Cloning and Characterization of Type III Iodothyronine Deiodinase from the Fish Oreochromis niloticus*

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ABSTRACT

Type III iodothyronine deiodinase (D3) catalyzes the inner ring deiodination (IRD) of T3 and T4 to the inactive metabolites rT3 and 3,3'-diodothyronine (3,3'-T2), respectively. Here we describe the cloning and characterization of complementary DNA (cDNA) coding for D3 in fish (Oreochromis niloticus, tilapia). This cDNA contains 1478 nucleotides and codes for a protein of 267 amino acids, including a putative selenocysteine (Sec) residue, encoded by a TGA triplet, at position 131. The deduced amino acid sequence shows 57–67% identity with frog, chicken, and mammalian D3, 33–39% identity with frog, fish (Fundulus heteroclitus) and mammalian D2, and 30–45% identity with fish (tilapia), chicken, and mammalian D1. The 3' UTR contains a putative Sec insertion sequence (SEClS) element. Recombinant tilapia D3 (tD3) expressed in COS-1 cells and native tD3 in tilapia brain microsomes show identical catalytic activities, with a strong preference for IRD of T3 (Km ~20 nM). IRD of [3,5-125I]T3 by native and recombinant tD3 are equally sensitive to inhibition by substrate analogs (T3 > T4 > rT3) and inhibitors (gold thioglucose >> iodoacetate >> propylthiouracil). Northern analysis using a tD3 ribo-probe shows high expression of a 1.6-kb messenger RNA in gill and brain, although D3 activity is much higher in brain than in gill. The characterization of tD3 cDNA provides new information about the structure-activity relationship of iodothyronine deiodinases and an important tool to study the regulation of thyroid hormone bioactivity in fish. (Endocrinology 140: 3666–3673, 1999)

T HE MAJOR secretory product of the thyroid is a pro-hormone, T4, which is activated in peripheral tissues by outer ring deiodination (ORD) to T3. T4 and T3 are converted by inner ring deiodination (IRD) to the metabolites rT3 and 3,3'-diodothyronine (3,3'-T2), respectively (1–5). Three iodothyronine deiodinases are involved in these processes (1–5). In mammals, the type I deiodinase (D1) is located in liver, kidney, and thyroid. It has both ORD and IRD activity, in particular toward rT3 and sulfated iodothyronines (1–5). The type II deiodinase (D2) only catalyzes ORD with T4 as the preferred substrate. In rats, D2 is expressed predominantly in brain, pituitary, and brown adipose tissue, and recent findings suggest additional expression in human thyroid, skeletal muscle and, perhaps, heart (1–7). D3 has only IRD activity with preference for T3 as the substrate. In mammals, D3 is mainly found in brain, skin, placenta, and fetal tissues (1–5). The three deiodinases have recently been cloned from different species, showing that they are homologous seleno-
plementary DNA (cDNA) coding for D1 in tilapia (20). In contrast to our hypothesis, we found that tilapia D1 contains a Sec residue in a position corresponding to the Sec residue in PTU-sensitive D1s, indicating that differences in PTU sensitivity are determined by other structural elements (20).

Simultaneous with the cloning of D1 from tilapia kidney, we also attempted to clone other deiodinases from tilapia liver. This involved RT-PCR of tilapia liver messenger RNA (mRNA) using oligonucleotide primers based on amino acid sequences (NFGSCTSecP, YEEAH and VVVDTM) highly conserved in the D1 and D3 sequences available at that time (8–13). The RT-PCR products were sequenced and used as probes for cDNA library screening. This resulted in the isolation of TL31, a cDNA clone coding for D3 (tD3).

**Materials and Methods**

Tilapia (O. niloticus) were obtained from CERER-University of Liège (Tihange, Belgium) and kept as described before (36, 37). TRIZol reagent was obtained from Life Technologies, Inc. (Breda, The Netherlands); oligo-dT-cellulose was from New England Biolabs, Inc. (Beverly, MA); SuperTaq DNA polymerase was from HT Biotechnology Ltd. (Cambridge, UK); AMV reverse transcriptase and pCI-Neo were from Promega Corp. (Madison, WI); Klenow DNA polymerase was obtained from Roche Molecular Biochemicals (Mannheim, Germany); pCR-II was from Invitrogen (San Diego, CA); synthetic oligonucleotides were from Amersham Pharmacia Biotech (Rooseendaal, The Netherlands) or Life Technologies, Inc.; Hybond membranes, [α-32P]dATP and [α-32P]UTP were purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK); polyethylene glycol (PEG6000) was from Merck (Hohenbrunn, Germany); DEAE-dextran and Sephadex LH-20 were from Amersham Pharmacia Biotech. Nonradioactive iodothyronines were obtained from Hen.

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**Fig. 1.** Nucleotide and deduced amino acid sequence of cDNA clone TL31. The Sec residue is denoted by X. The putative SECIS element in the 3' untranslated region is underlined.
ning Berlin R&D (Berlin, Germany), [3', 5'-125I]T4 (1200 Ci/mmol) and [3', 5'-125I]T3 (2000 Ci/mmol) from Amersham Pharmacia Biotech, and [3', 5'-125I]T3 (35 Ci/mmol) from Dr. R. Thoma (Formula GmbH, Berlin, Germany) courtesy of Dr. G. Decker (Henning, Berlin, Germany). (3', 5'-125I]T1 and the riboprobe was generated using the T 3 Ampliscribe kit (Epicentre Technologies, Madison, WI) and [α-32P]UTP. Hybridization of the Northern blot was performed in NorthernMax buffer (Ambion, Inc., Austin, TX) overnight at 67 C. Blots were washed once for 30 min at 50 C with 0.1 SSC, 0.1% SDS, and twice for 30 min at 70 C with 0.1 SSC, 0.1% SDS. Autoradiographs were prepared by exposure of the blots at 70 C to Fuji Photo Film Co., Ltd. RX film. Analysis of the ethidium bromide-stained gels indicating 20–30% variation in the amount of applied RNA.

Expression

cDNA was cut out of pBK-CMV with SalI/NotI and ligated into XhoI/NotI digested pCI-Neo and expressed in COS-1 cells grown in DMEM/F12 containing 10% FCS (Life Technologies, Inc.) and 40 mM Na2SeO3 (45). One day before transient transfection, COS-1 cells were seeded at 50% confluence in 55 cm2 cell culture dishes. Expression constructs (7 μg) isolated by alkaline lysis and polyethylene glycol precipitation (46) were added to serum-free DMEM/F12 medium containing 100 μg/ml DEAE-dextran. After 2 h, the medium was replaced by serum-free DMEM/F12 medium containing 100 μM chloroquine. Again, 2 h later the medium was replaced by DMEM/F12 containing 10% FCS and 40 mM Na2SeO3. After 3 days, the cells were rinsed with PBS, collected in 0.3 ml 0.1 M phosphate (pH 6.9), 1 mM EDTA and 10 mM DTT, sonicated, snap-frozen on dry-ice/ethanol, and stored at −80 C.

Northern blots

Total tissue RNA (20 μg per lane) was separated on 1% (wt/vol) formaldehyde-agarose gels and blotted onto Hybond-N+ membranes by overnight capillary transfer using 20 × SSC. For preparation of a riboprobe, the TL31-pCI-Neo plasmid was double-digested with EcoRI/ XhoI and religated to remove nonspecifically hybridizing repetitive 3'UTR sequences. The 3'UTR-deleted construct was linearized with NheI, and the riboprobe was generated using the T 3 Ampliscribe kit (Epiconcept Technologies, Madison, WI) and [α-32P]UTP. Hybridization of the Northern blot was performed in NorthernMax buffer (Ambion, Inc., Austin, TX) overnight at 67 C. Blots were washed once for 30 min at 50 C with 0.1 × SSC, 0.1% SDS, and twice for 30 min at 70 C with 0.1 × SSC, 0.1% SDS. Autoradiographs were prepared by exposure of the blots at −70 C to Fuji Film Co., Ltd. RX film. Analysis of the ethidium bromide-stained gels indicating 20–30% variation in the amount of applied RNA.

Enzyme assays

Tilapia tissue homogenates and microsomal fractions were prepared as described before (37). Deiodinase activities of native and recombinant enzyme preparations were determined by measuring the radioiodide released from either [3', 5'-125I]T4 or [3', 5'-125I]T3 by ORD, or from [3', 5'-125I]T3 or [3', 5'-125I]T3 sulfate (T5) by IRD (40, 41). In short, appropriate amounts of tissue or lysate protein were incubated in triplicate for 30–60 min at 37 C with 10 nM [125I]substrate in 0.2 ml, 0.1 mM sodium phosphate buffer (pH 7.2), 2 mM EDTA, and 10 mM DTT. Reactions were stopped and [125I]iodothyronines were precipitated by successive addition of 0.1 ml 5% BSA and 0.5 ml 10% TCA. Radioiodide was further isolated from the supernatant on Sephadex LH-20 minicolumns (40, 41). For HPLC analysis of the deiodination products, 1 nM [3', 5'-125I]T4 or [3', 5'-125I]T3 was incubated in duplicate for 1 h at 37 C with (1 mg protein/ml) or without cell lysate in 0.2 ml, 0.1 mM phosphate (pH 7.2),

![Fig. 2. Predicted stem-loop structure of the SECIS element in the TL31 3'UTR. Consensus nucleotides are indicated in bold.](image-url)
2 mM EDTA and 50 mM DTT. The reactions were stopped by addition of 0.2 ml ice-cold methanol. After centrifugation, 0.2 ml of the supernatant was mixed with 0.2 ml 0.02 M ammonium acetate (pH 4), and 0.1 ml of the mixture was applied to a 250 × 4.6 mm Symmetry C18 column (Waters, Etten-Leur, The Netherlands) connected to an Alliance HPLC system (Waters) and eluted isocratically with a mixture of acetonitrile and 0.02 M ammonium acetate (33:67, vol:vol) at a flow of 1.2 ml/min. Radioactivity in the eluate was monitored on line using a Radiomatic A-500 flow scintillation detector (Packard, Meriden, CT).

Results

By RT-PCR of tilapia liver mRNA using oligonucleotide primers corresponding to the conserved amino acid sequences NFGSCTSecP and VVVDTM, a 246-bp cDNA fragment was obtained, which showed the sequence of which the homology with the corresponding region in *Xenopus laevis* and *Rana catesbeiana* D3 (12, 13). The labeled PCR product was used as a probe to screen the tilapia liver cDNA library (200,000 independent clones). Seven double-positive clones were identified after plating 500,000 pfu’s of the amplified library. Using vector- and PCR product-specific primers, several possibly full-length clones were identified. One cDNA clone (TL31) was found to be 1479 bp long with a reading frame coding for a 267-amino acid protein, assuming that TGA at codon 131 is translated as Sec (Fig. 1). The protein has a calculated molecular weight of 30,356 kDa and an isoelectric point (pI) of 6.2. Analysis of the 3' UTR region of TL31 by RNA secondary structure prediction reveals a stem-loop structure containing consensus SECIS element nucleotides (Fig. 2). SECIS elements are essential for the incorporation of Sec at the in-frame UGA opal stop codon (47–50). Evidence presented below indicates that TL31 represents cDNA coding for D3.

Figure 3 shows the alignment of the deduced amino acid sequence of tD3 with the D3 sequences of *X. laevis*, *R. catesbeiana*, chicken, human, and rat. The amino acid identity of tD3 amounts to 62–65% with frog D3 (12, 13), 67% with chicken D3 (19), and 57% with mammalian D3 (14, 15). The amino acid sequence of tD3 shows 35% identity with tilapia D1 (20), 33% identity with chicken D1 (19) and 30–33% identity with mammalian D1 (8–11). The amino acid identity of tD3 with fish (*F. heteroclitus*) D2 is 36% (18), with frog D2 33% (16) and with mammalian D2 38–39% (6, 17).

![Fig. 3. Alignment of the cDNA-deduced amino acid sequences of tilapia (til), *X. laevis* (xen), *R. catesbeiana* (ran), chicken (chi), human (hum) and rat D3. The Sec residue is denoted by X. Identical amino acids are indicated by dots, and gaps are indicated by hyphens.](image-url)
trations of unlabeled T3, T4, and rT3 with IC50 values of ~10, ~100 and ~1000 nM, respectively (Fig. 7, A and B). The apparent Km value of T3 for both native and recombinant tD3 amounts to ~20 nM, which is close to the Km values found for other D3 enzymes (1–5). Native and recombinant tD3 also show equal patterns of inhibition by increasing concentrations of GTG, IAc, and PTU (Fig. 7, C and D). Under the conditions used, IC50 values for these inhibitors amount to ~1 μM, ~1 mM and ~1 mM, respectively.

Figure 8 compares the tissue distributions of D3 activity and mRNA levels in tilapia. Analysis of the IRD of T3 in tissue homogenates indicates high D3 activity in brain and much lower activities in other tissues (Fig. 8A). On the Northern blots, a prominent 1.6-kb mRNA species is detected in gill and brain by hybridization with the tD3 riboprobe (Fig. 8B). Gill shows extensive hybridization of progressively shorter mRNA species, with a prominent band of ~1.2 kb which is clearly visible after shorter exposure times (not shown). Much weaker bands are present in liver and kidney. Furthermore, smaller mRNA species of ~1 kb are observed in heart and spleen. Gut and muscle show very little tD3 mRNA.
Discussion

Evidence that the TL31 cDNA cloned and characterized in this study codes for tD3 may be summarized as follows. 1) The nucleotide and deduced amino acid sequences of TL31 show much higher homologies with D3 sequences from various species than with D1 and D2 sequences from fish and other species. 2) The catalytic properties of the enzyme expressed in TL31-transfected COS-1 cell lysates (B, D) by increasing concentrations of unlabeled T₃, T₄ and rT₃ (A, B) or PTU, IAc and GTG (C, D). Conditions were: 10 nM [125I]T₃, 10 mM DTT, 0.1–0.25 mg protein/ml, and 1 h incubation at 37°C. Results are the means of closely agreeing triplicates in a representative experiment.

![Fig. 7](#)

**Fig. 7.** Inhibition of the IRD of [125I]T₃ by native tD3 in tilapia brain microsomes (A, C) and recombinant tD3 expressed in TL31-transfected COS-1 cell lysates (B, D) by increasing concentrations of unlabeled T₃, T₄ and rT₃ (A, B), or PTU, IAc and GTG (C, D). Conditions were: 10 nM [125I]T₃, 10 mM DTT, 0.1–0.25 mg protein/ml, and 1 h incubation at 37°C. Results are the means of closely agreeing triplicates in a representative experiment.

(1–5). However, tilapia D1 is much less sensitive to inhibition by GTG and IAc and is virtually insensitive to PTU (20, 36, 37). D2 and D3 from different species are even less sensitive than tilapia D1 to the effects of these inhibitors (1–5). Because Sec is supposed to be the catalytic center in all deiodinases, the reason for their differential sensitivities to these inhibitors remains an enigma (1–20).

The alignment of tD3 with the human, rat, *R. catesbeiana*, *X. laevis*, and chicken D3s reveals regions of high homology. The Kyte and Doolittle hydrophilicity plot strongly suggests that the highly conserved N-terminal sequence from Ala^{16} to Ile^{41} in tD3 represents a hydrophobic membrane-spanning domain that anchors the protein in the membrane of the endoplasmic reticulum or in the plasma membrane. Such a transmembrane domain has also been identified near the N terminus of other deiodinases (6, 8–20). Studies of the topography of mammalian D1 suggested that the N terminus is hidden in the lumen of the endoplasmic reticulum or in the plasma membrane. Such a transmembrane domain has also been identified near the N terminus of other deiodinases (6, 8–20). Studies of the topography of mammalian D1 suggested that the N terminus is hidden in the lumen of the endoplasmic reticulum with the major part of the protein exposed to the cytoplasm (51). Such an orientation fits with the requirement of thiols as cofactors for the deiodination of iodothyronines which are abundant in the reductive environment of the cytoplasm (52). These studies of D1 topography have also shown that basic amino acids flanking the transmembrane domain, which are located in positions 11 and 42–44 of tD3, are essential for proper insertion in the membrane (51).
are conserved throughout the iodothyronine deiodinase family and have been shown in rat D1 to be essential for enzyme activity (53). One of these may directly participate in the catalytic process by forming a hydrogen bond with the selenol group, further increasing its nucleophilicity (53, 54). Phe65 in rat and human D1 (11, 41) has been shown to be involved in binding of rT3. The absence of Phe in a corresponding position of D3 may contribute to the low affinity of rT3 for this enzyme.

Although incorporation of Sec into tD3 has not been demonstrated directly, our findings strongly suggest that this enzyme features a Sec residue in a position corresponding to the Sec residue in other deiodinases. Sec is encoded by the UGA opal stop codon if the termination of translation normally signalled by this codon is suppressed in the presence of a SECIS element in the 3’ UTR of the mRNA (47–50). The stem-loop structure predicted in the 3’ UTR of the tD3 cDNA contains most but not all of the consensus nucleotides observed in other SECIS elements (47–50). The putative tD3 SECIS element contains the sequence GUGA (nucleotides 1268–1271) instead of AUGA in other SECIS elements (47–50).

The function of this first adenosine is not clear, since it is not involved in the nonWatson/Crick base-pairing proposed by Walczak et al. (50). A similar deviation from the consensus SECIS element was found in the second putative SECIS element in tilapia D1 cDNA (20). The consequences of this substitution for the efficiency of Sec incorporation are unknown. However, the tD3 SECIS element appears to function effectively in COS-1 cells not only in the context of wild-type tD3 cDNA but also in a chimeric construct combining the coding sequence of human D2 and the 3’UTR of tD3.

The TL31 cDNA clone represents most of the tD3 mRNA because the size of the largest and most prominent band observed on Northern blots is only slightly bigger than TL31. Smaller mRNA species are observed in heart and spleen. The significance of these multiple mRNA species is unknown, but they may represent mRNA processing intermediates. However, the smaller D3 mRNA species in heart and spleen (~1 kb) are not expected to translate into functional protein because they are too short to contain both the initiator codon and the SECIS element. The high-stringency conditions used in the Northern analysis preclude hybridization of the tD3 riboprobe with D1 and D2 mRNA. This is supported by the barely detectable hybridization with RNA from liver and kidney which show abundant expression of D2 and D1, respectively (5, 20, 36, 37). Furthermore, hybridization with D1 (20) and D2 (55) riboprobes shows different hybridization signals.

The translational efficiency of the tD3 mRNA apparently shows substantial differences between tissues. Brain contains high levels of both D3 activity (37) and D3 mRNA. Even higher D3 mRNA levels are found in gill, although this tissue contains only limited D3 activity (37). The tailing observed with D3 mRNA from gill suggests high mRNA degradation. It is also remarkable that the Northern blots showed very little expression of D3 mRNA in tilapia liver, although D3 cDNA fragments were produced by RT-PCR of liver mRNA, and the tilapia liver cDNA library contained several independent TL31-like clones.

In conclusion, we have cloned and characterized D3 cDNA from tilapia. Together with the human, rat, chicken, and frog D3 sequences, the elucidation of a fish D3 sequence helps to define the conserved regions of these proteins which are essential for IRD activity.

Acknowledgments

We thank Hans van Toor for expert assistance with the HPLC analyses.

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