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The signal peptide of human preproendothelin-1

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Synthetic mRNAs were produced using either the complete coding sequence of a human preproendothelin-1 cDNA clone or a truncated form in which the portion encoding the first 17 amino acids, representing a putative signal peptide for insertion into the endoplasmic reticulum, was replaced with a methionine codon. The mRNAs were translated in vitro in the presence or in the absence of microsomal membranes. Protection from trypsin digestion demonstrated that the full-length polypeptide, but not the truncated form, could be inserted into the membranes. Sequence analysis revealed that membrane insertion is accompanied by removal of the first 17 amino acids. These results indicate that the first 17 amino acids of human preproendothelin-1 are a functional signal peptide which allows the protein to enter the secretory pathway.

Endothelin biosynthesis; Endoplasmic reticulum; Signal peptide

1. INTRODUCTION

The in vivo biosynthetic pathway of the 21 amino acid peptide endothelin (ET), one of the most potent vasoconstrictors known, is not yet completely clarified [1]. The primary translation product, as deduced by sequencing of cDNAs of different mammalian sources [2,3], is a polypeptide of around 200 amino acids. Expression of a cDNA clone in *E. coli* resulted in the synthesis of a polypeptide with approximately the expected size of the primary translation product [4]. However, this precursor has never been identified in the native cells. The largest precursor of ET-1 so far identified in vivo is big ET-1, which differs from ET-1 in having an extension of 17 or 18 amino acids at the carboxy terminus [5,6]. The cleavage point for the conversion of big ET-1 into ET-1 is between Trp and Val, at an unusual processing site, while big ET-1 probably originates from endoproteolytic cleavage at paired basic residues [2]. The first 19 amino acids of the porcine precursor [2] and the first 17 amino acids of the human precursor [3] show the sequence characteristics of a signal peptide for the insertion into the endoplasmic reticulum (ER), as predicted by the algorithm of Von Heijne [7]. The precursor has therefore been termed preproET-1 [2]. The fact that big ET-1 and ET-1 were found in the cell culture media, and the presence of a putative signal peptide, suggest that processing steps may occur during protein transport through the ER-mediated secretory pathway or immediately after secretion. However, some proteins that do not carry a functional signal peptide may follow an alternative secretory mechanism, not ER-mediated [8]. In a first effort to elucidate the pathway of ET-1 synthesis and the subcellular organelles involved in the processing steps, we show here that the first 17 amino acids of preproET-1 indeed function as a signal peptide.

Abbreviations: ER, endoplasmic reticulum; ET, endothelin

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2. MATERIALS AND METHODS

2.1. *Construction of plasmids, transcription and translation*

The cDNA encoding preproET-1 was isolated from a human placenta polyA$^+$ library as previously described [9]. To remove the 5′ untranslated sequence, the 1.1 kb *Eco*RI fragment of M13mp18-ET(A) was amplified with the polymerase chain reaction using two specific oligonucleotides with *Sma*I linkers. The sense-orientation oligonucleotide (5′-GTCACTCCCGGGAGAATGGATTATTTGCTCATG-3′) begins at −3 of the coding strand whereas the antisense-orientation oligonucleotide (5′-GTCACTCCCGGGGATCCGAATGAGACTGTGTGTTTCTGCT-3′) complements the sequence starting from the end of the cDNA. The *Sma*I-digested fragment, after being controlled by sequencing, was subcloned into blunt-ended dephosphorylated *Bgl*II site of the plasmid pSP64T [10]. This expression plasmid was termed CNR1. To produce CNR2, the same strategy was used but a different sense-orientation oligonucleotide was synthesized (5′-GTCACTCCCGGGAGAATGGCTCCAGAAACAGCAGTCTTAGG-3′). In this way the codons encoding the first 17 amino acids in preproET-1 were replaced by one ATG. We used the PC/GENE PSIGNAL program to determine the potential cleavage site of the presequence.

In vitro transcription and translation in the presence of L-[4,5-^{3}H]leucine (5.62 TBq/mmol, Amersham International) were essentially as described by Fabbrini et al. [11], except that SP6 RNA polymerase (Promega Biotec, Madison) was used, at 0.6 U/μl final concentration. In vitro translation in the presence of microsomal membranes was performed as specified by the manufacturer (Promega Biotec, Madison).

2.2. *Trypsin treatment*

Transcripts of CNR1 or CNR2 were translated in a final volume of

30 μl in the presence or in the absence of microsomal membranes. At the end of the translation $CaCl_2$ in 20 mM HEPES, pH 7.5, was added to a 1.66 mM final concentration. Each sample was then incubated for 10 min on ice. Aliquots of 12 μl were made up to 15.5 μl with water or Triton X-100 (1% final) in the presence or absence of 50 μg/ml freshly prepared trypsin (Sigma, St. Louis). After incubation for 30 min on ice, 0.5 μg of soybean trypsin inhibitor (Boehringer, Mannheim) were added. After 10 additional min on ice, samples were analyzed by SDS-PAGE and fluorography.

2.3. *NH_2-terminal sequence analysis*

For protein sequence analysis, the CNR1 transcript was translated in the presence of microsomal membranes and labeled with [^{3}H]Leu in a final volume of 150 μl or with L-[2,3,4,5-^{3}H]proline (3.48 TBq/mmol, Amersham International) in a final volume of 30 μl. Samples were then treated with trypsin as described above. After SDS-PAGE, protein samples were electroblotted onto polyvinylidene difluoride membranes (Immobilon P, Millipore, Bedford) at 300 mA for 45 min using 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid, pH 11, 10% methanol as transfer buffer. The radiolabelled bands were excised from the membrane and arranged in the cartridge block of the sequencer, beneath a polybrene-preconditioned trifluoroacetic acid-treated glass-fiber filter [12]. Automated Edman degradation was performed on a Gas-Phase Sequencer Model 470A (Applied Biosystems, Foster City) using the manufacturer's program ATZ470-1. The radioactivity in each cycle fraction was determined by scintillation counting.

3. RESULTS AND DISCUSSION

The complete coding sequence of human preproET-1 was subcloned into the expression vector pSP64T [10]. The construct was termed CNR1 (Fig. 1). In a second construct (termed CNR2), the nucleotides encoding the first 17 amino acids, representing a putative signal sequence for insertion into the ER, were removed and replaced by the translation initiator codon ATG (Fig. 1). Synthetic RNAs were produced by in vitro transcription and then tested in the reticulocyte lysate translation system supplemented with [^{3}H]leucine (Fig. 2). The polypeptide synthesized by the CNR1 transcript had an apparent M_r around 23000, although the band was somehow smeared (lane 4 or 6). This is in good agreement with the expected size of preproET-1. Putative proET-1, synthesized upon translation of CNR2, behaved as a more defined band with slightly lower electrophoretic mobility on SDS-PAGE, instead of the expected higher mobility (lane 5). This may be due to abnormal SDS binding of the presequence in CNR1, as also suggested by the smeared pattern of the CNR1 product.

To test whether the first 17 amino acids of preproET-1 direct the insertion of the polypeptide into the ER, translation of the CNR1 transcript was performed in the presence of pancreatic canine microsomes. Under these conditions, CNR1 synthesized a major polypeptide which co-migrated with the product of CNR2 translated in the absence of membranes (Fig. 2, compare lane 3 with lane 5 and Fig. 3, compare lane 6 with lane 9). Conversely, the mobility of CNR2 product was not affected by the presence of membranes (Fig. 3, compare lane 1 with lane 2). These results strongly suggested that a large portion of preproET-1 underwent removal of its presequence in the presence of membranes. To determine whether CNR1 product was translocated into microsomes, we performed an assay of protection against tryptic digestion (Fig. 3). In the presence of membranes only the product of CNR1 (lanes 6 and 7), but not that of CNR2 (lanes 2 and 3), was partially resistant to trypsin. The protected form was that co-migrating with the CNR2 product (compare lanes 6 and 7 and lanes 9 and 10). Resistance was not intrinsic, since it could be abolished by Triton X-100 treatment (lane 8). Together these

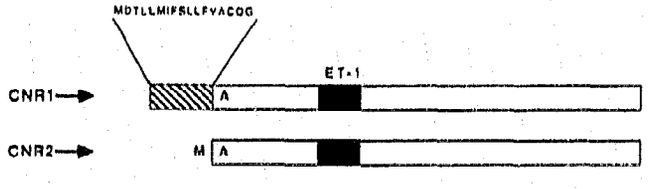

Fig. 1. Schematic representation of the constructs CNR1 and CNR2. Dashed box, putative signal peptide; black box, mature ET-1. Amino acids are indicated using the one-letter code.

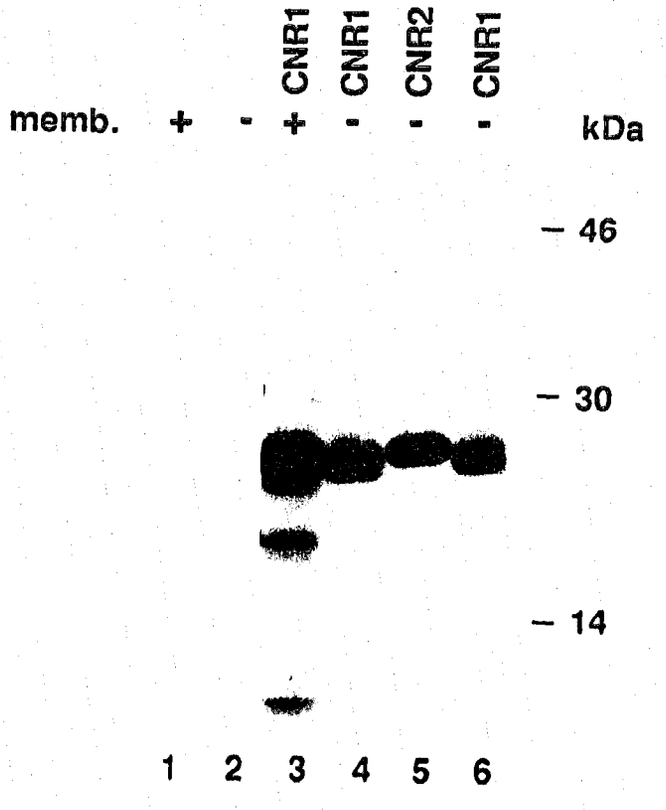

Fig. 2. In vitro translation of CNR1 and CNR2 transcripts with or without microsomal membranes. Analysis of the polypeptides was by SDS-PAGE and fluorography. Lanes 1 and 2: no RNA, as controls. Positions of molecular mass markers (in kDa) are indicated on the right.

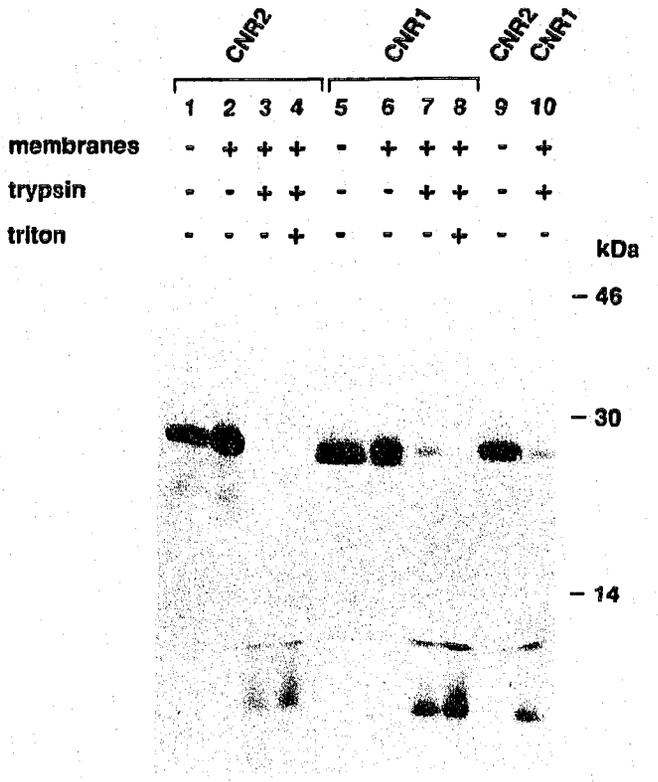

Fig. 3. Trypsin treatment of polypeptides synthesized in vitro by CNR1 and CNR2 transcripts. Analysis was by SDS-PAGE and fluorography. Positions of molecular mass markers (in kDa) are indicated on the right.

results indicate that the presequence directs insertion of preproET-1 into the microsomes.

Finally, we determined the positions of the first leucine and of the first proline present in the membrane-inserted CNR1 translation product. The CNR1 transcript was translated in the presence of microsomal membranes and either [^{3}H]Leu or [^{3}H]Pro. Samples were then treated with trypsin and, after enzyme inactivation, run on SDS-PAGE. The gel was then blotted onto a polyvinylidene difluoride membrane, the radiolabelled bands were excised, and protein subjected to automated Edman degradation. The potential cleavage site between position 17 and 18 conforms to the (−3, −1) rule of Von Heijne [7] and the highest score (9.18) is found at this position. As expected for cleavage at this site, the first [^{3}H]leucine appeared at the seventh cycle of Edman degradation, while the first [^{3}H]proline was removed at the second cycle (Fig. 4). This clearly indicates that upon insertion into microsomes the first 17 amino acids of preproET-1 had been removed.

In conclusion, we determined that the first step in the synthesis of human ET-1 consists of insertion into the ER. Insertion is mediated by a signal peptide of 17 amino acids, which is then removed. Most probably, in vivo proET-1 is then transported along the secretory pathway through the Golgi complex. At which positions along the pathway subsequent processing steps occur remains to be elucidated.

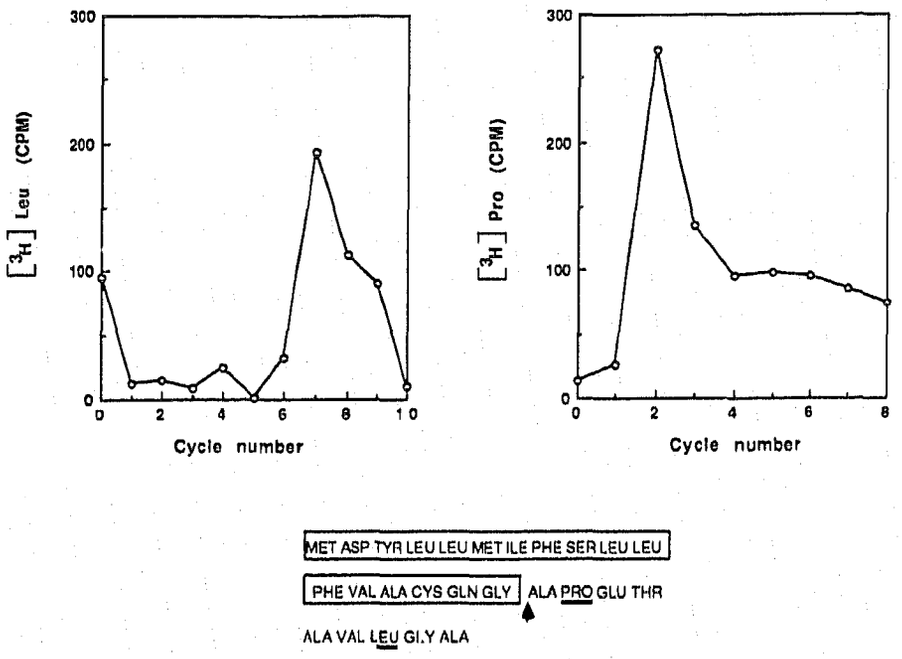

Fig. 4. NH_2-terminal sequence analysis of the polypeptide synthesized in vitro by CNR1 transcript and inserted into microsomal membranes. The graph at the left shows the recovery of [^{3}H]leucine in each sequencer cycle. The graph at the right shows the recovery of [^{3}H]proline in each sequencer cycle. Arrow in the sequence at the bottom indicates the deduced splicing site of prepro ET-1.

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