Vol. 161, No. 1, 1989 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 30, 1989 Pages 236-241

MOLECULAR CLONING AND AMINO ACID SEQUENCE OF RAT KIDNEY AMINOPEPTIDASE M: A MEMBER OF A SUPER FAMILY OF ZINC-METALLOHYDROLASES

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Received April 18, 1989

Using a polyclonal antibody, a partial cDNA clone for rat aminopeptidase M was identified in a λgt11 library from rat kidney. A synthetic oligonucleotide probe derived from the sequence of the insert was used to screen a randomly primed λgt10 library. This allowed the identification of several overlapping clones encoding the full sequence of the enzyme. The reading frame, 2898 base pairs in length, encodes a 966 amino acid polypeptide. A highly hydrophobic segment, 24 amino acids in length, located close to the aminoterminus, is proposed to serve as the membrane-spanning domain for this membrane-bound enzyme. The sequence includes nine potential N-linked glycosylation sites and one potential sulfation site. In addition, the rat aminopeptidase M sequence contains an eight amino acid consensus sequence believed to serve as the zinc binding domain in a family of zinc-metallohydrolases. Rat aminopeptidase M shows 77% similarity with the recently cloned human enzyme, as well as weaker but significant similarity with aminopeptidase N from E.coli (18%) and with human leukotriene A4 hydrolase (21%).

Peptidases play a key role in the metabolism of neuropeptides, both at the level of their maturation from larger precursors and their degradation following their release from nerve endings (1,2). We have been particularly interested in the degradation of the opioid pentapeptides enkephalins in the brain. A body of evidence has implicated two membrane-bound metalloenzymes in this process, namely enkephalinase (neutral endopeptidase; EC 3.4.24.11) (3) and

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0006-291X/89 $1.50

aminopeptidase M (APM; EC 3.4.11.2) (4). High affinity inhibitors of these enzymes have been shown to display analgesic properties, probably explained by the prolonged activity of endogenous enkephalins once released in synapses (5,6). In order to gain a better understanding of the biochemical properties of these enzymes, we have cloned the genes for rat and human enkephalinase (7,8), and we report here the cloning of rat kidney APM. We show that this enzyme, which shows 77% homology with the recently cloned human APM (9), is a member of a recently defined super family of zinc-metallohydrolases, and shows similarity with two members of this family, aminopeptidase N from E.coli (10,11) and human leukotriene A4 hydrolase (12).

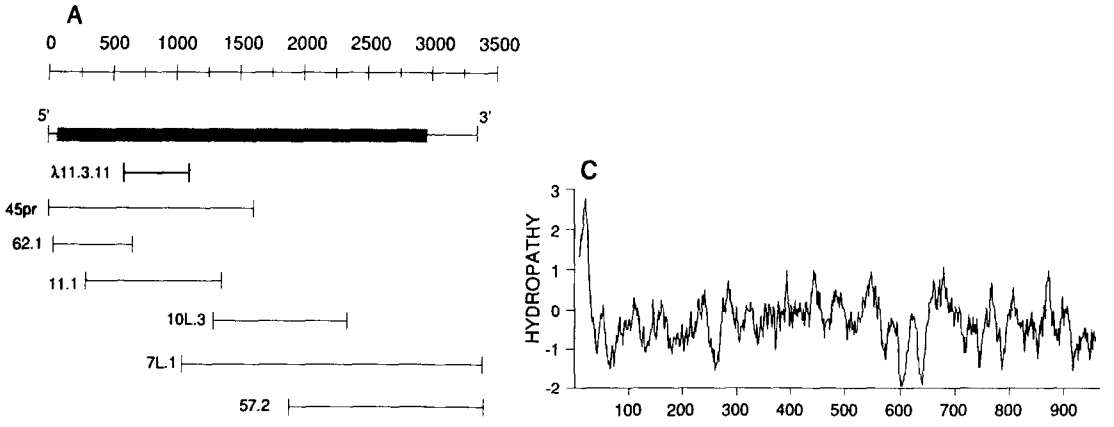
MATERIALS AND METHODS

A rabbit polyclonal antibody against purified rat kidney APM (13) was used at a dilution of 1:1,000 to screen a λgt11 library constructed from rat kidney mRNA (Clontech) according to published procedures (14). Two clones out of approximately 50,000 screened were identified and the EcoRI insert of the largest was sequenced. A 60 base oligonucleotide corresponding to the middle of this clone was synthesized, end labelled, and used to screen at high stringency a randomly primed λgt10 library constructed from rat kidney mRNA (7). Several clones were identified and sequenced. Oligonucleotides corresponding to the 5' and 3' extremities of the reading frame of two overlapping clones were synthesized, end labelled, and used to screen the same library. A total of 6 overlapping clones were necessary to yield a full length sequence, including a stop codon, and a start codon with an in frame stop codon further upstream (Fig.1A). All inserts were subcloned in M13 derivatives and sequenced by the chain termination method (15).

RESULTS AND DISCUSSION

The cloning strategy is summarized in Fig. 1A. We used a rabbit polyclonal antibody raised against highly purified rat kidney APM to screen a λgt11 library constructed from rat kidney mRNA. This led to the identification of two clones. The largest clone, 741 base pairs in length, had an open reading frame encoding a 247 amino acid polypeptide. A synthetic oligonucleotide probe based on this sequence was then used to identify other clones and this process was repeated until an entire reading frame was obtained. Of the 6 overlapping clones identified (Fig. 1A), one contained an in frame ATG codon and an in frame stop codon further upstream, while another had an in frame stop codon. This shows that the reading frame obtained is full length.

The nucleotide sequence of this reading frame as well as the translated amino acid sequence are shown in Fig. 1B. Rat APM is a



B

Figure 1. Nucleotide and deduced protein sequence for rat aminopeptidase M cDNA.

(A) Schematic representation of APM mRNA. Untranslated sequences are represented by a line; coding sequences are boxed. Overlapping cDNA clones used in sequence determination are shown below the diagram of the mRNA structure. Clone λ11.3.11 was obtained from a lgt11 library and all others were from a λgt10 library. (B) Nucleotide and predicted amino acid sequence of rat APM. The protein sequence is numbered from Met[1]. An inframe 5' stop codon (ATG) is found at the 5' end of the cDNA. A putative 24 amino acid transmembrane spanning domain is indicated by a dotted line. Nine potential N-linked glycosylation sites are shown by lines. A potential sulfation site is indicated by an asterisk. An 8 amino acid potential zinc binding site is shown by a dotted box. (C) Hydropathy analysis of APM protein sequence. The method of Kyte and Doolittle (27) was used with a window length of 10 residues. Hydrophobic regions (positive values) demonstrate the presence of a single transmembrane spanning domain between residues 11 to 34.

966 amino acid protein, which fits with its apparent molecular weight (110 Kd) as deduced from SDS polyacrylamide gel electrophoresis (13). It displays 77% homology with the recently cloned human APM (9). Landmarks in the amino acid sequence of rat APM are shown in Fig. 1B. They include a potential membrane-spanning domain, 9 potential N-linked glycosylation sites, one potential sulfation site and one 8 amino acid region which is likely to serve as a zinc binding site.

APM is a membrane-bound ectoenzyme, with its active site extracellular. A 24 amino acid long domain with a high hydropathy value is found close to its amino terminus (Fig. 1C). Since there are no other highly hydrophobic domains in its sequence, this amino terminal segment is likely to serve as the transmembrane region. The sequence on the amino end of this putative transmembrane region is very short (10 amino acids) and is very unlikely to contain the active site of the enzyme. It is thus most probably intracellular. This is the opposite orientation of many transmembrane proteins, which have their aminoterminus located extracellularly. However, several other enzymes found on kidney microvillar membranes, including enkephalinase (7,16-18), are also inserted through membranes with their aminoterminus intracellular and their carboxyterminus extracellular.

APM is a zinc-containing metallopeptidase. The potential zinc binding site HE--H similar to that originally identified in the bacterial zinc-metalloendopeptidase thermolysin (19) is present in its sequence ($H^{387}E^{388}$--H^{391}). This partial similarity with thermolysin extends to another three amino acids on the amino end of the first histidine residue, yielding the pattern V--HE--H. This pattern is found conserved in a number of other enzymes, including aminopeptidase N from E.coli (10,11), members of the mammalian collagenase family (20,21), angiotensin-converting enzyme (22), enkephalinase (7,8,16) and leukotriene A4 hydrolase (12). All but two of these enzymes are known to be zinc-metalloenzymes and, based on the occurrence of the octapeptide within their sequence, it can be inferred that the other two, aminopeptidase N from E.coli and human leukotriene A4 hydrolase, are also zinc-metalloenzymes. This octapeptide has been proposed to identify a super family of hydrolases sharing a common catalytic mechanism based on the presence of a zinc atom in their active site (23,24).

In order to gain more insight into this family of enzymes, we compared their sequence in a systematic fashion, using a sequence

	Thermo	N.Prot	APEColi	Collag	Collag4	Strom	Strom-2	ACE	Enkase	APM	LA4H
Thermo	1503	[585]	22	31	49	33	33	15	27	29	15
N.Prot		2332	27	8	8	13	13	20	18	17	23
APEColi			4295	11	25	12	12	23	9	[128]	[210]
Collag				2467	[969]	[1419]	[1414]	38	6	10	14
Collag4					3479	[1051]	[1051]	11	28	8	8
Strom						2486	[2481]	6	15	6	24
Strom-2							2485	6	15	6	27
ACE								6919	10	12	39
Enkase									3754	5	10
APM										4808	[310]
LA4H											3162

Thermo : thermolysin **Collag :** fibroblast collagenase **Strom-2 :** stromelysin-2 **APM :** aminopeptidase M

N.PROT : neutral protease **Collag4 :** type IV collagenase **ACE :** peptidyl-dipeptidase **LA4H :** leukotriene A4 hydrolase

APEColi : aminopeptidase N from *E.Coli* **Strom :** stromelysin **Enkase :** enkephalinase

Figure 2. Sequence similarity scores between proteins, members of a super family of zinc-metallohydrolases.
The method of Dayhoff et al. (28) was used with a deletion penalty identical to that used by Lipman and Pearson (29). Using this method, a similarity score between two random protein sequences is in the range of 10-20. Any score 100 and higher thus indicates significant similarity.

comparison program yielding a score of global similarity (25). As shown in Fig. 2, the only significant similarities were within the collagenase family, between thermolysin and neutral protease, and between aminopeptidase N from E.coli, leukotriene A4 hydrolase, and APM. While the partial similarity between human APM and aminopeptidase N from E.coli had already been described (9), that between APM and leukotriene A4 hydrolase was unexpected. Indeed, these enzymes catalyse very different reactions. APM hydrolyses a peptide bond and leukotriene A4 hydrolase hydrolyses the epoxide ring in leukotriene A4, and releases after further rearrangement leukotriene B4 (26). The global similarity between rat APM and aminopeptidase N from E.coli is 18%, between rat APM and leukotriene A4 hydrolase 21%, and between aminopeptidase N from E.coli and leukotriene A4 hydrolase 20%. The similarity between the three enzymes is particularly strong around their zinc binding site octapeptide. This indirectly points to the importance of this region in the activity of these enzymes. Further, this observation suggests that these three enzymes may have evolved from a common distant ancestor.

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