Note

RT-PCR- and MALDI-TOF Mass Spectrometry-Based Identification and Discrimination of Isoforms Homologous to Pufferfish Saxitoxinin- and Tetrodotoxin-Binding Protein in the Plasma of Non-Toxic Cultured Pufferfish (Takifugu rubripes)

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Four genes of Takifugu rubripes, tentatively designated Trl–Tr4, encoding homologs of pufferfish saxitoxinin- and tetrodotoxin-binding protein, were identified by BLAST search and 3′-RACE. RT-PCR and MALDI-TOF mass spectrometry allowed the identification and discrimination of Tr isoforms from the non-toxically cultured specimens. The expression of Trl and Tr3 mRNAs exclusively in the liver and the presence of their products as 120-kDa plasma proteins were confirmed.

Key words: Takifugu rubripes; tetrodotoxin; toxin-binding protein; (MALDI-TOF) mass spectrometry

Marine pufferfish generally possess the potent neurotoxin tetrodotoxin (TTX). Many recent reports have clarified that TTX in the fish is exogenous via the food chain (as reviewed by Noguchi and Arakawa, 2008).1) Thus, cultured with TTX-free diets by netcage or land transportation, accumulation, metabolism, and excretion remains largely unclear. Matsui et al. purified and partially characterized the TTX-binding protein from the plasma of Takifugu niphobles.2) Yotsu-Yamashita et al. isolated an acidic glycoprotein that binds to TTX and saxitoxin (STX) from the plasma of wild-caught Takifugu pardalis, and designated it pufferfish saxitoxinin- and tetrodotoxin-binding protein (PSTBP).3) Two cDNAs encoding distinct PSTBP isoforms (PSTBP1 and PSTBP2, with 93% amino acid sequence identity) were cloned and sequenced.4) Although it has been suggested that PSTBPs appear as non-covalently linked dimers of a single subunit,4) it is unknown whether PSTBPs form homodimers or heterodimers with both isoforms or either one (PSTBP1 or PSTBP2). Western blot analysis using IgG antibody against recombinant PSTBP1 suggested that a PSTBP homolog of 124 kDa (as estimated by SDS–PAGE) is present in the plasma of wild T. rubripes.5) Structurally, PSTBP is classified as a member of the lipocalin superfamily, and it shares weak sequence similarity with other lipocalin members such as tributyltin-binding proteins (TBT-bps), alpha1-acid glycoprotein, and male-specific proteins.6) The lipocalin protein family is a large group of extracellular proteins that bind small hydrophobic molecules trapping to the β-barrel pocket, to act in the transportation and clearance of endogenous and exogenous compounds.7) Recently, tissue-specific and ligand-responsive expression of a distinct lipocalin isoform was investigated in fish, suggesting that each of the lipocalin isoforms has a specific biological significance.8,9) On the other hand, the molecular diversity and biological significance of PSTBP isoforms have not yet investigated in detail. The tiger puffer (T. rubripes), which is known to possess the smallest genome size of any vertebrate, has become a model organism suitable for post-genomic study in molecular biology and molecular genetics, owing to the draft genome sequencing10) and large-scale expressed sequence tag (EST) analysis.11) Post-genomic researches, T. rubripes ortholog searches utilizing sequence databases, have been reported12,13) In the present study, we found four T. rubripes genes encoding PSTBP homologs in the published databases, of which three genes appeared to have partial open reading frames (ORFs). The complete ORFs of the two genes were deduced by 3′-RACE analysis. Based on RT-PCR and MALDI-TOF mass spectrometry, we achieved the identification and discrimination of individual PSTBP isoforms from the plasma of non-toxic cultured T. rubripes.

The nucleotide sequences of PSTBP homologs were searched with Takifugu pardalis PSTBP1 (accession no. Q90WJ7) as query sequence by BLAST at the JGI Takifugu (Fugu) rubripes v4 genome browser (http://genome.jgi.doe.gov/Takru4/Takru4.home.html), the Ensembl Fugu genome browser (http://www.fugu-sg.org/), and NCBI Basic BLAST (http://blast.ncbi.nlm.nih.gov/). The non-toxic puffers (T. rubripes) used in this study were obtained by netcage culture, as reported

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Abbreviations: PSTBP, pufferfish saxitoxinin- and tetrodotoxin-binding protein; Tr, toxin-binding protein-related; BLAST, Basic Local Alignment Search Tool; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TTX, tetrodotoxin; STX, saxitoxin; ORF, open reading frame; 3′-RACE, rapid amplification of 3′cDNA ends; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; PTH-AA, phenylthiobutyrylamino acid; RT-PCR, reverse transcription polymerase chain reaction
previously. Total RNA was isolated from frozen tissues (skin, muscle, liver, and ovary, stored at -80 °C) of a non-toxic specimen (female, body weight 439.1 g) using TRIzol reagent (Invitrogen, Boston, MA, USA) in accordance with the manufacturer’s instructions. To obtain first-strand cDNA, total RNA (2 μg) from the liver (for 3'-RACE) or from each tissue (for RT-PCR analysis of mRNA expression) was reverse-transcribed in a 20-μL reaction volume using a PrimeScript II first-strand cDNA Synthesis Kit (Takara, Shiga, Japan) with anchor-tagged oligo dT primer (AP: 5'-GCCACGCGG-TCGACTAGTAC-3' + dT17) for 3'-RACE, or with oligo dT primer (included in the kit) for RT-PCR of mRNA expression analysis. Gene-specific primers for 3'-RACE were designed from the published draft genome sequence of *T. rubripes*: Tr1F1 (5'-ATCAGAGACGCACCTTCTCC-3'), Tr1F2 (5'-ACTCTGCTGTCTCATCAAGTT-3'), Tr3F1 (5'-CGTCGCTAACACCAGCTGAAA-3'), and Tr3F2 (5'-GAGAGGGAGGTCACTGACAGCC-3'). 3'-RACE was performed with an initial combination of primer pairs for Tr1 (Tr1F1 and AP) and Tr3 (Tr3F1 and AP), followed by nested-PCR using primer pairs for Tr1 (Tr1F2 and AP) and Tr3 (Tr3F2 and AP). First-round PCR was performed with 1 cycle of 3 min at 94 °C, 30 cycles of 1 min at 94 °C, 1 min at 51 °C (Tr1) or 55 °C (Tr3), 1 min (Tr1) or 2 min (Tr3) at 72 °C, and 1 cycle of 7 min at 72 °C, in a 25-μL reaction mixture containing 12.5 μL of Go Taq Green Master Mix (Promega, Madison, WI, USA), 0.5 μL of primer pairs (1 μM each), 0.5 μL of first-strand cDNA, and 11 μL of nuclease-free water. Second-round PCR was performed in the same way, except for primer pairs (Tr1F2 and AP for Tr1, and Tr3F2 and AP for Tr3), amplification steps (20 cycles), and template DNA (the first-round PCR product). The PCR products were purified by the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA), and directly sequenced at the Division of Functional Genomics, Center for Frontier Life Sciences Nagasaki University, using an ABI PRISM 3130 sequencer (Applied Biosystems, Carlsbad, CA, USA). The 3'-RACE products were sequenced bi-directionally by primer walking, and the sequences were submitted to DDBJ/EMBL/GenBank under accession nos. AB719395 (Tr1) and AB719396 (Tr3). Plasma was prepared from the blood of five non-toxic *T. rubripes* (male and female, body weight 452.1 ± 45.8 g), as described by Yotsu-Yamashita et al.10 PSTBP-like protein was enriched from total plasma proteins by stepwise ammonium sulfate fractionation (50% saturation, followed by 50–70% saturation). The supernatant obtained at 70% saturation of ammonium sulfate was filtered through a centrifugal filter device (Ultrafree-MC, 0.45 μm filter unit, Millipore, Bedford, MA, USA) to remove insoluble buoyant particles, dialyzed against water, and concentrated by SpeedVac (Thermo Scientific, Kanagawa, Japan). SDS–PAGE was performed by the method of Laemmli.11 Reductive alkyla-
tion and in-gel digestion were carried out as described by Shevchenko et al.15 Peptide extraction and MALDI mass spectrometry were performed as previously described.16 MS and MS/MS spectra were obtained using a MALDI-QIT-TOF mass spectrometer (AXIMA Resonance, Shimadzu, Kyoto, Japan) in positive mode. Protein electrophoresis onto a polyvinylidene difluoride (PVDF) membrane (Sequi-Blot, Bio-Rad, Hercules, CA, USA) was performed using a tank-blotter (Criteron Trans-Blot Cell, plate electrode type, Bio-Rad, Hercules, CA, USA) in ice-cold blotting buffer (25 mM Tris, 192 mM glycine, pH 8.3) at 100 V for 30 min. Under these conditions, all the proteins of the pre-stained marker were transferred completely to the membrane. Protein sequencing was done employing a gas-phase protein sequencer (PSQ-33A, Shimadzu Biotech, Kyoto, Japan). BLAST searches identified four distinct *T. rubripes* genes significantly homologous to pufferfish saxitoxin- and tetrodotoxin-binding protein (PSTBP) from *T. par- dalis*, three from the genomic sequences of scaffold_533 (93081–93572), scaffold_10076 (632–1879), and scaffold_3348 (822–1688), and one from an mRNA sequence (an EST assembly of CA333584 and CA333650). Tentatively we designated these putative genes Tr1 to Tr4 (toxin-binding protein related genes 1–4). Since the 3'-portions of the Tr1 and Tr3 genes were not available from the draft genome sequence, 3'-RACE was performed to obtain 3'-side sequences. The open reading frame (ORF) of the Tr1 gene was obtained by overlapping the nucleotide sequence of scaffold_533 (93081–93572) with that of the RACE product (AB719395), and that of the Tr3 gene by scaffold_3348 (822–1688) and AB719396. The alignment of the deduced amino acid sequences of the Tr proteins with PSTBP1 is shown in Fig. 1. Unlike PSTBP1, which contains two lipocalin domains, Tr1 and Tr2 appeared to be novel PSTBP homologs having a single lipocalin domain. Having two lipocalin domains in tandem, Tr3 is probably a functional counterpart of PSTBP1. Tr4 is presumably a multi-domain PSTBP-like protein (Fig. 1). The expression of Tr mRNAs in non-toxic *T. rubripes* was analyzed by RT-PCR. As shown in Fig. 2A, liver-specific expression of the Tr1 and Tr3 transcripts was observed among the tissues tested. No amplification of Tr2 or Tr4 cDNAs was seen in our specimen. Two ESTs (CA333584 and CA333650), corresponding to cDNAs of the Tr4 were derived from the liver of an adult puffer, whereas our specimen was a juvenile (before sexual maturation). Tr4 mRNA expression could fluctuate in the process of sexual maturation and toxification, though this must be investigated further. We, then, examined Tr gene expression at protein level in the non-toxic cultured specimens. Since the presence of PSTBP-like protein (124-kDa) in wild *T. rubripes* has been suggested by immunoblot analysis,5 we separated the corresponding plasma protein by SDS–PAGE (a 120-kDa band, Fig. 2B), and analyzed its in-gel digest by MALDI-TOF mass spectrometry. The MS spectrum of the tryptic digest from the 120-kDa plasma protein is shown in Fig. 2C. By MS/MS analysis of the major peptide ions, the 120-kDa protein was identified as a protein product of Tr genes: m/z 1,115.69, assigned to Tr1, Tr2, and Tr3; m/z 1,237.85, to Tr2 or Tr3; and m/z 1,285.89 and 1,300.77, exclusively to Tr1 (Fig. 2C). Because the RT-PCR analysis indicated no expression of Tr2 mRNA, the 120-kDa protein might be a mixture of Tr1 and Tr3. The unambiguous N-terminal sequence of the 120-kDa protein (APSXEEHKLT-KAV) as determined by automated protein sequencer also confirmed the 120-kDa protein to be a mixture of Tr1 and Tr3, and this suggests that the signal peptide Toxin-Binding Protein Homologs of Non-Toxic Cultured Pufferfish 209
cleavage site is conserved between the Tr proteins and PSTBP1 (see Fig. 1). The theoretical masses calculated from the deduced mature polypeptides of Tr1 and Tr3 were 23.5 kDa and 43.6 kDa respectively, and the sizes were not consistent with the observed mass (120 kDa). This discrepancy was probably due to heavy glycosylation (Tr1 and Tr3) and the formation of an SDS-resistant dimer (Tr1). Yotsu-Yamashita et al. have reported that Glycopeptidase F-treated PSTBP-like protein appeared as an approximately 43-kDa band on immunoblot analysis. Since the size of the deglycosylated form (43 kDa) exactly matched the sequence mass of Tr3, mature Tr3 must be largely modified by N-glycan (Fig. 1, potential N-glycosylation sites). Tr1 might form an SDS-resistant dimer even under reducing conditions, as observed for other lipocalins, e.g., dimerization of α-1-microglobulin via in vivo covalent cross-linking with oxidized metabolites, and that of β-lactoglobulin via ex vivo denaturation/aggregation. The possibility that one of the 120-kDa proteins is the product of an unidentified gene that encodes tandem-repeated Tr1-like domains, however, cannot be ruled out. The concentrations of the 120-kDa proteins (Tr1 and Tr3) in the plasma of non-toxic T. rubripes was estimated to be 0.084 nmol/mL, calculated in terms of the initial yields of PTH-AA from blots of standard apomyoglobin (62.1 pmol/100 pmol blot) and the 120-kDa protein (Fig. 2B, a 10.41 pmol/band from 0.2 mL plasma). PSTBP accounts for 0.24% (w/w) of total plasma protein in wild T. pardalis, that is, the PSTBP concentration in the plasma is assumed to be 2.10 nmol/mL (43 kDa as polypeptide of the monomer). Although the concentration of Tr proteins in non-toxic cultured T. rubripes was 25-fold lower than that of PSTBP in the wild T. pardalis, constitutive expression of the two distinct isoforms (Tr1 and Tr3) among the four Tr genes was obvious. Tr1 and Tr3 may play roles in acute-toxin uptake and delivery, taking part in immediate toxin-recognition in the blood circulation and not requiring toxin-triggered gene expression. Recently, it was reported that intramuscular administration of TTX upregulates the expression of mRNAs encoding acute-phase response proteins, such as hepclin, complement C3, and serotransferrin, in the liver of
Fig. 2. Analyses of Tr Gene Products in Non-Toxic Cultured T. rubripes.

A. Tissue-specific expression of Tr transcripts. The gene-specific primers used for RT-PCR analysis were Tr1F3 (5'-CTGGGTTCTGGTCTGGTACATCAG-3'), Tr1R (5'-CTTCACCGTGCGCTTCGAGGTG-3'), Tr2F (5'-GGAAATGGGGGGTTGTCAAGAATAG-3'), Tr2R (5'-CTCCGTCTGAGATGAAAGCCTGT-3'), Tr3F (5'-CGTAAACCACTGAGAAGTGATC-3'), Tr3R (5'-CTTTGAGCATCTAGTACCTC-3'), Tr4F (5'-CTCGCCAACATGTGCTTGACCC-3'), and Tr4R (5'-GTCCTAGATGAGGCGTGATGCG-3'). The primer pairs used were Tr1F3 and Tr1R for Tr1, Tr2F and Tr2R for Tr2, Tr3F and Tr3R for Tr3, and Tr4F and Tr4R for Tr4. The expected sizes of RT-PCR products were Tr1F (242 bp), Tr2 (233 bp), Tr3 (353 bp), Tr4 (289 bp), and beta-actin (324 bp). The PCR conditions in RT-PCR analysis were 1 cycle of 3 min at 94 °C, 35 cycles of 10 s at 94 °C, 20 s at 58 °C, 30 s at 72 °C, and 1 cycle of 7 min at 72 °C, in a 25-μL reaction mixture. Beta-actin mRNA (accession no. U37499), a transcript of the housekeeping gene, was used as positive control following Kurokawa et al. 

B. CTGGGTTCTGGTCTGGTACATCAG-3' 

C, 30 s at 72 °C. The sequence homology between lipocalin domains of Tr proteins and their homologs is summarized in the Table 1. Tributyltin (TBT)-binding protein Trub.TBT-bp alpha, a member of the lipocalin family, has been isolated from the plasma of T. rubripes and characterized.19) Although observation of upregulated PSTBP homologs under TTX administration was not described,19) post-transcriptional regulation of Tr genes is an interesting open question.

The sequence homology between lipocalin domains of Tr proteins and their homologs is summarized in the Table 1. Tributyltin (TBT)-binding protein Trub.TBT-bp alpha, a member of the lipocalin family, has been isolated from the plasma of T. rubripes and characterized.19) Tr1 and Trub.TBT-bp alpha are similar in terms of domain architecture, containing a single lipocalin domain, but they show only 48% sequence similarity, whereas Tr1 shows 90% similarity with the C-terminal domain of PSTBP1 (Table 1). Thus the structure and function of Tr1 may be more similar to those of PSTBP1 than to those of Trub.TBT-bp alpha. The lipocalin domains in the Tr proteins showed high similarity (63–98%), and these domains are conserved in their C-terminal regions (Table 1 and Fig. 1). Therefore, discrimination of Tr isoforms by immunoblot analysis (e.g., with polyclonal antibody) would be difficult. The method presented in this paper, RT-PCR- and MALDI-TOF mass spectrometry-based identification and discrimination of closely related isoforms, is a reliable alternative strategy in studying the physiological significance of individual Tr isoforms and other PSTBP-

Table 1. Amino Acid Sequence Homology between the Lipocalin Domains of Tr Proteins and Their Homologs

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aDomain names of Tr proteins and PSTBP1 are referred in the legend to Fig. 1. Trub.TBT-bp, T. rubripes tributyltin-binding protein (TBT-bp) alpha; PoTBT-bp1, Paralichthys olivaceus TBT-bp type 1; PoTBT-bp2, P. olivaceus TBT-bp type 2.

bSignal peptide (Fig. 1, amino acid residues 1–20) was omitted before comparison by BLAST 2 SEQUENCES.

cA short N-terminal region (seven residues) of predicted mature protein might be missing due to incomplete ORF in an assembled EST sequence (see Fig. 1 and text).
related proteins. The ligand selectivity and affinity of Tr isoforms and PSTBP-related proteins are under investigation.

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References