Cleavage of arginyl-arginine and lysyl-arginine from the C-terminus of pro-hormone peptides by human germinal angiotensin I-converting enzyme (ACE) and the C-domain of human somatic ACE

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Mammalian germinal angiotensin I-converting enzyme (gACE) is a single-domain dipeptidyl carboxypeptidase found exclusively in male germ cells, which has almost identical sequence and enzymic properties with the C-domain of the two-domain somatic ACE. Mutant mice that do not express gACE are infertile, suggesting a role for the enzyme in the processing of undefined peptides involved in fertilization. A number of spermatid peptides [e.g. cholecystokinin (CCK) and gastrin] are processed from pro-hormones by endo- and exo-proteolytic cleavages which might generate substrates for gACE. We have shown that peptide hormone intermediates with Lys/Arg-Arg at the C-terminus are high-affinity substrates for human gACE. gACE from human sperm cleaved Arg-Arg from the C-terminus of the CCK5-GRR (GWMDFGRR), a peptide corresponding to the C-terminus of a CCK–gastrin prohormone intermediate. Hydrolysis of CCK5-

GRR by recombinant human C-domain ACE was Cl⁻ dependent, with maximal activity achieved in 5–10 mM NaCl at pH 6.4. C-Domain ACE cleaved Lys/Arg-Arg from the C-terminus of dynorphin-(1–7), a pro-TRH peptide KRQHPGKR, and two insect peptides FSPRLGKR and FSPRLGRR. C-Domain ACE displayed high affinity towards all these substrates with $V_{\max}/K_m$ values between 14 and 113 times greater than the $V_{\max}/K_m$ for the conversion of the best known ACE substrate, angiotensin I, into angiotensin II. In conclusion, we have identified a new class of substrates for human gACE, and we suggest that gACE might be an alternative to carboxypeptidase E for the trimming of basic dipeptides from the C-terminus of intermediates generated from pro-hormones by subtilisin-like convertases in human male germ cells.

INTRODUCTION

Angiotensin I-converting enzyme (ACE, EC 3.4.15.1) is primarily a dipeptidyl carboxypeptidase, responsible for removing dipeptides from the C-terminus of circulating angiotensin I (AI, DRVYIHPFHL), bradykinin (RPPGFSPFR) and the haemoregulatory peptide *N*-acetyl-SDKP (AcSDKP) [1–4]. Human ACE exists as two isoforms, somatic ACE [sACE, M_r (150–180) × 10³] and testicular or germinal ACE [gACE, M_r (100–110) × 10³], the latter enzyme being restricted to spermatids and spermatozoa [3,5,6]. The larger sACE is anchored to the plasma membrane by a hydrophobic sequence towards the C-terminus and has two catalytically active domains, named the N- and C-domains after their positions relative to the N- and C-termini of the protein [7,8]. The two domains have a high level of sequence homology, the structure of the mammalian ACE (dipeptidyl carboxypeptidase I) gene having clearly arisen from a gene duplication event during the course of evolution [9]. The amino acid sequence of the C-domain is identical with gACE, apart from a 68 amino acid N-terminal sequence. gACE is coded for by exons 13–26 of the ACE gene, with the short N-terminal sequence unique to gACE being encoded by exon 13, which is spliced from the somatic ACE mRNA [10]. The predicted pro-protein has a signal sequence for secretion, and when a full-length cDNA for human gACE is expressed in transfected Chinese hamster ovary cells, gACE is expressed as both a membrane-bound and a soluble secreted enzyme [11]. In rats, the final form of gACE in maturing spermatids and spermatozoa also differs from plasma membrane sACE in that the germinal enzyme is processed to a soluble protein with an intracellular

localization [12]. A study of the expression of gACE in mice and rats using *in situ* hybridization and immunocytochemical methods showed that gACE mRNA appears during the cap phase, with maximum expression occurring in the acrosome phase [13]. The enzyme is at first restricted to the Golgi region close to the acrosome, whereas at a later stage of spermatogenesis gACE protein is located in the cytoplasm and at the plasma membrane. Human gACE has also been localized to the Golgi apparatus, the Golgi-derived acrosomal cap, the acrosome and the plasma membrane [5,14].

Evidence for the importance of ACE in fertilization has been obtained from studying mice carrying an insertional mutation resulting in the inactivation of both sACE and gACE [15,16]. Male mice homozygous for the deficient mutation are infertile, despite having normal motile sperm and displaying normal mating behaviour. The infertility of these male mice appears to result from the lack of gACE, since adult males lacking active sACE, but retaining gACE activity, are fertile [17]. The effect of the absence of gACE on male fertility is not due to the failure to generate AII, since male mice that do not express the angiotensinogen gene are fertile [18].

During mammalian spermatogenesis a number of pro-hormone genes [pro-enkephalin, pro-cholecystokinin (pro-CCK), pro-gastrin and pro-pituitary adenylate cyclase activating peptide] are expressed and the gene products are processed to mature peptides, which in the case of enkephalin, CCK and gastrin accumulate in the acrosome [19–25]. The localization of gACE to the acrosome and the Golgi apparatus close to the acrosome, at a stage when this organelle is very active in secretion, led us to

Abbreviations used: ACE, angiotensin I-converting enzyme; sACE, somatic ACE; gACE, germinal ACE; AI, angiotensin I; AcSDKP, *N*-acetyl-SDKP; CCK, cholecystokinin; CCK5-GRR, GWMDFGRR; CCK5-G, GWMDFG; CPE, carboxypeptidase E; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; the one-letter symbols are used for amino acid sequences.

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consider the possibility that gACE is involved in the intracellular processing of spermatid peptide pro-hormones. Since ACE is first and foremost a dipeptidyl carboxypeptidase, we have investigated whether gACE can remove pairs of basic residues (Arg-Arg and Lys-Arg) from the C-terminus of hormone peptide intermediates. Peptides flanked by pairs of basic residues are common intermediates generated in the course of pro-hormone processing by the subtilisin-like pro-hormone (or pro-protein) convertases (PCs) [26], and are substrates for carboxypeptidase E (CPE), a known component of the secretory pathway [27–29]. A dipeptidyl carboxypeptidase with a preference for removing pairs of basic residues from the C-terminus of pro-hormone intermediates is appealing as an alternative processing enzyme, because the flanking basic amino acids would be removed by a single reaction, unlike the sequential cleavage performed by CPE. There are no reports in the literature describing the hydrolysis of basic dipeptides from C-termini by ACE, but it is well known that the ACE active site can accommodate basic residues at either the penultimate or ultimate positions at the C-terminus of peptide substrates. For example, the physiological ACE substrates AcSDKP and bradykinin have a basic residue in one of these positions and the penultimate histidine of AI may be partially charged at neutral pH.

We now report that a peptide (GWMDFGRR, CCK5-GRR), corresponding to residues 67–74 of human pre-progastrin and residues 79–86 of human pre-proCCK and matching the C-terminal sequence of a CCK and gastrin-processing intermediate, is processed to GWMDFG (CCK5-G) by gACE in human sperm and by the C-domain of human somatic ACE. We also show that human ACE C-domain displays a general hydrolytic activity towards a number of different peptide substrates with a common Lys/Arg-Arg C-terminal sequence and that these peptides are more efficiently hydrolysed compared with the physiological substrate, AI.

EXPERIMENTAL

Chemicals

Peptide substrates were either synthesized (Sussex Centre for Neuroscience, University of Sussex, Brighton, U.K.) or purchased from Sigma Chemical Co., Poole, Dorset, U.K.

Expression of recombinant human ACE C-domain

A soluble form of the C-domain of somatic ACE was expressed in *Pichia pastoris*, as described previously [30]. The soluble recombinant C-domain, encoded by exons 14–26 of the human *Ace* gene, is devoid of a membrane anchor and the C-terminal cytoplasmic tail, which are present in both somatic and germinal isoforms of human ACE. Apart from a small N-terminal sequence, encoded by exon 13, and the membrane anchor region, the recombinant ACE C-domain has an identical amino acid sequence with that of human gACE. The enzyme (M_r 80 × 10³) was purified by affinity chromatography using a Sepharose-lisinopril column [30].

Homogenate of human spermatozoa

Samples of spermatozoa, free of seminal fluid, were prepared from ejaculated human semen as described previously [31]. Spermatozoa were homogenized in 1% Triton X-100/50 mM Tris/HCl, pH 7.4, and a detergent-soluble enzyme preparation was obtained by centrifugation at 13 000 g for 10 min.

Hydrolysis of peptides

Peptides (4–40 µM) were incubated with either a homogenate of ejaculated spermatozoa (0.38 µg of protein) or the recombinant C-domain of sACE (300 ng) at 37 °C for 15–30 min in 0.1 M Mes buffer, pH 6.4/10 µM ZnSO₄/10 mM NaCl (final volume of 240 µl). Under these incubation conditions the extent of hydrolysis did not exceed 15% of the initial substrate and reactions were linear with time. The effect of Cl[−] and pH on the hydrolysis of CCK5-GRR by recombinant ACE C-domain was studied using either 0.1 M Mes (pH 5.4–6.4) or 0.1 M Mops (pH 6.5–8.4) buffers. Reactions were terminated by the addition of 20 µl of 8% (v/v) trifluoroacetic acid and the hydrolysis products were quantified by reverse-phase HPLC with UV detection (214 nm) using a Pharmacia Pep-S column and a linear gradient of acetonitrile in 0.15% trifluoroacetic acid (15–36% acetonitrile for AI; 12–32% acetonitrile for dynorphin-(1–7) and CCK5-GRR; 3–30% acetonitrile for the insect peptides FSPRLG(K/R)R; 1–5% acetonitrile for the TRH intermediate peptide (KRQHPGKR).

Identification of peptides

Peptides generated as the result of ACE hydrolysis were identified either by co-chromatography with authentic peptides ([Leu⁵]enkephalin, KRQHPG) or by matrix-assisted laser desorption ionization time-of-flight ((MALDI-TOF) MS of HPLC-purified metabolites using a matrix solution of 3,5-dimethoxy-4-hydroxycinnamic acid (10 mg/ml in 30% aqueous acetonitrile/0.1% trifluoroacetic acid) and a VG Analytical Micromass TofSpec instrument.

RESULTS AND DISCUSSION

Hydrolysis of CCK5-GRR by spermatozoa and somatic ACE C-domain

CCK5-GRR, corresponding to a common C-terminal region of the CCK and gastrin pro-hormone intermediate, was incubated with a detergent-solubilized homogenate of human spermatozoa. A single UV-absorbing metabolite was generated and identified as CCK5-G by HPLC/MALDI-TOF MS (Figure 1). The formation of the hexapeptide was totally inhibited by 200 nM captopril (Figure 1C), a potent inhibitor of human ACE, demonstrating that ACE was responsible for all of the activity leading to the removal of the arginine residues from the C-terminus of this peptide. The failure to detect any CPE-like activity towards CCK5-GRR in this experiment might have been due to the fact that CPE is resistant to solubilization in detergent solution, requires Co²⁺ for optimal activity and has a pH optimum around 5.6 [27]. In order to test for CPE activity towards CCK5-GRR in spermatozoa, the experiment was repeated using a homogenate of spermatozoa that had not been subjected to centrifugation and the assay was conducted in an incubation buffer of pH 5.6 (0.1 M Mes/10 mM NaCl/10 µM Zn²⁺) to which 1 mM CoCl₂ was added. However, even under these incubation conditions, which were optimized for CPE activity, only a captopril-sensitive cleavage of CCK5-GRR was detected in the homogenate (results not shown). This result does not necessarily indicate the absence of CPE-like activity in spermatozoa, but does demonstrate the relatively high efficiency of the conversion of CCK5-GRR into CCK5-G by gACE.

This novel pro-hormone processing activity of ACE was also studied using the C-domain of human sACE, which is almost identical with gACE in amino acid sequence and enzymic properties, and which was available as a soluble recombinant

Figure 1 Conversion of CCK5-GRR into CCK5-G by human gACE

CCK5-GRR (20 μ M) was incubated with either detergent-solubilized spermatozoa (**A–C**) or a recombinant form of human ACE C-domain (**D**) as described in the text. Samples of the reaction mixture were taken at the beginning of the experiment (**A**) and after 20 min incubation (**B**) for analysis by HPLC (see Experimental section for conditions). The 100% absorbance (y -axis) represents a full-scale absorbance of 0.2 with the UV detector set at 214 nm. The only peptide metabolite detected was identified as CCK5-G by MALDI-TOF MS ($[M + Na]^+$ ion at m/z 735), the formation of which was completely blocked by 200 nM captopril (**C**). Recombinant human ACE C-domain also converted CCK5-GRR into CCK5-G (**D**).

protein. Recombinant ACE C-domain converted CCK5-GRR into CCK5-G (Figure 1D) and the reaction was linear with time (0–30 min). No additional UV-absorbing peaks were detected, indicating that CCK5-G was not significantly metabolized to smaller peptide fragments during the course of the 30 min incubation period.

Effect of pH and chloride concentration on the hydrolysis of CCK5-GRR by recombinant ACE C-domain

The conversion of CCK5-GRR into CCK5-G by ACE C-domain was dependent upon the presence of sodium chloride in the reaction buffer. Near optimal hydrolytic activity was achieved with a chloride concentration of 5 mM (Figure 2A).

The pH dependence of the reaction was investigated using Mes and Mops buffers to cover pH ranges 5.4–6.4 and 6.5–8.4 respectively (Figure 2B). The results of these experiments revealed a pH optimum of 6.4 with strong activity maintained at pH 5.6 (80% of maximum) and relatively weak activity above pH 7.4 (< 30% of maximum).

Kinetic parameters for the hydrolysis of Lys-Arg and Arg-Arg from the C-terminus of peptides by recombinant ACE C-domain

In addition to CCK5-GRR, two other mammalian peptides [dynorphin-(1–7) and a pro-TRH-derived peptide, KRQHPGKR] and two insect hormone pro-hormone peptides (FSPRLGRR

and FSPRLGKR), all ending with C-terminal Lys/Arg-Arg, were tested as potential substrates for the recombinant ACE C-domain. Dynorphin-(1–7) was converted into [Leu⁵]enkephalin; KRQHPGKR was hydrolysed to KRQHPG and both FSPRLGRR and FSPRLGKR were converted into FSPRLG. K_m and V_{max} values for the hydrolysis of the various peptide substrates were compared with the kinetic parameters for the conversion of AI into AII by the same sample of recombinant enzyme. The K_m values for the hydrolysis of CCK5-GRR, dynorphin-(1–7) and FSPRLGKR and FSPRLGRR were between 5.8 and 12.7 μ M, demonstrating the high affinity of the enzyme for these substrates (Table 1). ACE C-domain had a slightly lower affinity for KRQHPGKR ($K_m = 23.7 \mu$ M).

The V_{max}/K_m provides an indication of the overall catalytic efficiency of the hydrolysis of the various peptides by the recombinant ACE C-domain. The V_{max}/K_m was greatest for the hydrolysis of CCK5-GRR; this value was more than two orders of magnitude greater than the V_{max}/K_m for the conversion of AI into AII by the same enzyme preparation. The other peptides ending with a C-terminal Lys/Arg-Arg sequence were also hydrolysed much more efficiently compared with the hydrolysis of AI (Table 1).

We have shown that ACE C-domain displays general hydrolytic activity towards five peptide substrates with C-terminal Arg-Arg or Lys-Arg that, apart from the insect peptides, are structurally very different. This fact, together with the generally high V_{max}/K_m values for the hydrolysis of these peptides compared

Table 1 Catalytic properties of human ACE C-domain

Peptides (4–40 μ M) were incubated with recombinant C-domain of sACE (300 ng) at 37 °C for 15–30 min, in 0.1 M Mes buffer, pH 6.4/10 μ M ZnSO₄/10 mM NaCl (see Experimental section for details). The rates of cleavage of either Arg-Arg or Lys-Arg from peptide substrates by recombinant human ACE C-domain were determined by quantifying the peptide product (CCK5-G for CCK5-GRR, [Leu⁵]enkephalin for dynorphin (1–7), AI for AI, KRQHP for KRQHPKR, FSPLG for FSPLGKR and FSPLGRR) by HPLC with UV (214 nm) detection. For all substrates, the assay conditions were such that there was no detectable hydrolysis of the primary reaction product as determined by HPLC. The identities of AI, [Leu⁵]enkephalin and KRQHPG were confirmed by co-chromatography with authentic marker peptides. FSPLRG was identified by MALDI-TOF MS of HPLC-purified peptide ($[M + H]^+$ at m/z 676). Enzyme assays and HPLC analyses were performed as described in the text and the results are the means of triplicate assays. Units of enzyme activity are defined as nmol of product formed per min and values are given as means $\pm$ S.E.M.

Peptide substrate	K_m (μ M)	V_{max} (units/ μ g of protein)	V_{max}/K_m	Ratio of V_{max}/K_m
GWMDFGRR (CCK5-GRR)	9.0 $\pm$ 0.2	37.6 $\pm$ 0.4	4.18	113
YGGFLRR [dynorphin-(1–7)]	5.8 $\pm$ 0.6	17.7 $\pm$ 0.6	3.05	82
KRQHPKR	23.7 $\pm$ 3.4	12.5 $\pm$ 0.7	0.53	14
FSPLGKR	12.7 $\pm$ 0.7	22.2 $\pm$ 0.5	1.75	47
FSPLGRR	8.2 $\pm$ 0.7	22.1 $\pm$ 0.8	2.70	73
DRVYIHPFHL (AI)	25.6 $\pm$ 3.0	0.95 $\pm$ 0.04	0.04	1

Figure 2 Effect of NaCl (A) and pH (B) on the hydrolysis of CCK5-GRR by recombinant human ACE C-domain

CCK5-GRR (20 μ M) was incubated with 300 ng of recombinant human ACE C-domain in 0.1 M Mes buffer, pH 6.4, 10 μ M ZnSO₄ and NaCl (0–20 mM) (A), and in either 0.1 M Mes buffer, pH 5.4–6.4/10 μ M ZnSO₄/20 mM NaCl (■) or 0.1 M Mops buffer, pH 6.5–8.4/10 μ M ZnSO₄/20 mM NaCl (●) (B). Enzyme assays were performed in triplicate as described in the Experimental section. Data are expressed as means and all S.E.M. values were $< \pm 2.5\%$.

with AI, suggests that we have identified, in the C-terminal Lys/Arg-Arg sequence, a motif defining a new class of potential ACE substrates. The ACE C-domain hydrolysed CCK5-GRR with optimal activity between pH 5.6 and 6.6, indicating that the enzyme would be active in the weakly acidic environment of

parts of the secretory pathway where pro-hormone processing is expected to take place. It is well known that mammalian ACE requires Cl[−] for maximal activity and it has been shown that the extent of activation is substrate and pH dependent [32–34]. The fact that the hydrolysis of Arg-Arg from CCK5-GRR approaches maximal activity at the low concentration of 5 mM, might reflect the relatively low intracellular concentration of Cl[−] in spermatids/spermatozoa as opposed to the generally high Cl[−] levels found in extracellular fluids.

The expression of genes for pro-CCK, pro-gastrin, pro-enkephalin and pro-pituitary adenylate cyclase activating peptide and the presence of processed peptides in spermatids and spermatozoa means that a pro-hormone processing pathway exists in developing male germ cells [19–25]. A germ cell-specific PC (PC4) has been characterized from mouse and this enzyme is essential for fertilization, since targeted disruption of the murine PC4 gene results in the failure of apparently normal-looking sperm to fertilize eggs [35,36]. The effect of the total loss of PC4 on fertility is very similar to what is found in ACE-deficient mice, which is consistent with both PC4 and ACE being components of the same pro-hormone or pro-protein processing pathway. That mammalian testes are the richest source of ACE has been known for over 30 years, but during this time there has been little progress in defining the biological role of the testicular enzyme [2,3,37]. We have reported a novel hydrolytic activity of gACE, which identifies a new class of peptides that should be considered as substrates for gACE in developing spermatozoa. The loss of fertility in male mice lacking gACE might therefore be due to the general disruption of pro-hormone or general pro-protein processing.

We thank Jeff Keen of the Department of Biochemistry and Molecular Biology, University of Leeds for MS work and Christopher Kowalczyk, Centre for Neuroscience, University of Sussex for peptide synthesis.

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