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CLONING AND EXPRESSION OF RAT PREPROENDOTHELIN-3 cDNA

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Summary: We report here the cloning and expression of a rat full-length cDNA encoding preproendothelin-3 (preproET-3). The predicted rat preproET-3 consisted of 167 amino acid residues. As in other ET-family peptides, the mature rat ET-3 was predicted to be produced through unusual processing from a 41-residue intermediate, the big ET-3 in rat. Transient transfection of COS-7 cells with the cloned preproET-3 cDNA resulted in the production of mature ET-3 and this production was inhibited by phosphoramidon, a metaloprotease inhibitor. This suggested that a phosphoramidon sensitive mechanism was involved in the production of ET-3 in the transfected COS-7 cells. Northern blot analysis showed that an approximately 3.0-kb rat preproET-3 mRNA was expressed in rat tissues, including the eye ball, submandibular gland, brain, kidney, jejunum, stomach and spleen. A 2.0-kb and a 3.3-kb mRNA were also detected in the eye ball and small intestine, respectively. The distinct distribution of rat preproET-3 mRNA from that of preproET-1 mRNA suggested that ET-1 and ET-3 played different roles.

Endothelins are a family of highly conserved 21 amino acid peptides known to possess strong vasoconstrictor activity. The first member of the endothelin (ET)-family, ET-1, was initially isolated from a culture medium of the porcine aortic endothelial cells (1), but it was subsequently found to have a wide variety of effects on both vascular and non-vascular tissues (2, 3). Subsequently, the existence of three distinctive genes which potentially encode for three isopeptides of the ET family, designated as ET-1, ET-2, and ET-3, was predicted from the discovery of three separate genes in human and other mammalian genomes (2-4). The three isopeptides each possess a diverse set of pharmacological activities with quantitatively different potencies (2-4). In particular, ET-3 was demonstrated to be less potent as a vasoconstrictor than ET-1 and ET-2, and it has been thought to be a novel neuropeptide in the central and peripheral nervous systems (6).

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Previously we reported on the cloning of rat ET-3 gene (5), however, the structure and tissue distribution of rat preproET-3 mRNA still remains to be determined. In this study, we cloned a cDNA encoding a rat preproET-3 to further examine into this point. Subsequently, we transfected this cDNA clone into COS-7 cells in order to proceed with the characterization of the processing mechanism of preproET-3.

MATERIALS AND METHODS

Preparation of RNA. Total RNA was prepared with selective precipitation in 3 M LiCl / 6 M Urea (7) from designated rat tissues, and poly $(A)^+$RNA was further purified by chromatography on oligo(dT) cellulose as described (8).

cDNA Cloning and Sequencing. Double strand cDNA was synthesized from 2 μg of poly $(A)^+$RNA prepared from rat eyeballs using oligo(dT) as the first strand primer by modified Gubuler and Hoffman's method and ligated to EcoRI/BamHI/NotI adaptors (Takara). The cDNA was size fractionated as described (12) and ligated into EcoRI site of λgt10 vector. Approximately 2.4×10^7 individual plaques from the unamplified library were screened by plaque hybridization method. Two synthetic oligonucleotides (5'-GCACGTGCTTCACT-TATAAGGACAAG-3' and 5'-AGTAGTAGACACACTCCTTGTCCTTA-3') were annealed to each other, and labelled to a specific activity of 10^9 c.p.m. / μg DNA with Klenow fragment of *E. coli* DNA polymerase I in the presence of [α-^{32}P]dCTP (3000 Ci / mmol, Amersham) and used as the hybridization probe. This probe corresponded to the partial sequence of rat genomic fragment which encoded rat preproET-3 (5). We detected one positive plaque under the condition of high hybridization stringency, and this clone (λrET-3) was subjected to further characterization. Restriction fragments from the 1.7-kb cDNA inserts of this clone were subcloned into pUC118 / 119 phagemids, rescued as single-strand DNAs (10), and sequenced by the dideoxy chain termination method using Sequenase™ T7 DNA polymerase (United States Biochemical) (11). Sequences of both strands of the entire cDNA were determined from overlapping subclones.

Transfection of COS-7 Cells and Enzyme-immunoassay for ET-3. Stock cultures of COS-7 cells were maintained in DMEM (Dulbecco's modified eagle medium) supplemented with 10% fetal calf serum in a humidified atmosphere containing 5% CO_2. The full-length insert of λrET-3 was ligated downstream of the SRα promoter of mammalian expression vector pcDL-SRα296 (pSRαrET-3) (13). A day before transfection, the cells were seeded in 12-well plates at 5×10^4 cells / well. pSRαrET-3 and the empty vector were transfected into the monolayer of COS-7 cells by the DEAE-dextran/chloroquine method as previously described (12). Two days after the transfection, the culture medium was changed to DMEM containing 0.3% bovine serum albumin (BSA). The cells were cultured further for 24 h with or without 30 μM phosphoramidon, and the content of immunoreactive ET-3 in the conditioned media of these cells was analyzed by an enzyme-immunoassay (EIA). This assay system detected ET-3 but not ET-1, ET-2 nor big ET-3 (14).

Northern Blot Analysis. Poly$(A)^+$RNA (10 μg / lane) from designated rat tissues were separated by a formaldehyde / 1.1% agarose gel electrophoresis, and transferred onto a nylon membrane (Hybond N^+; Amersham). The insert (1.7 kb) of λrET-3, which included the entire coding region of rat ET-3 was labelled to a specific activity of 8×10^8 c.p.m. / μg with [α-^{32}P]dCTP (3000 Ci/mmol, New England Nuclear) by the random priming method and used as a probe. Hybridization was performed at 42 °C in 1 M NaCl / 50% formamide / 1% SDS / 250μg / ml salmon sperm DNA. The membrane was washed finally in 0.1 x SSC / 0.1% SDS at 50°C, and autoradiographed with intensifying screens at -80 °C for 10 h.

RESULTS AND DISCUSSION

Nucleotide sequence of cDNA encoding rat preproET-3. The nucleotide sequence of the cDNA insert of λrET-3 is shown in Fig. 1a. The 5'-most ATG triplet, which was followed by a 504 bp open reading frame, was preceded by an in-frame stop codon (TAG; nucleotide 172-

175). The nucleotide sequence around this ATG triplet conformed reasonably well with Kozak's consensus for the favored sequence of translation initiation sites of eukaryotic mRNAs (15). The similarities between rat and human hypothalamic and placenta preproET-3 cDNA sequences were 72.2 and 68.8% in the coding regions and 51.7 and 48.7% in the 3' non-coding regions, respectively (16, 17).

Deduced amino acid sequence of rat preproET-3. Fig. 1a also shows the predicted amino acid sequence of rat preproET-3. The deduced rat preproET-3 consisted of 167 amino acid residues and was similar to human hypothalamic and placenta preproET-3, which had 45.4 and 49.2 % amino acid identities. In particular, the amino acid sequence of big ET-3 was 39/41 match

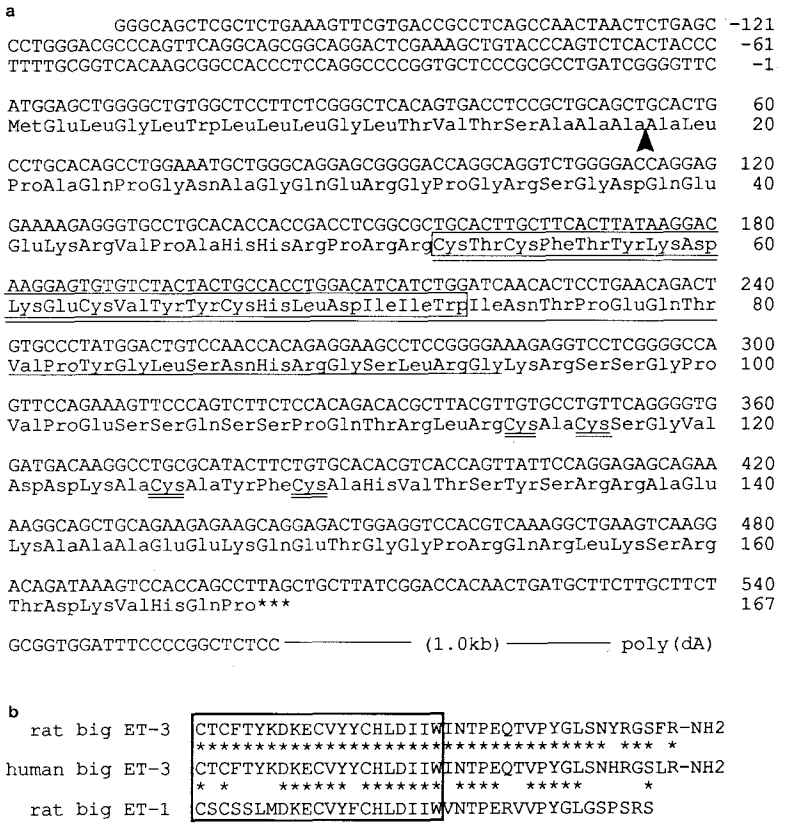

Fig. 1. a. Nucleotide and deduced amino acid sequence of rat preproET-3 cDNA. The putative signal sequence cleavage site predicted by the von Heijne's algorithm (18) is indicated by an arrowhead. Mature ET-3 and big ET-3 sequences are indicated by a box and an underline, respectively. Four Cys residues within the "Endothelin-like" region are doubly underscored. **b. Amino acid sequence of rat big ET-3 compared with human big ET-3 and rat big ET-1.** The deduced amino-acid sequence of rat big ET-3 is 39/41 identical to that of human big ET-3 (16, 17, 21). Mature ET sequences are indicated by a box.

with that of human big ET-3 (Fig. 1b), and mature rat ET-3 sequence was perfectly identical to that of human and rabbit ET-3. The first 18 residues of rat preproET-3 were predicted to constitute a secretory signal sequence by von Heijine's algorithm (18). Paired basic amino acid residues, Arg51-Arg52, which were recognized by processing endopeptidases, directly preceded the mature ET-3 sequence. However, no dibasic pair was found until Lys95-Arg96 which indicated that mature rat ET-3 was produced from a 41-residue rat big ET-3 with possibly a C-terminal amide (19). Thus the mature ET-3 was predicted to be produced through the unusual proteolytic cleavage between Trp73 and Ile74 which may have been catalized by putative ET-converting enzyme (1). The sequence that spanned amino acid residues 115-135 contained four Cys residues at the relative positions 1, 3, 11 and 15, which were identical to the positions of the Cys residues in mature ETs, represented an "endothelin-like" motif as in the other ET family members (Fig. 1a). The repeat sequence which was observed in human preproET-3 sequence was absent from rat preproET-3.

Production of immunoreactive ET-3 in the transfected COS-7 cells. Fig. 2 shows the production of recombinant immunoreactive ET-3 (ir-ET-3) by COS-7 cells transfected with the cDNA for rat preproET-3. The content of ir-ET-3 was under the detection limit (< 0.6 fmol/ml) in the medium from cells transfected with the empty vector. The cells transfected with the λrET-3 insert produced approximately 30 fmol/ml/24 h of ir-ET-3 in the culture medium. The production of ir-ET-3 by these cells was reduced to about 20% by the addition of phosphoramidon (30 μM). Phosphoramidon, a metaloproteinase-inhibitor, was reported to inhibit the conversion of big ET-1 to ET-1 (20). Our results suggested that ET-3 is also produced through a phosphoramidon-sensitive mechanism in the transfected COS-7 cells.

Tissue distribution of rat preproET-3 mRNA. Northern blot of poly $(A)^+$RNA from various rat tissues was hybridized with the cloned rat preproET-3 cDNA (Fig. 3). A 3.0-kb preproET-3 mRNA was detected in many of these tissues. In contradiction to preproET-1, the preproET-3 mRNA was not detectable in rat pulmonary macro- and microvascular endothelial cells in culture (data not shown). Among native tissues, preproET-3 mRNA was most abundantly expressed in the eye ball. The brain, submandibular gland, small intestine and kidney also contained relatively large amounts of preproET-3 mRNA. Small amounts of preproET-3 mRNA were also detected in the stomach and spleen. These findings were in concordance with the previously reported tissue distribution of immunoreactive ET-3 in the rat (21). Taken together

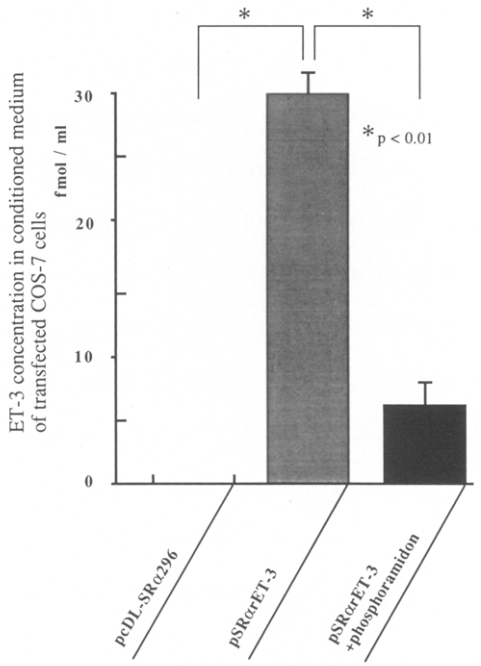

Fig. 2. Production of recombinant ET-3 by the transfected COS-7 cells and inhibition by phosphor amidon. Conditioned media were corrected from COS-7 cells following 24 h incubation and assayed for irET-3 by an EIA specific for mature ET-3 (14). Each point represents a mean of four wells; bars indicate the SD.

with the observations that the distribution of preproET-3 mRNA was similar to that of ET_B subtype of ET-receptor (12), the present observations suggested that ET-3 may have played important part as a locally acting mediator via ET_B receptor. On the other hand, the distribution of preproET-3 mRNA was quite different from that of preproET-1 mRNA (22) which suggested that ET-1 and ET-3 may have different physiological roles from each other. In the eyeball, a weak ~2.0 kb hybridizing signal, and in the small intestine a ~3.3 kb tissue specific mRNA were also detected. The cloned cDNA may probably corresponded to 2.0 kb species of mRNA, as suspected, from the polyadenylation position. This allowed for the possible speculation that the expression of plural mRNAs was likely to add further meticulosity to the tissue- and physiological state-dependent regulation of the ET-3. Although the 3.0 kb mRNA in the eyeball is more abundantly expressed than 2.0 kb mRNA, we consistently failed to detect cDNA clones that correspond to the 3.0 kb of mRNA, which suggested that the 3' non-coding region of the mRNA had a structure which was very difficult to be a template for reverse-transcriptation.

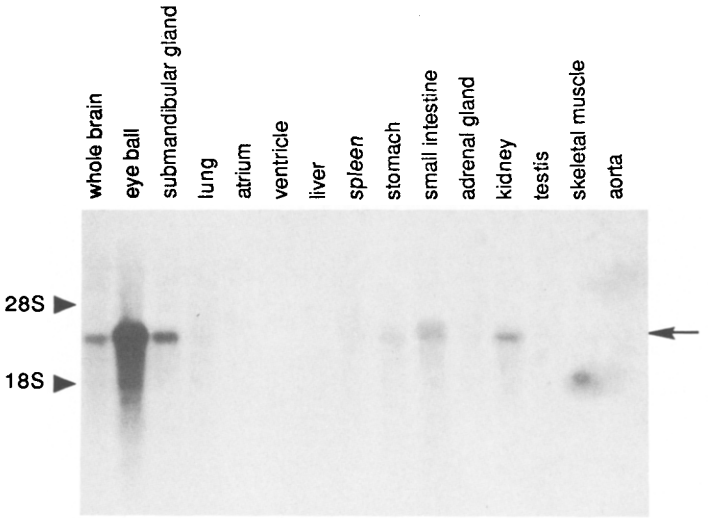

Fig. 3. Northern blot hybridization of preproET-3 mRNA in rat tissues. Poly(A)+RNA (10 μg / lane) from designated rat tissues was analyzed by Northern blot hybridization. The 3-kb preproET-3 mRNA is indicated by an arrow. A 2-kb and a 3.3-kb preproET-3 mRNA is also detected in eyeball and in small intestine, respectively. The presence of intact 28S ribosomal RNA was confirmed in all lanes (not shown).

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