents the nuclear fraction measured for all previous conditions after 60 min with steroid. Results are the mean ± SEM of six experiments for the aldosterone and 11-OP curves and three experiments for DOC treatment. All points from 5 to 20 min are significantly different ($p \leq 0.001$) from control curves (■, ▲) except the incubation with 11-OP for 20 min. (C) Effect of GA on MR-associated factors. Cells were treated with 0.1% DMSO (−) or 2.5 μM GA (+) for 20 min at 37 °C, MR was immunoprecipitated, and proteins were resolved by Western blotting. Bands from three independent experiments were scanned and the relative intensities ± GA were compared, yielding significant differences ($p \leq 0.005$) for all blotted proteins except for MR. NI, nonimmune antibody.

change upon steroid binding, which in turn may led to differential recruitment of soluble factors such as IMMs by the receptor. This property may have its correlate in the nucleus. We did measure the nuclear translocation rate of MR in the presence of both steroids, aldosterone and 11-OP, and found that it was as efficient as the maximum rate observed with the natural agonist (data not shown). However, we also observed that the distribution pattern of MR was slightly different. Figure 5 shows confocal microscopic images for nuclear MR activated with each steroid. While 11-OP/MR complexes showed a punctuated subnuclear pattern concentrated in large nuclear speckles (that are excluded from heterochromatin and nucleoli), aldosterone/MR complexes showed smaller and evenly distributed speckles. Interestingly, when cells were exposed to both steroids, the nuclear distribution of MR was intermediate

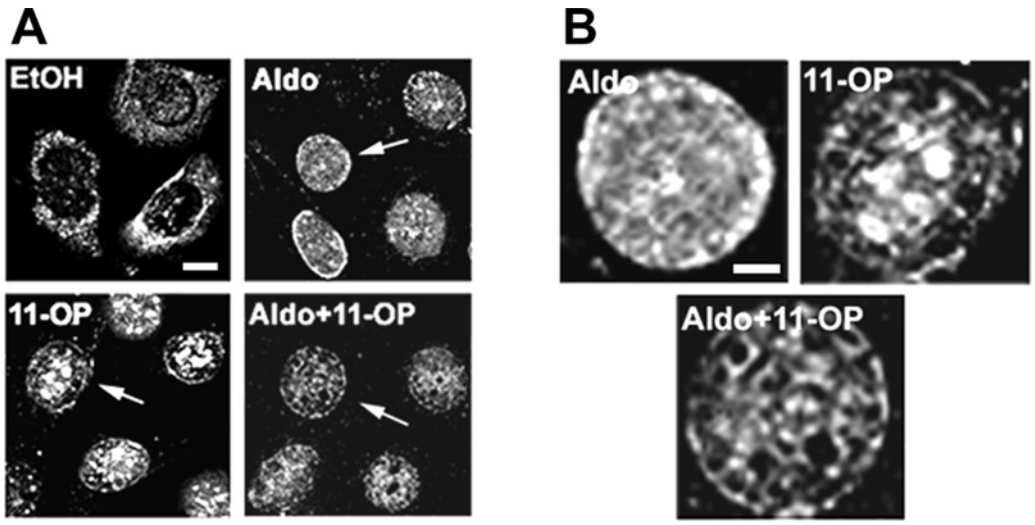

FIGURE 5: Nuclear pattern of aldosterone/MR and 11-OP/MR complexes by confocal microscopy. (A) NIH-3T3 cells were transfected with FLAG-MR and treated with 10 nM aldosterone (Aldo), 50 nM 11-OP (11-OP), or both steroids (Aldo + 11-OP). MR was visualized by confocal laser scanning microscope ($n = 4$). Bar, 10 μm. (B) Magnification of those nuclei indicated by arrows in panel A; bar, 2 μm.

between the two patterns, although it seems to be closer to that observed with aldosterone alone. This observation agrees with the potential association of each steroid/MR complex with different structures or factors in the nucleus, which in turn should be an obvious consequence of the particular conformation generated in MR upon ligand binding.

Nuclear Colocalization of FKBPs with MR. If the Hsp90·IMM complex is required for the cytoplasmic retrotransport of MR, the transformation of the receptor (i.e., dissociation of the Hsp90 complex) should occur at the nuclear pore when MR has reached the nuclear envelope. Alternatively, transformation may be a nuclear event that takes place immediately after the MR·Hsp90·IMM heterocomplex has translocated through the nuclear pore, such that the DNA-binding domain is exposed and the receptor can interact with hormone response elements. Accordingly, MR should exist associated with IMMs in the nucleus.

Figure 6 shows immunofluorescence images by confocal microscopy for nuclear MR (rhodamine-labeled) activated with aldosterone or 11-OP, FKBP51, and FKBP52 (Alexa-Fluor-488-labeled). Nuclear MR shows significant colocalization with FKBP52 regardless of the ligand bound (Figure 6Cb,d), whereas colocalization of MR and FKBP51 appears to be less evident (Figure 6Ca,c). Colocalization with FKBP52 was not affected when both steroids were used together (Figure 6E). These observations are in agreement with the release of FKBP51 from MR and the subsequent recruitment of FKBP52 upon binding of the steroids (Figure 3).

Inhibition of MR Transcriptional Activity by FKBP51. Since FKBP52 is recruited to the heterocomplex and FKBP51 dissociates from MR upon hormone binding (Figure 3), and because there is a strong colocalization of the former with the receptor in the nucleus, we studied the possible effects of both TPR proteins on MR transcription. In this regard, there are some conflicting antecedents with GR. Thus, it has been reported that increased levels of FKBP51 cause glucocorticoid resistance (*32*, *33*). Overexpression of FKBP51 reduces the nuclear translocation rate of GR (*34*) and GR transcriptional activity (*34*, *35*). With respect to FKBP52, the results are variable according to the experimental system assayed. While upregulation of FKBP52 in a tetracycline-inducible system caused an increase in both GR hormone-binding affinity and transactivation (*36*), it has also been reported that FKBP52 showed no significant effect on GR transcriptional activity in mammalian cells (*34*). On the other hand, overexpression of FKBP52 in yeast (*35*), a system that is devoid of endogenous steroid receptors, enhances GR-dependent transcription, whereas FKBP51 has no direct effect on GR, although it is able to prevent FKBP52-dependent enhancement.

On the basis of these contrasting studies and because essentially nothing is known to date about the effect of TPR proteins on MR transcriptional capacity, we analyzed whether the two highly homologous IMMs FKBP51 and FKBP52 show any effect on MR transactivation. Figure 7 shows inhibition of MR-mediated transcription with increasing concentrations of FKBP51, whereas cotransfection with FKBP52 did not significantly change the transcriptional activity. The Western blot for FLAG-MR shown below the bar graphs demonstrates that the levels of MR expression did not change upon cotransfection.

Even though both steroid/MR complexes are sensitive to overexpression of FKBP51, the inhibitory effect is less efficient for 11-OP/MR complexes than for aldosterone/MR complexes. This may be due to different types of MR−nuclear factor or MR−nuclear structure interactions that result from the specific conformation acquired by MR upon binding of each ligand. This interpretation is also in agreement with the differential nuclear distribution of MR observed by confocal microscopy in Figure 5.

Inasmuch as Figure 6 shows that the nuclear colocalization of MR and FKBP51 is poor, and because increasing amounts of FKBP51 are able to repress MR transcriptional activity, we wondered if the excess IMM is located with MR in the same nuclear foci. Figure 7D shows that nuclear MR largely colocalizes with IMM in cells overexpressing FKBP51, suggesting a possible nuclear interaction between both

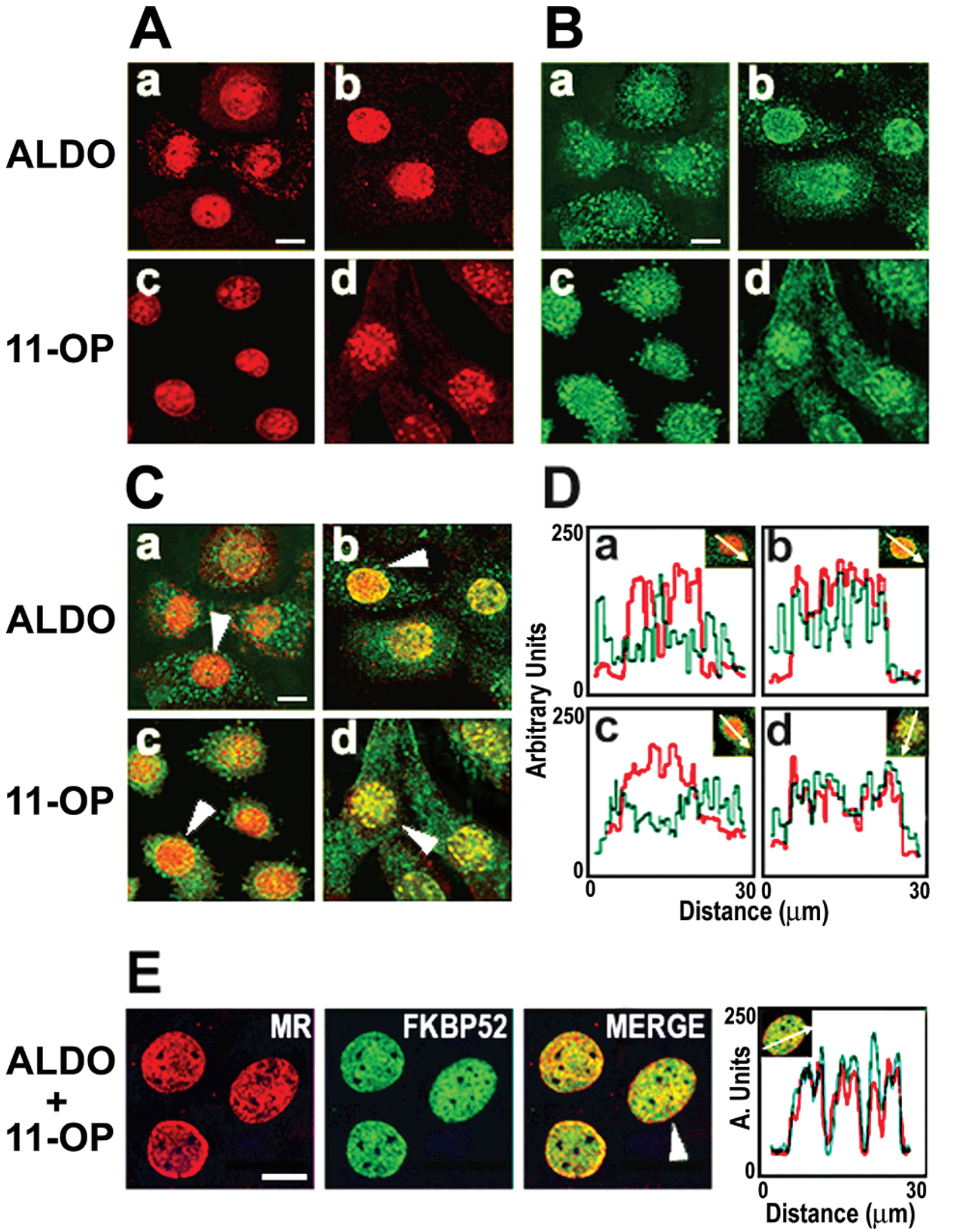

FIGURE 6: Nuclear colocalization of FKBPs and MR. NIH-3T3 cells were transfected with FLAG-MR, incubated with 10 nM aldosterone (all subpanels a and b) or 50 nM 11-OP (all subpanels c and d), fixed, and immunostained for MR (panel A) and either endogenous IMM, FKBP51 (panels B-a and B-c), or FKBP52 (panels B-b and B-d). Cells were visualized by confocal microscopy. Panel C is the merged image of panels A and B. Panel D shows the profile scans through the nuclei shown by arrowheads in panel C, the position of the line scan being shown by a white arrow in the insets. MR, red profile; FKBPs, green profile. White bar in subpanels a, 10 μm. Panel E shows colocalization of MR and FKBP52 in cells cotreated with both steroids. The scan profile corresponds to the nucleus shown with the arrowhead. These images were reproduced three times. Bar, 10 μm.

proteins. Note that FKBP51 and MR show colocalization only in the cytoplasm of cells incubated without steroid (Figure 7C). Because the IMM exerts a negative effect on mineralocorticoid action, all these observations may be

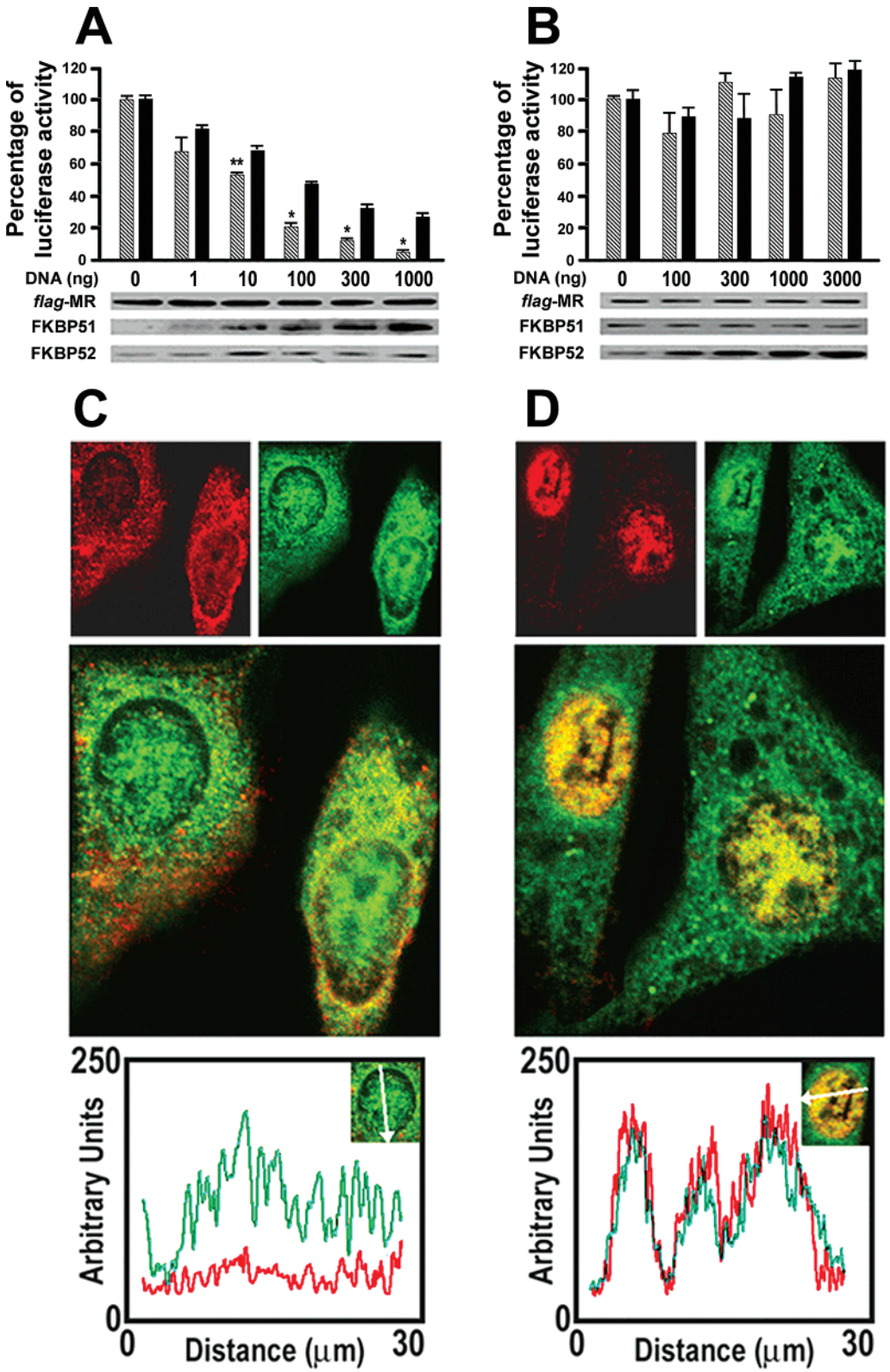

FIGURE 7: Inhibitory effect of FKBP51 on MR-dependent transcription. 293-T fibroblasts were cotransfected with MR and increasing concentrations of (A) FKBP51 or (B) FKBP52. MR-dependent induction of luciferase activity (mean ± SEM, $n = 5$) was measured with 0.05 nM aldosterone (hatched bars) or 10 nM 11-OP (solid bars). Western blots shown under the bar graphs show the expression of FKBPs and FLAG-MR. (A) Luciferase activity induced by aldosterone is different from 11-OP induction for a given DNA concentration at $*p < 0.01$ or $**p < 0.05$. (B) Luciferase activity is not significantly different among all conditions. The subcellular distribution of MR (red) and FKBP51 (green) is shown by confocal microscopy in NIH-3T3 cells transfected with pCI-Neo-hFKBP51. The cells were cultured in a hormone-free medium (C) or with 10 nM aldosterone (D). Scan profiles depicted at the bottom of each panel correspond to those FKBP51 overexpressing cells shown by an arrowhead in the respective merged images. These images were reproduced three times. Bars, 10 μm.

related to the functional reason FKBP51 is dissociated from the complex upon ligand binding when the mineralocorticoid response is required. In summary, FKBP51 functions as a regulator of the MR-dependent effect.

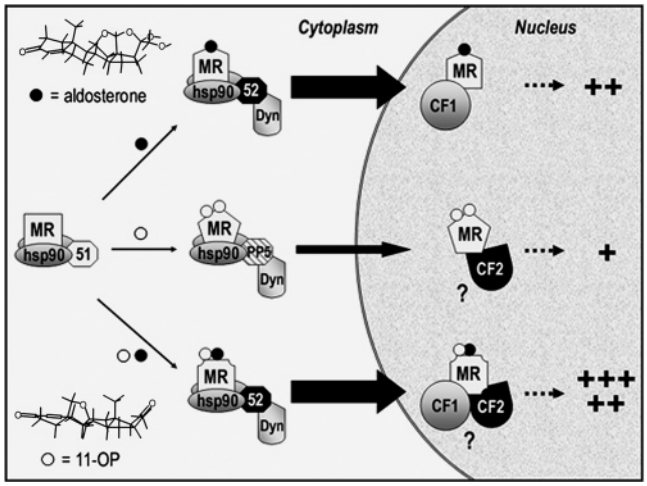

FIGURE 8: Recruitment of IMMs by ligand-activated MR.

DISCUSSION

In this work we provide direct evidence that (a) TPR proteins such as FKBP51, FKBP52, and PP5 are differentially recruited to the MR·Hsp90 heterocomplex in a ligand-dependent manner, (b) MR may be retrotransported by the Hsp90·FKBP52·dyenin complex, and (c) FKBP51 inhibits the ligand-induced transctivation of MR. An indirect extrapolation of this mechanism is that MR transformation could be a nuclear event rather than cytoplasmic.

Figure 8 shows an integrative model for MR nuclear translocation based on the results described here. Upon aldosterone or 11-OP binding, MR undergoes a specific conformational change that allows IMM swapping (i.e., FKBP52 or PP5 for FKBP51). Note that there are two binding sites for 11-OP, the aldosterone-binding pocket and a secondary site not recognized by aldosterone. When both steroids are bound to MR, the recruitment of FKBP52 and dynein is favored, so the nuclear translocation rate of MR is as fast as that measured with aldosterone (wide arrow). Then MR recruits different nuclear factors (or greater amounts of the same factor), which results in different MR transcriptional potency.

We emphasize the relevance of the finding that IMMs are recruited in ligand-dependent fashion. Because the Hsp90·FKBP52 interaction occurs via a TPR acceptor site that accommodates only one TPR protein (*30*, *37*), FKBP52 recruitment should promote the displacement from Hsp90 of other TPR proteins (e.g., FKBP51). Binding of ligand to MR causes FKBP52/FKBP51 swapping without affecting Hsp90, and dynein is corecruited. Dynein binds to the peptidylprolyl isomerase domain of the IMM (*22*), a property not shown by FKBP51 (*34*). Interestingly, the dynein-binding IMM PP5 is recruited by 11-OP/MR complexes only.

In agreement with the reduced recruitment of dynein by 11-OP/MR, MR nuclear accumulation is slower (thin arrow in Figure 8) than the rate measured for aldosterone/MR complexes. Moreover, cell treatment with the Hsp90 inhibitor geldanamycin impairs the association of Hsp90·IMM·dynein with MR (Figure 4C), due to a lower association of Hsp90 with MR and to occupation of the TPR acceptor site with Hop/p60. This functional property and the fact that dynein coimmunoprecipitates with MR favors the hypothesis that MR movement toward the nucleus is powered by this motor protein. In this sense, it has been positively demonstrated that IMMs are implicated in the retrotransport of GR (*22*) and p53 (*14*). However, there are no studies addressing the possibility that this is the molecular mechanism of movement for other nuclear receptors. Nonetheless, there is some indirect evidence suggesting that this may be the case for AAV-2 (adeno-associated virus 2) (*38*), poly(glutamine)-aggregated proteins in Kennedy disease cells (*39*), and possibly the brain-specific protein PAHX-AP1(*40*). The interaction between Dyn IC and IMMs has also been found in plants (*41*), suggesting that its functional role may have been preserved during evolution.

It is important to emphasize that the model depicted in Figure 8 is valid for all cell types used in this work and could presumably be extended to most cell types from different tissues. Here, 293-T human fibroblasts were used for transactivation assays because these cells are efficiently cotransfected with various plasmids without affecting the efficiency of expression. On the other hand, E82.A3 (a $GR^{-/-}$ L929-derived clone) and NIH-3T3 cells were more useful for microscopy imaging due to their flatter shape with higher ratio of cytoplasmic to nuclear volume. The E82.A3 cell line was the most appropriated biological reagent for the overexpression and subsequent immunoprecipitation of MR along with its heterocomplex, whereas NIH-3T3 cells exhibited more prominent nuclear speckles containing MR, which in turn are more difficult to observe in 293-T human cells. Nonetheless, all the properties described in this article for nuclear translocation rates of MR, subnuclear redistribution upon steroid binding, and composition of the Hsp90-based heterocomplex are shared for the three cell types.

In higher organisms, the nuclear receptor superfamily bears a close resemblance to a primordial predecessor (*2*). Therefore, it is tempting to think that many aspects of the molecular mechanism of action of these receptors are the same. In a pioneering report, Davies et al. (*18*) have also reported that hormone binding favors the recruitment of FKBP52 to GR, although no differences were observed upon binding of agonists or antagonists. In the case of MR, aldosterone binding does not affect the amount of PP5, which is recruited only upon 11-OP binding. Unlike GR (*36*), FK506 inhibits MR transcriptional activity without changing aldosterone affinity, the total number of binding sites, or MR trafficking properties (*42*). Clearly, these contrasting results between the receptors indicate that the molecular properties of GR and MR differ from one another, and no direct extrapolation to MR can be made from findings with GR.

Unfortunately, the proteolytic pattern of MR liganded with both steroids is not different from that shown with aldosterone alone (data not shown). This approach was attempted by using several proteases in addition to chymotrypsin and trypsin. Nonetheless, this negative result may be due to the fact that 11-OP binding to MR in the presence of aldosterone may stabilize an aldosterone-like, active conformation of the receptor, so the resultant conformation is similar (but not identical) to that generated with aldosterone alone. Therefore, limited proteolysis may not have enough sensitivity for detecting these slighter conformational changes generated when both steroids are bound to MR. In fact, no significant differences were detected for the recruitment of cytoplasmic factors to aldosterone/MR complexes as compared to aldosterone/11-OP/MR complexes, such that the nuclear translo-

cation rate of MR was as fast as that measured with aldosterone. This is expected if the phenomenon is analyzed from the perspective of a model where 11-OP binding stabilizes MR in an active, aldosterone-like conformation. This does not mean that the structure of MR bound to aldosterone and 11-OP must be necessarily the same as that generated by aldosterone binding alone. This is supported by differences in transcriptional assays (Figure 1), subnuclear redistribution of MR with both ligands (Figure 6), the differential effect observed by cotransfection with FKBPs (Figure 7), the competition curves in Figure 2E, the Scatchard plots in Figure 2F, and the kinetic assays of Table 1.

It is accepted that nuclear factors are highly mobile. However, positioning of transcriptionally active loci appears confined to certain subnuclear regions. Therefore, it is entirely possible that the aldosterone/MR complexes are able to recruit specific nuclear and cytoplasmic factors more efficiently than 11-OP/MR complexes in those foci. Figure 7 shows that the induction of luciferase activity for each ligand/MR complex is differentially affected by FKBP51. In similar fashion, this selective property for each steroid/receptor complex may explain the particular MR nuclear pattern observed in cells treated with aldosterone or 11-OP. It is quite remarkable that the nuclear pattern shown by MR in cells treated with both steroids is, in turn, different from that observed with each steroid. This may be in agreement with the hypothesis that MR may acquire at least three different conformational states: those induced by binding of aldosterone, 11-OP, or both steroids.

11-OP may mimic the characteristics of endogenous steroids that may serve other purposes today because during the evolutionary process they have acquired other roles. Interestingly, the naturally occurring steroid 5α-dihydroprogesterone, a progesterone derivative that promotes mitogenic and metastatic activity in breast cells (*43*), exhibits as flat a conformation as 11-OP and, consequently, is a strong mineralocorticoid (*26*). Preliminary studies have shown that 5α-dihydroprogesterone is also able to potentiate aldosterone action.

On the other hand, it is known that the enzyme 11β-hyroxysteroid dehydrogenase protects MR from cross talk with glucocorticoids in epithelial tissues (*44*). Nonetheless, it is still unclear why the mineralocorticoid effect is still preserved in tissues where the enzyme is not expressed. Perhaps the cooperative action of endogenous steroids on MR in a given tissue and the subsequent recruitment of specific TPR proteins may parallel the effects observed here on the differential conformation acquired by MR (Figure 2), the specific recruitment of other proteins to the complex (Figure 3), the effect of FKBP51 on transcription (Figure 7), and/or MR nuclear anchorage at the nuclear site of action (Figure 5).

In summary, the evidence shown in this work supports a model where the Hsp90·IMM complex links soluble proteins such as primarily cytoplasmic nuclear factors with dynein motors. Therefore, the retrograde movement of these signaling cascade factors throughout the cytoplasm is more efficiently achieved than that observed by other alternative mechanisms (e.g., simple diffusion). A good example of this property is evidenced when the molecular machinery of transport was disrupted by the Hsp90 inhibitor geldanamycin (Figure 4). From the mechanistic point of view, excluding factors from the nucleus not only relieves the organelle of congestion but also adds regulatory check-points along the pathway of those factors. In this sense, the IMM swapping triggered by hormone binding to steroid receptors may be a representative case for a novel regulatory mechanism for these and perhaps other soluble proteins.

ACKNOWLEDGMENT

We are very grateful to Dr. David F. Smith (Mayo Clinic, Scottsdale, AZ), Dr. Jack-Michel Renoir (Faculté de Pharmacie, Châtenay-Malabry Cedex, France), and Dr. Shigeaki Kato (University of Tokyo) for generously providing plasmids and to Dr. Gerald Litwack (Jefferson Cancer Institute, Philadelphia, PA) for the kind gift of the anti-MR antibody. We are indebted to Dr. Kimon Kanelakis for the kind review of the manuscript.

REFERENCES

1. Evans, R. M. (1988) The steroid and thyroid hormone receptor superfamily, *Science 240*, 889−895.
2. Escriva, H., Bertrand, S., and Laudet, V. (2004) The evolution of the nuclear receptor superfamily, *Essays Biochem. 40*, 11−26.
3. Nollen, E. A., and Morimoto, R. I. (2002) Chaperoning signaling pathways: molecular chaperones as stress-sensing 'heat shock' proteins, *J. Cell Sci. 115*, 2809−2816.
4. Pratt, W. B., Galigniana, M. D., Morishima, Y., and Murphy, P. J. (2004) Role of molecular chaperones in steroid receptor action, *Essays Biochem. 40*, 41−58.
5. Ratajczak, T., Ward, B. K., and Minchin, R. F. (2003) Immunophilin chaperones in steroid receptor signalling, *Curr. Top. Med. Chem. 3*, 1348−1357.
6. Robertson, N. M., Schulman, G., Karnik, S., Alnemri, E., and Litwack, G. (1993) Demonstration of nuclear translocation of the mineralocorticoid receptor (MR) using an anti-MR antibody and confocal laser scanning microscopy, *Mol. Endocrinol. 7*, 1226−1239.
7. Lombes, M., Binart, N., Delahaye, F., Baulieu, E. E., and Rafestin-Oblin, M. E. (1994) Differential intracellular localization of human mineralocorticosteroid receptor on binding of agonists and antagonists, *Biochem. J. 302*, 191−197.
8. Piwien-Pilipuk, G., and Galigniana, M. D. (1998) Tautomycin inhibits phosphatase-dependent transformation of the rat kidney mineralocorticoid receptor, *Mol. Cell. Endocrinol. 144*, 119−130.
9. Nishi, M., Ogawa, H., Ito, T., Matsuda, K. I., and Kawata, M. (2001) Dynamic changes in subcellular localization of mineralocorticoid receptor in living cells: in comparison with glucocorticoid receptor using dual-color labeling with green fluorescent protein spectral variants, *Mol. Endocrinol. 15*, 1077−1092.
10. Piwien Pilipuk, G., Vinson, G. P., Sanchez, C. G., and Galigniana, M. D. (2007) Evidence for NL1-Independent Nuclear Translocation of the Mineralocorticoid Receptor, *Biochemistry 46*, 1389−1397.
11. Harrell, J. M., Murphy, P. J., Morishima, Y., Chen, H., Mansfield, J. F., Galigniana, M. D., and Pratt, W. B. (2004) Evidence for glucocorticoid receptor transport on microtubules by dynein, *J. Biol. Chem. 279*, 54647−54654.
12. Galigniana, M. D., Harrell, J. M., Housley, P. R., Patterson, C., Fisher, S. K., and Pratt, W. B. (2004) Retrograde transport of the glucocorticoid receptor in neurites requires dynamic assembly of complexes with the protein chaperone hsp90 and is linked to the CHIP component of the machinery for proteasomal degradation, *Brain Res. Mol. Brain Res. 123*, 27−36.
13. Giannakakou, P., Sackett, D. L., Ward, Y., Webster, K. R., Blagosklonny, M. V., and Fojo, T. (2000) p53 is associated with cellular microtubules and is transported to the nucleus by dynein, *Nat. Cell Biol. 2*, 709−717.
14. Galigniana, M. D., Harrell, J. M., O'Hagen, H. M., Ljungman, M., and Pratt, W. B. (2004) Hsp90-binding immunophilins link p53 to dynein during p53 transport to the nucleus, *J. Biol. Chem. 279*, 22483−22489.
15. Burton, G., Galigniana, M., De Lavallaz, S., Brachet-Cota, A. L., Sproviero, E. M., Ghini, A. A., Lantos, C. P., and Damasco, M.

C. (1995) Sodium-retaining activity of some natural and synthetic 21-deoxysteroids, *Mol. Pharmacol. 47*, 535−543.
16. Czar, M. J., Galigniana, M. D., Silverstein, A. M., and Pratt, W. B. (1997) Geldanamycin, a heat shock protein 90-binding benzoquinone ansamycin, inhibits steroid-dependent translocation of the glucocorticoid receptor from the cytoplasm to the nucleus, *Biochemistry 36*, 7776−7785.
17. Galigniana, M. D., Scruggs, J. L., Herrington, J., Welsh, M. J., Carter-Su, C., Housley, P. R., and Pratt, W. B. (1998) Heat shock protein 90-dependent (geldanamycin-inhibited) movement of the glucocorticoid receptor through the cytoplasm to the nucleus requires intact cytoskeleton, *Mol. Endocrinol. 12*, 1903−1913.
18. Davies, T. H., Ning, Y. M., and Sanchez, E. R. (2002) A new first step in activation of steroid receptors: hormone-induced switching of FKBP51 and FKBP52 immunophilins, *J. Biol. Chem. 277*, 4597−4600.
19. Galigniana, M. D. (1998) Native rat kidney mineralocorticoid receptor is a phosphoprotein whose transformation to a DNA-binding form is induced by phosphatases, *Biochem. J. 333*, 555−563.
20. Galigniana, M. D., Vicent, G. P., Piwien-Pilipuk, G., Burton, G., and Lantos, C. P. (2000) Mechanism of action of the potent sodium-retaining steroid 11,19-oxidoprogesterone, *Mol. Pharmacol. 58*, 58−70.
21. Galigniana, M. D., Morishima, Y., Gallay, P. A., and Pratt, W. B. (2004) Cyclophilin-A is bound through its peptidylprolyl isomerase domain to the cytoplasmic dynein motor protein complex, *J. Biol. Chem. 279*, 55754−55759.
22. Galigniana, M. D., Radanyi, C., Renoir, J. M., Housley, P. R., and Pratt, W. B. (2001) Evidence that the peptidylprolyl isomerase domain of the hsp90-binding immunophilin FKBP52 is involved in both dynein interaction and glucocorticoid receptor movement to the nucleus, *J. Biol. Chem. 276*, 14884−14889.
23. Piwien-Pilipuk, G., Ayala, A., Machado, A., and Galigniana, M. D. (2002) Impairment of mineralocorticoid receptor (MR)-dependent biological response by oxidative stress and aging: correlation with post-translational modification of MR and decreased ADP-ribosylatable level of elongating factor 2 in kidney cells, *J. Biol. Chem. 277*, 11896−11903.
24. Galigniana, M. D. (1996) Stability study on renal type I mineralocorticoid receptor, *Life Sci. 59*, 511−521.
25. Galigniana, M. D., and Piwien Pilipuk, G. (2004) Activation of the ligand-mineralocorticoid receptor functional unit by ancient, classical, and novel ligands. Structure-activity relationship, *Vitam. Horm. (San Diego) 69*, 31−68.
26. Piwien-Pilipuk, G., Kanelakis, K. C., and Galigniana, M. D. (2002) Correlation between pregnanesteroid conformation, receptor affinity, and anti-natriuretic effect, *Eur. J. Pharmacol. 454*, 131−143.
27. Hultman, M. L., Krasnoperova, N. V., Li, S., Du, S., Xia, C., Dietz, J. D., Lala, D. S., Welsch, D. J., and Hu, X. (2005) The ligand-dependent interaction of mineralocorticoid receptor with coactivator and corepressor peptides suggests multiple activation mechanisms, *Mol. Endocrinol. 19*, 1460−1473.
28. Fuse, H., Kitagawa, H., and Kato, S. (2000) Characterization of transactivational property and coactivator mediation of rat mineralocorticoid receptor activation function-1 (AF-1), *Mol. Endocrinol. 14*, 889−899.
29. Galigniana, M. D., Harrell, J. M., Murphy, P. J., Chinkers, M., Radanyi, C., Renoir, J. M., Zhang, M., and Pratt, W. B. (2002) Binding of hsp90-associated immunophilins to cytoplasmic dynein: direct binding and in vivo evidence that the peptidylprolyl isomerase domain is a dynein interaction domain, *Biochemistry 41*, 13602−13610.
30. Silverstein, A. M., Galigniana, M. D., Kanelakis, K. C., Radanyi, C., Renoir, J. M., and Pratt, W. B. (1999) Different regions of the immunophilin FKBP52 determine its association with the glucocorticoid receptor, hsp90, and cytoplasmic dynein, *J. Biol. Chem. 274*, 36980−36986.
31. Housley, P. R., and Forsthoefel, A. M. (1989) Isolation and characterization of a mouse L cell variant deficient in glucocorticoid receptors, *Biochem. Biophys. Res. Commun. 164*, 480−487.
32. Hubler, T. R., Denny, W. B., Valentine, D. L., Cheung-Flynn, J., Smith, D. F., and Scammell, J. G. (2003) The FK506-binding immunophilin FKBP51 is transcriptionally regulated by progestin and attenuates progestin responsiveness, *Endocrinology 144*, 2380−2387.
33. Denny, W. B., Valentine, D. L., Reynolds, P. D., Smith, D. F., and Scammell, J. G. (2000) Squirrel monkey immunophilin FKBP51 is a potent inhibitor of glucocorticoid receptor binding, *Endocrinology 141*, 4107−4113.
34. Wochnik, G. M., Ruegg, J., Abel, G. A., Schmidt, U., Holsboer, F., and Rein, T. (2005) FK506-binding proteins 51 and 52 differentially regulate dynein interaction and nuclear translocation of the glucocorticoid receptor in mammalian cells, *J. Biol. Chem. 280*, 4609−4616.
35. 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) The Hsp90-binding peptidylprolyl isomerase FKBP52 potentiates glucocorticoid signaling in vivo, *EMBO J. 22*, 1158−1167.
36. Davies, T. H., Ning, Y. M., and Sanchez, E. R. (2005) Differential control of glucocorticoid receptor hormone-binding function by tetratricopeptide repeat (TPR) proteins and the immunosuppressive ligand FK506, *Biochemistry 44*, 2030−2038.
37. Young, J. C., Obermann, W. M., and Hartl, F. U. (1998) Specific binding of tetratricopeptide repeat proteins to the C-terminal 12-kDa domain of hsp90, *J. Biol. Chem. 273*, 18007−18010.
38. Zhao, W., Zhong, L., Wu, J., Chen, L., Qing, K., Weigel-Kelley, K. A., Larsen, S. H., Shou, W., Warrington, K. H., Jr., and Srivastava, A. (2006) Role of cellular FKBP52 protein in intracellular trafficking of recombinant adeno-associated virus 2 vectors, *Virology 353*, 283−293.
39. Thomas, M., Harrell, J. M., Morishima, Y., Peng, H. M., Pratt, W. B., and Lieberman, A. P. (2006) Pharmacologic and genetic inhibition of hsp90-dependent trafficking reduces aggregation and promotes degradation of the expanded glutamine androgen receptor without stress protein induction, *Hum. Mol. Genet. 15*, 1876−1883.
40. Li, P., Ding, Y., Wu, B., Shu, C., Shen, B., and Rao, Z. (2003) Structure of the N-terminal domain of human FKBP52, *Acta Crystallogr. D: Biol. Crystallogr. 59*, 16−22.
41. Harrell, J. M., Kurek, I., Breiman, A., Radanyi, C., Renoir, J. M., Pratt, W. B., and Galigniana, M. D. (2002) All of the protein interactions that link steroid receptor·hsp90·immunophilin heterocomplexes to cytoplasmic dynein are common to plant and animal cells, *Biochemistry 41*, 5581−5587.
42. Deppe, C. E., Heering, P. J., Viengchareun, S., Grabensee, B., Farman, N., and Lombes, M. (2002) Cyclosporine a and FK506 inhibit transcriptional activity of the human mineralocorticoid receptor: a cell-based model to investigate partial aldosterone resistance in kidney transplantation, *Endocrinology 143*, 1932−1941.
43. Wiebe, J. P., Souter, L., and Zhang, G. (2006) Dutasteride affects progesterone metabolizing enzyme activity/expression in human breast cell lines resulting in suppression of cell proliferation and detachment, *J. Steroid Biochem. Mol. Biol. 100*, 129−140.
44. Funder, J. W. (2000) Aldosterone and mineralocorticoid receptors: orphan questions, *Kidney Int. 57*, 1358−1363.

BI701372C