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Characterization of PC2, a mammalian Kex2 homologue, following expression of the cDNA in microinjected *Xenopus* oocytes

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A human insulinoma cDNA (PC2) that encodes a protein homologous to the Kex2/subtilisin-like proteinases has recently been described [1990, J. Biol. Chem. 265, 2997–3000]. In order to characterise the associated proteinase activity, mRNA encoding PC2 was synthesised in vitro and microinjected into *Xenopus* oocytes. The proteinase activity released into the media from oocytes microinjected with PC2 mRNA was assayed using small peptide fluorogenic substrates. Boc.Gln.Arg.Arg aminomethyl coumarin was hydrolysed in a Ca^{2+}-dependent manner, but substrate analogues bearing a single basic aminoacid were not. The substrate specificity, inhibitor profile, and pH optimum of 5.5 were compatible with an involvement of PC2 in prohormone processing in mammalian cells.

PC2; Prohormone processing; Subtilisin; Kex2

1. INTRODUCTION

Most polypeptide hormones are synthesised as larger precursors, from which the bioactive peptides are generated by limited proteolysis [1]. Cleavage usually occurs at sites marked by pairs of basic amino acids, although peptides can also be generated by cleavage at single basic amino acids [2]. An endopeptidase is thought to cleave on the COOH-terminal side of the pairs of basic amino acids and a carboxypeptidase then removes the COOH-terminal basic residues [3].

Some progress has been made in identifying the enzymes responsible for the intracellular proteolytic processing of prohormones. The prohormone processing carboxypeptidase (carboxypeptidase H) has been cloned and sequenced [4], and endopeptidase involved in the processing of proinsulin [5], proopiomelanocortin [6], prosomatostatin [7,8], proenkephalin [9] and prooxytocin/neurophysin [10] have been described and at least partially characterised. However, the only propolypeptide processing endopeptidase definitively identified and characterised at the protein and gene level is the *KEX2* gene product of *Saccharomyces cerevisiae* [11]. The Kex2 enzyme is a Ca^{2+}-dependent protease with a neutral pH optimum, which cleaves pro-α factor and pro-killer toxin at Lys-Arg and Arg-Arg sites during the transport of these precursors through the cellular secretory pathway [12].

Recently, a cDNA (PC2) encoding a mammalian homologue of Kex2 was cloned from a human insulinoma cDNA library [13]. PC2 encodes a 638 amino acid protein which contains an NH_2-terminal signal peptide and a 282 residue domain that is homologous to both Kex2 and related bacterial subtilisins (see [14] for review). PC2 and Kex2 [15] also showed similarity to the furin gene product, a putative membrane-associated protein [16]. This similarity suggests that furin might also be a candidate propolypeptide processing protease.

Here we describe the biosynthesis of PC2 in *Xenopus* oocytes microinjected with mRNA encoding PC2, and the characterisation of the enzymatic activity associated with the expressed protein.

Abbreviations: PMSF, phenylmethylsulphonylfluoride; SDS, sodium dodecyl sulphate; ALACK, Ala-Lys-Arg-chloromethylketone; TLCK, tosyl-L-Lysinechloromethylketone; TPCK, tosylphenylalaninechloromethylketone; MCA, aminomethyl coumarin

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2. EXPERIMENTAL

2.1. *Animals*

Xenopus laevis were purchased from Xenopus Ltd., Redhill, Surrey, UK.

2.2. *Chemicals and reagents*

Boc.Gln.Arg.Arg-MCA, Boc.Gln.Gly.Arg-MCA and Boc.Gly.Lys.Arg-MCA were from Peninsula Laboratories, St Helens, Merseyside, UK; 7-amino-4-methyl coumarin and protease inhibitors were from Sigma, Poole, Dorset, UK and Ep 475 was a gift from Dr K. Hanada, Taisho Pharmaceutical Co. Ltd., Saitama, Japan.

2.3. *General procedures*

In vitro transcription, in vitro translation, *Xenopus* oocyte microinjections, and SDS polyacrylamide gel electrophoresis (SDS PAGE) were performed as previously described [17].

2.4. *Assay for Boc.Gln.Arg.Arg-MCA hydrolysing activity*

Oocyte medium (20 μl) was incubated at 37°C for 18 h in a reaction (final volume 50 μl) containing 200 mM MES (pH 5.5), 1 mM $CaCl_2$, 1% (v/v) Triton X-100, and 100 μM Boc.Gln.Arg.Arg-MCA. The reaction was terminated by addition of 0.9 ml 0.125 M $ZnSO_4$ and the fluorescence of the samples measured at excitation 385 nm and emission 465 nm. A standard curve was prepared using 7-amino-4-methylcoumarin. In order to account for effects of additions and pH on the low level of fluorescence measured, results were expressed relative to the fluorescence measured in media samples from oocytes microinjected with H_2O.

3. RESULTS

In order to characterise the protein and enzymatic activity encoded by PC2, capped mRNA was synthesised in an in vitro transcription reaction using SP6 RNA polymerase. A comparison of the translation products in *Xenopus* oocytes and in a wheat germ cell-free translation system is shown in Fig. 1. In the wheat germ system a single radiolabelled protein of M_r 69 000 corresponding to pre(pro)PC2 was observed. In the media from *Xenopus* oocytes microinjected with PC2 mRNA, 2 proteins of M_r 71 000 and 68 000 were observed. These proteins were not present in media from oocytes microinjected with H_2O suggesting that they were encoded by the PC2 mRNA. Further studies have shown that PC2 is synthesised in the oocytes as a 75 kDa membrane-associated protein which undergoes proteolysis to generate the 71 kDa glycosylated protein. This protein is released into the media, where it in turn undergoes cleavage to generate the 68 kDa mature protein (Shennan, Seal, Smeekens, Steiner and Docherty, unpublished results).

The proteinase activity present in the media at different time points after microinjection of oocytes with PC2 mRNA was assayed using a small fluorogenic peptide substrate, Boc.Gln.Arg.Arg-MCA. Proteinase activity was detected in the media after 4 h, and this activity increased during a subsequent 20 h incubation of the oocytes (Fig. 2). The appearance of proteolytic activity in the media paralleled the release of the radiolabelled PC2-encoded peptides (data not shown). No proteolytic activity could be detected in media from oocytes microinjected with H_2O.

The proteinase activity released into the media from oocytes microinjected with PC2 mRNA was inhibited by EDTA and EGTA, by leupeptin, antipain and *p*-hydroxymercuribenzoate (Table I). The active-site-directed inhibitor Ala-Lys-Arg-chloromethylketone (ALACK) was a potent inhibitor, whereas tosyl-L-Lysinechloromethylketone (TLCK) and tosylphenylalaninechloromethylketone (TPCK) did not inhibit substantially. The secreted proteinase activity was not inhibited by 1,10-phenanthroline, pepstatin A (an aspartate protease inhibitor), PMSF (a serine protease inhibitor) nor by iodoacetic acid, iodoacetamide, E-64 or Ep475 (cysteine proteinase inhibitors). The slight activation by dithiothreitol and inhibition by *p*-hydroxy-

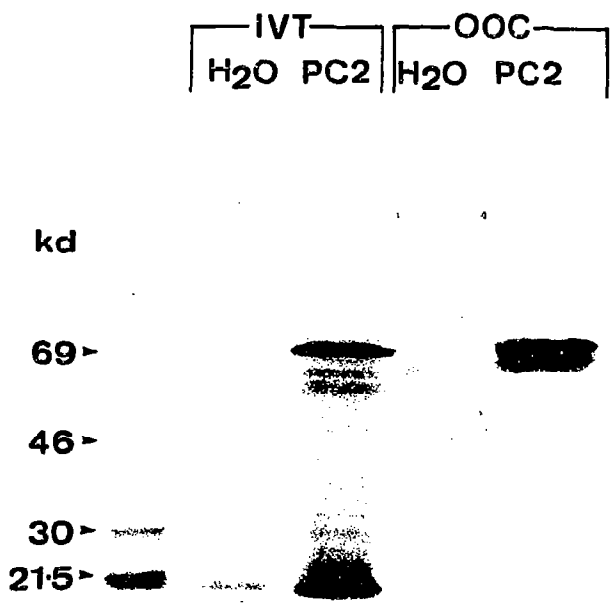

Fig. 1. Translation of PC2 mRNA. Translation was performed in a wheat germ cell-free system (IVT) and in microinjected *Xenopus* oocytes (OOC). For the wheat germ system, H_2O (track 1) or PC2 mRNA (track 2) was added to a reaction mixture containing [^{35}S]methionine (0.5 mCi/ml) in a final volume of 30 μl. 20 μl aliquots were analysed by SDS-PAGE and fluorography. *Xenopus* oocytes (10) were microinjected with 50 nl of H_2O (track 3) or PC2 mRNA (track 4) and incubated for 6 h in modified Barth's saline. The oocytes were then incubated in 30 μl of modified Barth's saline containing 1 mCi/ml [^{35}S]methionine for 18 h at 20°C. The media were removed and the volume adjusted to 100 μl with modified Barth's saline, and a 5 μl aliquot analysed by SDS-PAGE. ^{14}C-labelled molecular weight markers are shown on the left.

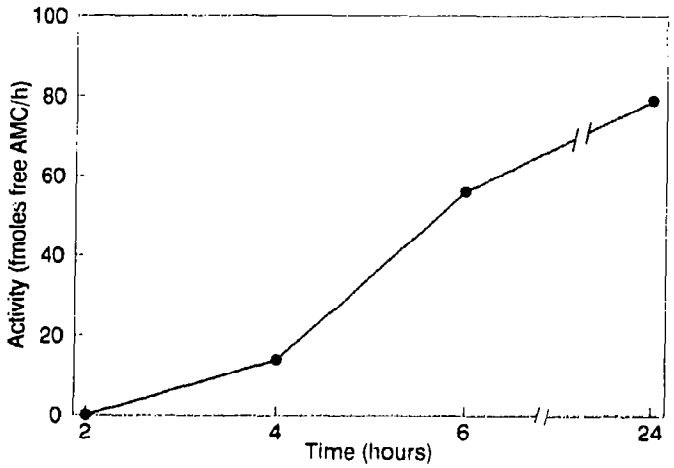

Fig. 2. Time course of secretion. *Xenopus* oocytes were microinjected with H_2O or PC2 mRNA, and incubated in modified Barth's saline for 18 h at 20°C. Groups of 10 oocytes were then incubated in 30 μl modified Barth's saline, and, at the indicated times, the media were removed and the volume adjusted to 100 μl with modified Barth's saline. 20 μl aliquots of media were then incubated in the standard proteinase assay with 100 μM Boc.Gln.Arg.Arg-MCA.

Table I

Effect of proteinase inhibitors

Inhibitor	Concentration (mM)	Inhibition (%)
EDTA	6	100
EGTA	6	95
1,10-phenanthroline	1	0
Iodoacetic acid	1	13
Iodoacetamide	1	0
2,2'-Dipyridyl disulphide	1	8
Dithiothreitol	1	−42
p-Hydroxymercuribenzoate	1	84
Leupeptin	1	91
Antipain	1	100
Ala-Lys-Arg-Chloromethylketone	0.1	81
Ala-Lys-Arg-Chloromethylketone	1	95
Tosyl-L-Lysine Chloromethylketone	0.1	29
Tosyl-L-Phenylalanine Chloromethylketone	0.1	7
Phenylmethylsulphonyl fluoride	0.1	0
Pepstatin A	0.1	0
E-64	0.1	0
EP-475	0.1	6

Oocytes were microinjected with PC2 mRNA, and 24 h later, 20 μl aliquots of media were assayed under standard reaction conditions (100 μM Boc.Gln.Arg.Arg-MCA) with the indicated final concentration of inhibitor. Inhibition was then calculated as a percentage of PC2-associated activity in the absence of any addition. The results represent the means of triplicate determinations performed on at least 3 separate occasions.

mercuribenzoate may be explained by the presence of a cysteine residue (Cys^{212}) close to the active site histidine (His^{208} [13].

The proteinase was activated by Ca^{2+} in the millimolar range ($K_{0.5}$ = 1 mM) (Fig. 3), while the pH optimum for the activity was 5.5 (Fig. 4). An extended pH profile demonstrated that there was no activity below pH 4.0 nor above pH 7.0 (data not shown).

The substrate specificity of the proteinase activity associated with PC2 was investigated using Boc.Gln.Gly.Arg-MCA which contained a single basic amino acid and Boc.Gly.Lys.Arg-MCA and Boc.Gln.Arg.

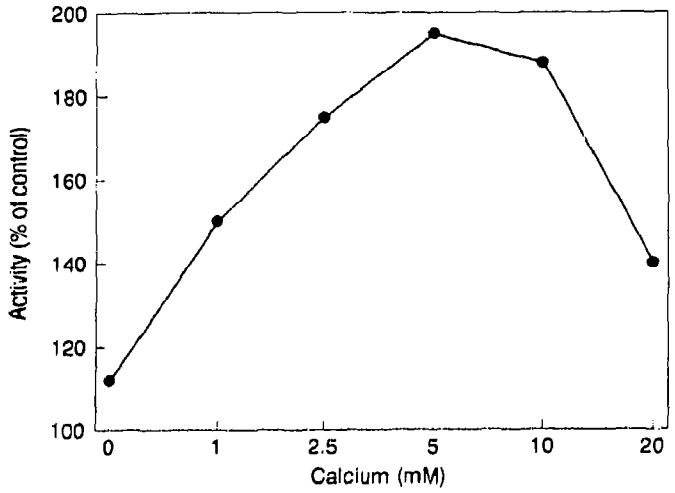

Fig. 3. Ca^{2+} dependence. *Xenopus* oocytes were microinjected with H_2O or PC2 mRNA and incubated in modified Barth's saline for 18 h. 20 μl aliquots of media were then assayed for proteinase activity under standard conditions with the indicated concentration of Ca^{2+}. Results are expressed as fluorescence obtained with media from oocytes microinjected with PC2 mRNA as a percentage of that obtained with media from oocytes microinjected in parallel with H_2O.

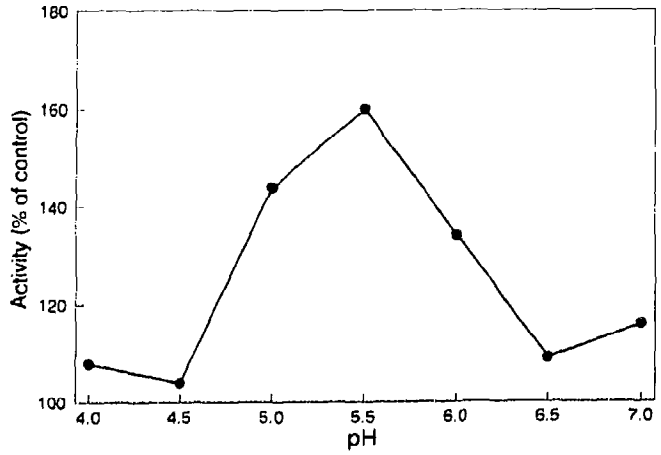

Fig. 4. pH optimum. *Xenopus* oocytes were microinjected with H_2O or PC2 mRNA and incubated for 18 h in modified Barth's saline at 20°C. 20 μl aliquots of media were then assayed for proteinase activity in a reaction mixture containing 1 mM $CaCl_2$, 1% (v/v) Triton X-100 and either 200 mM sodium acetate, 200 mM MES, or 200 mM BES adjusted to pH 4.0–7.0 with NaOH or HCl. Results are expressed as fluorescence obtained with media from oocytes microinjected with PC2 mRNA as a percentage of that obtained with media from oocytes microinjected with H_2O, and represent the mean of triplicate determinations performed on at least 3 separate occasions.

Table II

Substrate specificity

Substrate (0.1 mM)	Acticity (fmol free MCA/h)
Boc.Gln.Gly.Arg-MCA	0
Boc.Gln.Arg.Arg-MCA	48
Boc.Gly.Lys.Arg-MCA	150

Oocyte were microinjected with PC2 mRNA and 24 h later, 20 μl aliquots of media were assayed under standard conditions with either 100 μM Boc.Gln.Gly.Arg-MCA, 100 μM Boc.Gln.Arg.Arg-MCA or 100 μM Boc.Gly.Lys.Arg-MCA.

Arg-MCA which contained paired basic amino acids at the cleavage site. No activity could be detected with Boc.Gln.Gly.Arg-MCA while the activity against Boc.Gly.Lys.Arg-MCA was 3 times that detected with Boc.Gln.Arg.Arg-MCA (Table II).

4. DISCUSSION

We report here data on the enzymatic properties of PC2. Using small fluorogenic peptide substrates PC2 was shown to encode a protease with similar substrate specificity, inhibitor sensitivity, and Ca^{2+}-dependence to the yeast Kex2 enzyme, with the exception that PC2 exhibited a pH optimum of 5.5 while Kex2 was optimally active at neutral pH [12].

The inhibitor profile and pH optimum of PC2 were similar to those of a proinsulin converting activity present in a crude islet granule fraction [18] and purified secretory granules from rat insulinoma [19]. These results strongly support the hypothesis that PC2 is involved in prohormone processing. The difference in pH optima between PC2 and Kex2 is in keeping with the acidic pH of the secretory compartments within which prohormone processing is thought to occur in mammalian cells [20], and the neutral pH of the late Golgi body of yeast within which *KEX2* is thought to be active [11].

The proteolytic activity involved in the processing of proinsulin in β cells has been further characterised by Davidson et al. [5]. Two endoproteases have been described: one enzyme (type I) cleaves exclusively on the COOH-side of the amino acids Arg^{31}-Arg^{32} (B chain/C-peptide junction); the other enzyme (type II) cleaves preferentially on the COOH-side of Lys^{64}-Arg^{65} (C-peptide/A chain junction), while exhibiting some activity against the Arg-Arg site. In common with PC2, both the type I and type II enzymes have a pH optimum of 5.5–6, and exhibit the same sensitivity to inhibitors as PC2 [21]. The Ca^{2+}-dependence of the type I enzyme ($K_{0.5}$ = 1–1.5 mM) is more similar to that of PC2 than is the Ca^{2+} dependence of the type II enzyme ($K_{0.5}$ = 0.1 mM). On the other hand the substrate specificity of PC2 (i.e. a preference for Lys-Arg-containing substrates, while still exhibiting activity against Arg-Arg-containing substrates) is more similar to that of the type II enzyme than the type I enzyme. Establishment of the relationship between PC2 and the type I and type II enzymes must therefore await definitive characterisation of the PC2 enzymatic activity using proinsulin as substrate. In the present study we were unable to obtain such data because of the low level of PC2 activity expressed by the oocytes.

In summary, we have presented data on the expression of PC2 in *Xenopus* oocytes which strongly suggest that PC2 is a mammalian prohormone processing peptidase. Further high efficiency expression studies are required to characterise the cleavage specificity against various prohormone substrates.

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