cDNA Cloning and Sequence Analysis of Preproendothelin-1 (PPET-1) from Salmon, *Oncorhynchus keta*

HONGYU WANG,* JIEXIA QUAN,* EI-ICHI KOTAKE-NARA,*,† TSUYOSHI UCHIDE,‡ TADASHI ANDOH,§ AND KANAME SAIDA¶,[1]

*Institute for Biological Resources and Functions, National Institute of Advanced Industrial Science and Technology, Tsukuba, 305-8566 Japan; †New Energy and Industrial Technology Development Organization, Kawasaki, 212-8544 Japan; ‡Department of Toxicology, School of Veterinary Medicine and Animal Sciences, Kitasato University, Towada, 034-8628 Japan; §Hokkaido National Fisheries Research Institute, Fisheries Research Agency, Kushiro, 085-0802 Japan; and ¶Human Stress Signal Research Center, National Institute of Advanced Industrial Science and Technology, Ikeda, 536-8577 Japan

The presence of endothelin (ET)-like immunoreactivity and the cardiovascular effects of mammalian ET-1 in fish have been reported. To identify ET-related peptides in fish, we screened the cDNA library of the salmon (*Oncorhynchus keta)* stomach by means of rapid amplification of cDNA ends, and we cloned cDNAs encoding an ET-related peptide. The salmon ET-related sequence of 21 amino acids is identical to the trout ET-1 peptide recently purified from kidney specimens of *Oncorhynchus mykiss*. The deduced amino acid sequence of salmon pre-proET-1 (PPET-1) comprises 244 amino acids, including a putative signal sequence and mature ET-1, as well as big ET-1 and ET-1-like sequences. This precursor, the first reported PPET-1 sequence for Salmoniformes, Teleostei, has low homology with the sequences of human, mouse, frog (*Xenopus laevis*), and zebrafish (*Danio rerio*) PPET-1 (26%, 29%, 24%, and 39%, respectively). Exp Biol Med 231:709–712, 2006

Key words: salmon; endothelin-1; stomach; cDNA; RACE (rapid amplification of cDNA ends); precursor

Introduction

The family of endothelin (ET) ligands comprises ET-1, ET-2/vasoactive intestinal contractor, and ET-3. These ligands are 21-residue peptide hormones with varied and important roles in the regulation of mammalian physiological processes (1–12). ET-like immunoreactivity has been observed not only in mammals but also in fish (13, 14). The biological actions of exogenously administered mammalian ET-1 peptide are similar in fish and mammals (15, 16). Recently, trout (*Oncorhynchus mykiss*) ET-1 peptide was purified and its myotropic activity analyzed (17). Zebrafish (*Danio rerio*) ET-1 was identified as a morphogen that is expressed ventrally in the pharyngeal arches and required for correct patterning of pharyngeal cartilage development (18).

In the present article, the rapid amplification of cDNA ends (RACE) method (10) was used to screen ET-related peptides from specimens of salmon stomach. The cloned cDNA sequence and deduced amino acid sequence of a salmon precursor of the ET-related peptide were analyzed.

Materials and Methods

Reagents and software used are the same as those in the report by Quan *et al*. (10). The stomach was excised from a fresh-caught salmon preserved on ice. Isolation of mRNA, cDNA amplification, subcloning of RACE-PCR products, sequencing of the cloned products, and sequence analysis were performed as previously described (10). Two kinds of guess PCR primers, 5′-GSP (5′-GGBGTRTTGACCCA-GATKATRTCC-3′, where B = G, T, or C; R = A or G; and K = G or T) and 5′-NGSP (5′-GATGATRTCCARGTGR-CAGAART-3′), which were designed on the basis of the conserved region in the mature peptide (10), were used in 5′-RACE PCR. For 3′-RACE PCR, 3′-GSP (5′-

J.Q. is a recipient of the Japan Society for the Promotion of Science postdoctoral fellowship for foreign researchers. E. K.-N. is a recipient of a New Energy and Industrial Technology Development Organization fellowship. This work was supported by grants from the Ministry of Economy, Trade, and Industry of Japan (to K.S.) and the Japan Society for the Promotion of Science (17580089 to K.S.).

[1] To whom correspondence should be addressed at the Institute for Biological Resources and Functions, National Institute of Advanced Industrial Science and Technology, 1-1-1 Higashi, Tsukuba, Ibaraki 305-8566, Japan. E-mail: k.saida@aist.go.jp

Received October 6, 2005.
Accepted December 22, 2005.

1535-3702/06/2316-0709$15.00

CCTATGGGAAAAGAAACAGCGGTA-3′) and 3′-NGSP (5′-CAGTGCGCCACATCAGGAAC-3′), which were designed on the basis of the sequences of the 5′-RACE products shown in Figure 1, were used. Several clones of 5′- and 3′- RACE products were obtained, and the resulting sequences were the same as an identical cDNA shown in Figure 1.

The sequence data from this study has been deposited in the EMBL/GenBank/DDJB Data Bank with accession number AB175495.

Results and Discussion

Nucleotide and Deduced Amino Acid Sequences of Salmon *(O. keta)* PPET-1. To identify ET-related peptides in fish, we screened the cDNA in samples from salmon with RACE and cloned 5′-RACE cDNAs encoding the 5′-portion of an ET-related peptide. To obtain the 3′-portion of the ET-related mature peptide and the full-length precursor protein of the fish ET-related peptide, we screened and cloned 3′-RACE cDNAs from salmon stomach specimens and then combined them with the

```
gactgacagaacagagcaagtggtatgcttttgcgctccttcgtaaagaggtaacttgtatccaaacgtacgcagttgacactgtgtcgc    90
tttttggaacgttttccttgaccaaggaatattccattttgtgaaaaacttattctttctggactgaagaaagatcaagATGGATTTAAG   180
                                                                    M  D  L  R      (4)
AATATTTTTCTCCGTGTTGTCAATTGTGTACTCTGGAATTGTACAAACAGTTACATCTGCCCCTATGGGAAAAGAAACAGCGGTAGCCAC   270
 I  F  F  S  V  L  S  I  V  Y  S  G  I  V  Q  T  V  T  S ▲A  P  M  G  K  E  T  A  V  A  T    (34)
CCCAGCCTCCCCGGTGCCAGTGCGCCACATCAGGAACAAGCGATGCTCCTGCGCAACGTTCCTAGACAAGGAGTGCGTTTACTTCTGTCA   360
 P  A  S  P  V  P  V  R  H  I  R  N  K  R  C  S  C  A  T  F  L  D  K  E  C  V  Y  F  C  H    (64)
CCTGGACATCATATGGGTCAACACGCCTGAGCGCGTGGTTTCCTACGGACTGGGCAACGCATCTAGAAAGAAGCGCGCAATCAAGGATCC   450
 L  D  I  I  W▲V  N  T  P  E  R  V  V  S  Y  G  L  G  N  A  S  R  K  K  R  A  I  K  D  P    (94)
CGAGGTCTCCCAACGGGACCCTCGATGTAAGTGCGTCAGCGAAGATGACGGCACGTGTATAAGATTCTGTCAGATGGAAAATCACCTCAG   540
 E  V  S  Q  R  D  P  R  C  K  C  V  S  E  D  D  G  T  C  I  R  F  C  Q  M  E  N  H  L  R   (124)
ATACGGGACAGCAGCCTTGATCCATCCCGCGTCAGACACAGTGATCCGTCGCGTGCCAGACACCGTGATCCGTCCCGTGCCAGACACCGT   630
 Y  G  T  A  A  L  I  H  P  A  S  D  T  V  I  R  R  V  P  D  T  V  I  R  P  V  P  D  T  V   (154)
GATCCGTCCCGTGCCAGACACCGTGATCCGTCCCGTGCCAGACACCGTGATCCGTCGCGTGCCAGACACCGTGATCCGTCCCACCCTTGC   720
 I  R  P  V  P  D  T  V  I  R  P  V  P  D  T  V  I  R  R  V  P  D  T  V  I  R  P  T  L  A   (184)
TGAGTATGCTTGGAGGCGGCAGTGCAAACTCAAGCTGGCAGCCAAAACGTCTAGGATTCAAAGGGTGAAACACAGAGACCACAAAGCAGT   810
 E  Y  A  W  R  R  Q  C  K  L  K  L  A  A  K  T  S  R  I  Q  R  V  K  H  R  D  H  K  A  V   (214)
TGGCCCCTCAGCTCTAAGGGCCACAGTCAAAGCTTGCCTGCTGCTGGAGAAGTGGACGGTGAAACAACGACACAAGCCAAGAGAAGGGAG   900
 G  P  S  A  L  R  A  T  V  K  A  C  L  L  L  E  K  W  T  V  K  Q  R  H  K  P  R  E  G  R   (244)
Atgagaagagagatgaggagagagatgaggatacacaggcgcaccttgtgggagatgtccacatactccgtcacactcacagagggtaga   990
 *
gatccatccatctctttctatccacctgccattggggactcatatgctgtggtccctggagatgcattggattctccacaggaagcctgc  1080
cactggacccccatgttggctcaatggtgtgcgtcatgaaaggggatgaatgactgtgctggtgggtgccgcgtccagtctctccactga  1170
acgtgtccctcttcatcagtgagatgcagacggtcggagatacaagcaggagaaatcagaggaacaggtgtcaacgttactgacaccaca  1260
tcctgaagacaaggatggacacttgatggtcctcccatgtaccgtctgtggacacacccttggacttcaactgcaaccttcaaacagag  1350
cttcagatcatatctgatgacttgaatgactgaaacttgaatctctacattcctttgactgtcactgggtttgagacaccaggctacttc  1440
acacaacggcgtcagaagcactgttgctgctagcctgtccaaagctcttgtgtctgctcttttcaagaacatgttggattcaaagaagat  1530
cacaatctcatagacttgttgaattgactctatggcccagcataagtaactcaacagattgattccaggtgttgtcatagtttgacggag  1620
gtttctcacctttgacagacctaacctttccaaatgattcattagaacttgggagttttctgctaatattgatttctgtctctgtttgtc  1710
tgagatttctctatacacattgtataatattgaatcactatatatttaatacacattgtataataatgaatcactataaatgtaatacac  1800
attgtataataattcatcactataaatgtaatacacactgtataataattcatcactataaatgtaatacacattgtataataattaatc  1890
attatatatttatacacattgtataataattcatctctatatatttaatgcacattgttctgtccctatgtcttatttatttgcattcatt  1980
tatagtcatattggcccatgatatgataatatgttgtatgtaatttctgtgaaatcattttgtcgccaagatagttttggcaaaacttag  2070
ggaaataaattgtcacacttatcc-poly(A)                                                              2094
```

Figure 1. Nucleotide and deduced amino acid sequences of salmon (*O. keta*) preproendothelin-1 (PPET-1). An open reading frame and noncoding regions are shown in capital and small letters, respectively. The sequence of *O. keta* endothelin-1 (ET-1) (C^{49}–W^{69}) is underlined by a solid line and the C-terminus of big ET-1 (V^{70}–K^{87}) by a dashed line. The putative proteolytic processing site at the C-terminus of ET-1 is indicated by an arrow (between W^{69} and V^{70}). Paired basic amino acid residues are doubly underlined. The ET-1-like peptide (C^{103}–C^{117}) is underlined by a thick dotted line. Amino acids of the probable signal sequence are in italic. The arrowhead indicates the putative site of signal sequence cleavage (between S^{23} and A^{24}). Six occurrences of the DTVIRRVP repeat sequences are present in the C-terminal region of PPET-1, as indicated by dotted lines. The deduced amino acid sequence is given below the nucleotide sequence, which is numbered at the end of each sequence line. Amino acid residues are numbered in parentheses beginning with the initial methionine. The termination codon is marked with an asterisk. Five attta sequences, destabilizing signals for mRNA (4, 5), are present in the 3′-untranslated region, just before the poly $(A)^+$ additional signal aataaa. This cDNA was produced by combining two overlapping 5′- and 3′-RACE cDNAs.

```
Salmon      MDLRIFFSVLSIVYSGIVQTVTSAPMGKETAVATPASPVPVRHIRNKRCSC   51
            * *** * **    **       *  *  **      *   ** *******
Zebrafish   MHLRIIFPVLTMLTSGFFDFGAPASLGPGTA------P--ARHSRNKRCSC   43

Salmon      ATFLDKECVYFCHLDIIWVNTPERVVSYGLGNASRKKRAIKDPEVSQRDPR  102
            * ********************* ******** **** *  *      *
Zebrafish   ASFLDKECVYFCHLDIIWVNTPERTVSYGLGNAPRKKRSVTEPVVLES--R   92

Salmon      CKCVSEDDGTCIRFCQMENHLRYGTAALIHPASDTVIRRVPDTVIRPVPDT  153
            ***    * **  ***    *    *       **  *
Zebrafish   CKCADSQDKTCSSFCQADSALQFKAA------SDRAI--------------   123

Salmon      VIRPVPDTVIRPVPDTVIRRVPDTVIRPTLAEYAWRRQCKLKLAAKTSRIQ  204
                                        *          *** ***     *
Zebrafish   --------------------------RAAQGHDCAGKQCKHKLAETQTKIR  148

Salmon      RVKHRDHKAVGPSALRATVKACLLLEKWTVKQRHKPR----EGR         244
            *   *  *       *    *  ******     *       *
Zebrafish   RL--RTTKQTDV--LSGKKKIQLLLEKWRMRRNHRSQAWISENS         188
```

Figure 2. Comparison of PPET-1 amino acid sequences of salmon and zebrafish. The deduced amino acid sequence of salmon PPET-1 is aligned with that of zebrafish PPET-1. Identical amino acids are indicated by asterisks. Mature and similar peptides are indicated. Six occurrences of the DTVIRRVP repeat sequences of salmon PPET-1 are absent in the C-terminal region of zebrafish PPET-1, as indicated by a dotted line divided into six segments. Amino acid sequences are numbered at the end of each sequence row.

cloned cDNAs identified by 5′-RACE into a cDNA of 2094 base pairs (Fig. 1). The identified ET-related sequence of 21 amino acids (C^{49}–W^{69}) is identical to the sequence of fish ET-1 peptide recently determined in kidney specimens from steelhead trout (*O. mykiss)* (17). The cDNA includes the full length (732 base pairs) of the coding region of the salmon precursor of ET-1, preproET-1 (PPET-1), which encodes 244 amino acid residues. The first *N*-terminal 23 hydrophobic amino acids were predicted by Henrik Nielsen's algorithm to be a secretory signal sequence (19). Paired basic amino acid residues K^{47}–R^{48}, which are recognized by processing endopeptidases, directly precede a putative mature region of the salmon PPET-1. However, no dibasic pair is found until K^{88}–R^{89}, indicating that salmon ET-1 is produced from a 39-residue intermediate, salmon big ET-1 (C^{49}–K^{87}). Amino acid residues C^{103}–C^{117} of salmon PPET-1 encode an ET-1–like peptide of 15 amino acids. This sequence contains four cysteine residues (C^{103}, C^{105}, C^{113}, and C^{117}) at relative positions 1, 3, 11, and 15, an arrangement that is structurally related to mature ET-1 in salmon.

Comparison of Salmon PPET-1 Sequence with Zebrafish, Frog, Mouse, and Human PPET-1 Sequences. Salmon PPET-1 is the first ET-1 precursor sequence identified in Salmoniformes, Teleostei. Deduced amino acid sequences of salmon PPET-1 and zebrafish PPET-1 (18) are aligned in Figure 2. The salmon ET-1 (C^{49}–W^{69}) and zebrafish ET-1 sequences are different; one

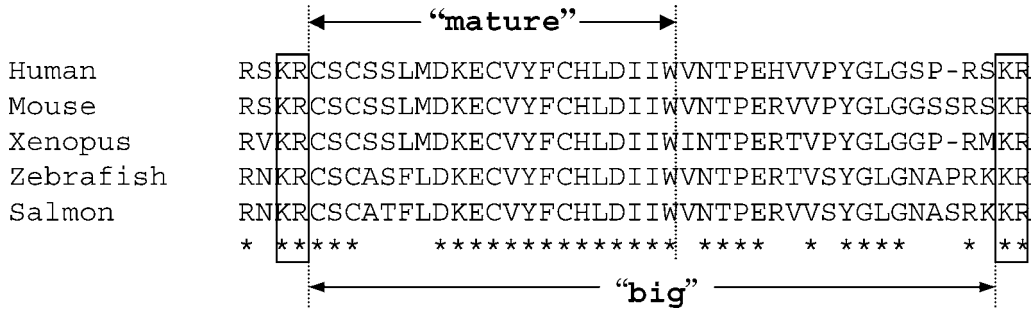

Figure 3. Comparison of amino acid sequences of mature ET-1 and big ET-1 from fish to mammal. Identical amino acids are indicated by asterisks. "Mature" and "big" ET-1 peptides are indicated by arrows. Paired basic amino acid residues on either side of big ET-1 are boxed. The ET-1-converting enzyme processing site at the C-terminus of mature ET-1 is indicated by a dotted line.

of 21 amino acid differs between the two sequences. Salmon big ET-1 (C^{49}–M^{87}) has a high homology with zebrafish big ET-1, with 36 of 39 amino acids identical (Figs. 2 and 3). The salmon PPET-1 sequence has low identity with zebrafish (96 [39%] of 244 amino acids; Fig. 2) (18), frog (*Xenopus laevis*) (24%) (10), mouse (29%) (4), and human (26%) (20) PPET-1 sequences. The dibasic amino acid pairs flanking the 5′- and 3′- regions of salmon big ET-1 (K^{47}–R^{48} and K^{88}–R^{89}) are identical to those in other PPET-1s, indicating that the generation of big ET-1 is conserved from fish to mammal (Fig. 3). Further studies based on this cDNA sequence, including expression and bioactivity experiments, are needed to better understand the biological characteristics of the ET-1 peptide in salmon.

K.S. gratefully thanks Drs. Kazunori Nakamura, Hiroshi Kuriyama, Masahiro Iwakura, and Etsuo Niki of AIST for their support. H.W. and K.S. thank Prof. Shiro Bannai (University of Tsukuba) and Dr. Hideyo Sato (Yamagata Univ.) for encouragement. H.W. is on leave from Hokkaido System Science Company (Sapporo, Japan).

1. Rubanyi GM, Polokoff MA. Endothelins: molecular biology, biochemistry, pharmacology, physiology, and pathophysiology. Pharmacol Rev 46:325–415, 1994.
2. Cody WL, Doherty AM. The development of potent peptide agonists and antagonists for the endothelin receptors. Biopolymers 37:89–104, 1995.
3. Saida K, Mitsui Y, Ishida N. A novel peptide, vasoactive intestinal contractor, of a new (endothelin) peptide family: molecular cloning, expression, and biological activity. J Biol Chem 264:14,613–14,616, 1989.
4. Kurama M, Ishida N, Matsui M, Saida K, Mitsui Y. Sequence and neuronal expression of mouse endothelin-1 cDNA. Biochim Biophys Acta 1307:249–253, 1996.
5. Saida K, Hashimoto M, Mitsui Y, Ishida N, Uchide T. The prepro vasoactive intestinal contractor (VIC)/endothelin-2 gene (EDN2): structure, evolution, production, and embryonic expression. Genomics 64:51–61, 2000.
6. Uchide T, Uchide T, Adur J, Yoshioka K, Sasaki T, Temma K, Saida K. Endothelin-1 in smooth muscle cells and mast cells of mouse uterus after parturition. J Mol Endocrinol 27:165–73, 2001.
7. Kozakai T, Sakate M, Masuo Y, Uchide T, Saida K. Increased gene expression of endothelin-1 and vasoactive intestinal contractor/endothelin-2 in the mammary gland of lactating mice. Biochem Biophys Res Commun 297:1339–1343, 2002.
8. Masuo Y, Ishikawa Y, Kozakai T, Uchide T, Komatsu Y, Saida K. Vasoactive intestinal contractor/endothelin-2 gene expression in the murine central nervous system. Biochem Biophys Res Commun 300: 661–668, 2003.
9. Adur J, Takizawa S, Quan J, Uchide T, Saida K. Increased gene expression and production of murine endothelin receptors after birth. Biochem Biophys Res Commun 305:700–706, 2003.
10. Quan J, Uchide T, Takizawa S, Adur J, Nara E, Saida K. cDNA cloning and sequence Analysis of *Xenopus laevis* preproendothelin-1. J Cardiovasc Pharmacol 1:S256–S259, 2004.
11. Kotake-Nara E, Takizawa S, Quan J, Wang H, Saida K. Cobalt chloride induces neurite outgrowth in rat pheochromocytoma PC-12 cells through regulation of endothelin-2/vasoactive intestinal contractor. J Neurosci Res 81:563–571, 2005.
12. Takizawa S, Uchide T, Adur J, Kozakai T, Kotake-Nara E, Quan J, Saida K. Differential expression of endothelin-2 along the mouse intestinal tract. J Mol Endocrinol 35:201–209, 2005.
13. Uemura H, Naruse M, Naruse K, Hirohama T, Demura H, Kasuya Y. Immunoreactive endothelin in plasma of nonmammalian vertebrates. J Cardiovasc Pharmacol 17:S414–S416, 1991.
14. Stenslokken KO, Sundin L, Nilsson GE. Endothelin receptors in teleost fishes: cardiovascular effects and branchial distribution. Am J Physiol Regul Integr Comp Physiol 290:R852–R856, 2006.
15. Olson KR, Duff DW, Farrell AP, Keen J, Kellogg MD, Kullman D, Villa J. Cardiovascular effects of endothelin in trout. Am J Physiol 260(4 Pt 2):H1214–H1223, 1991.
16. Poder TC, Silberberg SD, Rampe D. Contraction of reptile, amphibian, and fish blood vessels by endothelin-1. Can J Physiol Pharmacol 69: 215–217, 1991.
17. Wang Y, Olson KR, Smith MP, Russell MJ, Conlon JM. Purification, structural characterization, and myotropic activity of endothelin from trout, *Oncorhynchus mykiss*. Am J Physiol 277(6 Pt 2):R1605–R1611, 1999.
18. Miller CT, Schilling TF, Lee K, Parker J, Kimmel CB. Sucker encodes a zebrafish Endothelin-1 required for ventral pharyngeal arch development. Development 127:3815–3828, 2000.
19. Nielsen H, Engelbrecht J, Brunak S, von Heijne G. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Protein Eng 10:1–6, 1997.
20. Itoh Y, Yanagisawa M, Ohkubo S, Kimura C, Kosaka T, Inoue A, Ishida N, Mitsui Y, Onda H, Fujino M, Masaki T. Cloning and sequence analysis of cDNA encoding the precursor of a human endothelium-derived vasoconstrictor peptide, endothelin: identity of human and porcine endothelin. FEBS Lett 231:440–444, 1988.