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The variable region of iodothyronine deiodinases directs their catalytic properties and subcellular localization

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ABSTRACT

The stereospecific removal of iodine from thyroid hormones is an essential first step for T3 action and is catalyzed by three different deiodinases: D2 and D3 remove iodine only from the outer or inner ring, respectively, whereas D1 catalyzes both pathways. We used *in silico* predictions from vertebrate deiodinase sequences to identify two domains: the N-terminal variable region (VR) containing the transmembrane, hinge and linker domains, and the conserved or globular region (CR). Given the high sequence and structural identity of the CR among paralogs as well as of the VR among orthologs but not paralogs, we hypothesized that both the catalytic properties and the subcellular localization rely on the VR. We used shark D2 and D3 as templates to build the chimeric enzymes D2VR/D3CR and D3VR/D2CR. Biochemical characterization revealed that D3VR/D2CR has inner-ring deiodination activity and T3 as preferred substrate, whereas D2VR/D3CR showed no deiodinating activity. Also, D2VR/D3CR and D3VR/D2CR reside in the endoplasmic reticulum and plasmatic membrane, respectively, as do their D2 and D3 wild-type counterparts. We conclude that the VR determines the subcellular localization and is critical in defining the catalytic properties and activity of thyroid hormone deiodinases.

1. Introduction

Thyroid hormone (TH) deiodination is the critical first step of thyroid hormone action and is catalyzed by a family of selenoenzymes known as deiodinases (Ds). It is well accepted that deiodinase activity determines the relative concentrations of bioactive *vs.* inactive intracellular THs, thus playing a key role in homeostasis of the vertebrate thyroidal system (reviewed by Bianco, 2011; Gereben et al., 2008b). Three Ds have been identified: D1, a multifunctional enzyme that can cleave iodine from the outer as well as the inner ring of an iodothyronine, thereby activating or inactivating the TH; D2 which functions predominantly as an activating deiodinase *via* outer ring deiodination (ORD), and D3 which only inactivates TH through inner ring deiodination (IRD) (Bianco and Larsen, 2005; Gereben et al., 2008a). Consequently, the selectivity and stereo-specificity of iodine removal from the thyroid hormone molecule determine the function of the deiodinase isotype. Interestingly, the three deiodinases show considerable similarity and share a common structural organization, suggesting that they may have diverged from a common ancestral gene (Darras and Van Herck, 2012; Laudet, 2011; Orozco et al., 2012; Valverde-R et al., 2004). In this context, the *in silico* modeling of human Ds suggests that the molecular conformation of the three paralogs consists of four functional domains known as: transmembranal (TM), hinge (H), linker (L), and catalytic or globular (G) (Callebaut et al., 2003). Furthermore, we recently reported that when the amino acid sequences of most expressed vertebrate deiodinases are aligned, two distinct major regions are revealed, which we have identified as the variable region (VR) and conserved region (CR) (Orozco et al., 2012). The VR includes the TM, H and L domains and is highly variable among paralogs, presenting only 20% molecular identity; however, this region is relatively conserved among orthologs (percent identity: D1, 50%; D2, 55% and D3, 60%). The CR, which comprises only the G domain, is very similar among the 3 paralogs (60% molecular identity) as well as among orthologs (percent identity: D1, 75%; D2, 79% and D3, 73%).

As previously mentioned, the function of each deiodinase isotype is the stereo-specific removal of an iodine atom from the thyroid hormone. It would be reasonable to propose that, since the catalytic reaction takes place in the CR where the active site resides, this region would, at least in part, determine the stereo-specificity of TH iodine removal, but this has not been demonstrated to date. An argument against this proposal is the high sequence conservation of this region among deiodinase paralogs. Moreover, mammalian D paralogs show a different subcellular localization: D2 is inserted into the endoplasmic reticulum membrane, whereas D1 and D3 are

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plasma membrane residents (Baqui et al., 2000, 2003; Friesema et al., 2006). No structure–function studies are available regarding the domains of Ds that direct subcellular localization or confer catalytic properties. Here, we provide experimental evidence showing that both stereo-selectivity and subcellular localization of Ds are influenced, to some extent, by the VR molecular structure.

2. Materials and methods

2.1. Animals

Sharks (*Chiloscyllium punctatum*) ranging from 25 to 50 cm in total length were obtained from a commercial fish collector and held at 25 ± 1 °C in a seawater aquarium under a 12-h:12-h L:D photoperiod. Sharks were anesthetized in MS-222 (0.5 g L^{-1}) prior to sacrifice, and the livers were immediately dissected and quick frozen. All animal procedures were approved by the institutional animal care committee.

2.2. Cloning and sequencing of shark D2

Shark D2 (sD2) was cloned as previously described for shark D3 (sD3) (Martínez et al., 2008). Briefly, 10 μg of total RNA from shark liver was reverse transcribed (SuperScriptTM ll, RNase reverse transcriptase; Invitrogen, Carlsbad CA) with an oligo(dT) primer. Degenerate primers recognizing the sequence coding for the active site of deiodinases (Supplement Table S1) were used in touchdown PCRs (Platinum Taq DNA Polymerase, Invitrogen, Carlsbad CA), and the amplicon (130 bp) was subcloned into a pGEM-T vector (Promega; Madison, WI) and sequenced. The open reading frame (ORF) of sD2 was amplified by 3′ and 5′ rapid amplifications of cDNA ends (RACEs) in a series of nested PCRs using specific primers designed based on the initial 130 bp fragment, as previously described (Martínez et al., 2008; Orozco et al., 2002, 2003). The amplified products from both 3′ and 5' RACE were cloned and sequenced. In order to express a functional protein, the ORF of sD2 was linked to the selenocysteine insertion sequence (SECIS) from killifish D2 using a hybrid pair of oligonucleotides (Supplement Table S1), as previously described (Orozco et al., 2002). The entire cDNA sequence that included the SECIS was amplified using sense and antisense primers that contained NcoI and NotI sites, respectively, for site-directed cloning into a pXENEX1 vector (see discussion later).

2.3. Construction of deiodinase vectors

2.3.1. Chimeras

The chimeras were generated by PCR by fusing the entire VR of sD2 to the CR of sD3 and *vice versa*, the VR of sD3 to the CR of sD2. The resulting products were named D2VR/D3CR and D3VR/D2CR, respectively. To this end, internal hybrid primers (Supplement Table S1) were prepared to amplify those segments that correspond to the VR and CR of each enzyme as illustrated in Fig. 1b. VR and CR fragments form sD2 and sD3 were independently amplified (PCR), and the corresponding fragments (sD2 + sD3 and sD3 + sD2) were hybridized in two, sequential PCR reactions in which the first protocol was designed to amplify the template and did not include primers, whereas the second protocol included the corresponding external primers and extended the complete chimeras. In all cases, the entire chimeric cDNA sequences were amplified using sense and antisense primers that contained the NcoI and NotI sites, respectively, for site directed cloning into a pXENEX1 vector (see discussion later) and verified by sequencing.

2.3.2. GFP-tagged deiodinases

ORFs for D3, D2, D2VR/D3CR or D3VR/D2CR were subcloned using EcoRI/BamHI sites into a pEGFPN1 vector [Clontech (Mountain View, CA)]. In all cases, the selenocysteine coding codon was replaced by the cysteine codon to allow expression of GFP C-terminal GFP-tagged deiodinases. Constructs were confirmed by sequencing.

2.4. Expression of recombinant shark deiodinases

As previously described, cDNA constructs of the native sD2 and sD3, as well as the chimeras D2VR/D3CR and D3VR/D2CR, were digested and ligated into a vector (pXENEX1) designed to express RNA in *Xenopus* oocytes (Jeziorski et al., 1998). The cDNAs were verified by sequencing and linearized with HindIII [New England Biolabs (Ipswich, MA)], purified, and used to transcribe capped RNA using T7 polymerase. Stage V–VI oocytes were removed from *Xenopus* under MS-222 anesthesia and treated as previously described

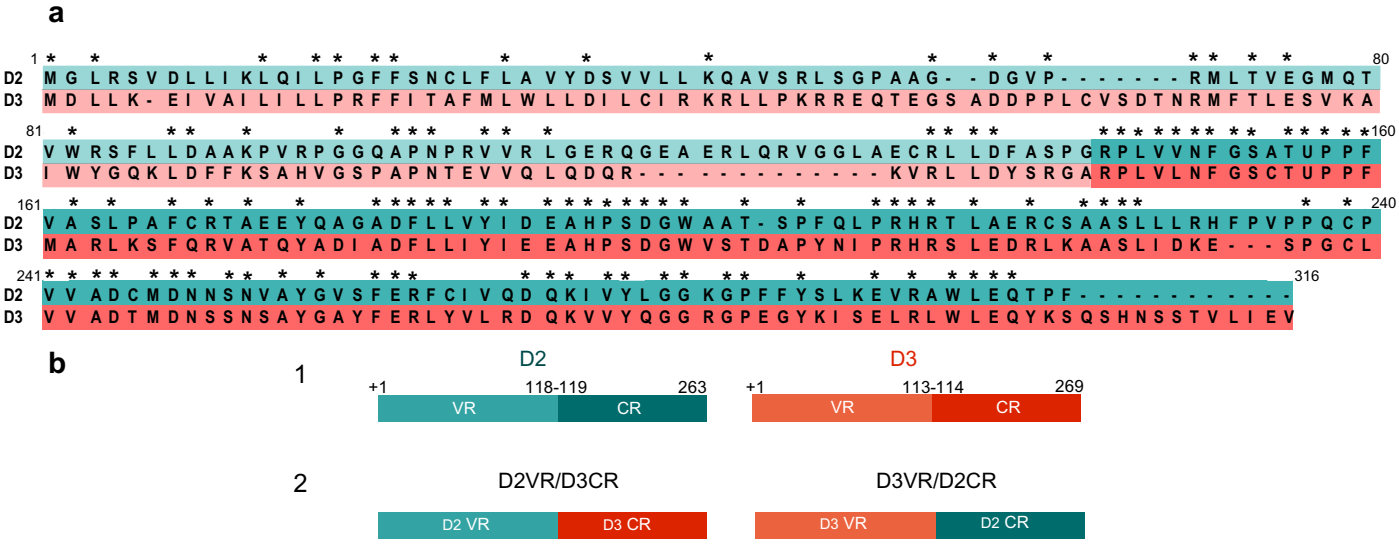

Fig. 1. Construction of shark deiodinase chimeras. (a) Full amino acid sequence of shark D2 (Accession number KP455983) and D3 (Accession number ABX60542.1). The invariant amino acids are indicated with an asterisk. Light green and light red highlight the VR while dark green and dark red highlight the CR. (b) Schematic representation of VR and CR from D2 and D3 with their corresponding amino acid numbering; D2VR/D3CR and D3VR/D2CR chimeras were constructed by exchanging the entire VR of D2 with the CR of D3, and *vice versa*. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)