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CLONING AND CHARACTERIZATION OF cDNA ENCODING A PRECURSOR FOR HUMAN ADRENOMEDULLIN[1]

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SUMMARY: Adrenomedullin is a novel hypotensive peptide recently isolated from human pheochromocytoma. Since a high concentration of immunoreactive adrenomedullin was found in pheochromocytoma tissue, the cDNA library of pheochromocytoma was constructed, and the cDNA clone encoding an adrenomedullin precursor was isolated and sequenced. The precursor for human adrenomedullin (human preproadrenomedullin) is 185 amino acids in length, including an adrenomedullin sequence. Proadrenomedullin (proAM) contains a unique twenty amino acid sequence followed by Gly-Lys-Arg in the N-terminal region. It is possible that a novel 20 residues peptide, termed "proadrenomedullin N-terminal 20 peptide" (proAM-N20) whose carboxy terminus may be Arg-NH_2, is processed from proadrenomedullin. By RNA blot analysis, human adrenomedullin mRNA was found to be highly expressed in several tissues including adrenal medulla, ventricle, lung and kidney as well as pheochromocytoma.

Adrenomedullin (AM) is a novel hypotensive peptide found in human pheochromocytoma (PC), discovered by monitoring the elevating activity of platelet cAMP (1). The peptide, consisting of 52 amino acids, has one intramolecular disulfide bond and shows a slight homology with calcitonin gene related peptide (CGRP). An intravenous bolus injection of AM caused a potent and long lasting hypotensive effect in anesthetized rat. In studies of the regional distribution of immunoreactive (ir-) AM in human tissue, it was abundant in normal adrenal medulla as well as PC tissue, but was not detectable in brain. AM is thought to be a candidate for a new hormone participating in circulation control, because it is found in blood in a considerable concentration (1).

To further understand the physiological implications and biosynthesis of AM, it is essential to analyze cDNA encoding human AM. In the present study, we have succeeded in cloning the cDNA encoding the precursor of human AM.

[1]The nucleotide sequence reported in this paper has been submitted to the DDBJ/EMBL/GenBank DNA database with accession number DI 4874.

Abbreviations used are: AM, adrenomedullin; proAM-N20, proadrenomedullin N-terminal 20 peptide; PC, pheochromocytoma; ir-, immunoreactive; RIA, radioimmunoassay.

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Materials and Methods

cDNA library construction: Total RNA was extracted from human PC (4 g) by the guanidine thiocyanate method (2). Poly(A)$^+$RNA was isolated by oligo(dT)-Latex (TAKARA SHUZO CO., LTD, Kyoto, Japan) (3). Double-stranded cDNA was synthesized by reverse transcription of 5 μg of human PC poly(A)$^+$RNA by the method of Gubler and Hoffman (4), using a primer which contained an oligo (dT) and a XhoI restriction enzyme recognition site. The cDNA was ligated to EcoRI adaptors and digested with XhoI for a uni-directional cloning site, then size-fractionated by Sephacryl S-400 column (Pharmacia). Size-fractionated cDNA was ligated to 1 μg of Uni-Zap XR vector arms and packaged *in vitro* (Stratagene). One round amplification was carried out to increase stability of the library.

cDNA cloning and sequencing: A porcine AM cDNA fragment (pPAM-2), which will be described elsewhere, was labeled by the random-primed method and was used to screen the human PC cDNA library by *in situ* plaque hybridization as previously described (5). A pBluescript with positive DNA inserts was recovered from the λ Zap II phage by the *in vivo* excision method using helper phage R408 (6). The clone (pHAM-3), which carried the longest cDNA insert, was used for the sequencing. Restriction fragments generated from the cDNA insert by digesting with relevant restriction enzymes (SmaI, NaeI, SacI and RsaI) were subcloned into Bluescript plasmids and were sequenced by the dyeprimer cycle sequencing method using an automated DNA sequencer (373A, Applied Biosystems).

RNA blot analysis: PC tissue was surgically removed from the patient, and the normal human tissue was obtained from cadavers. Total RNA was extracted from each tissue (1 g) by the guanidine thiocyanate method (2). Poly (A)$^+$ RNA (1 μg), isolated by oligo(dT)-Latex (3), was denatured using glyoxal and dimethylsulfoxide, and was electrophoresed on a 1.0% agarose gel, then transferred to a nylon membrane (Zeta Probe, Bio-Rad). A cDNA insert of pHAM-3 was labeled by the random-primed method and used as a probe for hybridization under conditions described (5).

Results and Discussion

In our previous study, we demonstrated that PC tissue is very rich in ir-AM (1). Therefore, a cDNA library for isolation of clones encoding an AM precursor was constructed with poly (A)$^+$RNA of human PC. A portion of the library (approximately 4 x 10^5 p.f.u.) was subjected to a first screening with a porcine AM cDNA probe to isolate 6 positive clones. By a second screening of these clones, 4 positive clones were finally isolated. The clone, which harbored the longest insert of approximately 1,400 base pairs, was designated pHAM-3 and was used for sequencing. According to the strategy indicated in Fig. 1, the complete nucleotide sequence of the AM precursor cDNA, which was 1293 bp long excluding poly(A), was determined from both strands.

Fig. 2 shows the complete nucleotide sequence and the deduced amino acid sequence encoded in the open reading frame. A putative initiation codon ATG is located at nucleotides 1-3, preceded by the consensus sequence for the initiation, while a termination codon TAG is found 185 codons later at nucleotides 556-558. The deduced amino acid sequence, shown in Fig. 2, indicates that human AM mRNA encodes a 185-residue protein. The nucleotide sequence of bases 238-438 corresponds exactly to the amino acid sequence of human AM isolated from PC. Nucleotides 439-441 encode glycine, which is likely to contribute an amide structure (7) to the C-terminal Tyr residue of AM. The first 21-residue peptide starting from the initial methionine is thought to be a signal peptide, as predicted by its characteristic hydrophobic feature and the

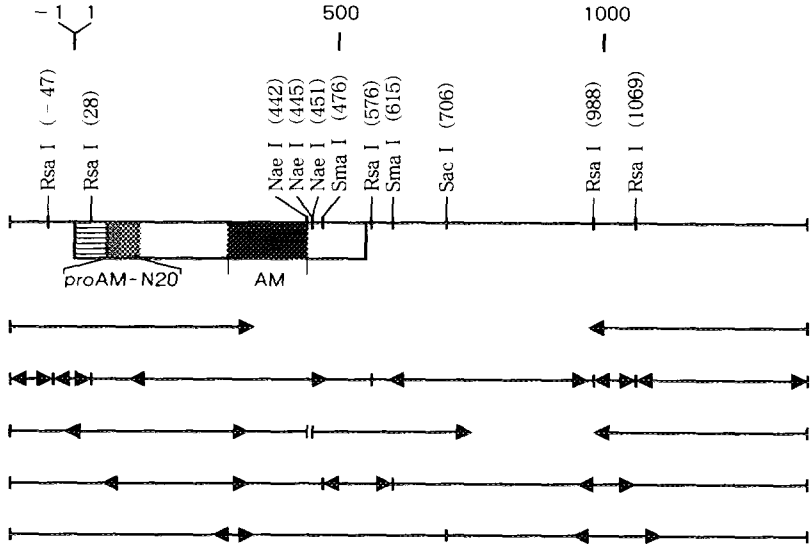

Fig. 1. Strategy of sequencing the cDNA insert in clone pHAM-3. The restriction map displays only relevant restriction sites. (▤) signal peptide; (▒) proAM-N20; (■) AM. Arrows show the direction and extent of sequence determination.

weight-matrix method (8). Therefore, it is likely that the first processing of the precursor takes place between Thr^{21} and Ala^{22} to generate 164-residue proadrenomedullin (proAM).

It is well known that many peptide hormones and neuropeptides are produced from larger, biologically inactive precursors through cleavage at pairs of basic amino acids, primarily Lys-Arg and Arg-Arg (9,10). As shown in Fig. 2, three typically paired basic amino acids, which represent sites for proteolytic processing signals, are found in the predicted mature precursor protein. The last two (Lys^{93}-Arg^{94} and Arg^{148}-Arg^{149}) flank the AM peptide and represent sites for proteolytic processing to release AM. The first paired basic amino acid, Lys^{43}-Arg^{44}, is a representative site for proteolytic cleavage, and follows the Arg^{41}-Gly^{42} residue. These sequences indicate the possibility that the site converts to Arg-NH_2 as the carboxy terminus, because the Gly followed by paired basic amino acid will contribute an amide structure to the C-terminus (7). Therefore, it is highly possible that a novel 20 residue peptide, termed "proadrenomedullin N-terminal 20 peptide" (proAM-N20), whose carboxy terminus is Arg-NH_2, is processed from the amino terminal region of proAM. This predicted peptide shows no significant homology with other known biologically active peptides. However, a computer search (PRF-SEQDB, Protein Research Foundation, Osaka, Japan) indicated that the amino acid sequence of a glucagon-like peptide 1 receptor [405-424] has significant homology with 45% identity to proAM-N20 (11). The sequence is not flanked on either side by the typical processing signal to release biologically active peptides. We cannot explain at present the physiological implications of this sequence homology. Although the biological activity of proAM-N20 is obscure, the peptide may have profound biological function, because the car-

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156                                                    CTG GAT AGA ACA GCT CAA GCC TTG CCA CTT CGG GCT   121
120  TCT CAC TGC AGC TGG GCT TGG ACT TCG GAG TTT TGC CAT TGC CAG TGG GAC GTC TGA GAC   -61
 60  TTT CTC CTT CAA GTA CTT GGC AGA TCA CTC TCT TAG CAG GGT CTG CGC TTC GCA GCC GGG    -1

  1  Met Lys Leu Val Ser Val Ala Leu Met Tyr Leu Gly Ser Leu Ala Phe Leu Gly Ala Asp    20
  1  ATG AAG CTG GTT TCC GTC GCC CTG ATG TAC CTG GGT TCG CTC GCC TTC CTA GGC GCT GAC    60
                 ↓
 21  Thr Ala Arg Leu Asp Val Ala Ser Glu Phe Arg Lys Lys Trp Asn Lys Trp Ala Leu Ser    40
 61  ACC GCT CGG TTG GAT GTC GCG TCG GAG TTT CGA AAG AAG TGG AAT AAG TGG GCT CTG AGT   120

 41  Arg Gly Lys Arg Glu Leu Arg Met Ser Ser Ser Tyr Pro Thr Gly Leu Ala Asp Val Lys    60
121  CGT GGG AAG AGG GAA CTG CGG ATG TCC AGC AGC TAC CCC ACC GGG CTC GCT GAC GTG AAG   180

 61  Ala Gly Pro Ala Gln Thr Leu Ile Arg Pro Gln Asp Met Lys Gly Ala Ser Arg Ser Pro    80
181  GCC GGG CCT GCC CAG ACC CTT ATT CGG CCC CAG GAC ATG AAG GGT GCC TCT CGA AGC CCC   240

 81  Glu Asp Ser Ser Pro Asp Ala Ala Arg Ile Arg Val Lys Arg Tyr Arg Gln Ser Met Asn   100
241  GAA GAC AGC AGT CCG GAT GCC GCC CGC ATC CGA GTC AAG CGC TAC CGC CAG AGC ATG AAC   300

101  Asn Phe Gln Gly Leu Arg Ser Phe Gly Cys Arg Phe Gly Thr Cys Thr Val Gln Lys Leu   120
301  AAC TTC CAG GGC CTC CGG AGC TTT GGC TGC CGC TTC GGG ACG TGC ACG GTG CAG AAG CTG   360

121  Ala His Gln Ile Tyr Gln Phe Thr Asp Lys Asp Lys Asp Asn Val Ala Pro Arg Ser Lys   140
361  GCA CAC CAG ATC TAC CAG TTC ACA GAT AAG GAC AAG GAC AAC GTC GCC CCC AGG AGC AAG   420

141  Ile Ser Pro Gln Gly Tyr Gly Arg Arg Arg Arg Arg Ser Leu Pro Glu Ala Gly Pro Gly   160
421  ATC AGC CCC CAG GGC TAC GGC CGC CGG CGC CGG CGC TCC CTG CCC GAG GCC GGC CCG GGT   480

161  Arg Thr Leu Val Ser Ser Lys Pro Gln Ala His Gly Ala Pro Ala Pro Pro Ser Gly Ser   180
481  CGG ACT CTG GTG TCT TCT AAG CCA CAA GCA CAC GGG GCT CCA GCC CCC CCG AGT GGA AGT   540

181  Ala Pro His Phe Leu ***                                                          185
541  GCT CCC CAC TTT CTT TAG GAT TTA GGC GCC CAT GGT ACA AGG AAT AGT CGC GCA AGC ATC   600

601  CCG CTG GTG CCT CCC GGG ACG AAG GAC TTC CCG AGC GGT GTG GGG ACC GGG CTC TGA CAG   660
661  CCC TGC GGA GAC CCT GAG TCC GGG AGG CAC CGT CCG GCG GCG AGC TCT GGC TTT GCA AGG   720
721  GCC CCT CCT TCT GGG GGC TTC GCT TCC TTA GCC TTG CTC AGG TGC AAG TGC CCC AGG GGG   780
781  CGG GGT GCA GAA GAA TCC GAG TGT TTG CCA GGC TTA AGG AGA GGA GAA ACT GAG AAA TGA   840
841  ATG CTG AGA CCC CCG GAG CAG GGG TCT GAG CCA CAG CCG TGC TCG CCC ACA AAC TGA TTT   900
901  CTC ACG GCG TGT CAC CCC ACC AGG GCG CAA GCC TCA CTA TTA CTT GAA CTT TCC AAA ACC   960
961  TAA AGA GGA AAA GTG CAA TGC GTG TTG TAC ATA CAG AGG TAA CTA TCA ATA TTT AAG TTT  1020
1021 GTT GCT GTC AAG ATT TTT TTT GTA ACT TCA AAT ATA GAG ATA TTT TTG TAC GTT ATA TAT  1080
1081 TGT ATT AAG GGC ATT TTA AAA GCA ATT ATA TTG TCC TCC CCT ATT TTA AGA CGT GAA TGT  1140
1141 CTC AGC GAG GTG TAA AGT TGT TCG CCG CGT GGA ATG TGA GTG TGT TTG TGT GCA TGA AAG  1200
1201 AGA AAG ACT GAT TAC CTC CTG TGT GGA AGA AGG AAA CAC CGA GTC TCT GTA TAA TCT ATT  1260
1261 TAC ATA AAA TGG GTG ATA TGC GAA CAG CAA ACC (poly A)                             1293
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Fig. 2. Nucleotide sequence of the cDNA insert in pHAM-3 with predicted amino acid residues. Nucleotide residues are numbered beginning with the first residue of ATG triplet encoding a putative initiating methionine, and those on the 5'-site of nucleotide 1 are indicated by negative numbers. The termination codon is marked with three consecutive asterisks. The arrow indicates the potential signal peptide cleavage site to generate 164 residue proAM. Typical sites for proteolytic cleavage to mature peptides are underlined. AM and proAM-N20 sequences are boxed. Gly residues which contribute to C-terminal amide structure are dotted boxed.

boxy terminal amide structure is often observed in many naturally occuring biologically active peptides. An investigation of the biological activity of proAM-N20 is now in progress.

Fig. 3 shows RNA blot analyses of human AM. When the cDNA insert of human AM clone (pHAM-3) was used as an AM probe, an intense band was observed in PC and adrenal medulla at 1.6 kilobases. High levels of AM mRNA were also found in lung, ventricle and kidney as well as in PC and adrenal medulla. It is not expected that AM mRNA expression in lung, ventricle and kidney is comparable with that in adrenal medulla, because we already re-

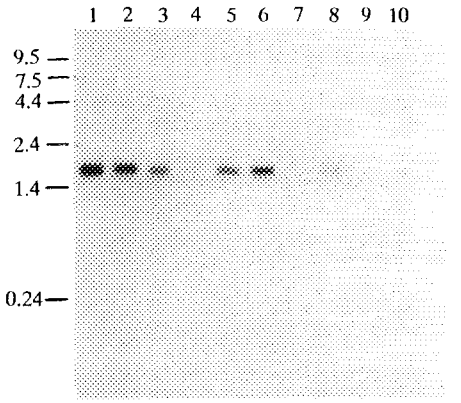

Fig. 3. RNA blot analysis of human AM transcript in human tissues. Each lane contained 1.0 μg of poly (A)$^+$RNA. Numbers on the left indicate kilobase as determined from RNA size markers. Lanes: (1) PC; (2) adrenal medulla; (3) ventricle; (4) brain; (5) kidney; (6) lung; (7) liver; (8) pancreas; (9) intestine; (10) spleen.

ported that the concentration of ir-AM in these tissues was less than 1% of that in adrenal medulla (1). This discrepancy may be explained by the possibility that AM biosynthesized in these tissues is rapidly released into the blood. In contrast, AM synthesized in PC and normal adrenal medulla is thought to be stored in the granule. The total production of AM in these organs must be much larger than that in the adrenal medulla and it may contribute to the rather high concentration of AM in human plasma. The very slight mRNA expression in brain is consistent with the fact that ir-AM was not detectable in human brain (1).

In conclusion, human AM cDNA has been cloned and its precursor structure was determined. The AM precursor contains an AM sequence which corresponds exactly to the amino acid sequence of human AM isolated from PC. In addition, it contains a candidate for a novel 20 residue biologically active peptide, proAM-N20, whose carboxy terminus may be amidated. Furthermore, human AM mRNA was highly expressed in several peripheral tissues including lung, ventricle and kidney as well as in adrenal medulla and PC. Evidence for the AM expression in these peripheral organs may indicate the possible existence of a novel system for circulation control.

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