Identification of amino acid residues in the C-terminal tail of big endothelin-1 involved in processing to endothelin-1

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ABSTRACT

Big endothelin-1 (big ET-1) is converted to 21-amino acid residue endothelin-1 (ET-1) via a specific cleavage at Trp²¹-Val²² by endothelin converting enzyme (ECE). This conversion is an essential step to produce bioactive ET-1 and represents a regulatory site in the biosynthesis of this potent vasoconstrictor. ECE-1a, a unique membrane-bound enzyme, processes big ET-1 more efficiently than other big ET isoforms, which mainly differ in the C-terminal tail (residues 22-38). In this study, each of the highly conserved residues, Val²², Pro²⁵, Pro³⁰, Gly³², Leu³³, and Gly³⁴ were replaced with Ala in the prepro-endothelin-1 (PPET-1) cDNA using site-directed mutagenesis. The mutant and wild-type cDNAs were transiently transfected into Chinese hamster ovary cells along with ECE-1a cDNAs, and concentrations of the resulting recombinant peptides, ET-1 and big ET-1, in the transfection media

were then measured. The concentration of immunoreactive ET-1 in the media from Val²², Pro²⁵, Pro³⁰, Gly³², and Leu³³ mutant PPET-1-transfected cells was 4- to 6-fold lower than that of wild type and (Gly³⁴→Ala)PPET-1. Moreover, with the exception of Gly³⁴, there was a corresponding increase in the concentrations of immunoreactive big ET-1 in the media from mutants. Similar results were obtained when His²⁷, Val²⁸, and Ser³⁵ of big ET-1 were substituted with the corresponding residues in big ET-2 and big ET-3. These findings suggest that the C-terminal tail has an important role in the intracellular processing of big ET-1 by ECE-1a. Herein we also report that a recombinant big ET-1 analog we previously generated and characterized, (Ala²¹)big ET-1, inhibits ECE-1a activity in a dose-dependent ($K_i=1\mu\text{M}$) and competitive manner.

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INTRODUCTION

Endothelin-1 (ET-1), the most potent vasoconstrictor known at present, is generated from post-translational processing of its 212-amino acid residue precursor, preproendothelin-1 (PPET-1), in multiple enzymatic steps (Yanagisawa *et al.* 1988). PPET-1, which contains the signal sequence in the first 17 amino acid residues, is processed to big ET-1 by dibasic-specific endopeptidases and a carboxypeptidase. The final step in the biosynthesis of ET-1 involves the selective hydrolysis of the Trp²¹-Val²² bond of the 39-amino acid residue peptide, big ET-1, by endothelin converting enzyme (ECE) (Yanagisawa *et al.* 1988). As big ET-1 is less potent than ET-1 (Kimura *et al.* 1989), the conversion of big ET-1 to ET-1 is believed to be essential for physiological activity. Therefore, ECE inhibitors that prevent the formation of active

ET-1 are potential candidates for therapeutic intervention.

Two distinct genes encoding ECE have recently been cloned (Shimada *et al.* 1994, Xu *et al.* 1994, Emoto & Yanagisawa 1995) and designated as ECE-1 (Xu *et al.* 1994) and ECE-2 (Emoto & Yanagisawa 1995). Both gene products are membrane-bound proteases that contain a zinc-binding motif and show sequence similarity to neutral endopeptidase-24.11 and KELL protein. ECE-1 and ECE-2 are 71% and 52% identical to each other within the C-terminal one-third of the extracellular domain and N-terminal region, respectively (Emoto & Yanagisawa 1995). ECE-2, which has an acidic pH optimum, is localized mainly in the lysosomes (Emoto & Yanagisawa 1995). The localization of ECE-1 has been very controversial, and it has recently been reported that there are two ECE-1 isoforms that are generated

FIGURE 1. The amino acid sequence comparison of big ET-1 isoforms and the list of mutations described in the current study. The residues that differ in big ET-1 are shown in bold and the mutated residues are indicated as bold and italics. The arrow indicates the cleavage site.

from the same gene by alternative splicing: ECE-1a and ECE-1b (Turner & Murphy 1996). Bovine ECE-1a has been reported to be primarily intracellular, whereas ECE-1b is found on the plasma membrane (Emoto *et al.* 1996). The neutral pH optimum and wide tissue distribution of ECE-1 mRNA suggest that ECE-1 isoforms are the physiologically relevant enzymes for the cleavage of big ET-1 (Turner & Murphy 1996).

Interestingly, the purified ECE-1 isoforms process big ET-1 more efficiently than big ET-2 and cleave big ET-3 very poorly (Xu *et al.* 1994). As the most variable residues in the big ET isoforms are located in the C-terminal region between residues 27 and 38, it is reasonable to conclude that these residues are important for recognition and cleavage by ECE-1, in addition to the Trp²¹-Val²² cleavage site. We have previously shown that, when a mutant PPET-1 cDNA, (Trp²¹→Ala)PPET-1, was expressed in COS-7 cells, both the processing and secretion of the recombinant (Ala²¹)big ET-1 was impaired (Ergul & Puett 1995). Thus transient expression of PPET-1 cDNA in mammalian cells provided additional information on the structural determinants for the intracellular processing events leading to secretion of bioactive ET-1 that cannot be obtained by use of synthetic big ET-1 analogs.

In an attempt to define the role of the C-terminal tail region of big ET-1 in the biosynthesis, recognition and cleavage by ECE, the highly conserved residues, Val²², Pro²⁵, Pro³⁰, Gly³², Leu³³, and Gly³⁴ (Fig. 1), were individually replaced by Ala using site-directed mutagenesis and the cDNAs to wild-type and mutant PPET-1s and ECE-1a were co-expressed in mammalian cells. In addition, to determine whether His²⁷, Val²⁸, and Ser³⁵ contribute to the specificity of ECE-1 for big ET-1, these amino acids were individually changed to Gln, Thr, and Asn respectively, which are the corresponding

residues in big ET-2 and big ET-3. Our results show that, with the exception of Gly³⁴→Ala, these replacements resulted in a decrease in the conversion of big ET-1 to ET-1 by ECE-1. Moreover, recombinant (Ala²¹)big ET-1 inhibited ECE-1a activity in a dose-dependent manner.

MATERIALS AND METHODS

Reagents

The cDNAs for bovine ECE-1a and human PPET-1 were kindly provided by Dr Masashi Yanagisawa (The University of Texas Southwestern Medical Center, Dallas, TX, USA) and Dr Carla Zoja (Mario Negri Institute for Pharmacological Research, Bergamo, Italy), respectively. Cell culture media and serum were purchased from Fisher Scientific (Pittsburg, PA, USA). Phosphoramidon, phenylmethylsulfonyl fluoride (PMSF), ethylenediaminetetra-acetic acid (EDTA), polyoxyethylene-10-lauryl ether, *p*-chloromercuriphenylsulfonic acid, and trypsin were from Sigma Chemical Co. (St Louis, MO, USA). ET-1 and big ET-1 enzyme-linked immunoassay (ELISA) kits and synthetic big ET-1 were products of American Research Products (Belmont, MA, USA) and Peninsula Laboratories (Belmont, CA, USA) respectively. *Pfu* DNA polymerase was obtained from Stratagene Cloning Systems (La Jolla, CA, USA).

Preparation of PPET-1 mutants

PPET-1 mutants were generated using the Quick-Change Site-Directed Mutagenesis protocol (Stratagene Cloning System). Briefly, the desired mutations were introduced into the pcDNA3/PPET-1 construct via two mutant primers, each complementary to opposite strands of the vector, in a polymerase chain reaction performed with *Pfu* DNA polymerase. The PCR product was then digested with *Dpn* I, which cleaves the methylated, wild-type parental strands. The remaining strands were then transformed into DH₅ α cells and mutant clones were identified by sequencing and restriction digestion mapping. The amino acid sequence comparison of big ET-1 isoforms and the replacements made are described in Fig. 1.

Cell culture and transient expression of mutant PPET-1 cDNAs

Chinese hamster ovary cells (CHO-K1), obtained from American Type Culture Collection (Rockville, MD, USA), were grown as recommended. Cells

between passage numbers 3 to 6 were used for transfections. To eliminate the ECE-1-like activities present in tissue-culture-grade trypsin preparations, a highly purified crystallized trypsin (Sigma, T7418) was employed in tissue culture procedures.

The CHO cells were transiently co-transfected with the eukaryotic expression vector pcDNA3/PPET-1, wild type and mutants, and pcDNA3/ECE-1a by incubating the cells with Lipofectamine-DNA mixture for 5 h at 37 °C. For a 75-cm² tissue culture flask, 1 µg wild-type or mutant PPET and 10 µg ECE-1a DNA were used. As controls, cells were also individually transfected with pcDNA3 vector (mock-transfection), pcDNA3/PPET-1, or pcDNA3/ECE-1a constructs. After transfection, the cells were incubated in growth medium supplemented with 10% (v/v) fetal bovine serum for 18 h, and then the serum concentration was reduced to 2%. After 48 h, the medium was collected and cell extracts were prepared for subsequent ET-1 and big ET-1 ELISA analysis. Both preparations were stored at -80 °C until the day of analysis.

Cell extracts were prepared by rinsing the cells with Buffer A (150 mM NaCl, 20 mM HEPES, pH 7.4, 1 mM PMSF, 5 mM EDTA) and then scraping them on ice into 1 ml Buffer A containing 1% Nonidet P-40 and 20% glycerol. The cells were then homogenized at 400 r.p.m. for 2 min with a Tekmar tissumizer and incubated on ice for 30 min to solubilize the membrane bound proteins. The homogenate was centrifuged at 15 000 *g* for 30 min and the supernatant was assayed for the presence of ET-1 and big ET-1-like immunoreactivity.

(Ala²¹)big ET-1 recombinant peptide was prepared and characterized as described previously (Ergul & Puett 1995).

ELISA of ET-1 and big ET-1

The concentrations of immunoreactive (ir) ET-1 and irbig ET-1 in the transfection media and cell extracts were measured using ELISA kits specific for ET-1 and big ET-1. In these assays, an immunoaffinity-purified polyclonal capture antibody and a monoclonal detection antibody, both highly specific for the peptide being measured, ET-1 or big ET-1, were used to provide maximum sensitivity (Ergul *et al.* 1996). The sensitivity of the ET-1 assay was 0.1-50 fmol/ml, and the cross-reactivities of the antibodies used were reported to be <1% with big ET-1 (1-38) and the C-terminal fragment (22-38), and <5% with big ET-2. The sensitivity of the big ET-1 assay was similar and ranged between 0.1 and 70 fmol/ml. The cross-

reactivity of the assay with both ET-1 and the C-terminal fragment (22-38) was <1%. Samples were diluted with the assay buffer to be in the range of standards and were analyzed three times in separate ELISA runs.

Preparation of soluble membranes

Forty-eight hours after transfection with ECE-1a cDNA as described above, approximately 10⁸ cells were rinsed with cold phosphate-buffered saline, scraped off the flasks and collected by centrifugation. All subsequent procedures were performed at 4 °C. Cells, resuspended in 10 × volume of Buffer A (20 mM Tris-HCl, pH 7.4, 20 µM pepstatin A, 1 mM PMSF, and 250 mM sucrose), were homogenized at 400 r.p.m. with a Tekmar tissumizer. The homogenate was then centrifuged at 1000 *g* for 10 min, and the resulting supernatant was centrifuged at 100 000 *g* for 60 min. The crude membrane fraction (pellet) was solubilized in buffer A containing 2.5% polyoxyethylene-10-lauryl ether for 30 min. The mixture was then centrifuged at 50 000 *g* for 60 min, and the supernatant was stored as solubilized membrane.

Measurement of ECE-1 activity

In order to determine whether (Ala²¹)big ET-1 has any inhibitory effect on ECE-1 activity, enzyme activity was measured by incubating various concentrations of big ET-1 (0.01-2 µM) with 30 µg of solubilized membrane preparation in 50-µl reaction mixtures in a 0.1 M sodium phosphate buffer, pH 6.8, containing 0.5 M NaCl for 1 h at 37 °C in the presence and absence of 1.5 µM (Ala²¹)big ET-1 recombinant peptide. In addition, to compare the inhibitory effects of the recombinant peptide and phosphoramidon, 30 µg membrane protein was incubated with 0.1 µM big ET-1 in the absence and presence of various concentrations of (Ala²¹)big ET-1 (0.1-2 µM) and phosphoramidon (0.1-100 µM). The reaction was terminated with 50 µl 5 mM EDTA and the mixture assayed for ET-1 by ELISA in three individual runs. Reaction conditions were set for the hydrolysis to be in the linear range with respect to time and protein concentration.

Statistical analysis

All transfections, enzyme assays, and ELISA assays were repeated at least three times unless otherwise indicated in the text, and the results are given as means ± S.E.M. The S.E.M. of the ratio and the sum of irET-1 (mean ± S.E.M.) and big

FIGURE 2. Production of ET-1 and big ET-1 by transiently transfected CHO cells. Cells were co-transfected with cDNAs for PPET-1, wild type or mutants, and ECE-1a as described in Materials and Methods; after 48 h, the medium was collected and assayed by ELISA kits specific for ET-1 and big ET-1. Data are given as means $\pm$ S.E.M. ($n=4$).

ET-1 (mean $\pm$ S.E.M.) were calculated as described (Kendall & Stuart 1977). The values obtained for wild type recombinant peptides were compared with those of mutants using unpaired Student's t -test and a P value <0.05 was considered significant.

RESULTS

In order to clarify the roles of highly conserved amino acid residues in the C-terminal tail of big ET-1, and of the residues that differ among the big ET-1 isoforms, on enzyme activity and specificity of ECE-1a, several PPET-1 mutant cDNAs were constructed (Fig. 1) and transiently transfected into CHO cells along with the ECE-1a cDNA. There was no detectable irET-1 or irbig ET-1 in the medium from mock-transfected cells, confirming that CHO cells do not produce ET-1 (Xu *et al.* 1994). In cells transfected with the PPET-1 cDNA alone, only irbig ET-1 was detected, suggesting that these cells are devoid of ECE-1 activity. As shown in Fig. 2, almost all the mutations introduced into the PPET-1 cDNA interfered with the processing of the big ET-1 to ET-1. The ratio of ET-1 to big ET-1 in the medium obtained from cells co-transfected with cDNAs to wild type PPET-1 and ECE-1 was 4 ± 1 . This ratio was about 3 for the Gly³⁴ $\rightarrow$ Ala and His²⁷/Val²⁸ $\rightarrow$ Gln/Thr

TABLE 1. Amount of total (irET-1+irbig ET-1) and the ratio (irET-1:irbig ET-1) of recombinant proteins in the media after transient co-transfection of CHO cells with wild-type and mutant PPET-1 and ECE-1a cDNAs

Mutation	irET-1+irbig ET-1 (fmol/ml)	irET-1:irbig ET-1
Wild type	1792 $\pm$ 157	4 $\pm$ 1
Val ²² $\rightarrow$ Ala	1106 $\pm$ 134	0.5 $\pm$ 0.1*
Pro ²⁵ $\rightarrow$ Ala	765 $\pm$ 71**	0.4 $\pm$ 0.1*
His ²⁷ $\rightarrow$ Gln	950 $\pm$ 160	0.4 $\pm$ 0.1*
Val ²⁸ $\rightarrow$ Thr	1294 $\pm$ 161	0.6 $\pm$ 0.1*
His ²⁷ /Val ²⁸ $\rightarrow$ Gln/Thr	2048 $\pm$ 545	2.8 $\pm$ 0.7
Pro ³⁰ $\rightarrow$ Ala	723 $\pm$ 132**	0.6 $\pm$ 0.2*
Gly ³² $\rightarrow$ Ala	1265 $\pm$ 280	0.4 $\pm$ 0.1*
Leu ³³ $\rightarrow$ Ala	2736 $\pm$ 1779	0.2 $\pm$ 0.1*
Gly ³⁴ $\rightarrow$ Ala	1052 $\pm$ 347	3.0 $\pm$ 1.7
Ser ³⁵ $\rightarrow$ Asn	1046 $\pm$ 127	0.6 $\pm$ 0.2*

Values significantly less than those in wild type and other mutants: * $P<0.05$, ** $P<0.005$.

mutants and in the range 0.2-0.6 for the other mutants (Table 1). These results also indicated that the replacement of proline residues by alanine led to a decrease in the amount of ET-1 and big ET-1 secreted into the medium. The total amount of recombinant protein in the medium (sum of irET-1 and big ET-1) of cells transfected with Pro²⁵ $\rightarrow$ Ala and Pro³⁰ $\rightarrow$ Ala mutants was significantly less ($P<0.05$) than that of those detected in the media of cells transfected with wild type and other mutants (Table 1). In order to determine whether these mutations interfere with the secretion of the peptides, extracts of cells were also assayed for Pro²⁵ $\rightarrow$ Ala and Pro³⁰ $\rightarrow$ Ala mutants, but there was no detectable irET-1 and big ET-1 (data not shown). Furthermore, to investigate whether co-transfection affects the transfection efficiency and if the enzyme activity is similar in all cases, solubilized membrane fractions were prepared from a single transfection with the ECE-1a cDNA or several co-transfections with wild type, Pro²⁵ $\rightarrow$ Ala, His²⁷ $\rightarrow$ Gln, Leu³³ $\rightarrow$ Ala, and Ser³⁵ $\rightarrow$ Asn PPET-1 cDNAs, and their associated ECE-1a activities measured. The respective values obtained were 14.5 ± 0.2 , 12.3 ± 0.2 , 11.4 ± 0.5 , 10.0 ± 0.3 , 11.2 ± 0.4 , and 12.6 ± 0.1 pmol/mg protein.h. These results indicate that co-transfection does not reduce the transfection efficiency. We have previously shown that COS-7 cells, unlike CHO cells, have endogenous ECE-like activity and when Trp²¹ was replaced with Ala in the PPET-1 cDNA and transiently expressed, the recombinant peptide (Ala²¹)big ET-1 accumulated in the cells, suggesting that this replacement interferes with both the secretion of big ET-1 and

TABLE 2. Expression of wild-type PPET-1 and (Trp²¹→Ala)PPET-1 mutant in COS-7 cells

	Medium	Extract		Total	
	irET-1 (ng)	irbig ET (ng)	irET-1 (ng)	irbig ET-1 (ng)	irET-1+big ET-1
Wild type	38.4 ± 2.0	9.0 ± 1.0	0.01 ± 0.00 ₂	0.2 ± 0.00 ₂	47.6 ± 1.6
(Trp ²¹ →Ala)PPET-1	6.5 ± 1.2	9.1 ± 1.3	0.01 ± 0.00 ₁	25.0 ± 0.6	40.6 ± 1.3
Co-transfection	21.0 ± 2.0	35.0 ± 1.0	0.01 ± 0.00 ₂	23.5 ± 1.0	80.0 ± 2.4

its cleavage by ECE (Ergul & Puett 1995). To investigate the possibility that the (Ala²¹)big ET-1, accumulating in the cells, interacts with the converting enzyme ECE, co-transfection experiments with wild-type PPET-1 and (Trp²¹→Ala)PPET-1 cDNAs were performed. Single transfections with only wild-type or (Trp²¹→Ala)PPET-1 cDNA were also repeated under the same conditions, and irET-1 and irbig ET-1 concentrations were measured. To be consistent with our previous results, constructs in the pSVL expression vector and COS-7 cells were used for this set of transfections. In the medium collected from cells co-transfected with wild-type and (Trp²¹[sA]la)PPET-1 cDNA, there was a decrease in irET-1 concentrations accompanied by a corresponding increase in irbig ET-1 concentrations as compared with that obtained from cells transfected with wild-type PPET-1 cDNA alone (Table 2). This observation strongly suggests that (Ala²¹)big ET-1 interacts with ECE and prevents the processing of wild-type big ET-1.

In order to characterize further the interaction between ECE-1a and (Ala²¹)big ET-1, enzyme assays were performed using the soluble membrane fractions prepared from CHO cells transfected with the ECE-1a cDNA and (Ala²¹)big ET-1 obtained from extracts of COS-7 cells transfected with this mutant PPET-1 cDNA (Ergul & Puett 1995). Irbig ET-1 in the extracts was characterized by a radioimmunoassay and HPLC as described previously (Ergul & Puett 1995). Initially, 30 µg soluble membrane incubated with 0.1 µM synthetic big ET-1 in the absence and presence of various concentrations of (Ala²¹)big ET-1 (0.01–2 µM) and the nonspecific metalloprotease inhibitor, phosphoramidon (0.1–100 µM). The *K_i* value obtained for (Ala²¹)big ET-1 (0.9 µM) was similar to that of phosphoramidon (0.6 µM) (Fig. 3). In order to ensure that these findings were specific for ECE-1a and (Ala²¹)big ET-1, a series of control experiments was performed. Because the recombinant (Ala²¹)big ET-1 was obtained from crude extracts of cells transfected with (Trp²¹Ala)PPET-1 cDNA, cell extracts and medium obtained from wild-type PPET-1 cDNA-transfected cells were also assayed

as controls, to eliminate the possibility that any inhibitory effect detected is nonspecific. As shown in Fig. 3, these preparations had no effect on enzyme activity. Although intact COS-7 cells exhibit ECE-like activity, there was no enzyme activity associated with the cell extracts. This finding was most probably a result of the loss of the activity during the preparation and concentration of the extracts.

Next, to characterize the inhibitory properties of (Ala²¹)big ET-1, the enzyme assays were repeated in the presence and absence of 1.5 µM (Ala²¹)big

FIGURE 3. Concentration-dependent inhibition of enzyme activity in solubilized membranes prepared from CHO cells transfected with ECE-1a cDNA by (Ala²¹)big ET-1, phosphoramidon, and medium containing recombinant wild type ET-1. Thirty micrograms membrane protein and 0.1 µM big ET-1 were incubated in the absence and presence of various concentrations of inhibitors and the ET-1 produced was measured. The data obtained in the presence of inhibitors were normalized to those obtained in the absence of inhibitors and expressed as the % inhibition of enzyme activity. The results represent means ± S.E.M. of three individual experiments.

FIGURE 4. The effect of (Ala²¹)big ET-1 on ECE-1a activity. Solubilized membrane preparation (30 μg) and the designated concentrations of big ET-1, (Ala²¹)big ET-1, and big ET-1+1.5 μM (Ala²¹)big ET-1 were incubated for 1 h at 37 °C. (A), Amount of ET-1 produced, measured by ELISA as described in Materials and Methods. (B), Double-reciprocal plot of the data.

ET-1 and various concentrations of synthetic big ET-1 (0.01–2 μM), to determine the K_m and V_{max} values. In the presence of the inhibitor, the ECE-1a activity, as measured by ET-1 produced, was significantly reduced (Fig. 4A). As shown in Fig. 4B, the double-reciprocal analysis of the same data showed that the change in K_m was quite subtle, from $0.23 \pm 0.06 \mu\text{M}$ to $0.53 \pm 0.33 \mu\text{M}$ in the absence and presence of the inhibitor respectively. However, the V_{max} value was reduced fivefold, indicating that (Ala²¹)big ET-1 behaves as a competitive inhibitor. To clarify whether (Ala²¹)big ET-1 acts as a partial substrate and contributes to the ECE-1 activity shown in Fig. 4A, the enzyme assays were performed using the recombinant protein as a substrate. As shown in Fig. 4A, even at the greatest concentration of the substrate (2 μM), the conversion to ET-1 was less than 8% of that of synthetic big ET-1.

DISCUSSION

The results described herein show that the C-terminal tail of big ET-1 is important in the recognition and cleavage by ECE-1 and that a big ET-1 analog, (Ala²¹)big ET-1, behaves as a potent ECE-1a inhibitor and only a weak substrate.

Reports on ECE purified from various tissues or cell lines revealed distinctive properties of its specificity. Recently, human ECE-1 has been reported to cleave another vasoregulatory peptide, bradykinin, with a K_m value of 978 μM, whereas its affinity for big ET-1 under the same conditions was 8 μM (Hoang & Turner 1997). Although one cannot rule out the possibility that ECE-1 might have a broader spectrum of substrates, thus far big ET isoforms appear to be the only biological substrate for this enzyme (Turner & Murphy 1996, Turner & Tanzawa 1997). Moreover, even the conversions of big ET isoforms to ET display marked differences, depending upon the sources from which the ECEs are purified. For instance, a partially purified preparation from EA.hy926 cells processed human big ET-1 much more efficiently than big ET-2 and failed to convert big ET-3 (Waxman *et al.* 1994). Similarly, the cloned and expressed recombinant enzymes cleaved big ET-1 more efficiently than big ET-2 and had almost no effect on big ET-3 (Schmidt *et al.* 1994, Shimada *et al.* 1994). However, the bovine endothelial ECE-1a cDNA, which was the ECE cDNA used in the present study, processed both big ET-2 and big ET-3 with lower velocities of hydrolysis than that of big ET-1 (Xu *et al.* 1994). These observations suggest that big ET-1 possesses unique structural elements required for recognition and cleavage by ECE-1. Our results, showing reduced conversion efficiencies after replacements of the highly conserved amino acid residues with Ala, and also of the residues unique to big ET-1 with the corresponding residues in big ET-2 and big ET-3, provide further support that the C-terminal tail of the big ET-1 molecule is involved in the recognition, cleavage, or both, by ECE-1.

Previously, it was reported that the minimal substrate for ECE was the sequence from Ile¹⁹ to Gly³⁴ of big ET-1, and that truncated big ET-1 analogs have substantially greater specific activity, thus indicating that the N-terminal loop structure of big ET-1 (residues 1–15) hinders the conversion (Okada *et al.* 1991, 1993). Alanine scanning of a synthetic truncated big ET-1, (19–34)big ET-1, suggested that His²⁷, Val²⁹, Pro³⁰, Tyr³¹, Gly³², Leu³³, and Gly³⁴ were more important than the other residues in the C-terminal tail of big ET-1 (Okada *et al.* 1993). Our results are in good

FIGURE 5. The ribbon diagram of the molecular model of big ET-1 (modified from Peto *et al.* 1996). The proposed hydrogen bonds in the structure are indicated by the dashed lines.

agreement with these studies. Using the full-length mutant big ET-1 analogs expressed in a mammalian expression system, we showed that Val²², Pro²⁵, His²⁷, Val²⁸, Pro³⁰, Gly³², Leu³³, and Ser³⁵ were involved in the recognition and cleavage by ECE-1a.

Recently, a molecular model for big ET-1 was reported in which the structure of the endothelin core was almost identical to the crystal structure of ET-1 (Peto *et al.* 1996). In their big ET-1 structure (Fig. 5), the C-terminal helix is extended and, because of a loop formed by a hydrogen bond between Asp¹⁸ and Thr²⁴, the chain folds back on itself, resulting in hydrogen bonds between residues 1 and 5 of the N-terminal region of big ET-1 and residues 27 and 29 of the C-terminal tail. The structure is further compacted by a turn generated by hydrogen bonding between Ser³⁵ and Ser³⁸ bringing the side chain of Ser³⁸ in close contact with Lys⁹. As a previous study (Corder 1996) reported that the conversion rate of big ET-1 analogs, in which Lys⁹ residue was chemically modified, was substantially reduced, it was argued that this residue forms a hydrogen bond with Ser³⁸ and stabilizes the big ET-1 structure (Peto *et al.* 1996). In the present study, the replacement of Ser³⁵ with Asn, which is the corresponding residue in big ET-2 and big ET-3, affected the cleavage and resulted in lower ET-1 production. This finding

supports the molecular modeling studies predicting a bond between Ser³⁵ and Ser³⁸. Interestingly, we previously demonstrated that, when (Lys⁹→Glu)PPET-1 and (Lys⁹→Ala)PPET-1 cDNAs were transiently expressed in COS-7 cells, the conversion of mutant big ET-1 analogs to ET-1 was similar to that of wild type, suggesting that these mutations did not interfere with the processing by ECE (Ergul *et al.* 1995). These findings, however, were different from those obtained with Lys⁹-modified big ET-1 analogs (Corder 1996). One possible explanation of this difference might be that functional groups incorporated into Lys⁹ are quite bulky and disrupt the bond between Ser³⁸ and Lys⁹, whereas Ala or Glu substitution did not affect the compact structure of big ET-1.

Interestingly, on the basis of the results obtained by two groups, who showed that the replacement of Val²² with Phe enhanced the cleavage substantially (Ohnaka *et al.* 1993) and that the substitution of Trp²¹ with Phe or Tyr did not alter the processing in *Xenopus* oocytes (Fabbrini *et al.* 1993), it was suggested that there was no strict requirement for Trp-Val at the cleavage site (Turner & Murphy 1996). It was also proposed that a possible requirement for substrate binding and catalysis by ECE-1 might be a large hydrophobic residue in the S₁' (i.e. Val²²) site and a rather flexible S₁ position (i.e. Trp²¹) (Hoang & Turner 1997). However, we previously demonstrated that when the (Trp²¹→Ala)PPET-1 mutant cDNA was expressed in COS-7 cells, which possess endogenous ECE activity, (Ala²¹)big ET-1 accumulated in the cells, suggesting that this mutation interfered with both the processing and the secretion of the recombinant peptide (Ergul & Puett 1995). In this study, we showed that co-transfection of wild-type PPET-1 with (Trp²¹→Ala)PPET-1 resulted in a reduction in the formation of wild-type ET-1 from big ET-1, providing further support for an inhibitory role of (Ala²¹)big ET-1 on ECE. Moreover, enzyme activity studies performed using soluble membrane preparations of ECE-1a and big ET-1 in the presence and absence of (Ala²¹)big ET-1 demonstrated that, at the concentrations used (1–2 μM), (Ala²¹)big ET-1 acts as a potent competitive inhibitor and only a weak substrate of ECE-1a. Interestingly, a big ET-1 analog, [D-Val²²]big ET-1(16–38) (Morita *et al.* 1994), has been reported to have inhibitory effects on the converting enzyme, demonstrating the importance of this region of the molecule in recognition by ECE.

Our cell culture expression system also provided information on the contribution of the individual residues on the synthesis of the recombinant peptides. For example, (Pro²⁵→Ala)PPET-1 and

(Pro³⁰→Ala)PPET-1 cDNAs expressed at lower levels than the wild-type and other mutant PPET-1 cDNAs. Possible explanations of this finding are at least twofold: (i) as proline residues are important for introducing turns into the proteins, Ala replacement might cause misfolding of the protein leading to faster degradation, or (ii) the mutations might have interfered with the recognition of anti-big ET-1 antibodies used in the ELISA experiments, resulting in low irbig ET-1 concentrations. Although we do not have direct evidence, we believe the former is more likely, because the irET-1 concentrations in the media obtained from cells transfected with Pro mutants were also lower than those of the wild type and other mutants. In addition, crossreactivity of the anti-big ET-1 antibody with ET-1 and C-terminal fragment, (22-38)big ET-1, were reported to be <1%, indicating that it recognizes a certain conformation of big ET-1 molecule and even a failure of recognition by the antibody would imply a conformational change.

In conclusion, the data presented herein strongly suggest that the amino acid residues between positions 21 and 35 contribute to the recognition and cleavage of big ET-1 by ECE-1a and that (Ala²¹)big ET-1 is a potent inhibitor and only a very weak substrate for ECE-1a. Although the replacement of the residues unique to big ET-1 by the corresponding residues in big ET-2 and big ET-3 reduced the conversion rate of these mutant big ET-1 analogs, detailed enzyme assays are required to determine whether these residues, His²⁷, Val²⁸, and Ser³⁵, confer the high specificity of ECE-1a for big ET-1.

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