Phosphorylation of Mineralocorticoid Receptor Ligand Binding Domain Impairs Receptor Activation and Has a Dominant Negative Effect over Non-phosphorylated Receptors

Rubén Jiménez-Canino, Miguel X. Fernandes, and Diego Alvarez de la Rosa

From the Departamento de Ciencias Médicas Básicas, Instituto de Tecnologías Biomédicas y Centro de Investigaciones Biomédicas de Canarias (CIBICAN), Universidad de La Laguna, 38071 La Laguna, Tenerife, Spain

Post-translational modification of steroid receptors allows fine-tuning different properties of this family of proteins, including stability, activation, or interaction with co-regulators. Recently, a novel effect of phosphorylation on steroid receptor biology was described. Phosphorylation of human mineralocorticoid receptor (MR) on Ser-843, a residue placed on the ligand binding domain, lowers affinity for agonists, producing inhibition of gene transactivation. We now show that MR inhibition by phosphorylation occurs even at high agonist concentration, suggesting that phosphorylation may also impair coupling between ligand binding and receptor activation. Our results demonstrate that agonists are able to induce partial nuclear translocation of MR but fail to produce transactivation due at least in part to impaired co-activator recruitment. The inhibitory effect of phosphorylation on MR acts in a dominant-negative manner, effectively amplifying its functional effect on gene transactivation.

Steroid receptors (SRs) are part of the nuclear receptor superfamily of ligand-dependent transcription factors that modulate gene transcription in response to changes in steroid hormone levels (1). SRs present a modular architecture, with three well defined domains: an NH$_2$-terminal activation domain (NTD), a central DNA binding domain, and a COOH-terminal ligand binding domain (LBD). Generally, the apo receptor resides in the cytosol forming a heterocomplex with other proteins including chaperones such as heat-shock protein 90 (Hsp90). Ligand binding alters SR conformation, releasing it from the complex and promoting translocation to the nucleus, where it binds specific DNA sequences as a dimer and alters transcription of target genes by recruiting transcriptional co-regulators (2). Post-translational modifications, including phosphorylation, ubiquitylation, sumoylation, and acetylation, modulate SR stability and different steps on the activation pathway, including receptor dimerization, DNA binding, and interaction with co-regulators (3).

Until recently, it was widely assumed that SR ligand affinity is an intrinsic property defined by the structure of the LBD and not subject to modulation by post-translational modifications. However, Shibata et al. (4) have shown that regulated phosphorylation of Ser-843 in the LBD of human mineralocorticoid receptor (MR), a canonical member of the SR subfamily of nuclear receptors, impairs its ability to mediate gene transactivation by lowering ligand affinity. MR is closely related to the glucocorticoid receptor (GR) and can be activated under physiological conditions by mineralocorticoids such as aldosterone and glucocorticoids such as cortisol or corticosterone. MR has a wide variety of physiological and pathophysiological functions, with a prominent role in regulating transepithelial ion and fluid transport, which is essential for extracellular volume homeostasis and, therefore, blood pressure control. Phosphorylation of Ser-843 in the LBD of MR has been shown to result in decreased ligand affinity and thus reduced ability to activate target genes under physiological conditions.

Working with an MR phosphomimetic mutant, Shibata et al. (4) showed that Ser-843 phosphorylation results in the loss of ligand-induced nuclear translocation at physiological concentrations of aldosterone (1 nM) and, consequently, a loss of gene transactivation. Ligand binding affinity is decreased by 2 orders of magnitude ($K_d$ for [3H]aldosterone changed from 0.64 nM in the wild-type receptor to 86 nM in mutant S843E (4)). Structural modeling of the LBD suggested that phosphorylation of residue Ser-843 may affect the interaction between helices H6 and H7 in the ligand-bound state.

Based on these results, we set out to answer two different but related questions. 1) Does the phosphorylation of Ser-843 result only in decreased binding affinity or does it also affect coupling between ligand binding and receptor activation? 2) How does the phosphorylation of Ser-843 affect receptor function? Our results demon...
strate that agonists at high concentrations are able to induce near-complete nuclear translocation of phosphomimetic mutant receptors, albeit at a much slower rate, but are ineffective in inducing agonist-dependent gene transactivation. This suggests altered coupling between ligand binding and receptor activation, resulting in impaired interaction with co-regulators. Moreover, phosphomimetic MR mutants have a dominant negative effect on WT receptor function, a feature that will amplify the physiological effect of LBD phosphorylation.

Results

This study used a previously described fluorescent derivative of mouse wild-type MR with insertion of yellow fluorescent protein (YFP) after amino acid 147 of the MR sequence (5). This construct is indistinguishable from the wild-type mouse receptor in hormone-induced nuclear translocation, apparent affinity for ligand-induced gene transactivation, DNA binding, and regulation of non-genomic pathways (5). The construct, originally named MR-YFP-147 but referred to as MR-WT in this study, was the basis to introduce phosphomimetic mutants S839D and S839E (mouse Ser-839 is the equivalent of human MR S843). To ensure that these constructs display the expected affinities for aldosterone and are comparable with those described by Shibata et al. (4) using human MR, we calculated equilibrium dissociation constants ($K_d$) with competitive binding assay in transfected COS-7 cells (Fig. 1). Results showed a $K_d$ of 1.07 nM for fluorescent MR-WT, which is in the expected range for a wild-type receptor ($K_d$ 0.5–2 nM; Ref. 6). Phosphomimetic mutants S839D and S839E presented decreased affinity for aldosterone, with $K_d$ values of 86.7 and 34.3 nM, respectively, which are in the same range of the $K_d$ value described for the equivalent mutation S843E in the human MR clone.

We then asked whether phosphorylation of MR at residue 843 impairs receptor activity exclusively by lowering ligand affinity or whether it also uncouples ligand binding from receptor activation. If the first option is correct, increasing aldosterone concentration should result in normal nuclear translocation and gene transactivation. To test this hypothesis we studied aldosterone-induced nuclear translocation using our fluorescent derivative of wild-type mouse MR (MR-WT) and compared it with the same receptor harboring phosphomimetic mutations. COS-7 cells transfected with WT, S839E, or S839D MR were left untreated or exposed overnight to increasing concentrations of aldosterone (0.1–100 nM) covering a range of physiological (0.1–2 nM) to supra-physiological levels. In the absence of ligand the vast majority of cells showed a predominantly cytosolic MR localization (Fig. 2, A and C) as previously described (7, 8). Mutations S839D or S839E did not change naïve MR subcellular localization (Fig. 2, A and C). When cells were stimulated overnight with increasing concentrations of aldosterone, MR-WT translocated to the nucleus, with >90% of the cells showing an exclusive MR nuclear localization with as little as 1 nM aldosterone (Fig. 2, A and C). In comparison, aldosterone partially translocated mutants MR-S839D and MR-S839E. The effect started to be apparent at 1 nM aldosterone and continued growing with up to 100 nM aldosterone, where ~90% of cells showed a predominantly nuclear localization (Fig. 2, B and C). These results suggests that phosphorylation of MR impairs nuclear translocation but only to a limited extent, with a significant amount of receptor present in the nucleus.

![Figure 1. Phosphomimicking mutants MR-S839D and MR-S829E show decreased affinity for aldosterone.](image-url)
To test whether decreased nuclear localization induced by phosphorylation arises from slower nuclear import, we examined the kinetics of this process in COS-7 cells transfected with MR-WT or phosphomimetic mutation S839D (both tagged with YFP). To that end, living cells were placed under a confocal microscope in a temperature-controlled chamber at 37 °C and stimulated with 10 nM aldosterone. Images of the same cells were taken every 2 min for up to 60 min after the addition of the hormone, and the proportion of nuclear receptor was calculated in every cell for each time point. Representative images of WT and S839D MR translocation are shown in Fig. 3A. When compared with the WT receptor, mutant S839D showed very slow translocation kinetics, with 20% of the cytosolic receptor translocated over the period of 1 h (Fig. 3, A and B). This strongly suggests that impaired ligand-induced nuclear accumulation of the phosphomimetic MR mutant arises from slower nuclear import. This in turn may arise from lower affinity for the ligand but could also imply either decreased stability of aldosterone in the LBD or a defect in coupling ligand binding with receptor conformational change.

We then tested the functional consequences of mutating mouse MR residue Ser-839 for MR-mediated gene transactivation. We tested non-phosphorylatable mutant S839A and phosphomimicking mutants S839D and S839E. Aldosterone dose-response luciferase reporter assays showed an EC_{50} of 0.4 nM for MR WT, whereas S839A EC_{50} was 2.8 nM. Upon stimulation with cortisol, WT MR displayed an EC_{50} of 9.8 nM, and MR-S839A had an EC_{50} of 19.3. As described with human MR-S843E, mouse MR-S839E and mouse MR-S839D did not show partial ligand-induced nuclear translocation. A, representative images of YFP-tagged wild-type MR (WT) subcellular localization in COS-7 cells in the absence of ligand or after overnight treatment with 10 nM aldosterone. B, representative images of YFP-tagged MR-S839D subcellular localization in COS-7 cells in the absence of ligand or after overnight treatment with 10 nM aldosterone. C, quantitative analysis of MR-WT, MR-S839D, and MR-S839E subcellular localization in the absence of ligand or after 16 h stimulation with the indicated concentrations of aldosterone. Cells were scored into five different categories reflecting the localization of MR (C, exclusively cytosolic; C > N predominantly cytosolic; C < N, predominantly nuclear; N, exclusively nuclear). Bars represent the average percentage of cells ± S.E. scored under each category (n = 3 independent experiment, with at least 75 cells scored in each group and experiment). No significant differences between mutants S839D and S839E were detected in any of the conditions tested (unpaired t test).

FIGURE 3. MR-S839D shows a drastically reduced ligand-induced nuclear translocation rate. A, representative images of cells transfected with YFP-tagged wild-type MR (MR-WT) or mutant S839D (MR-S839D) and examined under confocal microscopy at 37 °C to detect nuclear translocation induced by 10 nM aldosterone. Numbers refer to time after the addition of aldosterone (min). B, quantitative analysis of MR-WT or MR-S839D nuclear translocation. Individual points represent average ± S.E. (n = 9) percentage nuclear fluorescence intensity versus total cellular fluorescence (F). Images taken once every 2 min after the addition of 10 nM aldosterone, up to 60 min. Data points were fitted to Boltzmann sigmoid curves. The dashed line indicates the basal level of percentage nuclear localization.

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FIGURE 2. Phosphomimetic mutants MR-S839D and MR-S839E show partial ligand-induced nuclear translocation. A, representative images of YFP-tagged wild-type MR (WT) subcellular localization in COS-7 cells in the absence of ligand or after overnight treatment with 10 nM aldosterone. B, representative images of YFP-tagged MR-S839D subcellular localization in COS-7 cells in the absence of ligand or after overnight treatment with 10 nM aldosterone. C, quantitative analysis of MR-WT, MR-S839D, and MR-S839E subcellular localization in the absence of ligand or after 16 h stimulation with the indicated concentrations of aldosterone. Cells were scored into five different categories reflecting the localization of MR (C, exclusively cytosolic; C > N predominantly cytosolic; C < N, predominantly nuclear; N, exclusively nuclear). Bars represent the average percentage of cells ± S.E. scored under each category (n = 3 independent experiment, with at least 75 cells scored in each group and experiment). No significant differences between mutants S839D and S839E were detected in any of the conditions tested (unpaired t test).
display aldosterone or cortisol-dependent gene transactivation even at high doses of ligand (Fig. 4, A and B). Western blotting analysis showed that the lack of MR-S839D or MR-S839E transactivation and reduced MR-S839A transactivation were not due to impaired protein expression (Fig. 4C).

The lack of any gene transactivation by MR-S839E or MR-S839D at ligand concentrations where there is significant nuclear translocation in the same time frame (compare Fig. 2 and Fig. 4) supports the idea that phosphorylation of MR LBD not only lowers MR affinity for agonists but has a profound impact on the ability of the ligand to activate the receptor. Therefore, we hypothesized that the phosphomimetic mutant may prevent efficient interaction with co-regulators. To investigate this possibility we tested ligand-induced MR interaction with a well known co-activator, steroid receptor co-activator 1 (SRC-1) (9–11). SRC-1 displays a predominantly nuclear localization both in control and aldosterone-stimulated conditions, whereas MR shows the expected ligand-induced trafficking from cytosol to nucleus (Fig. 5A). To quantitatively assess MR-SRC-1 interaction, we used a proximity ligation assay (PLA). Co-transfection of MR-WT and SRC-1 resulted in a prominent PLA signal in the cell nucleus only in the presence of aldosterone (Fig. 5B). Omission of either one of the transfected plasmids resulted in the absence of signal (Fig. 5, B and C). Quantitative analysis demonstrated that PLA signal was drastically reduced (to ~15% of the WT signal) when the phosphomimetic mutants MR-S839D or MR-S839E were co-transfected with SRC-1 (Fig. 5, B and C).

Based on MR LBD crystal structure, Shibata et al. (4) predicted that phosphorylation of residue Ser-843 could affect ligand binding and/or receptor activation. Indeed, their experimental measurements of WT and phosphomimetic mutant S843E $K_d$ for aldosterone showed decreased affinity (from 0.63 nM to 86 nM) (4). To further investigate the possible effects of phosphorylation at Ser-843, we modeled this post-translational modification using available crystal structures of MR LBD as templates (12). Using this model we performed docking calculations to estimate the energy of interaction of aldosterone, cortisol, and spironolactone to MR LBD in the WT form with phosphorylated Ser-843 or introducing phosphomimetic mutation S843D (Table 1). To validate docking parameterization, we compared the position of aldosterone as calculated by docking with the position of aldosterone as determined by x-ray crystallography. Both were practically superimposable (not shown). Docking interaction energy for aldosterone was only slightly affected by phospho-Ser-843, S843D or S839E modifications (Table 1), suggesting that the decreased affinity observed by Shibata et al. (4) may have a kinetic basis rather than a change in equilibrium interaction. Given that intercalated cells, where Ser-843 phosphorylation has been described, lack 11-β-hydroxysteroid-dehydrogenase type 2 and its MR may, therefore, be activated by cortisol (4, 13), we also calculated docking energy for this hormone. In contrast to aldosterone, cortisol appears to bind slightly more stably to the modified LBD (Table 1), again suggesting that the absence of cortisol transactivation of phosphomimetic mutants observed previously (4) and confirmed here (Fig. 4B) does not arise from impaired equilibrium interaction with MR LBD. Spironolactone...
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tone showed a decrease in docking energy interaction with Ser-843 phosphorylation and S843D mutation but not with S843E mutation (Table 1). Interestingly, interaction energies for the three ligands were almost identical in the model incorporating a phosphorylation of Ser-843 when compared with mutation S843D (Table 1) but not compared with mutation S843E, suggesting that using phosphomimetic mutation S843D may be a better approximation to study the effects of Ser-843 phosphorylation in MR function.

To investigate the functional impact of partial MR phosphorylation we co-expressed different proportions of WT and S843D receptors in COS-7 cells. To ensure that the different proportions of plasmids produced the desired protein expression levels, we performed Western blotting analysis of receptor expression. Both receptors were recognized by the same

![Figure 5](image)

**Figure 5.** Phosphomimetic mutants MR-S839D and MR-S839E impair interaction with co-activator SRC-1 after aldosterone treatment. A, subcellular distribution of YFP-tagged wild-type MR (MR-WT) and HA-tagged SRC-1 transfected in COS-7 cells and treated or not with 10 nM aldosterone (aldo) for 24 h. B, representative images of proximity ligation assay results examining the interaction of MR with SRC-1. C, quantitative analysis of MR-SRC-1 interaction when WT or mutant MR were co-transfected with SCR-1 and treated with 10 nM aldosterone. Bars represent the average number of puncta/cell ± S.E. (n = 36–40 cells per condition). Negative controls consisted on the omission of aldosterone treatment (–aldo) or leaving out either MR (–MR-WT) or SRC-1 (–SRC-1) from the transfection mix. ****, p < 0.0001; n.s., no significant difference when compared with MR-WT + aldo condition; Kruskal-Wallis test followed by Dunn’s multiple comparisons test.

**Table 1**

Docking interaction energies (kcal mol$^{-1}$) of ligands with modeled MR WT, mutant, or phosphorylated ligand binding domain

| MR LBD variant | Aldosterone | Cortisol | Spironolactone |
|----------------|-------------|----------|----------------|
| Ser-843        | −11.3       | −9.9     | −13.2          |
| S843D          | −10.8       | −10.8    | −12.2          |
| S843E          | −11.3       | −11.2    | −13.5          |
| Ser(P)-843     | −10.8       | −10.6    | −12.3          |

Calculations assume an error of ± 0.3 kcal mol$^{-1}$.
anti-MR antibody but could be differentiated due to their different molecular weight produced by fusing YFP to MR-S839D. Co-expression of both receptors using 1:3, 1:1, and 3:1 plasmid proportions gave the expected ratios between WT and mutant MR (Fig. 6A). To confirm that S843D mutation does not disrupt dimerization and it is able to form dimers with WT MR, we used PLA (Fig. 6B). Quantification of PLA results showed that MR dimerization was identical in the absence or presence of S839D mutation (Fig. 6C). This result indicates that WT MR subunits do not discriminate between WT and S839D-contain-
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TABLE 2

Expected subunit composition of wild type and S839D heterodimers

Probabilities of different dimers are shown below for each possible plasmid combination, expressed in percentage. Assuming that both plasmids are processed equally to produce protein and that dimerization is not affected by S839D mutation, it is expected that the probability of heterodimer composition follows a binomial distribution given by the expression \[ p_{AB} = p_A p_B / (p_A + p_B) \]. Where \( p_i \) is the probability of \( i \) (0, 1, or 2) wild type subunits in a dimer \( (n = 2) \), and \( p_A \) and \( p_B \) are the probabilities of encountering a wild type or S839D mutant in the total protein pool depending on the proportions of plasmids transfected.

| Transfected proportions | Type of dimer formed |
|-------------------------|----------------------|
| WT-WT | WT-S839D | S839D-S839D |
| 3:1 | 56.25% | 37.5% | 6.25% |
| 1:1 | 25 | 50 | 25 |
| 1:3 | 6.25% | 37.5% | 56.25% |

It has previously been described that MR is able to heterodimerize with GR and affect its function. Using COS-7 cells as an expression model, Liu et al. (14) demonstrated that co-expression of MR diminishes GR-dependent transactivation, likely due to weak transcriptional activity of heterodimers. To test whether phosphorylation of MR residue Ser-839 affects GR activity, we co-transfected COS-7 cells with MR, WT MR, or phosphomimicking mutant MR-S839D and assessed dose dependence of cortisol-induced transactivation. MR and GR co-expression at a 1:1 ratio decreased luciferase induction by 50% at saturating cortisol concentrations when compared with GR expressed alone (Fig. 8A), consistent with previously published data (14). When GR was co-expressed with MR-S839D at a 1:1 ratio luciferase, transactivation further decreased to 25% that of maximum activity achieved when GR was expressed alone (Fig. 8A). The effect of MR-S839D co-expression on cortisol-induced MR activity was the same as the one detected with aldosterone (Fig. 8B). These observations are consistent with a model where MR-GR heterodimers possess very low or null transactivation capacity, with most of the detected cortisol-induced transactivation being conducted by GR-GR and MR-MR heterodimers. Because phosphorylated MR is inactive and acts as dominant negative toward non-phosphorylated MR, this post-translational modification would only further decrease cortisol-mediated gene transactivation in cells co-expressing MR and GR.

TABLE 3

Subcellular localization of MR and MR-S839D co-expressed in COS-7 cells at different proportions and stimulated with 10 nM aldosterone for 16 h

Values express average percentage of cells in each category as obtained in the experiments shown in Fig. 6C. Rows corresponding to unmixed conditions (only one plasmid transfected) contain dashes to indicate the missing MR form (WT or S839D mutant).

| MR-MR-S839D | N | N>C | N+N>C |
|--------------|---|-----|-------|
| 1:0          | 91.5 | 10.0 | 8.6 |
| 0:1          | 93.7 | 16.7 | 4.4 |
| 3:1          | 90.4 | 3.8 | 6.8 |
| 1:1          | 81.7 | 1.9 | 17.6 |
| 1:3          | 81.7 | 1.9 | 17.6 |

Proportions of transfected plasmids.
Values obtained by adding the percentage of cells in N and N>C categories.

Discussion

Our results show that physiological and supra-physiological doses of aldosterone are able to induce at least partial translocation of phosphomimetic mutants MR-S839D and S839E, but the mutants are totally impaired for gene transactivation. This correlates with an inability to recruit a common MR co-activator, SRC-1. In silico modeling of the effect of Ser-839 phosphorylation on ligand docking energy suggests that this modification displays minor effects on steady-state ligand interaction. When co-expressed with WT receptor, MR-S839D decreased gene transactivation to an extent consistent with a dominant negative role in the SR dimer, consistent with a model where only one phosphorylated unit in the dimer will completely impair MR activity. Finally, our data suggest that MR phosphorylation at residue S839D will further impact cortisol-induced gene transactivation in cells co-expressing MR and GR.

Based on the previous work by Shibata et al. (4) and the data presented in this work, we can clearly see how there is good agreement between WT receptor \( K_d \) (1.07 nM) and \( EC_{50} \) (0.4 nM) for aldosterone. In contrast, there is a large difference when
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Phosphomimetic mutants S839D or S839E (Ser–843 in the human sequence) are examined, with a $K_d$ of 86.7 and 34.3 nM, respectively, but no gene transactivation detectable, even at 1 μM aldosterone (see Fig. 4A). This discrepancy between $K_d$ and EC₅₀ is reminiscent of MR activation by cortisol. $K_d$ for cortisol and aldosterone are in the same order of magnitude (0.5–2 nM), but EC₅₀ for cortisol is 10 nM (for review see Ref. 6). The discrepancy between $K_d$ and EC₅₀ for cortisol-induced MR activation has been explained by the fast off-rate of cortisol from the complex (15–17). If this applies to the case of MR-Ser–839 phosphorylation, then one would expect to see a decreased nuclear translocation rate along with decreased apparent affinity for aldosterone-induced transactivation. Shibata et al. (4) found no aldosterone-induced translocation of the phosphomimetic mutant, but they used a physiological dose of 1 nM aldosterone.

In our experiments we could demonstrate partial translocation of the mutant receptor, with an increasing proportion of nuclear receptor following a dose-response relationship with aldosterone concentration (Fig. 2D). Surprisingly, no transactivation was detected, even at a high dose of aldosterone. Kino et al. (18) found that cyclin-dependent kinase 5-mediated neuronal MR phosphorylation at residues Ser–128, Ser–250, and Thr–159 (all placed in the NTD) dramatically interferes with MR activity but not with ligand-dependent nuclear accumulation. The authors interpreted this effect as a phosphorylation-dependent block of co-regulator interaction with the NTD without affecting hormone binding and receptor transformation associated to translocation. In the case of MR phosphorylation at Ser–839, it appears that, in addition to reduced agonist affinity, ligand binding-induced conformational changes are sufficient for nuclear translocation (even though it is performed at a much lower rate). However, ligand binding is clearly unable to support appropriate co-activator recruitment and gene transactivation, suggesting that the conformational change is either incorrect or unstable. This idea is further supported by recent work by Mani et al. (19) showing that mutation of human MR residue Ser–843 to proline (the amino acid present in the equivalent position in GR) does not affect MR affinity for aldosterone or cortisol but dramatically increases gene transactivation EC₅₀, suggesting an important role for this residue on receptor activation.

The above model is supported by our in silico analysis. Docking calculations of steady-state energy of interaction show that ligand binding is not greatly altered (in the case of cortisol, it is even more stable with the phosphorylated LBD), although an additional effect on ligand access to the binding pocket cannot be excluded. The fact that agonist binding was able to induce nuclear translocation suggests that MR LBD phosphorylation at residue Ser–839 may alter LBD conformational dynamics so that LBD-co-regulator protein-protein interaction interphase is altered (11, 16, 20).

The lack of transactivation function in mutants S839D and S839E made us wonder what would be the functional consequence of dimer formation between WT and phosphomimetic mutant receptors. Shibata et al. (4) showed that cells co-expressing wild-type MR and MR-S843E and treated with 1 nM aldosterone for 1 h display full wild-type MR translocation and no MR-S843E translocation. Therefore, the authors proposed that loss of aldosterone binding and lack of subsequent nuclear translocation produces the loss of gene transactivation. However, to explain the nuclear translocation data, one must assume that WT MR and MR-S843E do not dimerize and, therefore, act independently of one another. We examined this question in detail by co-transfecting MR WT and S839D mutant. Western blotting and PLA data (Fig. 6) demonstrate that both receptors were expressed in the expected proportions and, most importantly, that they appeared to dimerize freely. This is not surprising as the main determinant of receptor dimerization is the DNA binding domain, not the LBD (14). Under these conditions, the effect of S839D on aldosterone-induced MR activity fitted perfectly with a model where only
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WT-WT dimers are active, strongly suggesting that heterodimers of phosphorylated and non-phosphorylated MR would be functionally inactive. This may be explained by both units in the dimer adopting a productive conformation to be able to simultaneously recruit co-regulators. Given that MR-S839D shows impaired interaction with co-activator SRC-1, it could be the case that asymmetrical recruitment of co-activators by only one unit in the dimer (the WT one) is not sufficient to promote gene transactivation. A very important consequence of this fact is that at physiological levels of circulating aldosterone, which are in the low nm range, 50% of phosphorylated receptor would totally prevent aldosterone-dependent gene transactivation (see Fig. 6D). In addition, the effect of MR on decreasing GR activity (14) combined with MR phosphorylation at residue Ser-839 would imply a highly reduced cortisol-mediated gene transactivation in cells that co-express MR and GR.

In summary, we have shown that inactivation of an SR by phosphorylation in the LBD most likely arises from an uncoupling between ligand binding and receptor conformational changes, partially impairing nuclear translocation but mainly interfering with co-activator recruiting. The physiological effect of this post-translational modification will likely be amplified by the fact that it acts in a dominant-negative fashion when modified and unmodified receptors dimerize.

Experimental Procedures

Plasmid Constructs

Generation and use of a functional fluorescent derivative of MR with insertion of YFP after amino acid 147 has been previously described (5, 21). A derivative of that construct substituting YFP by three copies of the HA epitope was generated by commercially available monoclonal antibody (clone HA.11, Covance, catalogue number MMS-101R, Madrid, Spain). Anti-mouse or anti-rabbit peroxidase conjugates (GE Healthcare) were used at 1:10,000 dilution. Western blots were developed with Immun-Star WesternC kit (Bio-Rad), and signals were detected with a Chemidoc imaging system (Bio-Rad).

Western Blotting Analysis

MR protein expression was analyzed by Western blotting as previously described (7). Anti-MR 1D5 monoclonal antibody developed by Gomez-Sanchez et al. (23) was obtained from the Developmental Studies Hybridoma Bank, created by the NICHD, National Institutes of Health and maintained at The University of Iowa Department of Biology. Rabbit polyclonal anti-GFP antibody was the kind gift of Dr. Raimundo Freire (Hospital Universitario de Canarias, Spain) and has been previously described (24). HA-tagged proteins were detected using a competitive binding assay essentially as described (19). Briefly, transfected cells were incubated for 1 h in serum-free medium and then treated with [3H]aldosterone (PerkinElmer Life Sciences; 1 nM for wild-type MR or 20 nM for S839D and S839E mutants) for 2 h at 37 °C in the presence of increasing concentrations of unlabeled aldosterone. Bound aldosterone was extracted with 80% ethanol, and radioactivity was measured by liquid scintillation. Specific binding was calculated by subtracting disintegrations per minute (dpm) obtained in the presence of 10 μM unlabeled aldosterone. Half-maximal inhibitory concentrations (IC50) were then calculated using nonlinear regression analysis of Prism 5 (GraphPad, San Diego, CA). Equilibrium dissociation constants (Kd) were then calculated from the IC50 using the Cheng-Prussoff equation (25) in Prism 5 and assuming that the inhibition constant (Ki) are the same.

Analysis of MR Subcellular Localization and Nuclear Translocation Dynamics

Semiquantitative analysis of subcellular distribution in the absence of aldosterone was performed as previously described (7, 8, 26). Briefly, cells were fixed, images were taken under a confocal microscope, and at least 75 cells per condition were scored into five categories (N, exclusive nuclear localization; N > C, predominant nuclear localization; N = C, even distribution throughout cytosol and nucleus; N < C, predominant cytosolic localization; C, exclusive cytosolic localization). Data are shown as the percentage of cells in each category from the total amount of cells scored. To detect MR-3×HA, cells were immunostained following previously published procedures (26) using a monoclonal antibody against the HA epitope (clone
HA.11, Covance) and goat anti-mouse secondary antibodies conjugated to Alexa fluor 594 (Invitrogen). Images were collected using a Fluoview 1000 confocal microscope (Olympus, Barcelona, Spain). Kinetic analysis of aldosterone-induced MR nuclear translocation was performed as previously described (21, 26). Briefly, cells were transfected with MR-YFP and grown for 48 h in DMEM supplemented with charcoal-stripped FBS. Cells were then transferred to extracellular saline (137 mM NaCl, 4 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose, 10 mM HEPES, pH 7.4), placed under the confocal microscope in a temperature-controlled environmental chamber set at 37 °C, and treated by adding 10 nM aldosterone to the medium. Images were collected at a sampling rate of 1 every 2 min for 1 h. Quantitative analysis of MR-YFP distribution was performed frame-by-frame using the manufacturer’s software (Olympus). Controls in the absence of aldosterone were performed to control for photo-bleaching of YFP. Data processing and sigmoid curve-fitting were performed using Prism 5 (GraphPad) according to Equation 1:

\[
F = F_0 + \frac{F_{\text{max}} - F_0}{1 + \exp \left( \frac{t_{1/2} - t}{V_n} \right)}
\]

where \(F_0\) is the initial nuclear fluorescence, \(F_{\text{max}}\) is the maximal nuclear fluorescence reached, \(t_{1/2}\) is the time (min) at which fluorescence is halfway between \(F_0\) and \(F_{\text{max}}\), and \(V_n\) is a factor determining how steeply nuclear accumulation changes with time.

**Analysis of MR Transactivation Function**

Different proportions of WT and mutant MR were co-transfected with a plasmid containing firefly luciferase under the control of a synthetic promoter with two copies of the glucocorticoid/mineralocorticoid-responsive element (GRE2X; kindly provided by Dr. Rainer Lanz, Baylor College of Medicine, Houston, TX). Transfection efficiency was controlled by co-transfection of a plasmid encoding GFP antibody (a kind gift from Dr. Raimundo Freire) and mouse monoclonal anti-HA antibody (clone HA.11, Covance, Madrid, Spain) and were validated by immunocytochemistry using previously described procedures (5, 7). Negative controls consisted on omitting one of the transfected plasmids. When indicated, cells were treated with 10 nM aldosterone overnight. Results were quantified using the software provided by the manufacturer (Duolink Image Tool) and are expressed as average number of puncta/cell area.

**Structure Modeling and Ligand Docking**

**General**—All calculations were performed on a Windows 7 PC with six core Intel i7–4930K processor (3.4GHz) with 16 gigabytes total RAM using Schrödinger’s Biologics and Small-Molecule Drug Discovery suites of software programs (Schrödinger, LLC, New York).

**Ligand Preparation**—LigPrep was used to produce low energy three-dimensional conformations of all docked compounds, and Epik was used to generate their ionization/tautomeric states. The chiralities of the compounds were retained from the original state, and ligands conformations were minimized using OPLS-2005 force field.

**Protein Preparation**—The receptor ligand binding domain crystallographic structure was loaded from the Protein Data Bank (PDB) and prepared by using Protein Preparation Wizard. We used the structure with the PDB ID 3VHU (12). We assigned bond orders, added missing H atoms, and filled in the missing loops and the side chains using Prime. Water molecules beyond 5 Å from co-crystallized ligand were deleted, and ionization/tautomeric states were generated at pH 7.0 ± 2.0 using Epik. Afterward, the protein structures were refined by optimizing hydrogen bonds (H-bonds) and sampling water molecules orientations. At the end, a restrained minimization (<0.30 Å for each heavy atom) was performed using OPLS-2005 force field.

**Ligand Docking**—All the docking calculations were performed with Glide SP algorithm with the final scoring using the GlideScore. Docking grids were generated by Glide using the co-crystallized ligand at the center of the grid box. The compounds were docked flexibly, and after docking we kept the 20 best docked conformations for each pair ligand/receptor.

**Receptor Modeling**—Receptor variants of interest (the phosphomimetic and the phosphorylated ones) were generated using BioLuminate, which uses a rotamer library in Prime for side-chain sampling with a variable dielectric treatment of polarization from the protein side chains and an implicit solvent surface generalized born model with the OPLS-2005 force field for energy evaluations. The receptor variants were generated by sampling and refining all residues side chain and backbone atoms within 5 Å of the mutated residues using Prime side-chain prediction combined with backbone sampling.

**Author Contributions**—R. J.-C. conducted most of the experiments. R. J.-C. and D. A. R. analyzed the results and wrote most of the paper. M. X. F. conducted in silico simulations and contributed to analyzing and interpreting data and writing of the paper. D. A. R. conceived the idea for the project. All authors corrected and approved the final version of the manuscript.

**Acknowledgments**—We thank Dr. Nicolette Farman, Dr. Raimundo Freire, Dr. Fátima Gebauer, and Dr. Rainer Lanz for the kind gift of reagents.
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