Cloning of a cDNA for the Type II Iodothyronine Deiodinase*

(Received for publication, August 3, 1995, and in revised form, August 23, 1995)

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Three types of iodothyronine deiodinase have been identified in vertebrate tissues. cDNAs for the types I and III have been cloned and shown to contain an in-frame TGA that codes for selenocysteine at the active site of the enzyme. We now report the cloning of a cDNA for a type II deiodinase using a reverse transcription/polymerase chain reaction strategy and RNA obtained from Rana catesbeiana tissues. This cDNA (RC5DI) manifests limited but significant homology with other deiodinase cDNAs and contains a conserved in-frame TGA codon. Injection of capped in vitro synthesized transcripts of the cDNA into Xenopus laevis oocytes results in the induction of deiodinase activity with characteristics typical of a type II deiodinase. The levels of RC5DI transcripts in R. catesbeiana tadpole tail and liver mRNA at stages XII and XXIII correspond well with that of type II deiodinase activity but not that of the type III activity in these tissues. These findings indicate that the amphibian type II 5'-deiodinase is a structurally unique member of the family of selenocysteine-containing deiodinases.

Intracellular concentrations of the thyroid hormones, T91 and T93, are profoundly influenced by the activity of three iodothyronine deiodinases, classified as types I, II, and III (1). In mammals, the type I enzyme (5DI) catalyzes 5'-deiodination (5'D), the removal of iodine from the 5' (or 3') positions of T94 and its derivatives. The enzyme can also catalyze 5-deiodination (5D), the removal of an iodine located at either the 5 (or 3) positions of iodothyronines, but does so efficiently only with sulfated iodothyronine substrates (2). The type II enzyme (5DII) also catalyzes 5'D, but it is readily distinguished from the SDI by its kinetics, substrate specificity, sensitivity to propylthiouracil (PTU) and aurothioglucose (ATHG) (1, 3), and response to thyroid status (1). The type III enzyme (5DIII) catalyzes primarily 5D activity (1), a process that results in derivatives with little or no thyromimetic activity (1).

The primary function of the types I and II deiodinases is to convert T94 to its metabolically more active derivative, T93. However, the tissue distribution and physiological roles of the two enzymes are very different. The principal role of the 5DI in mammals is to provide a source of plasma T93 by deiodination of T94 in peripheral tissues such as liver and kidney. In contrast, the 5DII is responsible for the majority of the intracellular T93 in tissues such as the pituitary, brain, and brown fat by mediating local deiodination of T94 and is considered to be of major importance in regulating thyroid hormone action in these tissues (1, 3). The 5DII also plays a major role during development. 5DII is the principal 5'-deiodinase expressed in the mammalian fetus, and it is notable that 5DII activity in brain peaks in the neonatal period, the time that is critical for thyroid hormone-dependent development in this tissue (4). Moreover, 5DII is the only 5'-deiodinase present in the developing frog in which the orderly progression of developmental processes is dependent on the ability to attain appropriate intracellular levels of T93 (5). Thus, the 5DII appears to play an essential role in intracellular T3 production in those circumstances where thyroid hormone-dependent processes take on critical significance.

cDNAs for the type I enzyme of rat (6), dog (7), and human (8) have been cloned. These cDNAs contain an in-frame TGA codon for selenocysteine, which is necessary for maximal enzyme activity (6). Three cDNAs for the type III enzyme have also been cloned; we have shown that XL-15, a cDNA isolated by Wang and Brown (9) from a Xenopus laevis tadpole tail cDNA library, encodes a 5DII (10) and, using XL-15 as a probe, we have isolated 5DII cDNAs for Rana catesbeiana (11) and rat (12). These cDNAs exhibit significant sequence homology to the mammalian 5DI cDNAs including the in-frame TGA codon, which codes for selenocysteine.

Isolation of a cDNA for a 5DII has yet to be reported. To this end we predicted that this enzyme would share significant sequence homology with other deiodinases. Close examination of the sequences of the known 5DI and 5DII cDNAs revealed that, although the overall similarity between the two types is relatively low, there are three limited regions that are highly conserved. One is near the TGA codon that codes for selenocysteine, and the other two are approximately 60 and 230 bp 3' of this codon. We hypothesized that these regions would also be conserved in the 5DII gene.

This hypothesis proved to be correct. In the present report we describe the cloning of a cDNA for the 5DII of R. catesbeiana using a reverse transcription/polymerase chain reaction (RT/PCR) strategy, oligonucleotide primers based on the sequences of these conserved regions, and RNA from R. catesbeiana tissues that contain relatively high levels of type II 5'D activity. Once a portion of the putative coding region of the 5DII cDNA was obtained, gene-specific primers were used to synthesize the 3'- and 5'-ends of the cDNA using rapid amplification of cDNA ends (RACE) procedures (13). The resulting cDNA (RC5DII) contains the conserved TGA codon and codes for a protein with characteristics typical of a 5DII.
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Materials and Methods

Animals—R. catesbeiana tadpoles, stages XX–XXIV (Taylor and Kilross (14)), were obtained from Charles D. Sullivan, Inc., Nashville, TN. Maintenance of tadpoles and preparation of total and poly(A) mRNA from their tissues were carried out as described previously (15).

Synthesis of a Partial cDNA for the 5DII from R. catesbeiana Tissues—The sequences of the primers used in the synthesis of the cDNA for the 5DII of R. catesbeiana are shown in Table I. Labeled primers (A, B, C, and D) are based on the conserved sequences of the 5D and 5DII cDNAs; numbered primers (1–6) are specific to the 5C5 II cDNA. Their locations relative to each other in 5C5 II are shown in Fig. 1A. A schematic representation of the RT/PCR-based cloning strategy is shown in Fig. 1B. Total RNA obtained from the hindlimb of tadpoles was reverse transcribed using primer C, and the resulting cDNA was amplified by PCR using primers A and C. The following cycling conditions were used in this and all subsequent PCR reactions: 94°C for 45 s; 52°C for 45 s; 72°C for 60 s for each of 30 cycles. This was followed by a 10-min extension at 72°C. The 110-bp product obtained was reamplified with primers A and B to yield a 98-bp product whose sequence, including the exons, exhibited 46% identity with the corresponding sequence in the R. catesbeiana 5C5 II cDNA (RC5 II). Then skin RNA from stage XXIII tadpoles was reverse transcribed using the oligo(dT) adapter primer from the 3′-RACE kit (Life Technologies, Inc.), and the resulting cDNA was amplified using primers 1 and D to yield a 238-bp product, whose sequence was 48% identical to that of RC5 II. This sequence information revealed that the 32 nucleotides in the “conserved” region used to design primers B and C are 84% identical to those in RC5 II.

Synthesis of the 3′- and 5′-Ends of 5C5 II Using the RACE Procedure—The 3′- and 5′-RACE procedures were carried out as recommended by the manufacturer (Life Technologies, Inc.) with one exception. Since in our experience the universal amplification primer (UAP), which is used in both procedures, routinely makes multiple products, a modified UAP (UAP*) was designed. The 12 nucleotides at the 5′-end, which are only necessary if the kit’s cloning procedure is employed, were eliminated and, with the aid of a computer program (OLIGOTM 4.0, National Biosciences, Inc., Plymouth, MN), the remaining UAP was modified to 5′-CTGCGACGCACGACAC-3′, which is used in both procedures, routinely makes multiple products, and is only necessary if the kit’s cloning procedure is employed. Sense primers are placed above, and antisense primers below, the line.

Table I

| Location | Strand | Sequence | Name | Location |
|----------|--------|----------|------|----------|
| A 368–384 | Sense | 5′-GGCCAGCTGACCACTGGACC-3′ | B 446–465 | 5′-ATGGATGATAAGGAGGCT-3′ |
| C 461–476 | Antisense | 5′-TGGACCTTCTCCAAATGTA-3′ | D 605–622 | 5′-CAATCTGCACCCCTCA-3′ |
| 1 385–403 | Sense | 5′-TCCCTTATATACGGCGTTG-3′ | 2 395–413 | 5′-ACCAGCTGGCCAGCTCA-3′ |
| 3 427–457 | Sense | 5′-GCCATGGAGCCACACC-3′ | 4 546–563 | 5′-TGGCGACGACACAGATG-3′ |
| 5 713–731 | Antisense | 5′-GGAGGTTGATTGAGAAGAG-3′ | 6 732–755 | 5′-GGCTCACCACATGCGCTTCTCT-3′ |

*Location in the 5C5 II cDNA (bp).

Fig. 1. Schematic representation of the RT/PCR-based technique used to synthesize and clone 5C5 II. A, a “generic” deiodinase cDNA coding region showing the approximate locations, relative to each other, of all the oligonucleotide primers employed. The sequences of primers A–D are based on the “conserved” regions of the three 5D and three 5DII deiodinase cDNAs; the sequences of primers 1–6 are specific to 5C5 II. B, PCR-based approach used to synthesize the full-length 5C5 II cDNA (see “Materials and Methods” for details).

and membrane fractions were prepared as described by Sharifi and St. Germain (17), and 5′ and 5D activities were measured according to published methods (17, 18). For the 5′D assay, the substrate was 1 nM [125I]T4 with 0–120 nM nonradioactive T3, and the cofactor was 20 μM diithiothreitol; for the 5D assay, 1 nM [125I]T3 was used as substrate and 50 μM diithiothreitol as cofactor. [125I]Iodothyronines (Dupont de Nemours & Co., Boston, MA) were purified by chromatography using Sephadex LH-20 (Sigma) before use. In some experiments the effect of PTU (0.001–0.1 mM) and AT3G (0.1–100 μM) on 5′D activity was examined.

Functional Analysis of 5C5 II by Deletions and Mutations—5C5 II was truncated in the 3′ untranslated region at position 906 using Dral. Mutants of 5C5 II were made in which the in-frame TGA codon (bp 380–382) was changed either to a TAA (stop) or to a TGT (cysteine) codon. The sequences of these mutants were verified. The ability of cDNA in vitro synthesized RNA transcripts of these cDNAs to induce 5′D activity following injection into X. laevis oocytes was then determined.

Analysis of RNA—Samples of poly(A) RNA from tissues of premetamorphic tadpoles (stages X–XI) and tadpoles undergoing metamorphic climax (stages XXIII–XXIV) were examined for the presence of 5C5 II-specific transcripts by Northern and slot blot analyses using methods previously described (19). Hybridizations and washings were carried out, respectively, at 42 and 50°C. Signals were visualized by autoradiography and quantitated with the 628E Phosphorimagr (Molecular Dynamics). Data are reported as arbitrary densitometric units.

Results and Discussion

The nucleotide and deduced amino acid sequences of 5C5 II are shown in Fig. 2A. 5C5 II is a 1459-bp cDNA with an open reading frame extending from bp 11 to 802 and an in-frame TGA codon at bp 380–382 that, by analogy with the cDNAs for the 5D and 5DII, is likely to code for selenocysteine. A schematic comparison of the 5C5 II protein with those of R. catesbeiana type III and the rat type I is shown in Fig. 2B. The areas of homology are indicated, and it is also
noted that the proteins exhibit a hydrophobic region at the amino-terminal end and two histidine residues to the TGA.

In the rat 5DI, these histidines have been shown to be critical for 5D activity (20).

5D activity was induced in X. laevis oocytes after injection of capped RNA transcripts obtained by in vitro transcription of RC5DII (Fig. 3A). No 5D activity was detected (data not shown). The 5D activity was not inhibited by PTU (0.1 mM), but the percent deiodination of the [125I]rT3 was greatly reduced in the presence of 3 nM non-radioactive rT3, indicating that the enzyme manifests a low Km for this substrate. We have shown previously that the 5D activity in tadpole tissues exhibits a low Km and is resistant to inhibition by PTU (5, 21), characteristics that are typical of the type II 5D activity described in mammals (1). In contrast, capped transcripts of the 5DI cDNA, G21, induced activity that was highly sensitive to PTU and appeared to have a relatively high Km. In addition, the 5D activity induced by the RC5DII transcripts was relatively insensitive to inhibition by AthG (Fig. 3B). The 5D activity induced in oocytes by RC5DII transcripts was considerably lower than that induced by transcripts of the G21 cDNA. The reason for this difference is not known. However, 5D activity is only minimally induced in X. laevis oocytes by rat brown adipose tissue mRNA, a tissue containing considerable type II 5D activity, and it is possible that other factors important for type II 5D activity are not optimal in this oocyte system.

In view of the indirect evidence suggesting that the mammalian 5DII is not a selenoprotein (3), including the fact that it catalyzes 5D activity that is relatively insensitive to PTU and AthG, the possibility that the protein coded by RC5DII is not a selenoprotein or that selenocysteine is not involved in the activity of the enzyme was investigated. It was found that 5D activity in oocytes injected with capped transcripts derived from mutant RC5DII cDNAs, where the TGA codon had been changed to TAA (stop) or TGT (cysteine), was essentially the same as levels obtained in uninjected oocytes. Furthermore, no induction of activity was observed after injection of transcripts derived from RC5DII truncated at bp 906, suggesting that the deleted 3′-untranslated region contains a selenocysteine insertion sequence. These findings provide strong evidence that RC5DII codes for a protein with selenocysteine at its catalytic site. These findings provide strong evidence that RC5DII codes for a protein with selenocysteine at its catalytic site. Previous studies by Berry and Larsen (3) had led to the suggestion that sensitivity of a deiodinase to PTU and AthG could serve as a marker for the presence of selenocysteine at the enzyme's active site. That this is not a valid criterion is demonstrated by the presence of the TGA codon for selenocysteine in the RC5DII cDNA described herein and in three recently isolated typeIII deiodinase cDNAs, all of which encode enzymes resistant to PTU and AthG (10–12). Thus other structural properties of these enzymes or differences in kinetic
mechanisms may dictate PTU and AT3H sensitivity.

The ontogenic profiles of 5'D and 5D activities in R. catesbeiana tadpole tail and liver are very different; 5'D activity is minimal in tadpole tail until the onset of metamorphic climax when it increases markedly reaching a maximum by stage XXIII, while liver is devoid of 5'D activity at all stages of development (21). In contrast, 5D activity is present in both tail and liver during metamorphosis, and in liver it is greatly reduced when the tadpoles reach metamorphic climax (22). To obtain additional evidence concerning the identity of RC5DII, the size and relative abundance of RC5DII-related transcripts in tail and liver poly(A)\(^\text{+}\) RNA obtained from premetamorphic (stage XII) tadpoles and metamorphosing (stage XXIII) tadpoles were determined by Northern analysis (Fig. 4). No RC5DII transcripts were detected in liver mRNA at either stage of development. This finding is consistent with the absence of 5'D activity in this tissue at all stages of the life cycle (5, 21). However, a major RNA species of approximately 1.5 kb was detected in tail RNA; two minor species were just discernible at approximately 1.8 and 2.2 kb. The 2.2-kb species may represent cross-hybridization with RC5D transcripts since comparable hybrids were detected in tail RNA; two minor species were just discernible at approximately 7.4 kb. The level of the 1.5-kb species was much higher in RNA from metamorphosing tail than in that from premetamorphic tail. Thus the profile of RC5DII transcripts in liver and tail corresponds closely to that of 5'D but not 5D activity in these tissues. The increase in RC5DII mRNA species in tail during metamorphic climax was quantified using slot blot analysis. Densitometric analysis indicated that the hybridization signals in tail and liver at stages X–XII and stages XXIII–XXIV were, respectively, 280 \pm 50 (S.E.) and 5151 \pm 345 units (p < 0.001). As with the Northern blot, no signal was observed in liver RNA. Reprobing of the blot with RC5DII transcripts, which were clearly evident in both liver and tail at both stages of development, were not increased during metamorphic climax (data not shown). Thus, the observed increase in the level of RC5DII-related transcripts on the blot cannot be attributed even in part to cross-hybridization of RC5DII with RC5D transcripts.

Additional evidence that RC5DII is an amphibian type II deiodinase is provided by our recent identification of rat and human homologues of this cDNA. Both are highly homologous to RC5DII within the coding region (rat, 71% human, 73%). Furthermore, the tissue distribution of their related mRNA transcripts, as determined by Northern analysis, is characteristic of the mammalian type II enzyme.

We thus conclude that RC5DII is the cDNA for the 5DII in R. catesbeiana. The characteristics of the deiodinase for which it codes are comparable with those of the 5'D activity in R. catesbeiana tadpole tissues (5) and in mammalian brain, pituitary, and brown fat (1). The fact that type I 5'D activity has not been detected in tissues of this amphibian species (5, 21) makes it highly unlikely that RC5DII codes for a form of the type I deiodinase. In addition, the data strongly suggest that the type II deiodinase coded by RC5DII is a selenoprotein. We have compared the protein sequences deduced from the seven cloned deiodinase cDNAs, and it is evident that the RC5DII protein has limited but significant homology with both the type I and the type III enzymes. Thus the amphibian type II deiodinase represents a structurally unique member of this family of selenocysteine-containing enzymes.

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