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ANÁLISIS DE LAS PROTEÍNAS CORREGULADORAS ASOCIADAS AL COMPLEJO 3,5-T2+TRβ1 LARGO

T E S I S

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RESUMEN

Las hormonas tiroideas (THs) son esenciales para el correcto desarrollo y el mantenimiento de múltiples procesos biológicos en vertebrados. Los efectos de las THs son mediados principalmente a través de mecanismos genómicos, que involucran la unión de la TH a receptores de hormonas tiroideas (TR). En vertebrados, los genes *THRA* y *THRB*, codifican para los receptores TRα y TRβ, así como a sus correspondientes isoformas. Los TRs actúan como factores de transcripción dependientes del ligando. Los TR reconocen e interactúan con elementos de respuesta a TH (TRE) localizados en las regiones promotoras de los genes responsivos estas hormonas y reclutan grupos de proteínas correguladoras que modifican el estado de la cromatina para aumentar o reprimir la expresión génica. Aun cuando la 3,3´,5-triiodotironina (T3) es considerada la principal TH bioactiva debido a su alta afinidad por los TRs, ahora se reconoce que, al igual que la T3, la 3,5-T2 (T2) regula la expresión de genes y promueve el reclutamiento de diferentes poblaciones de proteínas correguladoras al TRE. En el presente trabajo analizamos el mecanismo de acción de la T2. Se realizaron ensayos de unión o "binding", analizando la interacción de T3 y T2 con distintas isoformas de TRβ1 de teleósteo [TRβ1 largo (L-TRβ1) y TRβ1 corto (S-TRβ1)] y de humano (hTRβ1). Asimismo, se analizaron las proteínas correguladoras que se asocian al complejo T2 + TRβ1 y se estudiaron los mecanismos transcripcionales de la comunicación cruzada de las THs y el cortisol sobre la regulación del gen *thrb* en tilapia. Los experimentos de "binding" mostraron que la T2 es un ligando alternativo de ambas isoformas L-TRβ1 y hTRβ1, pero a las que se une con una afinidad diferencial. Se identificó a la proteína c-jun binding domain interacting protein 1 (Jab1) como un corregulador dual ligando-dependiente de las isoformas L-, S- y hTRβ1, siendo un coactivador en presencia de T2 y un correpresor en presencia de T3. Por último, se observó que la T2 antagoniza los efectos del cortisol sobre la regulación del gen *thrb* en tilapia, a través de un novedoso mecanismo de transrrepresión, distinto al de T3. En conjunto, los resultados de la presente tesis muestran que la T2: i) se une al TRβ1, promoviendo el reclutamiento de proteínas correguladoras para modular la transcripción de forma específica, y ii) activa mecanismos de comunicación cruzada con otras vías de señalización hormonales. Los mecanismos de acción de la T2 podrían explicar, en parte, la pleiotropía de los efectos biológicos de las THs.

SUMMARY

Thyroid hormones (THs) are essential for the correct development and maintenance of multiple biological processes in vertebrates. TH effects are mediated by genomic mechanisms, that involve the binding to thyroid hormone receptors (TR). In vertebrates, *THRA* and *THRB* genes encode for the TRα y TRβ and their respective isoforms. TRs act as ligand-dependent transcription factors. TRs interact directly with TH-response elements (TRE) located at the promoter regions of TH-responsive genes and recruit a set of coregulator proteins that induce changes in the chromatin to up-regulate or repress gene expression. Even though, 3,3´,5-triiodothyronine (T3) is considered the main bioactive TH due to its high affinity for TRs, it is now recognized that, in the same manner as T3, 3,5-T2 (T2) regulates gene expression and promotes the recruitment of different populations of coregulator proteins to the TRE. In the present work, we analyzed the mechanism of action of T2. Binding experiments were performed by analyzing the interaction between T3 and T2 with the different teleost [TRβ1 long (L-TRβ1) y TRβ1 short (S-TRβ1)] and human TRβ1 isoforms (hTRβ1). Furthermore, coregulator proteins associated to the T2 + TRβ1 complex were analyzed as well as the transcriptional mechanisms of THs and cortisol cross-talk over *thrb* gene regulation in tilapia. Binding experiments showed T2 is an alternative ligand of both L-TRβ1 and hTRβ1 isoforms, but it binds with a differential affinity to each isoform. The c-jun binding domain interacting protein 1 (Jab1) was identified as a dual ligand-dependent coregulator of L-, S- and hTRβ1 isoforms, working as a coactivator in the presence of T2 and a corepressor in the presence of T3. Finally, we observed that T2 antagonizes cortisol effects over *thrb* gene regulation in tilapia, through a novel transrepression mechanism, different from that of T3. Altogether, results from the present thesis showed that T2: i) binds TRβ1, promoting the recruitment of coregulator proteins to modulate transcription in a specific fashion, and ii) activates cross-talk mechanisms with other hormonal signaling pathways. T2 action mechanisms could at least in part explain the pleiotropy of THs biological effects.

INTRODUCCIÓN

Las hormonas tiroideas forman parte de un conjunto de compuestos yodados de gran importancia en vertebrados, pues participan en el crecimiento y diferenciación celular, así como en el metabolismo energético. Dichos procesos están mediados principalmente por los efectos nucleares de la T3, la cual regula la transcripción de diversos genes mediante su unión a sus receptores nucleares. Además de la T3, la T2 -molécula que se genera a través de la desyodación del anillo externo de la T3- tiene efectos extra-nucleares sobre la regulación del metabolismo energético a nivel mitocondrial; sin embargo, poco se conoce sobre sus efectos genómicos. En este aspecto, estudios realizados en nuestro laboratorio han demostrado que la T2 tiene la capacidad de regular la actividad de genes responsivos a hormonas tiroideas. Aun cuando los mecanismos involucrados en dicha regulación no han sido dilucidados totalmente, existe evidencia que sugiere la participación de complejos transcripcionales o isoformas de receptores de hormonas tiroideas distintos a los que median los efectos de T3. Por esta razón, en este estudio nos proponemos investigar si la T2: i) se une a las isoformas de TRβ1 de teleósteos y de humano, ii) recluta proteínas correguladoras específicas al TR, distintas a aquellas reclutadas por T3, y iii) tiene comunicación cruzada con otras vías de señalización hormonales, específicamente con la del cortisol.

ANTECEDENTES

1- Hormonas Tiroideas.

Los sistemas endocrinos están presentes en todos los vertebrados y juegan un papel esencial en etapas del desarrollo temprano de los individuos, así como durante su vida adulta en procesos como el crecimiento, el funcionamiento adecuado de varios órganos, la reproducción y el mantenimiento del balance energético (producción, utilización y almacenamiento de energía) (Anexo 1). Las hormonas son los mensajeros del sistema endocrino e inducen efectos tejido-específicos. El presente trabajo se enfoca en el sistema tiroideo, específicamente en el estudio del mecanismo de acción de las hormonas tiroideas (TH), las cuales son esenciales en la historia de vida de los organismos (Tata, 2011). Las THs están formadas por dos anillos fenólicos, derivados del aminoácido tirosina unidos por un enlace éter; y pueden poseer hasta cuatro átomos de yodo en su estructura (Figura 1).

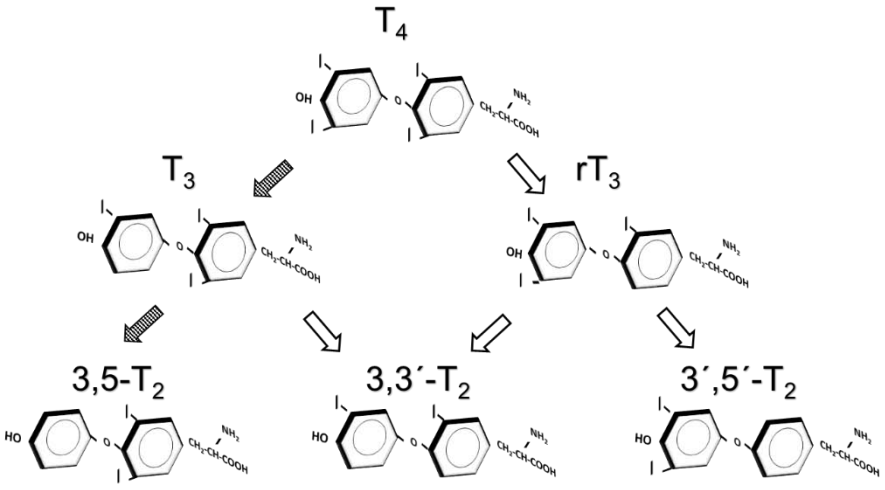

Figura 1. Estructura de las Hormonas tiroideas. Se indican las vías de activación (flechas rayadas) o inactivación (flechas blancas).

La síntesis y secreción de THs está regulada por el eje endocrino hipotálamo-hipófisis-tiroides, que culmina con la síntesis de 3,3´,5,5´-tetrayodotironina ó T4, la cual posee cuatro átomos de yodo en su estructura y es considerada una pro-hormona pues a partir de ella se produce la 3,3´,5-triyodotironina ó T3, que es considerada como la principal TH (Figura 1). Así, la remoción esteroespecífica de un átomo de yodo del anillo externo de la molécula de T4 genera T3, y esta reacción está

catalizada por la acción de enzimas denominadas desyodasas (Ds). Hasta ahora se reconocen a tres distintas Ds: desyodasa tipo 1 (D1), desyodasa tipo 2 (D2) y desyodasa tipo 3 (D3); la D2 cataliza exclusivamente la desyodación del anillo externo o vía de activación, la D3 cataliza la desyodación del anillo interno o vía de inactivación, mientras que la D1 cataliza ambas reacciones de desyodación del anillo externo e interno. Además de la producción de T3, la desyodación del anillo interno de la T4, produce la hormona T3 revesa o rT3; posteriormente la desyodación de T3 y rT3 produce otras yodotironinas como la 3´,5´-T2 y 3,3´-T2 a las cuales hasta el momento no se les ha atribuido actividad biológica, con la excepción de la 3,5-diyodotironina (3,5-T2), la cual resulta de la desyodación del anillo externo (vía de activación) de la T3 (Figura 1) y como se describe más adelante, la 3,5-T2 es una hormona tiroidea bioactiva y es objeto de estudio de la presente tesis.

Las THs inducen sus efectos biológicos a través de mecanismos genómicos y no genómicos, los cuales han sido estudiados desde hace varios años y han mostrado gran complejidad en sus elementos. La mayoría de los efectos a nivel genómico se han atribuido a la T3, mientras que los efectos a nivel no genómico se han atribuido tanto a la T3, como a la T4 y a la rT3.

Respecto a los efectos a nivel no genómico, las THs pueden activar receptores localizados en membrana plasmática, mitocondria o citoplasma y sus efectos pueden culminar o no en la regulación de la expresión génica, sin embargo, no involucran la participación de receptores nucleares *per se*. Por ejemplo, T3 y T4 pueden activar a la proteína estructural de membrana integrina αvβ3 y estimular vías de señalización que culminan en la activación de las vías de las MAPK (*mitogen activated phosphokinase*), promoviendo división celular y angiogénesis. T4 y rT3 participan en el proceso de polimerización de actina, al promover la formación de actina fibrosa, a través de un mecanismo que podría involucrar la participación de una isoforma truncada que solo contiene la porción C-terminal del LBD del receptor a hormonas tiroideas alfa 1 (TRΔα1). Por otro lado, la T3 también puede activar a otra isoforma del TRα1, denominada p30 TRα1, ubicada en membrana plasmática o en citoplasma e inducir modificaciones estructurales en el citoesqueleto y proliferación en células óseas. La actividad de las bombas de Ca^{2+}- y Na^{+}-K^{+}-dependientes de ATPasa, puede ser estimulada por T4 y T3, respectivamente, siendo la activación por T3 a través de la vía PI3K (*phosphatidylinositol 3-kinase*).

Se requieren más estudios para comprender los efectos de las THs a nivel no genómico y los mecanismos moleculares que los subyacen, pues resultan de gran importancia para el mantenimiento de funciones celulares básicas (Davis et al., 2015).

A continuación, se describirá de forma general el mecanismo de acción de las THs con especial énfasis en el mecanismo genómico.

2- Mecanismo de Acción de las THs.

El mecanismo de acción clásico de la T3 involucra su unión a los receptores nucleares de hormonas tiroideas (TR, *thyroid hormone receptor*), los TR actúan como factores de transcripción dependientes de ligando y se encuentran unidos a secuencias específicas de ADN denominadas TRE (*T3-responsive* e*lement*) que se encuentran localizadas en las regiones promotoras de genes TH-dependientes. Los TREs están formados por dos medios sitios de la secuencia hexamérica consenso 5´-(G/A)GGT(C/G)A-3´ y difieren en su orientación y espaciamiento. Se han descrito al menos tres arreglos distintos de TRE: el palindrómico (PAL), el repetido directo (DR) y el palindrómico invertido (IP).

Los TRs se encuentran asociados al TRE en forma de homodímeros, aunque usualmente forman heterodímeros con otros receptores nucleares como el receptor de retinoides X (RXR), receptor de vitamina D (VDR) y receptores de retinoides (RAR). La heterodimerización con el RXR aumenta la estabilidad de la interacción TR-TRE y aumenta la actividad transcripcional del TR (Cheng et al., 2010). La unión del ligando al TR, induce una serie de cambios conformacionales en la estructura del receptor, que permite reclutar proteínas correguladoras que realizan modificaciones en el estado de la cromatina y subsecuentemente reclutar a la maquinaria general de la transcripción para regular la tasa de transcripción de proteínas que participan en procesos biológicos dependientes de THs (Yen, 2001; Cheng et al., 2010) (Figura 2).

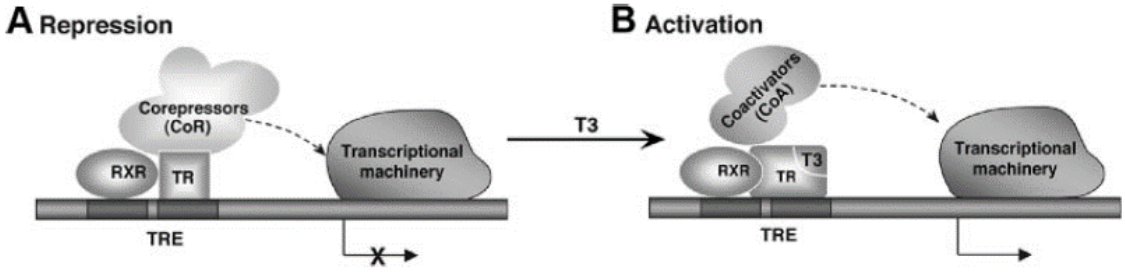

Figura 2. Mecanismo general de regulación de la transcripción por T3. **A.** Mecanismo de represión, en ausencia de la hormona, los complejos correpresores (CoR) son reclutados hacia el TR. **B.** La presencia del ligando T3, permite el reclutamiento de complejos coactivadores (CoA) para iniciar el proceso de transcripción. RXR, receptor de retinoides X. Tomado de Cheng et al., 2010.

Además de la T3, otros de los principales elementos involucrados en el mecanismo de acción de las THs son los TRs y los correguladores, por lo que en el siguiente apartado se describirán algunas características estructurales y funcionales de ambos pues forman parte medular de nuestro estudio.

2.1- Estructura de los TRs.

Los TR forman parte de la superfamilia de receptores nucleares (NR, *nuclear receptors*) que incluye a los receptores de esteroides, de vitamina D y del ácido retinoico, entre otros. Todos ellos se caracterizan por compartir una organización de dominios funcionales constituida por un dominio amino-terminal (NT); un dominio de unión al ADN, DBD (*DNA binding domain*); una región bisagra (D), y un dominio carboxilo terminal LBD (*ligand binding domain*) (Germain et al., 2006) (Figura 3).

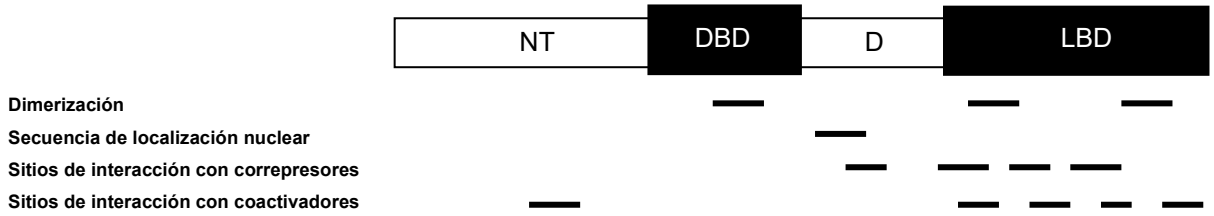

Figura 3. Esquema de la estructura y organización funcional de los NRs. NT, dominio N-terminal; DBD, dominio de unión al DNA; D, dominio bisagra; LBD, dominio de unión al ligando. Se indican las regiones importantes en la función-NR.

El dominio NT, es el dominio menos conservado entre los NR (Germain et al., 2006), es una región altamente desordenada estructuralmente (Chandra et al., 2008), y es susceptible a diversas modificaciones post-traduccionales, como la fosforilación y la sumoilación que afectan la actividad y estabilidad del NR (Hu et., al 1996). El dominio NT es esencial en la activación dependiente del ligando de los NR (Hollenberg et al., 1995; Mendoza et al., 2013) por ser una superficie de interacción con proteínas correguladoras.

El DBD es una región muy conservada (50% homología entre los NR); contiene dos dedos de zinc formados por dos alfa-hélices coordinadas por átomos de Zn^{2+}, que contienen aminoácidos esenciales para el reconocimiento de secuencias específicas en el DNA, denominadas elementos de respuesta (RE), que se localizan en las regiones promotoras de los genes blanco (Kumar & McEwan, 2012; Dudazy-Gralla et al., 2013). Al extremo C-terminal del DBD se encuentra la región CTE (*C-terminal*

extension), la cual presenta un tamaño variable (de 30 a 200 aminoácidos) dependiendo del NR, y le confiere al receptor una superficie de contacto con el ADN que proporciona estabilidad a la interacción NR-RE. Cambios puntuales en la secuencia de amino ácidos de ésta región disminuyen significativamente la interacción con el ADN.

La región bisagra (D) es otro dominio estructuralmente desordenado que contiene la señal de localización nuclear y la mayor parte de la región CTE. La bisagra es una región susceptible a modificaciones post-traduccionales (Kemper et al., 2009) y participa en la dimerización del receptor.

El LBD está formado por 12 α-hélices organizadas en tres capas antiparalelas que forman un "sándwich", en donde, las hélices 1 a la 3 forman la primera capa, seguidas de las hélices 4, 5, 8 y 9 que corresponden a la parte central y por último las hélices 6, 7 y 10 que forman la tercera capa del "sándwich"; mientras que las hélices 11 y 12, se encuentran en la región externa del LBD. En este dominio, se encuentra la región conocida como bolsa hidrofóbica o *LBP (ligand binding pocket)*, formada por aminoácidos hidrofóbicos que conforman el sitio de unión del ligando y su configuración depende de la conformación tridimensional del LBD, por lo que su estructura es variable entre los NRs.

En la porción carboxilo terminal del LBD se encuentra la región de activación dependiente del ligando o AF-2 (*activation function- 2*), localizado en la hélice 12, la cual, como se detallará más adelante, tiene un papel crucial en la activación transcripcional inducida por NRs (Kumar & McEwan, 2012).

2.2- Isoformas de TRs y su expresión.

La clonación inicial de los TRs en distintos vertebrados mostró diferencias estructurales entre éstos, especialmente en el dominio NT. Posteriormente, al realizar el análisis de las secuencias se determinó que existían múltiples isoformas del TR, las cuales son codificadas por dos genes: el *THRA* (*nr1a1*) y el *THRB* (*nr1a2*), presentes en todos los vertebrados.

La expresión del gen *THRA* genera dos isoformas del receptor mediante el mecanismo de splicing alternativo: TRα1 y TRα2, que difieren en la longitud y composición de aminoácidos en la región carboxilo terminal. Por otro lado, el gen

THRB codifica para las isoformas TRβ1 y TRβ2, mediante el uso de promotor alternativo, ambos receptores β difieren en la longitud y composición de aminoácidos en la región amino terminal. Aunque existen diferencias estructurales entre los receptores, todos ellos pueden unir T3 y asociarse al DNA con una afinidad similar, con excepción de TRα2, el cual actúa como un apo-receptor en etapas tempranas del desarrollo (Figura 4).

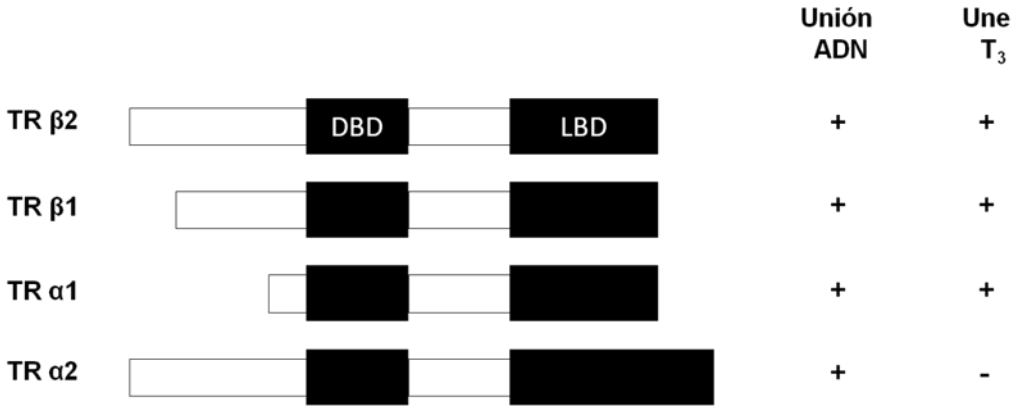

Figura 4. Estructura general y propiedades funcionales de los principales TRs. Modificado de Yen, 2001.

Cabe isoformas señalar que, dependiendo de la especie, los genes *THRA* y *THRB* generan diversas de TR; las cuales varían en su composición, principalmente en el dominio NT y poseen diferentes propiedades de unión al TRE y al ligando (Nelson y Habibi, 2009). Por mencionar algunas, en rata se han encontrado las isoformas TRβ3 y ΔTRβ3, la primera difiere en la región NT con el resto de las isoformas β, mientras que la segunda carece de dominio NT, DBD y de una porción del LBD (Cheng et al., 2010), su relevancia funcional aún queda por dilucidarse. En anfibios se expresan las isoformas TRβ-A y TRβ-B, las cuales varían en su porción NT y capacidad de unión a T3. En ratón, se ha descrito la isoforma TRΔα1, la cual únicamente posee la porción C-terminal del LBD (por lo tanto no se une al DNA).

En cuanto a su expresión, las isoformas de TR poseen un patrón de expresión temporal y tejido-específico: TRα1 se expresa constitutivamente en sistema nervioso durante el desarrollo embrionario y neonatal, mientras que TRα2 se expresa únicamente durante la última etapa del desarrollo embrionario. En etapa adulta, ambos TRα1 y TRα2, tienen una alta expresión en cerebro y una menor expresión en corazón, riñón, hígado, músculo esquelético, tejido adiposo café y pulmón. En

contraste, TRβ1 tiene una expresión muy baja durante el desarrollo embrionario y en etapa adulta se expresa primordialmente en riñón, hígado, cerebro, tiroides y corazón; encontrándose en menor proporción en músculo esquelético, pulmones, y vaso. Mientras que TRβ2 se expresa principalmente en cerebro (hipófisis), retina, oído interno, y en menor proporción en pulmón y corazón (Cheng et al., 2010).

Por otro lado, en teleósteos se han descrito múltiples isoformas de TR que varían primordialmente en su porción carboxilo terminal. Entre ellas, dos isoformas del gen *thrb* denominadas: TRβ1 largo (L-TRβ1) y TRβ1 corto (S-TRβ1); el primero posee un inserto de 9 aminoácidos (SAAGVKETK) localizados entre las hélices 2 y 3 del LBD, mientras que el segundo carece de dicho inserto. Ambos receptores son producto de splicing alternativo del gen *thrb* y están presentes en diversos teleósteos (Orozco et al., 2014). Es importante mencionar que la presencia de L-TRβ1 solo ha sido descrita en peces, lo que sugiere un papel relevante en estos organismos. Las isoformas L-TRβ1 y S-TRβ1 se expresan de forma diferencial en hígado de tilapia, siendo mayor la expresión de L-TRβ1 *vs* S-TRβ1 (Mendoza et al., 2013; Navarrete et al., 2014). La presencia de cada isoforma de TRα y TRβ varía dependiendo del tejido y la etapa de desarrollo del organismo (Nelson & Habibi, 2006).

Además, en los últimos años se han descrito distintos roles para los receptores α y β, a pesar de que tienen características bioquímicas similares (Figura 4). Por ejemplo, TRα1 y TRβ1, pueden modular la magnitud de la respuesta transcripcional del mismo gen o incluso pueden regular la expresión de un conjunto específico de genes (Chan & Privalsky, 2009). Entre isotipos de TR, se han descrito diferencias funcionales, por ejemplo, se ha observado que TRβ1 y TRβ2 pueden reclutar diferentes proteínas coreguladoras (Hamh et al., 2014); lo que sugiere que la diversidad estructural entre receptores está relacionada a su papel funcional.

2.3- Estructura de los correguladores.

Los correguladores son un grupo de proteínas que participan en la regulación transcripcional a través de su asociación con los NR. Hasta la fecha han sido identificados más de 450 correguladores (http://www.nursa.org) con características estructurales y funcionales específicas. Cada uno de ellos, puede participar en la activación o represión de la transcripción; es por ello que se han clasificado en dos grandes grupos: coactivadores (CoA) y correpresores (CoR) (Tabla 1).

Tabla 1. Principales correguladores y su actividad enzimática.

	Coregulador	Nombre	Actividad
Coactivadores	*p160 family* SRC-1, 2 y 3	*steroid receptor coactivator*	Acetiltransferasa
	p300/ CBP	*CREB binding protein*	
	pCAF	*p300/ CBP associated factor*	
	CARM1	*coactivator-associated arginine methyltransferase 1*	Metiltransferasa
	PRMT1	*arginine methyltransferase 1*	
	DRIP/TRAP	*vitamin D receptor interacting protein/ coactivator complex and the thyroid hormone receptor-associated proteins*	Asociación con NR y RNA polimerasa II
Correpresores	N-CoR	*nuclear receptor corepressor*	Asociación con NR y maquinaria de transcripción basal
	SMRT	*silencing mediator for retinoid and thyroid hormone receptors*	
	SUN-CoR	*small ubiquitious nuclear corepressor*	
	mSin3	*seven in absentia 3*	Desacetilasa de histona
	HDAC1, 3 y 4	*histone deacetylase 1, 3 y 4*	
	RPF1	*ring finger domain 1*	Ubiquitinasa
	Ubch7	*ubiquitin-conjugating enzyme*	

Información recopilada de Privalsky et al., 2004; Bulynko & O´Malley, 2011; Fondell, 2013.

La actividad de cada corregulador se debe a la presencia de dominios funcionales, de los cuales a la fecha se han identificado aproximadamente 43 distintos tanto en CoA como CoR (Bulynko & O´Malley, 2011); aun cuando muchos han sido caracterizados, todavía quedan muchos por analizar. En la Tabla 2, se muestran los dominios más estudiados y se ilustran en las Figuras 5 y 6.

Tabla 2. Dominios o motivos estructurales y su función en CoA o CoR.

Dominio/motivo		Características	Función
Siglas	**Nombre (en inglés)**		
RID o NR box	*Receptor interaction domain or nuclear receptor box*	α-hélice Secuencia LXXLL	Permite la interacción con NR
AD	Activation domain	Secuencia rica en leucinas	Superficie de interacción con otros CoA
HAT	*Histone acetitransferase*	Presente en diversos coactivadores primarios y secundarios	Actividad acetilasa de histonas
bHLH-Pas	*Basic helix-loop-helix- Per-ARNT-Sim*	Presente en algunos factores de transcripción	Permite la dimerización con el ADN
RD	*Repression or silencing domain*	Composición variable	Superficie de interacción con otros CoR. Cada CoR puede poseer de 2-3 RDs
ND o CoRNR box	*Nuclear receptor interaction domain*	Secuencia LXXX(I/L)XXXL	Permite la interacción selectiva con NR. Cada CoR puede poseer de 3-4 NDs

Información recopilada de Privalsky et al., 2004; Bulynko & O´Malley, 2011; Fondell, 2013. L=leucina, I=isoleucina, X=cualquier aminoácido.

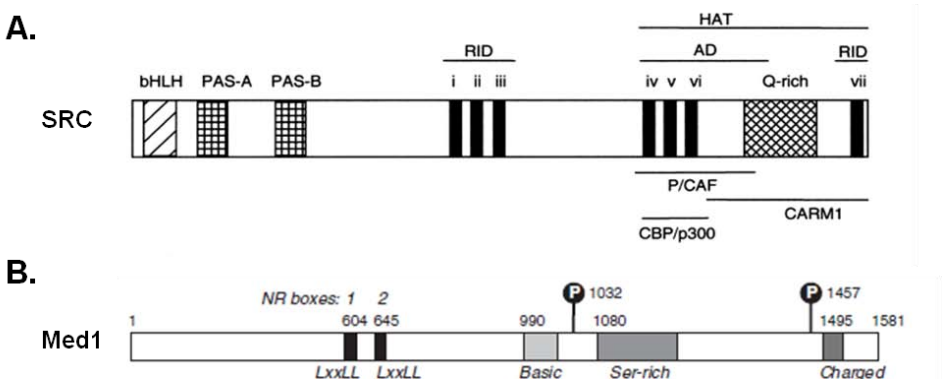

Figura 5. Motivos característicos de CoA. **A.** SRC. **B.** Med1. En ambos casos se muestran los motivos de interacción con receptor nuclear RID, motivo *LXXLL* y dominios de activación AD, así como regiones de interacción con CoA y factores de transcripción; dominio bHLH y PAS; regiones ricas en serinas (Ser-rich y Q-rich) y sitios de fosforilación (P). Modificadas de Fondell, 2013.

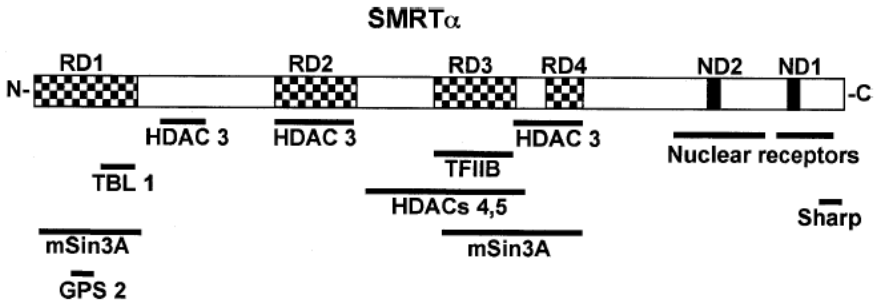

Figura 6. Motivos característicos de CoR. Se muestran algunos dominios de interacción de SMRTα con receptor nuclear (RD), dominios de represión (ND) y regiones de interacción con otros CoR. Tomado de Privalsky, 2004.

Los diferentes motivos o dominios estructurales de los correguladores permiten su interacción con los diferentes elementos del complejo regulador de la transcripción, que incluye a los NRs, a otros correguladores y al DNA. El proceso de regulación de la transcripción depende del ensamblaje correcto de cada uno de los componentes para formar complejos multiprotéicos cuya composición dictará el efecto final sobre la regulación génica. En la siguiente sección se resumen los mecanismos moleculares por los cuales las THs inducen el aumento o la represión de la transcripción a través de TRs y correguladores.

2.4- Función del complejo ligando/TR/corregulador.

La unión del ligando (T3) al LBD del TR es el primer paso en la regulación transcripcional. Como se mencionó anteriormente, el LBD está constituido por 12 alfa-hélices que forman una cavidad hidrofóbica conocida como LBP. En ausencia del ligando, se exponen superficies de interacción a CoR, localizadas en las hélices 3, 5, 6 y 10-11 del LBP (Renaud et al., 1995; Bourget et al., 1995; Heinzel et al., 1997; Marimuthu et al., 2002). Posteriormente, la unión del ligando induce la rotación de la hélice 12 hacia las hélices 3, 4 y 5, formando una superficie denominada “caja CoR” que posee afinidad por el motivo *LXXLL* presente en algunos correguladores (Tabla 2) (Darimont et al., 1998; Feng et al., 1998); de esta forma disociando la interacción de CoR y permitiendo la interacción con CoA; por lo que la posición de la hélice 12 es importante para el intercambio CoA-CoR con el NR y por ello es conocida como región de activación AF-2 (Glass y Rosenfeld, 2000) (Figura 7).

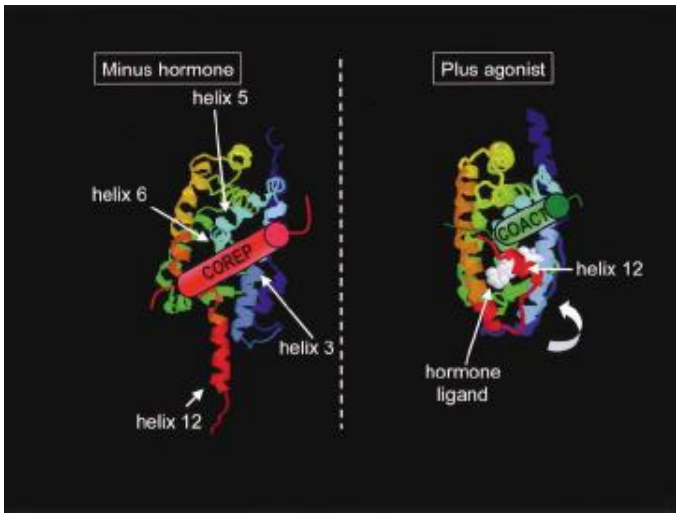

Figura 7. Estructura del dominio LBD. Se indica la ubicación de las hélices 3, 5, 6 y 12 bajo dos condiciones: Izquierda, en ausencia de T3 y en interacción con correpresores (rojo). Derecha, en presencia del ligando y en interacción con coactivadores.

Los CoA que interaccionan directamente con NR son conocidos como CoA primarios. Entre los más estudiados se encuentra la familia p160, formada por tres miembros: SRC-1, SRC-2 y SRC-3, que poseen dominios RID y AD; estos dominios les permiten asociarse a las regiones AF-2 o AF-1 del TR y a su vez reclutar a otros CoAs que inducen modificaciones sobre la cromatina, potenciando significativamente la actividad de los TR. A estos últimos, se les conoce como CoA secundarios, al no interaccionar directamente con NRs.

Entre los primeros complejos de CoAs secundarios reclutados por el TR se encuentran: i) complejo SWI/SNF- dependiente de ATP, el cual posee actividad DNA helicasa dependiente de ATP y promueve el desenrrollamiento del DNA; ii) complejo con actividad acetiltransferasa (HAT) y iii) complejo con actividad metiltransferasa (MET), los cuales realizan acetilaciones o metilaciones específicas de las histonas que inducen el relajamiento del nucleosoma y permiten la interacción de factores de transcripción y de la maquinaria general de la transcripción con el DNA (Jenuwein & Allis, 2001). Entre los principales miembros de estos complejos se encuentran CBP/p300, p/CAF (GCN5), TAFii250, CARM1 y PRMT1, entre otros (Chen et al., 1999) (Figura 8).

Existen otras modificaciones post-traduccionales como la fosforilación, ubiquitinación y sumoilación de histonas que, junto con la acetilación y metilación, representan señales traducidas por la célula para inducir un estado de activación o represión del gen, a este conjunto de modificaciones se les denomina “código de histonas” (Jenuwein & Allis, 2001).

Una vez que se induce la apertura de la cromatina, el complejo DRIP/TRAP o complejo mediador, formado por 30 subunidades aproximadamente (MED1-30) es reclutado por el TR y media la interacción con la RNA polimerasa y subunidades del complejo de pre-inicio de la transcripción como TFIIB, TFIID, TFIIE y TFIIH que dan inicio a la transcripción (Figura 8).

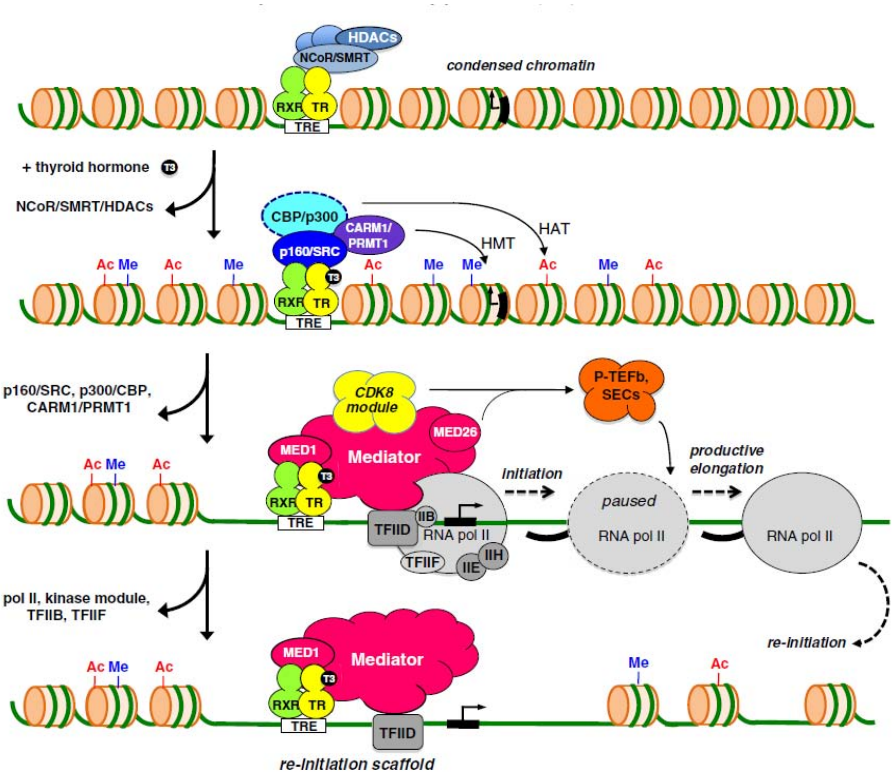

Figura 8. Mecanismos generales de la participación de los correguladores sobre la transcripción TH-dependiente. La ausencia de T3 induce el reclutamiento de complejos represores hacia el complejo RXR-TR-TRE, que van a promover un estado de represión de la cromatina. Posteriormente, la interacción con T3, permite el reclutamiento de complejos de CoAs que modifican el estado de la cromatina y posteriormente el reclutamiento del complejo mediador para la interacción con la maquinaria transcripcional para iniciar el proceso de transcripción. RXR, receptor de retinoides X. Tomado de Fondell, 2013.

Al concluir el proceso de transcripción, la T3 se disocia del TR, se induce un re-arreglo de la estructura del LBD que permite la interacción de correpresores como N-CoR y SMRT que carecen de actividad enzimática *per se*, pero reclutan a otras proteínas CoR a través del dominio RD y forman complejos represores. Dichos complejos pueden estar formados por distintos componentes, algunos de los cuales poseen actividad de desacetilasa de histonas, como las HDAC (3, 4, 5 y 7) y mSin3; algunos otros carecen de actividad enzimática, pero se cree que podrían participar

dando estabilidad al complejo represor, tales como Alien, TBL1 y TBLR1 (Privalsky et al., 2004).

2.5- Mecanismos alternos de regulación génica.

El mecanismo previamente descrito explica la regulación positiva de genes TH-dependientes, en donde la presencia de la hormona induce la transcripción del gen y su ausencia induce la represión del mismo. Sin embargo, existe otro tipo de regulación, en donde la expresión génica aumenta en ausencia de la hormona y disminuye en presencia de la misma, a través del reclutamiento de coactivadores y correpresores, respectivamente; esto resulta interesante pues se opone al mecanismo clásico de regulación positiva, haciendo surgir la siguiente pregunta: ¿un corregulador puede actuar como CoA y CoR?. Al respecto se han descrito algunos ejemplos, como el CoA SRC-1, el cual facilita la regulación negativa del gen de la subunidad beta de la TSH (hormona estimulante de la tiroides) en presencia de T3 (Sadow et al., 2003). Otra proteína que actúa como un CoA/CoR es RIP140, como CoA al interaccionar con el receptor de andrógenos y como CoR al interaccionar con el receptor de glucocorticoides; y aunque no se ha descrito un mecanismo específico para dichos efectos, los autores sugieren que podría deberse a modificaciones post-traduccionales de RIP140 (Subramaniam et al., 1999).

Lo anterior muestra que los mecanismos de regulación de la transcripción por TH son muy variados y por lo tanto la combinación ligando/TR/corregulador dictará el efecto final sobre el gen blanco. En este contexto, desde hace varios años se ha descrito que otras THs poseen actividad biológica relevante; de forma particular, nuestro grupo de trabajo ha estudiado la bioactividad de la T2, producto de desyodación de la T3, la cual actúa como un ligando alterno de TR y activa una vía de señalización específica, distinta a la de T3. A continuación, se resumen los más recientes hallazgos respecto a los efectos biológicos y los mecanismos de acción de la 3,5-T2.

3- Conocimiento actual de la 3,5-T2.

Desde hace varias décadas, los efectos biológicos de la 3,5-T2 (T2) han sido descritos en varias especies de vertebrados (Horst, et al., 1989; Orozco et al., 2014; Goglia et al., 2015) y se han agrupado en dos tipos de acuerdo al mecanismo que media sus efectos en: genómicos y no genómicos.

Los efectos no genómicos se han descrito en relación a la regulación del metabolismo energético en mamíferos, en donde se ha mostrado que la T2, induce un aumento en el metabolismo energético y a diferencia de T3, sus efectos se presentan de forma más rápida y no dependen de transcripción *de novo*. Aun cuando los mecanismos no han sido totalmente dilucidados, se ha mostrado que la T2 estimula directamente la actividad de diversas proteínas que participan en la cadena respiratoria y en el metabolismo de ácidos grasos para aumentar la capacidad oxidativa mitocondrial y el consumo de oxígeno (Goglia et al., 2015; Davis et al., 2015; Moreno et al., 2016), por lo que se ha implicado a la T2 en la inducción de respuestas rápidas ante estímulos como la temperatura o la dieta (Lanni et al., 1998; Lanni et al., 2005; de Lange et al., 2011; Moreno et al., 2011). Debido a lo anterior, actualmente la T2 es empleada como un suplemento alimenticio para reducir grasa corporal de forma rápida en humanos (https://redsupplements.com/t2-best-legal-thyroid-supplement/); desafortunadamente su venta no se encuentra regulada y los efectos secundarios debido a su consumo aún se desconocen.

Estudios recientes han mostrado que la T2 induce efectos tiromimeticos, tales como represión del eje endócrino H-H-T y aumento de la expresión hepática de genes tironino-dependientes involucrados en el metabolismo de lípidos en un modelo de obesidad en rata (Jonas et al., 2011; 2015). La T2 también modifica la expresión de genes involucrados en el metabolismo de xenobioticos, THs y esteroides en el hígado de ratones obesos y ratones no obesos (Lietzow et al., 2016). Asimismo, en modelos *in vitro,* la T2 disminuye la secreción de TSH y la expresión de los receptores TRβ2 (Ball et al., 1997), además estimula la síntesis de los mRNAs que codifican para la hormona de crecimiento (GH) (Moreno et al., 1998) y la desyodasa D1 (Baur et al., 1997), aunque la magnitud de estos efectos es hasta 100 veces menor a los observados con dosis equivalentes de T3. Sin embargo, toda la evidencia experimental sugiere que es necesario realizar más investigaciones para evaluar la seguridad del uso de la T2 como suplemento alimenticio (Hernández, 2015).

Por otro lado, nuestro grupo de trabajo ha empleado el modelo del teleósteo para mostrar que la T2 es una TH bioactiva capaz de regular la expresión de genes tironino-dependientes de la misma forma y con la misma potencia que la T3 (García-G et al., 2007), así como de regular la actividad de las desyodasas D1 y D2 (García-G et al., 2004). También se ha mostrado que la T2 induce la formación de complejos

transcripcionales proteína-TRE distintos a aquellos complejos que se forman en presencia de T3. Lo anterior se observó empleando un modelo de hipotiroidismo y reemplazo en *Fundulus heteroclitus (killifish)*, que consistió en dar un co-tratamiento con metimazol (MMI) -un inhibidor de la síntesis de TH- y dosis de reemplazo de T3 ó T2 (30 nM), durante cinco días. Posteriormente, se evaluó el efecto de la T2 sobre la formación de complejos transcripcionales proteína-TRE, incubando los extractos de proteínas nucleares hepáticas de los peces tratados con oligonucleótidos cuyas secuencias corresponden a los TRE canónicos y resolviendo los complejos transcripcionales mediante ensayos de retardo en la movilidad electroforética (EMSA). Como se muestra en la Figura 9, los extractos de peces eutiroideos (no tratados) formaron dos complejos proteína-DNA con el TRE, mientras que el tratamiento con MMI, un inhibidor de la síntesis de TH prácticamente eliminó estos complejos proteína-TRE. En los animales hipotiroideos (MMI) reemplazados con T3, únicamente se observó el complejo de menor peso molecular, y su intensidad fue similar a la del animal eutiroideo. En contraste, en el caso del tratamiento de MMI + T2 solo se observó el complejo de mayor peso molecular (García-G et al., 2007). La diferencia que se observa en el peso de los complejos formados sugirió que cada TH podría reclutar a un grupo específico de correguladores.

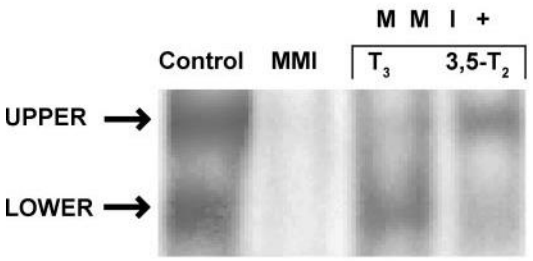

Figura 9. T2 y T3 inducen la formación de complejos transcripcionales distintos. EMSA representativo de extractos nucleares de killifish tratados con MMI y reemplazados con T2 o T3. Los extractos nucleares fueron incubados con el TRE DR4-marcado con P^{32}. Modificado de García-G et al., 2007.

Otros estudios realizados en *Oreochromis niloticus* (tilapia) mostraron que la exposición a dosis de 1 nM de T2 o T3 durante un mes (8 h/día, 3 días/semana), aumenta el crecimiento de los organismos hasta un 30% más que aquel observado en peces no tratados y que éste proceso ocurre a través de la activación de genes TH-dependientes involucrados en el proceso de crecimiento como GH, el factor de crecimiento insulínico tipo 1 (IGF-1) y la desyodasa D2. De forma interesante, se encontró que la T2 activa únicamente la expresión del receptor L-TRβ1, mientras que

T3 activa la expresión del S-TRβ1 (Figura 10) (Navarrete-Ramírez et al., 2014), sugiriendo una regulación diferencial en la expresión de cada receptor por sus respectivos ligandos y que los efectos de cada TH, son inducidos a través de vías de señalización específicas.

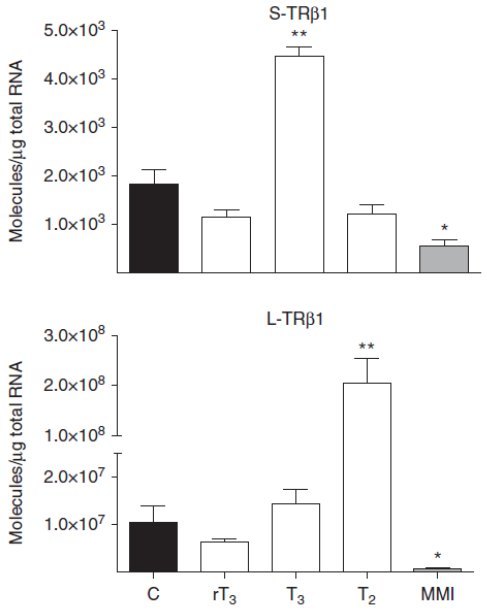

Figura 10. T2 y T3 regulan diferencialmente la expresión hepática de L-TRβ1 y S-TRβ1. Modificado de Navarrete-Ramírez et al., 2014.

El mecanismo de acción que subyace los efectos genómicos de la T2 en teleósteos y en mamíferos ha sido abordado mediante estudios de estructura-función de las isoformas TRβ1 de teleósteo y de humano (hTRβ1), los cuales mostraron que la T2 activa específicamente al L-TRβ1 y hTRβ1, mientras que T3 activa a las tres isoformas: L-TRβ1, S-TRβ1 y hTRβ1. Además, se encontró que la capacidad de activación por la T2, depende de la configuración tridimensional específica adoptada por el TR, que es condicionado por la presencia del inserto de 9 aminoácidos del LBD y del dominio NT, a diferencia de la activación dependiente de T3 (Figura 11) (Mendoza et al., 2013).

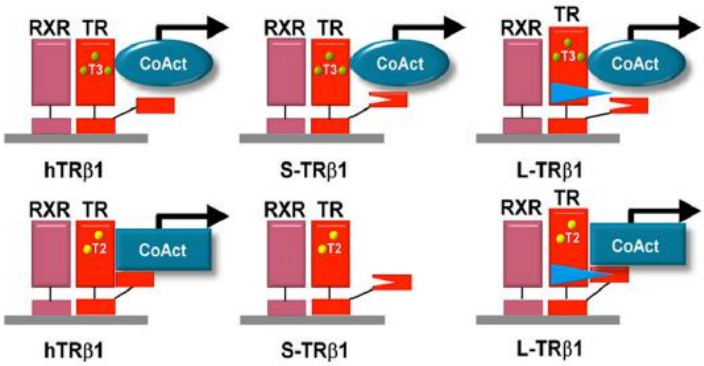

Figura 11. T2 activa a los receptores L-TRβ1 y hTRβ1. La activación por T2 depende de la presencia del inserto de 9 aminoácidos en el LBD (en el caso de L-TRβ1), así como de la configuración tridimensional que adopta el dominio LBD al unirse la T2 y de la interacción con el dominio NT de ambos receptores L-TRβ1 y hTRβ1. Modificado de Mendoza et al., 2013.

Lo anterior muestra que la T2 podría activar vías transcripcionales a través de TRs en teleósteos y en mamíferos. Otros grupos de trabajo han mostrado actividad de T2 en otras especies de peces; por ejemplo, en pez cebra la T2 participa en la regulación del proceso de respuesta termal a través de la modulación de la expresión de genes en el músculo (Little y Seebacher, 2013). En conjunto, es claro que otras THs, además de T3, en particular la T2, participa en la inducción de los efectos biológicos a través de mecanismos específicos ligando/TR/corregulador; por lo que podría tener un rol fisiológico relevante en otros vertebrados, además de los peces.

4- Comunicación cruzada entre las hormonas tiroideas y la vía de señalización del cortisol.

El cortisol es una hormona glucocorticoide que participa en la regulación de múltiples procesos biológicos en vertebrados, como la osmoregulación, la reproducción, el crecimiento y el metabolismo. Su producción es regulada por el eje endócrino hipotálamo-hipófisis-adrenal (H-H-A) y sus efectos biológicos son inducidos a través de los receptores a glucocorticoides (GR) (*nr3c1*). Los GRs pertenecen a la familia de NRs que reconocen secuencias específicas en el ADN denominadas GRE (*glucocorticoid response element)*. Los GREs están compuestos por dos medios sitios con la secuencia consenso 5´-AGAACA-3´- en configuración IR3 (inverso repetido, espaciado por tres nucleótidos).

Al igual que los TRs, los GRs median mecanismos de regulación de la transcripción a través de vías genómicas y no genómicas. El mecanismo genómico involucra la activación de homodímeros de GRs que se encuentran asociados a

complejos de proteínas chaperonas en citoplasma. La unión del cortisol induce el transporte de los GRs hacia el núcleo, en donde los GRs regulan la tasa de transcripción de genes blanco a través de la interacción directa con GREs o de la activación de otros factores de transcripción a través de interacciones proteína-proteína, mecanismo conocido como "*tethering"* (Ratman et al., 2013).

Existen mecanismos de comunicación cruzada entre el cortisol y las THs, en los cuales ambas hormonas pueden regular de forma positiva los efectos de la otra, un claro ejemplo de ello es la metamorfosis en anfibios (Denver, 2009a, 2009b), proceso durante el cual, el cortisol y las THs promueven la morfogénesis en anfibios. Estudios en el modelo de metamorfosis muestran que la presencia de ambas hormonas tiene un efecto sinérgico sobre la expresión de genes tironino-dependientes como el TRβ1, TRα1 y la desyodasa D2 (Bonett et al., 2010). Además, T3 puede regular la expresión del GR de forma tejido-específica, aumentando o reprimiendo su expresión en la cola y cerebro de renacuajo, respectivamente (Krain & Denver, 2004). Por lo que los efectos biológicos del cortisol y las THs parecen depender del contexto celular y fisiológico, más estudios en esta área nos permitirán comprender la interacción funcional entre ambos sistemas endocrinos.

JUSTIFICACIÓN

Nuestro grupo de trabajo y otros han mostrado la bioactividad de la T2 en modelos mamífero y en teleósteos, por lo que es claro que otras THs, además de T3, ejercen efectos biológicos en vertebrados y podrían explicar la pleiotropía de las THs. Sin embargo, los mecanismos moleculares que subyacen los efectos de la T2 aún no se han dilucidado, por lo que es necesario realizar estudios que nos ayuden a comprenderlos. La evidencia experimental actual apoya la idea que, en peces, la T2 actúa a través de vías de señalización distintas a aquellas de T3; dado que T2 activa y regula la transcripción de L-TRβ1, mientras que T3 activa y regula la transcripción de S-TRβ1. Asimismo, ensayos *in vitro* han permitido sugerir el reclutamiento de distintas poblaciones de correguladores al TRE, en presencia de T2 o T3. Es bien aceptado que la composición de los complejos de correguladores determina el efecto final sobre la transcripción génica. No obstante, la identidad de las proteínas implicadas en la señalización T2-dependiente se desconoce. Por ello, en el presente trabajo se inició el estudio de la identificación de los correguladores que participan en la vía de señalización de T2, con el fin de generar información sobre las vías de señalización que median los efectos de la T2, y así comprender los efectos genómicos de la T2 y sus implicaciones en la fisiología tiroidea en mamíferos y en peces. En la presente tesis se muestran los hallazgos obtenidos en el estudio del mecanismo de acción de la T2 a nivel de la interacción ligando/TR/corregulador y su posible comunicación cruzada con otras vías de señalización hormonales, específicamente con la del cortisol.

HIPÓTESIS

La T2 se une al L-TRβ1 y recluta a una población específica de correguladores, distinta a aquella reclutada por T3.

OBJETIVOS

Objetivo General

Analizar a las proteínas correguladoras asociadas a los complejos T2 + L-TRβ1 y T3 + S-TRβ1.

Objetivos Particulares

1. Determinar la afinidad de la T2 y la T3 por las isoformas L- TRβ1, S- TRβ1 y hTRβ1, mediante ensayos de unión.

2. Analizar a las proteínas correguladoras asociadas a los TRβ1 largo y corto, mediante ensayos de doble híbrido y ensayos de GST “pull down”.

3. Analizar la comunicación cruzada entre las vías de señalización de la T2 y el cortisol sobre la regulación del gen *thrb*, mediante ensayos de retardo en la movilidad electroforética.

MATERIALES Y MÉTODOS

1- Construcciones.

Las secuencias codificantes de las isoformas de teleósteo L-TRβ1 y S- TRβ1, isoforma de humano, hTRβ1; Jab1 (c-jun binding domain interacting protein 1) y Jab1A (proteína mutante de Jab1) fueron obtenidas previamente en el laboratorio. Cada una fue insertada en distintos vectores de expresión, como se detallará más adelante, para inducir la expresión de las proteínas en los sistemas heterólogos requeridos. Todas las construcciones fueron verificadas mediante secuenciación y se muestran esquemáticamente en la Figura 12.

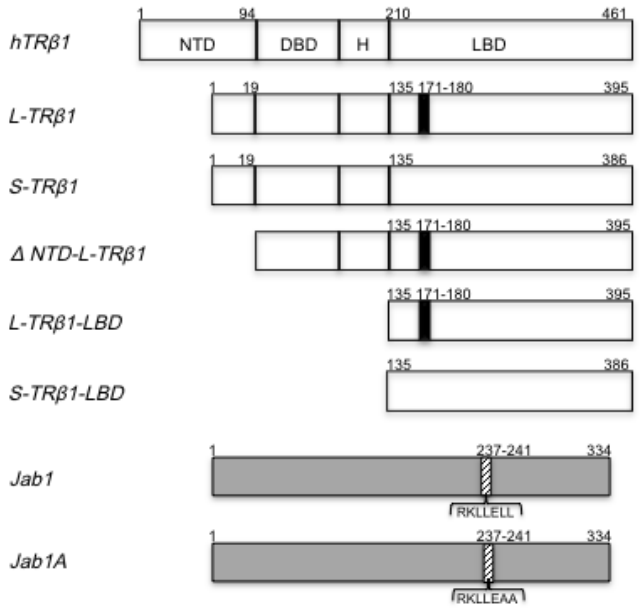

Figura 12. Esquema de las distintas isoformas de TRβ1: hTRβ1, L- TRβ1 y S-TRβ1; isoforma truncada Δ NTD-L-TRβ1 (carece del dominio NT). L-TRβ1-LBD y S-TRβ1-LBD (ambos corresponden a los dominios LBD de su respectivo receptor). Proteínas Jab1 y Jab1A. En todos los casos se indican los dominios estructurales y el número de aminoácidos.

2- Diseño Experimental.

2.1- Ensayos de unión o "binding".

Para determinar la afinidad de T2 y T3 por los receptores L-TRβ1, S-TRβ1 y hTRβ1, se realizaron ensayos de unión de radioligandos, empleando como trazador I^{125}-T3. Los receptores fueron expresados en ovocitos de *Xenopus laevis*: las clonas de TRβ1 fueron insertadas en el vector de expresión pXENEX1, el cual posee un promotor T7 y sitios de reconocimiento de la maquinaria del ovocito que facilitan la expresión de proteínas heterólogas (Jeziorski et al., 1998). Se realizó la transcripción

in vitro empleando el kit T7/T3 In vitro Transcription Kit (Invitrogen), de acuerdo a las instrucciones del fabricante. Se verificó la pureza e integridad del mRNA obtenido mediante espectrofotometría (Nanodrop®) y cada ovocito fue inyectado con 50 nL mRNA (100 ng/ovocito) (n=20 ovocitos por construcción). Tras la inyección, los ovocitos fueron incubados en solución de Barths normal (88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.33 mM $Ca(NO_3)$, 0.41 $CaCl_2$, 0.82 $MgSO_4$, 5 mM HEPES, pH 7.4) a 18°C durante 48 h y posteriormente fueron homogenizados con pistilo. Los homogenados se resuspendieron en buffer de dilución (0.5 M HEPES, pH 7.9) en proporción 1:10 y la concentración de proteínas fue cuantificada mediante el método de Bradford. Los homogenados fueron almacenados a -70°C hasta su uso.

Se realizaron dos tipos de ensayos de unión, de saturación y de competencia, para calcular las constantes de disociación (Kd) y las de inhibición (Ki), respectivamente. Para los ensayos de saturación se utilizaron concentraciones crecientes de I^{125}-T3 (0.01 - 2 nM) y una concentración constante de TRβ1 (20 fmoles) fueron incubados en solución amortiguadora buffer de unión (400 mM NaCl, 20 mM KPO_4 [pH 8], 0.5 mM EDTA, 1.0 mM $MgCl_2$, 10 (v/v) glicerol, 1 mM monotioglicerol y 50 µg de histonas de timo de cabra) a 4°C durante 18 h. Posteriormente, la I^{125}-T3 unida se separó por flujo gravitacional empleando una columna de 2-mL de Sephadex G-25, la radioactividad se cuantificó en un contador γ. El número aproximado de sitios de unión por unidad de volumen de homogenado fue calculado a partir de la actividad específica de la I^{125}-T3 (3825 cuentas/min=1 fmol). Los valores de Kd fueron calculados ajustando las curvas de saturación a las ecuaciones Scatchard (Cunha-Lima et al., 2011).

Los ensayos de competencia se realizaron bajo las mismas condiciones, excepto que se utilizaron 0.2 nM I^{125}-T3 y concentraciones de competidor no marcado radioactivamente en un rango de concentración de 0.01 a 500 nM de T2 o T3. Los valores de las constantes de inhibición, Ki; de T3 y T2 fueron calculados mediante análisis de regresión no lineal usando el modelo de one-site competition (Graphpad Prism 4.0) (Cunha-Lima et al., 2011).

2.2- Construcción de la biblioteca y ensayos de doble híbrido.

Para identificar a las proteínas correguladoras asociadas al complejo T2+TRβ1 se realizaron ensayos de doble híbrido en levadura (Y2H), tamizando una biblioteca de

cDNA de hígado de tilapia como fuente de proteínas reconocibles por el L-TRβ1. La biblioteca de cDNA se construyó a partir de mRNA de hígado de tilapia, empleando el kit MATCHMAKER Library Construction and Yeast two-hybrid assay (Clontech). La biblioteca fue co-transformada con el vector de expresión de pGADT7-Rec (Clontech) en la cepa haploide de levadura Mat-a (Y187), mientras que el vector de expresión L-TRβ1-pGBKT7 fue transformado en la cepa haploid Mat-α (Y2H Gold).

El Y2H se realizó cruzando las dos cepas haploides de levadura, de acuerdo a las instrucciones del fabricante (Clontech) y las células diploides que expresaban a las proteínas de interacción fueron identificadas mediante marcadores de selección nutricionales usando cuatro reporteros (ADE2, TRP, LEU y MEL1), en presencia de 10 μM T2. Después de la selección, las clonas positivas fueron rescatadas en *Escherichia coli* y secuenciadas para identificar su identidad usando el programa Basic Local Alignment Search Tool (BLAST) (National Center for Biotechnology Information; NCBI). La interacción de L-TRβ1 con la proteína Jab1 fue confirmada por Y2H; brevemente, el vector Jab1-pGADT7-Rec fue transformado en la cepa de levadura Y187, y el vector L-TRβ1-pGBKT7 fue transformado en la cepa Y2H Gold. Ambas cepas fueron sembradas en medio selectivo, en ausencia o presencia de 10 μM T2 y la interacción fue confirmada mediante activación de los cuatro genes reporteros.

2.3- Ensayos de GST (glutatión S-transferasa) "pull-down".

Para evaluar la interacción entre la proteína Jab1 y los TRβ1 de teleósteo se realizaron ensayos de GST "pull-down", que permiten evaluar la interacción proteína-proteína en un sistema *in vitro*. En el presente estudio, los ensayos se realizaron con los receptores completos: L-TRβ1 y S-TRβ1, o con las porciones LBD de dichos receptores: L-TRβ1-LBD y S-TRβ1-LBD, con el fin de evaluar la importancia de los dominios estructurales del TRβ1 en su interacción con correguladores. Las proteínas Jab1 y Jab1A (ver Figura 12) se emplearon para evaluar su interacción con los TRβ1 de teleósteo y analizar la importancia del posible dominio de interacción con NR '*LXXLL*' presente en Jab1, el cual fue mutado (LXXLL→LXXAA) para inhibir su interacción con NRs (Jab1A).

Las secuencias de TRβ1, Jab1 y Jab1A fueron insertadas en el plásmido pGEX4-T (Amersham Pharmacia) para expresarlas como proteínas de fusión a GST. Las proteínas fueron expresadas en *E. coli* cepa BL21 inducidas con 0.4 mM IPTG a

37 ºC por 3.5 h e incubadas con perlas de glutatión agarosa (Sigma) para purificar las proteínas de fusión a GST. Las proteínas Jab1 y Jab1A fueron digeridas posteriormente con 0.1 U/µg trombina (Amersham Pharmacia) para remover la porción GST. La presencia de la proteína digerida fue evaluada por tinción con azul de Coomasie.

Para los ensayos de GST "pull-down" las proteínas Jab1 o Jab1A fueron incubadas con las proteínas de fusión GST, GST-L-TRβ1, GST-S-TRβ1, GST-L-TRβ1-LBD y GST-S-TRβ1-LBD unidas a columnas de glutatión agarosa en buffer de unión Ppi [*Protein-protein interaction*] (20 mM HEPES [pH 8], 0.01 mM NaCl, 1 mM EDTA, 4 mM $MgCl_2$, 0.01 (v/v) NP-40, 10 (v/v) glicerol, 1 mM DTT, 0.1 (v/v) PMSF saturado y 10 mg/mL BSA) e incubados por 4 h a 4 ºC en ausencia o presencia de 100 nM T2 o T3.

Después de la incubación, las columnas fueron lavadas en tres ocasiones con buffer de lavado (50 mM Tris [pH 8.0], 200 mM NaCl, 1 (v/v) Triton X-100, PMSF solución saturada). Las muestras de glutatión-agarosa fueron resuspendidas en un volumen mínimo de buffer de unión Ppi, se agregó buffer Laemli 4X (Biorad) en proporción 1:1 y las muestras fueron incubadas a 95°C por cinco minutos. Las proteínas Jab1 o Jab1A unidas a la agarosa fueron analizadas por "inmunoblot" usando un anticuerpo primario monoclonal anti-Jab1 de ratón (Abcam, Inc., ab124720) y revelado con un anticuerpo secundario anti-IgG de ratón acoplado a peroxidasa de rábano (HRP) (Genetex, GTX213111-01).

2.4- Extracción de Proteínas Nucleares.

Para estudiar los mecanismos de regulación transcripcional de T2, se realizaron aproximaciones experimentales *in vitro* que requerían de poblaciones de proteínas nucleares, éstas fueron extraídas de hígado de tilapia de acuerdo al siguiente protocolo: hígados de juveniles de tilapia fueron resuspendidos (proporción 1:5 (p/v)) en buffer de sacarosa (320 mM sacarosa, 25 mM KCl, 5 mM $MgCl_2$, 5 mM EDTA, 50 mM Tris-HCl [pH 7.5], 2 mM PMSF) y homogenizados, seguido de una centrifugación a 12,000 g por 1 min. El sedimento se resuspendió en buffer hipotónico (10 mM HEPES [pH 7.9], 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT) y después de disrupción mecánica con ayuda de pistilo, los núcleos fueron recolectados por centrifugación a 12,000 g por 1 min; y la calidad de los núcleos fue evaluada con tinción azul tripano.

El sedimento se resuspendió en buffer hipertónico (20 mM HEPES [pH 7.9], 0.4 M NaCl, 1.5 mM $MgCl_2$, 25 (v/v) glicerol, 0.2 mM EDTA, 1 mM DTT, 0.5 mM PMSF) e incubó en agitación a 4°C por 30 min. Después de una centrifugación a 12,000 g por 20 min, los sobrenadantes fueron recolectados y diluidos en un buffer de dilución en una proporción 1:1 (20 mM HEPES [pH 7.9], 50 mM KCl, 25 (v/v) glicerol, 0.2 mM EDTA, 1 mM DTT, 0.5 mM PMSF) y la concentración de proteínas fue determinada mediante el método de Bradford. Es importante realizar el procedimiento de extracción a 4°C para mantener la integridad de las proteínas. Los extractos de proteínas nucleares obtenidos fueron almacenados a -70°C hasta su uso.

2.5- Ensayo de retardo en la movilidad electroforética (EMSA).

Para evaluar la posible comunicación cruzada entre T2 y la hormona cortisol, se realizaron una serie de experimentos *in vitro* para caracterizar los efectos de las THs y el cortisol sobre el promotor del gen *thrb* de tilapia, mediante EMSAs. La técnica EMSA permite estudiar la interacción DNA:proteína, en la cual, extractos de proteínas nucleares son incubados con una sonda de DNA y los complejos formados son separados por electroforesis.

En el presente estudio se analizaron los complejos DNA-proteína que se forman con elementos de respuesta TREs y GREs localizados en la región promotora del gen que codifica para el TRβ1, en presencia de T2 o T3 y/o cortisol. De acuerdo al siguiente protocolo: extractos de proteínas nucleares (50 µg) de hígado de tilapia fueron incubados en presencia o ausencia de hormona (100 nM de TH o cortisol) por 2 h en hielo, en un buffer de unión que contenía: 20 mM HEPES, 50 mM KCl, 20 (v/v) glicerol, 0.2 mM EDTA, 0.5 mM PMSF, 1 mM DTT, 1 mg/mL BSA y 1 mg/mL poli dI/dC (Pharmacia). Los oligonucleotidos correspondientes a las regiones del promotor analizadas fueron marcados con [P^{32}] y agregados a la reacción de unión o “binding”, la cual fue incubada por 20 min en hielo y 20 min a temperatura ambiente. La reacción fue cargada en un gel nativo de poliacrilamida 6.5 % y resuelto a 120 V por el curso de 2 h. Los geles fueron secados y los complejos DNA-proteína fueron visualizados por exposición en una pantalla sensible a fósforo (Molecular Dynamics, Sunnyvale, CA). Las pantallas fueron escaneadas en el equipo Storm Phosphorimager y analizadas con el software ImageQuant (Molecular Dynamics). Como controles positivos para verificar la interacción específica de TR con los TREs, se siguieron dos

aproximaciones: EMSAs fueron realizados con i) extractos de proteínas nucleares de hígado de tilapia y 8 µg de anticuerpo anti-TR (abcam ab2743; Bhat et al., 1993), y ii) 200 ng de proteína de fusión GST-L-TRβ1.

2.6- EMSA tipo "super-shift".

Para evaluar la presencia de Jab1 en complejos TR-TRE se empleó la técnica de EMSA tipo "super-shift", que permite determinar la presencia de la proteína de interés en complejos DNA:proteína. En el presente estudio los complejos DNA:proteína analizados fueron los complejos TRE:TR, en los cuales se determinó la presencia de la proteína correguladora Jab1. En esta técnica, extractos de proteínas nucleares son incubados con una sonda de DNA y un anticuerpo contra la proteína de interés, y los complejos formados son separados por electroforesis.

Los ensayos de "super-shift" se realizaron empleando el kit Dig Gel Shift Kit 2nd Generation (Roche). Extractos de proteínas nucleares de hígado de tilapia (10 µg) fueron incubados en hielo por 30 min con 2 µg de anticuerpo contra Jab1 (Abcam, Inc., ab124720) y/o 8 µg de anticuerpo contra TR (Abcam, Inc., ab2743) en buffer de unión (1 mM Tris-HCl pH 8.0, 30 mM KCl, 10 (v/v) glicerol, 20 mM $MgCl_2$, 0.5 µg/µL BSA y 1 µg/µL poli dI-dC). El elemento de respuesta Repetido Directo 4 (DR4, 5´-AGC TTC AGT CAC AGG AGG TCA GAG AG-3´) marcado con digoxigenina (DIG) se agregó a las reacciones de unión o "binding" y estas fueron incubadas por 15 min en hielo seguido de una incubación de 15 min a temperatura ambiente. La reacción fue cargada en un gel nativo de poliacrilamida 6.5 % y resuelto a 120 V por el curso de 2 h. Los complejos DNA:proteína fueron visualizados de acuerdo a las instrucciones del fabricante. Para evaluar la unión especifica e inespecífica del ensayo, un exceso de oligonucleótidos no marcados con las secuencias DR4 o de β-actina de pollo (5´-CTG GGA TGA TAT GGA GAA GAT CTG GCA CC-3´) fueron agregados a la reacción de unión o "binding", respectivamente.

3- Análisis Estadístico.

Los resultados fueron analizados con la prueba de ANOVA acoplada a una prueba post-hoc Tukey (control *vs* tratamientos) usando el software GraphPad Prism 6. Las diferencias fueron consideradas estadísticamente significativas a valores de $P < 0.05$.

RESULTADOS

Los resultados de la presente tesis se muestran en los siguientes artículos científicos:

- Mendoza, A., Navarrete-Ramírez, P., Hernández-Puga, G., Villalobos, P., Holzer, G., Renaud, J.P., Laudet, V., Orozco, A. 2013. 3,5-T_2 is an alternative ligand for the thyroid hormone receptor β1. Endocrinology. 154, 2948-2958.

Adobe Acrobat Document

- Hernández-Puga G, Mendoza A, León-del-Río A, Orozco A. 2017. Jab1 is a T2-dependent coactivator or a T3-dependent corepressor of TRB1 mediated gene regulation. Journal of Endocrinology. 232, 451-459.

Adobe Acrobat Document

- Hernández-Puga, G., Navarrete-Ramírez, P., Mendoza, A., Olvera, A., Villalobos, P., Orozco, A. 2016. 3,5-diiodothyronine-mediated transrepression of the thyroid hormone receptor beta gene in tilapia. Insights on cross-talk between the thyroid hormone and cortisol signaling pathways. Molecular and Cellular Endocrinology. 425, 103-110.

Adobe Acrobat Document

3,5-T_2 Is an Alternative Ligand for the Thyroid Hormone Receptor β1

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Several liganded nuclear receptors have alternative ligands acting in a tissue-specific fashion and playing important biological roles. We present evidence that 3,5-diiodothyronine (T_2), a naturally occurring iodothyronine that results from T_3 outer-ring deiodination, is an alternative ligand for thyroid hormone receptor β1 (TRβ1). In tilapia, 2 TRβ isoforms differing by 9 amino acids in the ligand-binding domain were cloned. Binding and transactivation studies showed that T_2 activates the human and the long tilapia TRβ1 isoform, but not the short one. A chimeric human TRβ1 (hTRβ1) that contained the 9–amino-acid insert showed no response to T_2, suggesting that the conformation of the hTRβ1 naturally allows T_2 binding and that other regions of the receptor are implicated in TR activation by T_2. Indeed, further analysis showed that the N terminus is essential for T_2-mediated transactivation but not for that by T_3 in the long and hTRβ1, suggesting a functional interaction between the N-terminal domain and the insertion in the ligand-binding domain. To establish the functional relevance of T_2-mediated TRβ1 binding and activation, mRNA expression and its regulation by T_2 and T_3 was evaluated for both isoforms. Our data show that long TRβ1expression is 10^6-fold higher than that of the short isoform, and T_3 and T_2 differentially regulate the expression of these 2 TRβ1 isoforms in vivo. Taken together, our results prompted a reevaluation of the role and mechanism of action of thyroid hormone metabolites previously believed to be inactive. More generally, we propose that classical liganded receptors are only partially locked to very specific ligands and that alternative ligands may play a role in the tissue-specific action of receptors. **(*Endocrinology* 154: 2948–2958, 2013)**

Most nuclear receptors (NRs) are ligand-activated transcription factors that provide a direct link between small extracellular ligands and transcription. The first NRs were discovered as receptors of well-known hormones such as 17β-estradiol, corticosteroids, or thyroid hormones (THs), and it was demonstrated that transcription of these receptors was activated by a very specific ligand. This formed a strong conceptual framework to understand the mechanism of action of natural hormones. However, some NRs (eg, pregnane X receptors [PXR] and farnesoid receptors [FXR]) are more promiscuous and are able to bind different ligands with affinities in the micromolar range. This discovery was a first sign that the strong relationship between a specific receptor (eg, TH receptor [TR]) and a specific ligand (eg, T_3) could not be generalized to the entire family of NRs. Recently, evidence has accumulated suggesting that even for the classical endocrine receptors such as the estrogen receptor (ER) or vitamin D receptor (VDR), this relationship may be an oversimplification. The testosterone metabolite 5α-androstane-3β,17β-diol (3βAdiol) competes with 17β-estradiol for binding to ERβ in the prostate, the growth of

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Abbreviations: ER, estrogen receptor; FCS, fetal calf serum; hTRβ1, human TRβ1; LBD, ligand-binding domain; L-TRβ1, long TRβ1 isoform; MMI, methimazole; NR, nuclear receptor; NTD, N-terminal domain; SERCA, sarco(endo)plasmic reticulum Ca^{2+} ATPase; S-TRβ1, short TRβ1 isoform; T_2, 3,5-diiodothyronine; TH, thyroid hormone; TR, TH receptor; TRE, TH response element; VDR, vitamin D receptor.

which it controls (1). Similarly, the VDR is activated by lithocholic acid in the intestine, whereas in other organs, its natural ligand is the bona fide 1α,25-dihydroxyvitamin D_3 (2, 3). Thus, classical NRs can have different ligands acting in a tissue-specific fashion that can play important biological roles.

T_3 has long been considered the main TH that exerts its effects by interacting with the 2 vertebrate paralogs, TRα and TRβ. However, we and others have shown that 3,5-diiodothyronine (3,5-T_2 or T_2), a naturally occurring iodothyronine that results from T_3 outer-ring deiodination (4, 5), is a bioactive molecule (6–8). The initial reports describing T_2 bioactivity showed nongenomic effects upon oxygen consumption different from those induced by T_3 (9, 10). Later, T_2 was also shown to act at the genomic level (6, 7, 11–15), suggesting that T_2 could be, as are lithocholic acid and 3βAdiol for VDR and ERβ, respectively, an alternative ligand for TRβ1 and may play a significant role in vivo.

Previous work from our laboratory showed transcriptional effects of both T_2 and T_3 upon hepatic mRNA levels of *GH*, *D2*, and *TRβ1* in fish (6, 7). Besides its transcriptional effects, we have demonstrated that hepatic nuclear proteins from hypothyroid killifish replaced with T_2 or T_3 form protein complexes of different weights when incubated with the TH response element (TRE), suggesting that T_2 exerts its transcriptional effects by interacting with a different isoform of the TR (7). Furthermore, we and others have shown that some teleosts express 2 isoforms of the TRβ1 that differ only by an insertion of 9 amino acids located at the beginning of the ligand-binding domain (LBD: α-helixes 2 and 3) in the long TRβ1 isoform (L-TRβ1), whereas the short TRβ1 isoform (S-TRβ1) lacks the insert (16–18). We here analyzed whether the genomic effects of T_2 were mediated by its interaction with a specific isoform of the TRβ1 in teleosts. Our data show that T_2 binds to and activates L-TRβ1 and regulates TRβ gene expression in vivo. Taken together, our results prompted a reevaluation of the role and mechanism of action of TH metabolites previously believed to be inactive. More generally, we propose that classical receptors are only partially locked to very specific ligands and that these alternative ligands may play a role in the tissue-specific action of receptors.

Materials and Methods

In vitro experimental design

*Cloning of the full-length isoforms of the long and short TRβ1 from the tilapia (*Oreochromis niloticus*)*

Total RNA from tilapia liver was reverse transcribed (oligodeoxythymidine). Based on available sequences of teleost TRβ1 cDNAs (accession number AF302247.1) (16, 19), degenerate primers that included or not the 27-bp insert were used in touchdown PCRs. The 230- and 257-bp clones obtained had around 94% identity to other teleost TRβ1 genes reported. Specific primers were used in 5′- and-3′ rapid amplification of cDNA ends in a series of nested PCRs. Two different cDNAs that encode 2 isoforms of the TRβ1 were obtained that differ only by 27 bp in their open reading frame: L-TRβ1 (1188 bp) and S-TRβ1 (1161 bp). The entire TRβ1 cDNA sequences were amplified using specific oligonucleotides for the 5′- and 3′-flanking regions and ligated into pcDNA 3.3-TOPO-TA (Invitrogen, Carlsbad, California).

Constructs of chimeric and truncated TRβ1 isoforms

All TRβ1 isoforms used in the present work are depicted in Figure 1. Once generated by PCR, each TRβ1 isoform was subcloned into pcDNA 3.3-TOPO and checked by sequencing.

Transient transfection assays

Two T_3-responsive vectors were used. sarco(endo)plasmic reticulum Ca^{2+} ATPase (SERCA) contains 5 TREs (2 DR4s and one half-site) inserted upstream of the skeletal muscle-specific SERCA1 minimal promoter subcloned into the pGL3-basic firefly luciferase reporter vector (20), and Luc-DR4 contains 2 ideal DR4 TREs inserted upstream from the TK minimal promoter subcloned into the pGL2 (16). Three cell lines were used: GH3 cells (T_3- and T_2-responsive [12, 14] and express low levels of TR [NURSA]), HEK293T (T_3-responsive) (21), and CV1 cells (lack NR expression) (22). All cell lines were maintained in DMEM supplemented with 10% fetal calf serum (FCS), 100 U/mL penicillin, and 100 μg/mL streptomycin and kept at 37°C in a humidified incubator with 5% CO_2. GH3 cells were plated on 12-well plates (4×10^5 cells per well) 24 h before transfections

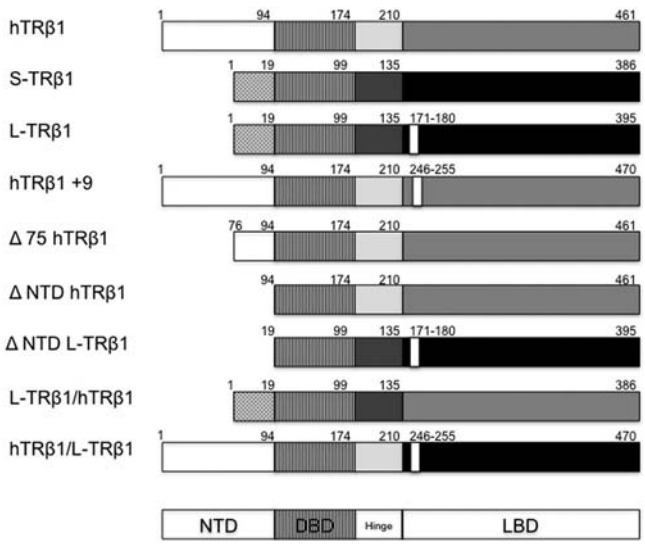

Figure 1. TRβ1 constructs. Schematic representation of the TRβ1 isoforms hTRβ1, S-TRβ1, and L-TRβ1 expressed in tilapia. The 9–amino-acid insert is represented by a rectangle in the LBD. hTRβ1+9 is the hTRβ1 into which the 9 amino acids found in the L-TRB1 were inserted; Δ75 hTRβ1, ΔNTD hTRβ1, and ΔNTD L-TRβ1 are mutated forms of the TRβ1 created to analyze the role of the NTD in transactivation. In the chimeras, the full LBD was exchanged between hTRβ1 and L-TRβ1. Abbreviation: DBD, DNA-binding domain.

using DMEM supplemented with 10% dialyzed FCS, which contained nondetectable levels of T_3 (RIA). Cells were cotransfected (Lipofectamine 2000) with 500 ng of either SERCA or Luc-DR4 reporters and 300 ng of one of the TRβ1 constructs inserted into pcDNA3.3 (Figure 1). Empty pGL3 basic and pcDNA 3.3 vectors were used as controls. Media were replaced 24 hours after transfection with fresh culture medium containing 10% dialyzed FCS plus T_3 or T_2 (100nM) or vehicle (final concentration, 10nM NaOH). Cells were lysed 48 hours later; the lysates were assayed for luciferase activity and protein concentration. All experiments were carried out independently at least 3 times.

T_3 binding assays

The TRβ1 cDNA sequences that encode the 3 native isoforms (long, short, and human) were ligated into the vector pXENEX1 (23). *Xenopus* oocytes were injected with 50 nL (100 ng/oocyte) of the different RNAs and incubated for 2 days. T_3 and T_2 binding affinities (K_d) were determined as previously described (24). Briefly, dilutions of expressed protein were incubated overnight at 4°C with L-3,5,3-[^{125}I]T_3 in 100 μL binding buffer (400mM NaCl, 20mM KPO_4 [pH 8], 0.5mM EDTA, 1.0mM $MgCl_2$, and 10% glycerol) containing 1 mM monothioglycerol and 50 μg of calf thymus histones. Bound [^{125}I]T_3 was isolated by gravity flow through a 2-mL column of Sephadex G-25 to separate bound from free ligand, and bound ligand was quantified using a γ-counter. The approximate number of binding sites per unit volume in the protein preparation was calculated from specific activity of [^{125}I]T_3 (3825 counts/min = 1 fmol). Saturation binding analyses were performed with about 20 fmol of TR protein and varying concentrations of [^{125}I]T_3 (0.05nM–2nM). K_d values were calculated by fitting saturation curves to the Scatchard equations. A concentration range of 0.1nM to 500nM was used to evaluate competition between T_3 and T_2.

In vivo experimental design

Juvenile tilapias of around 2 g were kept in freshwater tanks at a temperature of 28°C on a 12-hour light, 12-hour dark cycle and fed ad libitum with a commercial diet. Treatments (n = 10 fish per experimental group) were administered by immersion as previously described (6, 7). After treatment, fish were killed by decapitation, and the liver was quickly removed and divided into 2 segments for mRNA (quantitative PCR) and intrahepatic T_3 quantification (RIA) (6). All animal experimentation protocols and procedures were reviewed and approved by the Animal Welfare Committee of our Institute.

Two experimental approaches were used to evaluate the effects of T_3 and T_2 upon TH-dependent gene regulation. The effects of TH challenge were analyzed by treating fish groups with 100nM T_3 or T_2 for 24 hours, as previously described (7). To dissect the effects of T_3 and T_2, TH synthesis was blocked (4.5mM methimazole [MMI]) and fish groups were simultaneously cotreated with T_3 or T_2 at a final concentration of 30nM for 5 days (7). This MMI treatment significantly reduces the intrahepatic TH content in tilapia, facilitating measurement of the effects of exogenous T_3 or T_2 upon gene regulation (7). For both experiments, control and experimental groups were handled in the same manner, and at least 2 independent treatments were performed.

Quantitative PCR

Total RNA was extracted from tilapia livers and cDNA was reverse transcribed from 2 μg of total hepatic RNA using an oligo-deoxythymidine primer. Quantitative PCR was carried out in duplicate using tilapia β-actin as an internal standard as previously described (7). For β-actin determination, a 234-bp fragment was cloned (accession number AY116536.1) with oligonucleotides 5′-GTGACATCAAGGAGAAGCT-3′ (sense) and 5′-CGACGTCACACTTCATGAT-3′ (antisense) and used to construct a standard curve. Internal oligonucleotides were used (sense 5′-ACTTCGAGCAGGAGATGG-3′ and antisense 5′-GGTGGTTTCGTGGATTCC-3′) to amplify a product of 170 bp (3 seconds at 95°C, 7 seconds at 52°C, 8 seconds at 72°C for 45 cycles). To clone and quantify L-TRβ1, a single pair of specific primers (sense 5′-GTGAAGGAAGCTAAGCCTGA-3′ and antisense 5′-CACAAGGCAGCTCACAGAAC-3′) was designed. The 232-bp clone obtained was used to construct a standard curve, and experimental mRNA samples were analyzed (3 seconds at 95°C, 10 seconds at 52°C, and 10 seconds at 72°C for 55 cycles). For quantification of S-TRβ1, a 141-bp fragment was cloned (sense 5′-GCGGAAATTCCTGCCTGAG-3′ and antisense 5′-CACAAGGCAGCTCACAGAAC-3′), and a standard curve was constructed. A 135-bp mRNA fragment was measured with internal primers 5′-GCGGAAATTCCTGCCTGAG-3′ (sense) and 5′-GCAGCTCACAGAACATGGGC-3′ (antisense) (2 seconds at 95°C, 8 seconds at 52°C, and 7 seconds at 72°C for 45 cycles). For *D2* mRNA quantification, a pair of specific primers (sense 5′-GAA ACT TGG TCG TGA GGC-3′ and antisense 5′-GCT GGT CTA CAT CGA CGA-3′) was used to clone and quantify a 255-bp fragment (2 seconds at 95°C, 7 seconds at 58°C, and 10 seconds at 72°C for 50 cycles). *GH* mRNA quantification was performed as previously described (7). In all cases, the standard curve ranged from 10^2 to 10^9 molecules/μL. Real-time PCR detection and data analyses were carried out on a Light Cycler instrument (Roche Molecular Biochemicals, Indianapolis, Indiana), as previously described (7).

Statistical analysis

Results were analyzed using ANOVA coupled to a Tukey posttest (control vs treatments) and the software GraphPad Prism version 5. Differences were considered statistically significant at P values $\leq .05$.

Results

Tilapia expresses 2 isoforms of TRβ1

We have cloned 2 cDNA sequences from tilapia liver that encode for 2 distinct TRβ1 isoforms (GenBank accession numbers: KF224971 and KF224972). Both sequences lack 75 amino acids at the N-terminal domain (NTD), as compared with human TRβ1 (hTRβ1). Most importantly, one of them, designated as the L-TRβ, contains a 9–amino-acid insert in the LBD, whereas the other one, named the S-TRβ1, lacks it. The amino acid sequences for S-TRβ1 and L-TRβ1 show 95% and 97% identity, respectively, to other teleostean TRβ1. The L-TRβ1 had been described initially in the Japanese floun-

der (25) and later in other teleosts (16); however, just a partial (296–amino-acid) sequence was available for tilapia, which included only the LBD (GenBank accession number AF302247.1). The genome analysis from several teleosts (Ensemble) shows that the 9–amino-acid insert is encoded in a single 27-bp exon, suggesting that the long isoform is produced by an alternative-splicing event.

T_2 inhibits the binding of T_3 to L-TRβ1 in vitro

As depicted in Figure 2 and Table 1, T_3 binds to all 3 TRβ1 isoforms. In contrast, T_2 binds to L-TRβ1 and hTRβ1, but the binding to S-TRβ1 is very low. IC_{50} values calculated from binding assays are in the same range for T_3 bound to the 3 TRβ1 isoforms as well as for T_2 bound to L-TRβ1. However, these values are higher for T_2 bound to hTRβ1 and even more so when bound to S-TRβ1. These results clearly show that 1) T_2 binds with a high affinity to L-TRβ1, 2) S-TRβ1 binds only to T_3, and 3) hTRβ1 can bind both ligands, but the binding affinity for T_3 is around 40-fold higher than that for T_2.

T_2 stimulates L-TRβ1 and hTRβ1 in GH3 cells

Cotransfection of T_3-responsive GH3 cells with the SERCA reporter gene together with an expression vector

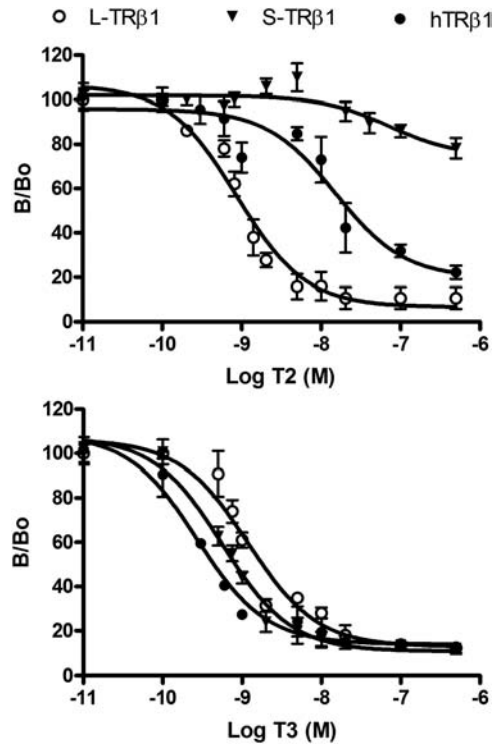

Figure 2. Displacement curves of [^{125}I]T_3 by T_2 (top panel) or T_3 (bottom panel) bound to L-TRβ1, S-TRβ1, and hTRβ1. Translation products were incubated with 1nM of [^{125}I]T_3 alone or in the presence of increasing concentrations of T_3 or T_2 (98 and 99% purity, respectively) as described in Materials and Methods. Data are shown as a percentage of specific binding (B) with respect to binding in the absence of competitor (Bo). Results represent the means ± SEM. The 95% confidence intervals were different for all receptors.

encoding hTRβ1 or tilapia S- or L-TRβ1 transfected in the absence of hormone elicited repressed expression of the SERCA reporter below the basal levels, suggesting that all isoforms recruit corepressors (See Supplement Figure 1 published on The Endocrine Soceity's Journals Online web site at http://endo.endojournals.org.). EC_{50} values from dose-response curves (Table 1) show activation of hTRβ1 and L-TRβ1 by both T_3 and T_2. In contrast, S-TRβ1 showed activation only in the presence of T_3 (Table 1 and Figure 3). Although the response was relatively modest, these results have been replicated in 6 independent experiments, each in triplicate, and they were statistically significant; moreover, the effect of T_2 on SERCA promoter activation was equal to the well-documented effect of T_3. Interestingly, the same effects were observed with the *Fundulus* TRβ1 (data not shown), suggesting that the ability of T_2 to selectively activate L-TRβ1 may be conserved across teleost fish species. It is important to note that GH3 cells express both endogenous and the transfected TR genes, but the expression of endogenous TRs in GH3 is blocked by T_3 and T_2 treatment (12), which allows the effects of the ectopically introduced TRβ1 to be detected above the endogenous background.

We then tested whether the effects were seen in other cell lines or when using a different reporter gene. When SERCA was transfected into CV1 or HEK293T cells, a robust activation by T_3 was observed for all 3 TRβ1s only in the HEK293T cell line and for S-TRβ1 in CV1 cells (Figure 4). This suggests that T_2 activates L-TRβ1 in a cell-specific manner. Similarly, when we used a LUC-DR4-based reporter construct in GH3 cells, no T_2-mediated transactivation was observed; on the contrary, T_2 repressed this reporter when bound to L-TRβ1 in this cell line (Figure 4). Thus, the transactivating effect induced by T_2 in vitro is specific to the reporter gene system employed (SERCA) together with GH3 cells and the long isoform of the tilapia TRβ1.

The 9–amino-acid insert impairs the activation of hTRβ1 in the presence of T_2 and T_3

Interestingly, we noticed that hTRβ1, used as a control, was also activated by T_2 in a promoter- and cell-type–specific manner. This was astonishing because, in terms of sequence, hTRβ1 is equivalent to S-TRβ1 because it does not contain the 9–amino-acid insertion found in L-TRβ1. To further characterize the role of this insert upon the ability to transactivate in the presence of T_2, hTRβ1+9 was constructed. This chimeric receptor showed no response to T_2 (Figure 5), suggesting that the conformation of hTRβ1 naturally allows T_2 binding, whereas the inser-

Table 1. K_d, IC_{50}, and EC_{50} Values for L-TRβ1, S-TRβ1, and hTRβ1 When Ligated to T_3 or T_2

	L-TRβ1		S-TRβ1		hTRβ1	
	T_3	T_2	T_3	T_2	T_3	T_2
Binding assays						
K_d, nM	0.2	0.2	0.2	17	0.2	8
IC_{50}	9×10^{-10}	8×10^{-10}	5×10^{-10}	6×10^{-6}	3×10^{-10}	1×10^{-8}
Transactivation assays						
EC_{50}	4×10^{-9}	2×10^{-13}	3×10^{-10}	1×10^{-5}	9×10^{-12}	4×10^{-7}

tion of the 9–amino-acid sequence disrupts such conformation and impairs T_2 stimulation of the TR. Taken together, our results suggest that both L-TRβ1 and hTRβ1 independently reach a conformation that allows activation by T_2 and that, in the context of the tilapia receptor, the 9–amino-acid insert regulates this conformation.

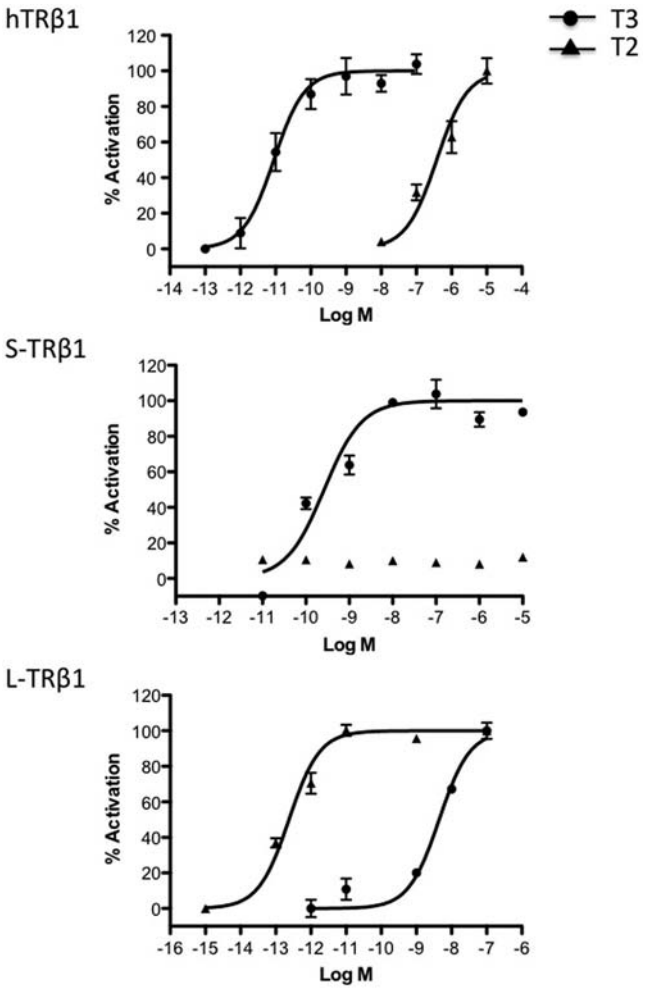

Figure 3. Dose-response curves for T_3 or T_2 activation of hTRβ1, L-TRβ1, and S-TRβ1 isoforms. Transactivation activity was tested using a reporter gene (SERCA) in GH3 cells with increasing concentrations of T_3 or T_2. Results represent the mean ± SEM of at least 3 independent experiments, each in triplicate. X-axis represents the logarithm of T_3 or T_2 molar concentration.

The NTD of L-TRβ1 and hTRβ1 is essential for transactivation in the presence of T_2

The results described above suggest that regions of the receptor, in addition to the 9–amino-acid insert, are implicated in TR activation by T_2. Another important difference between L-TRβ1 and hTRβ1 is their NTD, which contains a transactivation function region (AF-1) that is often cell type- and promoter-specific (26, 27). The hTRβ1 NTD comprises 94 amino acids, whereas that of L-TRβ1 has only 19. Three TRβ1 constructs were tested to explore whether the NTD participates in T_2 activation. As shown in Figure 5, ΔNTD L-TRβ1, which lacks the full NTD, lost T_2 but not T_3 transactivating capacity, suggesting that the NTD is indeed required for the T_2 response. However, Δ75 hTRβ1, which still retains the 19 amino acids that are present in the teleostean NTD and includes the putative AF-1, showed statistically significant T_2 stimulation (Figure 5). Furthermore, when the complete NTD was removed from the hTRβ1 (ΔNTD hTRβ1), T_2-mediated transactivating capacity was lost. Together, these results suggest that the NTD of L-TRβ1 and hTRβ1 is required for T_2-mediated transactivation, but not for that of T_3. This further suggests that a specific interaction may exist between this region and the 9–amino-acid insert present in L-TRβ1 that would account for the specific effect of T_2 on L-TRβ1 but not S-TRβ1. This result indicates that in the presence of T_2, L-TRβ1 and hTRβ1 require the NTD to effectively reach their active conformations, possibly by acting as a stabilizer of the TR-coactivator interaction.

The NTD of L-TRβ1 and hTRβ1 regulates the effect of T_2 in an isoform-specific fashion

To test whether a specific tertiary structure is required to modulate the transactivating capacity of L-TRβ1 and hTRβ1 in the presence of T_2, 2 chimeric TRβ1s were constructed in which the LBDs of the 2 receptors were exchanged. Both chimeric TRβ1s were activated only by T_3 and not by T_2 (Figure 5), reinforcing the notion that the conformation of L-TRβ1 and hTRβ1 plays an important role in allowing activation by T_2, most probably via spe-

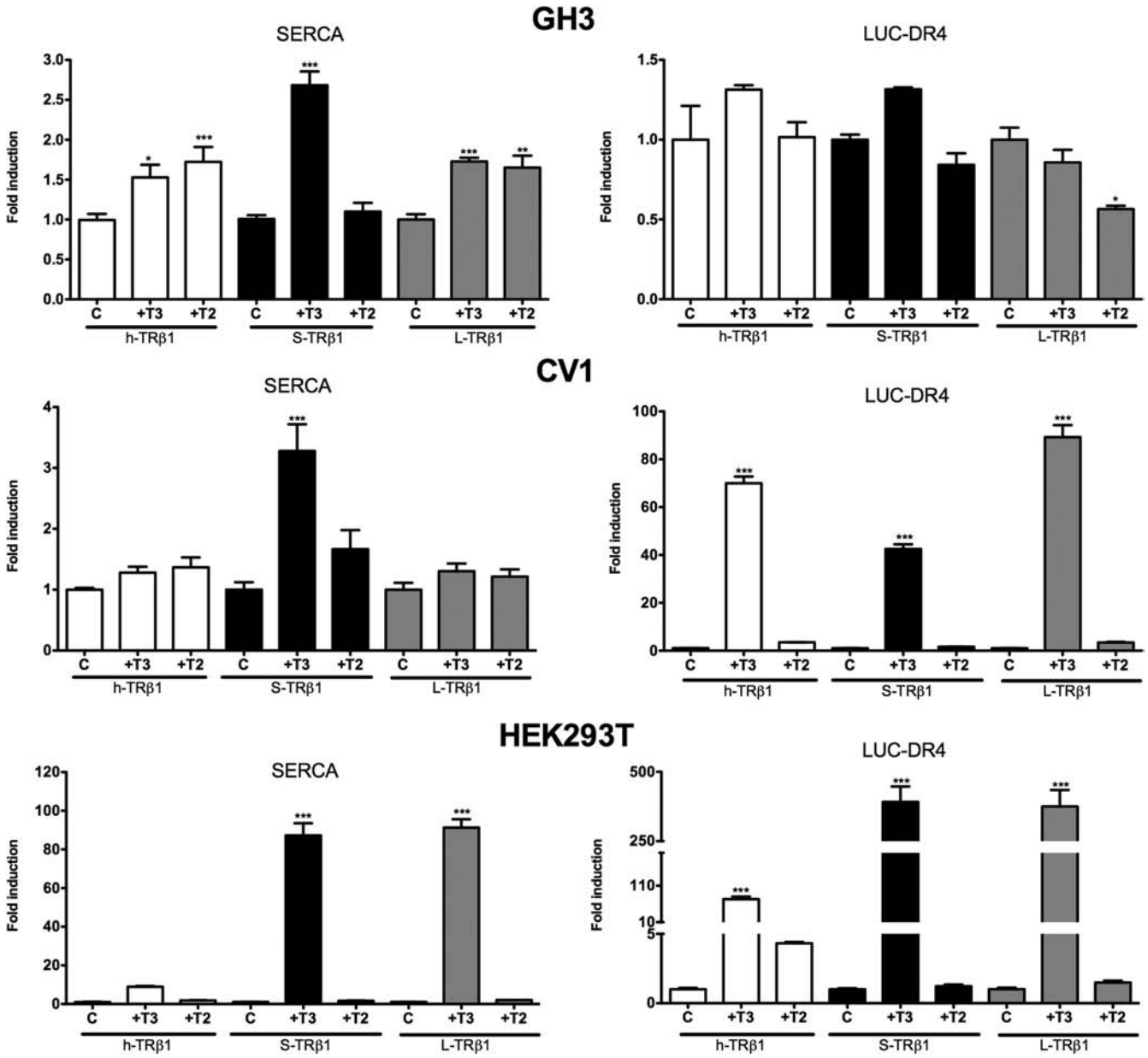

Figure 4. T_2 transcriptional effect is selective for the reporter gene and cellular matrix. GH3, CV1, or HEK293T cells were cotransfected with either a SERCA or a LUC-DR4 reporter gene together with hTRβ1, S-TRβ1, or L-TRβ1 and treated with vehicle (c) or 100nM T_3 or T_2. The luciferase assay was performed 48 hours after transfection. Data are expressed as fold induction versus vehicle at 100% activation. Results represent the mean ± SEM of 2 to 3 independent experiments, each in triplicate. *, $P < .1$; **, $P < .001$; ***, $P < .0001$.

cific interactions between the NTD and the N-terminal part of the LBD in which the 9–amino-acid insert is located. Moreover, these results suggest that T_2 and T_3 induce different conformational changes, explaining their different promoter and cell-type activities. Without discounting putative posttranslational modifications of the TR, these results indicate that the binding of T_2 to L-TRβ1 or hTRβ1 is indeed promoting the activation of the reporter gene.

L-TRβ1 is regulated by T_2 in vivo

As shown in Figure 6 (top panel), the expression of L-TRβ1 is 10^6-fold higher than S-TRβ1. TH challenge significantly increased intrahepatic T_3 levels in those tilapias exposed to T_3 (21.80 ± 4.8 pg/mg tissue) but not in those treated with T_2 (7.46 ± 0.2 pg/mg tissue), as compared with the control group (6.9 ± 0.4 pg/mg tissue). T_3 treatment decreased only S-TRβ1 mRNA expression, whereas in contrast, T_2 treatment exclusively decreased L-TRβ1 mRNA levels. The 2 TH-regulated genes *GH* and *D2* were up- and down-regulated, respectively, by both T_3 and T_2 (Figure 6).

When treated with MMI alone or coadministered with T_2, fish showed significantly decreased intrahepatic T_3 concentrations (1.02 ± 0.3 and 1.30 ± 0.3 pg/mg tissue,

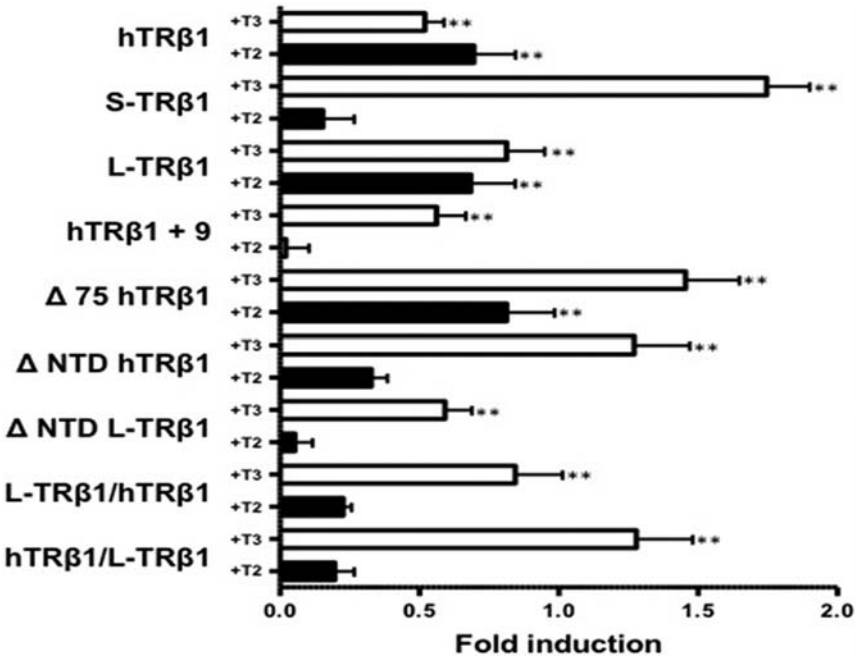

Figure 5. T_2 activates L-TRβ1 and hTRβ1 in a specific, NTD-LBD–dependent manner. A *SERCA* luciferase reporter, a pCDNA3.3 internal control, and the TRβ1 expression vectors indicated were transfected into GH3 cells. The cells were treated with 100nM T_3 or T_2 and lysed; relative luciferase activity was determined, and for each construct, induction was calculated with respect to a transfected control without treatment. Results represent the mean ± SEM of 3 to 6 independent experiments, each in triplicate. Mean and SEM are indicated. **, $P < .001$.

respectively) as compared with controls and with fish cotreated with MMI plus T_3 (7.2 ± 0.3 and 6.34 ± 0.2 pg/mg tissue, respectively). Even though MMI treatment did not completely deplete intrahepatic TH, T_3 levels were lowered enough to induce a response in T_3-dependent genes. Indeed, MMI treatment significantly increased hepatic mRNA expression levels of L-TRβ1, S-TRβ1, and *D2*, whereas it decreased that of *GH* (Figure 6). Interestingly, T_3 replacement restored euthyroid expression of S-TRβ1, and T_2 had a similar effect upon L-TRβ1 expression, whereas both T_3 and T_2 replacement restored that of *GH* and *D2* (Figure 6). Together, results show that T_2 and T_3 specifically regulate expression of L-TRβ1 and S-TRβ1 mRNA, respectively.

Discussion

In the present study, we show that T_2 binds and activates, in a cell- and promoter-specific manner, both the long fish isoform as well as the hTRβ1, whereas T_2 is inactive and has low affinity for the short fish TRβ1 isoform. We explored the mechanistic basis of this selective activation pattern and found that it is dependent upon a specific functional interaction between the NTD and the N-terminal part of the LBD that contains the insertion present in L-TRβ1. In addition, we observed that in vivo, T_2 exerts a specific regulatory effect on the expression of L-TRβ1. All these data suggest that T_2 should be considered as a ligand of TRβ1; thus, it is an active molecule that can exert specific physiological or developmental effects.

T_2 binds to and activates L-TRβ1 and hTRβ1 in vitro

The question of whether T_2 binds to the TR has been unresolved since the early reports analyzing T_2 bioactivity. Here we show for the first time that T_2 is as potent a ligand as T_3 when binding to L-TRβ1. Furthermore, T_2 binds to hTRβ1 with lower affinity than T_3, as previously shown for rat TRβ1 (11). Our present results suggest that T_2 is only a partial agonist of hTRβ1, whereas for fish T_2 is a physiologically relevant T_3 derivative. The facts that T_2 induces higher transactivating activity in L-TRβ1 than T_3 and that it specifically regulates L-TRβ1 expression in vivo (see below) suggest that T_2 is the main ligand for this TR isoform in fish. Thus, T_2 is another natural ligand for TRβ1, thereby increasing the possible pleiotropic effects of THs.

T_2 is a cell- and promoter-specific TRβ1 activator

This is the first report showing that the effects of T_2 are in part mediated by a TR. Here we show that T_2 is a selective activator of L-TRβ1 in tilapia as well as of hTRβ1. In vivo, T_2 up-regulates transcription of its receptor; however, results from the in vitro studies show that T_2 activity is cell- or promoter-specific. The most obvious explanation for these results is that T_2 could interact with the ligand-binding pocket of the receptor and produce a specific conformation that would allow the recruitment of a specific set of coactivators. In accord with this model, we have previously shown that nuclear proteins from fish exposed to T_2 or T_3 form different complexes with the TRE (7), suggesting that T_2 effectively alters the conformation of the receptor. Given the observed cell-type specificity, we can predict that various coactivators will be present in some cell types (such as GH3) but not in others (CV-1 and HEK293). It is likely that the promoter structure adds an additional layer of complexity to this model, perhaps by influencing the specific TR conformation generated by T_2, because the structure of the TRE as well as the TR isoform

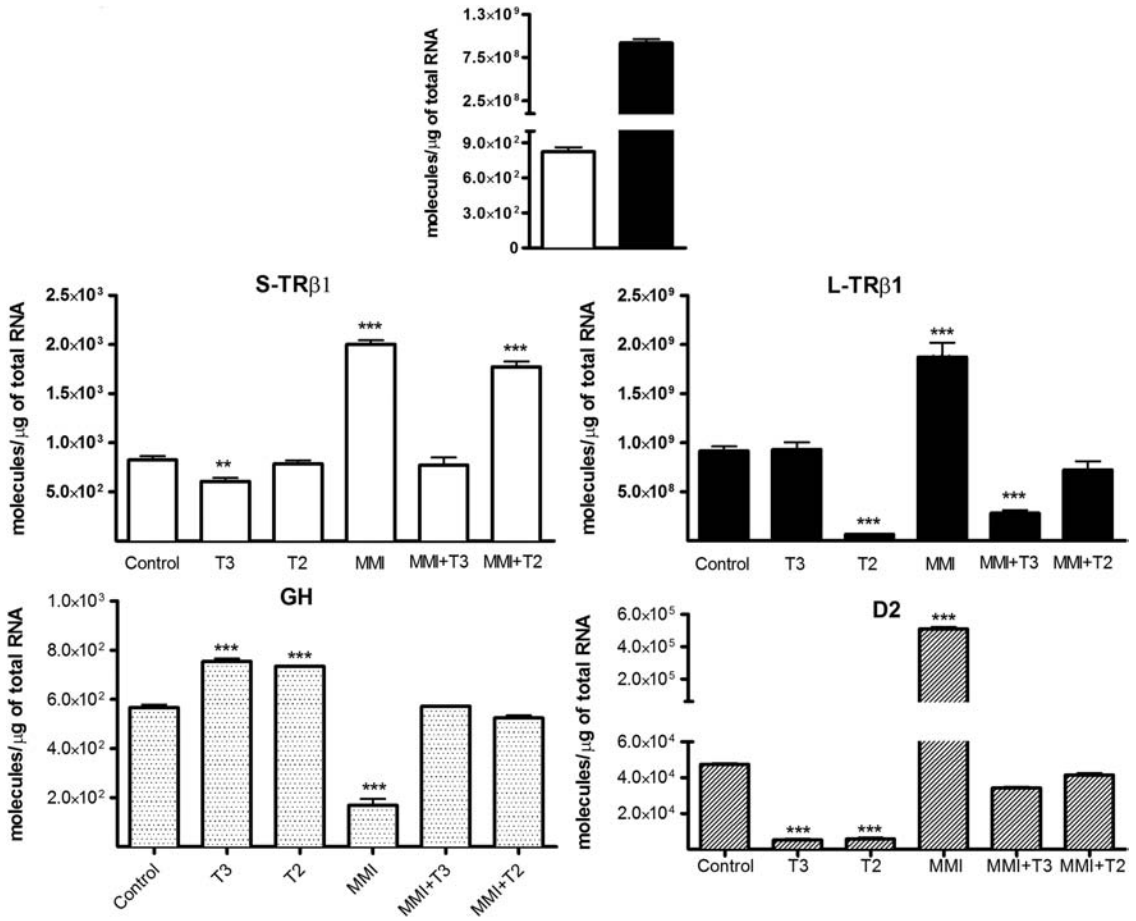

Figure 6. T_2 modulates the expression of L-TRβ1 in tilapia. For TH challenge, tilapias were treated with 100nM T_3 or T_2 for 24 hours. To dissect the effects of T_3 and T_2 upon gene regulation, TH synthesis was blocked by treating fish groups for 5 days with 4.5 mM MMI alone or together with 30nM T_3 or T_2. All treatments were administered by immersion. mRNA levels of S-TRβ1, L-TRβ1, D2, and GH were measured by RT-PCR. Top panel, mRNA levels of L-TRβ1 and S-TRβ1 in the control group; mRNA levels of S-TRβ1, L-TRβ1, D2, and GH in both experimental conditions are shown. n = 10 per experimental group. ** $P < .001$, *** $P < .0001$.

could determine the degree of inhibition of a specific gene, as seen in our present results (T_2 repression of L-TRβ1 in GH3 cells) or as observed in the generalized resistance to TH syndrome (28). Thus, it has to be emphasized that the SERCA promoter on which we see an effect has a complex organization with 2 direct repeats and a monomeric site that might contribute to generating a very distinct conformation of the TRβ1 with bound T_2 (20). It must be recalled that the structure of the response element can interfere with the pharmacological response of other NRs (eg, ERs and glucocorticoid receptors) (29). In the case of the glucocorticoid receptor, this effect has been explained on a structural basis (30, 31). Thus, we propose that 3,5-T_2 should be viewed as a selective TH modulator (STORM) that may play a very specific role in regulating a set of genes in a physiologically or developmentally specific fashion (32). A transcriptomic analysis of the T_2 response would be welcomed to determine the set of genes regulated by this compound.

Mechanistic basis of T_2 selectivity for fish TRβ1 isoforms

In the present study, we show that L-TRβ1 mediates transactivation in the presence of T_2 or T_3, whereas S-TRβ1 does so only in the presence of T_3. Despite the fact that L-TRβ1 and S-TRβ1 differ only by a 9–amino-acid insert in the LBD of L-TRβ1, the functional consequence of this insert seems remarkable because it allows the differential activation of the receptor by other ligands. The ability of L-TRβ1 to activate transcription in the presence of T_2 can be explained through at least 2 possible mechanisms: 1) the 9–amino-acid insert could stabilize the L-TRβ1-T_2 interaction, and/or 2) T_2 bound to L-TRβ1 could induce the recruitment of a set of coactivators different from those recruited by the TR bound to T_3. Present results suggest that the 9–amino-acid insert of L-TRβ1 modifies the conformation of the LBD, allowing a strong interaction with T_2 or T_3 and, as discussed later, promoting transactivation through the L-TRβ1 or hTRβ1.

The fact that T_2 activates both L-TRβ1 and hTRβ1 but not S-TRβ1 is puzzling, because hTRβ1 lacks this insert but is stimulated in the presence of T_2. Our results showed that when the 9 amino acids were inserted into the LBD of hTRβ1, T_2 responsiveness was abolished, raising the question of why the 9–amino-acid insert changes TR ligand preference in tilapia but not in human. A plausible explanation is that although the 9–amino-acid insert is fundamental for T_2-mediated activation in the teleostean TR, it changes the conformation of hTRβ1, preventing activation by T_2. In this context, aside from the 9–amino-acid insert, the LBDs of hTRβ1 and L-TRβ1 differ at 17 amino acids distributed throughout the domain. These amino acids could modify the LBD structure, allowing both the stimulation of hTRβ1 by T_2 or T_3 as well as T_2-mediated activation of hTRβ1 without requiring the 9–amino-acid insert. The analysis of this possibility is beyond the scope of this work; however, the LBD of hTRβ1 and L-TRβ1 is only one of the domains related to the T_2 responsiveness observed in these TRs (see below).

Our results clearly show that the NTD may also be related to the activation of L-TRβ1 and hTRβ1 in the presence of T_2. The NTD, like the LBD, has been established as essential for T_3-dependent TR transactivation, heterodimer formation, and coactivator recruitment, at least in mammals (33, 34). Indeed, no activation is observed in the presence of T_2 when the NTD is deleted from hTRβ1 or L-TRβ1. Most interestingly, significant activity is observed in the presence of T_2 when the NTD of hTRβ1 is truncated to only 19 amino acids, resembling the length of the L-TRβ1 NTD. This domain contains the AF-1, which modulates the activity of the receptor (26). Moreover, the chimera in which the hTRβ1 and L-TRβ1 LBDs were interchanged showed significant activity only in the presence of T_3 but not of T_2 (Figure 5). Together, these results suggest that when bound to T_2, L-TRβ1 and hTRβ1 require their native NTD to adopt the active conformation. Another possibility would be that T_2 binding shapes the LBD in a way that allows the interaction with a different set of coactivators, which in turn would require the AF-1 region to stably interact with L-TRβ1 or hTRβ1 and promote gene expression. Thus, as illustrated in Figure 7, a specific and cooperative interaction between the NTD and LBD on L-TRβ1 and hTRβ1 seems necessary to initiate T_2-mediated transactivation, whereas the binding of T_2 or T_3 could induce the recruitment of 2 different sets of coactivators. This mechanism is reminiscent of the situation found for other NRs such as the androgen receptor (35), but to our knowledge, it has never before been observed for TRs.

The structure of the ligand could also explain the difference of effects between T_2 and T_3. In the [hTRβ1 LBD/

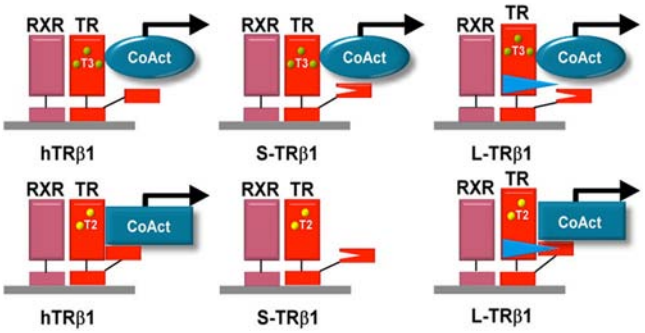

Figure 7. Schematic representation of the putative action mechanism of L-TRβ1, S-TRβ1, and hTRβ1 when ligated to T_3 or T_2. T_3 activates all 3 TRβ1 isoforms without the participation of the NTD, whereas T_2 activates hTRβ1 and L-TRβ1 in an NTD-dependent fashion that includes a putative interaction of this domain with the 9–amino-acid insert of the L-TRβ1. RXR, retinoid X receptor; CoAct, coactivators.

T_3] complex (PDB entry 1XZX), the 3′ iodine atom of T_3 is surrounded by a hydrophobic cluster: Phe269 CE1 (4.1 Å), Phe269 CD1 (4.0 Å), Phe272 CB (4.4 Å), Phe272 CD1 (4.3 Å), and Thr273 CG2 (5.0 Å) from H3; Gly345 CA (4.5 Å) and Leu346 CD2 (4.0 Å) from loop 6-7; Met442 SD (4.1 Å) from H11; and Phe455 CZ (4.9 Å) from H12; it also makes a Van der Waals contact with Gly344 O from loop 6-7 (4.0 Å, slightly too long to be a halogen bond, although the geometry would be correct: C-I●●●O angle 147.7°, I●●●O═C angle = 116.6°) (36) (Supplement Figure 2). Therefore, the 3′ iodine atom of T_3 contributes to the stabilization of the coactivator-binding surface involving H12. The residues of hTRβ1 that contact the 3′ iodine atom of T_3 are conserved in S- and L-TRβ1, in good agreement with the similar level of binding affinity of T_3 for S- and L-TRβ1 compared with hTRβ1 (Table 1). When T_2 is bound to hTRβ1 LBD, the absence of the 3′ iodine atom probably destabilizes H12 slightly, resulting in its suboptimal orientation and thus in suboptimal coactivator recruitment, explaining the partial agonist character of T_2. In the absence of experimental structural data, there is no obvious explanation for the loss of T_2 activity with S-TRβ1 and for its very high activity with L-TRβ1; however, it can be hypothesized that the presence of the 9–amino-acid insert in L-TRβ1 (located in loop 2-3) causes a conformational change in loop 2-3 and/or in the N-terminal part of H3 that could compensate the destabilization of the coactivator-binding surface induced by the absence of the 3′ iodine atom in T_2. A possible explanation is that in hTRβ1, the N-terminal part of H3 (and thus indirectly H12) is stabilized by van der Waals contacts between Ile250 in loop 2-3 and Glu267 and His271 from H3. In S-TRβ1, the residue corresponding to Ile250 is a cysteine, a much smaller residue that could not form similar van der Waals contacts; but in L-TRβ1, the presence of the 9–amino-acid insert could restore stabilizing con-

tacts between H3 and H12 either per se or through an additional stabilizing interaction with the NTD.

T_2 modulates the expression of L-TRβ1 in vivo

We had previously reported that TRβ1 was up-regulated in the absence of T_3 or T_2 and down-regulated with an excess of ligand (7). However, that study did not distinguish between the two TRβ1 isoforms. The novel contribution of the present work relies on the individual evaluation of the expression of L-TRβ1 and S-TRβ1. We found a striking difference in the basal levels of mRNA for the 2 isoforms; L-TRβ1 expression is 1×10^6-fold more than S-TRβ1 in the control group, suggesting that L-TRβ1 modulates most of the TH effects, at least in tilapia liver. Moreover, L-TRβ1 is more sensitive to T_2 (EC_{50}, 2×10^{-13}M) than S-TRβ1 is to T_3 (EC_{50}, 3×10^{-10}M). Interestingly, when the effects of T_2 and T_3 upon L-TRβ1 and S-TRβ1 expression were analyzed separately, we found that T_2 regulates L-TRβ1, whereas T_3 mainly regulates S-TRβ1. However, T_3 also affects the expression of L-TRβ1, an effect that could be due to T_2 possibly generated by outer-ring deiodination of T_3. The differential effects of T_2 and T_3 upon TR isoform expression can be explained by the alternative splicing of TRβ1 pre-mRNA in tilapia by at least 2 different mechanisms: T_2 and T_3 could differentially regulate the expression of the splicing factors in tilapia liver, or they could induce different post-translational modifications on splicing factors, which in turn would modify the alternative splicing of TRβ1 pre-mRNA. Both options remain to be analyzed; however, studies in HepG2 cells show that T_3 regulates the splicing of *THRA*, modifying the TRα1 to TRα2 ratio and increasing TRα2 during hyperthyroidism (37). The fact that T_2 and T_3 modulate the expression of L-TRβ1 and S-TRβ1, respectively, strongly suggests a different signaling pathway for each hormone in tilapia and leads us to propose that there is an extra level in the TH signaling cascade and that 3,5-T_2 is made and regulated specifically for this purpose.

Alternative ligands, a neglected paradigm for NR activation?

Our result should be added to the growing list of endogenous molecules that act as selective NR modulators (SNuRMs) in vivo (38). Such alternative ligands have been demonstrated to elicit relevant effects for several NRs. One of the first examples was lithocholic acid, which was demonstrated to bind the VDR in addition to FXR (3, 39). Another well-known example is 3Adiol, an ERβ ligand in the prostate (1). Interestingly, in both cases, these ligands exhibit a very narrow, tissue-specific action, and the affinity of the receptor for the alternative ligand is relatively low but is consistent with locally higher levels of the alternate than of the classical ligand. There are several other cases of NRs that may be activated by such alternative ligands, including the retinoic acid receptor, which has been shown to bind and be activated by S-4-oxo-9-*cis*-13,14-dihydro-retinoic acid (40) and the binding of dehydroepiandrosterone and its metabolites to steroid receptors (still speculative) (41) or of several nonaromatic steroids to ERs (42). We propose that T_2 should be added to this list and viewed as a tissue-specific endogenous activator of TRs. The existence of these endogenous ligands is perhaps reminiscent of the various steps during which high-affinity NRs evolved from less selective, low-affinity sensors (43, 44).

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Jab1 is a T_2-dependent coactivator or a T_3-dependent corepressor of TRB1-mediated gene regulation

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Abstract

Thyroid hormones (THs) induce pleiotropic effects in vertebrates, mainly through the activation or repression of gene expression. These mechanisms involve thyroid hormone binding to thyroid hormone receptors, an event that is followed by the sequential recruitment of coactivator or corepressor proteins, which in turn modify the rate of transcription. In the present study, we looked for specific coregulators recruited by the long isoform of the teleostean thyroid hormone receptor beta 1 (L-Trb1) when bound to the bioactive TH, 3,5-T_2 (T_2). We found that jun activation domain-binding protein1 (Jab1) interacts with L-Trb1 + T_2 complex. Using both the teleostean and human TRB1 isoforms, we characterized the Jab1–TRB1 by yeast two-hybrid, pull-down and transactivation assays. Our results showed that the TRB1–Jab1 interaction was ligand dependent and involved the single Jab1 nuclear receptor box, as well as the ligand-binding and N-terminal domains of TRB1. We also provide evidence of ligand-dependent, dual coregulatory properties of Jab1. Indeed, when T_2 is bound to L-Trb1 or hTRB1, Jab1 acts as a coactivator of transcription, whereas it has corepressor activity when interacting with the T_3-bound S-Trb1 or hTRB1. These mechanisms could explain some of the pleiotropic actions exerted by THs to regulate diverse biological processes.

Key Words
- thyroid hormone receptor
- 3,5-diiodothyronine
- Jab1
- nuclear receptor
- coregulator

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Introduction

Thyroid hormone (TH) signaling is essential for multiple biological processes including growth, development, reproduction and energy balance. The genomic effects of THs respond to intracellular T_3 levels and are transduced by the thyroid hormone receptors (TRs), which promote the activation or repression of a wide collection of genes (Grøntved *et al.* 2015). Two isoforms for the thrb gene have been identified in teleosts: long or L-Trb1 and short or S-Trb1, which differ by the presence of a nine amino-acid insert located in the ligand-binding domain (LBD) of L-Trb1. Previous work from our laboratory has shown that 3,5-diiodothyronine (T_2) is a specific ligand for L-Trb1, whereas S-Trb1 activates transcription only in the presence of 3,3′,5-triiodothyronine (T_3) (Mendoza *et al.* 2013). In a current consensus model, ligand binding

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to the TR induces conformational changes facilitated by the packing of the C-terminal helix-12 against the LBD into an active position (Brélivet *et al*. 2012, Billas & Moras 2013). This molecular event results in the dismissal of corepressors and the exposure of the coactivator-binding surface, which in turn facilitates the interaction of the primary coactivators (Figueira *et al*. 2011). Primary coactivators interact directly with active TRs through the nuclear receptor (NR) recognition motif (NR box) '*LxxLL*' (Savkur & Burris 2004). This allows the recruitment of secondary coactivators to ultimately form a transcriptional complex that harbors a vast enzymatic tool chest, which modulates chromatin structure and transcription-associated proteins through post-translational modifications (PTM) (Lonard & O'Malley 2007).

Previous findings from our laboratory using *in vivo* teleost models (García-G *et al*. 2007) as well as structure–function studies with teleost and human TRB1 isoforms (Mendoza *et al*. 2013) suggest that the binding of T_2 or T_3 differentially affects TRB1 conformation to promote the recruitment of specific sets of coregulators. To follow-up on this hypothesis, in the present study, we aimed to identify possible ligand-specific recruitment of coregulators to TRB1 isoforms. Here, we identified Jab1 as a TRB1 partner and showed that this protein acts as a coactivator or corepressor of TRB1 and that this Jab1-mediated activation or repression of gene transcription is ligand specific, suggesting that T_2 and T_3 could elicit opposite effects on a specific promoter when bound to the TRB1–Jab1 complex.

Materials and methods

Plasmids

Native and mutated TRB1 clones used in the present work were previously described (Mendoza *et al*. 2013). Full-length tilapia (*Oreochromis niloticus*) Jab1 was cloned as follows: total RNA from tilapia liver was reverse transcribed (oligo dT). Based on the available tilapia sequence (Accession No. XM_003443454.2; RefSeq Genome), the entire tilapia Jab1 ORF (1005 bp, 334 aa) was amplified using specific oligonucleotides for the 5′- and 3′-flanking regions. The sequence obtained presented 95.2% amino acid identity with the human counterpart. A Jab1 chimera (Jab1A) was generated by PCR-based site-directed mutagenesis using internal hybrid primers that introduced a substitution of the last two leucines of the NR box 'RKLLELL' by alanines (RKLLEAA). All constructs were ligated into pcDNA 3.3-TOPO-TA (Invitrogen) for transient transactivation assays; TRB1 clones were inserted into pGEX4-T (Amersham Pharmacia) for pull-down assays; L-Trb1 and L-Trb1-LBD were subcloned into pGBKT7 for yeast two-hybrid assays (see below). All clones are shown schematically in Fig. 1.

Library construction and yeast two-hybrid assay

The MATCHMAKER Library Construction and Yeast two-hybrid assay (Clontech) was used to construct a cDNA library using tilapia liver mRNA. The cDNA library was cotransformed with pGADT7-Rec vector into the haploid Mat-*a* yeast strain (Y187), whereas the L-Trb1-pGBKT7 expression vector was transformed into the haploid Mat-α yeast strain (Y2H Gold). The yeast two-hybrid assay (Y2H) was performed by mating the two transformed haploid yeast strains, according to the manufacturer's protocol. Diploids expressing the interacting proteins were identified by nutritional selection using four reporters (ADE2, TRP, LEU and MEL1) in the presence of 10^{-6} M T_2. After selection, positive clones were rescued in *Escherichia coli* and sequenced to identify their molecular identity by using the Basic Local Alignment Search Tool (BLAST)

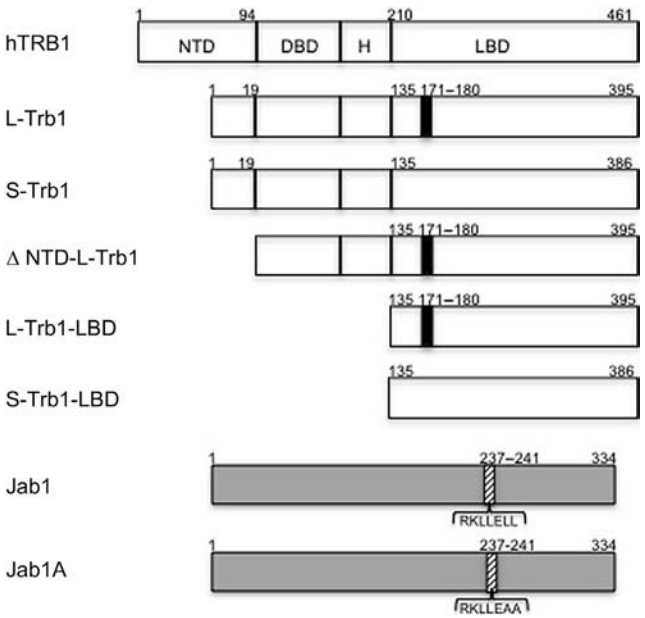

Figure 1
TRB1 and Jab1 constructs. Schematic representation of the TRB1 isoforms (hTRB1, S-Trb1 and L-Trb1); Jab1 and Jab1A constructs. The 9-amino-acid insert is represented by a black rectangle in the LBD. The plasmids ΔNTD L-Trb1 and Jab1A were constructed to analyze the role of the NTD and NR box in the TRB1 + Jab1 interaction.

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program (National Center for Biotechnology Information; NCBI). The L-Trb1+Jab1 interaction was confirmed by yeast mating. Briefly, the Jab1-pGADT7-Rec vector was transformed into the Y187 yeast strain, and the L-Trb1-pGBKT7 vector was transformed into the Y2H Gold yeast strain. Both strains were seeded on selective medium, in the absence or presence of 10^{-6} M T_2; interaction was confirmed by reporter activation.

GST pull-down assays

GST fusion proteins were expressed in *BL21 Escherichia coli* induced by 0.4×10^{-3} M IPTG at 37°C for 3.5 h and incubated with glutathione–agarose beads to purify the GST fusion protein. Jab1 and Jab1A proteins were also expressed as GST fusion proteins as described previously, but were further digested with 0.1 U/μg thrombin (Amersham Pharmacia). The presence of the digested protein was evaluated by Coomassie staining (Supplementary Figure 1, see section on supplementary data given at the end of this article). Jab1 or Jab1A proteins were incubated with GST, GST-L-Trb1, GST-S-Trb1, GST-L-Trb1-LBD and GST-S-Trb1-LBD fusion proteins bound to glutathione–agarose columns in binding buffer (2×10^{-2} M Hepes pH 8, 1×10^{-5} M NaCl, 1×10^{-3} M EDTA, 4×10^{-3} M $MgCl_2$, 0.01 (v/v) NP-40, 10 (v/v) glycerol, 1×10^{-3} M DTT, 0.1 (v/v) saturated PMSF and 10 mg/mL BSA) and incubated for 4 h at 4°C with or without 10^{-7} M T_2 or T_3. After incubation, the beads were washed, and the Jab1 or Jab1A agarose-bound proteins were analyzed by immunoblot using a primary monoclonal Jab1 antibody from Abcam (ab124720) and probed with a secondary anti-mouse IgG antibody coupled to horseradish peroxidase (HRP) (Genetex, GTX213111-01).

Transactivation assays

GH3 cells (1×10^4 cells/well) were seeded onto 96-well, white-wall plates and maintained in F12-K media, which was supplemented with 5 (v/v) of dialyzed fetal calf serum during the 24 h prior to transfection. All transfections were performed using Lipofectamine 2000 (Invitrogen), as previously described (Mendoza *et al.* 2013, Hernández-Puga *et al.* 2016). Cells were transfected with 125 ng pGL3-empty plasmid (Promega) as control reporter gene or SERCA-pGL3 plasmid (Promega) as TH reporter gene and 62 ng of one of the following plasmids: L-Trb1, S-Trb1, hTRB1 or delta N-terminal domain of L-Trb1 (ΔNTD-L-Trb1). Jab1 or Jab1A was cotransfected in increasing amounts ranging from 4 to 64 ng. Renilla pRL-CMV (12.5 ng) (Promega) was used as a reference reporter gene. Cells were treated with vehicle (NaOH, 0.05 N) or 10^{-7} M T_3 or T_2 added in the culture media for 24 h, after which a dual-luciferase assay (Promega) was carried out using DLR-ready Varioskan (Thermo Scientific). For every experiment, renilla luciferase activity was used to control the number of cells transfected per well, whereas the empty pGL3 vector was used for subtracting the background activity from the SERCA-pGL3 reporter gene. Then, data were normalized as fold induction using the vehicle-treated cells as control. For clarity, the charts only show the results for TH-treated cells to depict the effect of increasing amounts of Jab1. All experiments were carried out independently at least three times.

Western blotting

Western blot analysis of cell lysates was performed to evaluate protein expression levels of L-Trb1, S-Trb1 and hTRB1 in GH3-transfected cells (see above). Total proteins were extracted with the ProteoJET Cytoplasmic and Nuclear Protein Extraction Kit (Fermentas) following the instructions of the manufacturer. Protein concentrations were determined by the Bradford Protein Assay. Subsequently, 50 μg of total protein were separated by 12% SDS-PAGE and transferred onto a nitrocellulose membrane using a standard protocol. The membrane was incubated with a primary monoclonal TR antibody from Abcam (ab2743) and probed with a secondary anti-IgG antibody coupled to HRP (Genetex, GTX213111-01). The membrane was exposed to an Amersham Hyperfilm ECL (GE Health Care Lifesciences), and the images obtained were digitalized. Actin was used as loading control using a polyclonal antibody from Santa Cruz Biotechnology (sc-1616).

Supershift assays

Supershift assays were carried out using the Dig Gel Shift Kit 2nd Generation (Roche). Nuclear proteins from tilapia liver (10 μg) were obtained as previously described (García-G *et al.* 2007) and incubated on ice for 30 min with 2 μg of Jab1 antibody (Abcam, ab124720) and/or 8 μg of TR antibody (Abcam, ab2743) in binding buffer (1×10^{-5} M Tris–HCl pH 8.0, 3×10^{-4} M KCl, 10 (v/v) glycerol, 0.2×10^{-3} M $MgCl_2$, 0.5 μg/μL BSA and 1 μg/μL poly dI-dC). A DIG-labeled Direct Repeat 4 response element (DR4, 5′-AGC TTC AGT CAC AGG AGG TCA GAG AG-3′)

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was added, and the binding reactions were incubated for 15 min on ice followed by a 15-min incubation at room temperature. The reaction was loaded onto a 6.5% native polyacrylamide gel and resolved at 120 V over the course of 2 h. The DNA–protein complexes were visualized by following the instructions of the manufacturer. Excess cold DR4 or chicken β-actin oligonucleotides (5′-CTG GGA TGA TAT GGA GAA GAT CTG GCA CC-3′) were added to the binding reaction to evaluate specific and non-specific binding, respectively.

Quantitative PCR

Total RNA was extracted from tilapia tissues and cDNA was reverse transcribed from 2 µg of total RNA using oligo (dT) primer. Quantitative PCR was carried out in duplicate using tilapia β-actin and ubiquitin-conjugating enzyme E2Z (UBCE) as reference genes. The following oligonucleotides were used: β-actin, 5′-ACT TCG AGC AGG AGA TGG-3′ and 5′-GGT GGT TTC GTG GAT TCC-3′; UBCE, 5′-CTC TCA AAT CAA TGC CAC TTC C-3′ and 5′-CCC TGG TGG AGG TTC CTT GT-3′; Jab1, 5′-GAT CCC ACT CGG ACT ATT TCT G-3′ and 5′-GGA GTT CCA GGA GCT TTC TAT C-3′; L-Trb1, 5′-GTG AAG GAA GCT AAG CCT GA-3′ and 5′-CAC AAG GCA GCT CAC AGA AC-3′. All products were amplified at 10′ at 95°C, 10″ at 95°C, 10″ at 61°C and 10″ at 62°C for 40 cycles and were cloned into pGEM-T vector (Promega). A plasmid standard curve that ranged from 10^2 to 10^9 molecules/µL was constructed. In all cases, reactions contained 1 µL of the reverse transcribed reaction, 6 µL Maxima SYBR Green/ROX qPCR Master Mix (Fermentas, Waltham, MA, USA), and 500 nM forward and reverse oligonucleotides in a final volume of 12 µL. A Step One instrument was used for detection and data analysis according to the manufacturer's instructions (Applied Biosystems). The absolute mRNA concentration was expressed as molecules per microgram of total mRNA used in the RT reaction (2 µg) and obtained by interpolation with the standard curve and normalized to the concentration of β-actin or UBCE in each experimental sample. The data obtained with both reference genes were similar (Fig. 5).

Statistical analysis

Results were analyzed using ANOVA coupled to a Tukey *post hoc* test (control vs treatments) with the software GraphPad Prism 6. Differences were considered statistically significant at P values ≤0.05.

Results

Jab1 interacts with teleost L- and S-Trb1 isoforms

The screening of the tilapia liver cDNA library using L-Trb1 as bait in the presence of T_2 identified Jab1 as an interacting partner. This interaction was confirmed by yeast mating, where an activation of the reporter genes is observed in the absence of T_2, but enhanced when this hormone is present (Supplementary Figure 2). To characterize TRB1–Jab1 interaction, pull-down assays were performed using Jab1 and two full-length TRB1 isoforms, L-Trb1 and S-Trb1, in the presence or absence of T_2 or T_3. The results showed L-Trb1–Jab1 interaction in the absence of THs; however, the presence of T_2 or T_3 significantly enhanced the interaction between Jab1 and L-Trb1 (Fig. 2A).

We then analyzed the TRB1 and Jab1 domains that participate in the interaction. Most primary coregulators interact directly with the active coactivator-binding surface located at the NR LBD (Feng *et al*. 1998, Moras & Gronemeyer 1998). To elucidate if Jab1 interacted directly with this TR domain, we performed pull-down

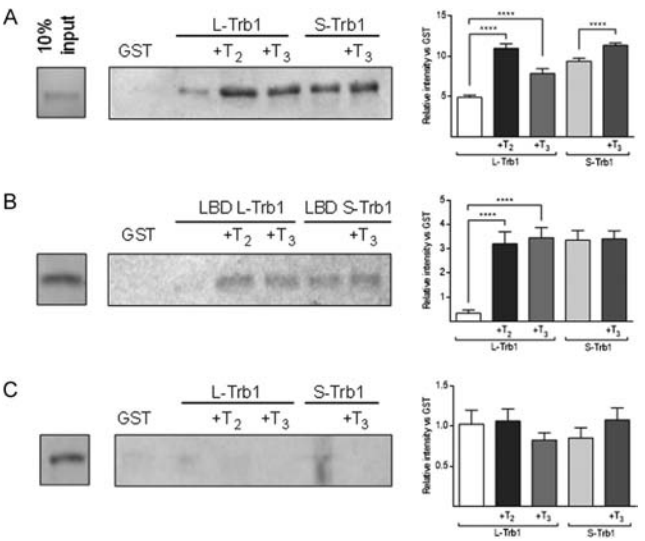

Figure 2
Jab1 + L-Trb1 or S-Trb1 interaction is ligand sensitive and depends on the full-length receptor configuration. GST pull-down assays of Jab1 with (A). recombinant GST or GST-fusion proteins L-Trb1 and S-Trb1 or (B). L-Trb1-LBD and S-Trb1-LBD. (C) GST-pull down assay of Jab1A with GST-fusion proteins L-Trb1 and S-Trb1. Jab1 or Jab1A proteins were incubated with recombinant proteins bound to a glutathione agarose matrix in the presence or absence of 10^{-7} M T_2 or T_3. Bound protein was detected using a monoclonal Jab1 antibody. Representative immunoblots of bound protein and 10% input of total protein used are shown ($n=3$). Densitometric analysis of the bound protein was performed using MYImage Analysis Software (Thermo Scientific). Values shown are the mean density (average intensity/area) ± SE. ****$P<0.0001$.

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assays using Jab1 and the LBD of L-Trb1 (L-Trb1-LBD) in the presence or absence of T_2 or T_3. Jab1 indeed interacted with L-Trb1-LBD (Fig. 2B); however, signal intensity was lower than that observed with the full-length TRB1 (Fig. 2A), suggesting that other TRB1 domains participate in stabilizing Jab1 recruitment.

Coregulators, on the other hand, exhibit different NR-interacting domains; such is the case of the NR box '*LxxLL*', one of the best-characterized interaction motifs present in several primary coactivators and known to directly associate with the coactivator-binding surface (Heery *et al*. 1997). Jab1 sequence analysis revealed the presence of a single '*LxxLL*' motif (Fig. 1), raising the question of whether Jab1 could interact with the TRB1-LBD through this motif. Pull-down assays were performed using a NR box-mutated Jab1 protein (Jab1A) in which the last two leucines of the NR box '*RKLLELL*' were substituted by alanine (*RKLLEAA*), together with full-length L-Trb1 isoform. As shown in Fig. 2C, Jab1A does not interact with L-Trb1, suggesting that the *LxxLL* motif is necessary for the interaction with L-Trb1.

To analyze if Jab1 was a T_2-specific interacting partner, the S-Trb1–Jab1 interaction was also evaluated. We previously reported that S-Trb1 only binds T_3, and experimental evidence suggests that T_3 effects in teleosts are mainly mediated through this short Trb1 isoform (Mendoza *et al*. 2013, Navarrete-Ramírez *et al*. 2014). Pull-down assays performed with Jab1 and S-Trb1 revealed a strong protein–protein interaction in the presence or absence of T_3 (Fig. 2A). Furthermore, pull-down assays showed reduced signal intensities when Jab1 interacted with the LBD of S-Trb1 (S-Trb1-LBD), and no signal was obtained when full-length S-Trb1–Jab1A interaction was tested (Fig. 2B and C), suggesting that the S-Trb1–Jab1 interaction requires binding surfaces similar to those of L-Trb1.

Jab1 is a dual-coregulator of TRB1

To explore the possible coregulatory effect of Jab1 on the transactivation activity of the two isoforms of TRB1, we transiently transfected the thyroid-responsive GH3 cells with increasing concentrations of Jab1 and treated them with a single dose of TH (10^{-7} M/48 h). The results showed that Jab1 enhanced L-Trb1 transactivation in the presence of T_2 in a concentration-dependent manner, revealing Jab1 to be a coactivator; this response seems to be T_2 specific as Jab1 did not affect the expression of the TH-reporter gene in the presence of T_3 (Fig. 3). On the other hand, when Jab1 was cotransfected with S-Trb1, a receptor that binds only to T_3, a surprisingly opposite response was observed: repression of the transactivating activity was evident from the lowest Jab1 concentration used (Fig. 3).

We then explored this apparent coactivator/corepressor capacity of Jab1 by employing the human TRB1 (hTRB1), a receptor activated by both T_2 and T_3 (Mendoza *et al*. 2013). Our results revealed that Jab1 increased hTRB1 transactivation activity in the presence

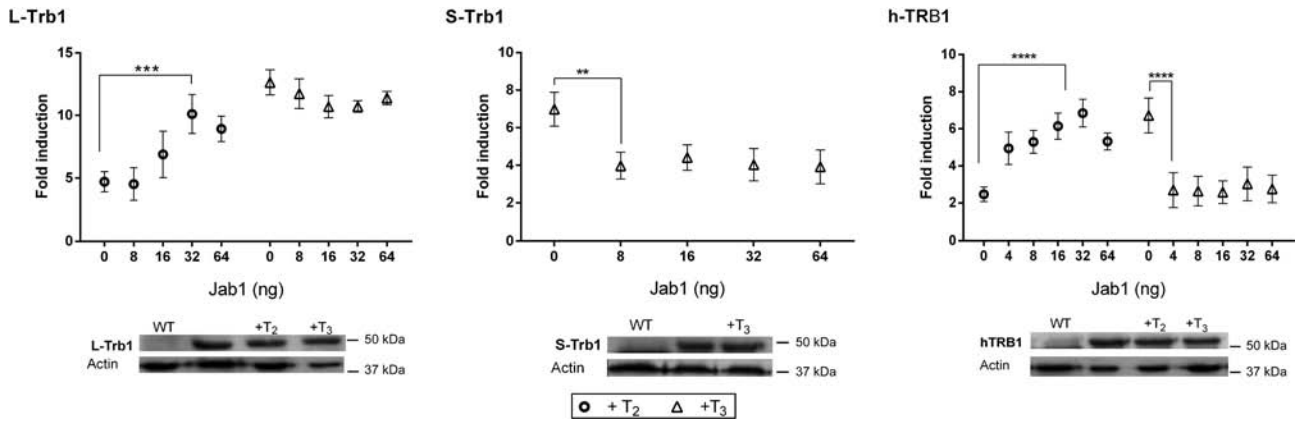

Figure 3
Jab1 is a ligand- and TR isoform-specific coregulator. Transactivation assays were performed in GH3 cells cotransfected with a SERCA-pGL3 luciferase reporter vector and 62 ng of one of the following TRs inserted into pcDNA 3.3 expressing vectors: L-Trb1, S-Trb1 or hTRB1 and treated with 10^{-7} M T_2 or T_3. A Jab1-pcDNA 3.3-expressing vector was cotransfected in increasing amounts (8, 16, 32 or 64 ng). Cells were lysed 24 h after transfection, and a DLR-ready Varioskan was used to perform luciferase assays. Results represent the mean ± S.E.M. of 3 independent experiments, each in triplicate. $^{**}P<0.002$; $^{***}P<0.0006$; $^{****}P<0.0001$. L-, S- or hTRB1 protein levels of transfected GH3 cells were evaluated by Western blotting using a monoclonal TRB1 antibody as described in 'Materials and methods' section. Actin served as loading control. Representative blots of one experimental condition (32 ng of Jab1-pcDNA 3.3) are shown ($n=3$).

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of T_2, whereas it induced a significant suppressing effect upon hTRB1 activity when the cells were stimulated with T_3 (Fig. 3). The L-, S- and h-TRB1 protein levels from transfected GH3 cells were comparable in all experimental situations, as showed by Western blot (Fig. 3).

Jab1 NR box and L-Trb1 N-terminal domain are determinant for a functional TRB1–Jab1 interaction

As pull-down assays showed that the *LxxLL* motif was essential for TRB1–Jab1 interaction, we evaluated the functional relevance of this coregulator NR box. Transactivation assays were performed by co-transfecting Jab1A with L-Trb1, S-Trb1 or hTRB1 in GH3 cells, in the presence of T_2 or T_3. In contrast to our observations with Jab1 (Fig. 3), Jab1A did not modify the transactivating capacity of either the L-Trb1 + T_2 and hTRB1 + T_2 or the S-Trb1 + T_3 and hTRB1 + T_3 complexes (Supplementary Figure 3), demonstrating that a functional TRB1–Jab1 transactivating complex requires the interaction through the Jab1 NR box.

Pull-down assays suggested that the LBD was not the only TR domain involved in TRB1–Jab1 interaction (Fig. 2B); thus, we evaluated the participation of the TRB1 NTD, a domain known to function as a coregulator-binding surface (Tian *et al.* 2006). Transactivation assays were performed using NTD-truncated L-Trb1 (ΔNTD L-Trb1) co-transfected with either Jab1 or Jab1A in the presence of T_2 or T_3. The lack of the NTD practically abolished the transactivating capacity of the T_2-bound L-Trb1, and Jab1 did not induce the previously observed upregulatory effect on the transactivating capacity of this TRB1 isoform (Supplementary Figure 4); neither Jab1 nor Jab1A had an effect upon its transactivating capacity in the presence of T_3. Together, these results suggest that both the NR box and NTD are necessary for the T_2-liganded L-Trb1 to adopt the holoreceptor conformation. As NTD is identical in L- and S-Trb1, it could be predicted that in the absence of NTD, Jab1 repressor effect over S-Trb1 transactivation capacity would be abolished; however, further experiments are required to evaluate this idea.

Jab1 is present in TR-transcriptional complexes

As an initial approach to gain insights on Jab1 relevance in the context of teleost TR signaling pathways, we looked for the presence of Jab1 and TR in transcriptional complexes. Supershift assays were performed using nuclear proteins from tilapia liver incubated with Jab1 and/or TR antibody and a canonical DR4-response element. The results showed that TR and Jab1 are indeed present in the same transcriptional complexes (Fig. 4). TR-complex specificity was probed with specific and non-specific competition assays (Fig. 4 and Supplementary Figure 5).

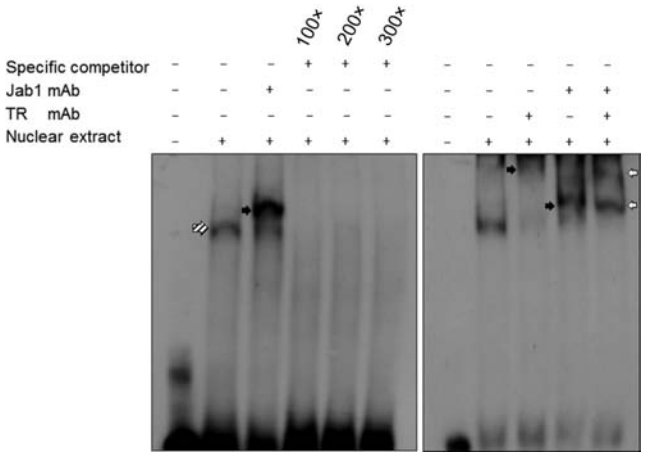

Figure 4
Jab1 is present in tilapia TR-transcriptional complexes. Supershift assays were performed with nuclear extracts of tilapia liver and a DIG-labeled DR4-response element incubated with or without a monoclonal Jab1 and/or TR antibodies, as described in 'Materials and methods' section. EMSA-specific competition assays were performed by adding an excess of non-labeled DR4-response element. Representative supershift images are shown ($n=3$). Black, white and dashed arrows indicate supershift, double supershift and TR-DR4 complexes, respectively.

Jab1 is expressed *in vivo*

Jab1 expression was detected in juvenile tilapia spleen, gill, heart, liver, gut, muscle, skin, kidney and brain. In all

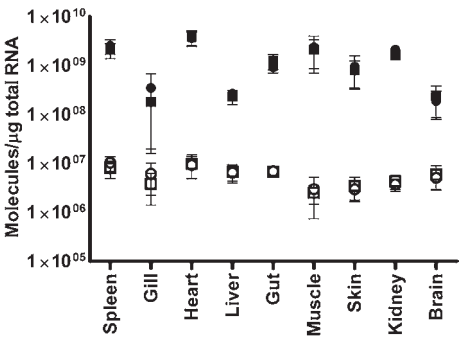

Figure 5
Jab1 and L-Trb1 are expressed in tilapia tissues. Jab1 and L-Trb1 mRNA levels were measured in several tilapia tissues by qPCR as described in 'Materials and methods' section. Graph shows the absolute expression of Jab1 (black symbols) and L-Trb1 (white symbols) normalized with β-actin (squares) or UBCE (circles) ($n=6$ individuals).

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tissues, Jab1 expression surpassed that of Trb1 for at least two orders of magnitude (Fig. 5).

Discussion

In the present study, we show that Jab1 acts as a dual coregulator when interacting with different TRB1 isoforms. Furthermore, this activation or repression of gene transcription is ligand dependent, suggesting that T_2 and T_3 have differential roles in gene modulation, adding further evidence that T_2 is a physiologically relevant ligand.

Our previous results showed that L-Trb1 and hTRB1 are active in the presence of T_2 and suggested that TRB1 could adopt different conformations in the presence of T_2 or T_3, exposing binding surfaces that favor the interaction with specific sets of coactivators (Mendoza *et al.* 2013). To test this last idea, we took advantage of the high affinity of L-Trb1 for both T_3 and T_2 to search for possible TRB1 ligand-specific coregulators. A yeast two-hybrid assay approach revealed Jab1 as a binding partner of L-Trb1+T_2. Jab1 (AKA COPS5) has been primarily described as part of the COP9 signalosome complex (Cope *et al.* 2002) and is known to be a major modulator of E3 ubiquitin ligase involved in protein degradation through the proteosome pathway (Wei *et al.* 2008, Kato & Yoneda-Kato 2009). However, some subunits of the COP9 signalosome have also been shown to have independent activity as monomers; e.g., COPS2 (AKA Alien) has been characterized as a corepressor of TR (Papaioannou *et al.* 2007) and Jab1 was previously identified as a coactivator of several liganded NRs, including TRa1 (Chauchereau *et al.* 2000).

Our study shows that Jab1 exhibits opposite roles upon gene regulation. As we found Jab1 to be bound to L-Trb1 in the presence of T_2 (Supplementary Figure 2), we expected it to be a coactivator of the complex. Indeed, Jab1 enhanced T_2-dependent transactivation when interacting with L-Trb1 and also with hTRB1, and the two isoforms that bind and are activated by T_2. However, Jab1 had no effect upon gene transactivation when interacting with L-Trb1 bound to T_3 (Fig. 3), whereas it repressed S-Trb1 and hTRB1 transactivation activity below T_3-induced levels when bound to this TH (Fig. 3). Thus, in teleosts that express two TRB1 isoforms, one of which is preferentially activated by T_2 (L-Trb1) and the other exclusively by T_3 (S-Trb1), Jab1 exerts a ligand- and isoform-specific function. Interestingly, in the case of hTRB1, which is activated by both T_3 and T_2, Jab1 induced ligand-dependent dual effects. Other studies have shown ligand-specific coregulatory effects; for example, tesmin acts as a coactivator of mineralocorticoid receptor when bound to aldosterone but not to cortisol (Rogerson *et al.* 2014); likewise, repressor of tamoxifen transcriptional activity protein, RTA, acts as a corepressor of estrogen receptor-a when bound to selective hormone response modulators like tamoxifen, but not when bound to pure agonists (Norris *et al.* 2002). As far as we know, a dual coactivator and corepressor effect has only been reported for the receptor-interacting protein 140 (RIP140), which repressed gene activation mediated by glucocorticoid receptor and peroxisome proliferator-activated receptor gamma but enhanced androgen receptor activity. Although the mechanisms were not described, the authors suggested that PTM of RIP140 might be involved in the coregulator duality (Subramaniam *et al.* 1999). Because Jab1 participates in the proteasome pathway, the observed repression of gene activation when interacting with the T_3 bound to S-Trb1 or hTRB1 could be interpreted as TR degradation. However, this possibility is precluded by the results obtained after the overexpression of Jab1 and the different TR isoforms in GH3 cells (Fig. 3). Thus, to our knowledge, this is the first description of a dual coregulator that activates or represses, in a ligand-dependent manner, the activity of isoforms of the same NR. These findings raise the question of how Jab1 induces such opposite transcriptional effects. One possibility is that T_2 and T_3 induce specific conformational changes in the LBD of the TR that would expose particular coactivator-binding surfaces influencing the functional outcome, as seen with other NR bound to different ligands (Moore *et al.* 2004, Nettles & Greene 2005, Jeyakumar *et al.* 2008). This hypothesis is consistent with the findings of Chauchereau and coworkers (Chauchereau *et al.* 2000) who showed that Jab1 can bind to both TR and steroid receptor coactivator-1 (SRC-1), possibly participating as a functional bridge between the TR and other coregulators. In contrast, the repressor effects of Jab1 could be secondary to its interaction with corepressor proteins, such as NCoR, which has been detected as a binding protein in MCF7 cells (Lu *et al.* 2016). However, further studies must be performed to unravel the dual effect mechanism of Jab1.

We then characterized the Jab1–TRB1 interaction by using Jab1A, a protein with a mutated NR box (Fig. 1), in transactivation assays. Interestingly, both Jab1-mediated activation (T_2) and repression (T_3) activities were lost when Jab1A was co-expressed with TRB1 isoforms (Supplementary Figure 3), showing that Jab1 requires its unique NR box to interact as a primary

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coregulator with the receptors. On the other hand, the TR coactivator-binding surface located in the LBD of the receptor is known to provide a primary coactivator-binding surface (Feng *et al*. 1998, Moras & Gronemeyer 1998). This protein domain was not sufficient for TRB1–Jab1 binding as only a weak interaction was observed between Jab1 and the LBDs of both L-Trb1 and S-Trb1 in the presence of T_2 and T_3 (Fig. 2), suggesting that other protein domains are essential for TRB1–Jab1 binding. In support of this idea, our functional assays showed that when a NTD-truncated L-Trb1 was tested, the L-Trb1 + Jab1 complex lost all activity when bound to T_2 (Supplementary Figure 4), confirming the importance of the full conformation of the receptor to provide a functional coactivator-binding surface, as reported previously by others (Hollenberg *et al*. 1995, Tomura *et al*. 1995, Blessing *et al*. 2015). Overall, the present results suggest that ligand binding induces a specific, three-dimensional conformation of the TRB1, as shown for other NR agonists and selective nuclear receptor modulators (SNuRMs) (Margeat *et al*. 2003, Kremoser *et al*. 2007), promoting a cooperative interaction between the NTD and LBD to provide a stable coregulator interaction surface. Thus, ligand-specific TRB1 'shaping' could determine the orientation of the bound primary coregulator (Jab1), which would in turn influence the recruitment of secondary coregulators that could play a central role in modulating gene expression. Whether the ligand- or TR isoform-specific interaction with Jab1 triggers the recruitment of specific secondary coregulators remains an open question.

Finally, in the context of a functional correlation of the L-Trb1–Jab1 interaction, we found that Jab1 was recruited to TH-transcriptional complexes (Fig. 4), strongly suggesting that Jab1 could be a relevant coregulator in tilapia TR signaling and T_2 a potent transcription regulator. In support of this idea, Jab1 expression was detected in all analyzed juvenile tilapia tissues (Fig. 5) and is also ubiquitously expressed in human tissues (http://www.genecards.org/cgi-bin/carddisp.pl?gene=COPS5). Although the physiological role of Jab–TRB1 remains to be elucidated, both transcription factors are co-expressed in the same tissues (Fig. 5 and Ref. Cheng *et al*. 2010). This could allow their joint participation in the regulation of positively or negatively TH-regulated genes. In this context, ongoing transcriptomic analysis in tilapia liver and cerebellum show Jab1 to be a TH-sensitive gene as both T_2 and T_3 regulate its expression in a tissue-specific manner, suggesting a role of Jab1 in TH-dependent signaling pathways. Further experiments are required to shed light on the physiological implication of this novel mechanism.

Overall, the present work supports the idea that the pleiotropic effects of thyroid hormones could be explained in part by a mechanism for the activation or repression of gene transcription by specific ligands such as T_2, which would in turn recruit a specific set of primary and/or secondary coregulators to differentially modulate chromatin structure and gene transcription.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/JOE-16-0485.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

G Hernández-Puga and A Mendoza performed the experiments; G Hernández-Puga, A Mendoza, A León-del Río and A Orozco designed the research and wrote the paper.

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3,5-Diiodothyronine-mediated transrepression of the thyroid hormone receptor beta gene in tilapia. Insights on cross-talk between the thyroid hormone and cortisol signaling pathways

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ABSTRACT

T3 and cortisol activate or repress gene expression in virtually every vertebrate cell mainly by interacting with their nuclear hormone receptors. In contrast to the mechanisms for hormone gene activation, the mechanisms involved in gene repression remain elusive. In teleosts, the thyroid hormone receptor beta gene or *thrb* produces two isoforms of TRβ1 that differ by nine amino acids in the ligand-binding domain of the long-TRβ1, whereas the short-TRβ1 lacks the insert. Previous reports have shown that the genomic effects exerted by 3,5-T2, a product of T3 outer-ring deiodination, are mediated by the long-TRβ1. Furthermore, 3,5-T2 and T3 down-regulate the expression of long-TRβ1 and short-TRβ1, respectively. In contrast, cortisol has been shown to up-regulate the expression of *thrb*. To understand the molecular mechanisms for *thrb* modulation by thyroid hormones and cortisol, we used an *in silico* approach to identify thyroid- and cortisol-response elements within the proximal promoter of *thrb* from tilapia. We then characterized the identified response elements by EMSA and correlated our observations with the effects of THs and cortisol upon expression of *thrb* in tilapia. Our data show that 3,5-T2 represses *thrb* expression and impairs its up-regulation by cortisol possibly through a transrepression mechanism. We propose that for *thrb* down-regulation, ligands other than T3 are required to orchestrate the pleiotropic effects of thyroid hormones in vertebrates.

1. Introduction

Thyroid hormones (THs) are major modulators of gene expression in virtually every vertebrate; they act by interacting with the thyroid hormone receptors (TRs), which in turn, recruit coactivators and/or corepressors in a hormone-dependent fashion. In TH up-regulated genes, it is well accepted that TRs are bound to the TH-response elements (TREs) and in the absence of hormone, they recruit corepressors, facilitating histone deacetylation and gene repression. Upon TH binding, the TR undergoes conformational changes which include the reorientation of helix-12, triggering corepressor release and the formation of a coactivator binding surface, ultimately leading to histone acetylation and gene expression (Fondell, 2013). In contrast, TH-dependent gene repression remains poorly understood despite the fact that most of the TH-responsive genes are known to be negatively modulated (Morte and Bernal, 2014). Furthermore, TH-responsive genes are differentially regulated during vertebrate ontogeny. In this context, the *thrb* gene (*nr1a2*) is dynamically modulated by the intracellular T3 concentrations in vertebrates. For example, *thrb* is highly up-regulated during teleost and amphibian metamorphosis in parallel with the well-described increase of T3 levels; however, *thrb* is negatively regulated by THs in teleost adulthood (Kawakami et al., 2008; Das et al., 2010; Navarrete-Ramírez et al., 2014).

Previous studies from our laboratory (Orozco et al., 2014) and others (Lehmphul et al., 2014) have shown that 3,5-diiodothyronine (3,5-T2 or T2) acts as a bioactive thyroid hormone. Teleosts express two isoforms of the TRβ1 which differ by the presence [long (L-)TRβ1] or absence [short (S-)TRβ1] of a nine-amino-acid insert at the beginning of the ligand-binding domain. Interestingly, the long TRβ1 has been shown to activate

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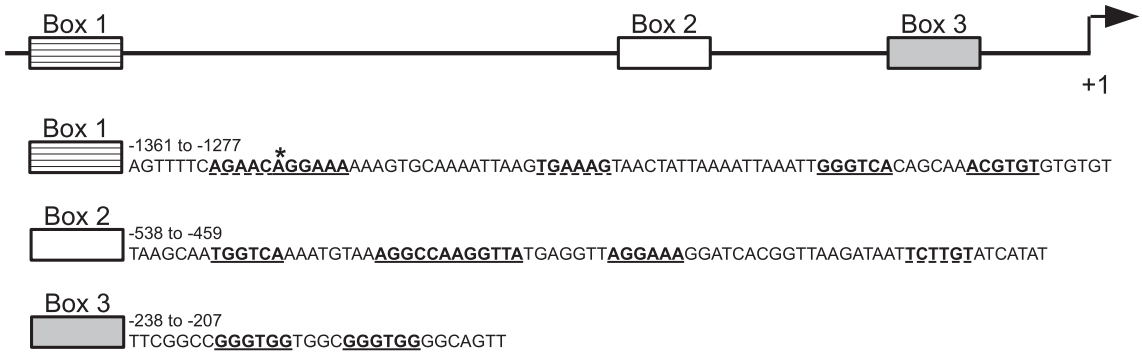

Fig. 1. Putative response elements for TR and GR. Promoter region (−3000 bp before the start site of transcription) of thrb from tilapia *O. niloticus*. The boxes represent the regions of the promoter that were used as primers. Identified putative response elements are in bold letters and underlined in bold (TREs) or dash (GREs). An asterisk marks a nucleotide shared between two response elements.

transcription in the presence of T2, whereas the short TRβ1 is active only in presence of T3. In addition, we observed that T2 specifically modulates the expression of the long TRβ1; on the other hand, T3 may function as a splicing modulator of the *thrb* transcript to shift synthesis towards the short TRβ1. Together, these results support the notion that 3,5-T2 is a bioactive TH with a signaling pathway independent of the T3 pathway (García-G et al., 2007; Mendoza et al., 2013; Navarrete-Ramírez et al., 2014).

Most TH-responsive gene promoters are targets of multiple transcription factors in addition to the TRs, which, in concert with the physiological requirements of the organism, modulate the rate of mRNA synthesis. Due to the differential regulation of the teleostean *thrb* by T2, we initially performed an *in-silico* screening of the first 3 kb upstream of the promoter from tilapia *thrb* to identify putative transcription factor binding sites. Interestingly, putative glucocorticoid response elements were identified in close proximity to TREs. Since THs and cortisol have been shown to promote permissive, synergistic, antagonistic, or co-regulatory interactions in numerous vertebrates (Ratman et al., 2013; Bonett et al., 2010; Terrien et al., 2011), we analyzed if their interaction could ultimately impact *thrb* regulation. Here we provide evidence that T2 is a negative modulator of *thrb* and in addition, we show that T2 interferes with *thrb* up-regulation by cortisol.

2. Materials and methods

2.1. thrb promoter region analysis

A putative promoter region of the *Oreochromis niloticus thrb* gene was identified by locating the start site of transcription in the ENSEMBL database (location: GL831306.1:535383-542493 in reverse strand), and the 3 Kb upstream were analyzed. A virtual, online tool PROMO software (Farré et al., 2003), as well as a simple algorithm with the "grep" command through the linux terminal were used to identify putative TREs and glucocorticoid response elements (GREs) with sequences of nucleotides previously reported (Meijsing et al., 2009; Ratman et al., 2013; Chatonnet et al., 2013; Paquette et al., 2014) as follows: (A/G)(C/G)G(A/G/C/T)(A/G/C/T)(A/G/C/T) and (A/T)(G/C)AA(A/C/T)(A/G/C/T), respectively. No full, canonic response elements were identified within the 3-Kb promoter of the *O. niloticus thrb* gene, but 12 putative half-sites were found within the 1.5-Kb proximal promoter, and probes containing these identified response elements (REs) were denominated Box 1, Box 2 and Box 3, as illustrated in Fig. 1.

2.2. Transient transfection assays

DNA fragments containing Boxes 1, 2 or 3 sequences were cloned into a pGL3-basic vector (Promega) (p-*thrb*-pGL3). We then characterized each specific Box as follows: GH3 cells (1×10^4 cells/well) were seeded onto 96-well white-wall plates and maintained using F12-K culture medium supplemented with 5% hormone-depleted dialyzed fetal calf serum (Gibco) 24 h prior to transfection. As previously described (Mendoza et al., 2013), cells were transfected using lipofectamine 2000 (Invitrogen) with 125 ng pGL3-empty (control reporter gene) or Box 1-pGL3, Box 2-pGL3 or Box-pGL3 and 62 ng of pcDNA3.3-L-TRβ1; renilla pRL-CMV (12.5 ng) was used as a reference reporter gene. Cells were treated with T3 or T2 with or without cortisol or cortisol alone at a final concentration of 100 nM in the culture media for 24 h. Optimal TH concentrations were previously established for these assays (Mendoza et al., 2013) and equivalent concentrations of cortisol were used to analyze equipotent effects. A dual luciferase assay (Promega) was carried out using DLR-ready Varioskan (Thermo Scientific). Data are reported as fold induction normalized to renilla luciferase, pGL3-empty light units and hormone-untreated controls. All experiments were carried out independently at least three times.

2.3. Animals

Tilapia juveniles (~0.8 ± 0.02 g) were kindly provided by the Quarentene Unit for Tilapia and Catfish at the Universidad Michoacana de San Nicolás de Hidalgo, México. Fish were kept in 10-L tanks containing constantly aerated fresh water at a temperature of 25 °C and maintained on a 12-h:12-h light–dark cycle. The fish were fed once a day to satiation with a commercial diet. All animal experimentation was conducted in accord with accepted procedures regarding handling and euthanasia of animals, as reviewed and approved by the Animal Welfare Committee of our Institute.

2.4. Nuclear extracts

Nuclear proteins were obtained as previously described (García-G et al., 2007) with minor modifications. In brief: untreated fish were sacrificed, and the livers were resuspended 1:5 (wt/vol) in sucrose buffer (320 mM sucrose, 25 mM KCl, 5 mM $MgCl_2$, 5 mM EDTA, 50 mM Tris–HCl, pH 7.5, 2 mM PMSF) and homogenized, followed by centrifugation at 12,000 *g* for 1 min. The tissue pellets were resuspended in hypotonic buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT); after mechanical disruption, the nuclei were collected by centrifugation at 12,000 *g* for 1 min, and their quality was evaluated with a Trypan blue stain. The pellet was resuspended in hypertonic buffer (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1.5 mM $MgCl_2$, 25% glycerol, 0.2 mM EDTA, 1 mM DTT, 0.5 mM

Table 1
Oligonucleotide sequences and real-time PCR protocols used to amplify the different genes.

Gene	Amplicon (bp)	Forward primer	Reverse primer	Real-time PCR protocol
β-actin	233	GCG ACA TCA AGG AGA AGCT	CGA CGT CGC ACT TCA TGA T	3 s @ 94 °C; 8 s @ 61 °C; 9 s @ 72 °C for 45 cycles
L-TRβ1	232	GTG AAG GAA GCT AAG CCT GA	CAC AAG GCA GCT CAC AGA AC	3 s @ 95 °C; 10 s @ 52 °C; 10 s @ 72 °C for 45 cycles
S-TRβ1	135	GCG GAA ATT CCT GCC TGA G	GCA GCT CAC AGA ACA TGG GC	2 s @ 95 °C; 8 s @ 54 °C; 7 s @ 72 °C for 55 cycles
GR	180	ATG GAT AAA GGT GGA GTG AAC	CTG TCC TGG CTG CAT CAA AG	2 s @ 95 °C; 10 s @ 60 °C; 7 s @ 72 °C for 55 cycles
MR	300	GGA AAG GAC ACA GAG AAC AG	GTC CAT TCC TGC TTA TGA CC	2 s @ 95 °C; 9 s @ 60 °C; 10 s @ 72 °C for 50 cycles

*The specific primers for the two TRβ1 isoforms are located in the ORF of the transcripts as follows: L-TRβ1: 5′ 526–545 pb, 3′ 678–697 and S-TRβ1: 5′ 501–519 pb, 3′ 657–670. Accession numbers: KF224971.1 and KF224972.1, respectively.

PMSF) and agitated at 4 °C for 30 min. After centrifugation at 12,000 *g* for 20 min, supernatants were collected and diluted in an equal volume of dilution buffer (20 mM HEPES, pH 7.9, 50 mM KCl, 25% glycerol, 0.2 mM EDTA, 1 mM DTT, 0.5 mM PMSF) for protein quantification by the Bradford method.

2.5. Electrophoretic mobility shift assay (EMSA)

Nuclear protein extracts (50 μg) of tilapia liver were incubated with or without hormone (100 nM of TH or cortisol) for 2 h on ice, in a buffer containing 20 mM HEPES, 50 mM KCl, 20% glycerol, 0.2 mM EDTA, 0.5 mM PMSF, 1 mM DTT, 1 mg/mL BSA, and 1 mg/mL polydI/dC (Pharmacia). The [^{32}P]-labeled oligonucleotides corresponding to Boxes 1, 2 and 3 (Fig. 1) were added, and the reaction was incubated on ice for 20 min and then at room temperature for another 20 min. The reaction mixture was loaded onto a 6.5% non-denaturating polyacrylamide gel and resolved at 120 V over the course of 2 h. The gel was dried, and the DNA-protein complexes were visualized by exposing in a Storage Phosphor Screen (Molecular Dynamics, Sunnyvale, CA). The screens were read in a Storm *Phosphorimager* and analyzed with the ImageQuant software (Molecular Dynamics). As positive controls to verify specific TR binding to the REs contained in Boxes 1, 2 and 3, two different approaches were followed: EMSAs were performed using the different probes together with i) whole tilapia nuclear extracts (50 μg) with 8 μg of TR antibody (abcam ab2743; Bhat et al., 1993), and ii) 200 ng recombinant GST + L-TRβ1 fusion protein.

2.6. Organotypic cultures of tilapia liver

Juvenile tilapia livers were dissected and placed in ice-cold Hank's Balanced Salt Solution (Gibco), which contained Minimum Eagles's Medium (MEM, Gibco), 10 mM HEPES, and 8 mM glucose (pH 7.2). Livers from an average of 5 individuals/experiment were sectioned at 400 μm thickness on a McIlwain tissue chopper. Slices were randomly placed onto semiporous membranes [six slices per membrane, (Millipore, Herts, UK)] and maintained in an incubator at 18 °C in 5% CO_2. The culture medium consisted of MEM, 10 mM HEPES, and Hank's Balanced Salt Solution supplemented with 10% horse serum (Gibco), 8 mM glucose, and 5% penicillin-streptomycin (Gibco).

2.6.1. Treatments

After 48-h of stabilization, the medium of each well (1.1 mL) was removed and replaced by the same volume of fresh medium, which contained the different hormone treatments. The optimal response was previously determined to be at 1 nM TH (Navarrete-Ramírez et al., 2014); dose–response curve experiments of cortisol previously solubilized in ethanol at concentrations of 0.1, 1, 10, and 100 nM were performed, and 1 nM was identified as optimal (data not shown). Subsequent experiments included 1 nM T3 or T2 previously solubilized in NaOH and/or 1 nM cortisol. In all cases, after a 24-h incubation, slices from each plate were removed and pooled for mRNA extraction and gene quantification. All experiments were performed independently at least twice, each in duplicate.

2.7. Quantitative PCRs

mRNA levels of S- and L-TRβ1, the glucocorticoid receptor [GR (*nr3c1*)], and mineralocorticoid receptor [MR (*nr3c2*)] were measured. In all cases, mRNA was quantified by real-time PCR as previously described (Mendoza et al., 2013) with certain modifications. Briefly, total RNA was extracted from tilapia liver slices used in the organotypic culture experiments (TRIzol, Invitrogen)

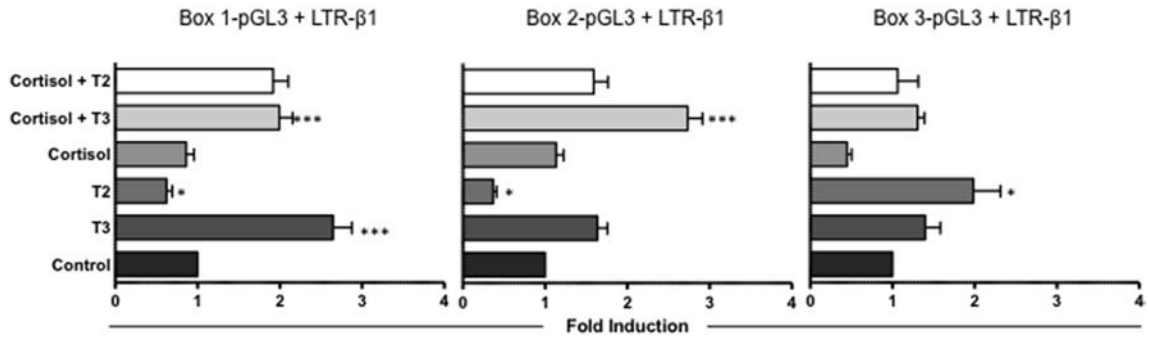

Fig. 2. Tilapia thrb Proximal Promoter Regions (Boxes 1, 2, and 3) are transcriptionally functional. Box 1, Box 2 and Box 3 sequences were inserted into the pGL3 basic vector. GH3 cells were co-transfected with Box 1-pGL3, Box 2-pGL3, or Box 3-pGL3 together with pcDNA3.3-L-TRβ1 and treated with 100 nM T3 or T2 and/or cortisol. The luciferase assay was performed 24 h after transfection. Data are reported as fold induction after normalizing to renilla luciferase, pGL3-empty light units and hormone-untreated controls. Results represent the mean ± SEM of 3 independent experiments, each in duplicate. Letters indicate statistical differences vs hormone-untreated controls. ***P < 0.001, **P < 0.01 and * P < 0.05.

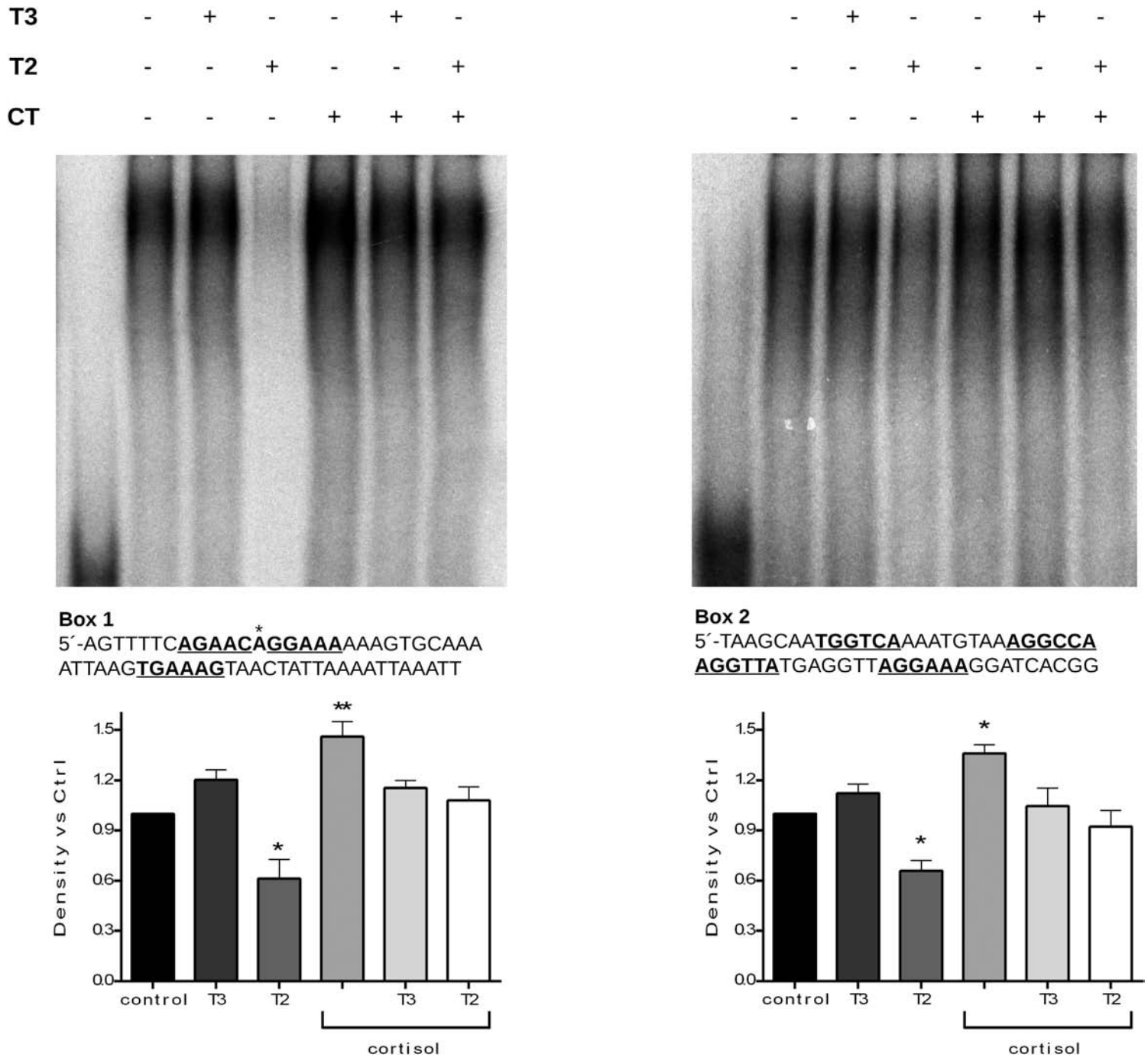

Fig. 3. T2 and cortisol impair or enhance, respectively, the interaction of tilapia nuclear proteins with specific thrb promoter regions. Top panels: representative EMSAs (n = 4 independent experiments) of nuclear protein extracts from tilapia liver incubated with ^{32}P-labeled oligonucleotide probes of Box 1 and Box 2 in the presence of 100 nM T3, T2, or cortisol (C). Sequences for probes are indicated as in Fig. 1 in which identified putative response elements are in bold letters and underlined in bold (TREs) or dash (GREs). An asterisk marks a nucleotide shared between two response elements. Bottom panels: Densitometric analysis of the DNA-protein complexes was done using the MYImage Analysis Software (Thermo Scientific). Values are means of density (average intensity/area) ± SE. Significance is indicated (**P < 0.01, *P < 0.05).

and reversed transcribed (RT) (M-MLV, Promega) from 2 μg of total hepatic RNA using an oligo(dT) primer (final volume of 25 μL). β-Actin was used as an internal standard (García-G et al., 2007; Yang et al., 2013; Mendoza et al., 2013; Navarrete-R et al., 2014) in reactions that contained 1 μL of the RT reaction, 6 μL Maxima SYBR Green/ROX qPCR Master Mix (Fermentas, Waltham, MA, USA), 1 μL DNase (Invitrogen) and 500 nM forward and reverse primers in a final volume of 12 μL. PCR protocols and oligonucleotides used for gene amplification are specified in Table 1. In all cases, the standard curve ranged from 10^2 to 10^9 molecules/μL, and a Step One instrument was used for detection and data analyses according to the instructions from the manufacturer (Applied Biosystems®). The mRNA concentration was expressed as molecules per microgram of total mRNA used in the RT reaction (2 μg) and obtained by comparison with the standard curve, normalized to the concentration of β-actin in each experimental sample.

2.8. Statistical analysis

Results obtained in transactivating assays were analyzed using a two-way analysis of variance (ANOVA) coupled with a Bonferroni post-test. Results obtained from qPCR mRNA determinations were analyzed using a one-way ANOVA coupled with Tukey's Multiple Comparison Test. Differences with $p \leq 0.01$ were considered statistically significant.

3. Results

3.1. thrb promoter region analysis

Within the proximal (1.5 Kb) promoter of the *O. niloticus thrb* gene we identified 12 putative half-sites [TRE (9) and GRE (3)] which we grouped in 3 regions or boxes as shown in Fig. 1. GRE-like sequences corresponded to individual half-sites, whereas TRE-like sequences were found in Boxes 1 (IP6), 2 (DR0), and 3 (DR4). To test if Boxes 1, 2 and 3 contained functional REs, we evaluated L-TRβ1 transactivating capacity in the presence of T3, T2 and/or cortisol (Fig. 2). T3 and T2 induced the activation of Box1-pGL3 and Box3-pGL3, respectively. Cortisol alone had no activating effects upon any of the Boxes, but it activated Box1-pGL3 when co-administered with T3 and T2, and Box2-pGL3 only when combined with T3. Furthermore, T2 induced the repression of the reporter gene in cells transfected with Box1-pGL3 and Box2-pGL3. To analyze if the putative response elements found in the tilapia *thrb* promoter could bind transcription factors, we first incubated the

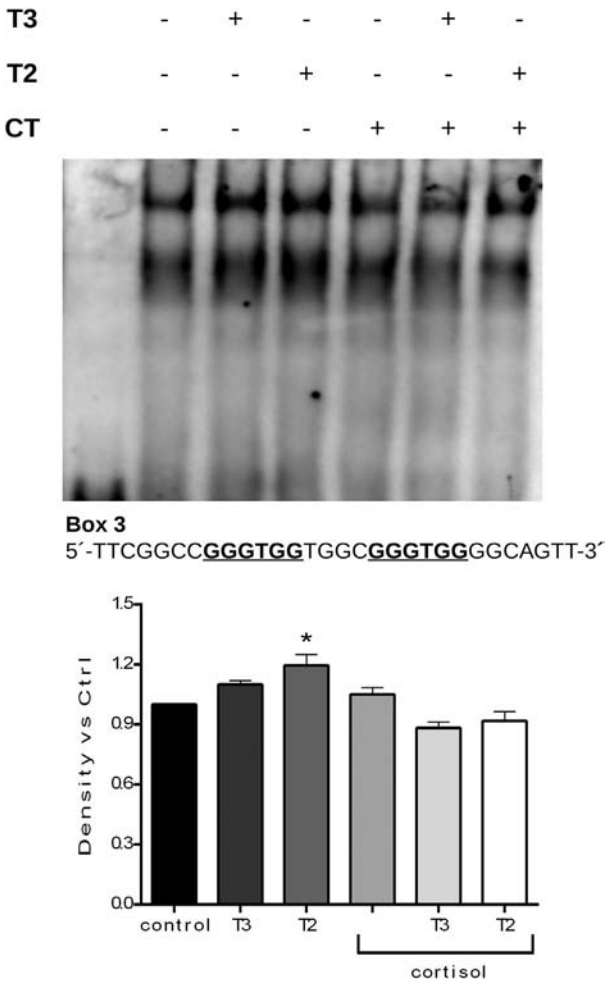

Fig. 4. T2 enhances the interaction of tilapia nuclear proteins with a native DR4-like response element. Top panel: representative EMSA (n = 4 independent experiments) of nuclear protein extracts from tilapia liver incubated with ^{32}P-labeled oligonucleotide probe of Box 3 in the presence of 100 nM T3, T2, and/or cortisol (C). The sequence of the probe is indicated in Fig. 1; the identified putative response element (DR4) is in bold letters and underlined. Bottom panel: Densitometric analysis of the DNA-protein complexes was done using the MYImage Analysis Software (Thermo Scientific). Values are means of density (average intensity/area) ± SE. Significance is indicated (*P < 0.05).

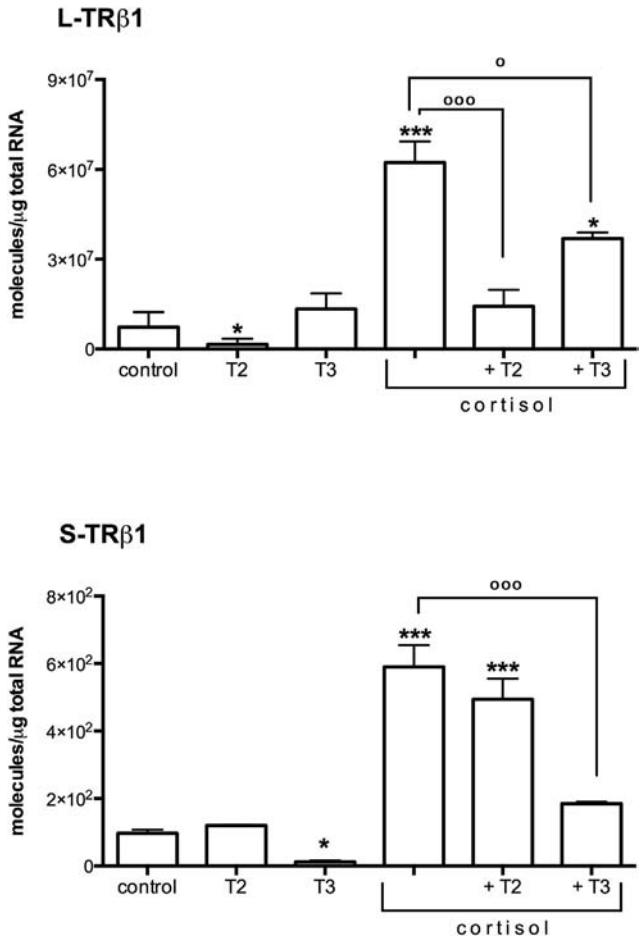

Fig. 5. Expression of short- and long-TRβ1 mRNAs from organotypic liver cultures. Organotypic tilapia liver cultures were treated for 24 h with 1 nM T3, T2 either alone or together with 1 nM cortisol (n = 6 slices/experimental group from an average of 5 individuals/experiment). Values are means ± SEM of three independent experiments. Significance is indicated (*P < 0.01; ***P < 0.0001 vs control) and ($^{○}$P < 0.01; $^{○○○}$P < 0.0001 vs cortisol).

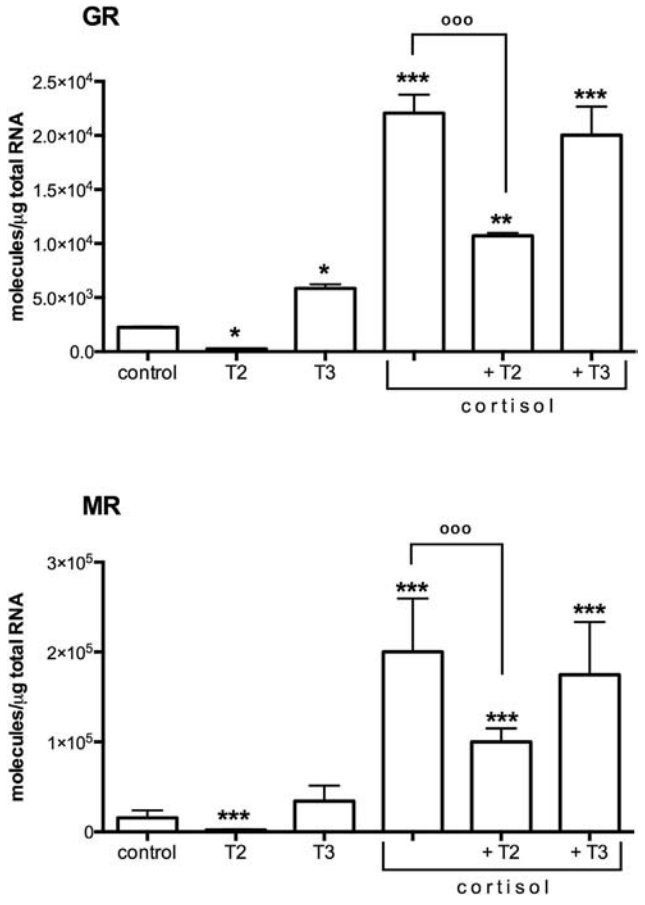

Fig. 6. Expression of GR and MR mRNAs from organotypic liver cultures. Organotypic tilapia cultures were treated for 24 h with 1 nM T3, T2 either alone or together with 1 nM cortisol (n = 6 slices/experimental group from an average of 5 individuals/experiment). Values are means ± SEM of three independent experiments. Significance is indicated (*P < 0.01; **P < 0.001; ***P < 0.0001 vs control) and (^{ooo}P < 0.0001 vs cortisol).

tilapia nuclear protein extracts with T3, T2 and/or cortisol in the presence of the radiolabeled probes (Boxes 1, 2 and 3) and resolved complex formation by EMSA. Nuclear proteins incubated with Box 1 or Box 2 probes plus either vehicle or T3 formed a single complex, whereas T2 destabilized the DNA-nuclear protein complex. Cortisol alone increased the intensity of the complex, but T3 and T2 slightly destabilized it (Fig. 3). Under control conditions the Box 3 probe formed two complexes that, in contrast, showed an increased stability only in the presence of T2 (Fig. 4). To test if the tilapia nuclear extracts contained TRs, GRs and MRs, we performed EMSAs using the radiolabeled canonical TRE and GRE probes. Two DNA-protein complexes were formed on both DR4 and GRE, and treatment with T3, T2 or cortisol slightly stabilized the DR4-protein interaction (Supplemental data 1). We then demonstrated that TRs in tilapia whole nuclear protein extracts do indeed bind to the TREs in the *thrb* specific promoter regions, since a super-shift was observed when these proteins were incubated with a TR antibody. Furthermore, L-TRβ1 was able to bind to the TRE-like sequences contained in Boxes 1, 2 and 3, as shown by the single complex formed when recombinant GST + L-TRβ1 fusion protein interacted with all Boxes (Supplemental data 2).

3.2. Regulation of thrb expression by THs and cortisol

We evaluated T2 and T3 cross-talk with cortisol by analyzing their effects upon the expression of the two TRβ1 isoforms in tilapia liver organotypic cultures. In line with our previous findings, only T2 exerted a down-regulatory effect upon L-TRβ1 after a 24-h treatment. In contrast, T3 but not T2 treatment down-regulated S-TRβ1. Cortisol alone significantly up-regulated the expression of both TRβ1 isoforms after 24 h of exposure. T2 or T3 prevented the cortisol effects upon L-TRβ1 or S-TRβ1 up-regulation, respectively (Fig. 5).

3.3. Regulation of GR and MR expression by THs and cortisol

In contrast to the results obtained on *thrb* regulation, T2 down-regulated both GR and MR expression after 24 h of exposure. Interestingly, although T3 did not affect MR expression, it elicited a small but significant up-regulation of GR expression. Cortisol treatment up-regulated both GR and MR expression after 24 h of exposure, an effect that was only blocked after a co-treatment with T2 (Fig. 6).

4. Discussion

In the present study, we identified for the first time in teleosts, putative TREs and GREs within the proximal promoter of the *thrb*. We confirmed that the regions that contain these response elements are functional and differentially respond to TH and/or cortisol treatments. Moreover, different DNA-protein complexes were formed between the distinct regions of the *thrb* promoter and the nuclear proteins from euthyroid tilapia liver incubated with THs (T3, T2), cortisol, or a combination of both. Notably, only T2 repressed Box 1(IP6)- and Box 2 (DR0)-mediated gene expression (Fig. 2). In line with these results, we observed a destabilizing effect of T2 as well as a stabilizing effect of cortisol upon DNA-protein complexes formed on promoter regions corresponding to Boxes 1 and 2. These observations lead us to propose that, by binding to the TR, T2 could repress *thrb*, possibly by sequestering coactivators away from an up-regulator complex, which could be cortisol dependent (Fig. 3). This possible TH-repression mechanism was suggested previously (Weitzel, 2008). In support of our proposal, T2 exerted a negative effect upon the expression of L-TRβ1 (the TR isoform that binds T2), whereas the positive effect of cortisol on the expression of this TRβ1 isoform was prevented by the presence of T2 but not T3 (Fig. 5). Interestingly, similar responses to T2 and cortisol were observed when we evaluated the mRNA expression of the two receptors that bind cortisol in teleosts, GR and MR (Bury and Sturn, 2007; Arterbery et al., 2011). These results suggest that T2 and cortisol could modulate gene expression through similar mechanisms. The fact that cortisol stabilized complex formation in Boxes 1 and 2 is unexpected; these two regions do not contain putative full-length GREs, as only isolated half-sites were predicted. Thus, the protein composition of the complexes formed at Boxes 1 and 2 may include GRs or MRs that could directly occupy composite GRE-TRE sites and cross-interact with unligated TRs at the promoter site (Ratman et al., 2013). In support of this proposal is the presence of at least one putative composite GRE-TRE predicted within Box 1 (Fig. 1). The other promoter region analyzed was Box 3, which exhibited a different pattern of DNA-protein complex formation. Notably, both the complexes formed in Box 3, as well as gene expression mediated by this promoter region were enhanced by T2. The predicted TRE in Box 3 resembles a DR4, a response element generally associated with TH-dependent gene up-regulation. In this context, *thrb* is up-regulated after an increase in the concentration of bioavailable TH during acute growth (Navarrete-Ramírez et al., 2014) and metamorphosis (Yamano and Miwa, 1998; Isorna et al., 2009) in teleosts, as well as during amphibian metamorphosis (Das et al., 2010). These effects could

occur with a different chromatin organization during these critical periods that would differentially expose response elements in the proximal promoter.

In a functional setting, both the thyroid and corticosteroid hormones are the end products of two endocrine axes conserved in all vertebrates. Aside from independently exerting potent metabolic effects in a large number of tissues, several studies have suggested an intimate functional linkage between THs and cortisol such that one influences the biological effect of the other (Bonett et al., 2010; Terrien et al., 2011; George et al., 2013; Arjona et al., 2008). This cross-talk has been demonstrated in the regulation of specific physiological processes including amphibian metamorphosis (Bonett et al., 2010; Denver, 2009), teleostean osmoregulation and development (Arjona et al., 2008; Applebaum et al., 2012), modulation of somatotroph abundance in both chick and rodents (Hemming et al., 1988; Liu et al., 2003), as well as regulation of some mammalian genes (Menjo et al., 1993; Molero et al., 1993; Nogami et al., 1995; Yamaguchi et al., 1999). These synergic effects have been attributed partly to the cross-regulation of the expression of their nuclear receptors, which would modulate their effects (Terrien et al., 2011). Our present results fit well with this proposal; THs and cortisol cross-modulate the expression of *thrb*, *gr*, and *mr*. Furthermore, our results show that in these TH down-regulated genes, the modulatory effect exerted by T2 prevailed over that of cortisol. Interestingly, T2 but not T3 seems to trans-repress *thrb* through a mechanism that is at least partly dependent on cortisol. In contrast to our observations in these TH down-regulated genes, for the TH up-regulated genes like GH and IGF-1, T3 and T2 were equally potent (data not shown; Navarrete-Ramírez et al., 2014; Mendoza et al., 2013).

Although a significant number of genes are negatively regulated by THs, a consensus action mechanism for TH-dependent gene down-regulation remains elusive. Findings in the present study suggest a multifactorial TH down-regulation mechanism that would require the presence of other ligands to orchestrate the pleiotropic functions of these endocrine messengers. Further studies are required to gain additional insight into the still elusive mechanism of TH-dependent gene down-regulation.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.mce.2016.01.023.

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DISCUSIÓN

En el presente trabajo se profundizó en el estudio del mecanismo de acción de la 3,5-T2, una hormona tiroidea bioactiva y con efectos biológicos en vertebrados. Las aportaciones de la presente tesis se resumen en tres puntos:

1- La T2 es una hormona que se une con alta afinidad al L-TRβ1 y con menor afinidad al receptor de humano (hTRβ1). Desde hace varias décadas, los efectos de la T2 sobre el metabolismo energético fueron reportados en mamíferos y hasta la fecha no se han descrito con claridad los mecanismos que subyacen dichos efectos, sin embargo, los estudios realizados sugieren que sus efectos se llevan a cabo a través de mecanismos no genómicos. Por otro lado, desde hace varios años, nuestro grupo de trabajo ha mostrado que la T2 induce efectos biológicos en teleósteos a través de mecanismos genómicos. Sin embargo, la unión de la T2 a un receptor a hormonas tiroideas solo lo habían demostrado Horst y colaboradores (1995), quienes encontraron que la T2 se unía con una baja afinidad al TRβ1 de rata. Aun cuando esos datos indicaron que, en mamíferos, la T2 podría ser un ligando de menor afinidad que T3, la información sobre la afinidad de T2 por otros receptores de vertebrados era inexistente; ya que la estructura de los TRs es variable dependiendo de la especie, era necesario determinar la afinidad de T2 por las isoformas TRβ1 de teleósteo.

Los resultados de los ensayos de “binding” mostraron que: a) T3 se une con alta afinidad a las isoformas de teleósteo y de humano (0.2 nM), b) T2 es un ligando más selectivo, ya que se une con una alta afinidad al L-TRβ1 (0.2 nM, similar a la de T3), y con una menor afinidad al hTRβ1 (8 nM, 40 veces menor que la afinidad por T3), mientras que no muestra afinidad por S-TRβ1 (Mendoza et al., 2013). Las explicaciones a dichas diferencias en afinidad radican en la estructura tridimensional de los TRs; estudios de estructura-función realizados en el laboratorio mostraron que: i) el inserto de 9 aa de L-TRβ1 es esencial para la activación por T2 y ii) aun cuando hTRβ1 carece del inserto, la composición de aminoácidos del LBD permite su interacción con T2. Asimismo, la estructura del ligando podría explicar las diferencias en afinidad, ya que predicciones de modelado molecular de los ligandos unidos a sus respectivos TRs, muestran que el átomo de yodo localizado en la posición 3’ del anillo externo de la T3 interacciona con un grupo de aminoácidos específicos de la porción

C-terminal de hTRβ1 (hélice12) y contribuye a la estabilización de la superficie de unión a coactivadores. Ya que estos aminoácidos también se encuentran conservados en L-TRβ1 y S-TRβ1, su presencia podría explicar la afinidad similar observada con las tres isoformas (Mendoza et al., 2013).

En el caso de la T2, la ausencia del átomo de yodo en la posición 3´ del anillo externo de la molécula podría desestabilizar la hélice 12 de hTRβ1, resultando en una baja activación del TR. Mientras que en L-TRβ1, la presencia del inserto produce un cambio conformacional en el LBD (loop 2-3 y porción N-terminal de la hélice 3) que podría compensar la desestabilización causada por la ausencia de un tercer átomo de yodo en la molécula de T2. En este sentido, la composición de amino ácidos en la porción hélice 3 de hTRβ1 sugiere que se podrían forman enlaces tipo van der Waals para estabilizar la estructura general del LBD y por tanto de la hélice 12. Por último, la naturaleza de los aminoácidos de la hélice 3 del LBD de S-TRβ1 impediría la estabilización de la hélice 12 en presencia de T2 y por lo tanto la activación del receptor por esta TH (ver Mendoza et al., 2013).

En conjunto la evidencia experimental apoya la idea de la T2 como un agonista parcial del receptor de humano. En esta especie se requerirían altas concentraciones de T2 en la célula para que esta hormona induzca sus efectos genómicos. Alternativamente, los efectos de T2 podrían ser de menor potencia que aquellos inducidos por T3 y estos dependerían de la disponibilidad de T2 en la célula; ésto fortalece la idea de que T3 y T2 induzcan efectos tejido-específicos. Por lo que es necesario realizar más estudios sobre la bioactividad de la T2 en mamíferos. En peces, la T2 es un ligando alternativo del L-TRβ1 y posiblemente es una hormona con relevancia fisiológica en estos organismos, pues como se mencionó anteriormente, L-TRβ1 solo se ha encontrado en peces (Orozco et al., 2014). Además, la alta capacidad de activación de L-TRβ1 en presencia de T2, la cual es mayor a la de T3 (Mendoza et al., 2013) y la regulación de la expresión de su receptor *in vivo* (Mendoza et al., 2013, Navarrete-R et al., 2016, Hernández-Puga et al., 2016) indican que la T2 es el principal ligando de esta isoforma en peces.

2- Jab1 es un corregulador dual- ligando dependiente de TRβ1. En la búsqueda de posibles correguladores reclutados por L-TRβ1 ligado a T2, se identificó a Jab1 (Hernández-Puga et al., 2017). Esta proteína, también conocida como COPS5, pues

se ha descrito que forma parte del complejo del signalosoma COP9 (Cope et al., 2002) y participa en el proceso de degradación de proteínas *vía* el proteosoma (Wei et al., 2008; Kato & Yoneda-Kato, 2009). Sin embargo, se ha mostrado que algunas de las subunidades de COP9 poseen actividad independiente como monómeros, tales como COPS2 [Alien], el cual ha sido caracterizado como un correpresor de TR (Papaioannou et al., 2007) o COPS5 [Jab1] el cual ha sido identificado como un coactivador de varios NR, incluyendo al TRα1 (Chauchereau et al., 2000). Por lo anterior, Jab1 resultó ser un buen candidato para estudiar su posible papel corregulador en las vías de señalización de T2.

La aproximación experimental incluyó estudios de interacción proteína-proteína y ensayos de transactivación empleando las dos isoformas de TRβ1 de teleósteo y la de humano. También se realizaron aproximaciones *in vivo* para estudiar el posible rol fisiológico de Jab1 en tilapia. Los resultados mostraron que Jab1 ejerce papeles opuestos sobre la regulación génica: al interactuar con L-TRβ1 y hTRβ1, funciona como un coactivador al aumentar la transactivación dependiente de T2, mientras que actúa como un correpresor al reprimir la actividad de transactivación de S-TRβ1 y hTRβ1 en presencia de T3 (Hernández-Puga et al., 2017). Lo anterior indica que Jab1 funciona como un corregulador dual de TRβ1 y sus efectos son ligando e isoforma-dependientes.

El efecto coactivador o correpresor específico del ligando ha sido descrito en algunos correguladores como la tesmina, la cual induce un efecto activador sobre el receptor de mineralocortiocides en presencia de aldosterona, pero no de cortisol (Rogerson et al., 2014); o el corregulador RTA (*repressor of tamoxifen transcriptional activity*) que actúa como un correpresor del receptor de estrógenos alfa en presencia de tamoxifen, pero no de agonistas como el estradiol (Norris et al., 2002). El único corregulador con propiedades duales que se ha descrito hasta la fecha es RIP140 (*receptor-interacting protein 140*), el cual induce o reprime la transcripción dependiendo del NR con el que interaccione (Subramaniam et al., 1999); sin embargo, nunca se había descrito el efecto dual-ligando dependiente de un corregulador sobre el mismo NR, como se observa con Jab1. Aunque los mecanismos que subyacen los efectos duales de Jab1 se desconocen, los resultados de la presente tesis apuntan a que la unión de diferentes ligandos al TR, T2 o T3 podría inducir cambios conformacionales específicos en el LBD. Dicha conformación específica expondría

superficies de interacción particulares del TR que impactarían en el resultado final de la regulación del gen blanco, como se ha visto con otros NRs (Nettles & Greene, 2005; Moore et al., 2004; Jeyakumar et al., 2008). En este contexto, los efectos duales de Jab1 podrían estar mediados por el reclutamiento de coactivadores y correpresores clásicos, pues se ha reportado que Jab1 puede interactuar directamente con SRC-1 (Chauchereau et al., 2000) o NCoR (Lu et al., 2016), ambos potentes correguladores de los NRs. Sin embargo, otros estudios serán necesarios para dilucidar dichos mecanismos.

En cuanto a las superficies involucradas en la interacción Jab1 + TRβ1, se encontró que Jab1 interactúa directamente con el LBD del TRβ1 a través de su único motivo LXXLL, como lo hacen los coactivadores primarios (Feng et al., 1998; Moras & Gronemeyer, 1998). Asimismo, como ya se ha mostrado con otros correguladores (Hollenberg et al., 1995; Tomura et al., 1995; Blessing et al., 2015), se requiere de una configuración completa del TR para la interacción estable con Jab1, la cual incluye la presencia del dominio NT, cuya importancia en la activación por T2 se había demostrado previamente (Mendoza et al., 2013).

Finalmente, en cuanto al rol fisiológico de Jab1 en tilapia, se encontró que Jab1 es reclutado en tilapia a complejos transcripcionales TR-TRE sugiriendo que puede ser un corregulador relevante en este organismo. Aunado a esto, Jab1 se co-expresa con L-TRβ1 en diversos tejidos de tilapia, lo que permitiría su participación en la regulación de genes TH-dependientes (Hernández-Puga et al., 2017).

En conjunto, nuestros datos sugieren que la unión de un ligando específico “modela” el LBD de tal forma que se exponen superficies de interacción con correguladores particulares en cooperación con otros dominios estructurales del NR. Esto influye en el grupo de correguladores que se asocian al NR y en el efecto sobre la regulación génica. De esta forma, la unión de ligandos específicos como T2, puede modular la activación o represión de la transcripción a través del reclutamiento de grupos de proteínas correguladoras específicas, idea que va de acuerdo con nuestra hipótesis de trabajo que postula que la T2 recluta a una población específica de correguladores, distinta a aquella reclutada por T3.

3- T2 y cortisol modulan la expresión génica a través de mecanismos similares. Es bien sabido que existe una conexión funcional entre THs y el cortisol, dicha conexión ha sido demostrada en diversos procesos biológicos como la metamorfosis en anfibios (Bonett et al., 2010; Denver, 2009) y el desarrollo en peces (Applebaum et al., 2012), entre otros. En el presente estudio se analizó la posible comunicación cruzada entre T2 y cortisol, a través de la caracterización de los efectos de ambas hormonas sobre la regulación del promotor del gen *thrb*. Inicialmente, se realizó un análisis *in silico* del promotor proximal del gen *thrb* (3 kb) y se buscaron los elementos de respuesta a THs (TREs) y a glucocorticoides (GREs). Se identificaron tres regiones que fueron denominadas como “Box1”, “Box2” y “Box3”, las cuales contienen medios sitios TRE o GRE, o TRE completos. Posteriormente, se analizaron los efectos de la T2 o la T3 y/o el cortisol sobre la formación de complejos proteína-DNA, empleando extractos nucleares de hígado de tilapia y las regiones “Box1”, “Box2” y “Box3” (Hernández-Puga et al., 2016).

Los resultados mostraron que la formación de complejos transcripcionales en la región promotora del gen *thrb* depende del elemento de respuesta en cuestión y además es susceptible a la presencia del ligando, pues se encontró que T2 reprime o promueve la formación de complejos transcripcionales dependiendo del TRE/GRE analizado. En contraste, T3 parece no inducir cambios en la formación de dichos complejos transcripcionales. De forma interesante se encontró que T2 antagoniza los efectos del cortisol, reprimiendo la expresión del gen *thrb* e induciendo la disociación de ciertos complejos transcripcionales, los cuales se favorecen en presencia de cortisol (Hernández-Puga et al., 2016). Así, el efecto de disociación del complejo TRE-TR/GR (receptor a glucocorticoides) sugiere un novedoso mecanismo de transrrepresión de T2 que al parecer podría depender de maquinaria celular compartida con cortisol, ya que la presencia de este último se reduce el efecto represor de T2, lo que sugiere la existencia de mecanismos de comunicación cruzada entre ambas hormonas, en donde las funciones de una hormona afectan a la otra.

Finalmente, se encontró que, en otras regiones del promotor, el efecto de T2 es opuesto, al promover la formación de complejos transcripcionales; lo que sugiere que uno de los posibles mecanismos de regulación a la alta o a la baja de la expresión de genes tironino-dependientes, podría ser a través del empleo de diferentes combinaciones de TREs y ligandos, como la T2 e incluso el cortisol. Lo que permitiría

orquestar finamente las funciones pleiotrópicas de ambos mensajeros endocrinos (Hernández-Puga et al., 2016).

En conjunto los datos obtenidos en el presente trabajo muestran a la T2 como una TH bioactiva que puede inducir sus efectos a través de mecanismos genómicos específicos, distintos a aquellos de T3 y que podrían explicar la gran pleiotropía funcional de las THs.

CONCLUSIONES

1. La T2 se puede unir con alta afinidad al L-TRβ1 y actuar como un ligando alternativo del L-TRβ1, y se puede unir con menor afinidad a hTRβ1 y actuar como agonista parcial del hTRβ1.

2. Jab1 es un corregulador dual del TRβ1 dependiente del ligando, siendo un coactivador en presencia de T2 y un correpresor en presencia de T3.

3. La T2 modula la expresión del gen *thrb* a través de un mecanismo de transrrepresión que involucra a la vía de señalización del cortisol.

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ANEXO

Efectos Biológicos de las THs.

Los efectos biológicos de las THs en vertebrados se clasifican en dos grandes grupos: aquellos relacionados a procesos de diferenciación y desarrollo, y aquellos relacionados al mantenimiento del balance energético.

A la fecha se ha reconocido que las TH tienen efectos en casi cualquier tipo celular; aunado a esto, los efectos son tejido-específicos y dependientes de la etapa de vida del organismo. A continuación, se mencionarán algunos ejemplos de los efectos biológicos más estudiados de las THs.

Las THs juegan un papel crucial durante etapas tempranas del desarrollo en procesos de crecimiento y diferenciación, entre los ejemplos más claros de esta función se encuentra la metamorfosis en anfibios, a través de la inducción de diversos cambios fisiológicos, bioquímicos y de diferenciación en la mayoría de los tejidos del renacuajo, que culminan en cambios morfo-funcionales que permiten la adaptación del organismo a un nuevo medio ambiente terrestre (Tata et al., 1998). En ausencia de TH, dicho proceso no se presenta y el organismo permanece como larva acuática durante toda su vida.

En organismos mamíferos y no mamíferos, las THs juegan un papel importante en la diferenciación y maduración de diversos órganos durante periodos pre y post-natales (Darras et al., 2011; Morvan-Dubois et al., 2013), un ejemplo de ello es el sistema nervioso, en donde participan en la regulación de procesos de mielinización, diferenciación, crecimiento axonal, arborización dendrítica, sinaptogénesis y migración neuronal (Horn and Heuer, 2010; Howdeshell, 2002; Decupeyre et al., 1990; Williams, 2008). Asimismo, regulan el crecimiento y desarrollo del hueso, mediante el control de la reabsorción de calcio, la regulación la actividad de osteoclastos y osteoblastos, la diferenciación de condrocitos, entre otras funciones (Williams 2009).

Otro de los tejidos blanco de las THs es el tejido adiposo, en donde participan en la diferenciación del tejido adiposo blanco, así como en la inducción de la termogénesis en tejido adiposo pardo, proceso esencial para el mantenimiento de la temperatura en mamíferos durante periodos post-natales (Obregón et al., 2014).

Durante la vida adulta, las THs participan en procesos de neurogénesis y plasticidad cerebral (Remaud et al., 2014; Sánchez-Huerta et al., 2016), así como en la regulación del gasto energético, a través de la regulación del metabolismo de macronutrientes (carbohidratos, lípidos, proteínas) movilización y almacenamiento de lípidos (Obregón et al., 2014) y consumo de oxígeno en hígado y tejido adiposo, principalmente (Mullur et al., 2014). En tejido cardiaco regulan el volumen sanguíneo e inducen efectos ionotrópicos y cronotrópicos (Klein and Ojamaa, 2001). Asimismo, las THs regulan el desarrollo y función de otros sistemas como el músculo-esquelético, piel, sistema inmune, entre otros (Yen et al., 2001; Cheng et al., 2010, Brent, 2012).

Por otro lado, las THs también son esenciales en etapas de desarrollo temprano y durante la metamorfosis de peces, un ejemplo claro de esta última función es la sorprendente metamorfosis del pez plano, en donde una larva simétrica se convierte en un juvenil asimétrico con ambos ojos en el mismo lado del cuerpo (Schreiber, 2013). Además, las THs participan en procesos de esmoltificación y crecimiento de diversas especies de peces (Eales, 2006; Power et al., 2001, Kawakami et al., 2008; Marchand et al., 2004; Walpita et al., 2007; Yang et al., 2007; Navarrete-R et al., 2014).