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TWO ENZYMES CONCERNED IN PEPTIDE HORMONE α-AMIDATION ARE SYNTHESIZED FROM A SINGLE mRNA

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SUMMARY: By expressing truncated rat pituitary 'peptidylglycine α-amidating enzyme' cDNAs in COS-7 cells, we found that the two reactions concerned in peptide carboxyl-terminal amidation, namely the peptidylglycine α-hydroxylation reaction and the peptidyl-hydroxyglycine amidation reaction, were catalyzed by 37- and 53-K proteins, which were derived from the 5'- and 3'-coding sequences, respectively. The full-length cDNA directed the expression of both the 37- and 53-K enzymes, and in the combined presence of the two enzymes the full conversion of a glycine-extended peptide into the amidated product was achieved. These results indicated that two enzymes concerned in peptide hormone α-amidation are generated from a common precursor protein encoded by a single mRNA.

A large number of endocrine and neuronal peptides such as oxytocin (1), vasoactive intestinal peptide (2) and pancreatic polypeptide (3) are synthesized from the glycine-extended precursors and amidated on their carboxyl termini. The α-amide structure is generally essential for their biological activities. The enzyme responsible for this post-translational modification was first identified in porcine pituitary by Bradbury et al. (4), and has so far been known as 'peptidylglycine α-amidating enzyme'. Recently we (5,6) and others (7-9) have suggested that the α-amidation reaction proceeds via a two-step mechanism, namely the conversion of peptidylglycine into an α-hydroxyglycine intermediate and the conversion of the intermediate into the mature α-amide product, and that each step should be catalyzed enzymatically. Here we show, by functional expression of

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truncated rat pituitary 'peptidylglycine α-amidating enzyme' cDNAs, that two distinct enzymes catalyzing the two steps are synthesized from a single mRNA molecule.

METHODS

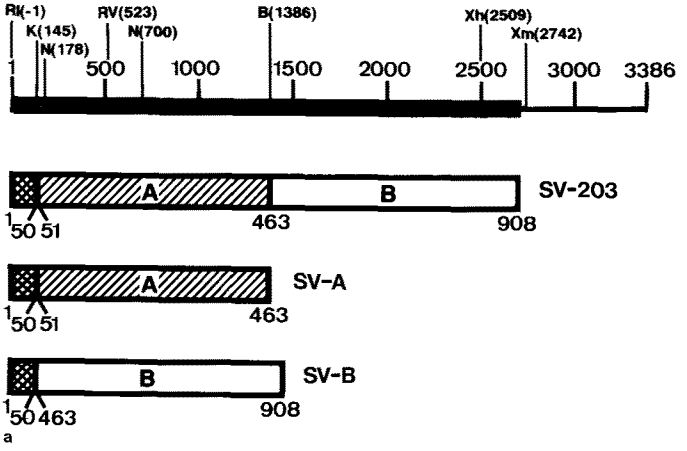
Construction and transfection of expression plasmids: SV-A was constructed from SV-203 (10) by deleting the 1,356-bp Bam HI-Xma I fragment (from positions 1,386-2,742) and ligating using oligonucleotide linkers (5'-GATCTGAAAC-3', 5'-CCGGGTTTCA-3') which contain a termination signal and Bam HI/Xma I sites. SV-B was constructed from SV-203 by deleting the 1,241-bp Kpn I-Bam HI fragment (from positions 145-1,386) and ligating using an oligonucleotide linker (5'-GATCGTAC-3') which fills in the Kpn I/Bam HI overhangs without causing frame shifts. The construction of the deleted cDNAs was confirmed by sequence analysis of the 5'- and 3'-terminal regions. COS-7 cells were transfected with 20 μg of each expression plasmid as described (11).

Northern blot analysis: After a 48-h transient expression, total RNAs were extracted from the transfected cells using the guanidinium/CsCl procedure (12), electrophoresed on a formaldehyde-agarose (1.5%) gel and then blotted onto a nitrocellulose filter. The filter was hybridized to the 5'-probe (the 345-bp Nsi I-Eco RV fragment, positions 178-523) or the 3'-probe (the 233-bp Xho I- Xma I fragment, positions 2,509-2,742) labelled with [α-^{32}P]dCTP by the random-priming method (13), washed, and autoradiographed as described (14).

Gel filtration and enzyme assays: After the 48-h transient expression in 10 ml of Dulbecco's modified Eagle's medium supplemented with 0.2% bovine serum albumin, the conditioned medium of each transfectant was concentrated 7-fold with collodion bags (Sartorius), applied on a Sephacryl S-200 (Pharmacia) column (1 x 95 cm) equilibrated with 10 mM Hepes-KOH (pH 7.0)/50 mM NaCl and eluted at a flow rate of 6 ml per h. Twenty μl aliquots of each fraction (1 ml) were assayed for peptidylglycine α-hydroxylating activity in 200 μl of 100 mM 4-morpholineethane-sulfonic acid (MES)-KOH (pH 5.6), 1 mM ascorbic acid, 0.1 mg/ml catalase, 5 μM copper sulfate and 15 μM Phe-Gly-Phe-Gly for 5 h at 37°C with shaking. Fifteen μl aliquots of each fraction were assayed for peptidyl-hydroxyglycine amidating activity in 35 μl of 100 mM MES-KOH (pH 5.6) and 15 μM Phe-Gly-Phe-α-hydroxyglycine for 2 h at 30°C without shaking. The reaction was stopped by the addition of 0.5% formic acid and 0.5 mM EDTA. Phe-Gly-Phe-α-hydroxyglycine was enzymatically prepared from Phe-Gly-Phe-Gly and HPLC-purified (5). The reaction products were separated and quantified as described (5).

RESULTS AND DISCUSSION

We constructed two deletion mutants from an expression plasmid SV-203, which carried the full-length cDNA for the rat pituitary enzyme (10). As shown in Fig. 1a, SV-A lacked the sequence corresponding to the carboxyl-terminal half (amino acid residues 464-908) of the protein encoded by SV-203 (10) and SV-B lacked the sequence corresponding to the amino-terminal half (residues 51-462). As the two mutants possessed the signal



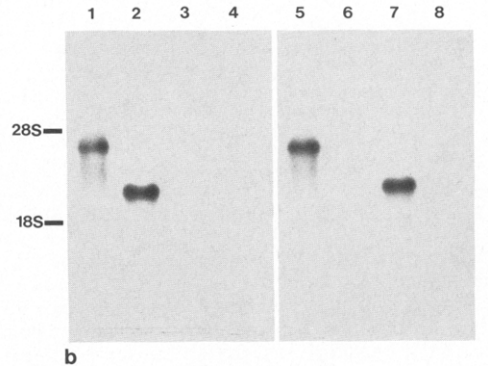


Fig. 1a. The predicted protein products from expression plasmids carrying full-length cDNA (SV-203), carboxyl-terminally truncated cDNA (SV-A) and amino-terminally truncated cDNA (SV-B). Restriction map of full-length α-amidating enzyme cDNA (10) is shown on the top, with nucleotide residue numbers beginning with the first residue of the ATG triplet encoding the initiator methionine. Cleavage sites for restriction enzymes, B (Bam HI), K (Kpn I), N (Nsi I), RI (Eco RI), RV (Eco RV), Xh (Xho I) and Xm (Xma I) are demarcated by vertical bars. The thick bar and the thin bar indicate the open reading frame and the 3'-noncoding sequence, respectively. Numbers below the predicted protein products refer to amino acid residues of the protein encoded by SV-203. The double-hatched box indicates the amino-terminal 50-amino acid region containing a 25-amino acid signal peptide (10). The single-hatched box (A) and the open box (B) represent protein segments encoded by SV-A and -B respectively.

Fig. 1b. Northern blot analysis of RNA from transfected COS-7 cells. RNA (7.5 μg of each) from SV-203 transfectant (lanes 1 and 5), from SV-A transfectant (lanes 2 and 6), from SV-B transfectant (lanes 3 and 7) and from pSV2 transfectant (lanes 4 and 8). RNA blots were hybridized with the 5'-portion (lanes 1-4) and the 3'-portion (lanes 5-8) of the full-length cDNA as probes. Positions of 28S and 18S rRNAs are given at the left.

sequence and lacked the membrane anchor-coding sequence (10), both were expected to drive transfectants to secrete expressed proteins into culture media as was the case with the parental plasmid SV-203. As shown in Fig. 1b, when each expression

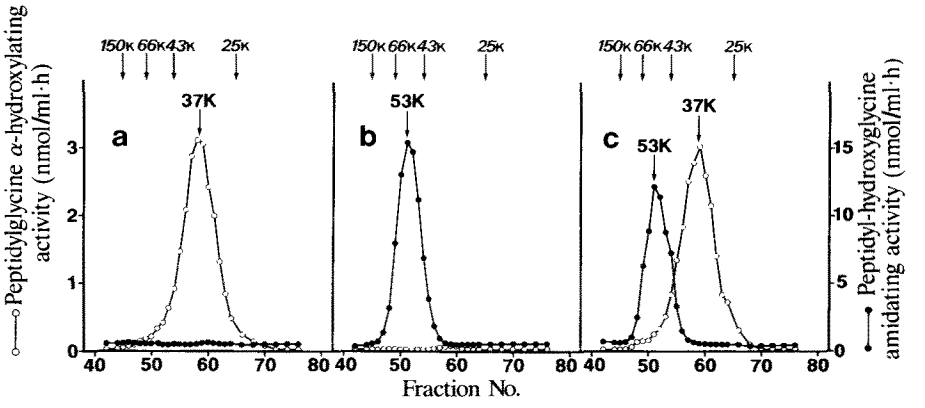


Fig. 2. Gel filtration analysis of the conditioned media of the SV-A transfectant (a), SV-B transfectant (b) and SV-203 transfectant (c). Peptidylglycine α-hydroxylating activity (o). Peptidyl-hydroxyglycine amidating activity (●). Two different experiments gave similar results. Peaks of each activity (arrows) were calculated from the elutions of alcohol dehydrogenase (Mr=150K), bovine serum albumin (66K), ovalbumin (43K) and chymotrypsinogen A (25K). The 37-K protein from SV-A and SV-203 transfectants, which exhibits peptidylglycine α-hydroxylating activity, may have resulted from each primary translation product by removal of the signal peptide and by a monobasic cleavage (20) at Pro^{382}-Lys^{383} (10). The 53-K protein from SV-B transfectant, which exhibits peptidyl-hydroxyglycine amidating activity, is almost equal to the molecular mass of the SV-B-encoded protein without the signal peptide. The 53-K protein from the SV-203 transfectant is probably derived from the precursor protein by a dibasic cleavage at Lys^{436}-Lys^{437} (see Fig. 3).

plasmid was introduced into COS-7 cells, a transcript of the expected size was synthesized, which hybridized to the corresponding sequence, but not to the deleted sequence of the cDNA.

We assayed the activity that converts Phe-Gly-Phe-Gly into Phe-Gly-Phe-α-hydroxyglycine (5) in the conditioned medium of each transfectant. As shown in Fig. 2a, the SV-A transfectant medium contained the peptidylglycine α-hydroxylating activity, which exhibited a single peak at 37 K on Sephacryl S-200 gel filtration. The SV-B transfectant medium contained no detectable peptidylglycine α-hydroxylating activity (Fig. 2b). On the other hand, we found the activity that converts Phe-Gly-Phe-α-hydroxyglycine into Phe-Gly-Phe-amide in the SV-B transfectant medium, peaking at 53 K on S-200 gel filtration, but not in the SV-A transfectant medium (Fig. 2a and b). The peptidylglycine α-hydroxylation reaction and the peptidyl-hydroxyglycine amidation reaction, driven by the SV-A and -B transfectant conditioned media, proceeded in dose-dependent and time-dependent manners, slowed down at a lower temperature (15°C), and were abolished when the medium had been heated at 90°C for 5 min (data not

Table 1. Reconstitution experiment using gel-purified recombinant proteins

	*Conversion of Phe-Gly-Phe-Gly to Phe-Gly-Phe-α-hydroxyglycine (pmol/h)	*Conversion of Phe-Gly-Phe-α-hydroxyglycine to Phe-Gly-Phe-amide (pmol/h)	†Conversion of Phe-Gly-Phe-Gly to Phe-Gly-Phe-amide (pmol/h)
37-K Protein	730	< 5	<1
53-K Protein	<1	800	<1
37-K Protein + 53-K Protein	380	750	440

Fractions (No. 56-60) of SV-A transfectant medium and those (No. 49-53) of SV-B transfectant medium in each gel filtration were collected (total 5 ml each) and concentrated 8-fold with collodion bags and referred to as 37-K Protein and 53-K Protein, respectively.
* Conversion rates were assayed at pH 5.6 using concentrated fractions (37-K Protein, 30 μl; 53-K Protein, 10 μl) as described in METHODS.
† Conversion of Phe-Gly-Phe-Gly to Phe-Gly-Phe-amide was assayed under the same conditions as that of Phe-Gly-Phe-Gly to Phe-Gly-Phe-α-hydroxyglycine.
Two different experiments gave similar results.

shown), indicating that the both reactions are enzyme-catalyzed. The peptidylglycine α-hydroxylation absolutely required Cu^{2+}, ascorbate and catalase as cofactors. On the other hand, the hydroxyglycine amidation was independent of these cofactors. The optimal pH of each enzyme reaction was 5.6. We then conducted a reconstitution experiment using the gel-purified recombinant proteins. As shown in Table 1, in the combined presence of the 37- and 53-K proteins from SV-A and -B respectively, both the α-hydroxylation and amidation reactions were successfully achieved, yielding the mature α-amide product from the glycine-extended precursor. The 37-K protein from SV-A transfectant alone was capable of catalyzing only the α-hydroxylation and the 53-K protein from SV-B transfectant only the hydroxyglycine amidation. As shown in Fig. 2c, cells transfected with the parental plasmid SV-203 released both the peptidylglycine α-hydroxylating activity and the hydroxyglycine amidating activity, which were recovered as 37- and 53-K peaks on the same column, indicating that the two enzymes are cleaved off from a precursor protein synthesized from the full-length cDNA (see Fig. 3).

In the present study, we have shown that the two reactions concerned in peptide α-amidation, namely peptidylglycine α-

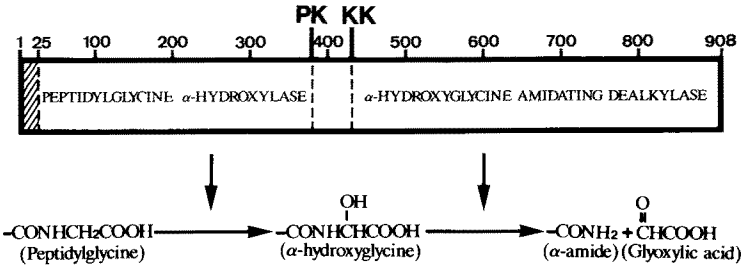


Fig. 3. Schematic representation of the organization of peptidylglycine α-hydroxylase and α-hydroxyglycine amidating dealkylase in the common precursor protein. Amino acid numbers beginning with the initiator methionine are indicated above the box. The hatched box indicates a 25-amino acid signal peptide (10). PK(Pro^{382}-Lys^{383}) and KK(Lys^{436}-Lys^{437}) indicate predicted endoproteolytic cleavage sites (10). The proposed reactions catalyzed by the two enzymes are also shown.

hydroxylation and peptidyl-hydroxyglycine amidation, are catalyzed by two different enzymes derived from a common precursor protein encoded by a single mRNA molecule. "Peptidylglycine α-hydroxylase" (7,15) and "α-hydroxyglycine amidating dealkylase" (9) should primarily correspond to the 37- and 53-K enzymes described in this study, respectively. A schematic diagram of the organization of these enzymes in the precursor protein is shown in Fig. 3, together with the proposed reactions that they catalyze.

'Peptidylglycine α-amidating enzymes' purified from various sources have been able to generate α-amide peptides under alkaline pH but incapable of doing so under acidic pH (6,16,17). This is probably because the purified enzyme was composed exclusively of "peptidylglycine α-hydroxylase" and devoid of "α-hydroxyglycine amidating dealkylase"; the conversion of peptidyl-hydroxyglycine into the α-amide product proceeds non-enzymatically to some extents under alkaline conditions (5). In the secretory vesicles where the α-amidation reaction takes place (18), pH is maintained at 5.5-6.0 (19). The peptidylglycine α-hydroxylation and peptidyl-hydroxyglycine amidation reactions catalyzed by the 37- and 53-K enzymes proceeded most efficiently at this acidic pH range. It is thus possible that the two enzymes facilitate the full conversion of the glycine-extended peptide to the α-amide product in the secretory vesicle.

That the two enzymes, "peptidylglycine α-hydroxylase" and "α-hydroxyglycine amidating dealkylase", are synthesized from a common precursor protein ensures that the two enzymes (i) are expressed by common regulatory mechanisms, (ii) are located in

the same subcellular compartment at the same time, and (iii) occur at the stoichiometric ratio (1:1) to achieve the efficient α-amidation of glycine-extended peptides.

REFERENCES

1. Land, H., Grez, M., Ruppert, S., Schmale, H., Rehbein, M., Richter, D., and Schutz, G. (1983) Nature 302, 342-344.
2. Itoh, N., Obata, K., Yanaihara, N., and Okamoto, H. (1983) Nature 304, 547-549.
3. Yamamoto, H., Nata, K., and Okamoto, H. (1986) J. Biol. Chem. 261, 6156-6159.
4. Bradbury, A.F., Finnie, M.D.A., and Smyth, D.G. (1982) Nature 298, 686-688.
5. Tajima, M., Iida, T., Yoshida, S., Komatsu, K., Namba, R., Yanagi, M., Noguchi, M., and Okamoto, H. (1990) J. Biol. Chem. 265, 9602-9605.
6. Takahashi, K., Okamoto, H., Seino H., and Noguchi, M. (1990) Biochem. Biophys. Res. Commun. 169, 524-530.
7. Bradbury, A.F., and Smyth, D.G. (1987) Bioscience Reports 7, 907-916.
8. Young, S.D., and Tamburini, P.P. (1989) J. Am. Chem. Soc. 111, 1933-1934.
9. Katopodis, A.G., Ping, D., and May, S.W. (1990) Biochemistry 29, 6115-6120.
10. Kato, I., Yonekura, H., Yamamoto, H., and Okamoto, H. FEBS Lett. in press.
11. Chen, C., and Okayama, H. (1987) Molec. Cell. Biol. 7, 2745-2752.
12. Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J., and Rutter, W.J. (1979) Biochemistry 18, 5294-5299.
13. Feinberg, A.P., and Vogelstein, B. (1983) Anal. Biochem. 132, 6-13.
14. Yonekura, H., Nata, K., Watanabe, T., Kurashina, Y., Yamamoto, H., and Okamoto, H. (1988) J. Biol. Chem. 263, 2990-2997.
15. Bradbury, A.F., and Smyth, D.G. (1987) Eur. J. Biochem. 169, 579-584.
16. Murthy, A.S.N., Mains, R.E., and Eipper, B.A. (1986) J. Biol. Chem. 261, 1815-1822.
17. Noguchi, M., Takahashi, K., and Okamoto, H. (1989) Arch. Biochem. Biophys. 275, 505-513.
18. Gainer, H., Russell, J.T., and Loh, Y.P. (1985) Neuroendocrinology 40, 171-184.
19. Russel, J.T., and Holts, R.W. (1981) J. Biol. Chem. 256, 5950-5953.
20. Schwartz, T.W. (1990) In Molecular Biology of the Islets of Langerhans (H. Okamoto, Ed.), pp. 153-205. Cambridge University Press, Cambridge.