J Med Sci 2007;27(6):245-250
http://jms.ndmctsgh.edu.tw/2706245.pdf

Insights from Post-translational Modifications of Orphan Nuclear Receptors TR2 and TR4

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Nuclear receptors (NRs) constitute one of the largest families of transcription factors that play important physiological roles. Among the 48 NRs identified in the human genome, approximately half are orphan members whose ligands remain to be identified. Testicular receptor-2 (TR2) and -4 (TR4) belong to the orphan category and represent a more ancestral subfamily. Classical molecular studies demonstrate their biological activities in regulating several hormone responsive genes; however, the molecular detail of their biological activity remained unresolved until recently. Gene knockdown studies suggest multiple physiological functions for TR4, and studies of stem cells reveal the physiological function of TR2 specifically in cell proliferation. More recent proteomic endeavor has uncovered extensive protein post-translational modification (PTM) of TR2 and TR4 and begun to elucidate the molecular basis underlying their ligand-independent biological activities in gene regulation. TR2 and TR4 can be modified by phosphorylation, sumoylation and lysine methylation, and each form of PTM modulates their biological activities in a distinct manner. Studies of PTM have considerably facilitated the verification of the biological activities of TR2 and TR4. With data gathered from recent studies of PTM, the biological relevance of this once disregarded family of transcription factors has been redefined.

Key words: orphan nuclear receptor, TR2/TR4, post-translational modification, gene regulation

A. Introduction to Nuclear Receptors

Nuclear receptors (NRs) comprise a superfamily of transcription factors that regulate gene transcription primarily through direct binding to their chromatin targets usually referred to as "hormone response elements" (HREs). In the human genome, this super-family consists of 48 members most of which are known to regulate complex gene net works involved in various physiological processes such as growth, development and differentiation[1,2]. These transcription factors are characterized by the presence of a conserved modular structure where three principal functional domains are assigned, i.e., the amino terminal activating function 1 (AF1) domain, the central DNA-binding domain (DBD) and the carboxyl terminal ligand-binding domain (LBD). The DBD constitutes the hallmark of this family of transcription factors and adopts a very similar finger-like structure coordinated by two zinc ions, therefore named zinc finger transcription factors[3]. This domain wraps around the target DNA, i.e., the HRE, which usually consists of multiple repeats of 6 base pairs with varying spacing sequences[4]. The LBD dictates the important biology of this family of transcription factors by determining the specificity of their ligands such as steroid hormones, thyroid hormones, vitamin A, vitamin D, and fatty acids, etc. The members with well-characterized ligands are named according to their ligands, i.e., retinoic acid receptors (RARs), vitamin D receptor (VDR), and estrogen receptor (ER) etc. Those members whose ligands have not been found are named orphan receptors. As the biological activity of LBD was initially thought to be an activation following ligand binding, this domain has also been referred to as the activating function 2 (AF2) domain. The amino-terminal AF1 domain is less characterized, but is believed to be involved in modulating the biological activity of the molecule by interacting with other proteins and the AF2-containing LBD. Despite two decades of intensive research attempting to identify the ligands of all the NRs, as of today, a merely two thirds of these members have been confirmed for their specific ligand binding abilities. As such, a large number of this family members remain as orphan NRs. Importantly, most of these orphan NRs do play physiological roles based upon gene knockout studies.

Received: June 6, 2007; Accepted: June 8, 2007
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Communicated by Pao-Luh Tao

The biochemical and molecular bases underlying the activities of NRs have been well studied, and the dogma describes a coregulator-triggered, "two-state" process where holo-receptors recruit coactivators to activate gene transcription whereas apo-receptors recruit corepressors to suppress gene transcription[5]. As of to date, more than 200 co-regulators have been identified for the 48 members of NRs. The coregulators for RARs have been reviewed[6]. Because of the discovery of co-repressors that appear to suppress gene transcription by acting together with the ligand-bound holo-receptors, the simple "two-state" mechanism has been challenged. One widely known ligand-dependent corepressor is named receptor interacting protein 140 (RIP140) that has been shown to suppress NR target genes even in the presence of specific ligands[7,8]. Further, despite the absence of known ligands, a number of orphan NRs are able to activate putative target genes, suggesting that certain biological events/factors, other than the hormones, may contribute to the biological activities of NRs.

B. Conventional views on orphan members TR2 and TR4

Among the two dozens of orphan NRs, testicular receptor-2 (TR2) and -4 (TR4) are among the first group that were cloned[9-11]. In the NR phylogenic tree, TR2 and TR4 are positioned distant from most of the remaining members[1]; however, they constitute a highly conserved and evolutionally related subfamily of their own. Despite the failure to identify specific physiological ligands of TR2 and TR4, a large volume of earlier studies have exploited reporter genes to gain insights into their biological activities and reported both repressive and activating functions for TR2 and TR4 in regulating various putative targets[12]. The repressive activities were initially ascribed to either the "competition" of these two receptors for binding to common HREs, such as in the case of TR2 action in regulating CRABPI gene via a thyroid hormone response element[13] as well as reporters carrying direct repeat-4 (DR4) or -5 (DR5)[14], or modulation of other transcription factors such as androgen receptor[15] and ER[16]. TR4 was also shown to regulate genes carrying certain HREs such as DR4 and DR1[17,18]. Further studies identified corepressors of TR2 and TR4, including histone deacetylases (HDACs)[19,20] and receptor interacting protein 140 (RIP140)[21], both constitute the repressive machinery for the repressive activities of TR2 and TR4. The activating function, however, has remained unresolved until we found that TR2 interacted with other transcription factors such as cyclic AMP response element modulator (CREMt) to elicit activation of the endogenous target RAR β 2[22], and that both TR2 and TR4 could recruit a specific coactivator p300/CBP-associated factor (P/CAF) following their post-translational modification (PTM)[23-25]. Intriguingly, unlike many other members of the NR family that can form heterodimer with the retinoid receptor X (RXR), neither TR2 nor TR4 can form heterodimer with RXR. In fact, TR2 and TR4 form TR2/TR4 heterodimer and it has been suggested that these two highly homologous NRs probably play roles in a distinct hormonal pathway that are different from, but probably related to, those pathways typically employing RXR as the partner[26].

With respect to the expression patterns, both TR2 and TR4 are highly expressed in embryonic stages[27,28], but in adult animals TR2 appears to be more selectively expressed and TR4 seems to be more ubiquitous. In adults, TR2 is elevated mostly in tissues containing stem cell populations, such as gonads, liver, lung and brain[29]. Detailed immunohistochemical studies reveal its specifically restricted expression in male germ cells that are actively proliferating, i.e., spermatogonia. Consistently, TR2 has been shown to activate specifically two important regulators for stem cells, or progenitor cells, the RAR β 2[22] and Oct 4[30] (see following). In adults, TR4 appears to be more ubiquitously expressed in many tissues including the central nervous system and the peripheral organs (Wei et al. unpublished).

Because of the lack of ligands, the physiological roles for TR2 and TR4 have been contended for a period of time. Gene knockout study reveals numerous phenotypes in TR4 knockout animals, including growth retardation, motor coordination, meiotic defect and altered expression of the apolipoprotein E/C-I/C-II gene cluster[31-34]. Recently, ApoE locus has been validated as a direct target of TR4 in studying its PTM[25]. It is believed that TR4 probably exerts a wide spectrum of physiological activities; however, the ultimate answer relies on the identification of its physiological ligands. With respect to the physiological roles of TR2, gene knockout animals exhibit no apparent phenotypes[35]. However, it is possible that TR2 is involved in some fundamentally important process such as cell proliferation as recently demonstrated[30,36], which is safeguarded by redundant or compensatory activities of other regulators including multiple NRs and other transcription factors. Therefore, based upon classical molecular and genetic studies, the physiological significance of TR4 is more conclusive, but it remains unclear as to whether it requires a physiological ligand. With respect to the significance of TR2, the results of the classical gene knockout studies are

not informative; but recent cell-based studies and examination of its PTM have shed lights on its potential role in stem cell proliferation. Again, it remains unresolved whether endogenous ligands of TR2 are present.

C. Post-translational modification (PTM) of TR2 and TR4

Despite intensive effort to identify the ligands of TR2 and TR4 and to crystallize the LBDs of these two NRs, these attempts have proven unfruitful. Our earlier studies of their biological activities have suggested that cellular factors could modulate their biological activities, presumably without specific ligands. Two approaches were taken, both attempting to uncover the missing link between the biological activities and the molecular features of these two mysterious NRs without ligand binding. First, a proteomic endeavor was launched to examine potential modifications of these receptor proteins. Second, a yeast two-hybrid screening was conducted to explore their interacting partners. The proteomic study was conducted by systemic mass spectrometric (MS) analyses of proteins either purified from insect cell cultures that are capable of modifying proteins in a manner reminiscent of that seen in mammalian cells or modified in vitro according to the software-predicted enzymatic modifications. Based upon the MS data, both TR2 and TR4 can be modified by phosphorylation and lysine methylation. The yeast two-hybrid screening result shows that TR2 can interact with the enzymatic machinery for sumo conjugation, suggesting the possibility of sumoylation of this protein, which has been validated (see following). In the following, the verified PTMs of TR2 and TR4 and the biological relevance established based upon studies are reviewed.

a. Phosphorylation of TR2

In vivo metabolic labeling experiments first demonstrated phosphorylation of endogenous TR2. Protein kinase C (PKC)-mediated phosphorylation was verified for Ser-568 and Ser-461 in its LBD. However, point mutation studies confirmed that Ser-568, but not Ser-461, significantly affected its biological activity and protein stability. It appeared that phosphorylation of this specific residue in the LBD protected TR2 from proteosome-mediated degradation[23]. In the DBD, two PKC target sites were predicted, Ser-170 and Ser-185, but only Ser-185 was confirmed by MS study. It appeared that phosphorylation of its DBD facilitated DNA binding and the recruitment of its co-activator P/CAF in exchange of its co-repressor RIP140[24]. An LC/MS study further validated phosphorylation at potential MAP kinase (MAPK) sites, such as Ser-203, Thr-208 and Thr-210 (Gupta and Wei, unpublished); however, the biological significance of these MAPK sites remains to be confirmed. Therefore, despite the absence of putative ligands, TR2 is able to activate its endogenous target genes including RARβ2 and Oct4, which is attributed to PKC-mediated phosphorylation on its LBD that stabilizes the protein, and PKC-mediated phoshporylation on its DBD that facilitates its recruitment of the specific coactivator P/CAF in exchange of the specific corepressor RIP140. More recent studies have further elucidated the mechanism underlying its activation potential that is triggered by phosphorylation-stimulated sumoylation (see following).

b. Phosphorylation of TR4

A systemic LC-MS study of TR4, with a 54% sequence coverage, has confirmed phosphorylation of TR4, but mostly in the amino terminal portion (AF1 domain) of the molecule. Using its target gene ApoE as a model, the biological significance of phosphorylation of TR4 was assessed, which revealed that the activating function of TR4, with respect to the ApoE gene, was suppressed by MAPK-mediated phosphorylation of its AF-1. Three phosphorylation sites were found in its AF-1 domain, Ser^{19}, Ser^{55}, and Ser^{68}; however, the functionality of phosphorylation could be verified only for Ser^{19} and Ser^{68}, but not Ser^{55} in site-specific mutation studies[25]. These point mutation studies uncover the mechanism of TR4 activation that is also triggered by the recruitment of coactivator P/CAF in exchange of co-repressor RIP140. However, in contrast to PKC-mediated phosphorylation of TR2 LBD, MAPK-mediated phosphorylation on the AF1 of TR4 suppresses its activating ability by reducing its recruitment of P/CAF coactivator.

c. Sumoylation of TR2 and TR4

In a yeast two-hybrid screening, multiple clones of SUMO ligase were found to interact specifically with the LBD of TR2. Sumoylation of endogenous TR2 was then verified using a SUMO-specific antibody, and a single site of sumoylation was confirmed at Lys-238[30]. It appeared that the unsumoylated TR2 served as a P/CAF-recruiting activator to activate an important regulator of stem cell proliferation, Oct4, by binding to a DR5 site of its promoter region. Further, the unsumoylated TR2 was recruited to the promyelocytic leukemia nuclear bodies (PML NBs) for sumoylation. The sumoylated TR2 seemed to be then released from the PML NBs and became a RIP140-recruiting repressor for the Oct4 gene[30]. Recently, the activator

function of TR2 in stem cells has been further confirmed in preadipocytes where the exchange of its co-activator P/CAF with co-repressor RIP140 was found to be mediated by a platform molecule GRIP1[36]. With regards to the mechanism of sumoylation, it is likely that a potentially important signal mediated by MAPK-phosphorylation of TR2 in its DBD is responsible (Gupta et al., unpublished). Therefore, the cellular environment can trigger a cascade of PTMs on TR2 to modulate its biological activity without apparent contribution of specific hormones or ligands. Whether and how this type of activation differs from classical hormone-triggered activation remains to be examined by structural studies.

Using the SUNO-specific antibody, we have also found that TR4 can be sumoylated. However, the sites of sumoylation and the functional significance of sumoylation on TR4 remain to be determined.

d. Lysine methylation

Based upon the LC-MS spectra, both TR2 and TR4 can be methylated on one of the lysine residues in the hinge region adjacent to the DBD. However, the biological significance of these PTMs remains to be validated. Interestingly, LC-MS study showed that the mouse RARα could be trimethylated on Lys[347] of its LBD[37]. Hypermethylation on this specific lysine residue facilitated its interaction with ligand-dependent coregulators including P/CAF co-activator and RIP140 corepressor, as well as its heterodimeric partner RXR. By replacing this lysine residue with a phenylalanine, a constitutive positive mutant of RARα was generated that partially mimicked the hypermethylated counterpart. This is the first example of lysine methylation on a non-histone protein that happens to be an important member of the NR family.

D. Future Perspectives

The conventional view of endocrinology focuses on the importance of "hormones" or "ligands" in terms of the biological activities of NRs. While it can not be overemphasized that hormones, or ligands, generate the first line of inputs to elicit specific biological responses in animals, and that the dogma of ligand-induced NR activation by forming complexes with coactivators remains largely correct, the presence of overwhelmingly large number of orphan NR members that seem to be able to elicit physiological activities strongly argues for an alternative pathway to regulate the biological activities of NRs. To this end, recent studies of PTM, not only for the orphan members but also for other NRs with well-characterized ligands, support the notion that the physiological action of certain NRs can be triggered by non-endocrine factors. This is particularly relevant to those members of more primitive origin in the phylogenic tree such as the TR2 and TR4 family.

Conceivably, this family of transcription factors evolve from certain ancestral members that probably do not absolutely need ligands to occupy their putative LBDs in order to trigger a conformational change; rather they rely on cellular signals to modify their amino acid residues, thereby changing their conformation in a way similar to that triggered by hormone binding. The definite answer requires structural information; however, the evidence for their PTMs is increasingly solid and the biological relevance is clear from a large number of reports. If PTM proves to be one of the principal ways to modulate NR activities without hormones in a physiological context, future studies and the interpretation of experimental results will be challenged by the uncertainty in numerous cellular factors that are present in various biological systems and known to drastically affect the cellular environment for protein modifications, such as extracellular signals, cell-cell communication and autonomous cellular events.

For technical reasons, these nuclear proteins are difficult to express and purify, therefore most studies using LC-MS, the most powerful and systemic methodology to uncover PTMs, have not been able to obtain full sequence coverage of the target protein. As such, the information about the PTM of most proteins that have been examined probably remains incomplete. Nevertheless, this approach has significantly advanced our understanding of how this mysterious NR family can be modulated by cellular factors and how they may act without ligands. This approach has also considerably speeded up the discovery phase in exploring the biological significance of these proteins while the identification of their ligands remains a challenge. The finding that TR2 plays an important role in cell proliferation, especially cells of a stem-ness property like embryonic stem cells and preadipocytes, further supports the notion that the family of NR probably represents the earliest member of NRs that are involved in fundamentally important and commonly shared biological processes. With the discovery and characterization of more PTMs on these orphan members, the biological relevance of this once disregarded family of transcription factors has been redefined.

ACKNOWLEDGEMENTS

This work is supported in part by NIH Grants DK54733, DK60521, DA11190, DA11806, and K02-DA13926, and by a Philip Morris award 20093127-A01 to LNW.

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