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CHROMAFFIN GRANULE ASPARTIC PROTEINASE PROCESSES RECOMBINANT PROOPIOMELANOCORTIN (POMC)

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SUMMARY: Our search for proteases responsible for proenkephalin (PE) processing in adrenal medulla led to the isolation of a 70 kDa aspartic proteinase that cleaves PE between the basic residues of the Lys-Arg processing site (1). Studies in pituitary have also identified a similar aspartic proteinase that processes POMC (2,3). To compare the chromaffin granule (CG) 70 kDa aspartic proteinase with that in pituitary, processing of recombinant POMC by the CG enzyme was examined. POMC was expressed in the T7 expression system in *E. coli*, and purified to homogeneity. The CG 70 kDa aspartic proteinase converted POMC to 27 and 22 kDa bands that were detected by anti-N-POMC immunoblots, and to 26, 22, and 14 kDa bands that were immunoreactive with anti-ß-lipotropin. POMC products represented by these bands indicate appropriate POMC processing by the CG 70 kDa aspartic proteinase. These results, combined with the similar biochemical properties of these two enzymes, suggest that the CG 70 kDa aspartic proteinase resembles the POMC-converting enzyme (PCE), an aspartic proteinase in pituitary (2,3).

The biosynthesis of peptide hormones and neurotransmitters requires proteolytic processing of prohormone precursors at paired basic residues (3-5). Enzymes belonging to the aspartic (1-3), serine (reviewed in 3,4,6,7), and cysteine (reviewed in 3,4) classes of proteases have been proposed as prohormone processing enzymes. Our studies of enkephalin precursor processing in bovine adrenal medullary chromaffin granules have indicated participation of a 70 kDa aspartic proteinase in

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proenkephalin processing (1). This proteinase cleaves recombinant proenkephalin at a Lys-Arg processing site, with a pH optimum of 5.5 that is consistent with the intragranular pH of 5.5-5.8 (8). In conjunction with the aspartic proteinase, enkephalin peptide production involves a novel cysteine protease, known as the 'prohormone thiol protease' (PTP), as an important proenkephalin processing enzyme (9-12). To a lesser extent, the subtilisin-like PC1/3 and PC2 (PC=prohormone convertases) proteases are also involved in proenkephalin processing in adrenal medulla (11-13).

Studies of POMC processsing in bovine pituitary intermediate lobe secretory granules have identified a 70 kDa aspartic proteinase as a primary POMC processing enzyme (2,3). Characterization of the chromaffin granule aspartic proteinase (1) indicates that it resembles the pituitary POMC converting enzyme (PCE) (2,3) with respect to molecular weight, glycoprotein nature, pH optimum, cleavage specificity for Lys-Arg processing sites, and inhibition by pepstatin A (an inhibitor of aspartic proteinases). These results suggest that prohormone processing in adrenal medulla and pituitary involves a 70 kDa aspartic proteinase.

In this study, the ability of the chromaffin granule (CG) 70 kDa aspartic proteinase to process POMC was examined to assess whether the CG aspartic proteinase resembles the pituitary PCE. Recombinant POMC was expressed with the T7 expression system in *E. coli*, and purified POMC was utilized as in vitro prohormone substrate. Results demonstrating appropriate POMC processing by the CG aspartic proteinase suggest the similarity of prohormone processing aspartic proteinases in adrenal medulla and pituitary.

Materials and Methods

Purification of 70 kDa aspartic proteinase from chromaffin granules (CG). Purification of the CG aspartic proteinase was conducted as described previously (1). Briefly, the soluble fraction of chromaffin granules was subjected to chromatography on concanavalin A-Sepharose, Sephacryl S-200, and pepstatin A columns. Proteolytic activity was monitored with ^{35}S-labelled enkephalin precursor as substrate, as previously reported (1). Expression and purification of recombinant POMC. The POMC/pET3c expression construct was generated by PCR of the POMC DNA fragment from the porcine pre-POMC cDNA (14), with subcloning into the pET3c vector (15). PCR primers (with NdeI and BamHI sites) were 5'-AAACATATGTGGTGCTTGGAGAGCAGCCAGTGTCAG-3' and 5'-AAAGGATCCCC-CTCACTGGCCCTTCTTGTGGGCGTTCTT-3'; PCR reactions (100 µl) utilized1 ng pre-POMC DNA template, 0.2 µM primers, and 30 cycles of 1 minute each at 94° C, 50° C, and 72° C, according to the protocol by Perkin-Elmer. The amplified POMC DNA was digested with NdeI and BamHI, ligated with T4 ligase to the pET3c vector digested with NdeI and BamHI, and transformed into DH5α *E. coli* cells. The POMC/pET3c plasmid DNA was subjected to

DNA sequencing, as described (16), to confirm that authentic POMC nucleotide sequence was amplified by PCR.

Expression of the POMC/pET3c construct in BL21(DE3) *E. coli* cells (in M9ZB medium with 100 µg/ml ampicillin) was induced with 0.2 mM IPTG (isopropyl-ß-thiogalactopyranoside). Expression was monitored by immunoblots with anti-ß-LPH (anti-ß-LPH serum was from the National Pituitary Agency, Gaithersburg, MD). POMC expressed from a 5 liter culture was solubilized by sonication of cells in 50 mM MOPS-HCl, pH 6.5, 6.0 M urea (buffer A). For purification of POMC, the solubilized POMC (collected as the supernatant after centrifugation at 27,000 x g for 30 minutes) was applied to a DEAE-Sepharose column (1.5 x 36 cm) and eluted with a 0-500 mM NaCl gradient (400 ml gradient) in buffer A. POMC positive fractions were pooled, dialyzed against 50 mM and then 0.1 mM Tris-HCl, pH 7.0, and concentrated to 0.5 ml by Speed-vac. POMC was then subjected to preparative SDS-PAGE gel electrophoresis in a Biorad model 491 cell, as previously described (11). After lypophilization, purified POMC was resuspended in H_2O for in vitro assays.

Chromaffin granule (CG) aspartic proteinase and POMC. The CG aspartic proteinase (2 µg) and recombinant POMC (4 µg) were incubated in 30 µl containing 0.1 M Na-citrate, pH 5.5, 1 mM DTT, 1 mM EDTA, and 10 mM CHAPS at 37° C for 6 hours. Aliquots (15 µl) of the enzyme reactions were subjected to SDS-PAGE gel electrophoresis, electrophoretically transferred to nitrocellulose membranes, and immunoblotted, as described (1,11), with anti-ß-LPH and anti-N-POMC antibodies (at 1:500 final dilutions).

Results and Discussion

Production of recombinant POMC. Recombinant POMC was successfuly expressed in *E. coli* to generate POMC for in vitro processing studies. POMC expression was induced by 30 to 120 minutes incubation of cells with IPTG, as demonstrated by anti-ß-LPH immunoblots (fig.1). The 35 kDa POMC corresponds to intact POMC. It is apparent, however, that two lower M_r bands appear to represent degraded forms of POMC. For this reason, a purification procedure was developed to purify intact 35 kDa POMC.

POMC was purified by DEAE-Sepharose and preparative SDS-PAGE gel electrophoresis. POMC was eluted from the DEAE-Sepharose column at 150 mM NaCl, with a recovery of approximately 40%. Preparative SDS-PAGE gel electrophoresis resulted in purification of POMC to apparent homogeneity, as demonstrated by a single 35 kDa POMC band on SDS-PAGE (fig. 2). Approximately 150 µg purified POMC was obtained, representing a recovery of 30% and a 250-fold purification.

POMC processing by the chromaffin granule (CG) 70 kDa aspartic proteinase. The 70 kDa CG aspartic proteinase was incubated with purified recombinant POMC (4 µM) for 6 hours at 37° C. Immunoblot analysis (fig. 3) of POMC products was performed with anti-N-terminal monoclonal antibody that recognizes the N-terminal fragment of POMC (17), and with

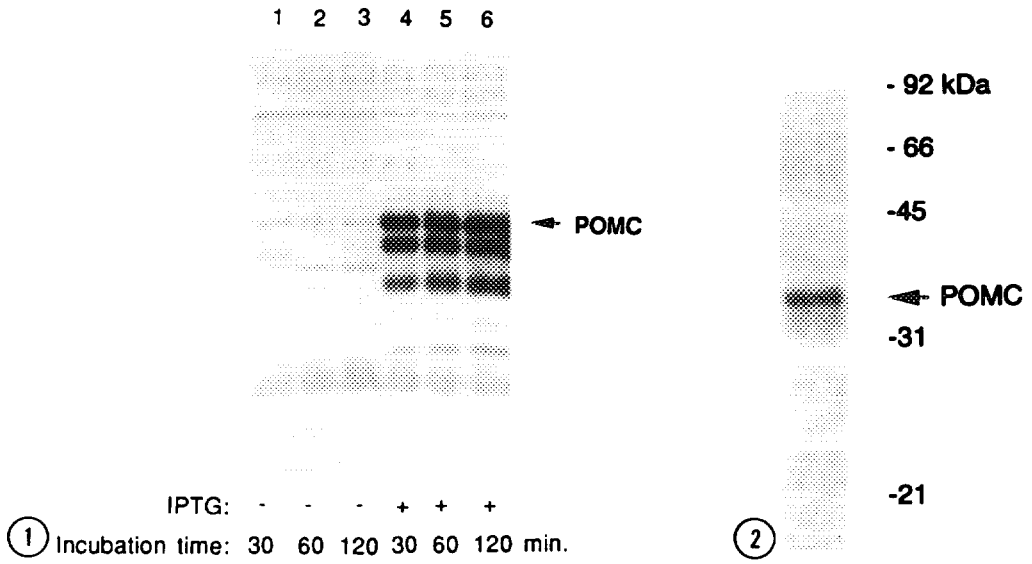

Figure 1. POMC expression in *E. coli.* Induction of POMC expression by IPTG was detected by anti-ß-LPH immunoblots. The arrow indicates expression of intact 35 kDa POMC.

Figure 2. Purified POMC. Recombinant POMC purified by DEAE-Sepharose and preparative gel electrophoresis is illustrated as a single 35 kDa band on SDS-PAGE gel stained with Coomassie Blue. The single band indicates purification of POMC to apparent homogeneity.

anti-ß-LPH serum that recognizes the COOH-terminal segment of POMC (fig.4). POMC was converted to N-terminal POMC positive product bands of 22 and 27 kDa (fig. 3a). Based on the known cleavage specificity of the CG aspartic proteinase for Lys-Arg sites (1), apparent M_r of products, and recognition of products by the anti-N-POMC antibody, the 22 kDa band (fig. 4) may correspond to 23 kDa ACTH that is also generated by PCE (2,3). The 23 kDa ACTH product results from processing at the Lys-Arg between CLIP and γ-LPH. Likewise, the 27 kDa band may represent 23 kDa ACTH + γ-LPH, that would result from cleavage at the Lys-Arg between γ-LPH and ß-endorphin.

Immunoblots with anti-ß-LPH serum indicated conversion of POMC to 14, 22, and 26 kDa bands that contain the ß-LPH region of POMC (fig. 3b). The 14 kDa band is consistent with a fragment containing ß-LPH and 4.5 kDa ACTH (fig. 4), which would be generated by cleavage at the Lys-Arg between the joining peptide (JP) and α-MSH. The 22 kDa band may represent a fragment containing junction peptide + ACTH (1-39) + ß-LPH, which may be generated by cleavage by at the Arg-Arg located within γ-MSH. The 26 kDa product is consistent with POMC lacking the N-terminal

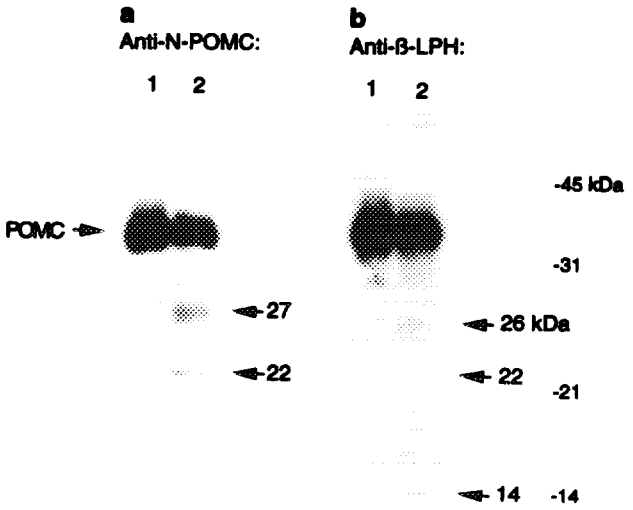

Figure 3. Chromaffin granule aspartic proteinase incubated with recombinant POMC: analysis of products by immunoblots.
(a) Anti-N-terminal POMC immunoblot. The monoclonal antibody recognizing the N-terminal fragment of POMC (fig. 4) (17) was used in immunoblot analyses of POMC products generated by the 70 kDa chromaffin granule aspartic proteinase. The arrows indicate 27 and 22 kDa product bands.
(b) Anti-ß-LPH immunoblot. The anti-ß-LPH serum was used in immunoblot analyses of POMC products generated by the 70 kDa chromaffin granule aspartic proteinase. The arrows indicate 26, 22, and 14 kDa product bands.

(1-49) segment, which would be generated by processing at a Arg-Lys site. Comparison of CG 70 kDa aspartic proteinase and pituitary PCE (POMC converting enzyme) in POMC processing. Based on the POMC products generated by the CG 70 kDa aspartic proteinase, and the known cleavage specificity of this aspartic proteinase for paired basic residues (1), it is predicted that the CG enzyme processed POMC at paired basic residue sites, as illustrated (by arrows) in figure 4. Comparison of POMC processing sites for the CG and pituitary PCE aspartic proteinases suggest that these enzymes process POMC in a similar fashion (fig. 4). Among the five paired basic residue sites predicted to be processed by the CG enzyme, the pituitary PCE cleaves four of these sites (2,3). Thus, the chromaffin granule and pituitary PCE aspartic proteinases process POMC similarly.

The adrenal medulla has been reported to contain several POMC products including N-terminal POMC, ACTH, α-amidated JP related peptides, ß-endorphin, and des-acetyl-α-MSH (18,19). Thus, the CG 70 kDa aspartic proteinase may participate in adrenal medullary POMC processing. Conclusions. Similar POMC processing by the CG and pituitary granule

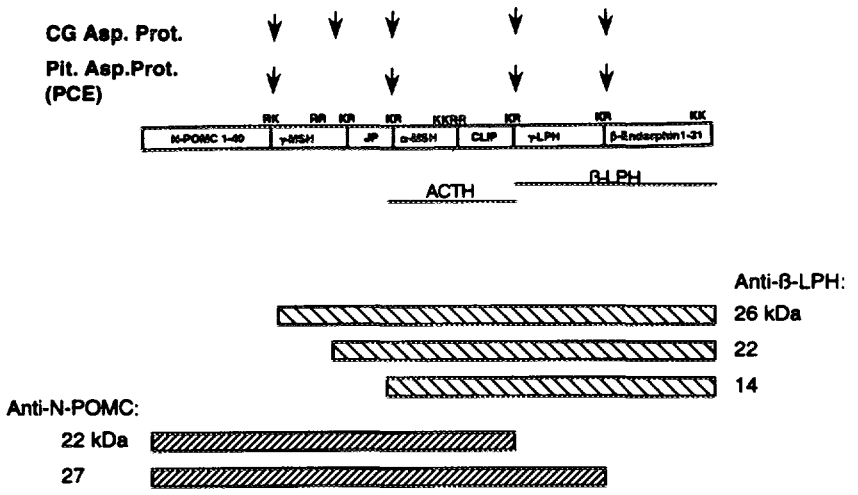

Figure 4. POMC structure and predicted products by chromaffin granule and pituitary PCE aspartic proteinases.

The POMC prohormone is illustrated to possess the following peptide segments: N-terminal POMC (1-49), γ-MSH (melanocyte stimulating hormone), JP (joining peptide), ACTH (adrenocorticotropin hormone), α-MSH, CLIP (corticotropin-like intermediate lobe peptide), ß-LPH (lipotropin hormone, γ-LPH, and ß-endorphin.

The predicted POMC products generated by the chromaffin granule 70 kDa aspartic proteinase detected by anti-ß-LPH immunoblots are illustrated as 26, 22, and 14 kDa products (▨). Predicted POMC products detected in anti-N-POMC immunoblots are indicated as 22, and 27 kDa products (▨).

Deduced cleavage sites for the chromaffin granule 70 kDa aspartic proteinase and pituitary PCE are illustrated by arrows above POMC.

aspartic proteinases is consistent with their parallel biochemical properties (Table I) with respect to M_r of 70,000, acidic pH optimum, inhibition by pepstatin A, cleavage at paired basic residues, and poor cleavage of peptide-MCA substrates (1-3). This study also indicates that both aspartic proteinases show no cleavage of the -KKRR- basic site within ACTH (2,3). The close resemblance in biochemical properties of the CG 70 kDa aspartic proteinase and pituitary PCE suggests a similar, if not identical, aspartic proteinase in adrenal medulla and pituitary for prohormone processing.

The CG and pituitary 70 kDa aspartic proteinases may represent members of an emerging group of aspartic prohormone processing proteases that also includes the yeast aspartic proteinase 3 (YAP3) (20,21) and a somatostatin-28 generating aspartic proteinase from anglerfish islets (22). It will be important in future studies to compare the relative roles of proteases of different mechanistic classes -- i.e., aspartic proteinases,

Table I. Similar Properties of Aspartic Proteinases from Chromaffin and Pituitary Granules

- 70 kDa single-chain glycoprotein
- Acidic pH optimum
- Cleaves paired basic residues within prohormones, but not in peptide-MCA substrates.
- Localized to secretory granules
- Inhibited by pepstatin A, an inhibitor of aspartic proteinases.

From references 1-3.

subtilisin-like PC1/3 and PC2 proteases, and the cysteine protease 'prohormone thiol protease' (PTP) -- in prohormone processing.

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References

1. Azaryan, A.V., Schiller, M., Mende-Mueller, L., and Hook, V.Y.H. (1995) J. Neurochem., in press.
2. Loh, Y.P., Parish, D.C., and Tuteja, R. (1985) J. Biol. Chem. 260, 7194-7205.
3. Loh, Y.P., Beinfeld, M.C., and Birch, N.P. (1993) In Mechanisms of Intracellular Trafficking and Processing of Proproteins (Y.P. Loh, Ed.), pp. 179-223, CRC Press, Boca Raton, FL.
4. Hook, V.Y.H., Azaryan, A.V., Hwang, S.R., and Tezapsidis, T. (1994) FASEB J. 8, 1269-1279.
5. Docherty, K., and Steiner, D.F. (1982) Annu. Rev. Physiol. 44, 625-638.
6. Steiner, D.F., Smeekens, S.P. Ohagi, S., and Chan, S.F. (1992) J. Biol. Chem. 267, 23435-23438.
7. Seidah, N.G., Day, R., Marcinkiewicz, Benjannet, S., Chretien, M. (1991) Enzyme 45, 271-284.
8. Pollard, H.B., Shindo, H., Creutz, C.E., Pazoles, C.J., and Cohen, J.S. (1978) J. Biol. Chem. 254, 1170-1177.
9. Krieger, T.K., and Hook, V.Y.H. (1991) J. Biol. Chem. 266, 8376-8383.
10. Azaryan, A.V., and Hook, V.Y.H. (1994) FEBS Lett. 341, 197-202.
11. Schiller, M.R., Mende-Mueller, L., Moran, K., Meng, M., Miller, K.W., and Hook, V.Y.H. (1995) Biochemistry 34, 7988-7995.
12. Tezapsidis, N., Noctor, S., Kannan, R., Krieger, T.J., Mende-Mueller, L., and Hook, V.Y.H. (1995) J. Biol. Chem. 270, 13285-13290.

13. Azaryan, A.V., Krieger, T.J., and Hook, V.Y.H. (1995) J. Biol. Chem. 270, 8201-8208.
14. Boileau, G., Barbeau, C., Jeannotte, L., Chretien, M., and Drouin, J. (1983) Nucl. Acids Res. 11, 8063-8071.
15. Studier, F.W., Rosenberg, A.H., Dunn, J.J., Budendorff, J.W. (1990) Meth. in Enzymol. 185, 60-89.
16. Hwang, S.R., Kohn, A.B., and Hook, V.Y.H. (1994) Proc. Natl. Acad. Sci. USA 91, 9570-9583.
17. Chevrier, D., Lemieux, E., Fyfe, M., Labont, N., Zollinger, M., Bennet, H.P.J., and Crine, P. (1991) Biochem. Cell Biol. 69, 58-65.
18. Gaspar, L., Chan, J.S., Seidah, N.G., and Chretien, M. (1989) Clin. Invest. Med. 12(2), 90-98.
19. Bjartell, A., Fenger, M., Ekman, R., Sundler, F. (1990) Peptides 11(1), 149-61.
20. Azaryan, A.V., Wong, M., Friedman, T.C., Cawley, N.X., Estivariz, F.E., Chen, H.C., and Loh, Y.P. (1993) J. Biol. Chem. 268, 11968-11975.
21. Egel-Mittani, M., Flygenring, H.P., and Hansen, M.T. (1990) Yeast 6, 1 27-137.
22. Mackin, R.B., Noe, D.B., and Spiess, J. (1991) Endocrinol. 129, 1951.