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MOLECULAR CLONING AND AMINO ACID SEQUENCE OF RAT ENKEPHALINASE

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cDNA clones encoding rat enkephalinase (neutral endopeptidase, EC 3.4.24.11) have been isolated in λgt10 libraries from both brain and kidney mRNAs and the complete 742 amino acid sequence of rat enkephalinase is presented. The enzyme possesses a single transmembrane spanning domain near the N-terminal of the molecule but lacks a signal sequence. Because enkephalinase has its active site located extracellularly and is thus an ectopeptidase, we suggest that the N-terminal transmembrane region of the enzyme anchors the protein in membranes and that the majority of the protein, including the carboxy terminus, is extracellular. Enkephalinase, a zinc-containing metallo enzyme, displays homology with other zinc metallo enzymes such as carboxypeptidase A, B and E, suggesting enzymatic similarities in these enzymes. © 1987 Academic Press, Inc.

Enkephalinase (neutral endopeptidase; EC 3.4.24.11) is a cell membrane-associated enzyme which hydrolyses the Gly^3-Phe^4 amide bond of enkephalins in vitro and in vivo (1-4). Thus, pharmacologically useful inhibitors of enkephalinase such as thiorphan (5) and phosphoryl-Leu-Phe (6) have been shown to produce naloxone-reversible analgesia in mice. In addition, acetorphan, a parenterally active enkephalinase inhibitor (7), displays analgesic properties in humans (8). Enkephalinase preferentially hydrolyses peptide bonds on the amino side of hydrophobic residues, and its substrate specificity is sufficiently broad that it is able to hydrolyse, in vitro, many short neuropeptides and peptide hormones (9-11). Although initially characterized in the brain (1), enkephalinase was later found to be present in many tissues, in particular the kidney (12), where it was shown to be identical to an enzyme identified several years before using the B chain of insulin as substrate (13), the so called "neutral endopeptidase" (14-17). We have isolated cDNAs encoding enkephalinase from both rat brain and kidney mRNAs and present the complete 742 amino acid sequence of rat enkephalinase.

MATERIALS AND METHODS

Purification and sequence analysis of rat kidney enkephalinase: Rat enkephalinase was purified as described (17) by differential solubilization of kidney membranes with Triton X-100, followed by DEAE Sephadex chromatography, Concanavalin A-Sepharose chromatography, and hydroxylapatite chromatography. Fractions eluted from the hydroxylapatite column and containing enkephalinase activity were loaded onto a 10x10 mm Concanavalin A-Sepharose column, and the enzyme was eluted in several 1ml fractions with 5 mM, pH 7.4 HEPES buffer containing 0.1% Triton X-100 and 500 mM methyl-a-D-Glucopyrannoside. Eight 1ml fractions, containing a total of 450 µg of protein as determined by the Coomassie blue method using bovine serum albumin as standard, were obtained. One of these fractions containing an estimated 100 µg of protein was concentrated to 200 µl using a Centricon 30 device, and loaded onto a Superose 6 (Pharmacia) column equilibrated in 5 mM, pH 7.4 phosphate/Na

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buffer containing 150 mM NaCl and 0.1% Triton X-100. The column was eluted in the same buffer at a flow rate of 200 µl/min and the fractions obtained (300 µl) were assayed for enkephalinase activity (18). Aliquots of fractions displaying enkephalinase activity were subjected to SDS-polyacrylamide (7.5%) electrophoresis and the gels, stained with coomassie blue, revealed a unique band of molecular weight ca 90 kD. These fractions were used for N-terminal amino acid analysis and for Lysine-C proteinase digest, of enkephalinase. N-terminal sequence was obtained by applying the purified enzyme to a vapour phase protein sequencer (Model 470A, Applied Biosystem) (19), equipped with an on-line amino acid PTH analyzer (Model 120A, Applied Biosystem). It was never possible to obtain reliable protein sequence after 15 cycles. For Lysine-C proteinase digest, the purified enkephalinase, as eluted from the Superose 6 column was digested overnight with the proteinase (10 ng per µg of enkephalinase) and the peptides released were separated by HPLC on a Synchrom 2x100 mm, 300 Å C4 column, eluted with a linear gradient of 0 to 70% 1-propanol (1% per min) in 0.1% trifluoroacetic acid at a flow rate of 400 µl per minute. The peptides displaying a large 280 nm to 214 nm absorbance ratio were rechromatographed on the same column, using acetonitrile instead of 1-propanol, and their amino acid sequence was determined using the vapour phase protein sequencer.

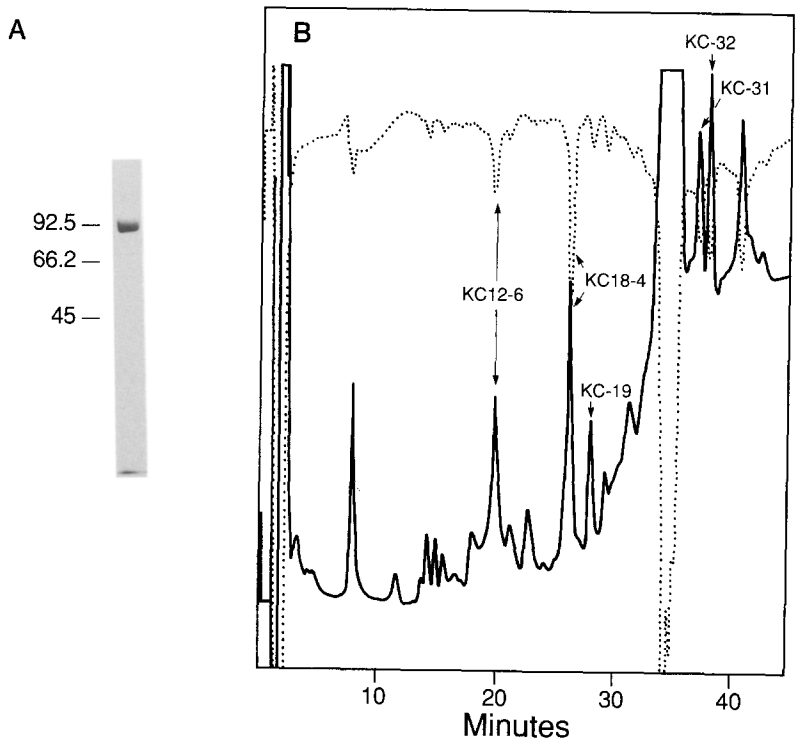
Molecular cloning: RNA was prepared from rat kidney or brain (20) and polyadenylated mRNA was obtained by oligo(dT) cellulose chromatography. High complexity cDNA libraries were constructed in λgt10 (21)as described elsewhere (22). Three libraries were generated: random 8-mers were used to prime rat kidney poly(A)$^+$ mRNA, generating a library of $\approx 6x10^6$ clones of size > 500 bp; oligo(dT) primed libraries of both rat kidney and brain poly(A)$^+$ mRNA, $\approx 5x10^5$ and $6x10^6$ clones of size > 1500 bp, respectively, were also generated. Oligonucleotide probes were designed based on the amino acid sequence of peptide KC2-18-4. A pool of 32x18-mers, 5'-YTGYTGNGTCCACCARTC-3' (Y = T, C; R = G, A; N = G, A, T, C) (short probe), covering all non-coding strand possibilities of the protein sequence DWWTQQ was used with the base composition independant method (23) and a single long probe (45-mer) 5'-GAAGTTGTTGGCGGACTGCTGGGTCCACCAGTCGACCAGGTCGCC-3', complementary to the entire sequence of peptide KC2-18-4 except for its C-terminal lysine residue was also used. Both the short and long probes were used to screen $5x10^5$ clones of the randomly-primed rat kidney cDNA library. Hybridization was carried out with ^{32}P-phosphorylated probes in 5xSSC, 20% formamide at room temperature. Filters were washed at room temperature in 0.5xSSC, 0.1% SDS for the long probe and in 3M tetramethylammonium chloride at 51 ° C for the short probe. Inserts of λ phage were subcloned into an M13 derivative and sequenced by the chain-termination method (24,25).

DNA- and RNA-blot analysis: Sprague-Dawley rat DNA, isolated by standard procedures, was digested with either PstI, EcoRI or BglII and electrophoresed on a 1% agarose gel. After Southern transfer (26) onto nitrocellulose the blot was hybridized with a ^{32}P-labelled (27) 466 bp EcoRI-BglII cDNA fragment of clone λK3, in 5xSSC, 50% formamide at 42 °C. The blot was washed at 65 °C in 0.1xSSC, 0.1% SDS, and exposed for 60 h. For Northern blots, polyadenylated mRNA (5 µg) was electrophoresed in a formaldehyde-1.2% agarose gel (28) and blotted onto nitrocellulose paper (29). The blot was hybridized with the 1459 bp EcoRI insert of λK3 in 5xSSC, 50% formamide at 42 °C and washed in 0.1xSSC, 0.1% SDS at 65 °C.

RESULTS AND DISCUSSION

Rat kidney enkephalinase was purified to homogeneity and subjected to amino acid sequence analysis either directly or after Lysine-C-proteinase digestion, followed by purification of peptide fragments generated by HPLC (Fig. 1). One such peptide, KC2-18-4, containing two tryptophan residues, was used to design oligonucleotide probes for screening cDNA libraries. Both a complementary short pool (32x18 mers) containing all possible nucleotide assignments, for screening by the base composition independant method (23), and a 45-mer long probe (30) were synthetized and used to screen, in duplicate, a randomly primed rat kidney cDNA library constructed in λgt10. Two positive clones were identified and the largest, λK3, was sequenced. This clone contains an open reading frame that encodes all peptide fragments sequenced, except the N-terminus. This cDNA clone was in turn used as a probe to screen oligo(dT) primed λgt10 cDNA libraries constructed from either rat brain or kidney poly (A)$^+$ mRNA. Two clones, λK2 and λK5, were identified from the kidney library, and two clones, λB10 and λB16, were identified from the brain library. Clones λK2, λK5 and λB10 contain a stop codon for the reading frame identified in clone λK3, while clone λB16 encodes the N-terminal protein sequence. Thus a complete reading frame coding for the enkephalinase molecule was obtained from overlapping clones, from brain and kidney (Fig. 2 a, b).

Brain and kidney cDNA clones overlap by a total of 2008 base pairs (bp) of the 3243 bp sequence. Two nucleotide sequence differences were detected. At nucleotide 1917, a T occurs in kidney clone λK2 and in brain clone λB10 while



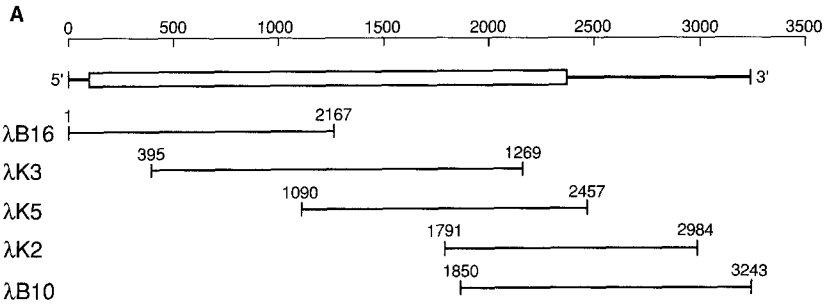
C

Peptides	Amino Acid Sequence
N-terminal	Asp-Ile-Thr-Asp-Ile-Asn-Ala-Pro-Lys-Pro-Lys-Lys-Lys-Gln-Arg
KC8	Leu-Leu-Pro-Gly-Leu-Asp-Leu-Asn-His-Lys
KC2-12-6	Glu-Arg-Ile-Gly-Tyr-Pro-Asp-Asp-Ile-Ile-Ser-Asn-Glu-Asn-Lys
KC2-18-4	XXX-Gly-Asp-Leu-Val-Asp-Trp-Trp-Thr-Gln-Gln-Ser-Ala-Asn-Asn-Phe-Lys
KC2-19	Glu-Glu-Glu-Tyr-Phe-Glu-Asn-Ile-Ile-Gln-Asn-Leu-Lys
KC2-31	His-Gln-Asn-XXX-Phe-Ser-XXX-Glu-Ile-Asn-Gly-Lys
KC2-32	Ala-Val-Val-Glu-Asp-Leu-Ile-Ala-Gln-Ile-Arg-Glu-Val-Phe-Ile-Gln-Thr-Leu

XXX: no residue identified.

Figure 1. Rat kidney enkephalinase purification and protein sequence analysis.
(A) Coomassie blue-stained SDS-polyacrylamide gel of purified enkephalinase. The positions of molecular weight standards are indicated. (B) Lysine-C proteinase map of purified enkephalinase. Upper trace is absorbance at 280 nm and lower trace absorbance at 214 nm. Arrows indicate peaks that were rechromatographed and sequenced. (C) Amino acid sequence of native enkephalinase (N-terminal) and of Lysine-C proteinase peptides.

a C is found in a second kidney clone, λK3; at nucleotide 2930, a G or an A is found in either the kidney (λK2) or brain (λB10) clones, respectively. Southern blot analysis (26) of rat genomic DNA using a small coding region cDNA probe was undertaken. The multiplicity of hybridyzing bands in only some restriction enzyme digests (Fig. 3a) suggests the presence of several intron sequences but not multiple genes. In the 3'-untranslated region of the cDNA clones there is over 650 bp of overlapping kidney and brain sequence with total homology, except for the single nucleotide change at position 2930. Since the 3'-untranslated region is typically least strongly conserved between related genes, this, and the Southern blot data, suggest that a single gene encodes both brain and kidney enkephalinase. The nucleotide differences are thus probably errors of reverse transcriptase. This identity of kidney and brain enkephalinase cDNA's fully supports the earlier contentions that the kidney and brain enzymes are identical (15,16,31).


A
0
500
1000
1500
2000
2500
3000
3500
5'
3'
λB16
1
2167
λK3
395
1269
λK5
1090
2457
λK2
1791
2984
λB10
1850
3243

B

Northern blot analysis of poly(A)$^+$ mRNA from rat kidney and brain reveals a complex pattern of hybridization (Fig. 3b). In kidney, the major message is 3.4 kilobases (kb) in size, with other messages of 6.5, 6.0, 3.2 and 2.6 kb in size. The brain messages are at least 10-fold less abundant and only two mRNA's, 6.5 and 3.4 kb in size, are detected. This difference in intensity is in agreement with the much higher specific activity of enkephalinase in kidney as compared to brain (12,16). Potential polyadenylation signals, ATTAAA and AATAAA at nucleotides 2485 and 3063, respectively (Fig. 2b), may correspond to the 2.6 and 3.2 kb kidney mRNA's.

The enkephalinase cDNA potentially encodes a 750 amino acid polypeptide, starting from Met^{-8} as numbered in Fig. 2. This reading frame has 78 bp of 5'-untranslated sequence and 912 bp of 3'-untranslated sequence. An in-frame stop codon is located 6 bp upstream of the AUG codon for Met^{-8}. A second, alternate initiation codon (Met^{-1}) is found downstream of the first ATG (Met^{-8}). Neither of these two potential initiation codons, at Met^{-1} or Met^{-8}, conform closely to the Kozak rule for predicting the site of protein initiation (32). Whereas most eukaryotic genes are translated using the first AUG encountered in the mRNA, a number of examples where a second AUG is used have been noted (32). N-terminal protein sequence data obtained using purified enkephalinase suggests that the second initiation codon (Met^{-1}) is used for the in vivo synthesis of mature enkephalinase. The polypeptide starting with Asp^1 at its amino terminus would be 742 amino acids in length, yielding a molecular weight of 85 kD. Molecular weight estimates of enkephalinase, obtained through SDS polyacrylamide gel electrophoresis, have yielded values in the 90-94 kD range (11). Since enkephalinase is known to be a glycoprotein, the difference between these estimates and the value deduced from the protein sequence presumably reflects N-linked glycosylation. There are six potential N-linked glycosylation sites (Asn-X-Thr/Ser) in the rat enkephalinase sequence (Fig. 2b). The failure to detect an asparagine residue in peptide KC2-31 (Fig. 1c) suggests that this residue, which forms a potential glycosylation site, is indeed glycosylated.

Enkephalinase has been shown to be an ectopeptidase, with its active site in the extracellular space (11,33). The hydropathic profile of the enzyme (Fig. 2c) shows the presence of a 23 amino acid long hydrophobic domain (residues 21-43) that is likely to span the lipid bilayer. Additionally, a very strong stop transfer sequence (34), PKPKKKQR,

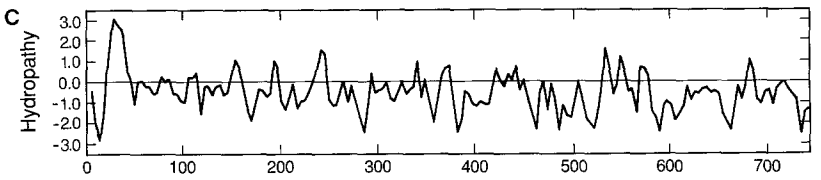


Figure 2. Nucleotide and deduced protein sequence for rat enkephalinase cDNA.
(A) Schematic representation of enkephalinase mRNA. Untranslated sequences are represented by a line; coding sequences are boxed. The scale is in nucleotides from the 5' end of the longest cDNA clone. Overlapping cDNA clones used in sequence determination are shown below the diagram of the mRNA structure. (B) Nucleotide and predicted amino acid sequence of rat enkephalinase. Nucleotides are numbered at the left, and amino acids are numbered throughout. The amino acid sequences determined by protein sequencing (see Fig. 1) are overscored. The amino acid sequence at position 592-608 was used for designing oligonucleotide probes. The protein sequence is numbered from Asp^1 as this is the N-terminal residue of the mature protein. Two potential initiation codons are Met^{-1} and Met^{-8} and are underscored, and an inframe 5' stop codon (TAG) is indicated by asterisks. The 8 amino acid stop-transfer sequence (PKPKKKQR) is indicated by a black bar and the putative 23 amino acid signal sequence/transmembrane spanning domain is indicated by an open bar. Six potential N-linked glycosylation sites are shown by cross-hatched bars. Potential poly(A) addition signals ATTAAA and AATAAA are underlined. (C) Hydropathy analysis of enkephalinase protein sequence. The method of Kyte and Doolittle (47) was used with a window length of 10 residues and a jump of 2 residues. Hydrophobic regions (positive values) demonstrate the presence of a single transmembrane spanning domain between residues 21 to 43.

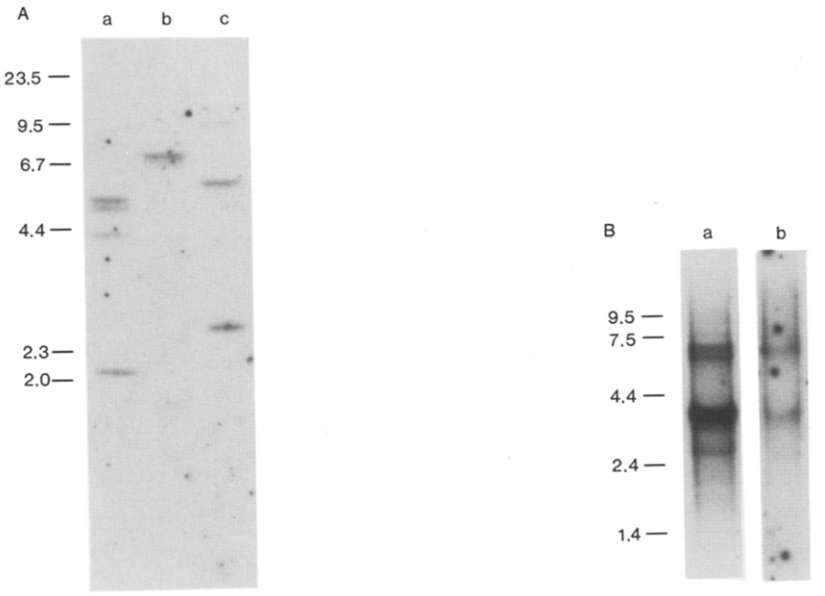


Figure 3. Southern and Northern blot analysis of rat DNA and RNA.
(A) Southern blot hybridization analysis of rat DNA, digested with PstI (lane a), EcoRI (lane b) or BglII (lane c) and hybridized with a 466 bp cDNA probe. Size standards (kb) are HindIII-cleaved λcI857 DNA. (B) Northern blot analysis of mRNA encoding rat enkephalinase. Rat kidney poly(A)$^+$ mRNA (lane a) and rat brain (minus cerebellum) poly(A)$^+$ mRNA (lane b) were hybridized with a ^{32}P-labelled 1459 bp cDNA probe (see Fig. 2) and exposed for either 4 or 80 h at -70 ° C. Size standards (kb) are from a RNA ladder (BRL).

is located at residues 8-15 on the N-terminal side of the transmembrane spanning region. This stop transfer sequence probably accounts for the fact that enkephalinase is very tightly bound to membranes (13,17). All these features suggest that the enkephalinase N-terminus is located in the cytoplasm whereas the majority of the protein (704 amino acids, which would contain the active site) including the C-terminus, extends into the extracellular space. The single transmembrane domain would therefore be required to act as a signal sequence as well as a transmembrane spanning sequence. Several membrane-bound proteins possess those features proposed for enkephalinase, namely an intracellular N-terminal sequence and an extracellular C-terminal sequence, a single hydrophobic domain acting as both a signal sequence and transmembrane-spanning sequence, and a stop-transfer sequence located N-terminally of the transmembrane-spanning sequence. These include transferrin receptor (35), asialoglycoprotein receptor (36), HLA-DR-associated invariant chain (37), and influenza virus neuraminidase (38), although the latter possesses an uncleaved signal sequence.

Enkephalinase is a zinc-containing metalloenzyme (13,39,40). Because it hydrolyzes peptide bonds at the amino side of hydrophobic residues in its substrates, it has been suggested that enkephalinase resembles two other zinc-containing peptidases, carboxypeptidase A and thermolysin (5,9-11,13,14,40-43). We compared the sequence of enkephalinase with that of bovine carboxypeptidases A (44), B (45) and E (46). The carboxy terminal region of enkephalinase (from residues 615 to 738) can be aligned with the N-terminal domain of all three carboxypeptidases (Fig. 4). The best alignment is with carboxypeptidase A (22% homology, plus 14% conservative changes). A weaker homology was also detected with the N-terminal region of thermolysin (12% homology, from residues 634 to 742 of enkephalinase; not shown). These homologies suggest that the C-terminal domain of the enkephalinase molecule may be functionally related to the N-terminal domains of the carboxypeptidases. The availability of cloned enkephalinase will facilitate an understanding of the substrate specificity and function of this enzyme.

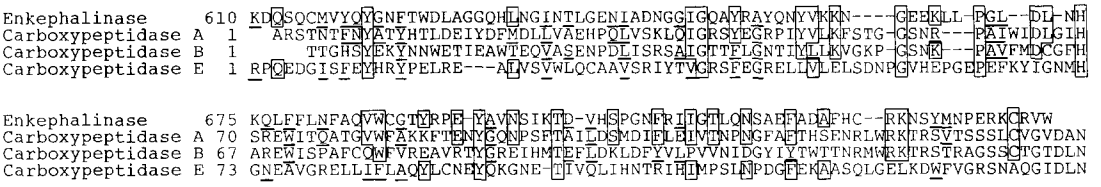


Figure 4. Sequence homologies of enkephalinase and bovine carboxypeptidases A, B and E.
Amino acids are shown in the one-letter code and numbered at the left. Identical residues are boxed whilst conservative changes, based on the following groupings: ILVM, DE, RKH, FWY, TS, GA, QN, P, C, are dotted.

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