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Mini Review in Molecular Endocrinology

Acetylation in Nuclear Receptor Signaling and the Role of Sirtuins

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Abstract

It has been known since the early 1970s that nuclear receptor complexes bind DNA in association with co-regulatory proteins. Characterization of these nuclear receptor coregulators has revealed diverse enzymatic activities which temporally and spatially coordinate nuclear receptor activity within the context of local chromatin in response to diverse hormone signals. Chromatin modifying proteins, which dictate the higher order chromatin structure in which DNA is packaged, in turn orchestrate orderly recruitment of nuclear receptor complexes. Modifications of histones include acetylation, methylation, phosphorylation, ubiquitylation, sumoylation, ADP ribosylation, deimination and proline isomerization (1). At this time, we understand how a subset of these modifications regulates nuclear receptor signaling. However the effects, particularly of acetylation and demethylation, are profound. The finding that nuclear receptors are directly acetylated and that acetylation in turn directly regulates contact-independent growth has broad therapeutic implications. Studies over the past 7 years have led to the understanding that nuclear receptor acetylation is a conserved function, regulating diverse nuclear receptor activity. Furthermore we now know that acetylation of multiple and distinct substrates within nuclear receptor signaling pathways, form an acetylation signaling network from the cell surface to the nucleus. The finding that NAD-dependent histone deacetylases, the sirtuins, are capable of deacetylating nuclear receptors provides a new level of complexity in the control of nuclear receptor activity in which local intracellular concentrations of NAD may regulate nuclear receptor physiology.

Nuclear Receptors Co-regulator Enzyme Complexes

In the early 1970s initial attempts at purifying nuclear receptors were confounded by the large number of co-associated proteins. The O'Malley laboratory had characterized the nuclear progesterone receptor/DNA complex and the thyroid hormone receptor associated with a heterogeneous group of proteins that was regulated in a ligand-dependent manner (2, 3). It was apparent that transcription factors contained transactivation domains which functioned as modular surfaces to regulate transcription independently of direct binding to DNA (4). The laboratory of Tjian and others characterized the TATA box binding protein-associated factors termed TAFs (5). Several cell-type specific activities were characterized and shown to regulate transcription factor activity. In this regard a B cell-specific activity designated Oct coactivator from B cells (OCA-B) regulated Oct-dependent B cell specific transcription (6).

Cross squelching experiments by the Chambon laboratory (7) suggested distinct classes of transcriptional activation domains existed within nuclear receptors. Consistent with the notion that nuclear receptors were capable of repressing transcription, formal evidence that nuclear receptors contain specific repression domains was provided by studies of the progesterone receptor and retinoic acid receptor (8, 9). These studies provided the rational basis for the identification of proteins mediating transcriptional activation and repression of nuclear receptors. Yamamoto identified the SWI protein as a key activator of the glucocorticoid receptor in yeast (10). In 1994, CBP was cloned as a coactivator of CREB (11) and p300 as an E1A-interacting protein (12, 13). Of fundamental importance was the identification of histone acetyltransferase

enzymatic activity within the p300 activation domain. These proteins were shown to function as rate-limiting co-activators of nuclear receptor activity partially dependent upon their intrinsic histone acetyltransferase activity.

A dynamic and rapidly evolving field has characterized diverse types of enzymes (14). Furthermore, the assembly of these enzymes was shown to be temporally coordinated. The histone acetyltransferase, p300, enhanced the efficiency of transcriptional initiation from an estrogen-regulated template assembled within chromatin. The reassembly of active complexes during subsequent rounds of reinitiation did not require p300 (14). Indeed consistent with these findings, chromatin immunoprecipitation experiments identified temporarily coordinated multi-protein complexes associated with ER α and with endogenous ER α DNA binding sites. These studies showed coactivators were recruited in a cyclical manner in association with local chromatin. p300 was recruited to the promoter region of the ER α -responsive genes in the first phase of ER α binding, but not in subsequent cycles of ER α recruitment (15).

Nuclear receptor acetylation governs cellular growth potential

Histone acetyltransferases have been shown to acetylate diverse substrates. The first evidence that nuclear receptors served as direct substrates for histone acetyltransferases were studies by Fu et al. (16). The residues of AR acetylated by p300 *in vitro* were conserved between species. Point substitution mutations of the acetylation sites identified *in vitro* regulated ligand-dependent transactivation. Subsequent studies demonstrated that the nuclear receptor acetylation site is conserved between a subset of nuclear receptors, including the ER α , TR β (17), PR and the GR

(18). With each of the nuclear receptors characterized to date, the acetylation sites regulate a subset of nuclear receptor functions with the AR currently being the best characterized. The addition of ligand, dihydrotestosterone, or other agonists such as bombesin, enhances androgen receptor acetylation (19). When reintroduced into AR-deficient human prostate cancer cells, gain of function point substitution of the AR acetylation site resulted in receptors that promote prostate tumor growth, both *in vitro* and *in vivo* (20). Characterization of the mechanism by which the androgen receptor acetylation site regulated contact-independent growth, indicated both enhancement of cellular proliferation and a reduction in cellular apoptosis (20, 21). The charge of lysine residues in the AR acetylation site regulated recruitment of p300, and in a reciprocal manner, disengagement of corepressor complexes including NCoR, HDAC, and Smad 3 (20).

The AR acetylation site regulates prostate cellular apoptosis. The Jun kinase kinase signaling pathway (MEKK1) induces cellular apoptosis in the presence of the AR. The AR acetylation site governs TRAIL-induced cellular apoptosis (21). Cells transduced with AR acetylation site gain-of-function mutants enhanced proliferation, associated with the induction of cell cycle markers such as Ki67 and cyclin D1. The relative abundance of cyclin D1 was increased in cells transduced with the AR activating mutants.

Chromatin immunoprecipitation analysis demonstrated that in addition to the enhanced recruitment of the AR to an androgen-responsive element binding site (ARE), the histone deacetylase inhibitor, TSA, enhanced AR recruitment in an AR acetylation site-dependent manner (22). AR acetylation site dead mutants showed reduced recruitment to the PSA promoter androgen-responsive element and reduced TSA-dependent recruitment. This suggests that the

acetylation site of the nuclear receptor regulated access in the context of local chromatin (22).

The ER α was shown to be acetylated at the same motif as the AR (23). Deletion analysis identified the primary site acetylated by p300 and a minimal peptide sufficient to serve as a substrate for p300 was characterized. The peptide encoding the ER α acetylation site conveyed similar substrate specificity as histone H3. Proteomic analysis including LC/MS and Edman degradation sequencing, demonstrated K302 and K303 were preferential sites of acetylation. Lys K303 is frequently mutated in human breast cancer (24). The ER α acetylation mutants function as hyperactive mutants in the presence of low concentrations of its ligand estradiol (23, 24). The ER α coactivator SRC1 is acetylated, and point mutation of the SRC1 acetylation site results in an ACTR hyperactive mutant that fails to be transcriptionally down regulated (25). As mutation of the ER α and the ER α coactivator ACTR acetylation site, enhances ligand-dependent acetylation may serve to repress hormone signaling by recruiting co-repressor activity as shown for PGC1 α (26), or through docking other as-yet-to-be-defined complexes. Consistent with this model ER α repression by BRCA1 (27, 28) is abolished by mutation of the ER α K302/303 acetylation site (RGP. CW unpublished data). Like PGC1 α and p53, in which multiple acetylation sites have been identified (29), ER α has recently been shown to be acetylated at the additional residue K266/K268 (30). In these studies, SRC1/p300 was used as the source of HAT. The relative importance of the K302/303 vs. the K266/K268 site in the diverse physiological roles of the ER α remains to be determined.

A body of evidence has contributed to the current understanding that PGC1 α , a transcriptional co-activator of nuclear receptors, is acetylated and the acetylation of PGC1 α

regulates cellular metabolism. The metabolic coactivator PGC1 α modulates the gluconeogenic pathway in fasting and diabetic states through interaction with several transcription factors (31, 32). PGC1 α forms part of a multi-protein complex that includes the TRAP/Mediator complex, including TRAP220 binding to its C-Terminus (33). The endogenous PGC1 α complex includes the histone acetyltransferase GCN5. p300 and SRC1 bind the N-terminal activation domain of PGC1 α (34). PGC1 α is directly acetylated by GCN5 resulting in a transcriptionally inactive protein which relocalizes from promoter regions to nuclear foci (26).

Acetylation of components within hormone signaling pathways

The addition of liganded nuclear receptor to a cell induces multiple downstream signaling cascades. A growing body of evidence has demonstrated that several additional components which regulate nuclear receptor signaling pathways are themselves substrates for histone acetyltransferases (Fig. 1). Additional components of nuclear receptor signaling cascade that are directly acetylated include MEKK1, Hsp90 (35, 36), and IKK (37). The acetylation of these nuclear receptor binding proteins, in turn, affects nuclear receptor function. Thus, for example, Hsp90-mediated glucocorticoid receptor (GR) maturation is regulated by acetylation of Hsp90 (36). Within the nucleus, components of the nuclear receptor coactivator or corepressor complexes are themselves substrates for histone acetyltransferases. Thus ACTR and MTA1 are histone acetylases, and ACTR and PGC-1 α serve as substrates for histone acetyltransferases (25, 26). Other transcription factors known to work in *trans* to regulate nuclear receptor signaling, such as the forked protein, are acetylated, providing an additional level of complexity for cross talk between (FKHR) and ER α signaling (38).

The NAD-dependent histone deacetylases regulate hormone signaling

The histone deacetylases are divided into three groups. The Class 1 and 2 HDACs share homology to the yeast Rpd3p and Hda1p proteins. The Class 3 HDACs are homologous to the yeast transcriptional repressor Sir2p. The Class 3 HDACs are nicotinamide adenine dinucleotide (NAD)-dependent. Mammalian Sirtuins are conserved with 7 genes homologous to the yeast *Sir2* gene. The nuclear sirtuins (SIRT1, SIRT6 and SIRT7), the mitochondrial sirtuin (SIRT3, SIRT4 and SIRT5) and cytosolic (SIRT2) regulate diverse metabolic function. The distinct substrates and subcellular localization of the mammalian Sirtuins suggests significant diversity of function (39, 40).

SIRT1 has been characterized in its interactions with nuclear receptors and nuclear receptor coactivators. The functional interactions between SIRT1 and nuclear receptor signaling has been examined for the cointegrators p300 (41) and PGC1 α (42-44), and for the nuclear receptor AR (45), PPAR γ (46) and ER α (47). SIRT1 has been shown to deacetylate coactivators PGC1 α and p300 and the nuclear receptors ER α and AR. SIRT1 regulates repression of the co-activator p300, providing a mechanism by which the NAD-dependent HAT deacetylases may regulate diverse nuclear receptor functions (41). The p300 coactivator functions as a rate-limiting protein in regulating diverse nuclear receptor functions. A direct link between the NAD-dependent histone deacetylases and the histone acetyltransferases was first demonstrated by Bouras *et al* (36) (Fig. 1). SIRT1 repressed p300 in an NAD-dependent manner. The lysine residues within the Cell Cycle Regulatory Domain (CRD1) of p300 were essential for SIRT1-mediated repression. Importantly Bouras *et al* demonstrated that lysine residues within p300

function as substrates for SIRT2 (41). SIRT1 also functions as a substrate for sumoylation (41). SSP3, a sumo specific protease, antagonized SIRT1-mediated repression of p300. Importantly these studies suggest a link between local metabolic changes which are anticipated to alter the NAD/NADH ratio and p300 activity. As sirtuins are regulated by NAD, endogenous levels of nicotinamide may limit SIRT1 function and thereby alter activity of many transcription factors and nuclear receptors which are regulated by p300.

Sirtuins have well characterized functions in regulating responses to caloric restriction and glucose metabolism, cellular growth and DNA repair. SIRT1 regulates gluconeogenesis by promoting the repression of glycolytic gene function (40). Importantly, recent studies have linked SIRT1-dependent deacetylation of the nuclear receptor coactivator PGC1 α to glucose homeostasis. PGC1 α is acetylated *in vivo* (43). Acetylation of PGC1 α was augmented by treatment with the SIRT1 inhibitor nicotinamide or by expression of the transcriptional coactivator p300 (28). The finding that SIRT1 associates with PGC1 α (28) led to studies examining the biological significance of PGC1 α acetylation. Fasting was shown to induce PGC1 α deacetylation in skeletal muscle. This important study, identifying SIRT1 as a functional regulator of PGC1 α to induce mitochondrial fatty acid oxidation *in vivo*, has broad implications for the understanding of adaptations to altered availability of nutrients. SIRT1 is increased in the fasted liver, thereby deacetylating and activating PGC1 α , enhancing hepatic glucose output (43).

These studies imply that PGC1 α abundance may in turn govern metabolic diseases such as obesity and diabetes. Recent studies using Resveratrol (RSV), a known activator of SIRT1 (48), demonstrated an increased aerobic capacity and induction of oxidative phosphorylation and mitochondrial biogenesis, primarily mediated by RSV-mediated inhibition of PGC1 α acetylation

and an increase in PGC1 α activity (49). Resveratrol was also shown to oppose the effects of a high calorie diet producing changes associated with longer lifespan (50). Caloric restriction enhances SIRT1 binding to and repression of PPAR γ -responsive gene promoters. SIRT1 repressed PPAR γ activity through binding the co-repressors NCoR and SMRT (46). Consistent with the physiological significance of SIRT1-mediated repression of PPAR γ activity, *SIRT1*^{+/-} mice demonstrated defective mobilization of fatty acids from white adipocytes upon fasting (46). Whether PPAR γ or components within a PPAR γ complex are direct substrates of acetylation, remains to be determined. Collectively, these studies demonstrate the important physiological role for PGC1 α acetylation and a key role for the NAD-dependent histone deacetylase SIRT1 in regulating fat metabolism.

Recent studies have provided indirect evidence for an additional potential functional interaction between nuclear receptors and the sirtuins. Studies from the laboratories of Rosenfeld and Glass demonstrated the induction of transient double-stranded DNA breaks upon the addition of estradiol, and the recruitment of ER α , PARP-1 and DNA topoisomerase II β (51). The *BRCA1* gene which plays an essential role in DNA repair, is known to bind the ER α in the context of local chromatin in a temporally coordinated manner (28). Although the role of ER α acetylation in recruitment to double-stranded DNA breaks is unknown, Sirtuins, in particular SIRT6, regulate DS-DNA break repair. SIRT6 plays a key role in resistance to DNA damage and suppression of genomic instability (52). The fibroblasts of *SIRT1*^{-/-} mice showed enhanced sensitivity to DNA damaging agents. Although the sirtuins and the ER α have both been independently implicated in DS-DNA break repair, it remains to be determined with sirtuins regulate this nuclear receptor function.

The use of Sirt1 inhibitors has strongly implicated endogenous SIRT1 in regulating AR activity in prostate cancer cells (37). SIRT1 colocalizes with the AR in nuclear subdomains (37). SIRT1 inhibits cellular proliferation in an AR-dependent manner. SIRT1 antagonists induce endogenous AR expression and enhance DHT-mediated AR-dependent gene expression. SIRT1 physically binds to and deacetylates the AR at a conserved lysine motif. The AR served as a substrate for the SIRT1 orthologs of several species including the mouse mSir2a, hSirt1 and two other enzymes *Cerevisiae* (SIR2p) and *Archaeoglobus fulgidus* (Sir2af2). Each of these enzymes were examined for deacetylation activity against a synthetic AR peptide known to be acetylated by p300. The deacetylation of the AR by the Sirtuins of several species was characterized by LCMS and MSMS fragmentation analysis. Thus the AR is deacetylated by SIRT1 to inhibit ligand-dependent AR activity to thereby inhibit DHT-dependent prostate cancer cellular proliferation.

What might be the physiological role of Sirtuin dependent regulation of nuclear receptor function? Sirtuin expression and activity is itself under complex control and may impact nuclear receptor function. The importance of NAD in Sir2 activity implies Sir2 activity may be regulated by intracellular NAD, by the NAD/NADH ratio, or nicotinamide (53, 54). Elevated lactate, which reduces the NAD/NADH ratio, is predicted to inhibit SIRT1 (55). Inhibition of SIRT1 may enhance AR function (45) with consequent anabolic repair effects within muscle (56, 57). During prostate cancer progression a shift towards cytosolic glycolysis occurs (58, 59). Increased local lactate concentration correlates with prostate cancer progression (60, 61), which may be predicted to inhibit SIRT activity and activate the AR (45). Sirtuins are located in diverse subcellular locations and it will be of interest to determine whether receptors in non-nuclear

compartments are regulated by Sirtuins.

Conclusion

Nuclear receptors serve as substrates for Sirtuins and play a role in glucose metabolism, cell growth and DNA-repair. Evidence suggests sirtuins regulate nuclear receptors and nuclear receptor coactivators to coordinate diverse physiological functions. Histones are modified by at least eight distinct types of enzyme. Post-translational modification of histones by specific enzymes determines their subsequent types of enzymatic modification. Acetylation of lysine residues provides docking sites for other enzymes which coordinate sequential enzymatic reactions. Distinct modifications of lysine residues are mutually exclusive. It has been proposed that post-translational modification of histones imparts specific genetic output and signaling specificity. Thus, post-translational modification of lysines within histone tails results in specific types of signaling. Nuclear receptors undergo acetylation, phosphorylation, sumoylation and methylation. It is likely that these post-translational modifications are part of a similar coordinated signaling cascade that occurs within the receptor. The mechanisms by which post-translational modification within nuclear receptors contributes to the specificity of hormone signaling has broad implications for health and disease, in particular hormone-responsive cancers.

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Figure Legends

Figure 1: The histone acetyltransferase (HAT) protein, p300, is capable of regulating gene expression through transcription factors (TFs) and nuclear receptors (NRs) binding to DNA specific sequences within euchromatin. This effect is governed by K1020 and K1024 acetylation within p300's CRD1 domain. SIRT1, the function of which is induced by NAD^+ produced as a byproduct of oxidative phosphorylation, serves as the master regulatory protein in this process.

Figure 1



