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Expression of Enzymatically Active Enkephalinase (Neutral Endopeptidase) in Mammalian Cells

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A cDNA encoding the rat enkephalinase protein (neutral endopeptidase; EC 3.4.24.11) has been constructed from overlapping λgt10 cDNA clones. This cDNA was inserted into an expression plasmid containing the cytomegalovirus enhancer and promoter. When transfected with this plasmid, Cos 7 cells transiently expressed the enkephalinase protein in a membrane-bound state. Recombinant enkephalinase recovered in solubilized extracts from transfected Cos 7 cells was enzymatically active and displayed properties similar to those of the native enzyme with respect to sensitivity to classical enkephalinase inhibitors.

Key words: enkephalinase, neutral endopeptidase, metallo peptidase

The signals conveyed by the opioid pentapeptides enkephalins when released in their synapses are turned off by degradation of the peptides by two membrane-bound metallopeptidases, enkephalinase (neutral endopeptidase; EC 3.4.24.11) and aminopeptidase M (EC 3.4.11.2) [1,2]. Aminopeptidase M hydrolyzes the Tyr^1 -Gly^2 amide bond of the pentapeptides, while enkephalinase hydrolyzes the Gly^3-Phe^4 amide bond, thus releasing the carboxy terminal dipeptide [3,4]. The study of the inhibitory potency of a number of peptides towards enkephalinase in its membrane-bound state helped to design potent inhibitors such as thiorphan [5] and phosphoryl-Leu-Phe [6], which proved to be invaluable tools to elucidate the physiological role of this enzyme. Thus, when these inhibitors are administered in the cerebral ventricles of rats or mice, both display naloxone-reversible antinociceptive activity, thereby demonstrating the involvement of enkephalinase in the in vivo degradation of opioid peptides, most likely the enkephalins [7].

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Although initially characterized in brain [3], enkephalinase activity was later found to be present in many peripheral organs [8], including the kidney, where activity is highest. It was soon realized that, in this organ, enkephalinase is identical with an enzyme identified several years before using the B chain of insulin as substrate, the so-called neutral endopeptidase [9–12]. The detailed characteristics and function of this enzyme had however remained obscure. Enkephalinase has now been purified from many organs. Estimates of the molecular weight of the enzyme in these studies have ranged from 90 to 94 Kd. This variation is thought to be due to the fact that enkephalinase is a glycoprotein, and that its degree of glycosylation may vary [13].

Recently, we have isolated cDNA clones encoding rat enkephalinase in λgtl0 libraries constructed from both brain and kidney mRNA [14], demonstrating the co-identity of the enzyme from both sources. In addition, Southern blot analysis of rat DNA indicated that a single gene encodes enkephalinase in the rat genome [14]. Rat enkephalinase is a 742 amino acid protein, which contains six potential sites for N-linked glycosylation. Its single putative membrane spanning region is located close to the amino terminus of the protein, suggesting that the bulk of this ectopeptidase, including its carboxy terminus, is located extracellularly [14,15].

We now report the production of enzymatically active recombinant rat enkephalinase from mammalian cells. We show that, based on the inhibitory potency of various compounds, the recombinant enkephalinase is similar to the native enzyme.

MATERIALS AND METHODS

A full-length cDNA for the rat enkephalinase gene was constructed from two overlapping partial clones, λB16 and λK5, described in Malfroy et al. [14]. An 1,190-base pair (bp) *Hind*III (site in Ml3 polylinker)-*Bgl*II fragment of λB16 was ligated to a 1,290-bp *Bgl*II-*Eco*RI (site in cDNA adapter) fragnient of λK5 at the *Bgl*II site after blunt ending the *Hind*III and *Eco*RI sites. The resulting 2,480-bp fragment was ligated into *SmaI*-cleaved pSP64 (prENKanti). The cDNA was further subcloned, from this intermediate, as a blunt ended 2,520-bp *Hind*III-*Sac*I fragment into a mammalian expression vector based on a human cytomegalovirus (CMV) immediate early gene promoter, yielding the recombinant pCISrENK. The vector, described by Eaton et al. [16], had the factor VIII cDNA removed by cleavage with *Cla*I and *Hpa*I and was blunt ended with T4 DNA polymerase. All subclonings were performed by standard techniques [17].

Transient expression in Cos 7 cells was carried out using the calcium phosphate transfection technique of Graham and van der Eb [18] as modified by Gorman [19]. Ten ug of pCISrENK DNA was used to make 1 ml of precipitate. One microgram of plasmid DNA containing the adenovirus VA RNA genes [20] was included in the precipitate. Culture dishes (60 mm, 80% confluent) were transfected with 0.5 ml of precipitate and the cells assayed for expression 36 h later. For detection by immunological methods cells were fixed with methanol:acetone (50:50) and stained with a rabbit polyclonal antibody to the rat enkephalinase protein followed by a peroxidase-conjugated second antibody (goat antirabbit from Dako Corp., Santa Barbara, CA). Detailed methods are described in Gorman et al. [20].

To assay for expression of enkephalinase, culture medium was removed, and 2 ml of 5 mM Hepes buffer (pH 7.4) containing 0.1% Triton X-100 were added to the

cells. The dishes were gently shaken until all cells had detached. The cells were then collected in plastic tubes and kept at 4°C for at least 16 h to maximize protein solubilization before "Western" blot analysis or measurement of enzymatic activity was undertaken. For Western blot analysis, the solubilized extracts were separated by polyacrylamide gel electrophoresis under denaturing conditions and electroblotted onto nitrocellulose as described by Towbin et al. [22]. The blot was incubated with a rabbit polyclonal antibody raised against a synthetic peptide fragment (residues 212 to 227) of the rat enkephalinase polypeptide chain conjugated to soybean trypsin inhibitor, and developed using a goat antirabbit second antibody conjugated with horseradish peroxidase (Bio-Rad). Enkephalinase activity was measured by the method of Llorens et al. [23] using ^{3}H-(DAla^2, Leu^5) enkephalin as substrate.

RESULTS

The expression vector for the rat enkephalinase cDNA, pCISrENK, uses the human cytomegalovirus (CMV) enhancer and promoter [24] to initiate transcription. This vector also contains a chimeric splice-intron region composed of sequences from the CMV immediate early gene and a synthetic splice acceptor [16]. Importantly, for high levels of transient expression in Cos 7 cells [25], this vector includes the simian virus 40 (sV40) origin of replication contained in the 8V40 early promoter region which, in this vector, directs expression of the mouse dhfr cDNA [26].

The full-length rat enkephalinase cDNA was constructed by appropriately fusing two partial cDNA clones, λB16 and λK5. Although the former clone was derived from brain mRNA and the latter from kidney mRNA, DNA sequence identity and Southern blot analysis had demonstrated that a single gene encodes enkephalinase in the rat genome [14]. The resulting cDNA thus contains 99 bp of 5′ untranslated sequence, the entire 742 amino acid coding region, and 128 bp of 3′ untranslated sequence. The structure of the enkephalinase expression construct, pCISrENK, is shown in Figure 1.

pCISrENK DNA was assayed for transient expression in COS 7 cells with the addition of the adenovirus virus-associated (VA) RNA genes [21]. The addition of the VA RNA genes has been shown to increase the level of protein expression during transient expression [27]. Expression of the rat enkephalinase protein in transfected mammalian cells was assayed by immunoperoxidase staining. Approximately 20% of the cells (Fig. 2) were found to express enkephalinase transiently.

Cells were harvested and solubilized with Triton X-100. After polyacrylamide gel electrophoresis, the solubilized extracts were subjected to Western blot analysis,

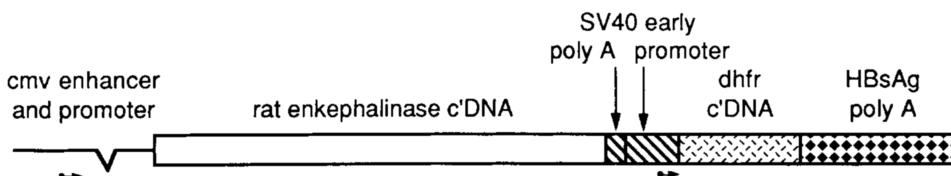

Fig. 1. pCISrENK expression plasmid. The vector diagram includes from left to right the cytomegalovirus enhancer and promoter [24], a splice donor-acceptor [16], the rat enkephalinase cDNA flanked by the SV40 poly(A) addition site, the SV40-dihydrofolate reductase (dhfr) transcription unit [26], and hepatitis B surface antigen poly(A).

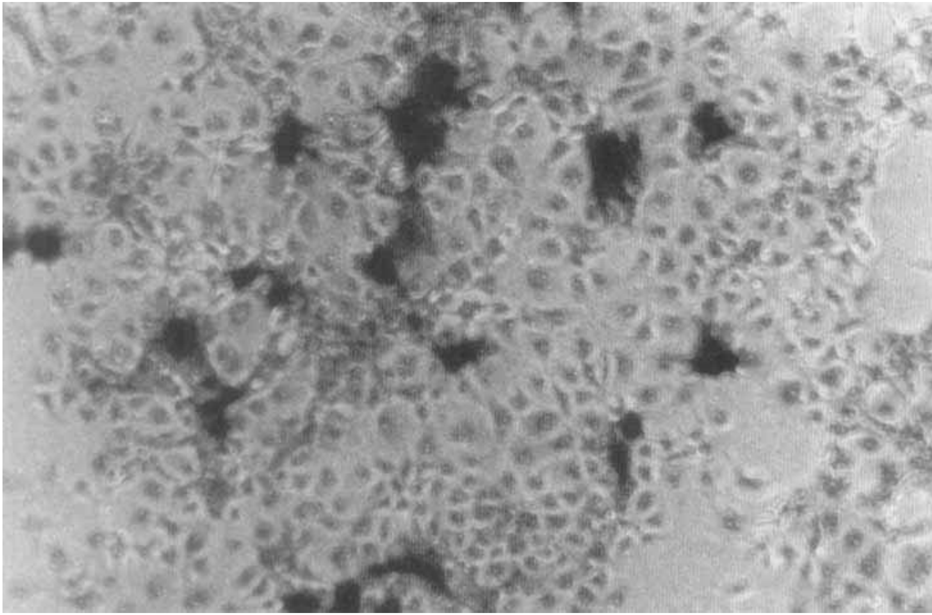

Fig. 2. Immunological detection of recombinant enkephalinase on Cos 7 cells. Cells were fixed with methanol:acetone (50:50) 36 h after transfection with the pCISrENK expression plasmid and stained with a rabbit polyclonal antibody to the rat kidney enkephalinase protein. (×100).

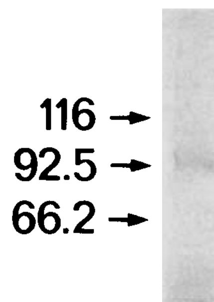

Fig. 3. Western blot analysis of solubilized extracts from pCISrENK-transfected Cos 7 cells. Triton X-100-solubilized Cos 7 proteins were separated by polyacrylamide gel electrophoresis under denaturing conditions and electroblotted onto nitrocellulose paper. The blot was incubated with a rabbit polyclonal antibody raised against a synthetic peptide fragment of the enkephalinase protein (fragment 212–227).

using the rabbit polyclonal antibody described above. These antibodies detected a 94-Kd protein (Fig. 3), further suggesting that the enkephalinase gene was being expressed by the transfected cells. This 94-Kd protein was not detected when preimmune serum was used.

To determine if the transfected cells were expressing enzymatically active enkephalinase, we assayed enkephalinase activity in solubilized Cos 7 cells transfected with pCISrENK, using a specific enkephalinase substrate, ^{3}H-(DAla^{2},Leu^{5}) enkephalin [23]. Enkephalinase activity could be detected as early as 1 day after transfection (Fig. 4). Activity was maximal (equivalent to the activity of about 2 μg native

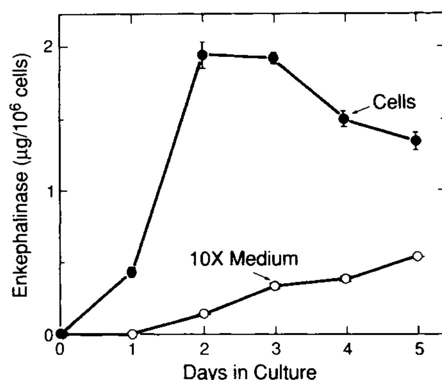

Fig. 4. Time course of expression of recombinant enkephalinase in pCISrENK-transfected Cos 7 cells. Enkephalinase activity was measured in solubilized Cos 7 cells or culture medium, following transfection with the pCISrENK expression plasmid. Activity is expressed as micrograms per 10^6 cells, assuming a specificity constant for the hydrolysis of ^{3}H-(DAla^2, Leu^5) enkephalin by recombinant enkephalinase similar to its value by the native enzyme (35 μmol$^{-1}\cdot$min^{-1}) [29]).

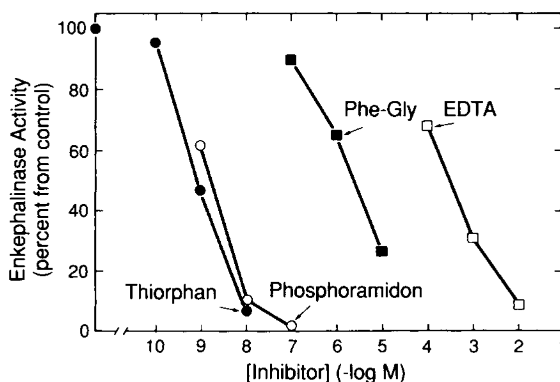

Fig. 5. Effect of various inhibitors on recombinant enkephalinase activity from solubilized Cos 7 cells. Enkephalinase activity (duplicate determinations varying by 5% or less) was measured using ^{3}H-(DAla^2, Leu^5) enkephalin as substrate, in the presence of increasing concentrations of inhibitors.

enkephalinase, in a 10^6 cells dish) after 3 days and slightly decreased afterwards. We also assayed enkephalinase activity in the culture media of the transfected cells and found a low activity that became detectable from the second day after transfection (Fig. 4). In some experiments, the transfected cells were harvested without added Triton X-100, lysed using a Polytron homogenizer, and a particulate fraction was prepared by centrifugation. Enkephalinase activity was only detectable in the particulate fraction (not shown) indicating that the enzyme is expressed in a membrane-associated state. Control, untransfected cells were totally devoid of enkephalinase activity.

To determine if recombinant enkephalinase activity has the same properties as the natural enzyme, we studied the effects of various known inhibitors of enkephalinase. As shown in Fig. 5, recombinant enkephalinase activity solubilized from Cos 7 cells 5 days after transfection could be inhibited by thiorphan (IC_{50} = 1 nM), phosphoramidon (IC_{50} = 2 nM), the dipeptide Phe-Gly (IC_{50} = 2 μM) and EDTA

(IC_{50}= 0.5 mM). These IC_{50} values agree with those obtained on the natural enzyme [5,11,12,27,28]. We also studied the effects of amidation in position P2′ on the sensitlvity of peptide substrates to hydrolysis. When both used at a concentration of 5 μM, the fluorescent substrate dansyl-Gly-Tyr-Gly-NH2 was hydrolyzed at a velocity 11% $\pm$ 1% of that of the carboxylated substrate dansyl-Gly-Tyr-Gly (mean and standard deviation from 3 determinations).

DISCUSSION

We have shown that the metallo peptidase enkephalinase (neutral endopeptidase; EC 3.4.24.11) can be expressed in its membrane-bound state in mammalian cells, and that the recombinant enzyme is enzymatically active and displays properties similar to those of the native enzyme with respect to sensitivity to inhibitors.

Three major post-translational modifications are known to occur in the biosynthesis of native enkephalinase: formation of disulfide bridges, glycosylation, and insertion of one atom of zinc. Twelve cysteine residues are found in the amino acid sequence of rat enkephalinase, and it has been suggested that there are at least four disulfide bridges in the native enzyme [30]. The location of these disulfide bridges has not yet been determined, but it is likely that their correct formation is necessary to maintain the tertiary structure of the enzymatically active enzyme.

Native enkephalinase is a glycoprotein. The extent of glycosylation has been reported to vary depending on the source of enzyme [13]. It is not yet clear if glycosylation plays any role in the enzymatic activity of the enzyme. However, the properties of enkephalinase from kidney and intestine, two organs where the enzyme is differently glycosylated, have been reported to be identical [31], suggesting that the degree of glycosylation of the enzyme may not have a critical importance for its enzymatic activity.

The final, major post-translational modification is the incorporation of one atom of zinc per enkephalinase molecule [32], and a potential binding site for the metal has been recently proposed [15] based on similarities with the binding site of zinc in the bacterial metallopeptidase, thermolysin. EDTA-treated enkephalinase has been shown to be easily reactivated upon addition of micromolar concentrations of zinc salts [32,33], suggesting that this step in the biosynthesis of the enzyme can occur spontaneously, provided a high enough concentration of the metal is present.

Active recombinant enkephalinase with properties similar to those of the native enzyme with respect to its sensitivity to various inhibitors is produced by the transfected Cos 7 cells (Fig. 5), showing that the folding of the polypeptide chain and the formation of the disulfide chains probably occurs correctly. Furthermore, the inhibition by EDTA of the enzymatic activity displayed by the recombinant enzyme implies the presence of a metal in its active site. Finally, we have found that the Triton X-100-solubilized enzyme binds to concanavalin-A (not shown), suggesting that the recombinant enzyme is glycosylated. This is also suggested by the molecular weight of the enzyme, 94 Kd (Fig. 3), which is higher than the predicted 85-Kd molecular weight of the unglycosylated enkephalinase polypeptide chain.

Native enkephalinase is a membrane-bound enzyme. The determination of the amino acid sequence of the enzyme, as deduced from cDNA clones [14,15] showed the presence of a single putative membrane spanning region. This transmembrane region, located close to the aminoterminus of the enkephalinase polypeptide chain

(residues 21–43) is preceded by a highly charged and conformationnally restrained region, Pro-Lys-Pro-Lys-Lys-Lys-Gln-Arg (residues 8 to 15), which probably serves as a stop transfer sequence [34] to terminate the transfer of the polypeptide chain through the cellular lipid membrane. In agreement with these hypotheses, we have found that, like the native enzyme, recombinant enkephalinase as produced in the Cos 7 cells is membrane-bound. The enkephalinase gene, as deduced from molecular cloning, does not encode a recognizable signal peptide [14]. It is likely that the single transmembrane region of the enkephalinase polypeptide plays the role of both signal peptide and membrane spanning/anchoring domain. The appearance of a small but significant amount of soluble enkephalinase activity in the culture medium, 2 days after transfection of the Cos 7 cells, suggests that enkephalinase may be released from the cell membranes by proteolytic activity. That such a process could yield enzymatically active enkephalinase is not unexpected since solubilization of native enkephalinase in an active form has been succesfully achieved by treating membranes with trypsin [32] or papain [30]. Additionally, it is possible to detect soluble enkephalinase activity in human plasma, cerebrospinal fluid [35] and urine (B. Malfroy, unpublished), suggesting that such a proteolytic solubilization of enkephalinase may also occur in vivo.

Recombinant enkephalinase as produced from Cos 7 cells displayed properties similar to those of the native enzyme as estimated by the potency of various inhibitors, with affinities ranging from nanomolar to millimolar concentrations (Fig. 5). We also found, using the fluorescent substrates dansyl-Gly-Tyr-Gly and dansyl-Gly-Tyr-Gly-NH_2 [36], that recombinant enkephalinase shows a clear preference for substrates displaying a free carboxyl end in position P2′, as has been shown to be the case for the native enzyme [12,29,36–38]. It has been suggested that this preference is due to the interaction of the free carboxyl group of substrates with an arginyl residue in the active site of enkephalinase [12,37–39], which would play a role similar to that of Arg 145 in bovine carboxypeptidase A [40]. The availability of a system to produce active recombinant enkephalinase will allow the examination of such questions by structure/function studies using site-specific mutagenesis.

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