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Synthesis of human endothelin-1 precursors in *Escherichia coli*

Takuya Watanabe, Yasuaki Itoh, Kazuhiro Ogi, Chiharu Kimura, Nobuhiro Suzuki and Haruo Onda

Tsukuba Research Laboratories, Takeda Chemical Industries, Ltd, Wadai-7, Tsukuba, Ibaraki 300-42, Japan

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Endothelin, the most potent vasoconstrictor found in nature, is thought to be important in the regulation of blood pressure and/or local blood distribution. Human placenta cDNA fragment encoding preproendothelin-1 (preproET-1) and its carboxyl terminal mature precursor (C-matured precursor) was expressed in *E. coli*. These products were characterized by both enzyme immunoassay and Western blot analysis.

Preproendothelin; Endothelin precursor; Vasoconstrictor peptide; Gene expression system; (*E. coli*)

1. INTRODUCTION

Recently, a novel potent vasoconstrictor peptide, endothelin, has been purified from culture medium of porcine aortic endothelial cells and its amino acid sequence determined [1]. Porcine and human preproET-1 cDNAs were isolated from cDNA libraries of porcine aortic endothelial cells [1] and human placenta [2], respectively. Recently, two other types of endothelin genes, termed ET-2 and ET-3, were found in human genomic DNA [3]. The nucleotide sequence of the human cDNA showed that preproET-1 contains 212 amino acid residues and is highly homologous to porcine preproET-1; mature ET-1, in particular, is identical with the porcine type. It is postulated that the mature ET-1 is synthesized through NH_2-terminal processing at the dibasic pair Lys-Arg and unusual COOH-terminal processing between Trp and Val [1]. The manner of processing for ET-1 maturation remains unknown. As an initial step in studying the secretion and maturation of human ET-1, we synthesized human ET-1 precursors in *E. coli*. To our knowledge, this is the first report in which the expression of ET-1 precursors is demonstrated.

Correspondence address: H. Onda, Tsukuba Research Laboratories, Takeda Chemical Industries, Ltd, Wadai 7, Tsukuba, Ibaraki 300-42, Japan

2. MATERIALS AND METHODS

2.1. *Construction of expression vectors*

A flow chart for the construction of preproET-1 and C-matured precursor expression vectors is shown in fig.1A. Plasmid pHET4-3 has a cDNA insert of preproET-1 isolated from a human placenta cDNA library [2]. After subcloning of the *Eco*RI-*Bgl*II fragment (1.01 kbp) into M13mp18, we generated a new *Nco*I restriction site at the initiation codon using a synthetic oligonucleotide (CCTTTTTTCCACCATGG-ATTATTTGC) [4]. We then changed the *Nco*I site to an *Eco*RI site by filling in and adding an *Eco*RI linker (GGAATTCC). The *Eco*RI fragment encoding preproET-1 was ligated to the expression vector pTB281 [5] containing a λ P_L promoter. The resulting plasmid, pTS4007, was introduced into *E. coli* N4830. The C-matured precursor expression vector was constructed from plasmid pGLD906-21, which has a preproET-1 cDNA with an artificial stop codon, TAA, substituted for Val^{74} by in vitro mutagenesis (Itoh, Y. et al., in preparation). The *Acc*I-*Xho*I fragment of pGLD906-21 and *Eco*RI-*Acc*I and *Eco*RI-*Sal*I fragments of pTS4007 were ligated (fig.1A). The resulting plasmid pTS4008 was transformed into *E. coli* N4830. Standard molecular cloning techniques were according to Maniatis et al. [6].

2.2. *Enzyme immunoassay (EIA)*

E. coli N4830 harboring pTS4007 or pTS4008 was screened for gene expression using anti-ET-1 antibodies. They were grown at 30°C for 5 h, and then for thermal induction, the temperature was increased to 42°C for a further 2 h. Cells were harvested and the products extracted using guanidine HCl. Guanidine HCl extraction was performed by suspending the cells in 1/50 vol. buffer [100 mM Tris-Cl (pH 8.0), 7 M guanidine HCl], and carrying out incubation for 1 h at 4°C. Cell-free extracts were diluted and assayed by sandwich EIAs.

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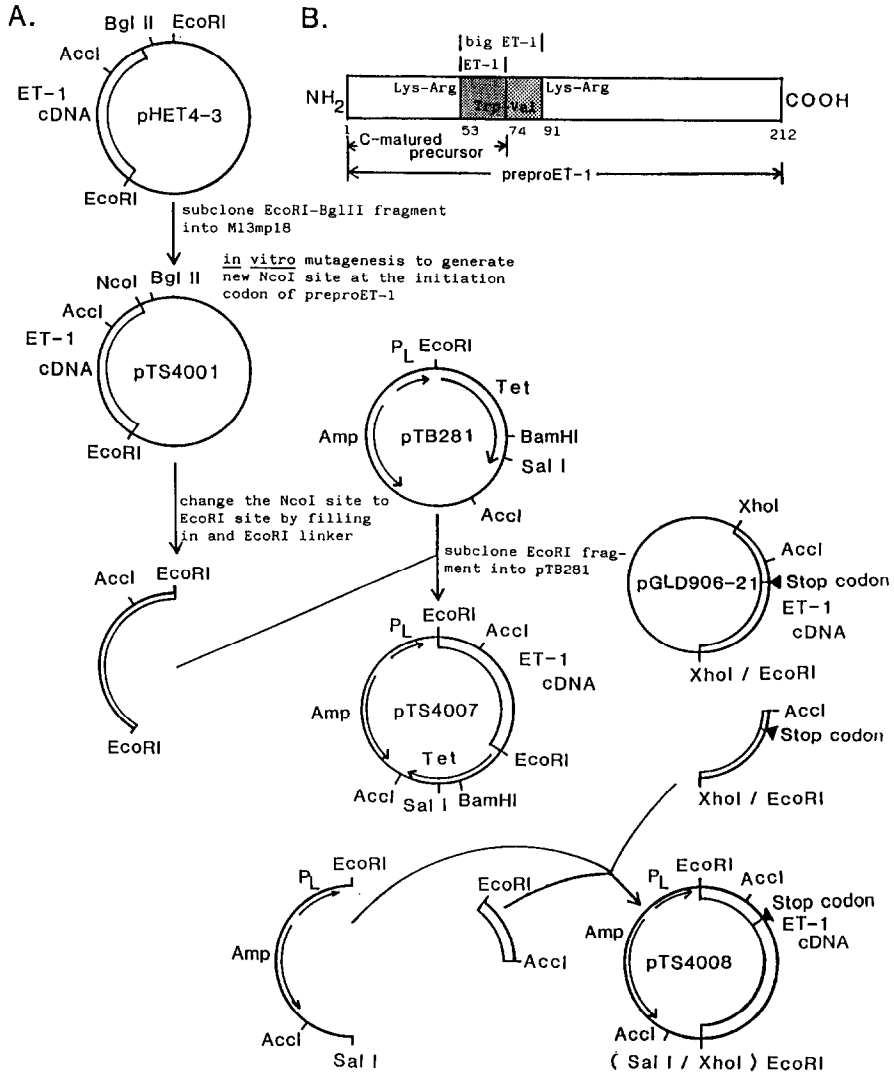

Fig.1. (A) Construction of expression vectors pTS4007 and pTS4008. (B) Structure of preproET-1 and C-matured precursor.

We have established two types of highly specific and sensitive EIA, one for ET-1 (preproET-1 Cys^{53}-Trp^{73}) [7] and the other for big-ET-1 (preproET-1 Cys^{53}-Ser^{90}) (Suzuki, N. et al., in preparation) (fig.1B). Both EIAs used a murine monoclonal anti-ET-1 antibody (AwETN40) [7] as an immobilized antibody. The second antibodies were peroxidase-labeled Fab' fragments of rabbit IgG against the C-terminal region of ET-1 (Cys^{53}-Trp^{73}) [7] and that of big-ET-1 (Val^{74}-Ser^{90}) for ET-1 and big-ET-1, respectively. We used the former to measure the C-matured precursor and the latter to measure C-extended precursor.

2.3. *Western blot analysis*

Cultured cells were harvested and suspended in eq. vol. buffer [62.5 mM Tris-Cl (pH 6.8), 0.1% SDS, 10% glycerol, 0.01% bromophenol blue], and heated for 10 min at 95°C. After centrifugation, bacterial lysates were separated by SDS-polyacrylamide gel (15%) electrophoresis and blotted onto nitrocellulose filter using a Sartorius transfer apparatus (Sartorius, Göttingen). The gene products were detected with AwETN40, and peroxidase-conjugated goat anti-mouse IgG (Organon Teknika Corp.-Cappel Products). Konica immunostaining HRP kit IS-50B (Konica, Tokyo) was used for the substrate of the conjugate.

3. RESULTS AND DISCUSSION

We constructed the expression plasmids pTS4007 for preproET-1 and pTS4008 for the C-matured precursor (fig.1). These plasmids were in-

troduced into *E. coli* N4830 and after thermal induction, the products were characterized by EIAs and Western blot analysis. As expected, the products of pTS4007 were only detected by the EIA system for the C-extended precursor while those of pTS4008 were only detected by that for the C-matured precursor (table 1). Based on calculation from the values of EIA, the amounts of preproET-1 and C-matured precursor expressed were estimated to be 187 and 0.87 ng per ml broth, respectively.

The products of pTS4007 or pTS4008 were subjected to Western blotting. Immunoreactive products of pTS4007 were observed with a major band at 28 kDa and three minor bands at about 26, 19 and 14 kDa (non-reducing conditions) (fig.2). Lane 2 of fig.2 shows that no immunoreactive proteins were observed in the lysate of *E. coli* harboring pTB281 (negative control). The 28 kDa protein, a major species of pTS4007 product, was within the expected size for preproET-1 (theoretical value, 24.4 kDa). The minor bands at 26, 19 and 14 kDa were considered to be hydrolysates of the major 28 kDa protein in *E. coli* cells, since all three appeared together with the 28 kDa product after induction. The products of pTS4008 were detected as a 7 kDa protein (fig.2) which is considered to be the C-matured precursor (theoretical value, 8.2 kDa).

EIA demonstrated that the amount of immunoreactive products of pTS4007 was larger than that of pTS4008 (table 1). Also, the Western blotting pattern indicated a similar result (fig.2). Because pTS4007 and pTS4008 have the same expression units and are believed to transcribe the same mRNAs except for the artificial stop codon of pTS4008, the transcription efficiency of these

Table 1

Enzyme immunoassay of extracts of *E. coli* cells

Plasmid	Assay system	
	C-matured[a]	C-extended[b]
pTS4007	0.036[c]	7.200
pTS4008	0.152	0.016

[a] Chemically synthesized ET-1; 1 ng = 0.601 A_{492}
[b] Chemically synthesized big-EG-1; 1 ng = 0.515 A_{492}
[c] A_{492}/ml broth

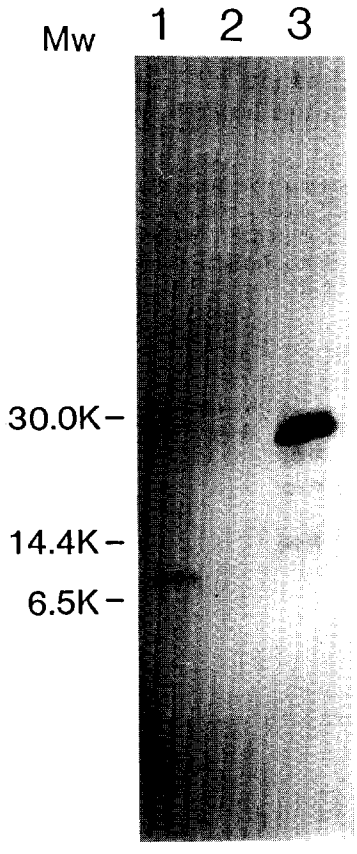

Fig.2. Western blot analysis of bacterial lysates. Lanes: (1) pTS4008, (2) pTB281, (3) pTS4007.

plasmids is considered to be similar in extent. Nilsson et al. [8] showed that premature termination of translation reduced the stability of mRNA. Therefore, this difference in expression levels between preproET-1 and C-matured precursor might reflect the stability of mRNAs in *E. coli* cells.

In endothelial cells, ET-1 is thought to proceed from a prepro form through unusual COOH-terminal processing between Trp^{73} and Val^{74} [1]. The maturation mechanism is at present unknown. PreproET-1 produced in *E. coli* can be a useful substrate for processing studies of ET-1. Our recombinant ET-1 precursors should also be useful for generating monoclonal and polyclonal antibodies, which can be used to investigate mechanisms of secretion in human tissues.

We also attempted to synthesize mature ET-1 in *E. coli* but without success thus far. Mature ET-1 (21 amino acid residues) may be too small to be

synthesized in *E. coli*. Therefore, the recombinant C-matured precursor provides good material for producing mature ET-1 through in vitro proteolytic cleavage between Arg^{52} and Cys^{53}.

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