Induction of Larval Tissue Resorption in Xenopus laevis Tadpoles by the Thyroid Hormone Receptor Agonist GC-1*

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A major challenge in understanding nuclear hormone receptor function is to determine how the same ligand can cause very different tissue-specific responses. Tissue specificity may result from the presence of more than one receptor subtype arising from multiple receptor genes or alternative splicing. Recently, high affinity analogs of nuclear receptor ligands have been synthesized that show subtype selectivity. These analogs can greatly facilitate the study of receptor subtype-specific functions in organisms where mutational analysis is problematic or where it is desirable for receptors to be expressed in their normal physiological contexts. We describe here the effects of the synthetic thyroid hormone analog GC-1 on the metamorphosis of the frog Xenopus laevis. The most potent natural thyroid hormone, 3,5,3′-triiodothyronine or T3, shows similar binding affinity and transactivation dose-response curves for both thyroid hormone receptor isotypes, designated TRα and TRβ. GC-1, however, binds to and activates TRβ at least an order of magnitude better than it does TRα. GC-1 efficiently induces death and resorption of premetamorphic tadpole tissues such as the gills and the tail, two tissues that strongly induce thyroid hormone receptor β during metamorphosis. GC-1 has less effect on the growth of adult tissues such as the hindlimbs, which express high TRα levels. The effectiveness of GC-1 in inducing tail resorption and tail gene expression correlates with increasing TRβ levels. These results illustrate the utility of subtype selective ligands as probes of nuclear receptor function in vivo.

Nuclear hormone receptors and their ligands are essential regulators of metazoan development, reproduction, and homeostasis. One of the most dramatic nuclear receptor-mediated events in nature is amphibian metamorphosis (1, 2). In the frog, metamorphosis occurs as a result of gene expression cascades induced by thyroid hormone (TH) secreted from the developing tadpole’s thyroid gland (3). These cascades result in dramatically different morphological responses such as the growth and differentiation of the limbs, the death and resorption of the larval gills and tail, and the remodeling of a large number of larval organs for new adult functions (1). As in all vertebrates, TH exerts its effects via a pair of TH receptor subtypes encoded by separate genes, TRα and TRβ (4). TRs are ligand-regulated transcription factors that are thought to repress target gene transcription in the absence of hormone and activate transcription upon hormone binding (5). The most detailed molecular studies on amphibian metamorphosis have been done on the frog Xenopus laevis. Xenopus TRs is expressed early in development (6, 7), long before the embryo and larval tadpole have a functional thyroid gland. Just prior to metamorphosis, xTRα is ubiquitously expressed, but particularly high levels of it are detected in the brain, limb buds, skin, and other tissues that are destined to respond to TH by proliferating and differentiating into adult organs (8, 9). In contrast, much lower levels of Xenopus TRβ mRNA are detectable prior to metamorphosis (6, 10). At metamorphosis, TRβ is strongly induced by TH in larval tissues such as the tail that will die and resorb. TRβ levels remain very low in the growing limbs, although expression in this tissue may be highly localized (11). In remodeling tissues, such as the intestine and cartilage of the head, TRβ mRNA is up-regulated in both dying and proliferating cells, suggesting a possible role for TRβ in both processes (8, 12, 13).

Xenopus TRs are extremely well conserved in comparison with their avian and mammalian counterparts (4). xTRα is >95 and 91% conserved in its DNA and ligand-binding domains, respectively, when compared with rat TRα. xTRβ is >96 and 94% conserved in those domains compared with rat TRβ. The major difference between the two Xenopus TR subtypes is the lack of an amino-terminal domain in xTRβ that may participate in cell-specific transcriptional activation or altered DNA binding properties in xTRα. A recently developed synthetic thyroid hormone analog, GC-1 (Fig. 1, bottom), preferentially binds and transactivates mammalian TRβ versus TRα (14). In human TRs, a single amino acid difference in the ligand binding pocket is responsible for this selectivity (15), and this amino acid difference is conserved in Xenopus TRα. In vivo, GC-1 selectively decreases plasma lipids and thyroid-stimulating hormone secretions without increasing the heart rate in hypothyroid rats (16). The effects of GC-1 are consistent with the known relative expression patterns of rat TR subtypes in the liver, the pituitary, and the heart. Recently, we have established that X. laevis metamorphosis provides a simple screening assay for the biological activity of thyroid hormone receptor

T3, 3,5,3′-triiodothyronine; BTEB, basic transcription element-binding protein; FAP, fibroblast activation protein; x, X. laevis (prefix).

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Animal Studies—X. laevis tadpoles were purchased from Nasco, Inc. (Fort Atkinson, WI) and staged according to Nieuwkoop and Faber (20). All chemicals were purchased from Sigma unless indicated otherwise. For hormone treatments, a 1 mM stock of T3 was dissolved in 4 mM NaOH, and a 10 mM GC-1 stock was made in dimethyl sulfoxide. GC-1 was synthesized as described elsewhere (14). For whole animal treatments, stage 52 and stage 53 tadpoles were placed in 1 X Steinberg’s solution (10 mM HEPES, 60 mM NaCl, 0.67 mM KCl, 0.34 mM Ca(NO$_3$)$_2$, 0.83 mM MgSO$_4$, pH 7.4) with 1 mM methimazole and the indicated hormone concentrations or appropriate vehicle and observed daily. After 1 week, animals were fixed in phosphate-buffered saline containing 10% formalin. Animals were photographed with a Nikon F-5 camera, and slides were scanned into Adobe Photoshop 5.0 using a Kodak RFS 3570 Plus film scanner. Images were then assembled in Adobe Pagemaker 6.5.

Tail Organ Culture—Tadpole tails were cultured in vitro as described previously (21), with some modifications. Staged tadpoles were pre-treated in 1 X Steinberg’s solution containing gentamycin (70 µg/ml) and streptomycin (200 µg/ml) 24 h before removal of the tail tips for culture. The tadpoles were anesthetized briefly in water containing 0.01% MS222 (aminobenzoic acid; Sigma), after which 6 mm of the tail tip was cut and placed in 1 ml of Steinberg’s solution with antibiotics and 5 µg/ml insulin in 12-well Falcon tissue culture dishes (BD Biosciences). Tail tips were left in the culture media for 24 h before the first hormone treatment. T3 (Sigma) and GC-1 were diluted to the appropriate final concentration. The same final dilution of the vehicle was added to control cultures. Afterward, the culture media with hormones were changed every 48 h. The length of each tail was measured every 24 h, and each experimental point consisted of the mean change of 4 to 6 tails. Each experiment was repeated twice.

RNA Analysis—Tadpoles of the indicated stage were treated with T3 or GC-1 in 1 X Steinberg’s solution for 48 h. Tails were harvested and frozen in liquid nitrogen. Total RNA was isolated and analyzed by Northern hybridization using cDNA probes as described previously (22). The amount of hybridized probe was determined using an Amersham Biosciences Storm PhorImager and ImageQuant software and normalized to the expression of the ribosomal protein rpL8 gene (23).

RESULTS

GC-1 Binds Preferentially to X. laevis TRβ over TRα—GC-1 is an iodine-free thyromimetic that preferentially binds mammalian TRβ over TRα (14). To determine the relative binding affinity of GC-1 for each of the Xenopus TRs, protease protection assays were performed. Protease protection assays have been used previously to both estimate binding affinity and reveal different conformations induced by various natural and synthetic nuclear receptor ligands (24). In addition, the assay is performed at equilibrium and bound and free ligands are not separated, so results tend to accurately reflect the relative transcriptional activation potency of the ligands in question (25). Increasing amounts of T3, the most potent form of TH (Fig. 1, top), induce a major protected band of ~35 kDa in both xTRα and xTRβ (Fig. 2A). The binding curves generated for xTRα and xTRβ were virtually identical (Fig. 2B). Using GC-1, a major protected band of the same size was produced; however, GC-1 bound to xTRβ at a concentration at least 10 times lower than that observed for xTRα (Fig. 2, A and B). Our protease protection assay revealed a preference of GC-1 for TRβ over TRα, which is remarkably consistent with the results of competitive ligand binding assays used for mammalian TRs. Therefore, GC-1 is a TRβ-selective ligand for Xenopus TRs.

Activation of X. laevis Thyroid Hormone Receptors by GC-1—We next tested the ability of GC-1 to activate TRs in X. laevis cells using a transient transfection assay. Using X. laevis XLA kidney cells, which express functional TRα, we examined the T3- and GC-1-regulated transactivation properties of the TRα and TRβ ligand-binding domains as Gal4 fusion proteins. The dose-response curves for T3 activation of TRα and TRβ on an upstream activation sequence-based reporter gene were virtually identical, whereas GC-1 activated TRβ at lower concentrations than TRα (Fig. 3, A and B). The leftward shift in the GC-1
FIG. 2. GC-1 binds preferentially to X. laevis thyroid hormone receptor β. A, in vitro translated, 35S-labeled xTRα or xTRβ were incubated with the indicated nanomolar concentrations of T3 or GC-1 and then treated with 4 μg of elastase. The resulting bands were resolved by 12% SDS-PAGE. I, input. B, T3 binding curves for xTRα (circles) and xTRβ (squares) were generated by quantitation of the protected bands shown in panel A and expressed as a percentage of the maximally protected bands. C, GC-1 binding curves for xTRα (circles) and xTRβ (squares) were generated by quantitation of the protected bands shown in panel A and expressed as a percentage of the maximally protected bands. Error bars in panels B and C represent S.D.
GC-1 preferentially activates X. laevis TRβ versus TRα relative in transient transfection assays. A and B, an upstream activation stream-containing promoter that drives luciferase reporter gene expression was co-transfected into X. laevis kidney epithelial cells (XLA) along with expression vectors for T3 or GC-1 only activated a TH response element-based reporter gene by <2-fold (see insets, Fig. 3, C and D). In response to T3, both TRα and TRβ strongly activated reporter gene transcription. In this case, the maximum level activation is different for TRα versus TRβ, but the EC50 (10 nM) does not change, as has been reported previously for mammalian TR (26). When GC-1 is used, the maximum activation is similar, but the EC50 value (~20 nM for TRβ versus 200 nM for TRα) is shifted in favor of TRα. (Fig. 3, C and D). Therefore, in both ligand binding and transactivation assays GC-1 is a TRβ-selective ligand for Xenopus as well as mammalian TRs.

Effect of GC-1 on X. laevis Tadpoles—An important advantage of metamorphosis as an assay system is that thyroid hormone can be added directly to the rearing water, causing accelerated effects that mimic what occurs during spontaneous metamorphosis. We treated premetamorphic tadpoles (stages 52 and 53) with increasing doses of both T3 and GC-1. These developmental stages are just prior to the time when the thyroid gland becomes active but after the animals are fully competent to respond to exogenous hormones. We observed the first clear response to GC-1 at 50 nM, where obvious gill resorption and modest tail resorption are induced (Fig. 4A). As the dose of GC-1 is increased, progressively more gill and tail resorption occurs. Very little change is observed in the limbs except for a loss of tissue around the forelimb buds, allowing them to extend away from the body. At high doses of GC-1 (>200 nM) the animals develop a beak-like appearance because of the expansion of Meckel’s cartilage. Finally, at 500 nM GC-1 the treated tadpoles are considerably smaller than the controls with an almost complete loss of the tail and gills. Internally, we noted extensive resorption of the intestine beginning above 100 nM GC-1, similar to what is observed with T3 treatments above 10 nM. Like T3, the effects of GC-1 on intact tadpoles are first apparent about 2 days after treatment begins, and most animals survive the 1-week treatments even at the highest doses of GC-1. In some batches of animals we observed limited limb growth and some widening of the brain at concentrations of GC-1 >200 nM, but these effects occurred infrequently and at higher concentration than those required for tail and gill resorption that were always observed.

Our results with GC-1 contrast significantly with the results obtained with the endogenous hormone T3. Low doses of T3 (2 nM) induced hindlimb and forelimb growth and differentiation without significant loss of tail tissue (Fig. 4A). In dorsal views (Fig. 4B), expansion of the brain is readily observed at low T3 doses; we observe a consistent 30–40% expansion of the brain in response to 2 nM T3. Higher T3 concentrations (10 nM)
induced limb growth to a greater extent, and gill loss becomes clearly noticeable. Both the growth of adult structures such as the hindlimbs and forelimbs and the resorption of larval tissues such as the tail and gills are apparent at the highest doses of T3 used in these studies (50 nM). A "hunchbacked" appearance is also apparent at these higher T3 doses that is not seen in GC-1 treated animals. This results presumably from the accelerated development of adult dorsal muscle (44). Toxicity was observed when T3 was used in excess of 50 nM, with many animals dying within a few days after treatment. Thus, when GC-1 and T3 dose responses are compared, GC-1 induces death and resorption of larval tissues at lower concentrations than are required to induce limb growth. This is completely opposite to the developmental program induced by the natural hormone T3, where growth responses generally precede larval tissue death and resorption.

The Sensitivity of Tadpole Tails to GC-1 Increases with Increasing TRβ Expression—Because higher levels of GC-1 than of T3 were required to cause tissue responses in premetamorphic tadpoles, we next tested whether the efficiency of GC-1 in inducing larval cell death would improve in later developmental stages when TRβ levels are significantly elevated. TRβ protein expression increases 8–10-fold as tadpoles develop from premetamorphosis (prior to thyroid gland maturation) to metamorphic climax when tail resorption commences in earnest (27). On the other hand, despite a modest increase in TRα message the TRα protein levels remain constant throughout metamorphosis. Therefore, we predicted a modest increase in sensitivity to T3 but a large increase in sensitivity to the TRβ-selective ligand GC-1 at two developmental stages as follows: (i) premetamorphic stages 52–54 with low TRβ levels; and (ii) prometamorphic stages 57 and 58, when TRβ levels were nearly maximal but tail resorption had not yet begun. We first established that isolated tail tips from premetamorphic tadpoles would resorb in a T3 dose-responsive and reproducible manner (Fig. 5A). Maximal resorption rates occurred at concentrations above 32 nM T3. We then measured the efficiency of tail resorption in response to increasing T3 and GC-1 at stages 53 and 54 and stages 57 and 58 tadpoles (open symbols) or stage 57 and stage 58 tadpoles (filled symbols). Squares, T3; circles, GC-1. Error is expressed as S.E.
compared. This increased sensitivity to GC-1 is especially apparent at low doses of GC-1 (50 nM) where there is no response in young tadpoles, but a robust response is observed in older tadpoles.

**Induction of TH Response Genes by T3 and GC-1**—A large collection of TH response genes has been cloned from *X. laevis* tadpole tails (28) as well as from several other tissues (29–31). Tail TH response genes include rapidly induced early genes that generally encode transcription factors and delayed genes that mostly encode membrane-bound, secreted, or intracellular proteases and extracellular matrix components and receptors (28, 32). Delayed gene expression is more tissue-specific than early gene expression and is especially strong in fibroblasts under the epidermis and between muscle fibers of the tail and in the gills (13). We have analyzed the induction of tail TH response genes by increasing doses of T3 and GC-1, again using stages of development with different levels of TR/βexpression.

xBTEB (33) is a zinc finger transcription factor, the expression of which is typical of early gene responses to both T3 and GC-1 (Fig. 6, A and B). xBTEB is quite sensitive to T3 because it is induced by as low as 1 nM T3 in stage 54 tadpoles. The induction of xBTEB by T3 at stage 57 is similar. GC-1 induces xBTEB above 100 nM in stage 54 tadpoles; however, at stages 57 and 58 GC-1 is unable to induce xBTEB expression above baseline. Gene 12, *TH*bZIP, and TR/β itself are other early genes that show a similar dose-response profile to T3 and GC-1 as xBTEB does (data not shown). We next looked at the delayed class of tail response genes, exemplified by the membrane-bound protease fibroblast activation protein α (FAPα) (32) (Fig. 6, C and D). First, a 5–10-fold higher level of T3 is required to activate delayed genes like FAPα than early genes like xBTEB. In response to GC-1, FAPα mRNA is first induced above baseline at 100 nM with a dose-response curve similar to that of xBTEB. Strikingly, FAPα becomes more, not less, sensitive to GC-1 in older animals that are expressing more TR/β. A clear induction of FAPα is seen in response to as low as 20 nM GC-1 in stage 57 and stage 58 animals. Other genes in this delayed class that show the same pattern of responses are the secreted matrix metalloproteinase collagenase-3 and the intracellular protease pepE (data not shown). We also noted that the dose-response curves for delayed gene expression closely parallel both the T3 and GC-1 dose-response curves in tail culture assays, further implicating these genes as key players in the process of tail resorption.

**Fig. 6. Northern blot analysis of thyroid hormone response genes induced by T3 and GC-1.** Two thyroid hormone response genes, BTEB (A and B) and FAPα (C and D), were assayed for induction by increasing doses of T3 (A and C, squares) or GC-1 (B and D, circles). RNA was analyzed from the tails of tadpoles at stages 53 and 54 (open symbols) or stages 57 and 58 (closed symbols). Northern data was normalized by stripping the blots and reprobing for constitutive rpL8 expression and is shown as a percentage of the maximum expression for each set of dose-response curves on a given blot.
DISCUSSION

A recently developed TRβ selective agonist, GC-1, preferentially induces a subset of TH-regulated developmental programs in X. laevis tadpoles, namely the death and resorption of larval tissues. Thus, we provide compelling evidence for different functional roles of the two TR subtypes during metamorphosis, extending earlier expression studies that were strictly correlative (8, 13, 28). Our results agree well with known TR isotype gene expression in that GC-1 induces TRα rich tissues poorly but is most effective in tissues that have low TRα levels but that strongly induce TRβ. TRβ expression in the tail is particularly high in fibroblasts under the skin and around the notochord that also strongly express delayed tail response genes like FAPα. The pronounced effects of GC-1 on gill and tail resorption are intriguing in light of recent experiments on the expression of TRα and TRβ in various salamander species. Obligatorily neotenic species like Necturus are unable to complete metamorphosis in that the external gills and tail fins of these animals are resistant to TH even though they have fully developed limbs. Interestingly, they express TRα in those tissues, but there is no detectable baseline or inducible TRβ message even though an apparently normal TRβ gene exists in the genome (34).

Most tail response genes are induced by similarly high doses of GC-1 in premetamorphic animals (>100 nM) but show very different responses at later developmental stages when TRβ is the predominant receptor isotype. Based on these results, we propose that there are two classes of genes in terms of TR subtype control. In young animals, sufficient TRα must be induced via TRα to activate delayed genes, but other early genes like xBTEB and gene 12 are also being induced in parallel. At later stages only the delayed genes become more sensitive to both T3 and GC-1, indicating that this gene class, at least in the tail, is preferentially controlled by TRβ. Our tail culture results are also most consistent with at least a partial requirement for activation for xTRα to induce sufficient xTRβ levels for a response in larval tissues like the tail. Indeed, the xTRβ gene contains a strong thyroid hormone response element near its transcription start site (35, 36), and TRβ can be induced in tadpoles even in the presence of protein synthesis inhibitors (10). However, there are other possibilities to account for the differences in tissue sensitivity to T3 and GC-1. There may be a less efficient uptake of GC-1 than of T3 from the rearing media in young tadpoles or stage differences in the metabolism of the ligands. In rats, GC-1 accumulates in the intestine (8, 12). In addition, the effects of GC-1 on metamorphic tadpoles are perhaps more dramatic than expected based on the dose-response profiles of transient transfection assays alone. It is possible that inappropriately timed TRβ activation may antagonize TRα function. The estrogen-inducible progesterone receptor antagonizes estrogen receptor function in the mammalian uterus (37). Another interpretation of our results is that the effects of GC-1 on metamorphic tadpoles may simply reflect where each isotype is most abundant as the tissue responds. In this model, the two TR isotypes differ quantitatively but not qualitatively. To test this possibility, transgenic approaches are now available in Xenopus, where it should be possible to express xTRβ in xTRα-dominated tissues and vice versa, depending on the availability of appropriate tissue-specific promoters.

There is growing evidence in other animals for isotype-specific functions of nuclear receptors. In insects, ecdysone receptor diversity is generated by alternate promoter usage of the same gene to create different amino-terminal transactivation domains (38). Three ecdysone receptor subtypes in Drosophila melanogaster are expressed in different ratios in different tissues, which correlate with different tissue fates at metamorphosis (38). Specific ecdysone receptor-B1 mutations prevent salivary gland histolysis and development of a subset of imaginal disks that cannot be rescued by overexpression of the ecdysone receptor-A subtype (39). In vertebrates, knockout studies of each TR gene alone and in combination in mice have demonstrated both overlapping and distinct functions for the two TR genes (40). For instance, proper functional development of the inner ear appears to require TRβ (41), and TRα appears to play a more dominant role in TH control of resting heart rate and body temperature (42, 43). However, other processes, such as negative feedback control of the thyroid stimulating hormone gene, were at least partially affected in both TR knockouts (40). It is possible that TR subtype-specific function is more distinct in taxa other than mammals. Certainly, the study of receptor signaling during development is easier in model systems with externally developing embryos. Our findings illustrate the utility of subtype-selective ligands as probes of nuclear receptor function in vivo and demonstrate the potential of natural systems such as amphibian metamorphosis for use as simple bioassays for the development of selective agonists and antagonists of thyroid hormone receptors.

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REFERENCES

Receptor Isotype Regulation of Xenopus Metamorphosis

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