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Nuclear Shuttling Precedes Dimerization in Mineralocorticoid Receptor Signaling

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SUMMARY

The mineralocorticoid receptor (MR), a member of the steroid receptor superfamily, regulates water-electrolyte balance and mediates pathophysiological effects in the renocardiovascular system. Previously, it was assumed that after binding aldosterone, the MR dissociates from HSP90, forms homodimers, and then translocates into the nucleus where it acts as a transcription factor (Guiochon-Mantel et al., 1989; Robertson et al., 1993; Savory et al., 2001). We found that, during aldosterone-induced nuclear translocation, MR is bound to HSP90 both in the cytosol and the nucleus. Homodimerization measured by eBRET and FRET takes place when the MR is already predominantly nuclear. In vitro binding of MR to DNA was independent of ligand but could be partially inhibited by geldanamycin. Overall, here we provide insights into classical MR signaling necessary for elucidating the mechanisms of pathophysiological MR effects and MR specificity.

INTRODUCTION

Steroid receptors are ligand-dependent intracellular receptors belonging to the nuclear receptor superfamily comprising the progesterone, estrogen, androgen, glucocorticoid, and mineralocorticoid receptor (MR). They possess a common structure with six domains A–F. The N-terminal A/B domain is the most variable among the receptors and contains an autonomous activation function. The C domain is highly conserved and possesses a DNA-binding domain with two zinc fingers. It is followed by a hinge region, D, and a carboxy-terminal ligand-binding domain. Previously, the MR with its classical role in water-electrolyte and blood pressure homeostasis was the least appreciated member of the steroid receptors. New interest in MR arose when clinical studies suggested that MR antagonists are beneficial for patients with cardiac remodeling and renal disease (Chrysostomou et al., 2006; Pitt et al., 2003). Since then, MR activation has been shown to be involved in a variety of pathophysiological effects in the renocardiovascular system including endothelial dysfunction, inflammation, hypertrophy, and fibrosis in animal experiments (Blasi et al., 2003; Brilla and Weber, 1992; Sartório et al., 2007). The exact mechanisms for these MR effects are still unclear.

The classical view is that MR resides in the cytoplasm in its unliganded form coupled to chaperone molecules like HSP90 (Sanchez et al., 1985). The interaction with HSP90 is a prerequisite for stabilization of the ligand-binding structure of the MR (Nemoto et al., 1993). Upon binding of ligand, the MR undergoes a conformational change and HSP90 dissociates from the receptor (Couette et al., 1998; Hellal-Levy et al., 2000). With the help of nuclear localization signals (NLSs), the MR is then transported to the nucleus (Piwien Pilipuk et al., 2007; Walther et al., 2005). After nuclear translocation, MR binds to hormone response elements as a homodimer and regulates the expression of its target genes (Drouin et al., 1992; Tsai et al., 1988; Wrange et al., 1989). The glucocorticoid receptor (GR) binds to the same hormone response elements (glucocorticoid response element; GRE) but nevertheless elicits different effects (Arriza et al., 1987). Recently, HSP90-dependent trafficking of steroid receptors was suggested, and an interaction between steroid receptors and molecular chaperones with nuclear pore components was demonstrated (Echeverría et al., 2009; Galigniana et al., 2010). Our aim was to elucidate the mechanism of MR translocation, dimerization, and DNA binding further and thereby extend the understanding of MR signaling.

RESULTS

Localization of MR in the Cell

To determine the localization of the MR in the cell, a fusion protein between MR and enhanced green fluorescent protein (EGFP) was utilized. HEK cells were transfected with this construct and localization of MR was tested by fluorescence microscopy 1.5 hr after incubation with either vehicle (DMSO), 10 nM aldosterone (MR agonist), 100 nM spironolactone (MR antagonist), 4 μM geldanamycin (HSP90 inhibitor), or 10 nM aldosterone with 4 μM geldanamycin (Figure 1A).

Unstimulated MR was predominantly located in the cytosol and resided in this location after incubation with spironolactone, geldanamycin alone, or geldanamycin + aldosterone. After incubation with 10 nM aldosterone, MR was mainly detected in the nucleus.

Translocation of MR to Nucleus and HSP90

Aldosterone-induced translocation of the MR into the nucleus was measured by time lapse recordings (Figure 1B). At a temperature of 37°C, a rapid translocation of the MR was visible with a half-life (T1/2) = 6.9 min (Figure 1C).

To verify that HSP90 is not only located in the cytosol but also in the nucleus, we detected HSP90 in MR-transfected HEK cells

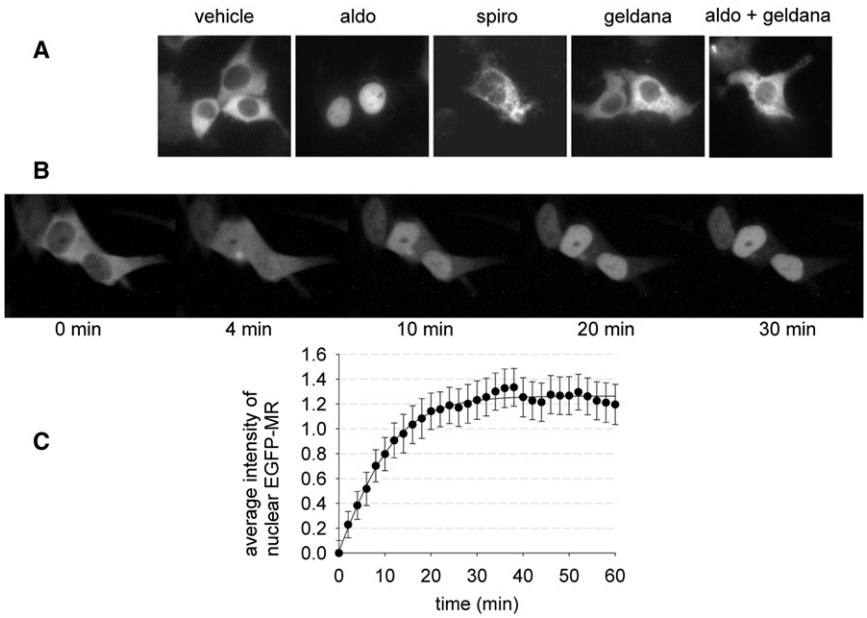

Figure 1. Localization of MR in Cell

(A) Localization of EGFP-hMR was detected by fluorescence microscopy in HEK293 cells transiently transfected with EGFP-hMR and incubated with vehicle (DMSO 1:1000), 10 nM aldosterone (aldo), 100 nM spironolactone (spiro), 4 μM geldanamycin (geldana), or 4 μM geldanamycin + 10 nM aldosterone (aldo + geldana) for 1.5 hr.

(B) For further information about the time course of the cytoplasmic-nuclear shuttling, transiently transfected EGFP-MR-HEK cells were incubated with 10 nM aldosterone and time lapse recordings were performed in an incubation chamber at 37°C.

(C) Increase in average intensity of the nucleus was measured in EGFP-MR-transfected HEK cells after incubation with 10 nM aldosterone (n = 10; data presented as mean ± SEM).

by immunofluorescence staining (Figure 2A). Cells shown were incubated with 10 nM aldosterone, but no visible difference in HSP90 distribution was found between aldosterone-stimulated or -unstimulated cells. These results were confirmed by western blot experiments in rigorously washed cytosolic and nuclear cell extracts of MR- and GR-transfected HEK cells (Figure 2B). These data show that HSP90 is not only located in the cytosol but also, to a lesser degree, in the nucleus. MR activation did not lead to a gross redistribution of HSP90.

To investigate the role of the chaperone HSP90 for MR nuclear translocation, we transfected HEK cells with EGFP-MR, immunoprecipitated the MR by its EGFP tag, and then detected coimmunoprecipited HSP90 by western blot. In whole cell lysates of unstimulated cells, MR was associated with HSP90. After incubation with aldosterone, the amount of coimmunoprecipitated HSP90 stayed unchanged within the first 6 min and then rapidly decreased to a minimum of 17% ± 5% after 30 min compared to cells without aldosterone stimulation. During the first 6 min of aldosterone incubation, nuclear translocation reached its half-maximal values while HSP90-MR coimmunoprecipitation was still unchanged. When immunoprecipitating the EGFP-MR in nuclear extracts and then detecting HSP90, the amount of MR-HSP90 complexes detected in nuclear extracts was not reduced but even enhanced (1.8-fold) after 6 min incubation with aldosterone compared to incubation without ligand as shown in a representative blot in Figure 2D. These data suggest that HSP90 does not dissociate from MR directly after its binding of aldosterone but that HSP90 is still attached to the MR when the MR enters the nuclear compartment.

Homodimerization of MR

The traditional GRE consists of 15 base pairs (bp) with an inverted repeat structure: 5′-AGRACAnnnTGTYCT-3′. Binding of MR to DNA classically involves formation of homodimers. To investigate MR homodimerization in vivo and in real time, we established an extended bioluminescence resonance energy transfer (BRET) system (eBRET) for monitoring MR-MR interactions. MR was coupled to either renilla luciferase or enhanced yellow fluorescent protein (EYFP). Four stably transfected pRLuc-MR HEK cell clones were chosen for further eBRET experiments. To optimize the amount of transiently transfected pEYFP-MR in our eBRET measurements, we determined BRET ratios for different concentrations of pEYFP-hMR when incubating cells with either vehicle or 10 nM aldosterone (Figure S1A available online). To exclude accumulation and unspecific interactions of proteins, we also measured BRET ratios for stable pRLuc (vector only) HEK clones with comparable concentrations of EYFP-MR (Figure S1B). With the optimized MR-MR eBRET system, we then measured BRET ratios after incubation with 10 nM aldosterone and compared them to the changes induced by the competitive MR antagonist spironolactone (Figure 3A). Aldosterone caused a significant increase in BRET ratio within the first 15 min of incubation, suggesting that MR-MR dimerization commences at this time point. Incubation with 100 nM spironolactone did not lead to an increase in BRET ratio, indicating that spironolactone does not induce MR homodimerization. Additional eBRET experiments were performed with geldanamycin (Figure 3B). It is interesting that BRET ratios increased within 15 min to levels comparable to those obtained after incubation with aldosterone. These results suggest that binding of the MR to HSP90 prevents MR dimerization and that dissociation from HSP90 enables MRs to come into close vicinity of one another, forming spontaneous homodimers even in the absence of aldosterone.

To gain information about the localization of the MR-MR dimerization, we correlated eBRET results with fluorescence microscopy and time lapse experiments. Nuclear translocation of MR occurred rapidly with T1/2 = 6.9 min (Figure 1C). The eBRET measurements show that homodimerization occurs within the first 15 min. For more rapid measurements, fluorescent resonance energy transfer (FRET) between EYFP-MR (excitation/emission maxima, 514 nm/527 nm) and RFP-MR (excitation/emission maxima, 558 nm/583 nm) was utilized (Figure 4A). The function of the generated fusion proteins for BRET and FRET was ensured by reporter gene assays and optionally

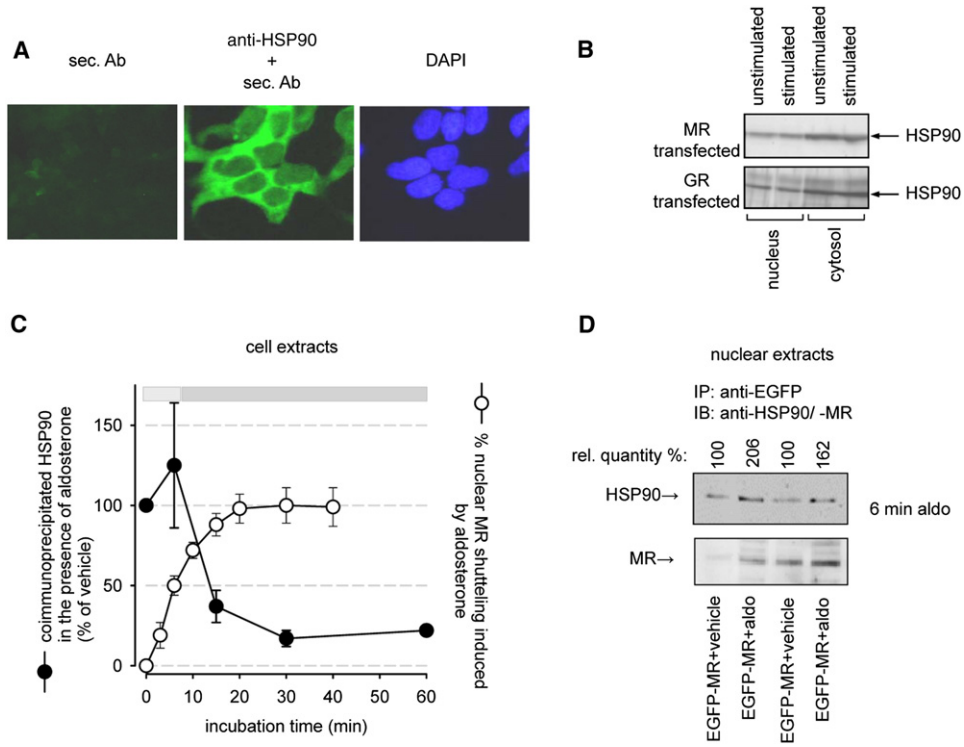

Figure 2. HSP90 and Nuclear Translocation of MR

(A) Transiently MR-transfected HEK cells were incubated with 10 nM aldosterone for 30 min. Immunofluorescent staining against HSP90 with Oregon Green secondary antibody was performed (middle) with additional DAPI staining to indicate the nuclei (right). Incubation with only secondary antibody did not lead to staining of cells (left). Images are taken from z-stacks obtained with a BZ-8000 microscope.

(B) HEK cells transiently transfected with MR or GR plasmid were incubated for 1 hr with DMSO (unstimulated) or 10 nM aldosterone for MR/100 nM dexamethasone for GR (stimulated). A cytosolic and a nuclear cell fraction were obtained, and western blotting against HSP90 was performed.

(C) Coimmunoprecipitation experiments of EGFP-MR-transfected HEK cells with EGFP-MR pulldown and subsequent HSP90 immunoblotting were performed with whole cell lysates after incubating the cells for different time points with 10 nM aldosterone. HSP90 was quantified densitometrically and depicted as percentage of unstimulated control incubated with vehicle alone. In the same graph, the percentage of nuclear MR shuttling after stimulation with 10 nM aldosterone is shown for different time points assessed by fluorescent microscopy of EGFP-tagged MR (data presented as mean ± SEM).

(D) Coimmunoprecipitation experiments of EGFP-MR-transfected HEK cells with EGFP-MR pulldown and subsequent HSP90 and MR immunoblotting were performed with nuclear extracts after incubating the cells for 6 min with vehicle or 10 nM aldosterone. A typical experiment is shown suggesting that the amount of MR-associated nuclear HSP90 increases after incubation with aldosterone.

displacement experiments (Figure S2). Within the first 10 min, no significant FRET ratio could be quantified. After 15 min and 60 min, FRET ratios indicating MR-MR dimerization were significantly increased. At this time, the MR has already translocated into the nucleus according to our time lapse experiments, supporting that homodimerization occurs in the nucleus. This was confirmed by single-cell FRET experiments, which show a positive FRET in the nucleus after incubation with aldosterone (Figure 4B). The HSP90 inhibitor geldanamycin also increased the FRET ratio compared to vehicle (Figure 4A). This indicates that MR dimerization is triggered by the dissociation of MR from HSP90—either in the nucleus by providing DNA for binding or in the cytosol promoted by geldanamycin.

Binding of MR to GRE

To investigate DNA binding of activated receptor, we applied a transcription factor ELISA with biotinylated GRE. In vitro translation of mineralocorticoid receptor, glucocorticoid receptor, and beta-galactosidase (Lacz) as negative control was performed in reticulocyte lysates. As assessed by western blot, similar amounts of receptor were translated (Figure 5A). In the transcription factor ELISA, MR and GR displayed comparable binding capacities at their response element GRE (Figure 5B). Next, we compared binding of MR and GR to GRE in the presence and absence of their respective ligands, aldosterone and dexamethasone (Figure 5C). Neither binding of MR nor that of GR was dependent on the presence of ligand. Because reticulocyte lysates contain HSP90 and we previously observed that HSP90 can be found in the nucleus, we investigated the effect of HSP90 on the binding of MR and GR to GRE (Figure 5D). No binding of HSP90 could be detected in MR-GRE or GR-GRE complexes (Figure 5E). Incubation with geldanamycin had no effect on binding of GR to GRE but significantly reduced binding of MR to GRE to 66 ± 8% of control (Figure 5F). To show that this effect is not mediated by enhanced protein degradation of the MR, western blots were performed and provided no evidence for enhanced MR or GR degradation by 1 hr incubation with geldanamycin. Thus, binding of MR or GR to DNA does not seem to involve recruitment of HSP90 to the DNA; however, binding of MR, but not GR, to GRE appears to be facilitated by

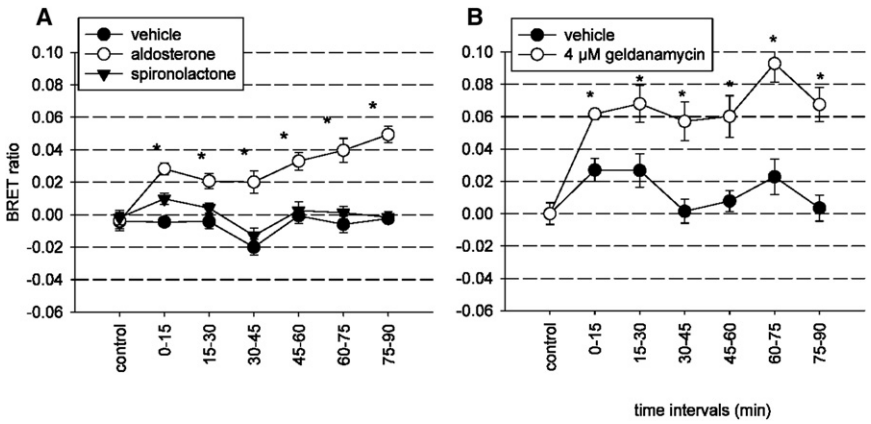

Figure 3. BRET of MR-MR

BRET ratios were determined for stably pRLuc-hMR-expressing HEK293 clones transiently transfected with pEYFP-MR. Cells were preincubated with 60 μM Enduren and then incubated with vehicle, 10 nM aldosterone, or 100 nM spironolactone (A) or with 4 μM geldanamycin (B) for 90 min at 37°C (n = 11–33 for each time interval; data are presented as mean ± SEM; *p < 0.05). See also Figure S1.

HSP90. This marks a difference between MR and GR that could be involved in the different effects of MR and GR at the GRE.

Transactivation of MR

Although translocation of MR to the nucleus, dimerization, and binding to GRE are all necessary for the function of the MR, they do not automatically lead to a transactivation. To test the actual transactivation of MR and GR, we used a reporter gene assay. As expected, transactivation of MR can be achieved by 10 nM aldosterone while GR is activated by 100 nM dexamethasone. Disrupting the function of HSP90 by incubation with geldanamycin prevented MR and GR transactivation in HEK cells (Figures 6A and 6B). These data suggest that the nuclear receptor dimers occurring in the cytoplasm in the presence of geldanamycin are not functionally active because they cannot be transported into the nucleus without HSP90. Dissociation of HSP90 is a prerequisite for dimerization, but transactivation can only occur if the dimers are formed in the nucleus because otherwise nuclear transport is not possible.

To exclude enhanced degradation of MR caused by geldanamycin, MR levels of unstimulated and aldosterone-stimulated cells were quantified after 24 hr and 48 hr in the presence and absence of geldanamycin. Geldanamycin had no visible effect on MR protein levels. Of note, incubation of cells with aldosterone led to an increased smear of the MR bands, suggesting an increase in posttranslational modifications of the stimulated MR. This change did not occur in the presence of aldosterone plus geldanamycin.

DISCUSSION

In the absence of ligand, the MR is predominantly located in the cytosol (Nishi et al., 2004; Piwien Pilipuk et al., 2007). Some groups also describe an equal distribution of MR between cytosol and nucleus in heterologous expression systems (Fejes-Tóth et al., 1998; Nishi et al., 2001; Walther et al., 2005). Additionally, constitutive nuclear localization was postulated in cardiomyocytes, suggesting a cell-type-specific behavior

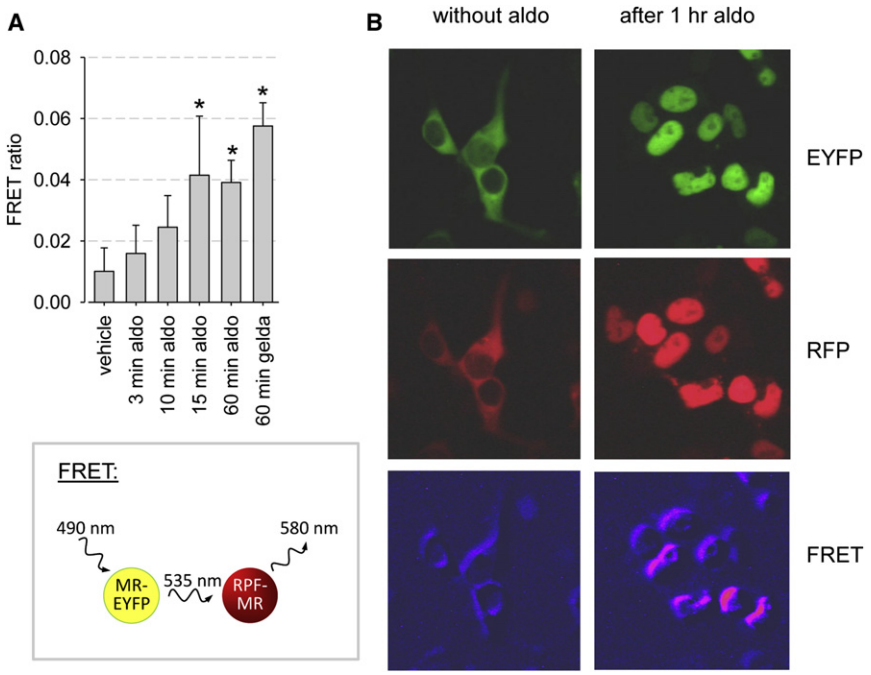

Figure 4. FRET of MR-MR

(A) To investigate the time course of dimerization in more detail, we performed FRET experiments in 96-well plates with RFP-MR and EYFP-MR transfected HEK cells after 0, 3, 10, 15, and 60 min incubation with aldosterone and after 60 min incubation with geldanamycin (n = 6; N = 3; data are presented as mean ± SEM; *p < 0.05) (top). Scheme of FRET experiment (bottom).

(B) FRET index images were acquired from single cells incubated with vehicle or 10 nM aldosterone for 1 hr. EYFP shows the donor emission after excitation of EYFP; RFP shows the acceptor emission after excitation of RFP. FRET displays the intensities of acceptor emission due to FRET with blue indicating no FRET and red indicating high FRET.

See also Figure S2.

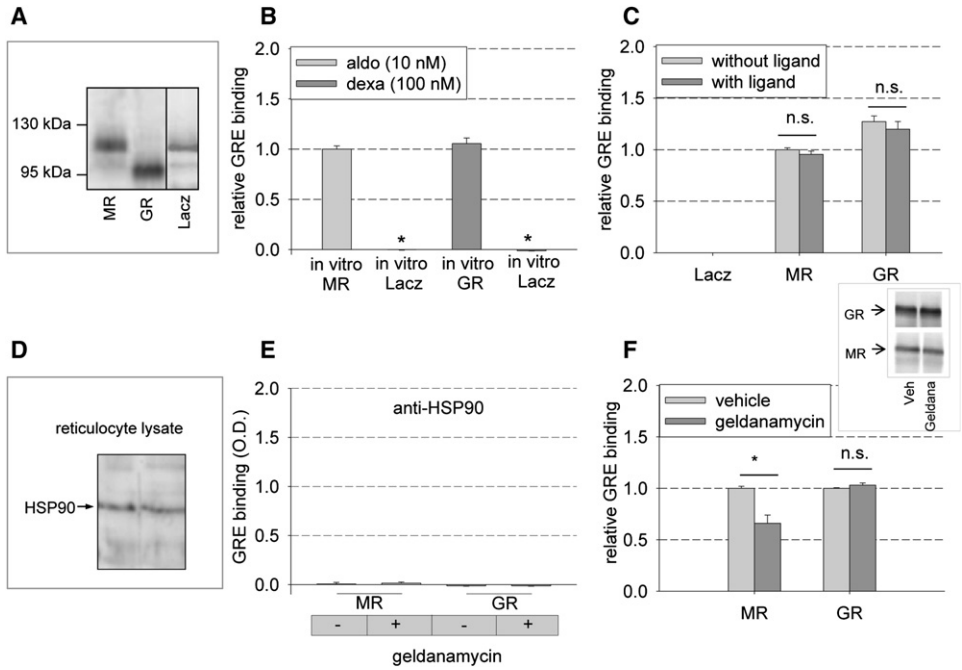

Figure 5. GRE Binding of MR/HSP90

(A) In vitro translation of MR, GR, and Lacz (negative control) was performed in reticulocyte lysates, and the resulting protein concentrations were compared by western blot with an antibody against a common Xpress tag.

(B) Binding capacities of in-vitro-translated MR, GR, and Lacz to GRE were compared in a transcription factor binding assay in the presence of the respective hormone receptor ligands aldosterone or dexamethasone (n = 9–13, N = 5–7; data are presented as mean ± SEM; *p < 0.05).

(C) Binding of in-vitro-translated MR, GR, and negative control Lacz to the glucocorticoid response element was assessed in a transcription factor binding assay in the presence or absence of the respective hormone receptor ligands aldosterone or dexamethasone (n = 8; N = 4; data presented as mean ± SEM; *p < 0.05).

(D) Reticulocyte lysates used for in vitro translation also contain HSP90 as shown here by western blot.

(E) Direct or indirect binding of HSP90 to GRE was tested in our GRE transcription factor ELISA with anti-HSP90 after incubating in vitro MR and GR in the presence or absence of 2 μM geldanamycin with biotinylated GRE (n = 6; N = 4; data are presented as mean ± SEM).

(F) The influence of HSP90 on hormone receptor DNA binding was investigated by testing the binding of MR and GR to GRE in the presence and absence of 2 μM geldanamycin (incubation time, 1 hr) (n = 8–12; N = 4–6; data are presented as mean ± SEM; *p < 0.05). Insert: Representative western blot showing in-vitro-synthesized MR and GR incubated with 2 μM geldanamycin or vehicle for 1 hr.

(Hernández-Díaz et al., 2010). The MR is associated with chaperones including HSP90, HSP70, and p23 and various immunophilins like FKBP51 (Galigniana et al., 2010; Sanchez et al., 1985). By binding to its chaperones, the MR is kept in a conformation that is able to bind aldosterone, and it is also prevented from rapid degradation (Binart et al., 1995; Caamaño et al., 1993). In agreement with our findings, aldosterone rapidly induces translocation of the MR into the nucleus under physiological conditions, i.e., 37°C and pH 7.4 (Fejes-Tóth et al., 1998; Piwien Pilipuk et al., 2007; Walther et al., 2005). Variations in the reported time courses can be attributed to different conditions because trafficking is, for example, highly dependent on incubation temperature (Nishi et al., 2004). Rapid nuclear translocation of MR is thought to be dependent on the NLS regions of the MR. So far, three potential NLS have been identified and a mechanisms involving importin-alpha has been proposed for penetrating the nuclear envelope (Pearce et al., 2002; Tanaka et al., 2005; Walther et al., 2005). Both NLS0 and NLS1 have been shown to be required for aldosterone-induced nuclear translocation of MR (Hernández-Díaz et al., 2010), although a slower additional NLS1-independent mechanism has also been postulated (Piwien Pilipuk et al., 2007). On the contrary, nuclear export takes several hours after steroid withdrawal for the GR and has not been addressed in detail for the MR, with Walther et al. even suggesting a unidirectional transport of MR to the nucleus (Galigniana et al., 1999; Savory et al., 1999; Walther et al., 2003).

Previously, it was hypothesized that binding of ligand causes dissociation of MR from HSP90 and thereby unmasking of NLS with associated nuclear translocation (Picard and Yamamoto, 1987; Walther et al., 2005). This was challenged by experiments showing that mutants without NLS but associated with a NLS-containing-HSP90 also get transported into the nucleus (Kang et al., 1994). Simultaneously, several investigators found that HSP90 is not exclusively located in the cytoplasm but also in the nucleus (Galigniana et al., 2010; Hernández-Díaz et al., 2010). Accordingly, we detected HSP90 in the nucleus when performing western blots with cell fractions and in z-stack images of immunofluorescence images. Our findings are supported by data suggesting that binding of ligand does not cause dissociation of MR from HSP90 but rather a shift in attached proteins from FKBP51 to FKBP52. FKBP52 then creates a link to dynein/dynactin motor proteins, which propagate nuclear translocation (Banerjee et al., 2008; Galigniana et al., 2010).

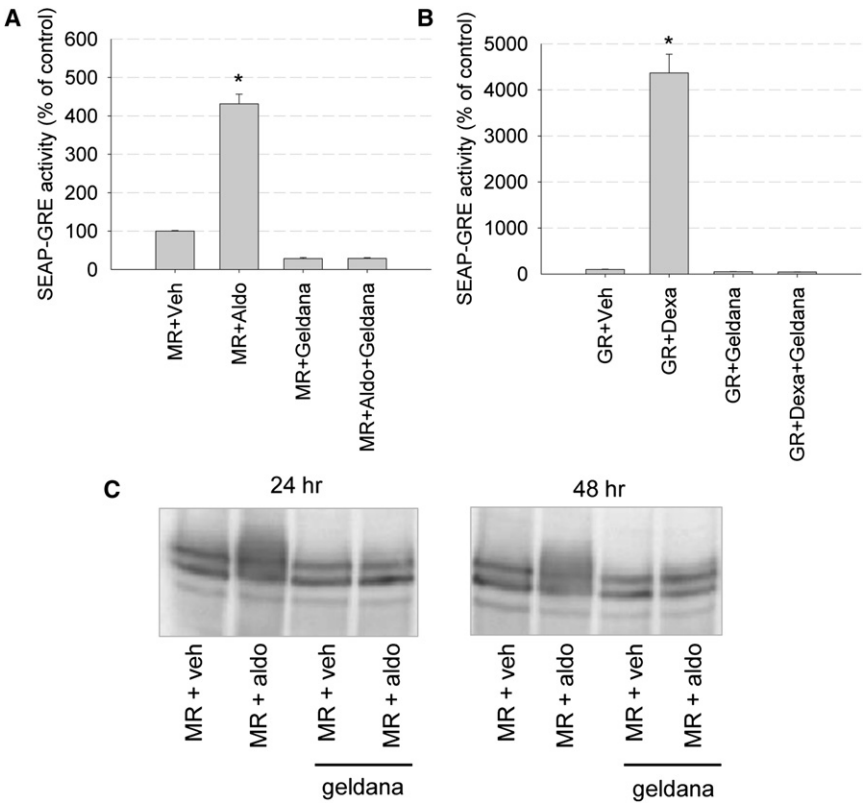

Figure 6. MR Transactivation Activity

(A) Transactivation activity was assessed in a GRE-SEAP reporter gene assay. HEK cells were transfected with MR and incubated for 48 hr with vehicle (control), 10 nM aldosterone, 2 μM geldanamycin, or a combination of 10 nM aldosterone and 2 μM geldanamycin (n = 9; N = 3; data presented as mean ± SEM; *$p < 0.05$).

(B) Transactivation activity was assessed in a secreted alkaline phosphatase (SEAP)-GRE reporter gene assay. HEK cells were transfected with GR and incubated for 48 hr with vehicle (control), 100 nM dexamethasone, 2 μM geldanamycin, or a combination of 100 nM dexamethasone and 2 μM geldanamycin (n = 9; N = 3; data are presented as mean ± SEM; *$p < 0.05$).

(C) Western blot analyses of MR in HEK cells transfected with EGFP-MR and incubated with vehicle (DMSO) or 10 nM aldosterone in either the presence or the absence of 2 μM geldanamycin were performed (n = 3).

This matches our own findings that HSP90 can be coimmunoprecipitated with MR from nuclear extracts and that when 50% of the MR has already translocated into the nucleus, the amount of HSP90 association with MR in cell lysates is still at a maximum level (Figure 2C). Nevertheless, colocalization of MR and HSP90 in the cytoplasm and the nucleus does not prove entirely but suggests that both molecules travel through the nuclear pore together. Alternatively, cytosolic HSP90 could also transport the MR to the nuclear pore, and nuclear HSP90 could then receive the MR after it has entered the nucleus. At present, these two options cannot be distinguished, whereas we regard the first option as more likely.

Formation of homodimers by steroid receptors has been well documented by several authors (Drouin et al., 1992; Tsai et al., 1988; Wrange et al., 1989). Furthermore, formation of heterodimers between MR and GR has been demonstrated, although the physiological relevance is still unclear (Liu et al., 1995; Nishi et al., 2004; Savory et al., 2001). However, the time point and localization of homodimer formation, i.e., whether the association takes place in the cytosol or the nucleus, is still under debate. Based on GR and progesterone receptor DNA-binding experiments, a model involving dimerization prior to binding of DNA is favored in literature instead of consecutive binding of monomers to DNA (Segard-Maurel et al., 1996; Tsai et al., 1988). One reason for this is the lower affinity to DNA of GR monomers compared to dimers (Tsai and O'Malley, 1994). With eBRET analysis, we found that aldosterone leads to MR dimerization within 15 min. To receive data for earlier time points with clear cytoplasmic MR localization, we additionally conducted FRET experiments. We observed an increase in FRET ratio after addition of aldosterone, which reached significance only after 15 min, a time point at which the MR is already predominantly nuclear according to our fluorescence microscopy experiments. Single-cell FRET experiments also indicate that dimerization occurs in the nucleus. This correlates with the exclusive nuclear heterodimerization of MR and GR reported by Nishi et al. (2004), applying FRET analysis in cultured hippocampal neurons and COS-1 cells after incubation with corticosterone. Additionally, GR mutants lacking the NL1 region were not able to translocate into the nucleus when coexpressed with intact MR, suggesting that heterodimerization does not occur in the cytosol. Savory et al. (2001) came to a somewhat different conclusion in COS7 cells after dexamethasone stimulation. They observed that more cells show a predominantly nuclear localization of GR-NLS1 mutants when MR is cotransfected simultaneously and suggested that this behavior can be explained by cytoplasmic dimerization. However, it could also be explained by stronger nuclear retention of GR after binding to MR in the nucleus. Furthermore, these differences could also be due to differences in experimental setup or might reflect an actual difference between GR and MR homodimers and heterodimers.

As a substance that influences steroid receptor function, the antibiotic geldanamycin was investigated in our eBRET system. Geldanamycin mediates its effects by binding with high specificity within the ADP/ATP binding pocket of HSP90, thereby inhibiting the function of HSP90 (Stebbins et al., 1997). Previously, it has been reported that geldanamycin hinders folding and thereby steroid binding activity of steroid receptors. In addition, HSP90 binding to receptor is impaired; therefore, degradation of steroid receptors is enhanced (Czar et al., 1997; Smith et al., 1995; Whitesell and Cook, 1996). Both a lack of and a slowing down of nuclear translocation have also been reported, as well as a loss in transactivation activity (Bamberger et al., 1997; Czar et al., 1997).

We used geldanamycin as an HSP90 inhibitor to depict the functional importance of HSP90 for trafficking and function of MR. We could show that HSP90 inhibition prevents aldosterone-induced nuclear translocation of the MR (Figure 1A), leads to spontaneous cytosolic dimerization of MR without aldosterone as measured by BRET and FRET (Figures 3B and 4A), partially inhibits DNA binding of MR but not GR (Figure 5F), and prevents transactivation of MR at a GRE element (Figure 6A). These results are in agreement with our model, in which MR is kept out of the nucleus without HSP90 even in the presence of aldosterone (Figure 1A). For MR, HSP90 also seems to further overall DNA binding.

Previously, analyses by fluorescence recovery after photobleaching (FRAP) and chromatin immunoprecipitation experiments suggest that binding of steroid hormone receptors to their promoter elements involves rapid cycling, perhaps with the rate of exchange influencing transcriptional levels. HSP90 has been shown either to initiate disassembly of the receptor from the promoter and the transcriptional regulatory complex or to stabilize binding of receptor to the promoter and thereby modify the receptor cycling at DNA (Freeman and Yamamoto, 2002; Kang et al., 1999; Stavreva et al., 2004). In both cases, HSP90 is involved in the highly dynamic process of steroid receptor binding to DNA, which implies that it also influences transactivation activity and possibly also degradation of receptors.

Furthermore, homodimerization of MR seems to occur as soon as MR is released from HSP90, even in the cytosol and therefore independently of DNA. The observation that dissociation of steroid receptor from HSP90 is a prerequisite for homodimerization is shared by Savory et al., who found that in-vitro-translated GRs form dimers independently of added DNA and that dissociation of MR from the chaperones by treatment with 0.4 M NaCl also leads to formation of dimers even in the absence of ligand (Savory et al., 2001). In our experiments, geldanamycin-induced cytosolic dimers did not lead to transactivation. Likewise, a divalent antibody enforcing progesterone receptor dimerization is able to promote DNA binding in the absence of ligand in vitro, but it cannot induce transcription (Allan et al., 1992). Overall, two kinds of dimers seem to form after dissociation from HSP90; however, only the dimers formed in the nucleus after ligand binding seem to be genomically functionally active.

As another substance, the MR antagonist spironolactone was tested in our experiments. Spironolactone prevented both nuclear translocation and dimerization assessed by eBRET. Similar results have been reported by others, thus showing a distinct behavior of antagonists versus agonists at steroid receptors (Couette et al., 1998; Fejes-Tóth et al., 1998; Huyet et al., 2007; Lombès et al., 1994; Tsai and O'Malley, 1994).

In our transcription factor ELISAs, geldanamycin reduced binding of MR to DNA while the binding of GR was not affected. Nevertheless, HSP90 enhances DNA binding of MR without being bound to DNA itself. A possible mechanistic explanation for this is the metastable structure of the N-terminal domain of steroid receptors (Bain et al., 2007). While smaller receptors like the hGR, with its 440-amino-acid (aa)-long N terminus, only require HSP90 for their stabilization in the cytosol, the larger hMR, with its 602-aa-long N terminus, presumably is more unstable and therefore requires the support of HSP90 for stabilization in both cytoplasm and nucleus until it is bound to DNA.

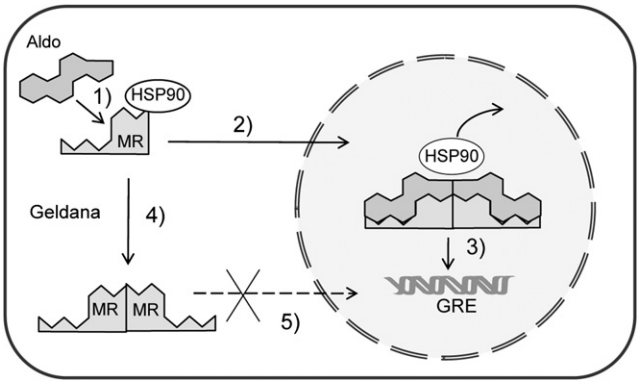

Figure 7. Model of Genomic MR Activation

Unstimulated MR resides in the cytosol bound to HSP90 (1). Aldosterone can bind to HSP90-bound MR, which then translocates into the nucleus together with HSP90 (2). In the nucleus, HSP90 dissociates from the MR, which then forms homodimers and binds to DNA (3). Alternatively, geldanamycin can inhibit HSP90 function, which causes the MR to form homodimers in the cytosol (4). These homodimers without HSP90 are not able to translocate into the nucleus and increase GRE activity (5).

Our findings conflict with previous results acquired by chromatin immunoprecipitation showing an interaction between GR and promoter of GRE liver genes. This discrepancy may arise from the different experimental settings. Our ELISA was performed in vitro with oligonucleotides containing only GRE. Possibly, a longer sequence would be needed for HSP90 association. Additionally, our in vitro system might lack cofactors necessary for binding of HSP90. Although binding of MR and GR to DNA does not depend on the presence of ligand, no transactivation of GR or MR occurs without ligand or after incubation with geldanamycin, because no facilitated translocation into the nucleus is possible in the absence of ligand or HSP90 (Figure 1A) (Nemoto et al., 1993).

Taken together, our results support the following model of MR activation (Figure 7): in its inactive form, the MR is located in the cytoplasm associated with HSP90 and other chaperones. At this time point, the chaperones are necessary to prevent MR degradation and to keep the MR in a form able to bind ligand. Upon ligand binding (1), a change in associated proteins takes place and the MR travels to the nucleus bound to HSP90 (2). Of note, nuclear trafficking has been also described in the absence of HSP90 but then occurs much slower (Galigniana et al., 2010). In the nucleus, HSP90 facilitates binding of the MR to DNA and then dissociates from the MR (3). Dissociation of HSP90 is a prerequisite for MR homodimerization. However, only homodimers formed in the nucleus seem to be able to regulate gene expression, while those formed in the cytoplasm after incubation with geldanamycin (4) do not possess the ability to translocate to the nucleus and influence transactivation (5). HSP90, therefore, is important for nuclear transport, and its absence is a prerequisite for dimerization. HSP90 itself does not bind to the DNA and presumably is recycled to the cytoplasm after binding of receptor to DNA. Ligand, on the other hand, seems to be necessary for the HSP90-dependent transport of MR into the nucleus but is not necessary for DNA binding. While HSP90 furthers the binding of MR to DNA, it does not affect DNA binding of GR.

Because MR and GR both bind to the same hormone response element but elicit very different effects, such differences are interesting for explaining MR specificity. Gaining further insights into non-classical MR signaling is of value to understand the mechanisms of the pathophysiological effects of the MR with the option of therapeutical intervention.

SIGNIFICANCE

In the present study, we mechanistically investigate the genomic signaling of steroids via nuclear receptors using aldosterone and the mineralocorticoid receptor (MR). Originally, the MR was thought to be important mainly for water-electrolyte homeostasis. New interest in MR signaling arose recently when clinical studies and experimental data demonstrated that the MR can convey pathophysiological effects including inflammation, remodeling, and endothelial dysfunction in the cardiovascular system and the kidneys. Nevertheless, the underlying mechanism for these pathophysiological MR effects is still unclear. Additionally, the mechanism for MR specificity over its closest relative the glucocorticoid receptor with which it shares a common hormone response element needs to be elucidated. We found that upon ligand binding, the MR shuttles into the nucleus and that during trafficking binding of MR to HSP90 is detectable both in the cytosol and the nucleus. MR homodimerization requires dissociation from HSP90 and is possible in the cytoplasm (induced by geldanamycin) and the nucleus (physiological). However, only the nuclear formed MR dimers induce transactivation at the hormone response element. Aldosterone, the endogenous MR ligand, initiates nuclear translocation but is not necessary for DNA binding of MR in an in vitro system. HSP90, on the other hand, furthers binding of MR to DNA but does not influence binding of the closely related glucocorticoid receptor, which is information of interest when investigating MR specificity. Overall, these findings contribute to the mechanistic understanding of the signaling of steroid receptors and facilitate the investigation for potential cytoplasmic interaction partners of the MR. This is of special interest for solving the enigma of MR specificity and to understand and ultimately develop strategies to prevent pathophysiological aldosterone effects.

EXPERIMENTAL PROCEDURES

Cell Culture and Vectors

HEK293 cells lacking MR expression were acquired from ATCC (Rockville, MD) and cultivated in DMEM/Ham's F-12 medium supplemented with 10% fetal calf serum at 37°C with 5% CO_2. Transient transfections were performed with Polyfect Reagent (QIAGEN), according to the manufacturer's instructions. Prior to experiments, cells were made quiescent by incubation in medium without serum or supplements for at least 24 hr.

pRLucC1 vector was purchased from Perkin Elmer. MR from pEGFP-hMR (a kind gift of N. Farman) was cloned into pRLucC1 with BglII and HindIII, into pDsRED2-N1 (Clontech) with BglII and KpnI to obtain pRFP-MR, into pEYFP-C1 (Clontech) with BglII and SacII to construct pEYFP-MR, and into pcDNA3.1HisC (Invitrogen) after restriction with BglII/BamHI and HindIII to acquire pcDNA3.1HisC-MR. We constructed pcDNA3.1His-GR by restricting pcDNA1-GR (a kind gift from M. Govindan) with EcoRI and cloning the fragment into pcDNA3.1HisB (Invitrogen). To obtain stable pRLuc-MR clones, HEK cells were transiently transfected with pRLuc-MR, selected with G418 (600 mg/l), and cloned by dilution.

Cell Fractionation

Cells were cultivated in 10 cm culture dishes and transfected with pcDNA3.1HisC-MR and pcDNA3.1HisB-GR. After 48 hr under quiescent conditions, cells were incubated with vehicle, aldosterone (10 nM), or dexamethasone (100 nM) for 1 hr. The isolation of the nuclear and cytoplasmatic extracts were performed using the Nuclear Extract Kit from Active Motif as recommended by the manufacturer. The protein content was determined using Bradford reagent.

Western Blot

Cells were washed, harvested, and lysed in Cell Signaling Technology (CST) buffer (20 mM Tris HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophysphate, 1 mM Na_3VO_4) supplemented with protease inhibitor cocktail I from Calbiochem. Subsequently, cell lysates were matched for protein content, separated by SDS-PAGE, and transferred to polyvinylidene fluoride (PVDF) membrane. Membranes were blotted with the respective antibodies (rMR1-18, DSHB; anti-GR, Santa Cruz; anti-HSP90, Santa Cruz; anti-Xpress, Invitrogen for pcDNA3.1HisMR, pcDNA3.1HisGR, and pcDNA3.1HisLacz).The bound primary antibody was visualized using horseradish peroxidase-conjugated secondary immunoglobulin G and the ECL system (Amersham Corp.). For the detection of western blots, the ChemiDoc XRS gel documentation system from Biorad was used together with the Quantity One software for quantification of bands. Only signals obtained within the linear range were analyzed.

Coimmunoprecipitation

Cells were transfected with EGFP or EGFP-hMR and then incubated with aldosterone or vehicle for the indicated amounts of time. Coimmunoprecipitations were performed with GFP-coupled microbeads and μ columns from Miltenyi as recommended by the manufacturer. Lysates from whole cells or nuclear extracts were used as indicated. Proteins were eluted from the columns and detected by western blot.

ELISA-Based Transcription Factor DNA Binding Assay

In-vitro-synthesized MR, GR, and Lacz (as a negative control) were synthesized using the TNT T7 Quick Coupled Transcription/Translation system (Promega). GRE probe was hybridized from biotinylated GRE oligonucleotides (sense: 5′-Biotin ctagcggtacattttgttctagaac-3′; antisense: Biotin-5′ gttctagaac aaaatgtaccgctag-3′) and was then immobilized in streptavidin-coated wells. Subsequently, hMR, hGR, or Lacz was added to the wells and incubated for 1 hr at room temperature with blocking buffer (5% bovine serum albumin [BSA] in PBS/Tween 0.05%). After three washes, the transcription factor or LacZ bound to the biotinylated GRE probe was detected by using a specific anti-XPRESS antibody (1:2,000; Invitrogen) and a horseradish peroxidase-coupled secondary antibody followed by a specific colored substrate reaction with o-phenylenediamine.

Fluorescence Microscopy

Cells were cultivated on coverslips and analyzed using an inverted microscope (Zeiss Axiovert 135) equipped with 40× and 100× flurorescence objectives. Images were obtained using an intensified charge-coupled device camera (Hamamatsu, Herrsching, Germany). Time lapse experiments and immunofluorescence images were analyzed with a Biozero 8000 fluorescence microscope from Keyence (Tokyo, Japan).

Time Lapse Experiments

Time lapse experiments were performed in an incubation chamber in microdishes (IBIDI) in HEPES buffer with a digital BZ-8000 fluorescence microscope with integrated camera and corresponding software from Keyence (Osaka, Japan).

Immunofluoresence

HEK cells were cultivated on glass coverslips, fixed with 4% formaldehyde (15 min), washed with PBS, and permeabilized with 0.5% Triton X-100. Subsequently, cells were incubated for 10 min in 1% SDS/PBS, followed by 10 min in 100 mmol/l glycine/PBS, and finally, 20 min in 10% serum/1% BSA/PBS. After 60 min incubation with anti-HSP90 (Santa Cruz), cells were

washed with PBS, incubated for 45 min with Oregon Green antirabbit secondary antibody and washed again with PBS. Subsequently, DAPI staining was performed. Cells were analyzed with the z-stack function of the Biozero 8000 fluorescence microscope from Keyence.

eBRET

For eBRET, stable pRLuc-hMR clones were seeded in 96-well plates and transiently transfected with pEYFP-hMR or pcDNAHisC-MR as described earlier. Prior to experiments, cells were cultivated in medium without supplements for 24 hr. For eBRET itself, cells were incubated with 60 μM EnduRen in Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 medium without supplements. After 90 min incubation time, basal luminescence was measured; vehicle (dimethyl sulfoxide; DMSO), 10 nM aldosterone, 100 nM spironolactone, or 4 μM geldanamycin was added; and further luminescence measurements were performed for up to 105 min with a 535 nm and a 460 nm filter in a multilabel counter (Victor^2, Wallac). Final concentration of DMSO added to the cells was $\leq$1:1,000. To take into account the overlap between emission and excitation spectra of donor and acceptor entities, BRET measurements were performed as BRET ratios (emission EYFP [535 nm]/emission RLuc [460 nm]). This eliminates variations in cell numbers per well, expression of proteins, and substrate concentrations. To account for spillover of light, the ratio emission (535 nm)/emission (460 nm) of donor-only transfected cells was subtracted (Pfleger and Eidne, 2006).

FRET

Cells were cultivated in 96-well plates and transfected with EYFP-MR and RFP-MR. After 48 hr, cells were incubated with vehicle, 10 nM aldosterone, or 2 μM geldanamycin for the indicated time span. Subsequently, cells were washed with PBS and fixed (4% paraformaldehyde, 60 min at 21°C). Fluorescence values ($F_{excitation/emission}$) were then measured using the Infinite 200 Multiwell Reader (Tecan): $F_{490/535}$ (Em1 for EYFP), $F_{558/580}$ (Em2 for RFP) and $F_{490/580}$ (Em3 for FRET determination). The excitation and emission wavelengths were set by two prisms with a bandpath of 2 nm for excitation and 15 nm for emission. FRET efficiency was calculated from the fluorescence values after background subtraction as described previously (Grossmann et al., 2010). Images for single-cell FRET were obtained with a Biozero 8000 fluorescence microscope from Keyence (Tokyo, Japan). The FRET image was calculated using the mean bleed through coefficient determined in the bleed through inlet for both the donor alone and the acceptor alone according to the FRET analyzer for ImageJ (http://rsbweb.nih.gov/ij/plugins/fret-analyzer/fret-analyzer.htm).

SEAP Reporter Gene Assay

The effect of geldanamycin on GRE transactivation and the function of both RLuc-MR and EYFP-MR were tested in a reporter gene assay as described previously (Pfau et al., 2007).

Statistics

The data are presented as mean values ± SEM. Significance of difference was tested by Student t test or an analysis of variance, with $p < 0.05$ considered statistically significant. N represents the number of individual experiments, and n represents the number of wells or culture dishes investigated per experiment.

SUPPLEMENTAL INFORMATION

Supplemental Information includes two figures and can be found with this article online at doi:10.1016/j.chembiol.2012.04.014.

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