Cell research (2003); 13(6):459-464
http://www.cell-research.com

Distinct expression profiles of transcriptional coactivators for thyroid hormone receptors during *Xenopus laevis* metamorphosis

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ABSTRACT

The biological effects of thyroid hormone (T_3) are mediated by the thyroid hormone receptor (TR). Amphibian metamorphosis is one of the most dramatic processes that are dependent on T_3. T_3 regulates a series of orchestrated developmental changes, which ultimately result in the conversion of an aquatic herbivorous tadpole to a terrestrial carnivorous frog. T_3 is presumed to bind to TRs, which in turn recruit coactivators, leading to gene activation. The best-studied coactivators belong to the p160 or SRC family. Members of this family include SRC1/NCoA-1, SRC2/TIF2/GRIP1, and SRC3/pCIP/ACTR/AIB-1/RAC-3/TRAM-1. These SRCs interact directly with liganded TR and function as adapter molecules to recruit other coactivators such as p300/CBP. Here, we studied the expression patterns of these coactivators during various stages of development. Amongst the coactivators cloned in *Xenopus laevis*, SRC3 was found to be dramatically upregulated during natural and T_3-induced metamorphosis, and SRC2 and p300 are expressed throughout postembryonic development with little change in their expression levels. These results support the view that these coactivators participate in gene regulation by TR during metamorphosis.

Key words: *transcription coactivators, thyroid hormone receptor, Xenopus laevis, metamorphosis, histone acetylation.*

INTRODUCTION

Metamorphosis in amphibians is absolutely dependent on the presence of thyroid hormone (T_3) [1, 2]. Rising levels of T_3 causes the remodeling of practically every organ in the body with respect its morphology and physiology. Some organs such as the gills and the tail are completely resorbed, whereas others viz. the limbs develop de novo. The transition of the larva to the adult involves precisely controlled gene regulatory events that occur at specific stages during development.

T_3 exerts these effects mainly, if not exclusively, through its receptor, the thyroid hormone receptor (TR), which can repress or activate genes depending on the absence or presence of T_3[3-9]. TR forms a heterodimer with the 9-cis-retinoic acid receptor (RXR) and binds to the thyroid hormone response elements (TREs) on T_3-responsive genes to regulate gene expression[6-12]. TR can interact with various transcriptional cofactor complexes[9, 13-19]. In the absence of hormone, TR/RXR interacts with corepressor complexes, which often contain histone deacetylases. When TR binds to T_3, the corepressors are released and coactivators are recruited. Many of the coactivators, but not all, are themselves histone acetyltransferases. Thus, it has been suggested that a change in cofactor complex recruitment may lead to local alterations in histone acetylation levels, resulting changes in transcription of the target genes.

The best-studied class of coactivators is the Steroid Receptor Coactivator (SRC) or p160 family, which comprises three members SRC1/NCoA-1, SRC2/TIF2/GRIP1 and SRC3/pCIP/ACTR/AIB-1/RAC-3/TRAM-1 [13, 14, 20]. These proteins interact with nuclear receptors directly through conserved LXXLL motifs in the presence of ligand[21-25]. These coactivators possess intrinsic histone acetyltransferase (HAT) activity. In addition, they also interact with other HATs, such as p300 and pCAF. These various HATs can acetylate histones, transcription factors, and other proteins in the transcriptional

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Received Sep-28-2003 Revised Oct-15-2003 Accepted Oct-25-2003

apparatus[26-30]. SRC proteins have two transcriptional activation domains, AD1 and AD2. AD1 has LXXLL motifs that interact with the coactivators p300 and CBP, also known as cointegrators[24, 31, 32]. Mutations in these motifs in the AD1 domain not only disrupt the interaction between SRC proteins and p300, but also abolish transcriptional activation by SRCs, indicating that the main function of AD1 is to recruit p300/CBP and p/CAF[25, 32]. The AD2 domain, present at the C terminus of the SRCs interacts with histone methyltransferases CARM-1 and PRMT-1[33, 34]. Thus, SRCs may facilitate nuclear receptor function through multiple mechanisms.

Despite a wealth of information available on the interactions of the coactivators with nuclear receptors in vitro, relatively much less is known about the *in vivo* contributions of particular coactivators in gene regulation by different transcription factors in development. We are interested to define the role of different coactivators during postembryonic development. *Xenopus laevis* metamorphosis offers a unique opportunity to study the role of coactivators in gene regulation by TR in various developmental processes because of its total dependence on T_3 and its ability to be manipulated by simply controlling the availability of T_3 to the tadpoles. As a first step towards understanding the involvement of coactivators in TR-dependent gene activation, we analyzed coactivator expression during natural and T_3-induced metamorphosis. Considering the implied role of SRC and p300 in TR function based on *in vitro* and tissue culture cell studies as summarized above, we focused our studies on the three coactivators that have been cloned in *Xenopus laevis*, i.e., SRC2, SRC3, and p300. Our results indicated that all of them are expressed during metamorphosis but with different patterns, supporting a role during metamorphosis.

MATERIALS AND METHODS

Animals and treatments

Wild type tadpoles of *Xenopus laevis* were obtained from Nasco (Fort Atkinson, WI.). Developmental stages were according to Nieuwkoop and Faber[35]. Transgenic tadpoles expressing a dominant negative TR (dnTR) were generated as described[36, 37]. When indicated, stage 54 tadpoles were treated, (2 tadpoles per liter) in 1 liter of dechlorinated tap water with 5 or 10 n*M* T_3 (Sigma). Tadpoles were sacrificed by decapitation after anesthesia (cooling on ice) for tissue isolation.

RNA isolation and RT-PCR

Tadpoles or dissected tissues/organs were homogenized in Trizol reagent (Invitrogen) and total RNA was isolated according to the manufacturer's protocol. The RNA in each sample was dissolved in DEPC-treated water and quantitated by measuring the OD at 260 nm. 500 ng of RNA was used per RT-PCR reaction in a volume of 25 ml using gene specific primers and primers for an internal control, ribosomal protein L8, rpl8[38]. PCRs were done for 25 cycles each consisting of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec. The primers used for the internal control rpl8 are: forward 5'-CGTGGTGCTCCTCTTGCC-3' and reverse 5'-AGACG-ACCAGTACGACGA-3'. The sequences of primers for detecting SRC2, SRC3 and p300 were designed according to the published sequences[39-41] and were as follows; SRC3 forward: 5-GGAATGAGTGGATTAGGGGAA-3'; SRC3 reverse: 5'-CACGGAT- CCCTACACATGTCATTAGA-3'; SRC2 forward: 5'-CGGCATGATGGCAACTCAGC- G-3', SRC2 reverse 5'-CGGGCATGCTGGTCTC- CAGC-3'; p300 forward 5'-ATGCTAACC-ACACCTGTACCACGCCA-3' and p300 reverse 5'-GCTACTGTCCTGTGTTCTT GCGTTT-3'. PCR was also done on RNA samples without reverse transcription as a control for genomic DNA contamination (data not shown). PCR products (5 μl) were electrophoresed on 2% agarose gels and stained with ethidium bromide.

RESULTS

Differential coactivator expression during natural metamorphosis

In order to study possible involvement of the coactivators SRC2, SRC3 and p300 in the postembryonic development (metamorphosis) of *Xenopus laevis*, we first analyzed their expression profiles in whole tadpoles from premetamorphic to postmetamorphic stages by RT-PCR with a pair of primers specific for each gene. To control for RNA quantity, quality, and RT-PCR, we included a pair of primers specific for the ribosomal protein L8 (rpl8) in the same RT-PCR reaction tubes, since rpl8 expression remains constant throughout metamorphosis[38]. In the whole tadpole, the expression of p300 and SRC2 was found to be fairly constant throughout metamorphosis (Fig 1A and B). On the other hand, SRC3 was upregulated by stage 62, the climax of metamorphosis, and remained at the higher levels at the end of metamorphosis (Fig 1C). These results suggest that SRC3 plays a role in metamorphosis, although the involvement of SRC2 and p300 cannot be ruled out.

Since the expression profiles seen in the whole animal reflects the sum of all different tissues and organs in the animals and the organ/tissue composition changes extensively during metamorphosis[1, 2], we were interested in determining whether the changes in SRC3 expression was due to T_3 regulation in individual organs/tissues. For this purpose, we chose the tail and intestine for analysis, as these two organs are two of the best-characterized

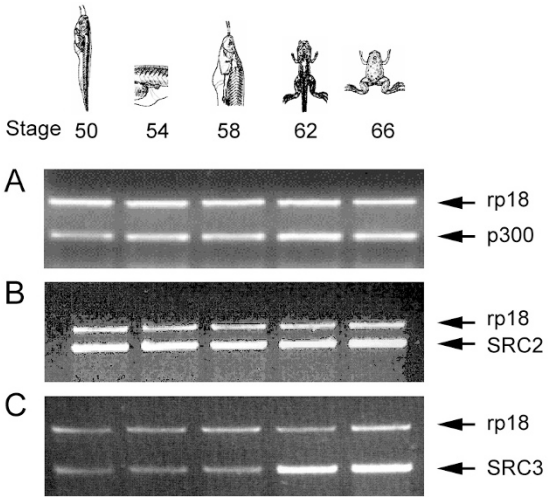

Fig 1. Distinct expression profiles of the coactivators p300, SRC2 and SRC3 in whole tadpoles during spontaneous metamorphosis. RNAs were isolated from tadpoles at the indicated developmental stages and used for the analysis of the expression of p300 (A), SRC2 (B), and SRC3 (C) by reverse transcriptase-polymerase chain reaction (RT-PCR). The rpl8 gene was used as an internal control. Note that SRC3 mRNA level increased dramatically in the whole animals at stage 62 to 66, i.e., from the climax of metamorphosis to the end of metamorphosis, while those of SCR2 and p300 did not change significantly.

organs at molecular and cellular levels during metamor phosis[1, 2]. In addition, both organs involve extensive cell death during metamorphosis[1, 2, 42, 43]. The tail is completely resorbed through programmed cell death or apoptosis during metamorphosis, whereas 70% of the intestinal cells, i.e., the larval epithelial cells, undergo apoptosis. Furthermore, both organs share many known T_3-induced genes[2, 44, 45]. Thus, we isolated RNAs from the intestine and tail of tadpoles at different stages and subjected them to RT-PCR analysis. In the intestine (Fig 2), SRC2 and p300 were again found to have constant mRNA levels (Fig 2A, B) while SRC3 were up-regulated during intestinal remodeling (stage 62) and in postmetamorphic frog intestine (stage 66) (Fig 2C). Similarly, in the tail, SRC3 was found to be dramatically upregulated at stage 62 (Fig 3C), which is the stage at which the tail begins to regress. In contrast, the levels of p300 and SRC2 remained unchanged (Fig 3A and B) (stage 66 was not used for tail analysis as tail is completely resorbed by stage 66). These results suggest that the enhanced levels of SRC3 coincide with the metamorphic changes occurring in the tail and intestine and that SRC3 expression is regulated by T_3 during metamorphosis.

SRC3 is a T_3 response gene while SRC2 and p300 are not

To directly test whether T_3 regulates the expression of SRC3 in individual organs, we treated premetamorphic tadpoles at stage 54 with 5-10 n*M* of T_3 for 3 days, close to the peak concentration during natural metamorphosis [46]. Such a treatment is known to induce some of the metamorphic events, such as intestinal epithelial cell death and limb development, but not many others such as the tail resorption, although many T_3 response genes are

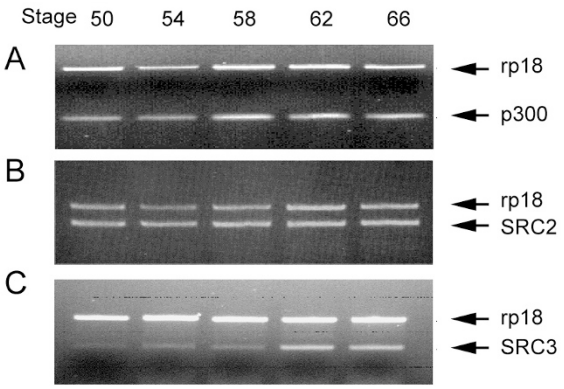

Fig 2. The expression of SRC3 but not p300 or SRC2 is upregulated during intestinal remodeling. Intestines of tadpoles at indicated stages were isolated and the contents were washed away. RNAs were isolated from the cleaned intestinal fragments and used for RT-PCR analysis of the expression of p300 (**A**), SRC2 (**B**), and SRC3 (**C**). The rpl8 gene served as an internal control.

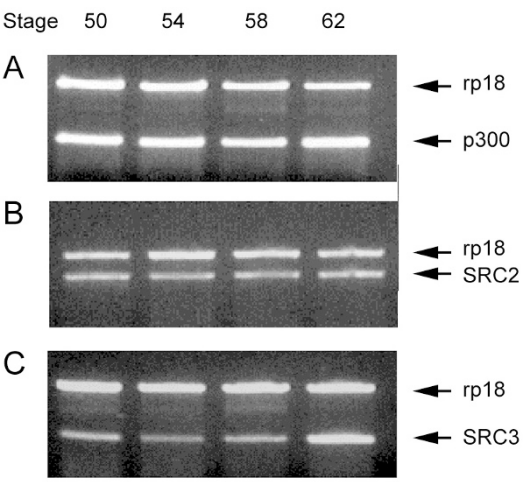

Fig 3. Tail resorption is accompanied by increased expression of SRC3 but not SRC2 or p300. RNAs were isolated from the tails of tadpoles of indicated stages and used for RT-PCR analysis of the expression of p300 (A), SRC2 (B), and SRC3 (C), with rpl8 as the internal control.

induced in the tail[1, 2]. To determine if coactivator expression was altered by the treatment, total RNA was isolated from the tail and intestine and subjected to RT-PCR analysis. The results revealed that SRC3 was upregulated in the intestine (Fig 4E) and tail (Fig 4F) by the T_3 treatment. In contrast, the expression of p300 and SRC2 remained unchanged (Figs 4A, B, C and D). The upregulation of SRC3 required a higher level of T_3 in the tail (Fig 4F). This is not unexpected since the tail requires a higher level of T_3 to regress and is one of the last changes to occur during metamorphosis[1, 2, 35]. Thus, SRC3 is upregulated by T_3 at least during intestinal remodeling and tail resorption, although our data does not show whether it is a direct T_3 response gene, i.e., regulated at the transcriptional level by TR, or indirect T_3 response gene.

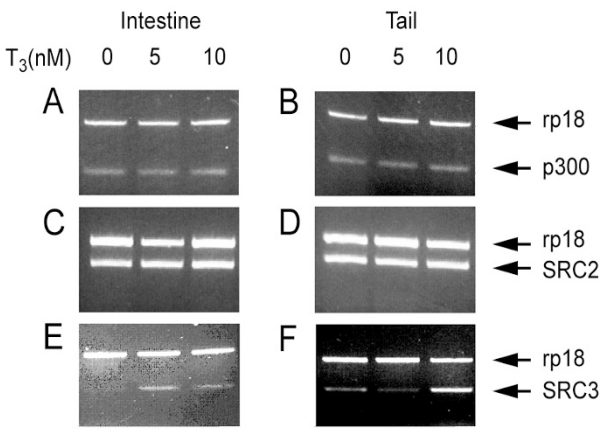

Fig 4. SRC3 is induced by T_3 treatment of premetamorphic tadpoles while SRC2 and p300 are expressed constitutively. Tadpoles at stage 54 were treated with 5 or 10 n*M* T_3 for 3 days. RNAs were isolated from the intestine and tail and used for RT-PCR analysis of the expression of p300, SRC2, and SRC3 as indicated, with rpl8 as the internal control.

Induction of SRC3 is impaired in transgenic tadpoles harboring a dominant negative form of TR

To further confirm the induction of SRC3 by T_3, its expression was studied in transgenic animals expressing a dominant negative form of TR (dnTR) that cannot respond to T_3. This dnTR has a deletion at the carboxyl terminus, lacking part of the T_3-binding domain[47]. Ourrecent studies showed that the dnTR inhibits T_3 regulation of both direct T_3-response genes, viz, TRβ, TH/bzip, stromelysin 3 (ST3), as well as indirect T_3-response genes[36]. Thus, if SRC3 were also regulated by T_3, the levels of its expression should not be altered by T_3 treatment of dnTR transgenic tadpoles. Thus, we subjected both wild type and dnTR transgenic tadpoles at stage 54 to 10 n*M* T_3 for 3 days. RT-PCR analysis on total RNA isolated from the tail and intestine of T_3-treated or untreated animals revealed that while SRC3 was induced in the wild type tadpoles by the T_3 treatment, this induction was greatly inhibited in the dnTR transgenic tadpoles (Fig 5). These results support the conclusion that SRC3 is regulated by the thyroid hormone.

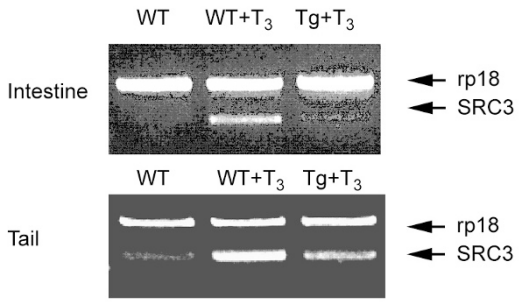

Fig 5. Transgenic expression of a dominant negative TR (dnTR) prevents the induction of SRC3 by T_3 in tadpoles. Wild type (WT) and transgenic tadpoles (Tg) at stage 54 were treated with 10 n*M* T_3 for 3 days. RNAs were isolated from the intestine and tail are used for RT-PCR analysis of SRC3 expression with rpl8 as the internal control.

DISCUSSION

In vitro and tissue culture cell studies have provided strong evidence for the involvement of SRC and p300 proteins in transcriptional activation by TR and other nuclear hormone receptors. However, few studies have addressed their involvement in the function of nuclear hormone receptors during vertebrate development, in part due to the lack of proper system for *in vivo* studies. Amphibian metamorphosis offers an opportunity to address the function of coactivators in TR function during development because of its total dependence on T_3. Here, we show that SRC2, SRC3, and p300 are all expressed during metamorphosis. Thus, they may be recruited by TR to direct T_3 response genes to activate their transcription, thereby inducing the metamorphic events. Interestingly, SRC3 is unique as being the only one of the three upregulated by T_3 in different organs/tissues during metamorphosis. Its induction by T_3 treatment of wild type tadpoles but not transgenic tadpoles expressing dnTR is

consistent with its upregulation during natural metamorphosis. Since in the presence of T_3, co-activators are recruited to the TR/RXR heterodimer at the TREs of direct T_3-response promoters, it is quite likely that SRC3 has little involvement in the initial events of metamorphosis due to its low level of expression prior to metamorphic climax, i.e., in premetamorphic tadpoles. At these earlier stages of metamorphosis, SRC2 or other similar coactivators, in conjunction with p300, may function with TR to activate direct T_3 response genes. On the other hand, metamorphosis depends on continuous presence of T_3, arguing the TR/RXR continuously activate the direct T_3 response genes and/or additional genes during later stages of metamorphosis. The upregulation of SRC3 is likely important for such late, T_3-dependent events. Alternatively, SRC3 may be utilized by other transcriptional factors in the cascades of gene regulatory events induced by T_3 during metamorphosis. Future studies using various molecular and genetic approaches should be able to address the nature of the involvement of SRC3 during this interesting postembryonic process.

ACKNOWLEDGMENTS

We thank Dr. JW Lee (Baylor College of Medicine, Houston, Texas), Dr. JL Gomez-Skarmeta (Centro de Biologia Molecular Severo Ochoa, Universidad Auto-noma de Madrid, Spain) and Dr. S Hirohashi (National Cancer Center Research Institute, Tokyo, Japan) for the *Xenopus* SRC3, SRC2 and p300 clones, respectively. We thank Dr. D Buchholz for the transgenic dnTR animals and other members of the group for discussions.

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