Chapter

Post-Translational Modification of MR Activity

Diego Alvarez de la Rosa and Natalia Serrano-Morillas

Abstract

The mineralocorticoid receptor (MR) is a ligand-activated transcription factor that transduces the effects of aldosterone and glucocorticoids in a tissue- and cell type-specific ways. Differential regulation of MR by post-translational modifications (PTMs) has been proposed to play a key role in modulating its function. In addition, modifications of other proteins that physically or functionally interact with MR add an additional layer of regulation to aldosterone or glucocorticoid signaling. In this chapter, we will summarize the main post-translational modifications of MR described so far, discussing their possible implications in the physiological and pathological roles of the receptor. We will also discuss post-translational modulation of other proteins impacting MR function such as heat shock protein 90 or 11ß-hydroxysteroid dehydrogenase type 2.

Keywords: mineralocorticoid receptor, aldosterone, glucocorticoids, steroid receptors, protein modification, phosphorylation, Ubiquitylation, SUMOylation, acetylation

1. Introduction

The mineralocorticoid receptor (MR) is widely expressed and performs different physiological and pathological roles depending not only on the activating ligand (aldosterone vs. glucocorticoids) but also on context [1–3]. This includes co-expression or not with 11ß-hydroxysteroid dehydrogenase (11ßHSD2) to control local levels of glucocorticoids [4, 5], differential interaction with co-regulators [6] or the physiological mechanism behind the increased circulating levels of aldosterone [7], to name a few.

Multiple levels of regulation controlling MR activity have been described. Transcription of the Nr3c2 gene, coding for the receptor, is modulated by different stimuli and depends on two alternative promoters [1, 8]. Transcriptional control depending on epigenetic mechanisms has also been described [1]. MR transcripts undergo alternative splicing, although the physiological significance of these variants is uncertain [9]. Post-translational control includes regulation of MR mRNA stability by proteins such as Tis11b and HuR [10, 11] or siRNAs such as miR-124 and miR-135a [12–14]. Some MR polymorphisms have been shown to produce different translational efficiencies *in vitro* [15]. Once synthesized, MR activation depends on ligand availability, which in turn can be modulated by co-expression of 11ßHSD2, which creates a low glucocorticoid-microenvironment by metabolizing biologically active glucocorticoids to their 11-keto, biologically inactive derivatives [4, 5]. Activation also depends on the interaction of MR with co-chaperones, including Hsp70 and Hsp90 [16–18]. MR can also be activated in a ligand-independent manner by the

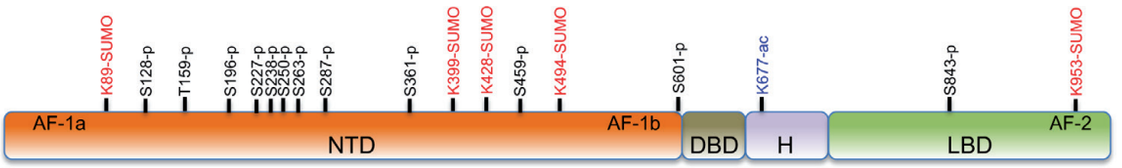

Figure 1.
Schematic representation of MR protein domains and the location of post-translational modifications with demonstrated functional effects. NTD, NH_2-terminal domain; DBD, DNA-binding domain; H, hinge domain; LBD, ligand-binding domain; AF, activation function. Phosphorylation sites (p) are shown with black letters; acetylation sites (ac) are shown in blue and SUMOylation sites (SUMO) are shown in red. The length of each domain and the position of each site are drawn to scale.

GTPase Rac1 [19] or by signaling through the angiotensin II receptor 1 [20]. Once activated, MR forms homodimers or, potentially, heterodimers with related steroid receptors including the glucocorticoid receptor (GR) [21, 22], translocates to the nucleus and interacts with DNA to modulate gene transcription with the help of co-regulators. This provides the biological readout of hormone signaling and involves multiple and complex regulatory steps. In addition, MR activation can initiate rapid signaling events outside the nucleus that modulate cell response and are essential to facilitate transcriptional responses [23, 24].

Every step of MR activation and its impact on cell responses is subject to be modulated by post-translational modifications (PTMs) of the receptor itself or of other proteins physically or functionally interacting with it. PTMs of MR have been previously reviewed in detailed [25] (**Figure 1**). It has long been known that MR is a phosphoprotein [26, 27] and multiple MR residues have been described as potential phosphorylation targets [25]. Most of these amino acids are serine residues, although threonine phosphorylation has been detected using phospho-specific antibodies [28]. One study reported phosphorylation in threonine residues T73[1] and S737 [29], although the functional impact of these modifications has not been studied. Of note, in a more recent study analyzing MR phosphopeptides by liquid chromatography with tandem mass spectrometry, Shibata et al. described 16 different phosphorylated serine residues, but found no evidence of threonine or tyrosine phosphorylation [7]. In addition, other chemical modifications of MR have been described, including ubiquitylation [30–33], SUMOylation [32, 34, 35], acetylation [36] and oxidation [37]. In this chapter, we will update the main known PTMs directly or indirectly affecting MR and focus on their consequences on MR activity.

2. Post-translational modifications that alter MR stability

The abundance of naïve MR likely impacts the potency of receptor-mediated cell responses. There are numerous examples in the literature describing alteration of MR steady-state abundance in different physiological or pathological situations [2]. Changes in receptor abundance can arise from changes in its synthesis or in its degradation rates. In addition, an activation-induced MR degradation also seems to participate in controlling hormone responses [33]. Several MR PTMs have been shown to affect receptor half-life in the cell, both in the basal state and after hormonal stimulation.

Basal level of MR expression appears to be controlled by ubiquitylation (**Figure 2**). In the naïve state, MR is monoubiquitylated at an unknown lysine. This modification is stabilized by association to Tsg101 (tumor suppressor gene 101), increasing the half-life of the receptor [31]. This mechanism is shared by related

[1] All amino acid numbers in this chapter refer to the human MR sequence (UniProt P08235).

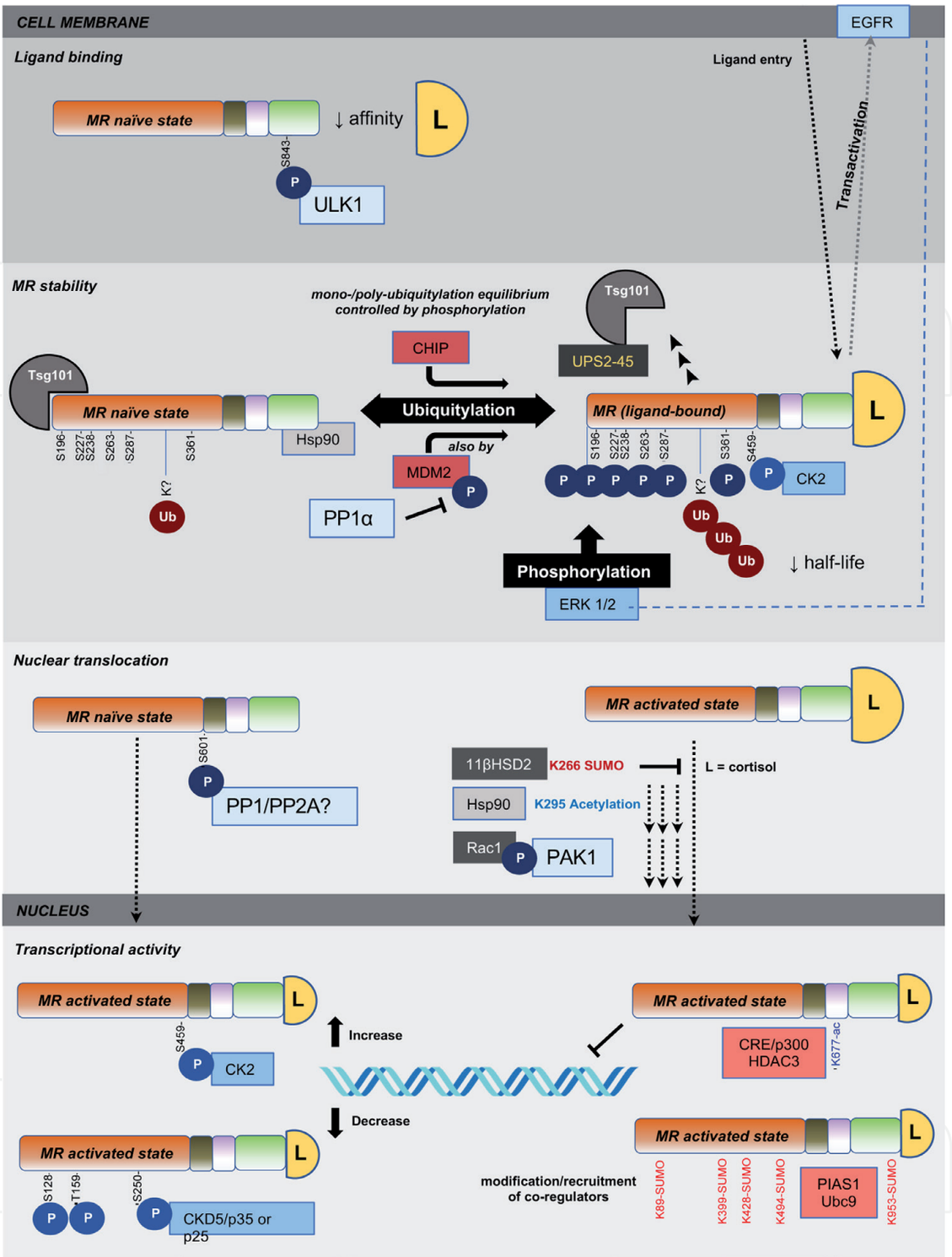

Figure 2.
Schematic representation of well-characterized post-translational modifications affecting mineralocorticoid receptor ligand binding, stability, nuclear translocation or transcriptional activity. MR domain are colored as in **Figure 1***. L, ligand.*

steroid receptors such as the androgen, glucocorticoid and estrogen receptors [38–40]. In addition, it has been shown that poly-ubiquitylation occurs when Hsp90 activity diminishes and the cytosolic heterocomplex recruits ubiquitin-ligase CHIP decreasing receptor expression level [41]. Taken together, these data suggest that equilibrium between mono- and poly-ubiquitylation contributes to regulating naïve MR abundance.

Ligand activation of MR induces receptor degradation through the proteasome, acting as a brake to attenuate aldosterone responses [33]. Subsequent studies

demonstrated that MR is poly-ubiquitylated in response to hormonal stimulation [30–32, 42], a signal that triggers proteasomal-mediated degradation. Interestingly, it has been proposed that the equilibrium between mono- and poly-ubiquitylation of MR is regulated by phosphorylation. Remarkably, MR antagonists such as spironolactone and finerenone totally block aldosterone-induced receptor phosphorylation, preventing the increased degradation rate associated to activation [43]. Aldosterone rapidly induces ERK1/2-mediated phosphorylation of MR at six different serine residues in the NTD (S196, S227, S238, S263, S287 and S361; **Figure 1**). The combined effect of these six phosphoserine residues is to promote the removal of mono-ubiquitylation from MR, triggering receptor destabilization [31]. Ubiquitin-specific protease 2-45 (USP2-45), an aldosterone-induced protein in the mouse distal nephron [44], is responsible for the ligand-induced loss of MR mono-ubiquitylation, simultaneously destabilizing MR/Tsg101 interaction [30] (**Figure 2**). in agreement with the model described above data obtained with *usp2* gene knockout mice showed increase expression of MR, although this change in abundance does not produce apparent alterations in sodium balance or blood pressure [45].

According to the model described above, phosphatases opposing MR ligand-induced phosphorylation should contribute to stabilize the receptor. Interestingly, it has recently been described that protein phosphatase 1α (PP1α) indeed stabilizes MR [42]. This study described PP1α as an MR cytosolic interaction partner. However, the effect of PP1α on MR appears to be indirect, mediated the dephosphorylation of ubiquitin ligase MDM2, which is inactivated, precluding MDM2-mediated MR proteasomal degradation [42] (**Figure 2**).

Modification of additional residues in MR contributes to the receptor stability. For instance, Ruhs et al. recently described that MR phosphorylation at residue S459 (**Figure 1**), catalyzed by casein kinase 2 (CK2), not only facilitates MR-DNA interaction, increasing aldosterone-induced gene transcription (see below), but also promotes rapid degradation of MR [46]. The mechanism involved in enhanced MR degradation by S459 phosphorylation is unknown.

As mentioned above, it has been previously shown that MR stability is controlled by Hps90 activity. When the co-chaperone is pharmacologically inhibited with tanespimycin, MR stability decreases through increase ubiquitylation mediated by CHIP [41]. In contrast, a different inhibitor of Hsp90, geldanamycin, did not produce any alterations in MR levels [17]. To further explore the role on Hsp90 on MR activation and stability, we tested the possible role of acetylation of Hsp90 at residue K295 [47], a modification that impairs interaction with co-chaperones and client proteins [48]. Surprisingly, we could not find any evidence for Hsp90 acetylation-induced alterations in MR stability [47], although this PTM did affect nuclear translocation dynamics (see below).

3. Post-translational modifications that directly or indirectly control ligand binding to MR

Ligand binding capacity of MR is not an exclusively intrinsic property of the ligand-binding domain (LBD) of this receptor. It has long been known that MR association to the chaperone Hsp90 is essential for aldosterone binding [49, 50]. In addition, the full-length MR sequence is required to bind aldosterone with high affinity, suggesting that areas outside the LBD contribute to folding of the receptor in a competent state [51]. The idea that PTMs may contribute to regulate MR ligand binding came from the observation that phosphatase treatment of cytosolic extracts greatly diminish aldosterone binding to MR [27]. This suggested that basal phosphorylation in serine/threonine residues is essential for the competency of the

receptor to bind ligands. However, these experiments did not allow distinguishing whether the phosphorylation takes place in the receptor itself or in other proteins of the cytosolic heterocomplex containing the naïve receptor. More recently, direct evidence implicating phosphorylation on ligand binding to MR has emerged. Phosphorylation of MR at residue S843 has been shown to diminish the affinity of the receptor for aldosterone and corticosterone [7]. This process is mediated by protein kinase ULK1 [52]. Interestingly, phosphomimetic mutants cannot be activated by aldosterone or cortisol even when the concentration of the hormones is one to two orders of magnitude higher than the calculated *Kd*, indicating that phosphorylation affects not only ligand binding but also ligand-induced receptor activation [53]. Importantly, phosphorylation of MR at residue S843 acts as a dominant-negative modification, inhibiting wild type receptors upon dimerization, which greatly amplifies the impact of this event on total MR activity [53].

Ligand binding to MR is also affected by oxidation. It has long been known that MR is a highly unstable protein and this has been ascribed to sulfhydryl oxidation, which prevents aldosterone binding [37, 54]. In particular, cysteine 849 and 942 appear to be responsible for this effect, since site-directed mutagenesis at these positions eliminate steroid binding to the receptor [55]. This phenomenon appears to be relevant in vivo, since inhibition of glutathione synthetase in mice abrogated aldosterone binding to kidney MR [56]. Since oxidative stress decreases aldosterone binding and activation and aldosterone binding decreases with age, it has been suggested that oxidation of MR could be an important mediator of aging in the kidney [57].

In classic aldosterone tissues like the kidney or the distal colon and in certain neurons, glucocorticoid accessibility to MR is crucially controlled by co-expression of 11ßHSD2, which metabolizes glucocorticoids to produce biologically inactive, 11-keto derivatives [4]. Therefore, transcriptional or post-transcriptional modulations of this enzyme potentially have a large impact on MR activity. We have recently described that 11ßHSD2 is modified by SUMOylation at residue K266 [58] (**Figure 2**). While the effect of SUMOylation on enzymatic activity is mild, its impact on MR activation process is puzzling. In spite of being enzymatically active, non-SUMOylatable mutant 11ßHSD2-K266R was unable to prevent MR nuclear translocation when cells were treated with cortisol, unlike the wild type enzyme [58]. The same was detected when 11ßHSD2 SUMOylation is reversed by co-expression of sentrin-specific protease 1 (SENP1), a protease that catalyzes SUMO deconjugation. However, MR translocated to the nucleus under these conditions does not increase transcriptional response to cortisol and shows diminished recruitment of co-activators [58]. Therefore, 11ßHSD2 SUMOylation drastically alters the ability of this enzyme to regulate MR subcellular localization, although the molecular mechanisms involved in this effect remain to be elucidated.

4. Post-translational modifications that alter MR nuclear translocation

Long-term, genomic actions of MR depend on its nuclear localization and interaction with chromatin. While some steroid receptor (ER and PR) are constitutively nuclear, naïve MR is considered to be mainly cytosolic, where it forms part of a heterocomplex with chaperones and other proteins, translocating to the nucleus after ligand binding. In fact, it is most common to find MR evenly distributed between nucleus and cytosol, with a clear nuclear shift when exposed to ligands. This mode of action fits well with data obtained in some models of cultured cell lines, such as COS-7 cells or HEK cells [17, 43, 47, 59] and native tissues [60–62]. In certain tissues and cell types, MR can be found in the nucleus even in the absence of ligand [18, 60, 61]. Therefore, it appears that nuclear translocation plays an important role

in MR regulation, although the physiological relevance of this step may strongly vary between different tissues or cell types.

Nuclear translocation has been studied with much more detail in the case of other steroid receptors, including GR, which shares significant sequence homology with MR. In the case of GR, two independent nuclear translocation pathways have been proposed, one that depends on Hsp90 and the attachment of the ligand-bound heterocomplex to microtubules and one where monomers or dimers of GR translocate independently of this machinery [63]. In the case of MR, there is strong evidence indicating that nuclear translocation occurs in an Hsp90-dependent way, with dissociation between the chaperone and the receptor occurring in the nucleus [16, 17]. Inhibition of Hsp90 abrogates hormone binding to MR and nuclear translocation of the receptor, although cells with low levels of Hsp90 expression such as cardiomyocytes present constitutively nuclear MR that can be activated by aldosterone or cortisol [18]. This result suggested that fine-tuning of Hsp90 activity could play a role in controlling MR subcellular localization. Therefore, we decided to explore the role of Hsp90 acetylation at residue K295 in MR nuclear translocation [47]. This modification, regulated by histone deacetylase 6 (HDAC6), inhibits Hsp90 and has been shown to decrease GR and AR activity [48]. In the case of MR, increased acetylation of Hsp90 does not affect ligand binding or transcriptional activity, but alters subcellular dynamics, accelerating MR nuclear import. Given the differential effects of Hsp90 K295 acetylation on MR and GR, it has been proposed that this modification may balance corticosteroid signaling between both receptors when co-expressed in the same cell [47].

Nuclear translocation of MR critically depends on nuclear localization signals (NLS) present in the sequence of the receptor. Three independent NLS has been identified in MR: NL0, 1 and 2 [64]. Among these three, NL0 has been implicated in nuclear localization of the naïve receptor. NL0 was mapped to amino acids 550–602 of human MR. In this area, a cluster of five serine residues and one threonine between amino acids 590 and 602 is important for NL0 activity, since its deletion significantly decreases naïve MR nuclear localization, although it does not abrogate ligand-induced translocation [64]. Interestingly, phosphomimicking mutation S601D (**Figures 1** and **2**) eliminated NL0 activity, resulting in a fully cytosolic MR localization in the absence of ligand. Conversely, non-phosphorylatable mutant S601A resulted in a significant nuclear shift of naïve MR [64]. Therefore, phosphorylation/dephosphorylation balance at S601 may be an important mechanism for controlling MR subcellular distribution in the absence of ligand. The effect of this equilibrium on the physiology of the receptor remains to be studied. Both *in vitro* and *in vivo* experiments indicate that a protein phosphatase from the PP1/PP2A subgroup regulates ligand-induced MR trafficking into the nucleus [65], although it is unclear whether this results from dephosphorylation of MR or other associated proteins such as Hsp90.

Aldosterone-induced nuclear translocation is potentiated by Rac1-mediated phosphorylation of p21-activated kinase 1 (PAK1) (**Figure 2**). This signaling pathway is relevant in the development of chronic kidney disease [19] and in cardiac remodeling and inflammation induced by blood pressure variability in the context of hypertension [66]. It remains to be determined whether PAK1 directly phosphorylates MR and whether it promotes its activity in addition to receptor translocation, as it has been described with estrogen receptors [67].

5. Post-translational modifications controlling MR activation and modulating gene transcription

In addition to the mechanisms discussed above modulating MR ligand binding, stability and subcellular localization, additional PTMs regulate the ability of MR to

modulate gene transcription, potentially altering the efficacy of the receptor without altering its affinity for agonists. This possibility was suggested after studying a human polymorphism introducing the mutation Y73C, which increases MR transactivation in response to aldosterone by twofold, without changing the EC_{50} [68]. Residue Y73 is placed in AF1a and therefore could modulate interaction with transcriptional co-activators. However, there are no further reports demonstrating that this potential phosphorylation site is actually modified in the protein. Le Moellic et al. proposed a possible role for phosphorylation in controlling MR transcriptional activity [28]. This study found that protein kinase C α (PKCα) mediates rapid MR phosphorylation at serine/threonine residues after stimulation with aldosterone, which presumably acts through a membrane receptor [28]. Blocking PKCα during this early, non-genomic phase precludes the development of the genomic phase. However, whether this effect is directly due to lack of activation of MR or, alternatively modulation of other aspects of the receptor such as aldosterone binding or nuclear translocation was not studied.

More direct evidence of phosphorylation involvement in controlling MR activity came from studying the effect of cyclin-dependent kinase 5 (CKD5) on the receptor. CKD5 has been proposed to phosphorylate two residues in the NTD of MR (S128[2], T159 and S250; **Figure 1**), producing a very powerful decrease in MR transcriptional activity without affecting nuclear translocation [69] (**Figure 2**). The phosphorylation is mediated by direct interaction of the CKD5/p35 or CKD5/p25 complexes with MR LBD in an aldosterone-dependent way [69]. T159 and S250 phosphorylation could be confirmed by mass spectrometry, while phosphorylation at residue S128 was inferred from mutagenesis studies. Mutation of all three residues to alanine was necessary to abolish CKD5-dependent MR inhibition [69].

As indicated above, CK2-dependent MR S459 phosphorylation (**Figure 1**) facilitates MR-DNA interaction, at least in an *in vitro* assay, and increases aldosterone-induced gene transcription [46]. This effect is partially mediated by the NTD of the receptor, where the phosphorylation site is located, possibly by promoting MR-CK2 interaction in a process that needs Hsp90 activity. Interestingly, phosphomimetic mutation S459D not only increased aldosterone-induced responses but also resulted in ligand-independent transcriptional activation (**Figure 2**). Modeling a pro-inflammatory environment by treating cultured cells with a cytokine cocktail increased CK2 expression, resulting in enhanced MR modification, leading to increase receptor activity and activating NFκB signaling and thus enhancing the expression of proinflammatory genes [46]. This could provide a mechanism to help explain the deleterious effects of MR activity in the context of inflammation, as demonstrated in endothelial cells [71]. The mechanism underlying altered MR activity by CK2 phosphorylation remains unclear. The authors speculated that S459 phosphorylation could induce a conformational change that promotes MR-DNA interaction or alternatively enhance MR association with transcriptional co-activators [46]. The latter possibility is plausible, since residue S459 lies in AF-1b within the NTD, a region involved in the interaction between MR and transcriptional co-regulators in a ligand-independent manner [72].

MR acetylation at residue K677 (**Figures 1** and **2**) inhibits its transcriptional activity by preventing MR and RNA polymerase II recruitment to target gene promoters. Surprisingly, K677 acetylation did not affect MR nuclear translocation [36], even though this residue is located in NL1. This study and subsequent work by

[2] Human MR has serine residues at positions 127 and 129, but not at position 128. The authors refer to this residue as residing "in the perfect motif of CDK5 phosphorylation site" [69], which is [S/T]PX[K/R/H] [70]. Residue S129 fits this description and therefore it is likely that the authors refer to it instead of residue 128, which is a methionine.

the same group identified CREB-binding protein (CRE)/p300 as the acetylase and HDAC3 as the deacetylase responsible for modifying K677 [36, 73] (**Figure 2**). The molecular basis for the lack of MR binding to target promoters when acetylated at residue K677, which is away from the DBD, is unknown.

Addition of small ubiquitin modifier (SUMO) proteins to five different lysine residues in MR (**Figure 1**), all located within a SUMOylation consensus site, has been proposed to alter its transcriptional activity and co-regulator recruitment [32, 34, 35]. The first description of MR SUMOylation followed the identification of protein inhibitor of activated STAT-1 (PIAS1), a SUMO E3 ligase, as an MR interacting partner using a yeast two-hybrid assay using the NTD of the receptor as bait [34]. The functional consequences of PIAS1-mediated SUMOylation are complex. Co-expression of PIAS1 with MR led to repression of receptor-mediated gene transactivation in two different model promoters, the mouse mammary tumor virus promoter (MMTV) and an artificial glucocorticoid response element (GRE) promoter [34]. However, introducing non-SUMOylatable mutations in the receptor did not produce the expected opposite effects in MMTV, which was unaffected. In contrast, the same mutations increased MR activity on the GRE promoter. Taken together, these results suggest that the effect of PIAS1 on MR is promoter-dependent and may occur through different mechanisms including direct receptor SUMOylation and perhaps SUMOylation of transcriptional co-regulators. In addition, PIAS1 may exert additional modulatory activities independent of its SUMO ligase activity. This possibility is suggested by the observation that a PIAS1 SUMO ligase-dead mutant W363A is still able to inhibit androgen receptor transcriptional activity [74]. The complexity of SUMOylation-dependent regulation of MR activity is illustrated by the fact that the E2 SUMO ligase responsible for its SUMOylation, Ubc9, is able to recruit steroid receptor coactivator-1 (SRC-1) to form a complex with MR and activate its transcriptional activity independently of addition of SUMO residues to the receptor [35]. Therefore, the two enzymes playing a role on MR SUMOylation, PIAS1 and Ubc9 (**Figure 2**), can have opposing effects on the receptor transcriptional activity in a promoter-dependent way, suggesting that many of these actions occur through modification/recruitment of co-regulators.

6. Summary and perspectives

Many PTM sites have been identified in MR, but only some of them have been experimentally linked to alterations in MR function (**Figures 1** and **2**). These include modulation of MR ligand-binding ability, stability, nuclear translocation and gene transactivation. In addition, MR-associated proteins such as Hsp90, 11ßHSD2 or ubiquitin ligases such as MDM2 are also modulated by PTMs, adding further regulatory possibilities for fine-tuning MR activity. This complex picture is not unexpected, given the near ubiquitous distribution of MR and the diverse functional roles played by this receptor in response to two different types of ligands, mineralocorticoids and glucocorticoids. Therefore, it will not be surprising to find new PTMs directly or indirectly implicated in the regulation of MR activity. In addition, ligand-dependency of MR PTMs needs to be addressed with more detail. In principle, changes in MR conformation induced by different ligands could affect accessibility to modification sites. Differential PTM in response to different agonists or antagonists could potentially underlie divergent effects of MR-mediated signaling events. Generally, detailed characterization of the functional effects of PTMs is feasible in cultured cells. However, the main challenge remains to elucidate the physiological or pathological importance of these modifications in whole organisms and the clinical relevance that they may have in humans.

Due to the nature of steroid receptors, including MR, they are inherently druggable targets. Excess signaling through MR has now been firmly established as an important factor in hypertension, heart failure and ocular diseases, with compelling evidence indicating further implication in brain, vascular, renal, metabolic and skin diseases [2]. Therefore, there is renewed interest in developing MR modulators with tissue-specific characteristics that may reduce or avoid undesirable side effects. Given the high degree of homology between LBDs of different steroid receptors, a significant effort to develop nonsteroidal inhibitors is underway [75]. In addition, it is possible that allosteric inhibitors or small molecules able to modulate MR protein-protein interactions may provide new strategies to manipulate the system. PTMs may have an important effect on drug binding and future drug development, both for competitive and allosteric modulators. In fact, recent *in silico* approaches have explored this possibility, exploiting the increasing availability of high-throughput PTM screenings and high-resolution protein three-dimensional structures [76]. It can be expected that improved PTM screening, combined with structural and computational methods will provide new testable hypotheses regarding the regulation of steroid receptors and possible new ways of pharmacological modulation of their activities.

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