In vitro elucidation of substrate specificity and bioassay of proprotein convertase 4 using intramolecularly quenched fluorogenic peptides

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The fourth member of Ca^{2+}-dependent mammalian secretory subtilase, PC4 (proprotein convertase 4), is primarily expressed in testicular germ cell and ovarian macrophage. Its role in sperm fertilization and in early embryonic development has been demonstrated earlier through several studies, including those with PC4 null mice. A number of physiological substrates found in reproductive tissues have been postulated or identified for PC4 by various biochemical studies. These include growth factors IGF-1 (insulin-like growth factor-1) and IGF-2, hormonal polypeptide proPACAP (where PACAP stands for pituitary adenylate cyclase-activating polypeptide) and a number of surface proteins of ADAM (A Disintegrin And Metalloproteinase-like) family such as ADAM-1 (fertilin α), ADAM-2 (fertilin β), ADAM-3 (procyritestin) and ADAM-5. To provide further evidence in support of this notion and also to study the substrate specificity and bioassay of PC4, a series of intramolecularly quenched fluorogenic peptides containing the cleavage sites and several mutants were prepared. A comparative kinetic analysis and measurement of V_{max} (app)/K_m (app) ratio of these fluorogenic substrates against PC4 and PC7 revealed that the mutant variants of h (human) proPACAP and m (mouse) ADAM-5 derived peptides Q-PACAP$^{141-151}$-mutant [Abz-^{141}RVKNKGRR↓I^{150}***P***^{151}***S***Y(NO_2)-A-$CONH_2$] (^{150}A^{151}Y replaced by ***PS***) and Q-ADAM-5$^{380-388}$-mutant [Abz-^{380}E^{381}***P***KPAR↓RP^{388}RY(NO_2)A-$CONH_2$] (^{381}R replaced by ***P***) are most efficiently and selectively cleaved by PC4. Using these two and Q-IGF-2$^{63-71}$ peptides, we showed that the sperm extract of normal adult mice is much higher when compared with that of PC4-null mice. This suggests that these fluorogenic peptides are useful for specific bioassay of PC4 activity. In addition, kinetic studies with various peptidyl-MCA indicate that the hexapeptide Ac-KTKQLR-MCA (where MCA stands for 4-methyl coumaryl-7-amide) is most efficiently and selectively cleaved by PC4 at R↓MCA, making it another effective agent for bioassay of PC4 activity. The study concludes that the most probable sequence motif for recognition by PC4 is KXKXXR or KXXR, where X is any amino acid other than cysteine and that it prefers proline at P3, P5 and/or P2′ positions. It was also revealed that PC4 is a good candidate processing enzyme for growth factors IGF-1 and -2, neuropeptide proPACAP and several ADAM proteins such as ADAM-1, -2, -3 and -5.

Key words: ADAM proteins, cleavage specificity, enzyme bioassay, intramolecularly quenched fluorogenic substrates, kinetic parameters, proprotein convertase 4, substrate specificity.

INTRODUCTION

Proprotein convertase 4 (PC4), first discovered in 1992 [1,2], belongs to a family of Ca^{2+}-dependent serine proteinases related to bacterial subtilisin and yeast kexin. Similar to other PCs, PC4 is produced in the inactive zymogen form and is activated by proteolytic removal of an N-terminal fragment known as prodomain. Evidence indicates that this is probably an autocatalytic process and involves cleavage after motifs of basic residues **Arg/Lys**-$(X)_n$-**(Lys/Arg)**↓, where $n = 0$, 2, 4 or 6 and X is any amino acid residue other than cysteine (reviewed in [3–5]). PC4 is highly expressed in germ cells along with PC7, suggesting a possible reproductive function for this enzyme especially in spermatogenesis and fertility [6–8]. PC7 is not linked to spermatogenesis and fertilization since silencing its activity had little effect on fertility [9]. Within the germ cells, PC4 is found in spermatocytes and round spermatids, but not in Sertoli and Leydic cells. On the basis of mRNA analysis, PC4 is restricted to testicular germ cells and to some extent within hormonally stimulated ovaries [6–8,10]. Western-blot analysis detected an immunoreactive PC4 protein in epididymal spermatozoa [6–8]. Fertility of homozygous PC4-null mouse is severely impaired [11] and PC4 is more important in males than females for achieving fertilization and for supporting early embryonic development in mice [11]. The phenotype of PC4-null mice is linked to lack of or insufficient processing of precursor proteins of spermatogenic cells. Several genes and their translated proteins have been linked to fertilization [12–22]. These proteins require proteolytic activation at selected sites. PC4 has been identified as one of the key processing enzymes. So far, several surface proteins, neuropeptides and growth factors found on sperm surface or in the acrosome have been confirmed or proposed as physiological substrates for PC4. These are fertilin α chain also called ADAM-1 (**A** **D**isintegrin **A**nd **M**etalloproteinase-like), fertilin β chain also known as ADAM-2, procyritestin or ADAM-3, ADAM-5, proPACAP (where PACAP stands for pituitary adenylate cyclase-activating polypeptide), proenkephalin, pro-nerve growth factor, proIGF-1 (pro-insulin-like growth factor-1), proIGF-2 and some of their receptors [12–22]. So far, the correct physiological sites of PC4-mediated cleavages of IGF-1 and -2 [23] as well as proPACAP [24,25] have been well established by biochemical studies involving *in vitro* and *ex vivo* experiments as well as PC4-null mice [11]. However, for the rest of the proteins, the exact sites of cleavages were either unknown or predicted from co-expression studies [26–31].

Abbreviations used: Abz, 2-amino benzoic acid; ADAM, A Disintegrin And Metalloproteinase-like; AMC, 7-amino 4-methyl coumarin; Boc, *t*-butyloxycarbonyl; But, *t*-butyl; Fmoc, fluoren-9yl-methoxycarbonyl; IGF, insulin-like growth factor; IQF, intramolecularly quenched fluorogenic; KO, knock-out; MALDI–TOF-MS, matrix-assisted laser-desorption ionization–time-of-flight mass spectrometry; MCA, 4-methyl coumaryl-7-amide; PACAP, pituitary adenylate cyclase-activating polypeptide; PC, proprotein convertase; RP, reverse-phase; TFA, trifluoroacetic acid; Tyx, 3-nitro tyrosine [Tyr(3-NO_2)]; WT, wild-type.

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Previously, we obtained a soluble secreted form of recombinant PC4 using the vaccinia virus expression system in BSC-40 cell line and studied in part its enzymic property [23]. Using IQF (intramolecularly quenched fluorogenic) peptides [32–37] that encompass the identified or proposed cleavage sites for PC4 substrates, we have now performed detailed *in vitro* kinetic evaluation and compared with that of PC7, another germ cell subtilase. The goal of the present study is to confirm or identify PC4 substrates as well as to develop a specific bioassay for the enzyme. A preliminary report of this study was presented earlier in the form of a short abstract [38].

MATERIALS AND METHODS

All Fmoc (fluoren-9-ylmethoxycarbonyl) amino acids (L-configuration), the coupling agents and the solvents were purchased from PE Applied Biosystems (Framingham, MA, U.S.A.), Calbiochem–Novabiochem (San Diego, CA, U.S.A.), Chem-Impex International (Wood Dale, IL, U.S.A.) and Aldrich Chemical Company (Milwaukee, WI, U.S.A.). All peptidyl-MCAs (4-methyl coumaryl-7-amides) used in the present study were purchased from Peptides International (Louisville, KY, U.S.A.) or Bachem (Torrance, CA, U.S.A.). Amino acids were analysed after 24 h hydrolysis in 6 M HCl at 110 °C *in vacuo* using a Dionex autoanalyser equipped with electrothermal detector (Dionex, Oakville, ON, Canada; http://www.dionex.com).

RP (reverse-phase)-HPLC was performed on a Rainin (Dynamax, peristaltic pump model RP-1; Rainin Instrument Company, Emeryville, CA, U.S.A.) instrument equipped with SD-1 pump, using a 10 μm, 300 Å (1 Å $= 10^{-10}$ m) C_{18} column [Jupiter; Phenomenex, Torrance, CA, U.S.A.; size, 0.46 cm × 25 cm (for analytical run) or 1 cm × 25 cm (for semipreparative run)]. The buffer system comprises an aqueous 0.1 % (v/v) TFA (trifluoroacetic acid) solution and an organic phase of CH_3CN containing 0.1 % TFA. Peptides were eluted with a 1 %/min linear gradient (5–60 %) of 0.1 % aqueous TFA/CH_3CN at a flow rate of 1 ml/min (analytical run) or 2 ml/min (semipreparative run), following a 5 min isocratic run at 5 % of 0.1 % TFA/CH_3CN. The elution of the peptides was monitored on line by UV absorbance with wavelength (λ) at 210, 214 or 230 nm and by fluorescence measurement (Rainin, Dynamax fluorescence detector, model FL-2) at λ_{ex} and λ_{em} fixed at 320 ± 5 and 420 ± 3 nm respectively.

Peptide synthesis

All peptides were prepared as C-terminal amides. Synthesis was performed on Fmoc-PAL-PEG [poly-amino linker poly-(ethylene glycol)] resin (ABI, Foster City, CA, U.S.A.) support using an automated solid-phase peptide synthesizer (model: pioneer; PE PerSeptive Biosystems, Framingham, MA, U.S.A.) and HATU (*O*-hexafluorophospho-[7-azabenzotriazol-1-yl]-*N*,*N*,*N*′,*N*′-tetramethyluronium) and diisopropyl ethyl amine mediated Fmoc chemistry [36,37]. The following side-chain protecting groups were used: Boc (*t*-butyloxycarbonyl) for lysine; Pbf (2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulphonyl) for arginine; (*t*-butyl) for serine, threonine, aspartate and tyrosine; and trityl for histidine, asparagine and glutamine respectively. The N-terminal free bound peptides were cleaved from the resin and fully deprotected by treatment for 3–4 h at room temperature (25 °C) with reagent K as described previously [36,37]. In certain cases, owing to high content of protecting groups, particularly Pbf of arginine residues, it was found necessary to repeat the deprotection procedure for a second time.

Peptide purification, isolation and characterization

The crude peptides were purified by RP-HPLC using a semi-preparative CSC column (Chromatography Speciality, St-Laurent, QC, Canada) as described earlier. The peptide purity and concentration were determined by quantitative amino acid analysis after 24 h hydrolysis in the presence of 5.7 M HCl *in vacuo* at 110 °C on an amino acid analyser (Dionex). The identity of each purified peptide was confirmed by MALDI–TOF-MS (matrix-assisted laser-desorption ionization–time-of-flight mass spectrometry; Voyager; Applied Biosystems, Foster City, CA, U.S.A.) [36,37].

Peptide-1, Q-h(human)proIGF-1$^{66-74}$: 12-mer, Abz-P^{66}AKSARSVR^{74}-Tyx-A (where Abz stands for 2-amino benzoic acid and Tyx for 3-nitro tyrosine), RP-HPLC: $R_t = 20.5$ min (20.5 % CH_3CN), MS: *m/z* 1370 $(M + H)^+$.

Peptide-2, Q-hproIGF-2$^{63-71}$: 12-mer, Abz-P^{63}AKSERDVS^{71}-Tyx-A, RP-HPLC: $R_t = 19.1$ min (19 % CH_3CN), MS: *m/z* 1386 $(M + H)^+$.

Peptide-3, Q-h/r(rat)proPACAP$^{141-151}$: 14-mer, Abz-R^{141}VKNKGRRIAY^{151}-Tyx-A, RP-HPLC: $R_t = 22.9$ min (23 % CH_3CN), MS: *m/z* 1760 $(M + H)^+$.

Peptide-3a, Q-h/rproPACAP$^{141-151}$mut: 14-mer, Abz-R^{141}VKNKGRRI***PS**^{151}-Tyx-A, RP-HPLC: $R_t = 20.1$ min (20 % CH_3CN), MS: *m/z* 1707 $(M + H)^+$.

Peptide-4, Q-hproPACAP$^{99-111}$: 16-mer, Abz-D^{99}DAEPLSKRHSDG^{111}-Tyx-A: RP-HPLC: $R_t = 21.1$ min (21 % CH_3CN), MS: *m/z* 1823.3 $(M + H)^+$.

Peptide-5, Q-m(mouse)proCyritestin$^{449-462}$: 17-mer, Abz-T^{449}IAERGRL*M*RKSKD^{462}-Tyx-A, RP-HPLC: $R_t = 26.4$ min (26 % CH_3CN), MS: *m/z* 2058 $(M + H)^+$.

Peptide-6, Q-mFertilin $\alpha^{428-440}$: 16-mer, Abz-E^{428}PGRQSR*M*RRAAN^{440}-Tyx-A, RP-HPLC: $R_t = 23.4$ min (23 % CH_3CN), MS: *m/z* 1928 $(M + H)^+$.

Peptide-7, Q-hFertilin $\beta^{438-450}$: 16-mer, Abz-F^{438}MSKERM*M*RPSFE^{450}-Tyx-A, RP-HPLC: $R_t = 25.4$ min (25 % CH_3CN), MS: *m/z* 2072.8 $(M + H)^+$.

Peptide-8, Q-mADAM 5$^{380-388}$: 12-mer, Abz-E^{380}RKPARRPR^{388}-Tyx-A, RP-HPLC: $R_t = 24.6$ min (23 % CH_3CN), MS: *m/z* 1561.2 $(M + H)^+$.

Peptide-8a, Q-mADAM 5$^{380-388}$-mutant: 12-mer, Abz-E^{380}***P**KPARRPR^{388}-Tyx-A, RP-HPLC: $R_t = 25.2$ min (23 % CH_3CN), MS: *m/z* 1503.2 $(M + H)^+$.

Source of recombinant enzymes

Recombinant r (rat) PC4 is obtained by stable transfection of Hi5 cells with an rPC4A vector, lacking the putative C-terminal domain that is responsible for its attachment to the plasma membrane via glycophosphoinositol anchor (ending at Pro^{523}). The medium was concentrated and dialysed by using centricon-10 with cut-off molecular mass of 10 kDa (Millipore, Bedford, MA, U.S.A.) as described previously for isolation of recombinant PC4 from concentrated medium of rat epithelial BSC40 cells infected with a recombinant vaccinia virus carrying the rPC4A cDNA [23]. The presence of immunoreactive PC4 in the concentrate medium was confirmed by Western-blot analysis and immunohistochemistry, which showed the presence of a 54 kDa mature PC4 band (M. Mbikay, unpublished work). Recombinant PC7 used in the present study was obtained as described in [40].

Enzymic assay

All *in vitro* enzymic assays were performed using either initial rate assays and/or end-time assays at room temperature in a final

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Table 1 List of various IQF peptides

Amino acid sequences are from human (h), mouse (m) or rat (r). The amino acids underlined are those derived from the WT sequence of the corresponding proteins. The vertical arrow indicates the identified or proposed cleavage site. The amino acid mutated in the WT sequence is indicated in bold italics. The important basic residues are shown in bold.

No.	Peptide name	Amino acid sequence P_{11} . . . . . P_1		P'_1 . . . . . P'_6
1	*Q-hproIGF-1$^{66-74}$	Abz-PA**K**SA**R**^{71}	↓	SVR-Tyx-A
2	*Q-hproIGF-2$^{63-71}$	Abz-PA**K**SE**R**^{68}	↓	DVS-Tyx-A
3	*Q-h/rproPACAP$^{141-151}$	Abz-**R**V**K**N**K**G**RR**^{148}	↓	IAY-Tyx-A
3a	*Q-hproPACAP$^{141-151}$-mut	Abz-**R**V**K**N**K**G**RR**^{148}	↓	I***PS***-Tyx-A
4	*Q-hproPACAP$^{99-111}$	Abz-DDAEPLS**KR**^{107}	↓	HSDG-Tyx-A
5	Q-mproCyritestin$^{449-462}$	Abz-TIAE**R**G**R**L***M***R^{458}	↓	KSKD-Tyx-A
6	Q-mFertilin $\alpha^{428-440}$	Abz-EPG**R**QS**R*M*RR**^{437}	↓	AAN-Tyx-A
7	Q-hFertilin $\beta^{438-450}$	Abz-FMS**KER**M***MR***^{446}	↓	PSFE-Tyx-A
8	Q-mADAM-5$^{380-388}$	Abz-ER**K**PA**R**^{385}	↓	RP**R**-Tyx-A
8a	Q-mADAM-5$^{380-388}$-mut	Abz-E***PK***PA**R**^{385}	↓	RP**R**-Tyx-A

* The numbering starts after the removal of signal peptide.

volume of 100 μl in 96 well flat-bottom black plates (Dynatec, Millipore). The buffer used contained 25 mM Mes, 25 mM Tris and 2.5 mM $CaCl_2$ (pH 7.4). Unless stated otherwise, all assays were performed using a final concentration of 100 μM of pERTKR-MCA as substrate. The release of highly fluorescent AMC (7-amino 4-methyl coumarin) was quantified on-line using a spectrofluorimeter (Gemini; Molecular Dynamics, Sunnydale, CA, U.S.A.) at emission wavelengths of 370 ± 5 and 460 ± 3 nm respectively [36,37]. For assays with all IQF peptides, the excitation and emission wavelengths were set at 320 ± 5 and 420 ± 3 nm respectively.

Kinetic analysis

For determining the kinetic parameters V_{max} (app) and K_m (app), enzyme samples, typically 5 μl, equivalent to an activity leading to the release of approx. 3.4 μmol/h of free AMC from pERTKR-MCA (100 μM), were added to increasing concentrations of each IQF peptide ranging from 1 to 20 μM in a total volume of 100 μl of buffer as described above in a 96 microtitre well plate at 37 °C. The rate of substrate hydrolysis was obtained from the changes in fluorescence readings and the values transformed into amounts of μmol cleaved/h by using the standard curve and quenching corrections as described in [36,37]. Since the quantum yield of Abz is somewhat dependent on the nature of the N-terminal amino acid residue (particularly for proline as present in IGFs) of the substrate [41,42], standard peptides corresponding to the N-terminal cleaved fragment of each IQF substrate were made for conversion purposes. Two types of standard curves were used for these peptides: one correlating fluorescence unit with the amount and the other on quenching of fluorescence of N-terminal fragment by each substrate. These curves were applied for quenching correction and for conversion of raw fluorescence units into μmol units. The correction in raw fluorescence unit is particularly necessary for higher concentrations of the substrate, where the quenching of the undigested substrate by the released N-terminal Abz peptide is more significant [36,37]. Values were calculated from non-linear regression analysis (Grafit v4.0, Erthacus Software, Distributor; Sigma) of plots of the hydrolysis rate versus the substrate concentration.

Digestion of IQF peptides by PC4 and sites of cleavages

Each peptide (10 μg) was dissolved in a buffer (buffer A), consisting of 25 mM Mes, 25 mM Tris, 2.5 mM $CaCl_2$, pH 7.4 (80 μl), and then treated with recombinant PC4 or PC7 (10 μl) and incubated in a shaker incubator at 37 °C for 18 h. The crude digest was purified by RP-HPLC using a C_{18}-analytical column (Phenomenex) under the conditions described above. The collected peaks were characterized by amino acid composition and MS using α-cyanohydroxycinnamic acid as the matrix. For the control experiment, each peptide was incubated under identical conditions with concentrated medium from empty vector obtained similarly.

Extraction of sperm from WT (wild-type) and PC4-null mice

Sperm were collected from adult mice of both WT- and PC4-KO type (where KO stands for knock-out) and kept frozen in storage buffer [8] at −80 °C until use. Frozen sperm (approx. 0.5 g) was first slowly warmed to 4 °C until it became semisolid. A buffer (buffer B) consisting of 30% acetonitrile in 100 mM NaCl in buffer A (400 μl) was added for extraction. All operations were conducted at 4 °C. It was then centrifuged at 10600 ***g*** (centrifuge 5417R). The clear supernatant was collected. The process was repeated three times and the combined extract (1.2 ml) was used directly for enzymic assay using various IQF peptides for the presence of PC4 activity.

Assay for PC4 activity in sperm extracts

The crude sperm extract as obtained above from WT and PC4-KO mice (195 μl) was incubated in a shaker incubator at 37 °C with 5 μl of 1 mM of each IQF peptide in DMSO (final concentration, 25 μM) in a 96-well plate. The release of fluorescence (excitation and emission wavelengths set at 320 ± 5 and 420 ± 3 nm) was monitored both on line and by end-time assay. After incubation for 18 h, the crude digests were subjected to RP-HPLC separation and the collected peaks were analysed by MALDI–TOF-MS for any cleaved products.

RESULTS

Design of IQF peptides

Several proproteins of growth factor, neuropeptide, receptor or ADAM family are implicated in fertility and found on sperm surface overlying the acrosome, where active PC4 is mostly present (M. Mbikay, unpublished work). Several proteins of these families were proposed as physiological substrates of PC4 (Table 1).

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Table 2 MALDI–TOF-MS data of various fractions obtained from RP-HPLC chromatograms of 18 h PC4 digests of various IQF peptides

Parent IQF peptide	RP-HPLC peak positions, R_t (min)	Calculated molecular mass (Da)	Observed mass $(M+H)^+$	Peptide identified
Q-proIGF-1$^{66-74}$	20.5	1369	1370	Abz-PAKSAR↓SVR-Tyx-A-CONH$_2$
	18.6	747	748.8	Abz-PAKSAR-OH
	14.2	639	640.1	SVR-Tyx-A-CONH$_2$
Q-proIGF-2$^{63-71}$	19.1	1385.2	1386.2	Abz-PAKSER↓D-VS-Tyx-A-CONH$_2$
	17.7	805.3	806.4	Abz-PAKSER-OH
	14.5	598	600	DVS-Tyx-A-CONH$_2$
Q-proPACAP$^{141-151}$	22.9	1758.3	1760	Abz-RVKNKGRR↓IAY-Tyx-A-CONH$_2$
	18.8	1131	1132.5	Abz-RVKNKGRR-OH
	16.2	644.3	645.5	IAY-Tyx-A-CONH$_2$
Q-proPACAP$^{141-151}$-mut	20.1	1706	1707	Abz-RVKNKGRR↓I***PS***-Tyx-A-CONH$_2$
	18.8	1132	1131.5	Abz-RVKNKGRR-OH
	14.8	594	592.5	I***PS***-Tyx-A-CONH2
Q-proCyritestin$^{449-462}$	26.4	2057	2058.1	Abz-TIAERGRL***M***R↓KSKD-Tyx-A-CONH$_2$
	21.3	1320	1321.8	Abz-TIAERGRL***M***R-OH
	18.5	754	755.2	KSKD-Tyx-A-CONH$_2$
Q-fertilin $\alpha^{428-440}$	23.4	1926.2	1928	Abz-EPGRQSR***M***RR↓AAN-Tyx-A-CONH$_2$
	18.3	1392	1393.7	Abz-EPGRQSR***M***RR-OH
	15.1	553	553.9	AAN-Tyx-A-CONH$_2$
Q-fertilin $\beta^{438-450}$	25.4	2071	2072.8	Abz-FMSKERM***M***R↓PSFE-Tyx-A-CONH$_2$
	19.3	1334	1335.4	Abz-FMSKERM***M***R-OH
	16.3	756	757.3	PSFE-Tyx-A-CONH$_2$
Q-ADAM-5$^{380-388}$	24.6	1561	1562.2	Abz-ERKPAR↓RPR-Tyx-A-CONH$_2$
	23	874	875.3	Abz-ERKPAR-OH
	19.2	706	706.7	RPR-Tyx-A-CONH$_2$
Q-ADAM-5$^{380-388}$-mut	25.2	1502	1503.5	Abz-E***P***KPAR↓RPR-Tyx-A-CONH$_2$
	23.9	815	816	Abz-E***P***KPAR-OH
	19.2	705	706	RPR-Tyx-A-CONH$_2$

Three of them, namely proIGF-1, proIGF-2 and proPACAP, were later confirmed by *in vitro* and *in vivo* studies to be activated specifically by PC4 [23–25]. Sequence alignment of these and rest of the proteins at their identified or proposed cleavage sites showed the presence of either a single basic (arginine) residue at P1 or paired basic (Lys/Arg-Arg) residues at P2–P1 positions. Moreover, they all contain an additional basic residue (mostly lysine) at P4 and/or P6 positions. This motif is compatible with that recognized by PC4 [23]. On the basis of this observation and tissue distribution pattern, PC4 is implicated in processing of the above proteins. This hypothesis is further strengthened by the observation that in PC4-KO mice testis, proPACAP remains largely unprocessed; ADAM-2 (fertilin β) is abnormally processed and the processed form of ADAM-3 (proCyritestin) is substantially decreased. To confirm that proIGF-1, proIGF-2, proPACAP, fertilin α, fertilin β, proCyritestin and ADAM-5 are all physiological substrates of PC4, and also to develop a specific bioassay of this enzyme, a number of IQF peptides and variants derived from these proteins at their processing sites were designed (Table 1). They all contain the cleavage sites corresponding to the sequences KSAR^{71}↓SV for hproIGF-1, KSER^{68}↓DV for hproIGF-2, KGRR^{148}↓IA and LSKR^{107}↓HSD for hproPACAP, RL***M***R^{458}↓KS for mproCyritestin, R***M***RR^{437}↓AA for mFertilin α, RM***M***R^{446}↓PS for hFertilin β and finally KPAR^{385}↓RP for mADAM-5. In designing IQF peptides from fertilins and proCyritestin, a cysteine to methionine mutation, indicated in bold italics at positions P3 for fertilin α and P2 for both fertilin β and proCyritestin (Table 1), was performed to prevent any potential self-polymerization and aggregation. We reasoned that such a mutation will not significantly alter the overall conformation and the charge distribution of the peptide around the processing site. Both residues would contain the same hydrophilic sulphur atom at the β-position. In addition, the sulphur atom in methionine residue is present in the bound form as in the physiological protein, where all cysteine residues are linked to one another via disulphide bridges. Along with these mutations for fertilins and proCyritestin, we introduced a double mutation (P2′ and P3′) for Q-hproPACAP$^{141-151}$, where the WT ^{150}Ala^{151}Tyr was substituted by Pro-Ser to examine the effect of introducing a P2′ proline residue as found in ADAM-5, one of the most potent *in vitro* substrates of PC4 (see later) and a turn structure following the cleavage site. A P5-proline variant, substituting arginine in Q-ADAM-5, was also prepared to test the effect of turn inducing proline residues in odd-numbered P positions in the sequence.

Peptide synthesis

All peptide syntheses were accomplished without any difficulty, except for h/rproPACAP$^{141-151}$, which yielded a product with molecular mass of 1.814 kDa, 56 mass units more than the expected mass of 1.758 kDa. This is due to the presence of the protecting *t*-butyl group (a 56 mass unit) of tyrosine residue that remained intact. The complete removal of this group could only be achieved after repeated (twice) 1 h treatments with Reagent K [36,37]. The particular complexity encountered in removing this *t*-butyl group may be due to its poor accessibility and strong interaction with the adjacent Tyx moiety.

Digestion of IQF-peptides by PC4

When incubated with recombinant PC4, all ten IQF peptides were cleaved although with different kinetic efficiencies. In all cases, the cleavage occurs at the proposed or known physiological sites as indicated by vertical arrows in Table 1. The precise sites of cleavages were determined by RP-HPLC performed on crude digests and MALDI–TOF-MS analysis of the products under the collected peaks (Table 2). A typical example depicting the RP-HPLC chromatogram of 18 h digests of Q-proIGF-2$^{63-71}$ with increasing amounts of PC4 enzyme is presented in Figure 1. As indicated in Figure 1(A), the undigested peptide is eluted at

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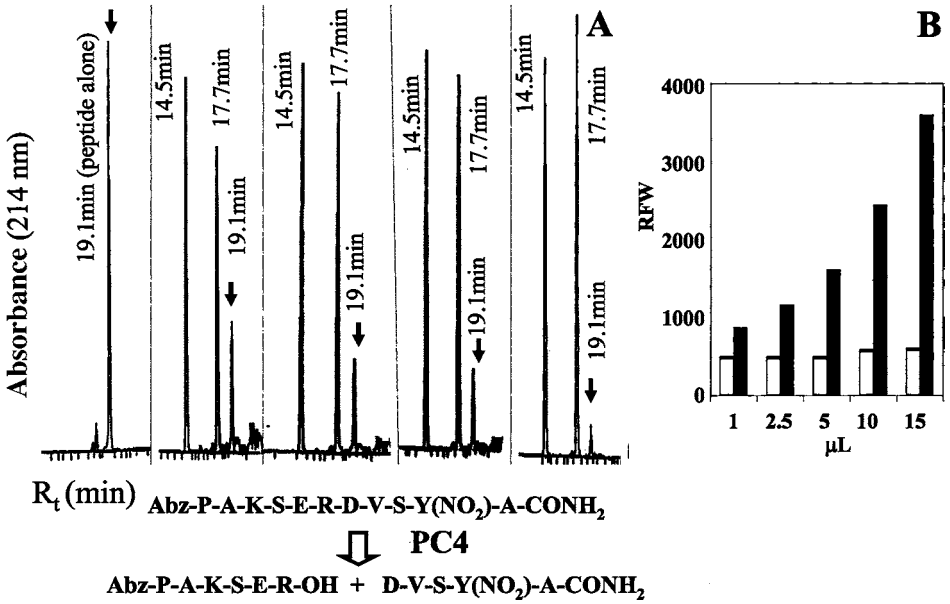

Figure 1 Digestion of Q-IGF-2$^{63-71}$ (left) and pERTKR-MCA (right) with different amounts of recombinant PC4

(**A**) The RP-HPLC chromatograms of Q-hIGF-2$^{63-71}$ (10 μg) alone (far left) and its crude digests after 18 h incubation with different amounts of PC4 enzyme (2.5, 5, 10 and 15 μl; subsequently from left). Various peaks with retention times (R_t) indicated were collected and analysed by amino acid composition and MALDI–TOF-MS. The peaks eluting at $R_t = 19.1$, 17.7 and 14.5 min (detected by UV absorbance at $\lambda = 210$ nm) were identified respectively as undigested Q-hIGF-2$^{63-71}$ peptide [Abz-PAKSERDVSY(NO$_2$)A-CONH$_2$] (*m/z* 1386), the highly fluorescent N-terminal fragment Abz-PAKSER-OH (*m/z* 806) and the non-fluorescent C-terminal fragment DVSY(NO$_2$)A-CONH$_2$ (*m/z* 600). (**B**) Fluorescence release on 18 h digestion of pERTKR-MCA (100 μM) with different amounts (0, 2.5, 5, 10 and 15 μl) of recombinant PC4 medium (black bars) and the WT medium (white bars) as indicated. The digestion is performed in a 96-well plate in 25 mM Tris, 25 mM Mes and 2.5 mM CaCl$_2$, pH 7.4 (total volume, 100 μl) and the raw fluorescence units (RFU) were measured at excitation and emission wavelengths fixed at 360 and 470 nm respectively as described in the Materials and methods section.

$R_t = 19.1$ min (observed *m/z* 1386.2, calculated, 1385.2) and is cleaved into two fragments eluting at $R_t = 14.5$ and 17.7 min. The late eluting highly fluorescent peak was identified by amino acid analysis and MS as the N-terminal fragment Abz-PAKSER-OH (observed *m/z* 806.4, calculated, 805.3), whereas the early eluting non-fluorescent peak was similarly characterized as DVSY(NO$_2$)A-CONH$_2$ (observed *m/z* 600, calculated, 598) (results not shown). It is evident from the figure that as the amount of PC4 enzyme is increased there were more cleavages as reflected in gradual increase in intensity of cleaved fragments ($R_t = 14.5$ and 17.7 min) and consequent decrease in intensity of peak for the undigested peptide ($R_t = 19.2$ min). This suggests that PC4 cleaved the proIGF-2 peptide at the correct physiological R^{68}↓D site. The presence of enzymic activity in the sample of recombinant PC4 used in the present study is confirmed in parallel by using the general PC substrate pERTKR-MCA, which exhibits a gradual increase in cleavage with the amount of PC4 used (Figure 1B). This is reflected in fluorescence measurements at fixed excitation and emission wavelengths of 360 ± 5 and 470 ± 3 nm respectively. Similarly, all other IQF peptides were also cleaved at the predicted or correct physiological sites by PC4 in a dose-dependent manner with the enzyme. As shown by MS (Table 2), all peptides on cleavage yielded two additional peaks with observed molecular masses consistent with the values calculated for the cleaved products. As a typical example, the cleavage of Q-ADAM-5-mutant, the most potent PC4 substrate (see later) upon incubation with recombinant PC4 is shown by RP-HPLC in Figures 2(A) and 2(B) and by MALDI–TOF-MS in Figure 2(C). The MS data on 18 h digest indicated a complete cleavage of starting peptide Q-ADAM-5$^{380-388}$-mut (mut stands for mutant) since no peak corresponding to its mass at *m/z* 1503 is noticeable. Instead, two peaks corresponding to the N-terminal fragment Abz-E***P***KPAR-OH (*m/z* 815) and the C-terminal segment RPRY(NO$_2$)A-CONH$_2$ (*m/z* 706) were observed showing a cleavage at KPAR^{385}↓RPR. RP-HPLC on 1 h digest (Figure 2A) showed three peaks at $R_t = 19.2$, 23.9 and 25.2 min, which were identified by MS and amino acid analysis as the C-terminal [RPRY(NO$_2$)A-CONH$_2$], N-terminal [Abz-E***P***KPAR-OH] and the undigested peptide respectively. It may be mentioned that, as expected, all peaks except the non-fluorescent C-terminal peptide eluting at $R_t = 19.2$ min are detectable by both fluorescence measurement and UV absorbance.

Kinetic parameters V_{max} (app) and K_m (app) for digestions of various substrates by recombinant PC4, furin and PC7

IQF peptide

The kinetic parameters V_{max} (app) and K_m (app) for cleavages of various IQF peptides by recombinant PC4 were obtained from the Michaelis–Menten curves (results not shown) and are presented in Table 3. Among the WT peptides, Q-ADAM-5$^{380-388}$ and Q-proIGF-1$^{66-74}$ are respectively the most and least potent substrates for PC4. However, among all those tested including the variants, Q-ADAM-5$^{380-388}$-mut is found to be the one most efficiently cleaved. In fact, it is cleaved approx. 2700-fold better than the least efficient substrate Q-proIGF-1$^{66-74}$. The next best substrate is Q-proPACAP$^{141-151}$-mut, which is cleaved approx. 2280-fold better than Q-proIGF-1$^{66-74}$. Q-fertilin $\alpha^{428-440}$, Q-fertilin $\beta^{438-450}$ and Q-proIGF-2$^{63-71}$ are all moderately cleaved with efficiencies 300–500-fold more than Q-proIGF-1$^{66-74}$. In contrast, both Q-proPACAP$^{141-151}$ and Q-proCyritestin$^{449-462}$ are cleaved with poor efficiencies.

Peptidyl-MCA

The measured kinetic parameters V_{max} (app) and K_m (app) for *in vitro* cleavages by PC4 of various peptidyl-MCA substrates

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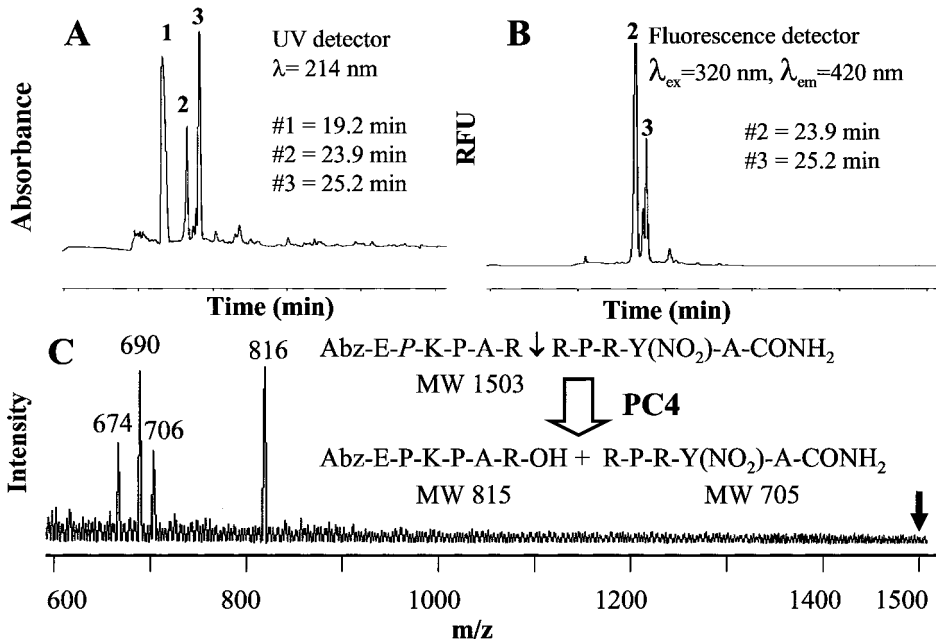

Figure 2 RP-HPLC chromatograms (A, B) on 2 h digest and MS analysis (C) on 18 h digests of Q-ADAM-5-mutant with rPC4

RP-HPLC chromatograms showing the separation of digestion products of Q-ADAM-$5^{380-388}$-mut (10 μg) by recombinant PC4 (10 μl, representing approx. 3.6 μmol of AMC released/h from 100 μM pERTKR-MCA) after 2 h incubation at 37 °C. Elution of the peaks was monitored on-line by UV absorbance at 214 nm wavelength (**A**) as well as by fluorescence measurement (**B**) (excitation and emission wavelengths fixed at 320 and 420 nm respectively). The three eluted peaks with $R_t = 19.2$, 23.9 and 25.2 min, marked as 1, 2 and 3 respectively, were characterized as the C- and N-terminal fragments and the undigested Q-ADAM-$5^{380-388}$-mut peptide. Note that the non-fluorescent C-terminal peptide ($R_t = 19.2$ min) could be detected only by UV absorbance and not by fluorescence measurement, whereas, as expected, the highly fluorescent N-terminal fragment ($R_t = 23.9$ min) and the weakly fluorescent undigested peptide ($R_t = 25.2$ min) were both noticeable under two detection systems. (**C**) MALDI–TOF-MS on 18 h crude digest of Q-ADAM-$5^{380-388}$-mut (10 μg) [Abz-E***P***KPAR↓RPRY(NO_2)A-$CONH_2$] by PC4 (10 μl). The peptide (*m/z* 1503) was fully digested yielding peaks at *m/z* 816 and 706 for the N-terminal fragment, Abz-E***P***KPAR-OH and the C-terminal fragment, RPRY(NO_2)A-$CONH_2$. The peaks at *m/z* 690 and 674 were generated due to the loss of one and two 16 mass units (NH_2) respectively from the C-terminal parent ion *m/z* 706.

Table 3 Kinetic parameters for the cleavage of IQF peptides by recombinant PC4

Substrate	V_{max} (app) ($M \cdot S^{-1} \times 10^{-6}$)	K_m (app) ($M \cdot L^{-1} \times 10^{-6}$)	V_{max} (app)/K_m (app) ($L \cdot S^{-1}$)	V_{max} (app)/K_m (app) (relative)
Q-hproIGF-1^{66-74}	1.6 ± 0.01	35.4 ± 1.1	0.045	1
Q-hproIGF-2^{63-71}	158 ± 1.2	6.9 ± 1.3	22.90	509
Q-hproPACAP$^{141-151}$	2.1 ± 0.02	1.3 ± 0.1	1.62	36
Q-hproPACAP$^{141-151}$-mut	780 ± 10	7.59 ± 0.5	102.77	2283
Q-hproPACAP$^{99-111}$	ND*	ND	ND	ND
Q-mproCyritestin$^{449-462}$	194 ± 0.7	83.9 ± 2.7	2.31	51
Q-mFertilin $\alpha^{428-440}$	455.5 ± 1.7	33.0 ± 1.3	13.80	307
Q-hFertilin $\beta^{438-450}$	256.5 ± 1.8	15.4 ± 0.3	16.65	370
Q-mADAM-$5^{380-388}$-mut	400.5 ± 6.0	3.3 ± 0.1	121.3	2696
Q-mADAM-$5^{380-388}$	38.5 ± 0.3	0.63 ± 0.07	61.10	1358

* ND, not determined, since it is very poorly cleaved.

indicated that among those tested, namely Boc-RVRR-MCA (I), pERTKR-MCA (II) and Ac-KTKQLR-MCA (III), Ac-KTKQLR-MCA (III) is by far the most efficiently cleaved (results not shown). This is followed in order by pERTKR-MCA and Boc-RVRR-MCA respectively. A similar conclusion is also reached when PC4 activity is monitored on-line by progress curves (Figure 3). As shown spectrofluorimetrically using 100 and 50 μM peptidyl-MCA and 10 μl of recombinant PC4 enzyme (representing an activity of approx. 3.6 μmol of AMC released/h from 100 μM pERTKR-MCA), the hexapeptide Ac-KTKQLR-MCA (III) exhibited a much higher release of free AMC compared with pERTKR-MCA and Boc-RVRR-MCA. At 100 μM concentration, the estimated slopes for (III), (II) and (I) are respectively approx. 22 300, 2800 and 1300 units, whereas at 50 μM concentration these are 12 100, 1200 and 600 units. This suggests that (III) is nearly 16–17- and 8–9-fold more potent substrate than (I) and (II) respectively. As expected, the control WT concentrate medium obtained from empty vector did not display any significant positive slopes under identical condition with any of the peptidyl-MCA used, suggesting a lack of cleavage at R↓MCA site (Figure 3).

Specificity of Ac-KTKQLR-MCA as a PC4 substrate

The efficiency of cleavage of Ac-KTKQLR-MCA by various mammalian convertases was examined *in vitro* and the measured

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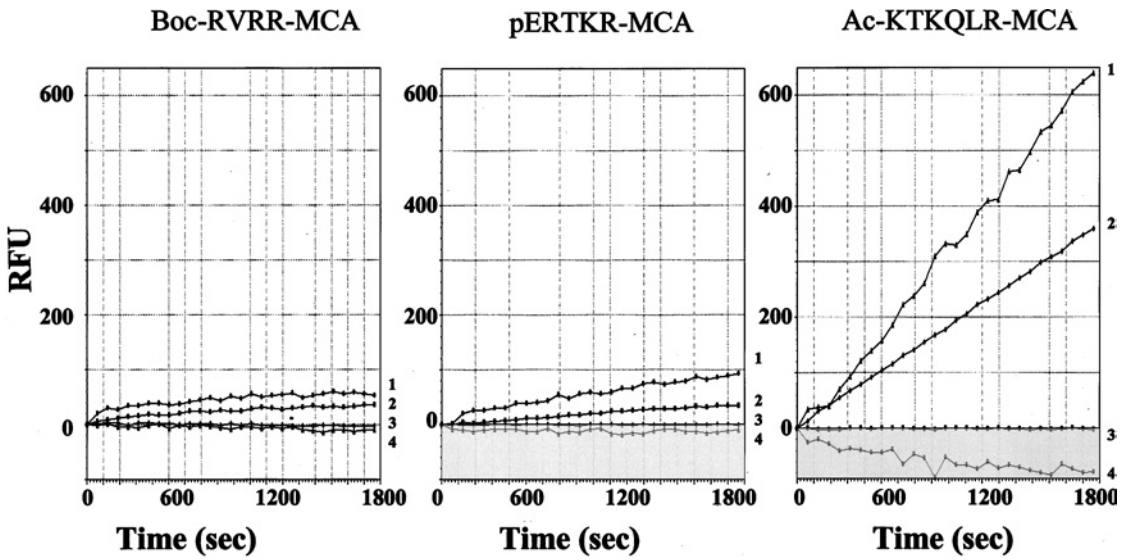

Figure 3 Progress curves showing the release of fluorescence on cleavages of various peptidyl-MCAs by recombinant PC4

Progress curves showing the release of fluorescence as a function of time on incubation of recombinant PC4 (10 μl) with various peptidyl-MCAs at 100 μM (curve 1) and 50 μM (curve 2) concentrations. The incubation was performed at 37 °C in a buffer consisting of 25 mM Mes, 25 mM Tris and 2.5 mM $CaCl_2$ (pH 7.4) and the fluorescence readings were measured at $\lambda_{ex} = 360$ and $\lambda_{em} = 470$ nm. For control, the progress curves were obtained for each peptidyl-MCA at 100 μM (curve 3) and 50 μM (curve 4) concentrations with WT concentrate medium (10 μl) under identical conditions. As expected, modest to strong positive slopes (enzyme activity) were obtained with PC4 samples (curves 1 and 2) depending on the nature of the peptidyl-MCA, whereas no significant slopes (no enzyme activity) were seen with WT medium for all the peptidyl-MCA used.

Table 4 A comparison of kinetic parameters for cleavage of Ac-KTKQLR-MCA by various recombinant proprotein convertases

Enzyme	V_{max} (app) ($M \cdot S^{-1} \times 10^{-6}$)	K_m (app) ($M \cdot L^{-1} \times 10^{-6}$)	V_{max} (app)/K_m (app) ($L \cdot S^{-1}$)	V_{max} (app)/K_m (app) (relative)
PC1	1881 ± 4.0	31.9 ± 2.1	59	9.2
Furin	149 ± 1.53	23.3 ± 1.3	6.4	1
PC4	1135 ± 0.5	32.6 ± 1.7	34.8	5.4
PACE4	532 ± 1.4	5.34 ± 0.5	99.6	15.6
PC5	1085 ± 6.1	146.9 ± 16.7	7.4	1.2
PC7	3389 ± 7.4	72.2 ± 3.9	46.9	7.3
Kexin	22 797 ± 77	26.9 ± 2.9	847.5	132.4

kinetic parameters V_{max} (app) and K_m (app) were compared with that of yeast kexin (Table 4). It is evident that yeast kexin cleaves this peptide-MCA with highest degree of efficiency. Among the mammalian PCs, PACE4 is the most effective cleaving enzyme followed respectively by PC7, PC1 and PC4. However, the substrate is cleaved poorly by both furin and PC5. Thus this substrate is not selective to PC4 activity. In fact, it appears to be more selective towards PACE4 with cleavage efficiency of more than 2.4-fold compared with that by PC4.

Specificity study of IQF peptides

Most IQF peptides (listed in Table 1) were investigated for their specificity as substrates towards PC4 activity as compared with PC7, the only other PC expressed in germ cells and the ubiquitously expressed convertase, furin. Our results indicated that among those tested including Q-ADAM-$5^{380-388}$-mut, only Q-proPACAP$^{141-151}$-mut was found to be most selective towards PC4. On the basis of the measured kinetic parameters against PC4, PC7 and furin (Table 5), it is evident that this substrate is cleaved *in vitro* by recombinant PC4 >1000-fold more efficiently when compared with both furin and PC7.

Table 5 Comparison of kinetic parameters for cleavage of Q-PACAP$^{141-151}$-mutant by the proprotein convertases, PC4, PC7 and furin

Kinetic parameters	PC4	Furin	PC7
V_{max} (app) ($M \cdot S^{-1} \times 10^{-6}$)	780 ± 10	0.52	0.96
K_m (app) ($M \cdot L^{-1} \times 10^{-6}$)	7.59 ± 0.5	6.3	10.1
V_{max} (app)/K_m (app) ($L \cdot S^{-1}$)	102.77	0.083	0.095
Fold	1238	1	1.14

Bioassay of PC4 activity

To examine the applicability of IQF peptides as specific substrates for bioassay of PC4 activity, sperm extracts of adult PC4-KO and -WT mice were incubated with 100 μM of each IQF substrate at 37 °C. The reaction was followed spectrofluorimetrically as well as by RP-HPLC and MS. On-line and stop-time assays (Figures 4A and 4B) for the release of fluorescence in duplicate samples using Q-ADAM-$5^{380-388}$-mutant as substrate indicated that WT sperm extract exhibited enzymic activity nearly 2-fold higher than that in the sperm extract of PC4-null mice. This is more consistent with PC4 activity. Interestingly, when Q-proIGF-2^{63-71} is used as a substrate, the KO-sperm extract showed only a negligible level of activity when compared with the WT sperm extract. In fact, there is a difference of at least 4-fold between the two. This suggests that, although less potent, Q-proIGF-2^{63-71} is more selective towards PC4 activity when compared with Q-ADAM-$5^{380-388}$-mutant. A similar observation is also seen with Q-proIGF-1^{66-74}. Fertlin α-, proCyritestin- and proPACAP-derived IQF peptides all exhibited higher activities in the KO-sperm extract compared with the WT-sperm extract (Figure 4C), suggesting that these fluorogenic peptides are not specific towards PC4 activity and are in fact more consistent with activities displayed by other convertases.

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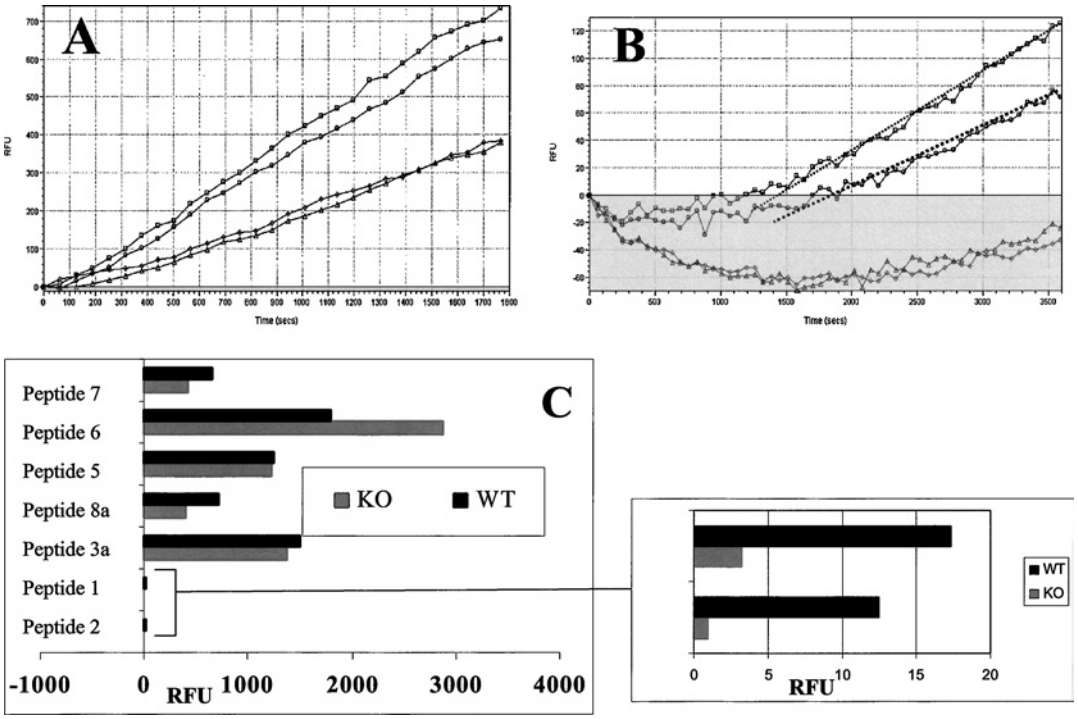

Figure 4 Progress curves showing on-line release of fluorescence and bar graphs (C) representing stop-time assay for PC4-like activity in sperm extracts of normal adult and PC4-null mice using various IQF peptides

Progress curves showing the time course of the cleavage of 100 μM of Q-ADAM-5$^{380-388}$-mut (**A**) and Q-IGF-2$^{63-71}$ (**B**) by sperm extracts of normal adult mice in duplicate (upper two curves represented by ○ and □) and PC4-null mice (lower two curves represented by △ and ◇). The release of fluorescence was measured at excitation and emission wavelengths set at 320 and 420 nm respectively. Note the differences in the pattern of the curves for two substrates. While the release of fluorescence is spontaneous with ADAM-5$^{380-388}$-mut as substrate, it is very slow and weak with Q-IGF-2$^{63-71}$ substrate. Besides, with the latter, there is a significant period of equilibration at the initial stage of incubation following which a steady release of fluorescence is observed. The slopes (indicated by ----) are measured after a steady state of fluorescence release is observed. (**C**) The bar chart representation of PC4-like activity in sperm extracts of normal adult mice (black bar) and PC4-null mice (shaded bar) using various IQF substrates (100 μM final concentration). The peptides used were Q-fertilin $\beta^{438-450}$ (Peptide 7), Q-fertilin $\alpha^{428-440}$ (Peptide 6), Q-proCyritestin$^{449-462}$ (Peptide 5), Q-ADAM-5$^{380-388}$-mut (Peptide 8a), Q-proPACAP$^{141-151}$-mut (Peptide 3a), Q-proIGF-1$^{66-74}$ (Peptide 1) and Q-IGF-2$^{63-71}$ (Peptide 2). The bar graphs for Q-proIGF-1$^{66-74}$ and Q-proIGF-2$^{63-71}$ were enlarged for better view (inset lower right). RFU, raw fluorescence unit.

DISCUSSION

This *in vitro* study reaffirms the earlier notion that the neuropeptide proPACAP is processed in reproductive tissues by PC4 although in another tissue such as endocrine/neuroendocrine, where proPACAP is also present, the same processing is performed by other convertases such as PC1 and PC2 [3,4]. Similarly, the study also confirms that both proIGF-1 and -2 are natural substrates for PC4, being cleaved at the correct physiological sites **K**SA**R**^{71}↓SVR and **K**SE**R**^{68}↓DVS respectively, generating the functionally active growth factors IGF-1 and -2 [43–45].

Results presented indicate that several proteins of the ADAM family that are expressed on the surface of sperm or acrosome may be processed by PC4 before they become functionally active. The study provides additional evidence in support of proposed sites of cleavages of these precursor proteins. Despite the fact that in the design of some model peptides, a cysteine residue was substituted by a methionine residue for reasons explained, it is concluded that fertilin α and β, also known as ADAM-1 and -2, are most probably cleaved by germ-cell-specific convertase PC4 within their cysteine-rich domains at R**R**^{437}↓AA and C**R**^{446}↓PS respectively. These cleavages are crucial for sperm-egg fusion and fertilization, where the cleaved matured forms of fertilin α and β play important physiological roles [29,30]. The above cleavages led to the formation of bioactive forms, consistent with those found under *ex vivo* conditions after maturation. Similarly, based on our results with synthetic peptides, it is most probable that two other testis-specific ADAM proteins, namely ADAM-3 (also called proCyritestin) and ADAM-5 are processed by PC4 at **R**LC**R**^{458}↓KS and **K**PA**R**^{385}↓RP respectively. Although various studies have been conducted on proteolytic maturation of proCyritestin and its implication in fertilization [46], not many results are available on the biochemistry and physiology of ADAM-5 processing [24,25]. The above cleavage site of proCyritestin is in conformity with that found in *in vivo* experiment [46]. It may be mentioned that in the three cases, namely proCyritestin, fertilins α and β, the results are based on cleavage of model WT peptides, where cysteine has been substituted by a methionine residue. This substitution is intended to avoid the potential problem of polymerization and aggregation of the WT peptide without affecting significantly the overall geometry and charge distribution of the molecule. We rationalize that both cysteine and methionine residues contain the same hydrophilic sulphur atom at β-position and therefore its effect, if any, on proteolytic event will be maintained despite substitution. Moreover, a methionine residue contains a blocked S atom, similar to disulphide bridged cysteine residues found in the physiological substrates. It is quite possible that there is many more ADAM or other surface proteins that may also serve as physiological substrates of PC4. In fact, the testis-specific ADAM-6 [47] is also believed to be processed by PC4 and initial study in our laboratory seems to indicate that this processing occurs at HNITKTYR^{418}↓FCGN site (A. Basak et al., unpublished work).

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The present study also revealed that IQF peptides, based on the physiological substrates of PC4 around the processing site, may offer a suitable avenue for development of efficient and specific bioassay for enzymic activity of PC4. In fact, based on our results using sperm extracts of adult normal and PC4-KO mice (Figure 4), we conclude that the IQF-peptides Q-mADAM-$5^{380-388}$-mut, Q-hIGF-2^{63-71} and Q-hIGF-1^{66-74} may serve as useful fluorogenic agents for bioassay of PC4 activity. In particular, the last two peptides, although much less sensitive, are more specific towards PC4 activity, whereas the former peptide is more potent but slightly less selective for PC4 activity since with this substrate the KO-sperm extract exhibited a significant basal activity. However, all other substrates displayed a higher level of activity for WT-sperm extract compared with KO-sperm extract (Figure 4).

It may be pointed out that Q-mADAM-$5^{380-388}$ contains two potential PC-cleavage sites at ^{380}E**RK**PA^{385}**R**↓^{386}**R**↓P^{388}**R** within the segment (380–388) selected for the present study. Of these, PC4 cleaves only the site R**K**PA^{385}**R**↓RPR, reinforcing our notion that PC4 cleaves best the sequence **K**XXR↓, which may be considered as the minimal sequence motif for PC4. This is further supported by the fact that Ac-**K**T**K**QL**R**-MCA, which contains the above motif, is also a potent fluorogenic substrate for PC4. Consequently, Ac-**K**T**K**QL**R**-MCA is another useful fluorogenic agent for bioassay of PC4 activity.

Among all those tested, Q-ADAM-$5^{380-388}$-mutant is by far the most potent *in vitro* substrate for PC4 followed closely by proPACAP$^{141-151}$-mutant. The common features of these substrates are the presence of a proline residue at P2′ position and the KXXR↓ motif. A higher degree of cleavage of Q-mADAM-$5^{380-388}$-mutant by PC4 compared with its WT and other sequences suggested that the presence of proline residues at P3 and P5 positions is also crucial for efficient recognition by PC4. In fact, a substitution of P5 arginine by proline in Q-mADAM-$5^{380-388}$ led to a 2.5-fold increase in PC4-cleavage efficacy. The present study indicated the crucial importance of not only P4 lysine and P1 arginine but also proline at P5, P3 and P2′ positions. In conclusion, we state that the overall best recognition motif of PC4 is represented by the sequence PKPXR↓XP. This information will become useful for our ongoing research efforts to design specific inhibitors of PC4 that may find useful applications in male contraception.

We thank Dr Nabil G. Seidah (Clinical Research Institute of Montreal) for the supply of recombinant PC7. We also thank Haidy Tadros and Charles Gyamera for the preparation of recombinant PC4 and sperm collection. Thanks are due to JoAnn MacDonald, OHRI, for secretarial assistance. This work is supported by grants from Natural Science and Engineering Research Council (M. M. and A. B.) and Canadian Foundation of Innovation and Ontario Innovation Trust (A. B.).

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Received 15 September 2003/5 January 2004; accepted 18 February 2004
Published as BJ Immediate Publication 18 February 2004, DOI 10.1042/BJ20031405

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