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AN ANTIBODY PURIFIED WITH A λGT11 FUSION PROTEIN PRECIPITATES ENKEPHALINASE ACTIVITY

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An antiserum was raised against the neutral endopeptidase "enkephalinase" in guinea pig and used to probe a rabbit kidney cDNA expression library. A positive clone has been isolated and sequenced. The identity of the corresponding fusion protein was ascertained by its ability to select, from the crude antiserum, antibodies which specifically immunoprecipitate neutral endopeptidase enzymatic activity. This approach eliminates the uncertainty inherent to clone identification obtained from oligonucleotide probe derived from a partial sequence of the protein.

The neutral endopeptidase 24.11 (NEP 24.11), also called "enkephalinase", is a membrane-bound zinc metallopeptidase which plays a crucial role in terminating the action of enkephalins released at synapses in the Central Nervous System (1). Inhibitors of the enzyme have been designed which produce naloxone-reversible analgesia (2). The *in vivo* activity of these compounds clearly demonstrates the physiological relevance of NEP-24.11 in enkephalin metabolism. The knowledge of the detailed structure of this peptidase should be a further benefit for both the design of even more potent and specific inhibitors and for investigations of its expression in various physiopathological conditions. As a first step, we report here the identification of a NEP 24.11 clone by screening of a

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λgt11 expression library constructed from rabbit kidney (3) using a specific polyclonal antibody. This approach complements a parallel study in which the cDNA identification was based on partial peptide sequencing of the enzyme and subsequent use of corresponding synthetic oligonucleotides (4).

MATERIALS AND METHODS

2.1. Purification of the NEP 24.11 and guinea pig immunization

NEP 24.11 was purified from rabbit kidney cortex as previously described by Almenoff and Orlowski (5). The band of an 8 % SDS-PAGE corresponding to the enzyme was excised, suspended (30 µg in complete Freund's adjuvent) and injected intradermally into male guinea pigs (2). Animals were boosted after three weeks (30 µg in incomplete Freund's adjuvent) and five weeks (30 µg in saline solution) with intrascapular and intraperitoneal injections respectively. Bleedings were carried out after a further two weeks.

2.2. Construction and screening of rabbit kidney cDNA library

Total RNA was extracted from the kidneys of three month old rabbits. Polyadenylated mRNA was prepared using oligo-dT affinity chromatography, and used to construct a cDNA library in the phage λgt11 as described by Watson and Jackson (6). About 100.000 independant clones were screened using the specific antiserum essentially as described by Huynh, Young and Davis (7), except that horseradish peroxidase conjugated anti-guinea pig antibodies were used.

2.3. Specific antibodies purification

Putative chimeric NEP 24.11 protein was attached to a nitrocellulose filter (ϕ 82 mm) according to Huynh and al. (7). The filter was then incubated overnight at 4°C with 10 µl of Ab.R. serum diluted in 5 ml of TBS (50 mM Tris-HCl pH 8.0, 150 mM NaCl) containing 20 % fetal calf serum, followed by washing for 5 min. successively with 5 ml TBS, 5 ml TBS containing 0.1 % Triton X100, 5 ml TBS and then 5 ml TBS containing 20 % fetal calf serum.

Specific IgGs against the fusion protein were recovered in 700 µl of 100 mM glycine-HCl at pH 2.5, and immediately neutralized with 300 µl 1 M Tris-HCl at pH 8.0 and few drops of 1 M HCl were added to obtain a final pH of 7.5.

2.4. Nucleotide sequence analysis

Positive clones were subcloned into the phage M13 and sequenced on both strands by the chain termination method of Sanger et al. (8).

2.5. Immunoprecipitation

Both sera and purified IgGs were assayed following the same procedure. Serum, or IgGs, were incubated with Protein A-Sepharose for 2 hours at 4°C and centrifuged for 5 minutes at 10.000 g. The pellet was then washed four times with 0.5 ml 50 mM Tris-HCl at pH 7.4 containing 3 % BSA, and resuspended in the same buffer. Aliquots were incubated overnight at 4°C with purified enzyme (total volume 200 µl). After centrifugation as above, the pellet was washed three times in 200 µl of the same buffer. The supernatants were pooled and aliquots were assayed for enzyme activity as previously described (9).

2.6. Western blot

Western blots were carried out as described by Towbin and al. (10). Crude enzyme extract was obtained by solubilizing kidney cortex membranes as described by Crine and al. (11).

RESULTS

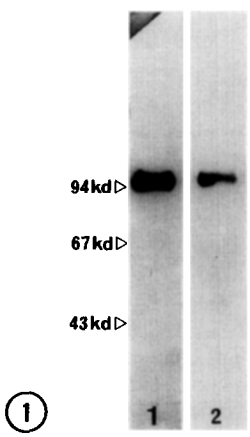
3.1. Characterisation of antibodies

Crude antisera (Ab. J. and Ab. R.) were first tested by Western blotting using both purified enzyme and a crude kidney cortex extract. Both sera recognized specifically a 94 kd protein as shown in fig. 1. Furthermore, these antibodies precipitated the enzymatic activity (table 1).

3.2. Characterisation of NEP-24.11 cDNA clones

Approximately 100.000 clones were screened with the Ab.R. serum. One clone, designated PK 123, which was positive after three rounds of screening was further analyzed. The corresponding cDNA insert was 210 bp long and its sequence is shown in fig. 2.

To fully ascertain that the clone encoded NEP 24.11, the β-galactosidase fusion protein was used to select specific antibodies from the crude Ab.

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  805                                                    840
.TT GGC ACT GAT GAT AAG AAC TCT ATG AAC CAT ATA ATT
... Gly Thr Asp Asp Lys Asn Ser Met Asn His Ile Ile

  844                                                    879
CAT ATT GAC CAA CCT CGA CTT GGC CTC CCT TCT AGA GAC
His Ile Asp Gln Pro Arg Leu Gly Leu Pro Ser Arg Asp

  883                                                    918
TAC TAT GAA TGC ACT GGA ATA TAT AAA GAG GCT TGT ACA
Tyr Tyr Glu Cys Thr Gly Ile Tyr Lys Glu Ala Cys Thr

  922                                                    957
GCA TAT GTG GAT TTT ATG ATT GCT GTG GCC AAA TTG ATT
Ala Tyr Val Asp Phe Met Ile Ala Val Ala Lys Leu Ile

  961                                                    996
CGT CAG GAA GAA GGA TTG CCT ATT GAT GAA AAC CAG ATT
Arg Gln Glu Glu Gly Leu Pro Ile Asp Glu Asn Gln Ile

 1000
TCT GTG GAA ATG AAT
Ser Val Glu Met Asn
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FIGURE 1. Western blot of crude NEP 24.11 extract using Ab.R. (lane 1) and Ab.J. (lane 2) antisera at a dilution of 1:100.

FIGURE 2. Nucleotide sequence of PK 123 clone insert and its corresponding polypeptide fragment using a three-letter code. Nucleotide numbering has been done with reference to the sequence described by Devault and al. (4).

Table 1

Precipitation of NEP 24.11 activity by antisera

Serum	% Activity
Pre-immune	98
Ab.J. (20 µl)	12
Ab.R. (20 µl)	15
"Purified" Ab.R. (150 µl)	10

In each experiment 8.8 ng NEP 24.11 were incubated with antiserum coupled to protein A-Sepharose as described in methods. After centrifugation, enzyme activity remaining in the supernatant was measured using [^{3}H] D-Ala^{2}-Leu-enkephalin as reported (9). Maximum enzyme precipitation was obtained using the amount of antisera indicated in parenthesis. All experiments were done in triplicate enzyme assays and the average activity is expressed as a percentage of a control, which consisted of enzyme incubated with protein A-Sepharose alone.

R. serum as described in materials and methods. These antibodies were first assayed by Western blot using a crude kidney preparation. As above, a single band was obtained at 94 kd (data not shown). They were then tested for their ability to immunoprecipitate the enzyme. As shown in table 1 about 90 % of the activity could be removed from a solution containing the purified native enzyme.

DISCUSSION

In this paper we describe the indentification of a cDNA clone corresponding to "enkephalinase" by use of specific antibodies directed against the enzyme. Enkephalinase was purified from rabbit kidney brush border membranes which provides the richest source available (3). Although some data suggest that the brain and kidney enzymes may not be completely identical (12), enzymatic and immunological considerations indicate that they are closely related and should therefore share major structural features.

Antibodies were prepared using the material contained in a single SDS

band. Their specificity was firmly established by two criteria. First they recognized a single 94 kd species on a Western blot performed with the puified enzyme and a crude rabbit kidney extract. Secondly, they precipitated the NEP 24.11 enzymatic activity. One of these sera were used to screen a λgt11 expression library and one clone yielded a positive signal. The corresponding fusion protein reacted specifically with antibodies which precipitated the enzyme. NEP-24.11 antiserum therefore provided a functional test that firmly established the identity of the corresponding clone. The sequence of the PK 123 clone identified by this strategy is in complete agreement with the data obtained by the peptide sequence strategy and eliminates the uncertainty of cDNA identification inherent to this parallel approach (4).

It should also be noted that the fusion protein represents a convenient material to obtain purified polyclonal antibodies and develop a radioimmunoassay. This approach can also be exploited to generate antibodies directed against various amino acid sequences of the enzyme. This method is essential to map the three dimensional structure of the neutral endopeptidase in solution and in the membraneous environment.

The availability of cDNA clones will be useful in establishing whether brain and kidney enzymes are identical. Regional diversity, resulting most generally from alternative splicing events, has been encountered in several instances (13). Clearly, tools are now available to investigate the regulation of expression of NEP 24.11 under various physiological and pharmacological conditions.

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