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Isolation and functional expression of pituitary peptidylglycine α-amidating enzyme mRNA

A variant lacking the transmembrane domain

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We demonstrate that, in rat pituitary, peptidylglycine α-amidating enzyme was encoded by at least 5 distinct mRNAs. Southern blot and ribonuclease protection analyses revealed that the mRNAs arose through alternative splicing. A variant lacking the transmembrane domain-coding sequence was a major mRNA species for the enzyme in the pituitary. When the cDNAs were expressed in COS-7 cells, the variant was the most efficient in producing a secretory form (37 kDA) of the enzyme.

Peptide α-amidating enzyme; Functional expression; Secretory form; Transmembrane domain; Alternative splicing; mRNA

1. INTRODUCTION

Peptide α-amidation represents an important step in the maturation of a large number of endocrine and neuronal peptides, and other biologically active peptides [1]. The enzyme responsible for this post-translational modification is known as peptide α-amidating enzyme (peptidylglycine α-amidating monooxygenase; EC 1.14.17.3), and was first identified in porcine pituitary [2]. Peptide α-amidating enzymes purified from various sources display a substantial heterogeneity in apparent molecular weights, ranging from 37 kDa to 75 kDa [3,4]. The distribution of the α-amidating activity in soluble and membrane fractions also varies among tissues [5,6]. These observations suggest the existence of a certain molecular diversity in the α-amidating enzyme. In the present study, we have identified five splice variants of α-amidating enzyme mRNA from rat pituitary and expressed them in COS-7 cells.

2. EXPERIMENTAL

2.1. *cDNA cloning and sequencing*

Using 5 μg of poly(A)$^+$RNA isolated from pituitaries of adult Wistar rats, cDNA libraries were constructed as described [7]. A cDNA library (4.8×10^5 independent clones) was screened with a synthetic 80-base complement of the bovine α-amidating enzyme cDNA (base numbers 389-468 in [8]), yielding one positive clone. As the cDNA insert of the clone lacked a coding sequence corresponding to the COOH-terminal two-thirds of the bovine α-amidating enzyme [8], the amplified cDNA library (3×10^6 clones) and another cDNA library (2.2×10^5 clones) were rescreened with the 5′ portion (285 bp) of the cDNA insert as probe. Cloned cDNAs were subcloned into pBS vectors (Stratagene) and sequenced by the dideoxy chain termination method [9].

2.2. *Southern blot analysis of genomic DNA*

High molecular weight DNA was prepared from rat liver, digested with various restriction enzymes, electrophoresed on 0.8% agarose gel and then blotted onto a nitrocellulose filter [7]. The filter was hybridized using ^{32}P-labeled *Eco*RI-*Eco*RV fragment (172 bp) of cDNA clone 202 (see Fig. 1) as probe [7].

2.3. *RNase protection analysis*

*Eco*RI(-1)-*Nsi*I (700) fragment of clone 205 and *Sph*I (1872)-*Xma*I (2948) fragment of clone 202 (see Fig. 1) were subcloned into pBS vector, and ^{32}P-labeled antisense RNAs were synthesized in vitro [10]. The ^{32}P-labeled RNA probe (1×10^6 cpm) was hybridized with 40 μg of rat pituitary total RNA or 10 μg of rat atrium total RNA, and treated with RNase A and RNase T_1 as described [11]. The protected fragments were electrophoresed in 8% polyacrylamide/urea gels followed by autoradiography.

2.4. *Construction of expression plasmids*

The 2.58 kbp *Eco*RI-*Xma*I fragment of the α-amidating enzyme cDNA clone 205 (see Fig. 1) was subcloned into the *Hin*dIII/*Bgl*II site of the pSV2 vector [12] using synthetic oligonucleotide linkers. The resulting plasmid, designated as SV-205, carries the entire coding sequence of the α-amidating enzyme cDNA (clone 205) downstream of the simian virus 40 early promotor. Expression plasmids SV-201, -202, -203 and -204 were constructed by replacement of *Nsi*I (700)-*Xma*I fragment of SV-205 with *Nsi*I (700)- *Xma*I fragments of cDNA clones 201, 202, 203 and 204 respectively: this was based on the finding that the 5′ coding sequences upstream of *Nsi*I site (700) were identical in each mRNA (see Figs. 1 and 3B). Thus, expression plasmids SV-201, -202, -203, -204 and -205 had unique open reading

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Abbreviations: kbp, kilobase pairs; bp, base pairs; Hepes, 4(2-hydroxyethyl)-1-piperazineethane sulfonic acid

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frames corresponding to cDNA clones 201, 202, 203, 204 and 205, respectively.

2.5. *DNA transfection*

COS-7 cells were transfected [13] with 20 μg of each expression plasmid per 5×10^5 cells. pSV2 vector with no insert was used as control. After incubation for 24 h at 35°C under 3% CO_2, cells were washed twice with 10 ml of Dulbecco's modified Eagle's medium (DMEM) supplemented with 0.2% bovine serum albumin (BSA) and then incubated for 48 h in 10 ml of DMEM/0.2% BSA at 37°C under 5% CO_2.

2.6. *Assay of expressed α-amidating enzyme*

After the 48 h transient expression, conditioned media were removed, and cells were washed and scraped gently with a rubber policeman in the presence of phosphate-buffered saline. After centrifugation at low speed for 4 min, cell pellets were weighed and homogenized in 9 volumes of 0.25 M sucrose containing 10 mM mannitol, 50 mM Hepes-KOH (pH 7.0) and 300 μg/ml phenylmethyl-sulfonylfluoride at 4°C using a teflon-glass homogenizer. Soluble and membrane fractions were prepared according to the method of May et al. [5] with some modifications. Homogenate was centrifuged at $600 \times g$ for 10 min. Supernatants were subjected to three cycles of freezing and thawing, followed by sonication for 1 min, and centrifuged at $100\,000 \times g$ for 60 min at 4°C. The supernatants from the high speed centrifugation were assayed for the soluble enzyme activity. The pellets were once washed with the homogenizing buffer containing 0.15 M KCl, resuspended in the homogenizing buffer containing 1% Triton X-100 and shaken for 30 min at 4°C. After centrifugation at $100\,000 \times g$ for 60 min at 4°C, the resultant supernatants were assayed for the membrane-bound enzyme activity. Amidation assay was performed as described [14] in 120 μl of 50 mM Hepes-KOH, pH 8.5, using 40 picomoles of ^{125}I-D-Tyr-Val-Gly as substrate.

3. RESULTS

3.1. *Isolation and characterization of cDNAs encoding pituitary peptide α-amidating enzyme*

Five cDNA clones isolated differed from each other by the presence or absence of 315-bp, 204-bp and 54-bp regions designated as region A, region B and region C, respectively (Fig. 1). clones 201 had no deleted segments. Clone 202 lacked region A, clone 203 region B and clone 204 region C. Clone 205 lacked both regions A and C. Deletions/insertions of these regions did not cause any translational frameshift. There were no base substitutions or deletions in the overlapping sequences of the 5 cDNAs. Sequencing analysis revealed that only clone 205 included the initiator Met as compared with the deduced amino acid sequence of bovine pituitary α-amidating enzyme [8]. The cDNA coded for 853 amino acid residues which terminated at position 2560 (Fig. 2). The NH_2-terminal sequence (residues 1–25) had the characteristics of a signal peptide with a consensus cleavage site [15]. The hydropathicity profile analysis indicated a clear hydrophobic segment consisting of 24 uncharged amino acid residues (residues 762–785) in the COOH-terminal portion of the protein (data not shown). This segment is contained in region B and is therefore missing in clone 203 (see Fig. 1).

3.2. *Southern blot and RNase protection analyses*

Only one band was recognized in each digestion of

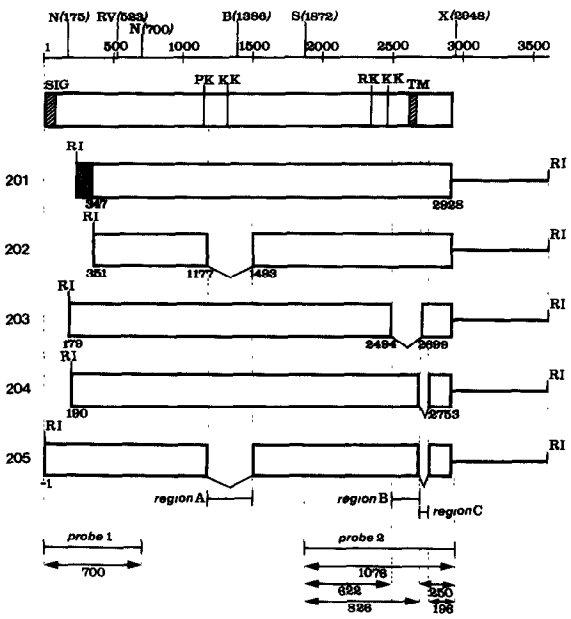

Fig. 1. Schematic representation of cDNA clones coding for peptide α-amidating enzymes. Nucleotide numbers are given at the *top*, and sites for restriction enzymes, B (*Bam*HI), N (*Nsi*I), RI (*Eco*RI), RV (*Eco*RV), S (*Sph*I), and X (*Xma*I) are demarcated by vertical bars (*Eco*RI sites are derived from *Eco*RI linkers). Nucleotide residues are numbered in the 5′ to 3′ direction, beginning with the first residue of the ATG triplet encoding the initiator methionine. Nucleotides on the 5′ side of residue 1 are indicated by negative numbers. The predicted protein product is shown below the restriction map. SIG, signal sequence. TM, transmembrane domain. PK (Pro-Lys), KK (Lys-Lys) and RK (Arg-Lys) are predicted endoproteolytic cleavage sites. Regions A, B and C are alternatively spliced regions. Horizontal bars indicate 3′-untranslated regions and poly(A) tracts. Clone 201 contained extraneous 130 bp at the 5′ end (cross-hatched area) which has been found to be a cloning artefact (data not shown).

genomic DNA (Fig. 3A). An antisense RNA probe corresponding to *Eco*RI(-1)-*Nsi*I (700) fragment of clone 205 yielded a single protected fragment of 700 bases in both pituitary and atrium (Fig. 3B). On the other hand, several fragments were protected with *Sph*I (1872)-*Xma*I (2948) fragment of clone 202 corresponding to the COOH-terminus of the enzyme. Protections of 1076-, 826-, 622-, 250- and 196-base fragments are exactly what would be predicted to result from the presence of mRNAs corresponding to cDNA clones 201–205 (Fig. 1). A densitometric analysis indicated that the 622- and 250-base fragments, corresponding to clone 203, are the major protected fragments in the pituitary, whereas in the atrium it is the 1076-base fragment, corresponding to clones 201 and 202, that is most abundant.

3.3. *Expression of α-amidating enzyme cDNAs in COS-7 cells*

Soluble fractions, membrane fractions and conditioned media of COS-7 cells transfected with expression plasmids carrying α-amidating enzyme cDNAs ex-

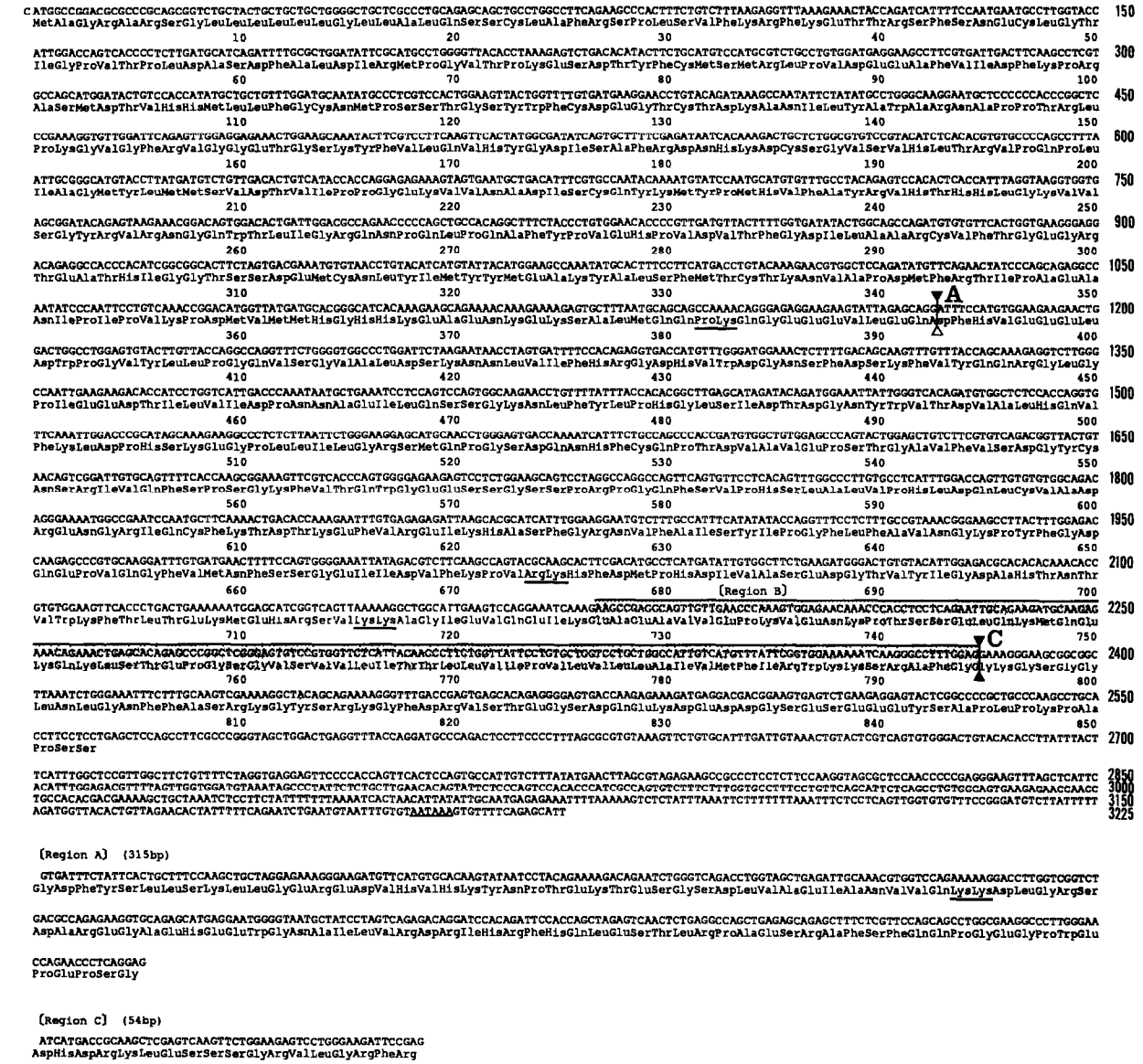

Fig. 2. cDNA sequence of clone 205 and deduced amino acid sequence. Region A is inserted at position A (▼) in clones 201, 203 and 204. Region B missing in clone 203 is hatched and overlined. Region C is inserted at position C (▼) in clones 201, 202 and 203. Amino acids are numbered below the sequence. The putative polyadenylation signal and predicted endoproteolytic cleavage sites are underlined.

hibited higher α-amidating activities than those of control cells (Table I). Cells transfected with SV-203, which lacks the COOH-terminal hydrophobic sequence, released a very much higher α-amidating activity into the culture medium than other transfectants. α-Amidating activity secreted from this transfectant was recovered as a single peak at approximately 37 kDa upon gel filtration (data not shown).

4. DISCUSSION

In the present study, we have identified 5 α-amidating enzyme cDNA species in rat pituitary cDNA libraries. As Southern blot analysis indicated that the enzyme gene is a single copy in the rat genome, the diversity of the mRNAs is thought to be due to alternative splicing. RNase protection assay suggested that tissue-specific alternative splicing events occur in the 3′ region of the mRNA and that the species lacking the COOH-terminal hydrophobic segment is a major form of α-amidating enzyme mRNAs in the pituitary. The enzyme cDNAs, which have so far been isolated from bovine pituitary [8] and from rat atrium [16], corresponded to clones 201, 202 and 204.

COS-7 cells transfected with the expression plasmids, SV-201, -202, -204 and -205, which contained the COOH-terminal hydrophobic segment, exhibited significantly higher α-amidating activities in the mem-

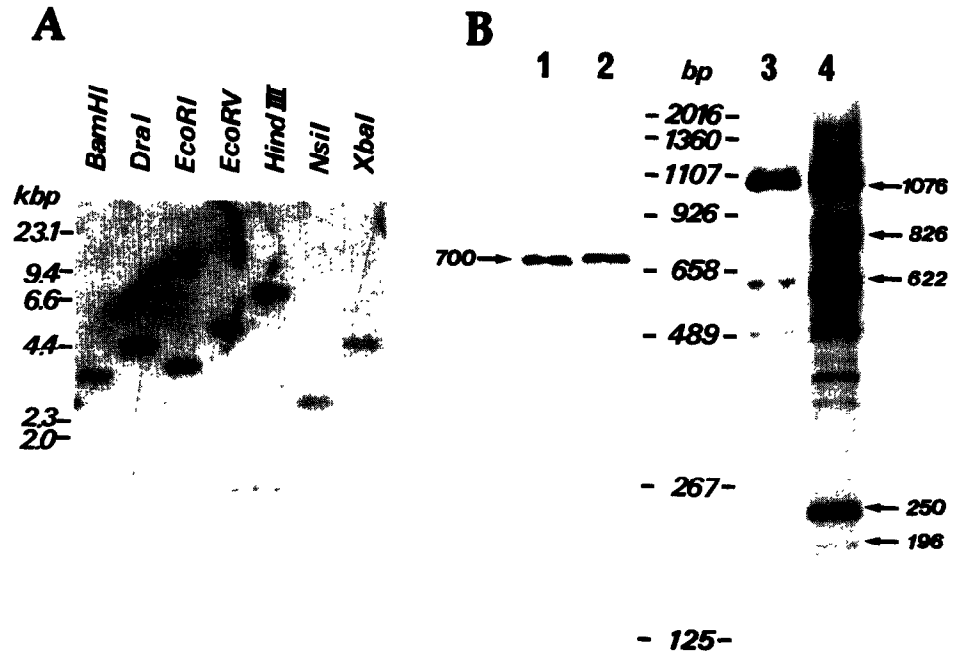

Fig. 3. (A) Southern blot analysis of genomic DNA. Ten μg of rat liver DNA was digested with *Bam*HI, *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Nsi*I. Positions of size markers are given at the left. (B) RNase protection analysis. Atrium RNA (lanes 1 and 3) and pituitary RNA (lanes 2 and 4) were hybridized with ^{32}P-labeled antisense RNA probe 1 (lanes 1 and 2) and with probe 2 (lanes 3 and 4). Positions of size markers are given at the center. Arrows indicate protected fragments. A diagram of probes 1 and 2 and of the expected protected fragments is shown in Fig. 1.

brane fractions. On the other hand, cells transfected with SV-203, which lacked the COOH-terminal hydrophobic segment, released a very much higher α-amidating activity into culture media than other transfectants and exhibited a lower activity in the membrane fraction. These results indicate that the COOH-terminal hydrophobic segment is a transmembrane domain and actually serves as a membrane anchor. Thus it is reasonable to assume that the enzyme protein lacking the transmembrane domain is primarily soluble and is most efficiently secreted as a 37 kDa protein, which may have resulted from the NH_2-terminal region of the precursor by a monobasic cleavage [1] at Pro-382-Lys-383 (see Fig. 1). The enzyme proteins encoded by clones 201, 202, 204 and 205 may be initially synthesized as membrane-bound forms, which are subsequently converted into soluble forms and secreted.

Table I

α-Amidating activity in transfected COS-7 cells

Plasmid	α-Amidating activity (fmol/h/10^6 cells)		
	Membrane-bound	Soluble	Conditioned medium
SV-201	392	1318	46800
SV-202	365	1230	38700
SV-203	90	327	214100
SV-204	410	1138	39700
SV-205	397	1273	47300
pSV2	42	162	7400

The activity is expressed as fmol of D-Tyr-Val-Gly converted into D-Tyr-Val-NH_2 per h per 10^6 cells. Similar results were obtained in 3 different experiments.

Substantial levels of peptide α-amidating activity have been detected in serum [17,18] and in cerebrospinal fluid [19]. The mRNA species lacking a transmembrane domain-coding sequence characterized in the present study may in a large part account for the extracellular nature of the distribution of the enzyme.

There is a practical interest in the present results, since it should be possible, by using the cDNA for the secretory form of peptide α-amidating enzyme built into expression vectors, to drive cell lines and yeast or prokaryotic cells so as to undertake large-scale synthesis of the enzyme.

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