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Molecular and genetic studies suggest that thyroid hormone receptor is both necessary and sufficient to mediate the developmental effects of thyroid hormone

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Abstract

Thyroid hormone (TH) affects diverse biological processes and can exert its effects through both gene regulation via binding the nuclear TH receptors (TRs) and non-genomic actions via binding to cell surface and cytoplasmic proteins. The critical importance of TH in vertebrate development has long been established, ranging from the formation of human cretins to the blockage of frog metamorphosis due the TH deficiency. How TH affects vertebrate development has been difficult to study in mammals due to the complications associated with the uterus-enclosed mammalian embryos. Anuran metamorphosis offers a unique opportunity to address such an issue. Using *Xenopus* as a model, we and others have shown that the expression of TRs and their heterodimerization partners RXRs (9-cis retinoic acid receptors) correlates temporally with metamorphosis in different organs in two highly related species, *Xenopus laevis* and *Xenopus tropicalis*. *In vivo* molecular studies have shown that TR and RXR are bound to the TH response elements (TREs) located in TH-inducible genes in developing tadpoles of both species. More importantly, transgenic studies in *Xenopus laevis* have demonstrated that TR function is both necessary and sufficient for mediating the metamorphic effects of TH. Thus, the non-genomic effects of TH have little or no roles during metamorphosis and likely during vertebrate development in general.

Keywords

thyroid hormone receptor; chromatin; histone acetylation; postembryonic development; metamorphosis; *Xenopus laevis*; *Xenopus tropicalis*

1. INTRODUCTION

Thyroid hormone (TH) plays an important role in vertebrate development and human pathology (Oppenheimer, 1979; Yen, 2001). The critical effects of TH on human development have been well documented. The most obvious and earliest known human abnormalities due to TH deficiency are the goiter (a lump in the neck due to thyroid gland enlargement) and cretinism (a form of severe mental deficiency together with retarded

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skeletal growth) (Hetzel, 1989). In humans, many of the developmental defects caused by TH deficiency prior to birth can be reversed if TH replacement is initiated shortly after birth (Larsen, 1989), indicating that TH influences neonatal development mainly by acting directly on the foetus, not through the mother. The most important period of TH action is the so-called postembryonic development, a few months before and several months after birth when TH levels are high (Howdeshell, 2002; Tata, 1993). This period bears many similarities to anuran metamorphosis (Shi, 1999; Tata, 1993), including the presence of high levels of TH. Such similarities coupled with the difficulties to manipulate the uterus-enclosed mammalian embryos have made anuran metamorphosis a highly valuable model to study TH action during vertebrate development. Here we will review some of the studies on the role of TH receptor (TR) during amphibian metamorphosis, with an emphasis on our own work in *Xenopus laevis* as presented at the meeting.

2. MECHANISMS OF TH ACTION

To affect target cells, circulating TH in the plasma needs to be taken up by cells through active transport (Hennemann et al., 2001; Shi et al., 2002). Upon entering the cells, TH can bind to a number of cytosolic proteins and enter the nucleus where it binds to TRs (Shi et al., 1996). In addition, TH can also bind to cell surface proteins, such as integrins (Bassett et al., 2003; Davis et al., 2005). Thus, it is not surprising that TH can affect cells through both the so-called non-genomic action via the binding to cell surface and cytoplasmic proteins and transcriptional regulation via TRs.

2.1. Non-genomic action of TH

TH affects diverse biological processes. Some of the effects of TH are too fast to be mediated through transcriptional regulation via TR in the nucleus (Bassett et al., 2003; Davis and Davis, 1996). For example, TH administration leads to acute improvement in cardiac output in human patients, TH can alter myocardial contractility and reduce systemic vascular resistance within minutes. At the cellular level, TH can affect cell morphology, respiration (mitochondrial function), and ion homeostasis, etc. TH appears to exert diverse non-genomic effects through multiple pathways (Bassett et al., 2003; Davis and Davis, 1996; Davis and Davis, 2002; Davis et al., 2005; Shi et al., 1996). First, it has long been known that TH can bind to cell surface proteins (Davis et al., 2005). The identities of these proteins are largely unknown except the integrin $\alpha V\beta 3$, which binds strongly T4 (3, 3', 5, 5'-tetraiodothyronine) and to a lesser extent T3 (3, 3', 5-triiodothyronine) (Davis et al., 2005). This binding of TH to the integrin is expected to affect cell-extracellular matrix interactions and trigger intracellular signaling processes rapidly. Second, within the cell, a number of cytosolic proteins are known to bind to TH (Davis and Davis, 1996; Davis and Davis, 2002; Parkison et al., 1991; Shi et al., 1994; Shi et al., 1996). Most of these cytosolic proteins have additional functions, often as enzymes. TH binding may thus affect the enzymatic functions of these proteins and conversely, regulating their enzymatic activity may influence their binding to TH. For example, a cytosolic thyroid hormone binding protein is the monomer form of M2 pyruvate kinase (Parkison et al., 1991). TH binding prevents the formation of the enzymatically active tetramer and conversely, the formation of the tetramer inhibits its binding to TH (Ashizawa and Chen, 1992; Ashizawa et al., 1991). Finally, while TR is predominantly nuclearly localized even in the absence of TH, a small fraction is present in the cytoplasm. It has been shown that one of the two TR isoforms, TR β , can form a complex with the signaling kinase MAPK in TH treated cells, which is likely responsible for the rapid activation of MAPK by TH (Davis et al., 2005). In addition, unliganded TR β can interact with phosphatidylinositol 3 kinase (PI3K) to activate the signaling pathway (Guigon and Cheng, 2009; Storey et al., 2006). Thus, TR β can also function as a mediator of the non-genomic effects of TH by interacting with these and other cytosolic proteins (Guigon and Cheng, 2009).

2.2. Nuclear action of TH

There are two types TRs in all vertebrates, TR α and TR β , both of which bind TH with high affinities (Davey et al., 1994; Puzianowsak-Kuznicka et al., 1996; Sap et al., 1986; Weinberger et al., 1986). TRs belong to the superfamily of nuclear hormone receptors (Evans, 1988; Laudet and Gronemeyer, 2002; Mangelsdorf et al., 1995; Tsai and O'Malley, 1994; Yen and Chin, 1994). TH can both activate and repress transcription through TRs. The mechanism for gene repression by TH is not well understood and thus will not be discussed here. Transcriptional activation by TH requires the binding of TRs, most likely as heterodimers with RXRs (9-cis-retinoic acid receptors), to the TH response elements (TREs) in TH-inducible genes. TR/RXR heterodimers bind to TREs constitutively, even in the context chromatin (Perlman et al., 1982; Tsai and O'Malley, 1994; Wong et al., 1995). They repress or activate transcription in the absence or presence of TH, respectively.

In vitro and cell culture studies involving different animal species by many laboratories have led to a fairly detailed understanding of the mechanisms of the gene regulation by TR. TR functions by recruiting cofactors. Many such cofactors have been isolated and characterized (Burke and Baniahmad, 2000; Glass and Rosenfeld, 2000; Huang et al., 2003; Ito and Roeder, 2001; Jones and Shi, 2003; McKenna et al., 1999; McKenna and O'Malley, 2001; Meng et al., 2003; Rachez and Freedman, 2000; 2001; Sato et al., 2009; Wahlstrom et al., 1999; Xu et al., 1999; Zhang and Lazar, 2000). In the absence of TH, TR recruits corepressors, such as the highly related proteins SMRT and N-CoR, which form multimeric complexes containing histone deacetylases (HDACs) (Burke and Baniahmad, 2000; Glass and Rosenfeld, 2000; Jones and Shi, 2003; Zhang and Lazar, 2000) (Fig. 1). This leads to the deacetylation of the promoter regions of the target genes to facilitate gene repression. When TH is present, the corepressor complexes are released and replaced by coactivator complexes. Many diverse groups of coactivators have been identified. Among them include ATP-dependent chromatin remodeling proteins, histone acetylases (HATs) such as p300 and SRCs, protein arginine methyltransferases, and TRAP/DRIP/mediator complex that associates with the recruitment and activation of RNA polymerase II (Chen et al., 1999; Demarest et al., 2002; Heimeier et al., 2008; Huang et al., 2003; Ito and Roeder, 2001; Li et al., 2000; Matsuda et al., 2009; Matsuda et al., 2007; McKenna and O'Malley, 2001; Rachez and Freedman, 2001; Sheppard et al., 2001; Yen, 2001; Zhang and Lazar, 2000) (Fig. 1). The recruitment of such cofactors to the target genes leads to histone acetylation, methylation, and chromatin remodeling, resulting in transcriptional activation.

3. ROLES OF TR IN *XENOPUS* METAMORPHOSIS

3.1. A model of TR in frog development

Early expression studies showed that the mRNA levels of TR, especially TR α , are upregulated shortly after hatching at stage 35 in *Xenopus laevis*, reaching peak levels by tadpole feeding stage (stage 45), when a free living tadpole is developed, although TR β expression parallels with plasma TH concentrations (Fig. 2) (Shi et al., 1994; Yaoita and Brown, 1990). In addition, RXR genes, in particular, RXR α , are also expressed in premetamorphic *Xenopus laevis* tadpoles (Fig. 2) (Wong and Shi, 1995). Similar expression patterns for TR and RXR genes have also been observed in *Xenopus tropicalis* (Wang et al., 2008). Based on these and the transcriptional properties of TR/RXR heterodimers, we have previously proposed a dual function model for TR during *Xenopus laevis* development (Fig. 2) (Sachs et al., 2000; Shi et al., 1996). According to the model, the unliganded TR expressed in premetamorphic tadpoles between stage 45 when a free feeding tadpole is formed (Nieuwkoop and Faber, 1956) and stage 55, just when endogenous TH becomes detectable (Fig. 2) (Leloup and Buscaglia, 1977), forms a heterodimer with RXR and the TR-RXR heterodimer binds to the TREs of TH-inducible genes, leading to the repression of

their expression. This then ensures proper tadpole growth before metamorphic organ transformations. After stage 55, availability of TH allows the binding of TH to chromatin-bound TR and the TH-bound TR-RXR then activates these target genes to initiate metamorphosis in different organs and tissues (Fig. 2).

3.2. TR binds to the TREs of endogenous target genes during frog development

We used the chromatin immunoprecipitation assays (ChIP) to analyze the binding of TR to target genes during *Xenopus laevis* development (Sachs and Shi, 2000). As the model predicted, there is little or no TR present at the TREs of two known direct TH-inducible genes, TR β and TH/bZIP genes, in embryos but TR is present on the TREs in premetamorphic tadpoles when analyzed either in whole animals or in individual organs like the intestine and tail (Buchholz et al., 2005; Havis et al., 2003; Matsuda et al., 2009; Paul et al., 2005a; Paul et al., 2005b; Sachs et al., 2002; Sachs and Shi, 2000; Tomita et al., 2004). Furthermore, quantitative ChIP assay showed that during metamorphosis or after TH-treatment of premetamorphic tadpoles, the binding of TR to the TREs increases, especially on the TH/bZIP TRE, which has a weaker affinity to TRs compared to the TRE in the TR β in direct DNA binding assays *in vitro* (Buchholz et al., 2005; Matsuda et al., 2009). Similar results were also observed in *Xenopus tropicalis* (Wang et al., 2008), a species highly related with *Xenopus laevis*. Thus, TR is bound to the TREs in premetamorphic tadpoles, especially those with high affinities for TR and during metamorphosis, the increase in TR expression, especially TR β due to auto-regulation (Kanamori and Brown, 1992; Machuca et al., 1995; Ranjan et al., 1994; Shi et al., 1992; Yaoita and Brown, 1990), leads to increased binding of the TR to the TREs in target genes, especially those with weaker affinities for TR. Such observations also suggest that the different temporal expression profiles of TR genes in different organs should lead to organ-dependent variations in the regulation of target genes by TR, which may contribute to the temporal regulation of metamorphosis in different organs (Shi et al., 1994; Shi et al., 1996).

3.3. RXR is important for TR function during frog development

Our ChIP assay also showed that like TR, RXR α is also present at the TREs in premetamorphic tadpoles but not during embryogenesis, suggesting that TR/RXR heterodimers are the functional complexes for frog development (Sachs and Shi, 2000). By making use of the fact that early *Xenopus* embryos have very low levels of endogenous TR and TH (Banker et al., 1991; Kanamori and Brown, 1992; Wong and Shi, 1995) (although such low levels are important for at least some aspects of embryogenesis (Havis et al., 2006)), we studied the effect of overexpression of TR upon microinjection of its mRNA into fertilized eggs (Puzianowska-Kuznicka et al., 1997). The results showed that TR α overexpression had distinct effects on embryonic development depending on the presence or absence of TH. Interestingly, the developmental effects of TR α were greatly enhanced by co-over-expression of RXR α . TR α or RXR α alone had little effect on embryonic development both in the presence or absence of T3. Coexpression of TR α and RXR α led nearly 90% of the embryos with deformity in the absence of T3 and in the presence of T3, 100% had deformity and/or embryonic lethality. More importantly, overexpression of TR and RXR together, but not either one alone, repressed endogenous TH-inducible genes while the addition of TH resulted in the reversal of the repression and further activation of the TH response genes. Thus, RXR is important for TR functions during frog development.

3.4. TR is required for TH-induced metamorphosis

The development of a sperm-mediated transgenic method (Kroll and Amaya, 1996) has made it possible to genetically determine the role of TR in mediating the metamorphic effects of TH. Schreiber et al. (Schreiber et al., 2001) reported the first transgenic study on TR where they ubiquitously overexpressed a dominant negative TR (incapable of binding

TH due to a small deletion at the C-terminus) in *Xenopus laevis* tadpoles and found that diverse aspects of TH-induced metamorphic changes were inhibited. The same conclusion was also reached in several labs in subsequent studies, where a dominant negative TR was overexpressed by using *in vivo* transfection or transgenesis with ubiquitous and tissue-specific promoters (Fig. 3) (Buchholz et al., 2003; Das et al., 2002; Nakajima and Yaoita, 2003; Schreiber and Brown, 2003; Schreiber et al., 2009). These *in vivo* studies also showed that the expression of the dominant negative TR inhibited TH-induced transcription of target genes. Mechanistically, we demonstrated that the dominant negative TR expressed in transgenic animals competed against endogenous TR for binding to endogenous target genes, leading to the retention of corepressors at the target genes even in the presence of TH (Buchholz et al., 2003). This caused a reduction in local histone acetylation, thus contributing to the inhibition of gene expression and frog metamorphosis. Thus, TR is essential for TH-induced gene regulation and metamorphosis.

3.5. TR is sufficient to mediate the metamorphic effects of TH

Given the ability of TH to affect cells through both non-genomic and nuclear pathways, it is important to determine whether TR is sufficient to mediate the metamorphic effects of TH. For this purpose, we generated a dominant positive TR that cannot bind to TH (due to a small deletion at the C-terminus) but constitutively activate transcription (due to the N-terminal fusion of the strong viral activator VP16) (Buchholz et al., 2004). As overexpression of liganded TR has adverse effects on early embryogenesis (see above), we placed the dominant positive TR under the control of a heat shock-inducible promoter for transgenesis. When transgenic tadpoles and their wild type siblings reached premetamorphic stages, we subjected them to daily heat shock treatment to induce the transgene expression and compared the effects of heat shock treatment with those due to TH treatment of the wild type sibling animals. As expected, TH-treatment led to typical metamorphic changes, including limb morphogenesis and gill resorption, etc. (Fig. 4). Interestingly, upon heat shock treatment, transgenic but not wild type tadpoles underwent the same changes as induced by TH treatment, including both external morphological changes such as limb morphogenesis and gill resorption (Fig. 4) as well as the remodeling of internal organs such as the intestine (Buchholz et al., 2004). Furthermore, gene expression studies showed that the expression of the dominant positive TR induced the expression of the same endogenous TH-inducible genes with the same organ-specificity as TH treatment (Buchholz et al., 2004). In addition, ChIP assays demonstrated the binding of the dominant positive TR to the TREs of endogenous TH-target genes (Buchholz et al., 2004). Thus, the binding of this constitutively active TR to these target genes activates them, leading to metamorphic changes in all organs/tissues. These results suggest that TR is sufficient to mediate the effects of TH during *Xenopus* metamorphosis, at least on all the parameters that were measured.

4. CONCLUSIONS

TH affects many biological processes and can elicit both fast, short-term effects as well as slow, long-term consequences. The fast effects have been generally observed and studied in cells and/or animal organs. It is generally believed that they are mediated through the non-genomic action of the hormone and that the underlying mechanisms likely vary depending upon the effects observed. It is unclear whether such non-genomic actions contribute to the developmental effects of the hormone, in part due to the lack of good model system. Studies on anuran metamorphosis have now provided strong evidence to show that TR is both necessary and sufficient to mediate the metamorphic effects of TH. Thus, the non-genomic action of TH appears to play little or no role during vertebrate development.

While the conclusions above are based on studies of frog development, there are many similarities between mammalian postembryonic development and anuran metamorphosis (Shi, 1999; Tata, 1993). For example, during mammalian development, TRs are also expressed prior to the synthesis of endogenous TH and TH levels in the plasma peaks during postembryonic development (Howdeshell, 2002; Tata, 1993). These and other conservations argue that in mammals as well as other vertebrates, the developmental effects of TH is most likely mediated by TR with non-genomic effects of TH contributing little. Such a conclusion may not be surprising given the slow and lengthy process of vertebrate development compared to the time scale of the non-genomic effects of TH.

An important question to be addressed is how TR mediates the developmental effects of TH in various tissues/organs. Here, studies in the *Xenopus laevis* model have offered some clues. In premetamorphic tadpoles in the absence of TH, corepressor complexes, such as those containing N-CoR and SMRT (Fig. 1) are present at the TREs of TH-inducible genes and are released upon TH-treatment or during natural metamorphosis when endogenous TH levels are high (Sachs et al., 2002; Tomita et al., 2004). More importantly, transgenic studies with a dominant negative corepressor have shown that unliganded TR recruits corepressors to regulate the timing for the initiation of metamorphosis (Sato et al., 2007). During metamorphosis, liganded TR recruits coactivator complexes containing SRC/p300 to the promoters to activate their transcription (Fig. 1) (Havis et al., 2003; Matsuda et al., 2009; Paul et al., 2005a; Paul et al., 2005b). Genetic studies indicate that TR needs to recruit coactivators to target genes in order to activate their transcription and induce metamorphosis (Paul et al., 2005a; Paul et al., 2005b) and that the levels of coactivators regulate the rate of metamorphic progression. It is tempting to speculate that organ-specific variations in cofactor concentrations and/or utilization of different cofactors, may be an important factor in determining the organ-specific temporal regulation of metamorphosis, such as the early completion of the limb development but the late resorption of the tail resorption (Shi, 1999; Shi et al., 1996). This is clearly one of the important questions that need to be addressed in the future. A possible approach to this is to use a combination of genetic and molecular analyses. This will involve the identification of tissue specific TH target genes and the cofactors participating in their regulation by using techniques such as genome-wide ChIP analyses of cofactor recruitment by TR in different organs, followed by genetic studies such as transgenic overexpression or knockdown to determine the role of cofactors in the metamorphosis of different organs. Parallel studies in other vertebrate species such as mouse will show whether such developmental mechanisms are conserved.

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Abbreviations

TH	Thyroid hormone
TR	Thyroid hormone receptor
HDAC	Histone deacetylase
HAT	Histone acetyl transferase
RXR	9-cis retinoic acid receptor
TRE	TH response element
ChIP	chromatin immunoprecipitation

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Fig. 1.

Mechanisms of transcriptional regulation by TR. For TH-inducible genes, TR heterodimerized with RXR constitutively binds the TREs in their promoters or enhancers. In the absence of TH, TR binds corepressor complexes, such as those containing histone deacetylase HDAC3 and the highly related protein N-CoR or SMRT to inhibit transcription from the promoters. This is accomplished in part through deacetylation of lysine residues of histone H3 and H4 to induce a "closed" chromatin state, as suggested by the folding of histone tails (red beaded structure) on to the DNA helix, because of the charge-charge interaction between the positively charged histone tails and negatively charged DNA. The binding by TH induces a conformational change in TR, leading to the binding of coactivator complexes, such as those containing coactivators SRC and p300, which are histone acetyltransferases (HATs). They will acetylate histones H3 and H4, facilitating the formation of an "open" chromatin state, as diagramed by the unfolding of histone tails (red beaded structure) away from the DNA helix due to the neutralization of the positive charges on the histone tails by acetylation. Liganded TR can also recruit other coactivator complexes, such as chromatin remodeling complexes and mediator complex (also known as DRIP/TRAP complex), with the latter directly contacting RNA polymerase, to activate transcription.

Fig. 2.

A dual function model of TR in frog development. During embryogenesis, TH response genes are expressed at basal levels in the absence of TR and TH to facilitate embryonic organ development. After tadpole hatching at stage 35/36, TR α expression increases, reaching high levels by stage 45 when tadpole feeding begins (Yaoita and Brown, 1990). RXR α is also highly expressed by this time (Wong and Shi, 1995), the TR/RXR heterodimers bind to TH response genes to repress their expression due to the lack of TH, thus ensuring proper tadpole growth and preventing premature metamorphosis. When endogenous TH level rises after stage 55 (Leloup and Buscaglia, 1977), the TH-bound TR/RXR heterodimers then activate TH response genes, such as the TR β genes, leading to metamorphosis.

Fig. 3.

TR is necessary for the metamorphic effects of TH. Transgenic expression of a dominant negative TR (dnTR) blocks TH-induced metamorphosis. Wild type animals treated with TH underwent characteristic changes, including gill resorption and limb morphogenesis (compared the middle panels to the ones on the left). The TH-treatment failed to induce such changes in the sibling transgenic animals (right panels, which resemble the left but not the middle ones) (Buchholz et al., 2003).

**Wild type
+ heat shock**

**dpTR
transgenic
+ heat shock**

**Wild type
+ TH**

Fig. 4.

TR is sufficient to mediate the metamorphic effects of TH. Wild type tadpoles and sibling tadpoles transgenic for a dominant positive TR (dpTR) under the control of a heat shock-inducible promoter were reared together in methimazole to block endogenous T3 synthesis and were heat-shocked daily for 8 days. For comparison, wild type tadpoles were treated with TH for 3 days. Note that the heat shock induction of dpTR expression resulted in metamorphic events, including gill resorption (bracket) and limb outgrowth (arrowhead) in the transgenic (middle) but not wild type (top) animals, just like TH-treatment of wild type sibling animals (bottom) (Buchholz et al., 2004).