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Kex2-LIKE PROTEOLYTIC ACTIVITY IN ADRENAL MEDULLARY CHROMAFFIN GRANULES

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SUMMARY: This study demonstrates the presence of boc-Gln-Arg-Arg-MCA cleaving activity in bovine chromaffin granule membranes that resembles yeast Kex2 proteolytic activity. The chromaffin granule boc-Gln-Arg-Arg-MCA cleaving activity, like Kex2 proteolytic activity, shows calcium dependence, optimum activity at pH 7.5-8.2, inhibition by serine protease inhibitors, and preference for cleavage at the COOH-terminal side of Arg-Arg and Lys-Arg, over Lys-Lys, paired basic residues. Potent inhibition by the active-site directed inhibitor [D-Tyr]-Glu-Phe-Lys-Arg-CK (20 μM) provided further evidence for dibasic residue cleavage site specificity. These results are the first report of endogenous mammalian Kex2-like proteolytic activity that may be related to PC1/PC3 and PC2 enzymes, the newly discovered mammalian homologues of Kex2 protease. It will be important to determine the role of this Kex2-like proteolytic activity in processing the precursors of adrenal medullary neuropeptides.

The posttranslational processing of prohormone precursors to biologically active peptides requires proteolytic cleavage of the prohormone at paired basic residues flanking the active peptide sequence

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Abbreviations: PC, prohormone convertase; MCA, methylcoumarin amide; AMC, 7-amino-4-methylcoumarin; boc, t-butoxycarbonyl; CK, chloromethyl ketone; -CHN_2, diazomethane; DFP, diisopropyl fluorophosphate; DTT, dithiothreitol; pCMB, p-chloromercuribenzoate; PMSF, phenylmethylsulfonyl fluoride; TLCK, tosyl-lysine chloromethyl ketone; TPCK, tosyl-phenylalanine chloromethyl ketone.

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(1, 2). Among several candidate endoproteolytic processing enzymes, only the yeast KEX2 gene product has been characterized at both the molecular and enzymatic levels. The Kex2 enzyme is a Ca^{2+}-dependent subtilisin-like serine protease that is required for in vivo processing of pro-α-mating factor (3-5) and pro-killer toxin (6) at the COOH-terminal side of Lys-Arg and Arg-Arg sites. Recently, three mammalian Kex2 homologues have been cloned and proposed as prohormone processing proteases; these are furin (7, 8), PC2 (9-11), and PC3, also known as PC1 (12,13). The expression of PC1/PC3 and PC2 appears to be restricted to neural and endocrine cells (10, 12, 13). However, endogenous Kex2-like enzymatic activity has not been demonstrated in mammalian tissue.

In this study, we find that adrenal medullary chromaffin granules (secretory vesicles) possess boc-Gln-Arg-Arg-MCA cleaving activity that resembles yeast Kex2 activity (4, 5). This is consistent with the presence of PC1/PC3 and PC2 mRNAs in bovine adrenal medulla (10, 12, 14), and provides support for the hypothesis that a Kex2-like protease(s) may be involved in the processing of adrenal medullary peptide precursors.

MATERIALS AND METHODS

Isolation of bovine chromaffin granule membrane and soluble fractions. Chromaffin granules were purified from fresh bovine adrenal medulla by discontinuous sucrose density gradient centrifugation as described previously (15). This procedure yields granules that are free from lysosomal contamination. After lysis of granules in 15 mM KCl by freeze-thawing, lysed granules in buffer A (50 mM Na-acetate, pH 6.0 and 100 mM NaCl) were centrifuged for 30 min at 100,000 x g. The supernatant was taken as the soluble fraction and the pellet (resuspended in buffer A) represented the membrane fraction.

Proteolytic enzyme assay. Enzymatic hydrolysis of peptide-MCA substrates was carried out as previously described (16). Boc-Gln-Arg-Arg-MCA and related peptides (100 μM final concentration) were each incubated with 50 μl chromaffin granule fraction in 0.1 mM Tris-HCl pH 8.0 at 37° C for 3 hours in a total volume of 160 μl, and the reaction was stopped by addition of 10 μl 1% trifluoroacetic acid. Two ml water was added to the supernatant and released AMC was measured at 465 nm (excitation 385 nm) with a Perkin-Elmer 650-40 fluorimeter. To examine the effect of protease inhibitors, the membrane fraction was preincubated with inhibitors at pH 8.0 for 30 min at 37° C before addition of substrate boc-Gln-Arg-Arg-MCA (100 μM), with further incubation for 3 hours at 37° C.

RESULTS AND DISCUSSION

The substrate boc-Gln-Arg-Arg-MCA was chosen to detect Kex2-like proteolytic activity in bovine chromaffin granules since it has been used

for studies of the yeast Kex2 protease (4, 5). The boc-Gln-Arg-Arg-MCA cleaving activity in membrane and soluble fractions of chromaffin granules showed a pH optimum of 7.5-8.2, and ~25% of maximal activity was detected at the intragranular pH of 5.5 (data not shown). Further characterization utilized pH 8.0.

Seventy percent of total chromaffin granule boc-Gln-Arg-Arg-MCA cleaving activity was present in the membrane fraction, and the soluble fraction possessed 30% of the granule activity (data not shown). Activity in the membrane fraction, and not in the soluble fraction, was stimulated by Ca^{2+} (17% above control, Table 1). The membrane localization and sensitivity to calcium resembles yeast Kex2 activity (4). These results are also consistent with the predominant localization of PC2 and PC3 protein to chromaffin granule membranes (17, 18).

TABLE 1. Effect of Protease Inhibitors and Modulators on Boc-Gln-Arg-Arg-MCA Cleaving Activity in Chromaffin Granule Membrane Fraction

Inhibitor	Concentration	Inhibition, %
Calcium	10 mM	-17
EGTA	2.5 mM	20
DFP	1.5 mM	70
α_1-antitrypsin	0.1 mg/ml	59
Soybean trypsin inhibitor	0.1 mg/ml	65
Benzamidine	1.0 mM	44
PMSF	0.1 mM	3
TPCK	0.1 mM	0
Chymostatin	0.5 mM	5
Leupeptin	2 mM	96
Antipain	1 mM	91
TLCK	0.1 mM	73
[D-Tyr]-Glu-Phe-Lys-Arg-CK	20 μM	90
Cystatin C	1 mM	0
Z-Arg-Leu-Val-Gly-CHN_2	1 mM	0
Z-Leu-Val-Gly-CHN_2	1 mM	0
DTT	1 mM	-37
Iodoacetamide	5 mM	35
$HgCl_2$	5 mM	30
Pepstatin A	0.1 mM	0
0-Phenanthroline	0.1 mM	10

Activity in chromaffin granule membrane fraction (150 μg protein) was assayed with boc-Gln-Arg-Arg-MCA (100 μM) at pH 8.0.

The effect of protease inhibitors and modulators (Table 1) showed that the serine protease inhibitors (at millimolar concentrations) DFP, α_1-antitrypsin, soybean trypsin inhibitor, and benzamidine were inhibitory; but PMSF, TPCK and chymostatin had no effect. These data indicate that the boc-Gln-Arg-Arg-MCA cleaving activity in the membrane fraction represents a serine protease, but not a chymotrypsin type protease. This activity was inhibited by leupeptin, antipain, and TLCK that typically inhibit proteases cleaving at Arg or Lys residues. Selectivity for paired basic residues was also demonstrated by potent inhibition of boc-Gln-Arg-Arg-MCA (as well as boc-Gly-Lys-Arg-MCA) cleaving activity by the active site-directed inhibitor [D-Tyr]-Glu-Phe-Lys-Arg-CK (20 μM). This inhibitor corresponds to the Glu-Phe-Lys-Arg sequence at the junction of ACTH and ß-lipotropin peptides within proopiomelanocortin that is cleaved by PC2, PC1/PC3, and Kex2 (11, 14, 19) in DNA cotransfection experiments. The boc-Gln-Arg-Arg-MCA cleaving activity is not a cysteine protease since cystatin C was not inhibitory; also, two peptidyl-diazomethanes Z-Arg-Leu-Val-Gly-CHN_2 and Z-Leu-Val-Gly-CHN_2 that mimick the inhibitory center of cystatin C (20) had no effect. Activation by 1 mM DTT (37% stimulation) and inhibition by iodoacetamide and $HgCl_2$ indicate the presence of exposed cysteine residue(s) near the enzyme active site. Pepstatin A, an aspartyl protease inhibitor, had no effect and 0-phenanthroline, an inhibitor of metalloproteases, had a minimal effect. Overall, the protease inhibitor study indicates that the chromaffin granule membrane boc-Gln-Arg-Arg-MCA cleaving activity represents a serine protease with specificity for paired basic residues.

Cleavage site preference studies (Table 2) showed that the peptides boc-Gln-Arg-Arg-MCA, boc-Arg-Val-Arg-Arg-MCA, boc-Gly-Lys-Arg-MCA, and boc-Gly-Arg-Arg-MCA were effectively cleaved, with boc-Gln-Arg-Arg-MCA as the best substrate. These results indicate cleavage on the carboxyl side of Arg-Arg and Lys-Arg pairs. Like the yeast Kex2 protease (4, 5), low activity against boc-Glu-Lys-Lys-MCA (Table 2) suggests a requirement for arginine in the P1 position.

Our demonstration of Kex2-like proteolytic activity in bovine chromaffin granule membranes is consistent with previous studies showing localization of PC1/PC3 and PC2 enzyme proteins in these membranes (17, 18). The chromaffin granule boc-Gln-Arg-Arg-MCA cleaving activity also resembles the yeast Kex2-like proteolytic activity with respect to calcium dependence, pH optimum, inhibitor profile, and preference for Arg-Arg and Lys-Arg pairs. These properties suggest that the chromaffin granule boc-Gln-Arg-Arg-MCA cleaving activity is related to the Kex2/furin/PC subtilisin-like superfamily of endoproteases.

TABLE 2. Hydrolysis of Peptide-MCA Substrates by Membrane Fraction of Chromaffin Granules

Substrate	Proteolytic activity (pmol AMC/hour/mg protein)
Boc-Gln-Arg-Arg-MCA	1326
Boc-Arg-Val-Arg-Arg-MCA	1031
Boc-Gly-Lys-Arg-MCA	844
Boc-Gly-Arg-Arg-MCA	562
Boc-Glu-Lys-Lys-MCA	375

Assays were performed in 0.1 mM Tris-HCl, pH 8.0 with substrates at a final concentration of 100 μM. Protein content of the membrane fraction was measured by the method of Lowry (26).

The presence of Kex2-like activity in chromaffin granules implicates a role for a subtilisin-like protease(s) in processing proenkephalin and other neuropeptide precursors in adrenal medulla. It will be important to purify the chromaffin granule Kex2-like protease(s) and define its relative role with the 'prohormone thiol protease' (21, 22), and other previously identified chromaffin granule serine proteases (23-25), in processing precursors of adrenal medullary neuropeptides.

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REFERENCES

1. Docherty, K. and Steiner, D.F. (1982) Ann. Rev. Physiol. 44 , 625-638.
2. Loh, Y.P., Brownstein, M., and Gainer, H. (1984) Ann. Rev. Neurosci. 7, 189-222.
3. Julius, D., Brake, A., Blair, L., Kunisawa, R., and Thorner, J. (1984) Cell 37, 1075-1098.
4. Fuller, R.S., Brake, A., and Thorner, J. (1989) Proc. Natl. Acad. Sci. USA 86, 1434-1438.
5. Brenner, C. and Fuller, R.S. (1992) Proc. Natl. Acad. Sci. USA 89, 922-926.
6. Bussey, H. (1988) Yeast 4, 17-26.
7. Bresnahan, P.A., Leduc, R., Thomas, L., Gibson, H.L., Brake, A.J., Barr, P.J., and Thomas, G. (1990) J. Cell Biol. 111, 2851-2859.

8. Hatsuzawa, K., Hosaka, M., Nakagawa, T., Nagase, M., Shoda, A., Murakami, K., and Nakayama, K. (1990) J. Biol. Chem. 265, 22075-22078.
9. Smeekens S.P. and Steiner D.F. (1990) J. Biol. Chem. 265, 2997-3000.
10. Seidah, N.G., Gaspar, L., Mien, P., Marcinkiewicz, M., Mbikay, M., and Chretien, M. (1990) DNA and Cell Biol. 9, 415-424.
11. Benjannet, S., Rondeau, N., Day, R., Chretien, M., and Seidah, N.G. (1991) Proc. Natl. Acad. Sci. USA 88, 3564-3568.
12. Seidah, N. G., Marcinkiewicz, M., Benjannet, S., Gaspar, L., Beaubien, G., Mattei, M.G., Lazure, C., Mbikay, M., and Chretien, M. (1991) Mol. Endocrin. 5, 111-122.
13. Smeekens, S.P., Avruch, A.S., LaMendola, J., Chan, S.J., and Steiner, D.F. (1991) Proc. Natl. Acad. Sci. USA 88, 340-344.
14. Thomas, L., Leduc, R., Thorne, B., Smeekens, S.P., Steiner, D.F., and Thomas, H. (1991) Proc. Natl. Acad. Sci. USA 88, 5297-5301.
15. Hook, V.Y.H. and Eiden, L.E. (1984) FEBS Lett. 172, 212-218.
16. Barrett, A. and Kirschke, H. (1981) Methods Enzymol. 80, 535-561.
17. Kirchmair, R., Gee, P., Hogue-Angeletti, R., Laslop, A., Fischer-Colbrie, R., and Winkler, H. (1992) FEBS Lett., in press.
18. Christie, D.L., Batchelor, D.C., and Palmer, D.J. (1991) J. Biol. Chem. 266, 15679-15683.
19. Thomas, G., Thorne, B.A., Thomas, L., Allen, R.G., Hruby, D.E., Fuller, R.S., and Thorner, J. (1988) Science 241, 226-230.
20. Grubb, A., Abrahamson, M., Olafsson, I., Trojnar, Y., Kasprzykowska, R., Kasprzykowski, F., and Grzonka, Z. (1990) Biol. Chem. Hoppe-Seyler 371, 137-144.
21. Krieger, T.J. and Hook, V.Y.H. (1991) J. Biol. Chem. 266, 8376-8383.
22. Krieger, T.J. and Hook, V.Y.H. (1992) J. Neurochem., in press.
23. Mizuno, K., Kojima, M., and Matsuo, H. (1985). Biochem. Biophys. Res. Commun. 128, 884-891.
24. Shen, F.S., Roberts, S.F. and Lindberg, I. (1989) J. Biol. Chem. 264, 15600-15605.
25. Laslop, A., Fischer-Colbrie, R., Kirschke, H., Hogue-Angeletti, R., and Winkler, H. (1990) Biochim. et Biophys. Acta 1033, 65-72.
26. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.