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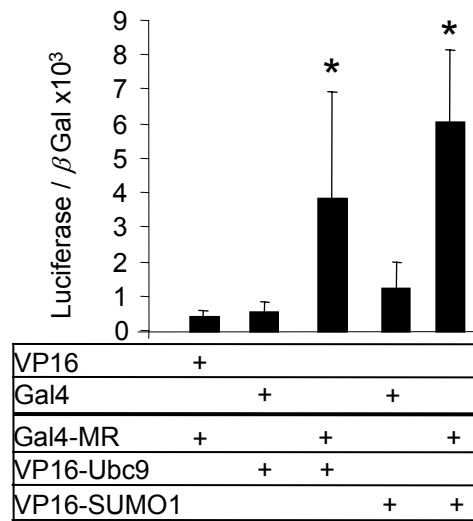
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Figure 1

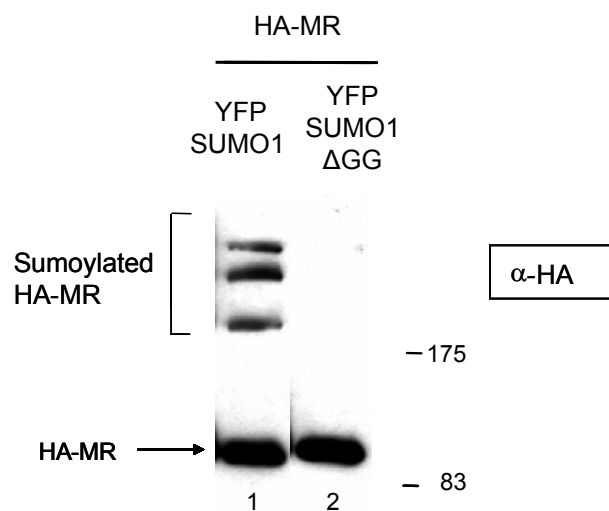
A.

Proteins (UniProt accession no.)	Incidence	Independent cDNAs
Ubc9 (P63279)	6	3
SUMO-1 (P63165)	6	4

B.



C.



D.

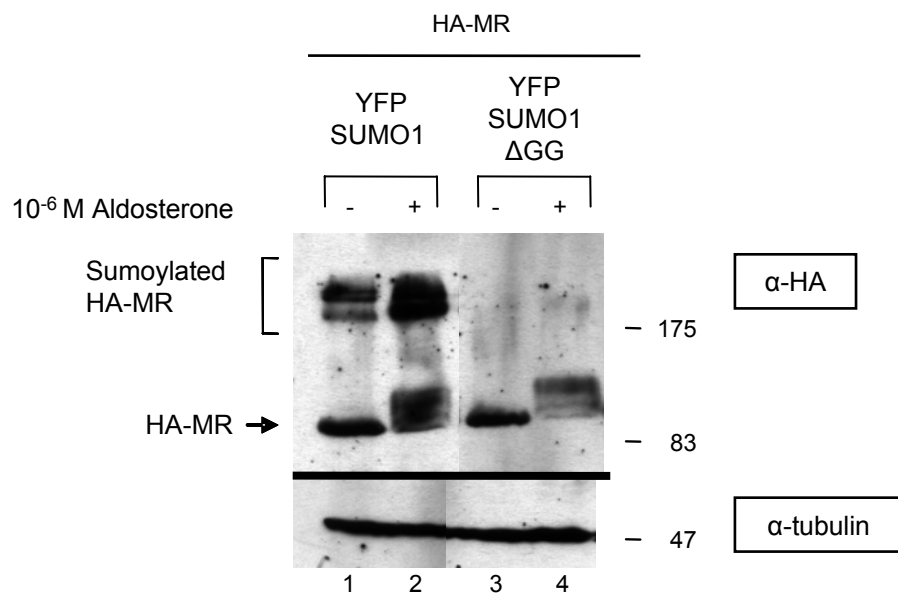


Figure 2

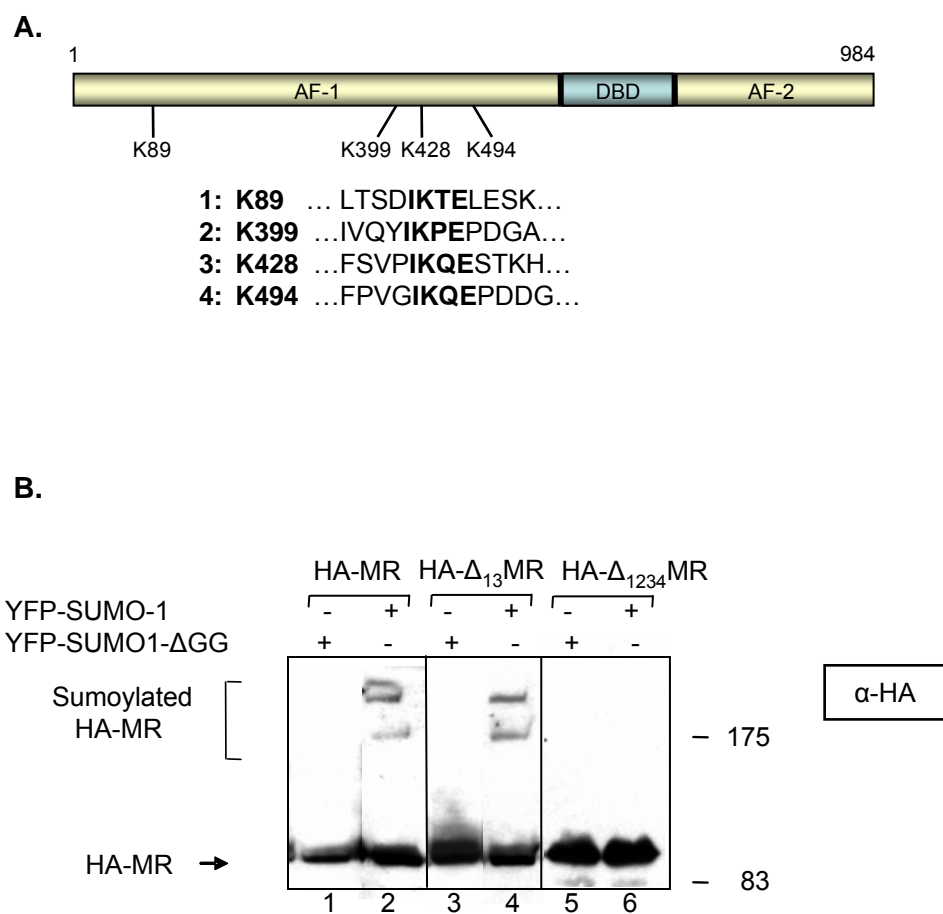
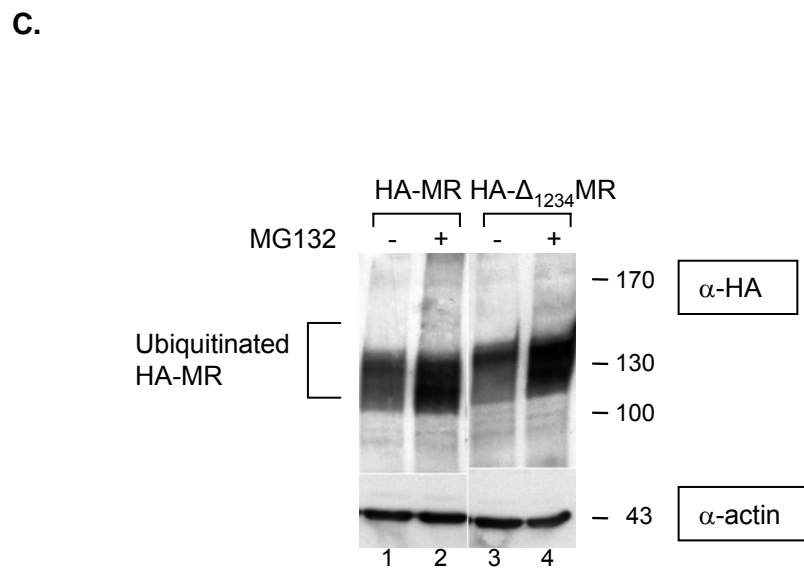
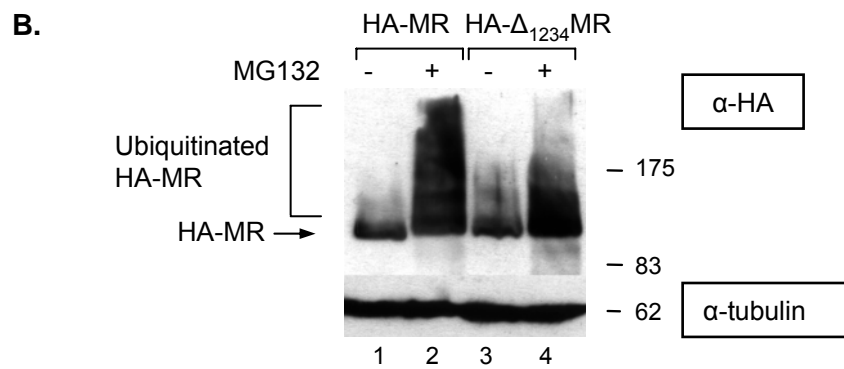
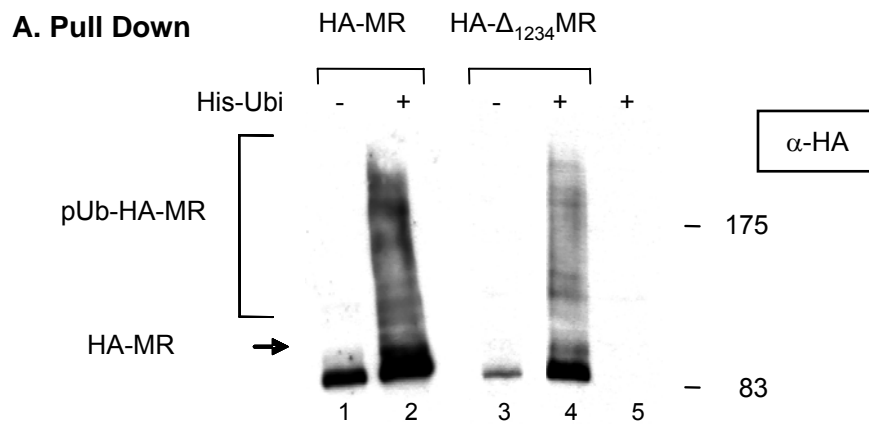
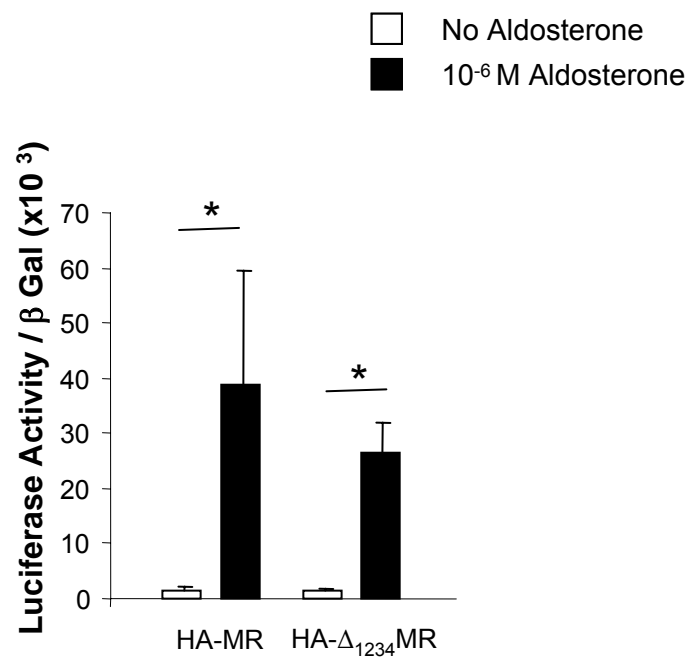


Figure 3



D. MMTV



E. MMTV

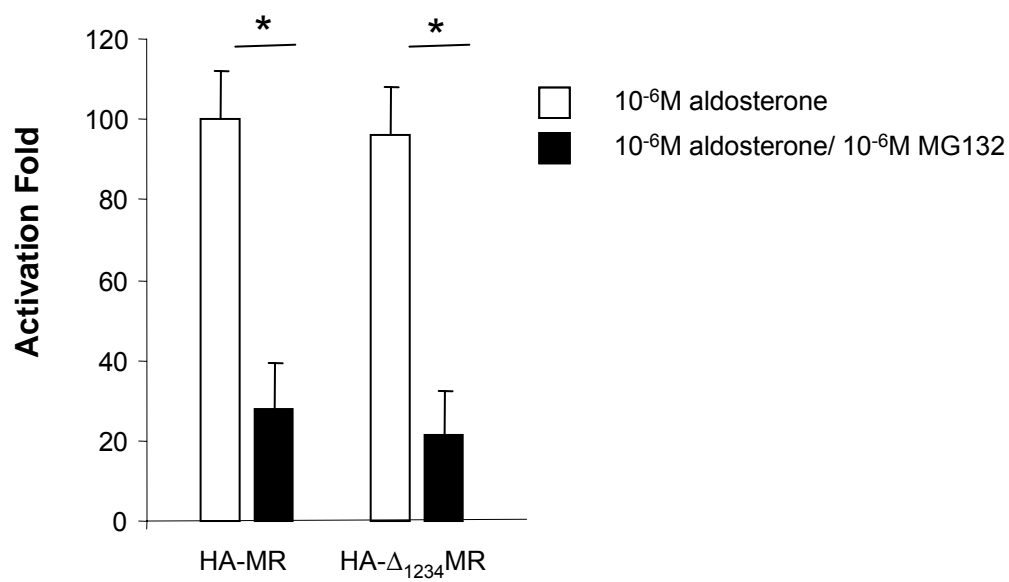
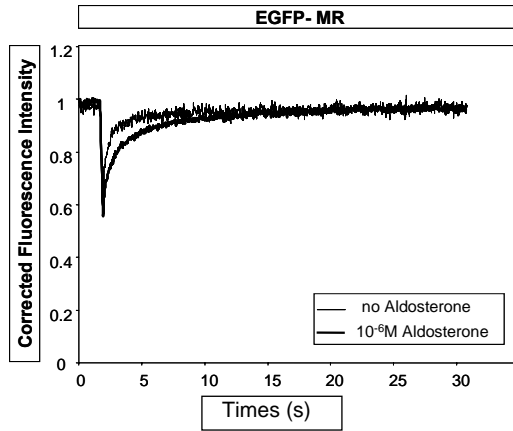
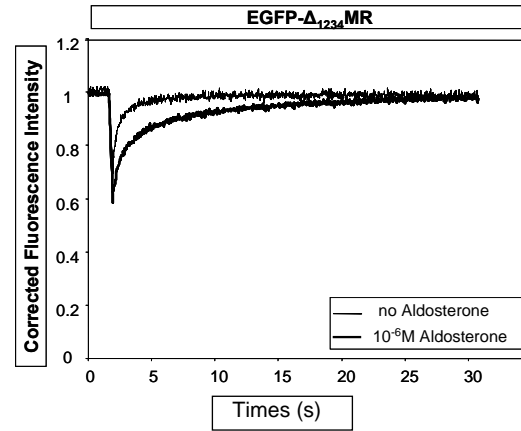


Figure 5 :

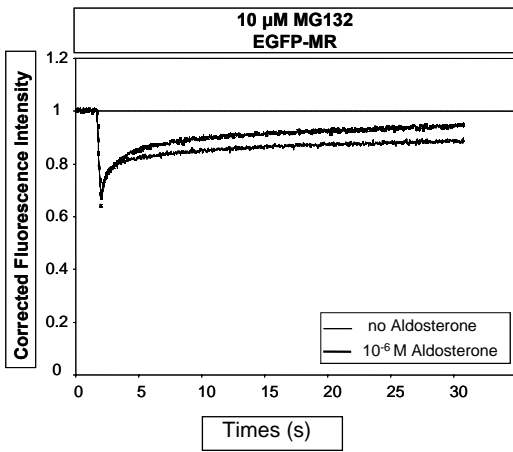
A.



B.



C.



D.

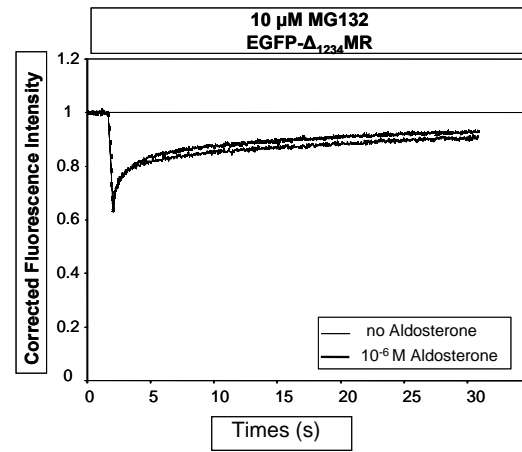
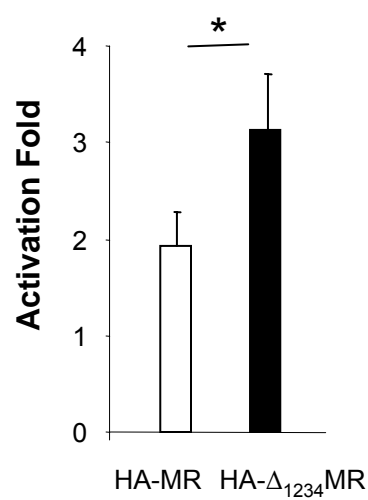


Figure 4

A. GREx3



B.

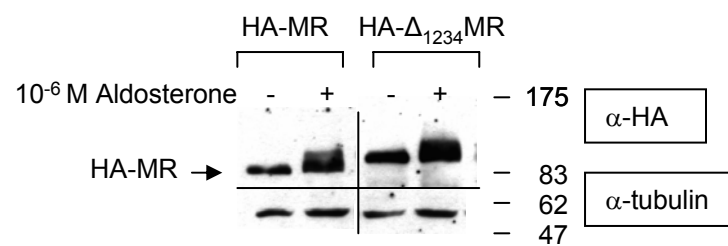


Table 1 : $t_{1/2}$ recovery

	HA-MR	HA- Δ_{1234} MR
No aldosterone	$0.5 \pm 0.06s$	$0.5 \pm 0.06s$
10^{-6} M aldosterone	$0.9 \pm 0.04s$	$1.1 \pm 0.07s$
No aldosterone 10^{-6} M MG132	$0.7 \pm 0.06s$	$0.7 \pm 0.07s$
10^{-6} M aldosterone 10^{-6} M MG132	$0.9 \pm 0.04s$	$1 \pm 0.08s$

Sumoylation and proteasomal activity determine the transactivation properties of the mineralocorticoid receptor

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Running title: Sumoylation and ubiquitination regulate MR transcriptional activity

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Abstract

MR is a hormone-activated transcription factor that carries a strong synergy inhibitory function at its N-terminus. Using this region as bait in a yeast two-hybrid screening, we isolated major components of the sumoylation pathway, including the SUMO-1-conjugating enzyme Ubc9, and SUMO-1 itself. We found that MR interacts with both, Ubc9 and SUMO-1 in mammalian cells, and that the receptor is sumoylated at four acceptor sites which are clustered within its AF-1 domain. We observed that MR can be poly-ubiquitinated and that proteasome activity is essential for MR-activated transcription. Disruption of the SUMO-1 attachment sites abolished MR sumoylation but interfered with neither the poly-ubiquitination of the receptor nor its transactivation potential on MMTV. However, the hormone-activated mutant displayed enhanced synergistic potential on a compound promoter and delayed mobility in the nucleus. FRAP analysis further showed that proteasome inhibition immobilizes a subpopulation of unliganded MR receptors in the nucleus, a phenomenon that is significantly attenuated in the presence of aldosterone. Interestingly, the ability of the hormone to counteract the immobilizing effect of MG132 requires the sumoylation-competent form of MR. Moreover, increasing exogenously SUMO-1 cellular levels, resulted in a selective, dose-dependent inhibition of the activity of the sumoylation-deficient MR. This effect was observed only on a synergy-competent promoter, revealing a mode for negative regulation of synergy that might involve sumoylation of factors different from MR. The data suggest that the overall transcriptional activity of MR can be modulated by its sumoylation potential as well as the sumoylation

level of MR-interacting proteins, and requires the continuous function of the proteasome.

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Introduction

The mineralocorticoid receptor (MR) is a hormone-activated transcriptional regulator with influence over a variety of physiological functions (De Kloet et al., 1998, Lombes et al., 1995, Bonvalet, 1998, Sousa and Almeida, 2002). Ligand binding to nuclear receptors induces changes in their conformation, triggers their translocation into the nucleus, and alters their subnuclear localization (DeFranco, 1999, Fejes-Toth et al., 1998, Htun et al., 1996, Pearce et al., 2002); regulation of target gene expression results from binding of the hormone-receptor complex to specific DNA response elements (Beato, 1989, DeFranco, 1999). The transcriptional regulatory regions of many steroid receptor target genes harbor multiple hormone response elements (HREs), and individual molecules of activated receptors bound on such compound promoters can act synergistically to produce greater-than-additive responses (Huang et al., 1999, Lee and Tsai, 1994, Adams et al., 2003). Nuclear hormone receptor signaling requires the regulated assembly and disassembly of transcriptional regulatory complexes. Recent advances in the field disclose a key role for post-translational modifications, such as receptor sumoylation and poly-ubiquitination in steroid hormone receptor signaling.

SUMO-1, -2 and -3 are small proteins, distantly related to ubiquitin but with a very similar secondary structure (Melchior, 2000, Yeh et al., 2000). Sumoylation and ubiquitination involve the covalent attachment of the C-terminus of the SUMO/ubiquitin polypeptide on the amino group of lysine residues of substrate proteins; the process is catalyzed by an enzymatic cascade through the action of

distinct enzymes (Melchior, 2000). In an ATP-dependent manner, the small peptide residue is first bound to an E1-activating enzyme (Johnson and Blobel, 1997) and is then transferred to target proteins through an E2-conjugating enzyme, directly or with the help of E3 ligases. In contrast to the large variety of ubiquitin E3 ligases (Pickart, 2001, Weissman, 2001), only a few SUMO E3 ligases are presently known (Melchior et al., 2003). E3 ligase activity can enhance the efficiency and selectivity of SUMO conjugation, both *in vitro* and *in vivo* (Hochstrasser, 2001, Kim et al., 2002). In the case of SUMO, the distinct subcellular localization of E3 enzymes (Kagey et al., 2003, Pichler et al., 2002, Sachdev et al., 2001), together with the dynamic and reversible nature of the sumoylation process (Melchior, 2000, Schwienhorst et al., 2000), is believed to contribute to precise, spatio-temporal regulation of substrate modification, necessary for the control of diverse cellular processes (Li and Hochstrasser, 1999). The functional consequences of SUMO attachment appear to differ from one protein to another and are still only poorly understood at the molecular level. One current view is that by modifying protein-protein and protein-DNA interactions, sumoylation can positively influence the assembly and stabilization of multi-protein complexes (Hoege et al., 2002, Mahajan et al., 1997, Seeler et al., 2001). Sumoylation may either enhance (Hong et al., 2001, Rodriguez et al., 1999) or attenuate (Tian et al., 2002, Abdel-Hafiz et al., 2002, Bies et al., 2002, Poukka et al., 2000, Sapetschnig et al., 2002) transcriptional activity, depending on the nature of the transcription factor, the promoter context and the cellular environment.

Protein ubiquitination is involved in numerous cellular processes, including protein degradation, membrane trafficking, intracellular localization as well as histone function, DNA replication and DNA repair. The transcriptional apparatus is also subject to the effects of ubiquitination (Dennis and O'Malley, 2005, Shcherbik and Haines, 2004) with the covalent modification of lysine residues by ubiquitination, sumoylation or acetylation representing an integral part of the finely-tuned control of transcription (Shcherbik and Haines, 2004). Ubiquitination of steroid hormone receptors can influence hormone response by priming the receptor for degradation by the proteasome machinery. For example, a cyclic, proteasome-mediated turnover of the estrogen receptor (ER α) on target promoters was recently shown to be critical for ER-mediated transactivation (Reid et al., 2003) and proteasome activity was found to be essential for the rapid exchange of GR on a responsive promoter (Stavreva et al., 2004). An intriguing effect of proteasome inhibition is that it reduces the mobility of nuclear receptors in the nucleus, as visualized by FRAP (fluorescence recovery after photobleaching) analysis (Deroo et al., 2002, Schaaf and Cidlowski, 2003, Stenoien et al., 2000), and increases their association with the nuclear matrix (Deroo et al., 2002, Stenoien et al., 2000, Stenoien et al., 2001).

In the present study we have identified Ubc9 and SUMO-1 as MR interacting partners and analyzed the transactivation properties as well as the nuclear mobility of the receptor as a function of its sumoylation potential and the activity of the proteasome.

Results

MR interacts with Ubc9 and SUMO-1 and can be sumoylated at four major acceptor sites which are located within the AF-1 domain.

Several components of the SUMO-1 pathway, including multiple independent cDNA clones for the SUMO-1-conjugating enzyme Ubc9 and the SUMO-1 polypeptide (Figure 1A) were isolated from a yeast two-hybrid screen of a human brain cDNA library using the region encompassing amino acid residues 170 to 433 of the AF-1 domain of the human MR protein as bait (Tirard et al., 2004). The interaction of Ubc9 and SUMO-1 proteins with the MR receptor was further examined using mammalian two-hybrid assays. SK-N-MC cells were transiently co-transfected with pM-MR (full-length MR fused to the GAL4 DNA-binding domain) and either pVP16-Ubc9 or pVP16-SUMO-1 (the VP16 transcription activation domain fused to full-length Ubc9 or SUMO-1), together with the pFR-Luc reporter plasmid. Co-transfection of pM-MR with the empty pVP16 vector or of the pVP16 fusions with the empty pM vector stimulated only weakly the pFR-Luc reporter activity (Figure 1B). However, co-expression of MR with the VP16-Ubc9 or the VP16-SUMO-1 fusion resulted in a significant activation of the reporter plasmid by approximately 8-fold and 12-fold, respectively (compared with the activity of the reporter observed when pM-MR was co-expressed with empty pVP16).

The interaction of MR with Ubc9 and SUMO-1 prompted us to test the sumoylation status of MR in mammalian cells. SK-N-MC cells were co-transfected with HA-tagged MR and YFP-SUMO-1 fusion-protein expression

vectors before isolating total protein extracts for analysis by Western blot, using an anti-HA antibody. As shown in Figure 1C (lane 1), co-expression of HA-MR and YFP-SUMO-1 resulted in the appearance of three bands with progressively higher molecular weights compared to the predicted size of HA-MR (~120 kDa). Considering that SUMO-1 does not generate polymeric chains on target proteins (Bylebyl et al., 2003), these bands most likely correspond to MR receptor conjugated with one, two and three YFP-SUMO-1 residues, respectively. A fourth band was observed after loading higher amounts of protein extract and longer film exposure (data not shown), suggesting that MR could bind up to four SUMO-1 residues in this system. Importantly, none of these bands could be detected when the HA-MR was co-expressed with a mutated form of SUMO-1, lacking the last two glycine residues that are required for conjugation of SUMO-1 to the target (YFP-SUMO1- Δ GG, Pichler et al., 2002, Figure 1C, lane 2). This observation further supported our interpretation that, the slow-migrating bands observed in the presence of the wild-type YFP-SUMO-1 represent distinct MR-SUMO-1 conjugates. We next co-transfected HA-MR together with YFP-SUMO-1 or the YFP-SUMO1- Δ GG mutant in SK-N-MC cells, and analyzed the sumoylation pattern of MR after treatment with aldosterone for 12 hr. As shown in Figure 1D, the presence of aldosterone consistently generated an upward-shift and a fuzzy band pattern corresponding to the liganded MR on the SDS/PAGE gels (lanes 2 and 4). This is most likely due to agonist-induced post-translational modifications of the protein, a phenomenon seen previously with other liganded steroid receptors, including MR (Galigniana, 1998, Lange et al., 2000, Tallec et

al., 2003). Interestingly, this experiment indicated that these aldosterone-induced modifications can occur also on the sumoylated forms of MR which might be favoured by the presence of the ligand (lane 2) as well as on the sumoylation-deficient mutant (lane 4). As expected, no sumoylated MR was detected when HA-MR was co-expressed with the mutated form of SUMO-1 protein (lanes 3 and 4). Incubation of these blots with an antibody against α -tubulin confirmed the equal loading of protein extracts on each lane.

Based on the tetrapeptide motif Ψ KxD/E (where Ψ is a hydrophobic residue, K the lysine targeted by SUMO-1, x any amino acid and D/E an acidic residue), we identified four putative SUMO-1 lysine acceptor sites in the N-terminal domain of the MR, at positions K89, K399, K428 and K494 (Figure 2A). Subsequently, we generated MR mutants in which the lysine residues within the SUMO-1 acceptor sites were replaced by arginines and evaluated the sumoylation pattern of the mutated proteins in transient transfection experiments in SK-N-MC cells. As shown in Figure 2B, co-expression of YFP-SUMO1 together with a construct expressing an MR protein carrying two mutated lysine residues at positions 89 and 428 (HA- Δ_{13} MR) resulted in formation of two of the three sumoylated MR forms detected previously (compare lanes 2 and 4). No sumoylation was observed when HA- Δ_{13} MR or intact HA-MR proteins were expressed together with YFP-SUMO1- Δ GG (lanes 1 and 3). Importantly, the typical sumoylation pattern of MR was completely lost when the cells were transfected with a construct carrying an MR mutant (HA- Δ_{1234} MR) in which all four SUMO-1 acceptor lysines at the N-terminal domain of the protein were replaced by

arginines (lane 6). It is worth-mentioning that the MR possesses a fifth consensus SUMO-1 acceptor site at the C-terminus, within the ligand binding domain (lysine 953). However, as no shifted band could be detected after SUMO-1 overexpression in cells transfected with the HA- Δ_{1234} MR mutant, the data clearly show that, at least under these experimental conditions, SUMO-1 is preferentially attached to the acceptor lysines which are located in the AF-1 domain of human MR.

Disruption of SUMO-1 attachment sites does not alter ubiquitination, proteasome dependence or normal activated transcription by MR on MMTV.

The turnover of many transcription factors including several steroid hormone receptors is regulated through the ubiquitin-proteasome pathway. Inhibition of the proteasome system can exert disparate effects on the transcriptional properties of individual steroid receptors, e.g. interference with ER α - or PR-mediated transcription, but enhancement of GR transactivation (Deroo et al., 2002, Lange et al., 2000, Lonard et al., 2000, Wallace and Cidlowski, 2001). In light of accumulating evidence suggesting a functional interplay between the ubiquitin and the sumoylation machineries (Desterro et al., 1997, Gill, 2004, Sachdev et al., 2001), we next sought to investigate the impact of proteasome activity on the transcriptional properties of MR and its sumoylation-deficient mutant. We first examined whether these two forms of MR can be poly-ubiquitinated. Pull down experiments were performed with extracts from cells co-transfected with His-tagged ubiquitin (His-Ubi) and either HA-MR or HA- Δ_{1234} MR expression vectors. Prior to lysis, the transfected cells were treated with 10 μ M MG132 for 4 h.

Lysates were passed through a metal resin and the bound proteins were analyzed for HA immunoreactivity by Western blot. As shown in Figure 3A (lanes 1 and 3), a small amount of MR was pulled down non-specifically. However, when either form of the receptor was co-expressed with His-Ubi, a strong HA-immunoreactive smear corresponding to poly-ubiquitinated MR was detected (lanes 2 and 4), which was not observed when His-Ubi was expressed alone (lane 5). Thus, disruption of the MR sumoylation sites does not interfere with poly-ubiquitination of the receptor. Consistent with these results, poly-ubiquitination of both forms of MR was also detectable after MG132 treatment (10 μ M for 6h) of SK-N-MC cells transfected with HA-MR (Figure 3B, lane 2) or HA- Δ_{1234} MR (lane 4). These poly-ubiquitinated forms of MR were not accumulated efficiently in the absence of MG132 (lanes 1 and 3). Finally, as shown in Figure 3C, also in the presence of the ligand, the disruption of the MR sumoylation sites did not affect the extent of MG132-induced MR poly-ubiquitination (lanes 2 and 4). Collectively, the above findings indicated that MR can be poly-ubiquitinated, irrespective of the presence or absence of SUMO-1 attachment sites.

We next analysed the effect of proteasome inhibition on the transactivation potential of MR and the sumoylation-deficient mutant on the MMTV promoter. As shown in Figure 3D, both HA-MR and HA- Δ_{1234} MR triggered a similar degree of transcriptional activation after aldosterone treatment (24- and 19-fold, respectively), suggesting that the inability of MR to be modified by SUMO-1 does not affect its transactivation potential on the MMTV promoter. Importantly,

inhibition of the proteasome resulted in a strong reduction (70%) of the transcriptional activity of the intact as well as of the sumoylation-deficient mutant, indicating that its continuous function is essential for MR transactivation in both cases (Figure 3E).

Disruption of the MR sumoylation sites enhances synergistic activation of transcription and reduces the nuclear mobility of the receptor.

The SUMO-1 conjugation sequence has been independently identified as a short motif that is involved in the regulation of transcriptional synergy (Iniguez-Lluhi and Pearce, 2000). Disruption of the SUMO-1 attachment sites has been consistently shown to enhance the ability of multiple DNA-bound steroid receptor molecules to activate transcription in a synergistic manner (Tian et al., 2002, Holmstrom et al., 2003, Iniguez-Lluhi and Pearce, 2000, Tallec et al., 2003). In order to evaluate the role of sumoylation on the synergistic properties of MR, we compared the activity of the intact receptor and its sumoylation-deficient mutant in transfection experiments, using as reporter the luciferase gene linked to a promoter bearing three aligned glucocorticoid response elements (pGREx3-Luc) that allow synergistic activation of transcription. In accordance with other steroid receptors, the sumoylation-deficient mutant displayed a higher potency on the GREx3 promoter, compared to the intact receptor (Figure 4A). Both MR forms were expressed at similar levels (Figure 4B), and no activation of the reporter was observed in the absence of exogenous MR (data not shown).

To gain further insight into the mechanism through which sumoylation can influence the transcriptional properties of MR, we analyzed the sub-nuclear dynamics of the receptor and its sumoylation-deficient mutant in living cells. For this, we generated EGFP-tagged receptors, which were expressed at comparable levels and displayed equipotent transcriptional activities to those seen with the corresponding HA-tagged versions (data not shown and ref. Fejes-Toth et al., 1998). No detectable differences were observed between EGFP- Δ_{1234} MR and EGFP-MR with regard to their subcellular distribution before or after hormone treatment. FRAP was then used to analyze the mobility of the two forms of MR in the nucleus of SK-N-MC cells that had been transiently transfected with pEGFP-MR or pEGFP- Δ_{1234} MR, together with an equal amount of pGREx3-Luc plasmid. The FRAP analysis was performed 24 h after transfection. The fluorescence recovery curves are shown in Figures 5A and 5B. Both forms of MR were highly mobile in the absence of hormone, as deduced from the very short half-maximal recovery times ($t_{1/2}$) of fluorescence after photobleaching (Table 1; 0.5 ± 0.06 s for both EGFP-MR and EGFP- Δ_{1234} MR). Addition of aldosterone (10^{-6} M for 30-45 min) resulted in a significant reduction in the mobility of both forms of MR, as demonstrated by the relative increase in the recovery of fluorescence (Table 1; $t_{1/2} = 0.9 \pm 0.04$ s for EGFP-MR and 1.1 ± 0.07 s for EGFP- Δ_{1234} MR); the recovery of the sumoylation-deficient MR mutant lagged slightly, but significantly lagged behind that of the intact receptor ($p < 0.05$).

Proteasome inhibition limits nuclear mobility of unliganded MR: ligand-mediated resistance requires a sumoylation-competent receptor.

The results from the proteasome inhibition experiments, implied a potential link between the ubiquitination of MR and the efficiency of its transcriptional activity. This interpretation is consistent with a central role of proteasome function on the continuous turnover of transcription factors on their responsive promoters, as has been demonstrated recently for the estrogen receptor (ER) (Reid et al., 2003). We therefore investigated how blockade of proteasome function affects the nuclear mobility of the MR receptors using FRAP analysis (Figure 5 C, D). Experiments were performed as described in Figure 5 (A, B); 24 h after transfection, cells were incubated for a further 6 hours with MG132 alone (10 μ M) or together with aldosterone (10^{-6} M) before analysis. After treatment with MG132, the MR showed a predominantly nuclear localization, in agreement with previous observations on the closely related receptor, GR (Deroo et al., 2002, Schaaf and Cidlowski, 2003). The same was observed for the sumoylation-deficient mutant, EGFP- Δ_{1234} MR. In neither case however, was the MG132-induced nuclear translocation sufficient to elicit transcriptional activation in the absence of hormone (data not shown). Interestingly, for both receptors (Figure 5C and D), the fluorescence intensity did not asymptote towards 1.0 during the recovery phase but stayed at around 0.8 even after 30 seconds, indicating an incomplete exchange of fluorescent molecules between the bleached region and the remaining parts of the cell. Thus, in both cases, proteasome inhibition generated a sub-population of receptor molecules that remained immobile during

the examined time-frame. Using the corrected fluorescence intensity observed at the end of the 30 s to calculate the $t_{1/2}$, we observed that treatment with the proteasome inhibitor slightly reduced the mobility of the remaining fraction of mobile MR and Δ_{1234} MR receptors, as indicated by the small, but consistent, increase in their corresponding recovery times (Table 1). No significant differences in the degree of immobilization were observed between the two forms of MR ($p > 0.5$). Co-administration of aldosterone efficiently counteracted MG132-induced MR immobilization (Figure 5C), and this newly-generated mobile fraction displayed a recovery time similar to that observed in the absence of MG132 (Table 1). Interestingly, in the case of the sumoylation-deficient mutant, the aldosterone-mediated resistance to the MG132-induced immobilization was less efficient compared to the intact MR (Figure 5D), suggesting that the competence of MR for sumoylation is functionally involved in this ligand-mediated resistance.

Transcriptional synergy depends on sumoylation of both, MR and a putative SC-motif-dependent, MR-interacting factor(s).

Given that we isolated SUMO-1 protein as an interacting partner of MR in our yeast two-hybrid screen (cf. Figure 1), we reasoned that, in addition to the modification of MR by SUMO-1, a potential interaction of MR with sumoylated components of the transcription machinery might further modulate its synergistic properties. To test this hypothesis, we took advantage of the fact that the synergistic activation of transcription by MR on the compound GREx3 promoter

was enhanced by the disruption of the SUMO-1 attachment sites (cf. Figure 4A), and examined whether increasing the intracellular levels of SUMO-1 protein can selectively affect the activity of HA- Δ_{1234} MR on the GREx3 promoter. Transfection experiments were performed using HA- Δ_{1234} MR or HA-MR, together with pGREx3-Luc or pMMTV-Luc as reporters, in the presence of increasing amounts of the pYFP-SUMO-1 expression plasmid. Indeed, the presence of exogenous SUMO-1 decreased selectively the HA- Δ_{1234} MR-mediated transactivation in a dose-dependent manner solely on the GREx3 promoter (Figure 6B, histograms 4, 5, and 6; $p \leq 0.05$) whereas it had no effect on the activity of HA-MR on either promoter (Figure 6A). Under these over-expression conditions, SUMO-1 was sufficient to enhance sumoylation of cellular substrates, as indicated by the dose-dependent increase in the sumoylated forms of HA-MR (Figure 6C, lanes 1 to 3) and the sumoylation of other proteins (Figure 6D); as expected, there was no conjugation of SUMO-1 to the HA- Δ_{1234} MR protein (lanes 4 to 6). These results suggested that the sumoylation of a factor(s) different from MR can specifically counteract the gain-of-function property of the sumoylation-deficient receptor on the GREx3 promoter. The effect of this putative factor(s) appears to be synergy-dependent, as no change in the transactivation potential of the HA- Δ_{1234} MR on MMTV was observed.

Discussion

Extending our previous work (Tirard et al., 2004), we show here that the MR receptor can interact with major components of the sumoylation machinery, such as Ubc9 and SUMO-1. The receptor can become sumoylated at four different lysine residues which are located at its N-terminal domain. This finding is in agreement with earlier observations in a different cell line (Tallec et al., 2003), thus pointing to the existence of a rather common sumoylation profile for MR. We further showed that MR is poly-ubiquitinated and that disruption of the SUMO-1 attachment sites does not interfere with this modification, indicating that ubiquitination and sumoylation occur at different lysine residues on MR, as has been previously described for the transcription factor C/EBP α (Subramanian et al., 2003).

We analyzed the transcriptional properties of the receptor under conditions where the proteasome is inhibited. These experiments showed that tonic proteasome activity is necessary for optimal MR transactivation on the MMTV promoter, consistent with current evidence for continuous proteasome-mediated turnover of highly active transcription factors on their cognate promoters (Lonard et al., 2000, Molinari et al., 1999, Reid et al., 2003, Stavreva et al., 2004). The absence of SUMO-1 attachment sites on MR influenced neither the receptor's transcriptional activity on the MMTV promoter nor its requirement for continuous proteasome function suggesting that the sumoylation potential of MR is not required for its regular cycling on MMTV.

As has been observed with many other transcription factors previously, including MR (Iniguez-Lluhi and Pearce, 2000, Tallec et al., 2003), disruption of the sumoylation sites enhanced the synergistic response of multiple DNA-bound MR receptors. Transcriptional synergy is defined as the greater-than-additive stimulation of transcription observed on promoters with multiple copies of a response element. It is essential for the efficiency and specificity of eukaryotic gene expression and is involved in the adaptation to developmental and environmental changes (Carey, 1998, Courey, 2001, Herschlag and Johnson, 1993). The discovery of synergy control motifs (Iniguez-Lluhi and Pearce, 2000), which overlap with sites at which SUMO-1 can be covalently attached (Mahajan et al., 1997, Mahajan et al., 1998), represented a major advance in our understanding of how transcriptional synergy is controlled. Such motifs selectively restrain transcriptional synergy on compound promoters and are found in variable numbers in numerous transcriptional regulators, indicating that they may serve as general modules for synergy control. Nevertheless, the mechanism(s) underlying SUMO1-mediated inhibition of synergy has remained unknown. In order to gain further insight into this process, we compared the nuclear mobility of the receptor as well as its sumoylation-deficient mutant in living cells. As revealed by FRAP, hormone activation, while enhancing their transcriptional response, reduced the nuclear mobility of both MR forms, suggesting a higher duration of promoter occupancy by the transcriptionally active receptors. The relative longer residence time of the sumoylation-deficient MR could indicate more competent interactions with components of the

transcription machinery and thereby, greater efficiency of polymerase loading, consistent with its unrestrained synergistic properties. Surprisingly, whereas no effect was observed on MMTV, increasing the cellular levels of SUMO-1 led to a substantial and selective decrease in the activity of the sumoylation-deficient MR on the synergy-competent promoter GREx3, which was extended beyond synergy. Thus, SUMO-1 overexpression might lead to recruitment of sumoylated regulators on the synergy-competent promoter that selectively inhibits the sumoylation-deficient MR *in trans*. During synergy, it is thought that multiple DNA-bound SUMO1-modified SC motifs at compound response elements may generate a multivalent recognition surface for a putative synergy control factor (SCF) (Holmstrom et al., 2003). The SCF, in turn, interferes with synergistic activation of transcription. Mutation of the SC motifs (Iniguez-Lluhi et al., 1997, Subramanian et al., 2003, Iniguez-Lluhi and Pearce, 2000) or conjugation with SUMO-1 (Le Drean et al., 2002) might block SCF binding, thereby allowing transcriptional synergy. Accordingly, the relative sumoylation states of MR and the sumoylation states of other, SC-motif-dependent transcriptional regulators (e.f. SCF) in a certain cell type or functional state may influence their mode of interaction, thereby determining the extent of the MR response. Such a model would explain the previously reported, opposing effects of SUMO-1 on the transcriptional activity of GR (Le Drean et al., 2002, Tian et al., 2002) and the failure of SUMO-1 overexpression to influence the activity of the intact MR in our system. The reversible nature of the sumoylation process (Li and Hochstrasser, 1999, Melchior, 2000, Schwienhorst et al., 2000), together with the potential

integration of multiple extracellular and intracellular signals (Manza et al., 2004), would provide precise control of the transcriptional output from a compound promoter.

Steroid receptors are characterized by high mobility in the nucleus (Deroo et al., 2002, McNally et al., 2000, Reid et al., 2003, Schaaf and Cidlowski, 2003, Stenoien et al., 2001) but they can nevertheless interact transiently with certain nuclear binding sites (Schaaf and Cidlowski, 2003, Stenoien et al., 2000, Stenoien et al., 2001, Tang and DeFranco, 1996, van Steensel et al., 1995). Moreover, MG132-induced immobilization has been previously correlated with increased association of nuclear receptors (Deroo et al., 2002, Schaaf and Cidlowski, 2003, Stenoien et al., 2001), including the MR (Fejes-Toth et al., 1998, Tang and DeFranco, 1996, van Steensel et al., 1995), with the nuclear matrix. Since MG132 treatment enhanced the poly-ubiquitination of MR and inhibited its transcriptional response, FRAP analysis was used to compare the nuclear mobility of the two forms of the receptor under these conditions. This analysis revealed that proteasome inhibition induces immobilization of both intact and sumoylation-deficient MR receptors in the nucleus. Additionally, the presence of aldosterone significantly, albeit not completely, attenuated the MG132-induced immobilization of MR; a similar effect was previously shown for the hormone-activated GR (Schaaf and Cidlowski, 2003). Interestingly however, in the case of the sumoylation-deficient mutant, the aldosterone-mediated resistance to MG132-induced immobilization was weaker compared to the intact MR. Proteasome inhibition as well as hormone activation, promote the association of

steroid receptors with certain nuclear structure(s) at which they become transiently immobilized (Fejes-Toth et al., 1998, Tang and DeFranco, 1996, van Steensel et al., 1995, Deroo et al., 2002, Schaaf and Cidlowski, 2003, Stenoien et al., 2001). Our above observations on MR indicate that this association can be strengthened by the disruption of the SUMO-1 attachment sites, implying a potential role of sumoylation on these nuclear interactions.

Materials and Methods

Plasmid constructs. The mammalian two-hybrid vectors used were pM, expressing the DNA-binding domain of *Saccharomyces cerevisiae* GAL4 protein, and pVP16, expressing the transcription activation domain of the herpes simplex virus VP16 (both from Clontech); pFR-luciferase (Stratagene) was used as a reporter. pRShMR was a kind gift from Dr. Ron Evans (La Jolla, CA). The *EagI* x *AflI* fragment of the human MR cDNA was inserted into *Bsp120I* x *XbaI* of pEGFP-C3 (Clontech) to generate pEGFP-hMR. The MR cDNA was excised from pEGFP-hMR using *SaII* x *BclI*(blunt) and inserted into the *SaII* x *XhoI*(blunt) sites of pCMV-HA (Clontech). Ubc-9 was excised from the yeast vector pACT2 and subcloned into the *BamHI* x *SaII*(blunt) of pVP16. SUMO-1 was excised from the yeast pACT2 vector and inserted into the *BamHI* x *SaII*(blunt) sites of pVP16. SUMO-1 constructs in pEYFP was prepared as described in Pichler *et al.* 2002 (Pichler et al., 2002). pHis-Ubiquitin was kindly provided by Dr. Bruce Stillman (Cold Spring Harbor Laboratory, Woods Hole, MA).

Mutagenesis. Lysine to arginine mutations were introduced according to the Quickchange Site-Directed Mutagenesis Kit manual (Stratagene), using the pCMV-HA-hMR as template. Mutations were verified by sequencing analysis. For the subcloning of the mutated form of MR (Δ_{1234} MR) in pEGFP, the mutated MR was amplified by PCR using as template the pCMV-HA- Δ_{1234} MR. The sense primers were the 5'GCACATCTCGAGATGGAGACCAAAGGC3' (for pEGFP);

the antisense primer was the 5'TGGAAGCTTCACTTCCGGTGAAGTAG3', in both cases.

Cell culture, transfections and luciferase assays. Human neuroblastoma SK-N-MC cells were maintained at 37°C and 5% CO₂ in DMEM (Invitrogen) containing 10% FCS (Invitrogen) and 1% kanamycin. For the luciferase reporter experiments, cells were seeded onto 24-well plates (8x10⁴ cells/well) one day before transfection with 375 ng/well of total DNA using jet-PEI (Qbiogene). Cells were maintained in DMEM containing 10% charcoal-stripped FCS (Sigma). Twenty four hours after transfection, cells were transferred to DMEM containing 1% charcoal-stripped FCS and treated with aldosterone (10⁻⁶ M in ethanol; Sigma) or MG132 (10 µM; Calbiochem). Cells were harvested 12 h thereafter in 100 µl of 1X lysis buffer (Promega) and centrifuged. The cleared supernatants were assayed for β-galactosidase and luciferase activity, as described previously (Tirard et al., 2004).

Western blotting. Cells were harvested by scraping in 100 µl of SDS-loading buffer complemented with 15 mM of N-ethylmaleimide (NEM; SIGMA), and sonicated briefly. Proteins were resolved by electrophoresis on 7% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. Membranes were blocked in PBS containing 5% fat-free milk and 0.2% Tween-20, and incubated with mouse anti-HA (1:1000; BabCo) or mouse anti-α-tubulin (1:1500; Chemicon), mouse anti β-actin (1:1000, SIGMA), mouse anti-GFP (1:5000, Roche). Antigens were revealed by enhanced chemoluminescence after

incubation with appropriate horseradish peroxidase-IgG conjugates (Amersham Biosciences).

Affinity pull-down assays. SK-N-MC cells were grown on 10-cm dishes and transfected with 3 μ g of each expression vector (pHA-hMR, pHA- Δ_{1234} MR, or pHis-Ubiquitin). Twenty four hours after transfection, cells were treated with 10 μ M MG132 for 4 hrs. After washing with PBS, cells were resuspended in Gua8 (6 M guanidine-HCl, 100 mM NaCl, 10 mM Tris, 50 mM NaH₂PO₄ [pH 8.0]), briefly sonicated and centrifuged. Cleared lysates were incubated at 4°C for 2 h with 25 μ l (bed volume) of sepharose beads (Clontech). Bound proteins were washed twice in Gua8 buffer and three times in Urea6.5 buffer (8 M urea, 100 mM NaCl, 50 mM NaH₂PO₄ [pH 6.5]). Finally, beads were washed once with ice-cold PBS, boiled in Laemmli buffer and supernatants were electrophoresed on 6% SDS-polyacrylamide gels.

FRAP experiments. FRAP experiments were carried out on a Zeiss 510 confocal microscope with a Plan-Apochromat oil objective (63x/1.4). Cells, in glass bottom dishes (MatTek P35GC-1.5-14C) were placed in a stage incubator (SPeCon GmbH) and maintained at 37°C under 5% CO₂. Bleaching was performed using the 488 nm wavelength of a 25 mW argon laser operating at 75 % laser power. Forty iterations were used for bleaching an average area of 8 x 4 pixels (9.1 x 4.6 μ m), resulting in a bleach time of about 300 ms. Pre- and post-bleach time series images were obtained from a 128 pixel x 16 pixel (146 μ m x 18 μ m) window at 34 ms intervals. For fluorescence excitation, 2% power of the

488 nm laser line was used; emission was detected using a 505-530 nm band-pass filter. Confocal filtering was performed using a pinhole of 1 Airy unit diameter, resulting in an imaged full width at half maximum (FWHM) slice thickness of 0.7 μm . The number of pre- and post-bleach images was selected with respect to the observed image noise and the expected $t_{1/2}$ value. Fluorescence signals from bleached and unbleached regions of interest (ROI) were calculated as mean-ROI-intensity at every time point using the LSM software AIM 3.0 (Carl Zeiss). The fluorescence in the bleached and non-bleached area, as well as in the whole nucleus was quantified for each cell (10-20 cells/experiment, 3 independent experiments). Corrected fluorescence intensities represent the ratio between background-subtracted fluorescence intensity of the bleached area and background-subtracted intensity of the total cell intensity at each time point. During the establishment of the FRAP experiments, we observed that co-transfection of MR with the plasmid carrying the MMTV promoter, on which the intact as well as the sumoylation-deficient receptor exhibit the same transcriptional activity, generated also identical recovery curves. Therefore all subsequent FRAP experiments were performed in the presence of the plasmid carrying the synergy-competent GREx3 promoter (pGREx3-Luc) on which the two forms of MR exhibit a differential response.

Statistics. Where appropriate, data were subjected to 1-way ANOVA, followed by appropriate *post hoc* tests. The level of significance was set at $p \leq 0.05$. For

Fig. 5, a two factorial analysis of variance was performed to compare the effect of aldosterone treatment on the mobilities of HA-MR and HA- Δ_{1234} MR.

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Figure Legends

Figure 1. MR interacts with Ubc9 and SUMO1.

A. Ubc9 and SUMO-1 were identified as interaction partners of the N-terminal domain of MR by yeast two-hybrid screen.

B. Two-hybrid assay in mammalian cells. SK-N-MC cells were seeded on 24-wells plates and transfected with 125 ng of pVP16-SUMO1 and pVP16-Ubc9 chimera expression plasmids and a luciferase reporter construct (pFR-Luc) together with the expression vector pM-MR; 50 ng pCMV- β Gal were added as internal control. Luciferase activity was measured twenty four hours after transfection and normalized for β -galactosidase activity and protein content. Luciferase activity of cells transfected with “empty” vectors pVP16 (VP16) and pM (Gal4) together with pM-MR and pVP16-SUMO1 or pVP16-Ubc9 are also shown. Each bar represents the mean \pm S.E.M. of three individual experiments performed in triplicate. $p < 0.05$ compared with control.

C. SK-N-MC cells seeded on 6-well plates were transfected with 500 ng of pHA-MR and an equal amount of pYFP-SUMO-1 (lane 1) or pYFP-SUMO1- Δ GG (lane 2) expression vectors. Twenty four hours after transfection, cells were directly lysed in SDS buffer and subjected to western blot analysis using anti-HA and anti α -tubulin antibodies.

D. SK-N-MC cells, seeded on 6-well plates, were transfected with 500 ng of pHA-MR and an equal amount of pYFP-SUMO1 (lane 1 and 2) or pYFP-SUMO1- Δ GG (lane 3 and 4) expression vectors. Twenty four hours after transfection,

cells were directly lysed in SDS buffer and subjected to western blot analysis using anti-HA and anti α -tubulin antibodies.

Figure 2: MR can become sumoylated at four major acceptor sites which are located within the AF-1 domain.

A. Schematic representation of MR, showing the functional domains of the receptor and the position of the four putative SUMO-1-acceptor lysine residues, in the N-terminal region.

B. SK-N-MC cells seeded on 6-well plates were transfected with 500 ng HA-MR (lanes 1 and 2), pHA- Δ_{13} MR (lanes 3 and 4) or pHA- Δ_{1234} MR (lanes 5 and 6) in the presence of either 500 ng pYFP-SUMO1- Δ GG (lanes 1, 3 and 5) or pYFP-SUMO1 (lanes 2, 4 and 6) expression plasmids. Thirty hours after transfection, cells were lysed and subjected to Western blot analysis using an anti-HA antibody.

Figure 3: MR can acquire poly-ubiquitin chains and proteasome activity is essential for activated transcription on MMTV promoter.

A. SK-N-MC cells were grown on 10-cm dishes and transfected with 3 μ g of pHA-MR (lanes 1 and 2) or pHA- Δ_{1234} MR (lanes 3 and 4), alone (lanes 1 and 3) or together with the pHis-ubi expression vector (lanes 2 and 4); on lane 5 pHis-Ubi was expressed alone. 24 hours after transfection, cells were treated with MG132 (10 μ M) for 4h. Lysates were subjected to His pull-down using metal

beads; bound proteins were subsequently analyzed by Western blot using anti HA antibody.

B. SK-N-MC cells were seeded on 6-well plates and transfected with 500 ng of pHA-MR or pHA- Δ_{1234} MR. Twenty four after transfection, cells were treated with MG132 (10 μ M, lanes 2 and 4) or vehicle (DMSO, lanes 1 and 3) for 4h and protein extracts were analyzed by Western blotting, using antibodies against HA or α -tubulin.

C. SK-N-MC cells were transfected as described in B. Twenty four after transfection, cells were treated with MG132 (10 μ M, lanes 2 and 4) or vehicle (DMSO, lane 1 and 3) in the presence of 10^{-6} M aldosterone (lane 1 to 4) for 4h and protein extracts were analyzed by Western blotting, using antibodies against HA or α -actin.

D. SK-N-MC cells were seeded on 24-well plates and grown in 10% steroid-stripped serum were transiently transfected with 125 ng of pHA-MR or pHA- Δ_{1234} MR and 125 ng of the pMMTV-Luc reporter plasmid; 50 ng pCMV- β Gal were added as internal control. After 24 h, cells were treated with 10 μ M MG132 and 10^{-6} M aldosterone or vehicle (ethanol) for 12 h before being assayed and luciferase activity was normalized for β -Gal activity. Results are shown as mean \pm S.E.M. of 3-5 experiments. Asterisks indicate $P \leq 0.05$.

E. SK-N-MC cells were transfected with the expression plasmids pHA- Δ_{1234} MR and pHA- Δ_{1234} MR together with the pMMTV-Luc as reporter. 24 h later they were treated with 10 μ M MG132 and 10^{-6} M aldosterone or vehicle (ethanol) for 12 h. 'Activation Fold' refers to the ratio between luciferase values observed after

hormone treatment and values observed in the absence of aldosterone. Results are shown as mean \pm S.E.M. of 3-5 experiments. MG132 treatment failed to elicit activation of the reporter in the absence of aldosterone (data not shown). Asterisks indicate $P \leq 0.05$. Aldosterone alone, white bar; aldosterone plus MG132, black bar.

Figure 4: Disruption of the MR sumoylation sites enhances synergistic activation of transcription

A. SK-N-MC cells were seeded on 12-well plates and transfected with 125ng of the expression plasmids pHA-MR or pHA- Δ_{1234} MR together with the pGREx3-Luc reporter and 50 ng pCMV- β Gal as internal control. Twenty-four hours later, the cells were treated with 10^{-6} M aldosterone or vehicle (ethanol) for 12 h and luciferase activity was normalized for β -Gal activity and protein concentration. 'Activation Fold' refers to the ratio between luciferase values observed after hormone treatment and values observed in the absence of aldosterone. Results are shown as mean \pm S.E.M. of 3-5 experiments. Asterisks indicate $P \leq 0.05$.

B. SK-N-MC cells were seeded on 6-well plates and transfected with 500 ng of pHA-MR (lane 1 and 2) or pHA- Δ_{1234} MR (lane 3 and 4). Twenty four after transfection, cells were treated with 10^{-6} M aldosterone (lane 2 and 4) or vehicle (ethanol, lane 1 and 3) for 12 h as described above, and protein extracts were analyzed by Western blotting, using antibodies against HA or α -tubulin.

Figure 5: Disruption of the MR sumoylation sites reduces the nuclear mobility of the receptor.

Cells were seeded on glass bottom dishes and transfected with 500 ng of pEGFP-MR (A and C) or pEGFP- Δ_{1234} MR (B and D) expression vectors. 24 h later, cells were incubated with aldosterone (10^{-6} M, 30-45 min) alone (A and B) or in combination with MG132 (10 μ M, C and D) for 6 hours before measuring the recovery of fluorescence. Plotted are the results from 3 independent experiments, consisting of analysis of 10-20 cells each. “Corrected Fluorescence Intensity” refers to the ratio between background-subtracted fluorescence intensity of the photobleached area and background-subtracted fluorescence intensity of all cells, at each time point. $t_{1/2}$ recoveries (time taken before fluorescence reached half of the maximum recovery) were calculated for individual cells using this plot, and the data are summarized on Table 1, as mean \pm S.E.M.

Figure 6: Over-expression of SUMO-1 selectively reduces the activity of HA- Δ_{1234} MR on the GREx3 promoter.

A, B. SK-N-MC cells seeded on 24-well plates and grown in 10% steroid-stripped serum were transiently transfected with 125 ng of pHA-MR (A) or pHA- Δ_{1234} MR (B) together with 125 ng pMMTV-Luc (black bars) or pGREx3-Luc (white bars) reporter plasmids together with increasing amounts of the pYFP-SUMO-1 expression vector (0, 50 and 125 ng), as indicated. pCMV- β Gal (50 ng) was added as internal control whereas an empty pEGFP vector was used to keep the

levels of total transfected DNA constant. After 24 h, cells were treated with 10^{-6} M aldosterone or vehicle (ethanol) for 12 h and assayed for luciferase activity. In the absence of SUMO1 (0 ng), the activation fold (i.e. the ratio of beta-galactosidase-normalized luciferase activity observed after aldosterone treatment with respect to the activity obtained in the absence of the hormone) was set as 100 for both promoters and the data in the presence of SUMO1 were expressed as percentage of this value (% Activation Fold). Results represent the mean \pm S.E.M. from at least 3 experiments. Asterisks indicate $P \leq 0.05$; double asterisks indicate $P < 0.005$.

C. SK-N-MC cells were seeded on 6-well plates and transfected with 500 ng of either pHA-MR or pHA- Δ_{1234} MR and increasing amounts of pEYFP-SUMO-1 (0, 200 ng and 500 ng), keeping the molar ratio between the MR and SUMO-1 expression plasmid DNA, equal to that used in A and B above. pEGFP was used to keep amounts of transfected DNA constant. Cells were directly lysed in SDS buffer and proteins were immunoblotted using antibodies against HA and α -tubulin.

D. SK-N-MC cells were seeded on 6-well plates and transfected with 500ng of pYFP-SUMO1 (lane 1) or empty vector (lane 2). Twenty-four hours after transfection, cells were lysed directly in SDS-loading buffer and the protein extracts were analyzed by western blot using anti-GFP antibody.

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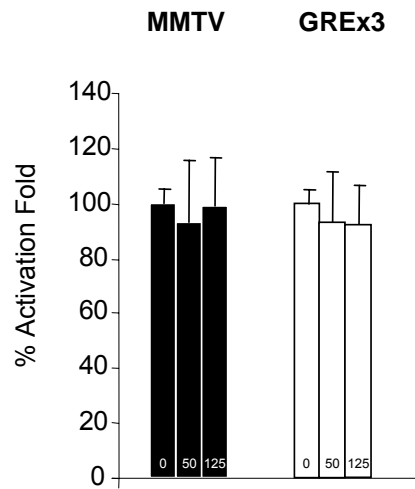
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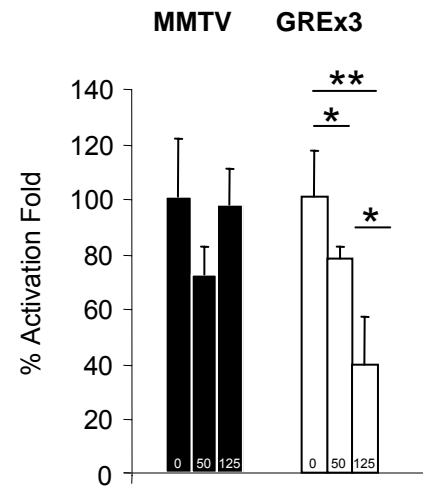
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Figure 6

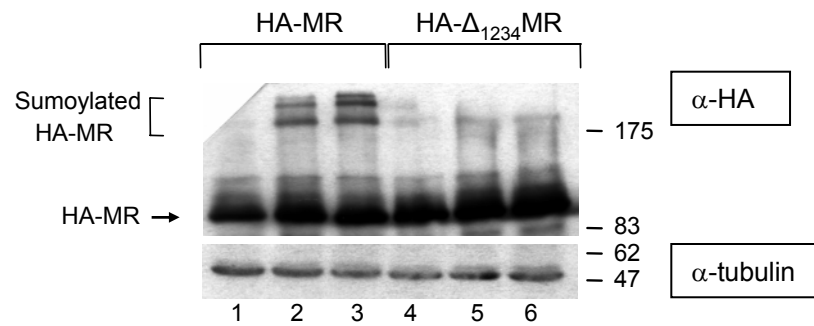
A. HA-MR



B. HA- Δ_{1234} MR



C.



D.

