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Research paper

Characteristics of the brown hagfish *Paramyxine atami* transthyretin: Metal ion-dependent thyroid hormone binding

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ABSTRACT

Transthyretin (TTR) is a vertebrate-specific protein involved in thyroid hormone distribution in plasma, and its gene is thought to have emerged by gene duplication from the gene for the ancient TTR-related protein, 5-hydroxyisourate hydrolase, at some early stage of chordate evolution. We investigated the molecular and hormone-binding properties of the brown hagfish *Paramyxine atami* TTR. The amino acid sequence deduced from the cloned hagfish TTR cDNA shared 33–50% identities with those of other vertebrate TTRs but less than 24% identities with those of vertebrate and deuterostome invertebrate 5-hydroxyisourate hydrolases. Hagfish TTR, as well as lamprey and little skate TTRs, had an N-terminal histidine-rich segment, allowing purification by metal-affinity chromatography. The affinity of hagfish TTR for 3,3′,5-triiodo-L-thyronine (T3) was 190 times higher than that for L-thyroxine, with a dissociation constant of 1.5–3.9 nM at 4 °C. The high-affinity binding sites were strongly sensitive to metal ions. Zn^{2+} and Cu^{2+} decreased the dissociation constant to one-order of magnitude, whereas a chelator, *o*-phenanthroline, increased it four times. The number of metal ions (mainly Zn^{2+} and Cu^{2+}) was approximately 12/TTR (mol/mol). TTR was also a major T3-binding protein in adult hagfish sera and its serum concentration was approximately 8 μM. These results suggest that metal ions and the acquisition of N-terminal histidine-rich segment may cooperatively contribute to the evolution toward an ancient TTR with high T3 binding activity from either 5-hydroxyisourate hydrolase after gene duplication.

1. Introduction

The gene encoding transthyretin (TTR), a plasma thyroid hormone (TH)-binding protein, is thought to have emerged by gene duplication from the gene for the ancient TTR-related protein, 5-hydroxyisourate hydrolase (HIUHase; EC 3.5.2.17) at some stage during chordate evolution. Genes for TTR have been identified in vertebrate groups from agnatha to mammals (Zanotti et al., 2006; Hennebry et al., 2006). In contrast, genes for TTR-related protein or HIUHase have been identified in a broad range of phylogenetic groups, including eubacteria and eukaryotes (Zanotti et al., 2006; Hennebry et al., 2006). HIUHase is an enzyme that catalyzes the formation of 5-hydroxy-2-oxo-4-ureido-2,5-dihydro-1H-imidazole-5-carboxylate from 5-hydroxyisourate (HIU) in the urate catabolic pathway (Ramazzina et al., 2006; Jung et al., 2006). Despite no functional relationship between the TTR and HIUHase families, they share approximately 35% identities in their primary sequences (Eneqvist et al., 2003). However, one or two amino acid substitutions at the active site of HIUHase generated two cavities for TH binding and abolished HIUHase activity simultaneously (Cendron et al., 2011; Li et al., 2013).

The extant agnatha consisting of the lamprey and hagfish groups stands in a basal phylogenetic position of vertebrate evolution, and separated from the gnathostome (jawed vertebrates) lineage 500–600 million years ago, probably after two rounds of whole-genome duplication (Kuraku et al., 2009). The thyroid system of vertebrates may have been gradually established at an early stage of chordate evolution (Paris and Laudet, 2008). There are limited data available regarding components constituting the hagfish

Abbreviations: BSA, bovine serum albumin; HIC, hydrophobic interaction chromatography; HIU, 5-hydroxyisourate; HIUHase, 5-hydroxyisourate hydrolase; HPLC, high performance liquid chromatography; K_d, dissociation constant; kDa, kilodalton(s); MBC, maximum binding capacity; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA end; RT, reverse transcription; rRNA, ribosomal RNA; rT3, reverse T3 (3,3′,5′-triiodo-L-thyronine); SDS-PAGE, sodium dodecyl sulfatepolyacrylamide gel electrophoresis; T3, 3,3′,5-triiodo-L-thyronine; T4, L-thyroxine; TBG, thyroxine-binding globulin; TBS, Tris-buffered saline; Tetrac, 3,3′,5,5′-tetraiodothyroacetic acid; TH, thyroid hormone; Triac, 3,3′,5-triiodothyroacetic acid; TTR, transthyretin.

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thyroid system: TH synthesis in the thyroid cells (Ohmiya et al., 1989; Chanet and Meunier, 2014), and the presence of THs in plasma (Henderson and Lorscheider, 1975; Henderson, 1976), deiodinases in several tissues (McLeese et al., 2000) and the identification of a TH receptor transcript (Genbank accession No. AY211768).

However, the proteins involved in TH transport or distribution in hagfish plasma are unknown. In the gnathostomes, most THs in plasma are distributed bound to protein. Major plasma TH-binding proteins are albumin, thyroxine-binding globulin, so-called TBG, TTR and lipoproteins in gnathostomes, which form a network for TH distribution in plasma (Richardson, 2007). TBG is only found in mammals, whereas the other TH-binding proteins are found in all groups of the gnathostomes (Yamauchi and Ishihara, 2009).

We have focused on elucidating the function of the hagfish TTR to gain insight into how TTR was recruited to the thyroid system as a TH-binding protein after the HIUHase gene duplicated. In this study, we cloned the cDNA for TTR from the brown hagfish *Paramyxine atami* liver, purified the recombinant and corresponding serum TTRs, and then determined their molecular characteristics and TH-binding properties.

2. Materials and methods

2.1. Reagents

3,3′,5-[^{125}I]-Triiodo-L-thyronine ([^{125}I]T3) (81 TBq/mmol, carrier-free) was obtained from PerkinElmer (Waltham, MA, USA). Unlabeled T3, L-thyroxine (T4), 3,3′,5′-triiodo-L-thyronine (rT3), 3,3′,5-triiodothyroacetic acid (Triac), 3,3′,5,5′-tetraiodothyroacetic acid (Tetrac) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ni-affinity resins were from Bio-Rad (Ni-IMAC Profinity, Hercules, CA, USA) and Invitrogen (Probond Nickel-Chelating Resin, Carlsbad, CA, USA), and Co-resin was from Clontech (Talon Metal Affinity Resin, Mountain View, CA, USA). Uricase and urate were purchased from Wako Pure Chemicals (Tokyo, Japan). All other chemicals used in this study were either chromatography grade or the highest grade available and were obtained from Nacalai Tesque (Kyoto, Japan) or Wako Pure Chemicals.

For the binding assay, unlabeled iodothyronines or their analogs were dissolved in dimethyl sulfoxide to a concentration of 2 mM and diluted with an appropriate buffer to give less than 0.3% (v/v) solvent. Although control assays without unlabeled iodothyronines were performed in the presence of dimethyl sulfoxide at the same concentrations, the solvent did not affect the assay results.

2.2. Animal care and ethics

Adult brown hagfish (*P. atami*), 40–50 cm in length, were caught at a depth of approximately 300 m in Suruga Bay, Shizuoka, Japan, in March 2010 and May 2012, by fishermen (Yaizu, Shizuoka, Japan), who have routinely caught them like the other commercial fishes in Suruga Bay, Shizuoka, Japan, and were transported to Shizuoka University. All animal experiments were performed according to the "Act on Welfare and Management of Animals" (Ministry of Environment of Japan), using procedures approved by the guideline for "Animal Research of Shizuoka University". All hagfishes were anesthetized using ethyl *p*-aminobenzoate (benzocaine; Sigma-Aldrich), before tagging, handling and sacrifice. All efforts were made to minimize animal handling and stress. Blood was collected by cardiac puncture from anesthetized animals. Chicken blood was also collected at a local abattoir in Shizuoka, Japan. Serum was separated from blood cells by centrifugation at 400*g* for 15 min at 4 °C, and was immediately used or stored at −35 °C. Tissues were harvested, and were immediately frozen in liquid nitrogen and stored at –84 °C until used.

2.3. cDNA cloning

Total RNA was prepared from the liver of adult *P. atami* using a kit (QIAamp RNA Blood Mini kit, Qiagen, Hilden, Germany). RNA integrity was confirmed by agarose gel (1%) electrophoresis containing 2.6 M formaldehyde, and 28S and 18S ribosomal RNAs (rRNAs) were visualized by ethidium bromide staining in an image analyzer (LAS-4000 miniEPUV, Fujifilm, Tokyo, Japan). Poly(A)$^+$ RNAs were prepared from the total RNAs using PolyATract® mRNA Isolation System (Promega, Madison, WI, USA) according to the manufacturer's instructions. Single stranded cDNAs were generated using reverse transcriptase (TaqMan® Reverse Transcription Reagents, Applied Biosystems, Foster City, CA, USA) and oligo $(dT)_{16}$ primer (2.5 μM) according to the manufacturer's instructions. Polymerase chain reaction (PCR) of the cDNAs was done using Taq DNA polymerase (TaKaRa Ex Taq™, Takara, Otsu, Shiga, Japan) with the primers F2 and R2 (each 1.0 μM; Table S1), which were designed using the lamprey (DQ855960 for *Petromyzon marinus* and DQ855961 for *Lampetra appendix*) and teleost TTR nucleotide sequences (BC164894 for *Danio rerio* and AF047443 for *Sparus aurata*). The approximately 0.3 kbp amplified PCR product was ligated into pMD20 T-vector (Takara).

To obtain 3′ and 5′ regions of the hagfish TTR cDNA, rapid amplification of cDNA end (RACE) was carried out using a 5′/3′ RACE kit 2nd Generation (Roche, Mannheim, Germany). After reverse transcription of the poly(A)$^+$ RNAs, RACE was done with oligo(dT)-anchor primer in the kit and specific primer (12.5 μM, F2 for 3′-RACE or R6 for 5′-RACE), and subsequently with the anchor primer in the kit and another specific primer (12.5 μM, F5 for 3′-RACE). Both approximately 0.45 kbp amplicons of the 5′- and 3′-RACE were ligated into pT7Blue T-vector (Novagen, Madison, WI, USA) or pMD20 T-vector (Takara). Finally, PCR reaction of the single stranded cDNA mixture was done using Taq DNA polymerase with the primers F8 and R11 to get a full length of the TTR cDNA. Plasmid DNAs were purified and the insert DNAs were sequenced (accession No. LC177972). Primers used for PCR cloning were listed in Table S1.

2.4. Phylogenetic analysis

Protein sequences were collected from original papers or the following databases: NCBI (http://www.ncbi.nlm.nih.gov/), Ensembl (http://www.ensembl.org/index.html). 5-Hydroxyisourate hydrolase sequences were obtained from the following species: purple sea urchin *Strongylocentrotus purpuratus* (XP_793771; 152-262 amino acid residues), acorn worm *Saccoglossus kowalevskii* (NM_001168202), Japanese lancelet *Branchiostoma japonicum* (JX878390), Florida lancelet *B. floridae* (XP_002600822, NW_003101473), zebrafish *D. rerio* (EH487643), rainbow trout *Oncorhynchus mykiss* (BX313165), Atlantic cod *Gadus morhua* (ES471358), gilt-head seabream *S. aurata* (AM952201), Nile tilapia *Oreochromis niloticus* (XM_003442328), West Indian Ocean coelacanth *Latimeria chalumnae* (ENSLACT00000025307), tropical clawed frog *Xenopus tropicalis* (XM_002933681), pig *Sus scrofa* (XM_013994248) and house mouse *Mus musculus* (NM_029821). Transthyretin sequences were obtained from the following species: brown hagfish *P. atami* (this study), American brook lamprey *L. appendix* (DQ855961), sea lamprey *P. marinus* (DQ855960), little skate *Leucoraja erinacea* (CV221819), *D. rerio* (NM_001005598), channel catfish *Ictalurus punctatus* (FD017171), *O. mykiss* (CX256523), *G. morhua* (ES240035), *S. aurata* (AF047443), Pacific bluefin tuna *Thunnus orientalis* (Kawakami et al., 2006), spotted green pufferfish *Tetraodon nigroviridis* (CR652101), *L. chalumnae*

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