Enzymic characterization *in vitro* of recombinant proprotein convertase PC4

Ajoy BASAK*†‡¹, Bakary B. TOURE§, Claude LAZURE‡, Majambu MBIKAY*, Michel CHRÉTIEEN*† and Nabil G. SEIDAH§

*Molecular Neuroendocrinology Laboratory, Clinical Research Institute of Montréal, Montréal, Qué, Canada H2W 1R7,

†Protein Chemistry Center, Loeb Health Research Institute, Ottawa Hospital Medical Hospital, 725 Parkdale Avenue, Ottawa, ON, Canada K1Y 4K9,

‡Biochemical Neuroendocrinology Laboratory, Clinical Research Institute of Montréal, Montréal, Qué, Canada H2W 1R7, and

§Structure and Metabolism of Neuropeptides Laboratory, Clinical Research Institute of Montréal, Montréal, Qué, Canada H2W 1R7

Proprotein convertase PC4A, a member of the subtilisin/kexin family of serine proteases, was obtained in enzymically active form following expression of vaccinia virus recombinant rat (r)PC4A in GH4C1 cells. It displayed maximal activity at pH 7.0 and a Ca^{2+} concentration of 2.0 mM. Using PC4-specific antibodies, Western blot analysis of the medium revealed a major band at ≈ 54 kDa, corresponding to the molecular size of mature rPC4A. Among the various peptidyl-[4-methylcoumarin 7-amide (MCA)] substrates tested, the one that was preferred the most by rPC4A was acetyl (Ac)-Arg-Lys-Lys-Arg-MCA, which is cleaved 9 times faster (as judged from V_{max}/K_m measurements) than the best furin and PC1 substrate, pGlu-Arg-Thr-Lys-Arg-MCA. Recombinant rPC4A, along with human (h)furin and hPC1, cleaved a 17-amino-acid synthetic peptide, YQTLRRRVKR↓SLVVPTD (where ↓ denotes site of cleavage, and the important basic residues are shown in bold), encompassing the junction between the putative pro-segment of rPC4A and the active enzyme, suggesting a possible auto-activation of the enzyme. In an effort to identify potential physiological substrates for PC4, studies were performed with pro-[insulin-growth-factor (IGF)]-derived synthetic peptides, namely Ac-PAKSAR↓SVRA (IGF-I^{66–75}) and Ac-PAKSER↓DVST (IGF-II^{63–72}), as well as two lysine mutants [(IGF-I^{66–75}Lys⁷⁰) and (IGF-II^{63–72}Lys⁶⁷)]. Unlike

PC1 and furin, rPC4A cleaved efficiently both IGF-I^{66–75} and IGF-II^{63–72}, suggesting a possible role of PC4 in the maturation of IGF-I and -II. In contrast, the peptides with a position 2 (P2) lysine mutation, IGF-I^{66–75}Lys⁷⁰ and IGF-II^{63–72}Lys⁶⁷, were cleaved more efficiently by PC1 and furin compared with rPC4A. Furthermore, using synthetic peptides containing the processing sites of pituitary adenylate-cyclase-activating polypeptide (PACAP)-38, we were able to confirm that, of the two testicular enzymes PC4 and PC7, PC4 is the best candidate enzyme for maturation of PACAP. Our data suggest that rPC4A is a functionally active convertase, with a substrate specificity somewhat different from that of other convertases, namely KXXR↓ (where X denotes any other residue). As expected, *p*-chloromercuribenzoic acid and metal chelators such as EDTA, EGTA and *trans*-1,2-diaminocyclohexane-*N,N,N'*-tetraacetic acid inhibit the proteolytic activity of rPC4A, whereas it is activated by dithiothreitol. PC4A was also inhibited by transition-metal ions ($\text{Cu}^{2+} > \text{Hg}^{2+} > \text{Zn}^{2+} > \text{Ni}^{2+} > \text{Co}^{2+}$), as well as by small peptide semicarbazones (SCs), such as Arg-Lys-Lys-Arg-SC (K_i 0.75 μM) and Arg-Ser-Lys-Arg-SC (K_i 11.4 μM).

Key words: inhibitor, protease activity, protein substrate, substrate specificity.

INTRODUCTION

Following the discovery of proprotein convertases (PCs), the mammalian counterparts of the serine proteases of yeast kexin and bacterial subtilisin, it became important to study their structural and functional properties. Consistent with their substrate specificity, these convertases share a common function of processing a wide variety of pro-hormones and/or pro-proteins, as established by both *in vivo* and *in vitro* studies. These include neuropeptides, biologically active proteins and their receptors, growth factors, viral glycoproteins, surface proteins, and others [1,2]. In all cases, cleavage occurs on the C-terminal side of either a single Arg or a pair of basic residues (mainly Lys-Arg or Arg-Arg). To date, seven members of this family have been discovered. These are furin [also called paired basic amino acid converting enzyme (PACE)], PC1 (also called PC3), PC2, PACE4, PC4, PC5 (also known as PC6) and the most recently discovered member, PC7, also called LPC, PC8 or SPC7 [1,2]. The analysis of both

tissue and cellular distribution, as well as gene regulation of the convertases, demonstrated a complex, but unique, pattern for each enzyme. Thus PC1 and PC2 are only expressed in endocrine and neuroendocrine tissues and cells. PC4 is found exclusively in germ cells, suggesting a possible reproductive function for this enzyme. PC5 and PACE4 are present in a wide array of tissue types [1,2]. Both furin and PC7 exhibit the most widespread tissue distribution. Varying degrees of overlap of proteolytic activity of convertases have been reported, indicating both redundancy and specificity in their biological functions [3–5].

Whereas the biosynthetic forms and *in vitro* properties of most convertases, including PC7 [6], have been examined quite extensively, little is known about PC4, which was discovered originally in 1992 [7,8]. In fact, the deduced cDNA sequence was the only criterion used for inclusion of this enzyme in the convertase family. Five mRNA transcripts (rPC4A–rPC4E) were identified, which differ with respect to their C- or N-terminal truncations [8,9]. It has been demonstrated that alternative

Abbreviations used: ac, acetyl; AMC, 7-amino-4-methylcoumarin; Boc, *t*-butoxycarbonyl; CDTA, *trans*-1,2-diaminocyclohexane-*N,N,N'*-tetraacetic acid; ECL, enhanced chemiluminescence; FAB, fast atom bombardment; h, human; m, mouse; MCA, 4-methylcoumarin 7-amide; PACAP, pituitary adenylate-cyclase-activating polypeptide; PACE, paired basic amino acid converting enzyme; PC(s), proprotein convertase(s); Pmc, 2,2,5,7,8-pentamethylchroman-6-sulphonyl; pro-IGF, proinsulin-like growth factor; r, rat; RP, reverse phase; SC, semicarbazone; VV, vaccinia virus.

¹ To whom correspondence should be addressed, at the Ottawa Hospital Medical School (e-mail abasak@lri.ca).

splicing is responsible for the generation of these 5' and 3' splice variants. The expression of mRNA for PC4 is largely restricted to testicular germ cells and, to some extent, within hormonally stimulated ovaries [7,8,10], whereas Western blot analysis revealed an immunoreactive protein in epididymal spermatozoa. The mouse (m)PC4 gene possesses an organization very similar to that of other convertases [8,11]. Recently, a PC4-null mouse has been obtained, and it was demonstrated that the fertility of homozygous null-animals is severely impaired [12]. It was also suggested that PC4 might be more important in the male than in the female for achieving fertilization, and for supporting early embryonic development in mice [12]. From their cDNAs, it was predicted that mPC4A and rat (r)PC4A would be soluble enzymes composed of 654 or 700 amino acids respectively. Co-expression of PC4 and several potential substrates, such as proenkephalins, pro-opiomelanocortin, 7B2, prorenin, amyloid precursor protein and pro-von Willebrand factor, by us and others [4,13] has so far yielded negative results. Until now, only one possible physiological substrate for PC4, namely pituitary adenylate-cyclase-activating polypeptide (PACAP), has been identified [14]. However, on the basis of localization in the testis and other studies, it is also likely that proinsulin-like growth factors (pro-IGFs)-I and -II could represent other potential substrates of PC4.

In the present study we report the first *in vitro* enzymic properties of recombinant rPC4A overexpressed in mammalian GH4C1 cells using the vaccinia virus (VV) expression systems. The processing of a large variety of synthetic fluorogenic substrates, as well as small peptides derived from pro-IGF-I, proIGF-II, PACAP and the junction of the pro-segment of PC4 and its catalytic subunit, were examined in order to elucidate the cleavage specificity and to identify possible substrates of PC4.

EXPERIMENTAL

Preparation of pCITE vector

The pCITE vector (Novagen; Madison, WI, U.S.A.) was digested with *Nco*I, blunted using the large fragment of DNA polymerase I (Klenow), and then digested with *Xba*I to generate a 3.6 kb band. All restriction and modification enzymes were purchased from Gibco Life Technologies (Burlington, ON, Canada).

Preparation of the 5'-end of rPC4

Two oligonucleotides (sense, 5'-GGCCCTCCCAGACAGCG-3'; antisense, 5'-AGATCTTGTCTATCTCCTTGTC-3') of the rat PC4 sequence were used to amplify a 386-bp cDNA fragment by PCR. A typical PCR reaction consisted of a rPC4 cDNA plasmid as template at a concentration of 5 ng, 1 μ M of both sense and antisense oligonucleotides, 2.5 mM $MgCl_2$, 1 $\times$ PCR buffer, 0.4 mM dNTPs and 0.5 unit of DNA *Taq* polymerase enzyme. The sense oligonucleotide was adapted with a linker (ATTACCATGG-) at its 5'-end, which was used to provide the initial methionine once the PC4 fragments were inserted into the proper open reading frame of the vector. The PCR product thus obtained was then blunted with T4 DNA polymerase, in the absence of dNTPs, to remove the 3' adenosine generated by the PCR reaction. A *Hinf*I digestion of the blunted PCR product yielded the 5' rPC4 fragment of 236 bp used in the final construct. The 5'-end of PC4 is quite unusual, in that it is relatively short (32 nt), and is almost exclusively made up of G and C bases [9]. Its replacement as described above significantly improved PC4 mRNA translation *in vitro* (unpublished results),

leading us to propose that this might increase in infected cells too.

Preparation of the 3'-end of rPC4

The 3'-end of rPC4 used in this construct came from an existing construction of rPC4 cDNA in the pBluescript vector [8], which was digested with *Hinf*I and *Xba*I to yield a 2.2 kb band.

All the fragments used to generate this 5'-modified rPC4 construct were separated by agarose gel electrophoresis; the DNA bands of interest were excised and purified from the agarose using the GeneClean purification technique (BIO 101 Inc., La Jolla, CA, U.S.A.). The rPC4 5'- and 3'-fragments were ligated together in a 10:1 molar ratio, and the ligation reaction was confirmed by gel electrophoresis. Ligation of the blunt ends was used to generate *Ssp*I sites; the ligation reaction mixture was then digested with *Ssp*I (blunt end) and *Xba*I restriction enzymes to yield the 5'-modified rPC4 fragment of 2.4 kb. The pCITE-digested vector and the 5'-modified rPC4 fragment were then ligated together in a 1:3 molar ratio, and were used to transform XL1-Blue competent cells (Invitrogen, Carlsbad, CA, U.S.A.). Individual clones were analysed by restriction-enzyme digestion, and sequenced to confirm the accuracy of the reading frame [15–17].

Cellular infections and isolation of recombinant rPC4A

Essentially, a procedure similar to that reported previously for PC1 [18] was followed. In brief, GH4C1 cells, a mammalian somatomammotrophic cell line (Biosystems, CA, U.S.A.), were grown to confluence in air/CO₂ (9:1) at 37 °C in 15-cm-diam. tissue-culture dishes, using minimum essential medium supplemented with 10% (v/v) fetal bovine serum (Life Technologies Inc.) and 28 μ g/ml gentamycin. Following a brief rinse with PBS, the cells were infected with recombinant viruses at 2–4 plaque-forming units/cell in 5 ml of PBS containing 0.01% (v/v) BSA for 30 min at room temperature. They were then returned to minimal essential medium plus fetal bovine serum, and incubated overnight at 37 °C. Cells were harvested twice, each time 3 h post-infection, by scraping into 20 mM Tris/acetate, pH 7.0. Suspensions from the Dounce homogenizer were centrifuged at 16000 *g* for 15 min at 4 °C in a microcentrifuge; the supernatants containing soluble cellular material were then removed and stored at –20 °C before further use. After collection of 2.5 l of medium, the frozen pools were thawed and concentrated to 3 ml ($\approx$ 840-fold) on a Centriprep-30 (Amicon, Danvers, MA, U.S.A.) cartridge. This was then centrifuged at 30000 *g* for 15 min to eliminate all the suspended cellular particles, and the clear supernatant was separated into 100- μ l aliquots that were stored at –20 °C. As a control, VV:WT/GH4C1 medium lacking rPC4A cDNA was similarly prepared, and stored for the purposes of comparison.

Antibodies against PC4

Three polyclonal antibodies raised against PC4, developed in the rabbit, were used in the present study (Figure 1). Antibody Ab-1 was raised against the synthetic peptide (D-Y⁷⁴+rPC4^{75–90}) of amino acid sequence D-Y⁷⁴QTLRRRVKRSLLVPTD⁹⁰ located in the N-terminal domain of rPC4A [8]; antibody Ab-2 was developed using a C-terminal fusion protein (rPC4A^{554–654}); and antibody Ab-3 was generated against the whole PC4A protein by injecting the plasmid containing PC4A cDNA, following the procedure reported previously [19].

Figure 1 Schematic representation of the structure of rPC4A and subtilisin BPN'

The various domains of these proteases are depicted according to the key shown at the bottom of the Figure. The total number of amino acids featured in each enzyme is indicated on the right. The various amino acid regions used to produce rPC4A-specific antisera are also shown.

Western blot analysis

Media from rPC4A-infected GH4C1 cells were analysed by using SDS/PAGE (10 % gels), and the separated proteins were electro-transferred on to PVDF membranes (Schleicher and Schuell, Keene, NH, U.S.A.). Protein bands were revealed by enhanced chemiluminescence (ECL) (Boehringer Mannheim, Indianapolis, IN, U.S.A.) using a primary rabbit antiserum [20] against either Ab-1 or Ab-2 (Figure 1). The specificity of the antiserum was verified by demonstrating that the bands made visible in the rPC4A-infected cells or medium could be blocked by pre-incubation with 100 µg/ml recombinant rPC4A protein.

Radioimmunoassays

For quantitative estimation of immunoreactive rPC4A protein in the concentrated medium and in cells obtained from VV:rPC4A-infected GH4C1 cells, three specific polyclonal antibodies, Ab-1, Ab-2 and Ab-3, were used in a procedure described previously [12,15]. Three separate samples of aliquots of various sizes were used, and the values were averaged.

Enzymic assay

The proteolytic activity of recombinant rPC4A was assayed using the commercially available synthetic peptidyl fluorogenic substrate, pGlu-Arg-Thr-Lys-Arg-[4-methylcoumarin-7-amide (MCA)] (Peptides International, Louisville, KY, U.S.A.) [18] as described below (hereafter, the residues are referred to by their single-letter codes). The enzyme sample (usually 10 µl) was routinely added to the assay mixture of a ternary buffer system

consisting of sodium acetate, Mes and *N*-ethylmorpholine (all at 30 mM final concentration) and 2 mM CaCl₂, pH 7.0. The reaction (in a final vol. of 100 µl) was initiated at room temperature in a microtitre-well plate by the addition of pERTKR-MCA at a final concentration of 100 µM. Fluorescence readings were recorded after 60 min of incubation using a Perkin-Elmer spectrofluorimeter (model LS 50B), with the excitation and emission wavelengths being set at 370 ± 3 and 460 ± 5 nm respectively.

pH, Ca²⁺ and inhibitor profiles

The protocols used were essentially the same as reported elsewhere [6,18]. For pH-sensitivity experiments, enzymic activity was measured using the previously described ternary buffer system, with the pH being adjusted in the range from 4.0 to 8.0 with 0.5 pH-unit increments by using either acetic acid or NaOH. In order to estimate the Ca²⁺ concentration required for optimal activity, increasing concentrations of Na₂EDTA were added initially to the assay mixture as described above in the absence of added CaCl₂ until no more enzymic activity could be detected. On the basis of this result, the assay mixture was supplemented with 1 mM Na₂EDTA and various concentrations of CaCl₂, ranging from 0–5 mM (final). For inhibition studies, the enzyme sample was preincubated with selected compounds in the above assay mixture for 30 min prior to the addition of pERTKR-MCA. The solutions of inhibitors were prepared in solvents as follows: EDTA, EGTA, *trans*-1,2-diaminocyclohexane-*N,N,N',N'*-tetraacetic acid (CDTA), *p*-phenylazophenylarsonic acid and 4-chloromercuribenzoic acid were prepared in 1.25 M

NaOH; PMSF, 4-amidino-PMSF and 3,4-dichloroisocoumarin were prepared in 2% (v/v) DMSO; and pepstatin was dissolved in 10% (v/v) methanol. The rest of the inhibitors used were dissolved in water at pH 6.8. For experiments involving EDTA, EGTA and CDTA, no CaCl_2 was added to the assay medium.

Fluorogenic substrates and determination of K_m (app) and $V_{\max}$ values

pERTKR-MCA was substituted with various other peptidyl-MCA substrates either obtained from commercial sources or made synthetically in the laboratory [21]. For the determination of K_m values, increasing amounts of peptidyl-MCA were added to the reaction mixture. The data, plotted as the rate of hydrolysis against concentration of peptidyl-MCA used, were subjected to non-linear regression analysis using the Enzfitter software (Elsevier-Biosoft, Cambridge, U.K.) to determine the K_m and $V_{\max}$ values.

Synthesis of peptide substrates

The following peptides were selected: residues 66–75 from pro-IGF-I [acetyl (Ac)-PAKSAR↓SVRA] (Ia), residues 66–75 from pro-IGF-I, with an Ala⁷⁰ → Lys substitution (Ac-PAKSKR↓SVRA) (Ib), residues 63–72 from pro-IGF-II (Ac-PAKSER↓DVST) (IIa), residues 63–72 from pro-IGF-II, with a Glu⁶⁷ → Lys substitution (Ac-PAKSKR↓DVST) (IIb), D-Tyr + residues 75–90 from pro-rPC4A (D-YQTLRRRVKR↓SLVVPTD) (III), residues 170–176 from PACAP (GRR↓IPYL) (IVa), residues 168–176 from PACAP (NKGRR↓IPYL) (IVb), residues 165–176 from PACAP (RVKNKGRR↓IPYL) (IVc), residues 128–135 from PACAP (LSKR↓HSDG) (Va) and residues 122–135 from PACAP (DDDSEPLSKR↓HSDG) (Vb). (Here, and subsequently throughout the paper, ↓ denotes the site of cleavage, and the important basic residues are shown in bold.)

The synthesis of these peptides was performed on an automated solid-phase peptide synthesizer (model 431A; Applied Biosystems, Foster City, CA, U.S.A.) using 2-(¹H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate-mediated Fastmoc chemistry [22,23]. All amino acids (L-configuration) were protected as fluoren-9-ylmethoxycarbonyl (Fmoc) derivatives at the N-terminal. The coupling agents and solvents were purchased from Calbiochem–Novabiochem (La Jolla, CA, U.S.A.), Bachem (Torrance, CA, U.S.A.) and PE-Biosystems (Foster City, CA, U.S.A.). The following side chain-protecting groups were used: t-butyloxycarbonyl (Boc) for lysine; t-butyl for serine, threonine, tyrosine, aspartic and glutamic acids; and 2,2,5,7,8-pentamethylchroman 6-sulphonyl for arginine. At the end of the synthesis, the peptides were cleaved from the resin and fully deprotected by treatment twice with reagent K at room temperature (5.0 ml) for 4 h each time [22,23]. The crude material obtained was purified by reverse phase (RP)-HPLC (vista 5500; Varian, Basel, Switzerland), which was performed on a C_{18} semi-preparative column (1.0 cm × 25 cm; Chromatographic Specialties Inc., St. Laurent, PQ, Canada) using, as the buffer system, an aqueous 0.1% (v/v) trifluoroacetic acid solution and an organic phase of CH_3CN , also containing 0.1% (v/v) trifluoroacetic acid. Elution was performed using a linear gradient from 5–60% of the organic phase in 60 min following a 5-min isocratic step at a flow rate of 2 ml/min, and the appearance of the eluate was monitored at a wavelength of 225 nm. The peptides were fully characterized by both fast atom bombardment (FAB)-MS and amino acid analysis. Amino acid analyses were performed following 18 h of hydrolysis in 5.7 M HCl at 110 °C in a sealed tube under a vacuum, and the hydrolysates were analysed using a modified Beckman 120C autoanalyser equipped with a Varian

DS 604 integrator/plotter, followed by post-column ninhydrin detection.

Incubation of synthetic peptides with various convertases

Incubations of various pro-IGF and pro-rPC4-related peptides (Ia, Ib, IIa, IIb and III) were carried out in a total vol. of 500 μl containing 50 μg of the peptide for time intervals ranging from 1 h to 24 h with hPC1, hPurin and rPC4A, representing activities of 15–16, 12–13 or 5–6 nmol of free 7-amino-4-methylcoumarin (AMC) released/h at 25 °C respectively. The final incubation mixtures contained, respectively, the ternary buffer and 5.0 mM CaCl_2 , pH 6.0, for hPC1, 1.0 mM CaCl_2 , pH 7.0, for Purin and PC7, and 2 mM CaCl_2 , pH 7.0, for rPC4A. The PACAP-based synthetic peptides (IVa–c and Va and Vb) were similarly incubated with only rPC4A and hPC7 [4].

Following the incubation, the aliquots were treated with 200 μl of 0.1M Na_2EDTA to terminate the reaction, and then were analysed on a Vydac 218TP54 C_{18} analytical column (0.1 cm × 25 cm) according to the conditions described above, except that the flow rate was maintained at 1.0 ml/min. The detected peptides were collected manually and subsequently analysed for amino acid composition, as well as by matrix-assisted laser desorption ionization–time-of-flight (MALDI–TOF) or ion-spray MS.

Synthesis of peptide semicarbazones (SCs)

The two tetrapeptide SCs, RSKR-SC and RKKR-SC, were synthesized as described previously [22]. The K_i values for these peptides against rPC4A were determined using Dixon's plot analysis [22,23].

Inhibition of rPC4A activity by various transition-metal ions

Five bivalent metal ions, mostly of the transition-metal series (namely Cu^{2+} , Co^{2+} , Zn^{2+} , Hg^{2+} , and Ni^{2+}) were examined for their ability to inhibit rPC4A activity. Freshly prepared aqueous solutions of CuCl_2 , CoCl_2 , ZnSO_4 , $\text{Hg}(\text{OAc})_2$ and NiCl_2 were preincubated with rPC4A for 5 min at room temperature in an assay mixture (100 μl) consisting of 30 mM each of Mes, sodium acetate and *N*-ethylmorpholine plus CaCl_2 (2 mM final concentration), pH 7.0, following which pERTKR-MCA (100 μM final concentration) was added. After another 4 h of incubation, the release of free AMC was monitored in a fluorimeter. Various concentrations of metal ions ranging from 50 to 1000 μM were used in the present study, and the residual activity was compared with the control carried out in parallel in the absence of any metal ions.

RESULTS

In vitro enzymic activity of rPC4A

Recombinant rPC4A, obtained from the medium of VV:rPC4A-infected GH4C1 cells upon concentration and dialysis, displayed a varying degree of enzymic activity towards fluorogenic peptidyl-MCA substrates such as benzyloxycarbonyl (Z)-ARR-MCA, Z-AKK-MCA, Z-KKR-MCA, Ac-KSKR-MCA, Ac-RKKR-MCA, ornithine-SKR-MCA, Ac-RSKR-MCA, Z-REKR-MCA, Boc-RVRR-MCA, pERTKR-MCA and Ac-YEKERSKR-MCA. Among them, Ac-RKKR-MCA was found to be the most potent, being cleaved at least 6 times more efficiently than Ac-RSKR-MCA or pERTKR-MCA, the commonly used fluorogenic substrates of most convertases [18,22,24,25]. In fact $V_{\max}/K_m$ values for the hydrolysis of Ac-RKKR-MCA are 10.3- and 8.4-fold greater than those for Ac-RSKR-

Table 1 Kinetic constants for the hydrolysis of synthetic peptidyl-MCA substrates by rPC4A

MCA substrate	$V_{\max}$ ($10^{-6} \cdot \text{M} \cdot \text{s}^{-1}$)	K_m (app) ($10^{-3} \cdot \text{M}$)	$V_{\max}/K_m$ ($10^{-3} \cdot \text{s}^{-1}$)	Fold
pERTKR-MCA	0.42 ± 0.05	4.21 ± 0.1	0.099	0.15
Ac-RKKR-MCA	0.12 ± 0.01	0.15 ± 0.02	0.83	1.0
Ac-RSKR-MCA	0.53 ± 0.09	6.6 ± 0.2	0.08	0.1

MCA and pERTKR-MCA respectively (Table 1). Surprisingly, even a tripeptidyl-MCA such as Z-ARR-MCA, lacking a peptidyl residue at the P4 position, was found to be as good a substrate as RSKR-MCA, REKR-MCA and RVRR-MCA, all of which possess an additional P4 arginine residue. This observation is somewhat different from that reported for other PCs, and suggests that PC4 is a distinct and functionally active convertase for which the P3 position may be more critical than for other convertases. This study also demonstrates the first *in vitro* enzymic activity of PC4.

pH profile

Employing a buffer system of different pH values ranging from 4.0 to 8.0 (see the Experimental section), it was noted that the pH optimum of rPC4A resides in the vicinity of 7.0 (Figure 2), very similar to that observed for furin [26–28] and PC7 [6]. Interestingly, both PC7 and furin maintained a significantly greater percentage of their maximal activities, even at pH 5.5, in contrast with PC4, which lost more than 90% of its maximal activity at this pH. Thus unlike PC7 and furin, PC4 displays a narrower pH optimum.

Ca²⁺ profile

A precise measurement of the Ca²⁺ requirement for maximal activity of rPC4A was accomplished, following prior measurement of endogenous Ca²⁺ in the enzyme preparation by titration with Na₃EDTA (results not shown). The Ca²⁺-activation profile of rPC4A at pH 7.0 is shown in Figure 2, which suggests that the optimal Ca²⁺ concentration for rPC4A activity is near 2.0 mM, again reminiscent of results reported for furin [26–28] and PC7 [6].

Inhibitory profile

On the basis of the study using various general and class-specific inhibitors, it was noted that the enzymic activity of rPC4A, as expected, is highly sensitive to cationic chelators such as EDTA, EGTA and CDTA, all of which, at 1–10 mM concentration, abolished almost completely the full enzymic activity. These observations confirm that this activity is indeed Ca²⁺-dependent. Furthermore, as found for other convertases, PC4A is also strongly inactivated by *p*-chloromercuribenzoate. Most cysteine protease and mixed serine/cysteine protease inhibitors had no significant effects on PC4A activity. *p*-Phenylazophenylarsonic acid, a known inhibitor of subtilisin [29], appears in this case to be a weak inhibitor of rPC4A, in contrast with its action towards PC1 and furin, both of which are strongly inhibited at a concentration of inhibitor of 0.1 mM (A. Basak and C. Lazure, unpublished work). Finally, a differential effect due to the presence of a reducing agent was observed: a 2–2.5-fold enhancement in PC4 activity occurred upon addition of 1 mM dithiothreitol. A similar effect was also noted for this compound on the activity of PC7 [6], although a completely opposite effect

Figure 2 pH-activation (A) and Ca²⁺-activation (B) profiles of recombinant rPC4A

(A) Preparations of VV:rPC4A-infected GH4C1 cells were assayed according to the protocol described in the Experimental section using pERTKR-MCA as a fluorogenic substrate. The results represent an average of three separate independent determinations for crude (●) and partially purified (○) rPC4A respectively. (B) Following prior incubation for 10 min with 1.0 mM EDTA to remove the endogenous Ca²⁺, enzyme samples from the medium of VV:rPC4A-infected GH4C1 cell lines were assayed in the presence of increasing concentrations of CaCl₂, as described in the Experimental section.

was reported with other convertases, namely ykexin and soluble furin [6].

Inhibitory influence of bivalent transition-metal ions

Consistent with earlier observations with PC1, furin [30] and PC7 [11], a strong inhibitory effect on rPC4A activity by the transition-metal ions such as Cu²⁺, Co²⁺, Ni²⁺, Zn²⁺ and Hg²⁺ was also noted; this inactivation was found to occur (in terms of decreasing inhibitory effect) in the order of Cu²⁺ > Hg²⁺ > Zn²⁺ > Ni²⁺ > Co²⁺, with IC₅₀ measurements for Cu²⁺ and Co²⁺ of

Figure 3 Immunodetection of rPC4A using Western blot analysis with Ab-1 antibody (A), or with Ab-2 antibody (B)

Samples from VV:rPC4A-infected GH4C1 cells were processed as described in the Experimental section, and analysed with SDS/PAGE (10% gels). Following electrotransfer to PVDF membranes, protein bands were made visible by ECL detection using a primary rabbit antiserum raised against the N-terminal peptide (Ab-1). (A) Left panel: VV:rPC4A-infected GH4C1 medium; right panel: VV:rPC4A-infected GH4C1 medium + rPC4A protein (100 µg/ml). Note that the band at 54 kDa, displaceable by PC4A protein, represents the full-length, active PC4A, whereas the band at 46 kDa is non-specific and not displaceable by PC4A protein. (B) The lanes were loaded as follows: left panel, first lane, VV:WT-GH4C1 medium; left panel, right lane, VV:PC4A-infected GH4C1 medium; and right panel, VV:rPC4A-infected GH4C1 medium + rPC4A protein (100 µg/ml).

12 ± 1.2 and 250 ± 4.5 µM respectively (data given as means $\pm$ S.D.). The possible cause for this inactivation may be related to interactions with metal-sensitive amino acid residues, such as cysteine, consequently leading to oxidation and/or complex formation [30].

Western blot analysis of rPC4A

Using the N-terminally directed antibody, Ab-1, Western blot analysis of the culture medium obtained from GH4C1 cells infected with VV:rPC4A revealed a distinct band at ≈ 54 kDa (Figure 3A). As expected, the appearance of this band can be

fully abolished by the presence of recombinant rPC4A protein (100 µg/ml), thereby confirming its identity. A similar band, also displaceable by rPC4A protein (100 µg/ml), was noted in the cell medium using the C-terminally directed Ab-2 antibody (Figure 3B). This ≈ 54 kDa band represents the full-length form of mature rPC4A (544 amino acids), and the molecular mass compares reasonably well with that of the endogenous enzyme (M_r approx. 52000) that is expressed in germinal cells of rat testes, and which is absent in similar cells of PC4-null mice [12]. It may be pointed out that a large majority of the recombinant rPC4A protein remained associated with the cell membrane as insoluble material, which was only partially extractable upon repeated cell lysis (results not shown).

Radioimmunoassay

Radioimmunoassays carried out on various samples of recombinant rPCA using the antibodies Ab-1, Ab-2 and Ab-3 indicated the presence of an extremely low amount of immunoreactive PC4A. On average, this ranged from 10–14 µg/l of medium, which was more than 100-fold less than that obtained in the case of PC1 [18].

Digestion profiles of synthetic peptides

The distribution of mRNA or protein levels of biologically active protein/hormone precursors in testicular germ cells [8,9,12,14,15,31] suggested a number of potential substrates of PC4A [12]. These include pro-nerve growth factor (THRSKR↓SS), pro-IGF receptor (PERKRR↓DV), (m)pro-IGF-I (PAKSAR↓SV), (m)pro-IGF-II (PAKSER↓DV), (m)pro-fertilin α -chain (QSRMRR↓AA), (m)pro-fertilin β -chain (SCKLKR↓RG), (m)ADAM5 (a disintegrin and metalloproteinase enzyme) (ARRPRR↓IC), (h)proenkephalin (GGFMKR↓HS), (r)pro-cytostatin (AERLCR↓KS) and hPACAP-related peptide (AVLGKR↓YK). In an effort to carry out chemical and enzymic characterizations, and also to determine potential substrates for PC4A, a number of synthetic peptides based on the sequences containing the putative cleavage site of pro-IGF-I (Ia and Ib), pro-IGF-II (IIa and IIb), pro-rPC4A (III) and PACAP (IVa–c and Va and Vb) were examined (Table 2). In addition to rPC4A, other convertases such as hPC1, hFurin and hPC7 were included in this study for comparison purposes.

As indicated in Table 2, both pro-IGF-I^{66–75} (Ia) and pro-IGF-II^{63–72} (IIa) were cleaved correctly by rPC4A at the expected physiological site, as confirmed by FAB-MS and amino acid analysis of the isolated products. In contrast, no significant processing of either (Ia) or (IIa) was noticed with hPC1 or hFurin, even after 24 h of incubation (Figure 4). As expected, the two lysine mutants [(Ib) and (IIb)] were both cleaved by PC4, PC1 and furin quite efficiently (Table 2), suggesting that, in the context of a monobasic cleavage, lysine at the P4 position within the sequence motif **KXXR** is recognized more easily by PC4 than by PC1 and furin.

Similarly, the pro-PC4-derived synthetic peptide (III) was also cleaved fairly efficiently by rPC4A, as well as by hFurin and hPC1 (Figure 4 and Table 2). The time required ($t_{1/2}$) for 50% cleavage of this peptide to take place by rPC4A, PC1 and furin, using an almost identical enzymic activity as measured against the fluorogenic substrate, pERTKR-MCA, showed that proPC4-derived peptide (III) was cleaved most efficiently by furin, followed by PC4 and PC1 respectively.

The synthetic PACAP-based peptides containing the N- and C-terminal processing sites (IVa, IVb, IVc and Va and Vb) were examined as possible substrates for both rPC4A and hPC7, since

Table 2 Relative cleavage rates of synthetic pro-PC4, pro-IGF-I, pro-IGF-II and PACAP-related peptides by various convertases, including rPC4A

The enzymic activity used in the digestion study of these synthetic peptides was 1.63, 1.38, and 1.45 nmol/h of released AMC from pERTKR-MCA (100 μ M final concentration) for rPC4A, hPC1 and hFurin respectively. nd, not determined. For further details of the procedure used, see the Experimental section.

Peptide sequence	Time ...	Extent of cleavage (%)							
		VV:rPC4		VV:hPC1		VV:hFurin		VV:hPC7	
		4 h	22 h	4 h	22 h	4 h	22 h	4 h	
pro-IGF-I (66–75) (Ia) (Ac-PAKSAR ↓ SVRA)	39	71	7	10	0	0			
pro-IGF-I (66–75 Lys ⁷⁰) (Ib) (Ac-PAKSKR ↓ SVRA)	nd	50	1	45	53	100			
pro-IGF-II (63–72) (IIa) (Ac-PAKSER ↓ DVST)	100	100	0	5	0	8			
pro-IGF-II (63–72 Lys ⁶⁷) (IIb) (Ac-PAKSKR ↓ DVST)	69	81	3	73	69	100			
rPC4(p-Tyr + 75–90) (III) (D-YQTLRRRVKR ↓ SLVVPTD)	25	67	15	44	84	100			
PACAP (170–176) (IVa) (GRR ↓ TPYL)	< 5								< 5
PACAP (168–176) (IVb) (NKGR ↓ IPYL)	78								< 5
PACAP (165–176) (IVc) (RVKNKGRR ↓ IPYL)	85								< 10
PACAP(128–135) (Va) (LSKR ↓ HSDG)	95								0
PACAP(122–135) (Vb) (DDDSEPLSKR ↓ HSDG)	90								2

Figure 4 (A) Treatment of IGF-I peptide (Ia) [Ac-PAKSAR ↓ SVRA] with hPC1, hFurin and rPC4A, and (B) treatment of rPC4A peptide (III) (D-YQTLRRRVKR ↓ SLVVPTD) with rPC4A, hFurin and hPC1

(A) RP-HPLC analysis of the digestion products was performed as described in the Experimental section. For furin and PC1, the amount of peptide injected was twice the amount injected for PC4-digested material. The peaks in the chromatogram for PC4-digested peptide were characterized as follows: the earliest eluting peak ($R_t = 23.9$ min) was N-terminal peptide (Ac-PAKSAR), the peak eluting next ($R_t = 24.7$ min) was C-terminal peptide (SVRA) and the last peak to be eluted ($R_t = 25.2$ min) was undigested peptide (Ia). The newly generated peaks upon digestion are indicated by asterisks. (B) RP-HPLC analysis of the digestion reactions was carried out as described in the Experimental section. The peptide (III) ($R_t = 32.6$ min) was cleaved at the expected physiological site by rPC4A, hFurin and hPC1 to yield N-terminal peptide (D-YQTLRRRVKR) ($R_t = 27.1$ min) and C-terminal peptide (SLVVPTD) ($R_t = 30.2$ min). With PC4A, two additional peaks (indicated by asterisks) at $R_t = 26.3$ min and $R_t = 28.0$ min were observed. These were attributed to the cleaved peptides D-YQTLRR and RVKRSVVPTD respectively, suggesting a second site of cleavage for PC4 as indicated (D-YQTLRR ↓ RVKRSVVPTD).

these convertases were found to be co-expressed with PACAP in germ cells [14]. Our study revealed that both (IVb) and (IVc) were cleaved by PC4, but not by PC7, whereas (IVa) was not cleaved by either convertase, again reinforcing the result that,

independently of other positions, the P4 lysine plays an important role in the substrate recognition by PC4. Again, both (Va) and (Vb) were cleaved fairly well by PC4, but not by PC7. These data *in vitro* suggest that PC4 is a better candidate enzyme for the

Figure 5 Dixon plot showing the inactivation of proteolytic activity of rPC4A by RSKR-SC (A) and RKKR-SC (B)

The activity of PC4A was followed at different substrate (pERTKR-MCA) concentrations for each inhibition study, namely 50 μM (S1) and 100 μM (S2).

processing of PACAP to PACAP-38, and possibly to PACAP-27, a notion already proposed by previous studies [14].

Ca²⁺-dependency of rPC4A activity

The dependency of proteolytic activity of rPC4A on Ca²⁺ is demonstrated by the observation that PC4-mediated cleavage of pro-IGFs and pro-PC4-related synthetic peptides can be fully abolished by the addition of EDTA (50 μM) (results not shown).

Effect of RKKR-SC and RSKR-SC on rPC4A activity

Both tetrapeptides RKKR-SC and RSKR-SC exhibited a strong inhibition of rPC4A activity in a reversible, competitive manner. This was confirmed by Dixon plot analysis, as shown in Figure 5, which indicates further that the K_i values for these peptides are 0.75 and 11.4 μM respectively. Thus RKKR-SC is nearly 66-fold more potent as an inhibitor of rPC4A than is RSKR-SC. It may be pointed out that these peptidyl-SCs were shown to be fairly strong inhibitors of both PC1 and furin, with K_i values ranging from 1.4 to 15 μM [22].

DISCUSSION

Among all the members of convertases, PC4 is probably the most unique because of its exclusive distribution in testis and ovarian cells. On the basis of mRNA distribution in various cell lines and in rat tissues, it was observed that, within the germinal cells, PC4 is found in pachetene spermatocytes and spherical spermatids of the testis [7,8]. In mouse, expression is limited only to the round spermatids of germ cells [7]. These observations, and the latest study that shows that PC4 knock-out mice possess reduced

fertility, sperm motility and are hyperactive compared with normal ones [12], suggest that PC4 probably plays an important role in reproductive biology, even though its mode of action has yet to be determined. Until now, very little was known about the enzymic properties of PC4 and its physiological substrates. The present study has now provided some important leads in this direction.

In vitro treatment of various synthetic peptides with rPC4A, hPC1 and hfurin confirmed that the peptide representing the junction between pro-PC4A and PC4A (YQTLRRRVKR ↓ SLVVPTD) was cleaved (as indicated) not only by rPC4A, but also by hPC1 and hfurin. This result indicated that, like other convertases, PC4 could also be autocatalytically activated. Both pro-IGF-I- and pro-IGF-II-derived peptides, Ac-PAKSAR ↓ SVRA (Ia) and Ac-PAKSER ↓ DVST (IIa), were cleaved at the site indicated by rPC4A, but not by PC1 and furin. It was further noted that the pro-IGF peptides that were mutated to lysine at the P2 position, namely Ac-PAKSKR ↓ SVRA (Ib) and Ac-PAKSKR ↓ DVST (Iib), were cleaved by all three convertases. These results suggest that a tetrapeptide sequence motif (KXXR) containing a P4 lysine and a P1 arginine residue is best recognized by rPC4A. The results indicate further that precursors of both IGF-I [32] and IGF-II [33,34] could represent potential substrates for PC4. It is known that these two important growth factors are generated from their precursors by the proteolytic removal of 35 and 38 amino acids respectively from their C-terminus (known as the E-domain), followed by the removal of Arg⁷¹ or Arg⁶⁹ for IGF-I or IGF-II respectively by the action of carboxypeptidase. In both cases, there is a unique 13-residue prohormone cleavage motif, characterized by the presence of five basic residues: KXXKXXR^{71/69} ↓ XXXXXR. Consistent with the PC4 distribution, IGF-I mRNA is highly expressed in germ cells, particularly after stimulation by follicle-stimulating hormone and, to a lesser extent, after treatment with growth hormone or luteinizing hormone [35]. Although IGF mRNA is largely localized specifically in Leydig cells, these cells are not the sole site of origin in the testis. In fact, non-Leydig cell expression might be an important component of testicular IGF-I production [36]. Numerous developmental abnormalities, such as growth deficiencies, delayed bone development, infertility and high mortality rate, in addition to many forms of cancer, have been linked to the expression of both IGF-I and -II [37–39]. Because of these important biological functions of IGF-I and -II, particular attention was taken to identify their processing enzymes. The studies performed to date [40,41] are sufficient to exclude furin as being the processing enzyme involved in IGF maturation, since furin-deficient Lovo cells were able to process pro-IGF-I at the correct Arg⁷¹ ↓ site, whereas transiently transfected HEK-293 cells process pro-IGF-I at the Arg⁷¹ ↓ site, as well as at another site C-terminal to this, possibly Arg⁷⁷ ↓. In a recent study of co-expression employing constructs of furin, PACE4, PC5A, PC5B and PC7, Duguay et al. [41] demonstrated that PACE4 and PC5A/PC5B are the most likely candidates for being the pro-IGF convertase. Our studies have now added PC4A to the list as another candidate for being the processing enzyme of pro-IGF-I and pro-IGF-II in the testis, and have also confirmed the earlier conclusion that neither furin nor PC1 is able to produce mature IGF-I from its precursor.

Recently, PC4 has been implicated in the processing of PACAP [14]. PACAP was isolated from the hypothalamus, and is known to exist as two amidated forms with 38 or 27 amino acids (PACAP-38 or PACAP-27 respectively). Studies on the immunohistochemistry of rat testis demonstrated strong PACAP-like immunoreactivity in spermatids in both cap and acrosomal phases [41,42]. An almost identical pattern of expression of PC4

transcripts and PACAP in germ cells during spermatogenesis was reported, and experiments involving co-transfection of precursor PACAP and PC4A in a GH4C1 cell line suggested that the PACAP precursor in the testis might be a possible substrate for PC4A, being cleaved to generate biologically active PACAP-38, which stimulates adenylate cyclase much more potently than vasoactive intestinal peptide [43,44]. Our data concerning the PC4-mediated processing of synthetic PACAP-derived peptides (IVa-c and Va and Vb), containing the activation sites, confirm that PACAP represents another physiological substrate for PC4.

In this study, we have also demonstrated that small synthetic peptide SCs may represent strong inhibitors of PC4A. Thus RKKR-SC inhibits PC4A activity with an estimated K_i of $0.75 \mu\text{M}$, which is 6 times better than the inhibition obtained with PC1, and 16-fold better than that of RSKR-SC (K_i $11.4 \mu\text{M}$) against rPC4A. These results confirm that, for an efficient cleavage at the post-dibasic site to take place, PC4 requires a P3 lysine residue (XKKR).

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REFERENCES

- Seidah, N. G. and Chtien, M. (1997) *Curr. Opin. Biotechnol.* **8**, 602–607
- Steiner, D. F. (1998) *Curr. Opin. Chem. Biol.* **2**, 31–39
- Day, R., Schfer, M. K. H., Watson, S. J., Chtien, M. and Seidah, N. G. (1992) *Mol. Neuroendocrinol.* **6**, 485–497
- Marcinkiewicz, M., Ramla, D., Seidah, N. G. and Chtien, M. (1994) *Endocrinology* (Baltimore) **135**, 1651–1660
- Dong, W., Marcinkiewicz, M., Vieau, D., Chtien, M., Seidah, N. G. and Day, R. (1995) *J. Neurosci.* **15**, 1778–1796
- Munzer, J. S., Basak, A., Zhong, M., Mamarbachi, A., Hamelin, J., Savaria, D., Lazure, C., Benjannet, S., Chtien, M. and Seidah, N. G. (1997) *J. Biol. Chem.* **272**, 19672–19681
- Nakayama, K., Kim, W. S., Torii, S., Hosaka, M., Nakagawa, T., Ikemizu, J., Baba, T. and Murakami, K. (1992) *J. Biol. Chem.* **267**, 5897–5900
- Seidah, N. G., Day, R., Hamelin, J., Gaspar, A., Collard, M. W. and Chtien, M. (1992) *Mol. Endocrinol.* **6**, 1559–1570
- Mbikay, M., Raffin-Samson, M. L., Tadros, H., Sirois, F., Seidah, N. G. and Chtien, M. (1994) *Genomics* **20**, 231–237
- Torii, S., Yamagishi, T., Murakami, K. and Nakayama, K. (1993) *FEBS Lett.* **316**, 12–16
- Mbikay, M., Seidah, N. G., Chtien, M. and Simpson, E. M. (1995) *Genomics*, **26**, 123–129
- Mbikay, M., Tadros, H., Ishida, N., Lerner, C. P., Lamirande, E. D., Chen, A., El-Alfy, M., Clermont, Y., Seidah, N. G., Chtien, M. et al. (1997) *Proc. Natl. Acad. Sci. U.S.A.* **94**, 6842–6846
- De Strooper, B., Creemers, J. W. M., Moechers, D., Huylebroek, D., Van de Ven, W. J. M., Van Leeuwen, F. and Vam den Berghe, H. (1995) *Biochim. Biophys. Acta* **1246**, 185–188
- Li, M., Nakayama, K., Shuto, Y., Somogyvari-Vigh, A. and Arimura, A. (1998) *Peptides* (Tarrytown, N. Y.) **19**, 259–268
- Seidah, N. G., Hamelin, J., Gaspar, A., Day, R. and Chtien, M. (1992) *DNA Cell Biol.* **11**, 283–289
- Moss, B., Elroy-Stein, O., Mizukami, W. A., Alexander, W. A. and Fuerst, T. R. (1990) *Nature* (London) **348**, 91–92
- Fuerst, T. R., Niles, E. G., Studier, F. W. and Moss, B. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 8122–8126
- Jean, F., Basak, A., Rondeau, N., Benjannet, S., Hendy, G. N., Seidah, N. G., Chtien, M. and Lazure, C. (1993) *Biochem. J.* **292**, 891–900
- Donnelly, J. J., Ulmer, J. B. and Liu, M. A. (1994) *J. Immunol. Methods* **176**, 145–152
- Basak, A., Boudreau, A., Chen, A., Chtien, M., Seidah, N. G. and Lazure, C. (1995) *J. Peptide Sci.* **1**, 385–395
- Jean, F., Boudreau, A., Basak, A., Seidah, N. G. and Lazure, C. (1995) *J. Biol. Chem.* **270**, 19225–19231
- Basak, A., Jean, F., Seidah, N. G. and Lazure, C. (1994) *Int. J. Pept. Protein Res.* **44**, 253–261
- Basak, A., Schmidt, C., Ismail, A. A., Seidah, N. G., Chtien, M. and Lazure, C. (1995) *Int. J. Pept. Protein Res.* **46**, 228–237
- Zhou, Y. and Lindberg, Y. (1993) *J. Biol. Chem.* **268**, 5615–5623
- Molloy, S. S., Bresnahan, P. A., Leppla, S. H., Klimpel, K. R. and Thomas, G. (1992) *J. Biol. Chem.* **267**, 16396–16402
- Vidricaire, G., Denault, J. B. and Leduc, R. (1993) *Biochem. Biophys. Res. Commun.* **195**, 1011–1018
- Fuller, R. S., Brake, A. J. and Thorner, J. (1989) *Science* **246**, 482–486
- Hatsuzawa, K., Nagahama, M., Takahashi, S., Takada, K., Murakami, K. and Nakayama, K. (1992) *J. Biol. Chem.* **267**, 16094–16099
- Glazer, A. N. (1968) *Proc. Natl. Acad. Sci. U.S.A.* **59**, 996–1002
- Boudreau, A., Seidah, N. G., Chtien, M. and Lazure, C. (1995) *J. Cell. Biochem.* **19B**, 243
- Duguay, S. J., Milewski, W. M., Young, B. D., Nakayama, K. and Steiner, D. F. (1997) *J. Biol. Chem.* **272**, 6663–6670
- Rinderknecht, E. and Humbel, R. E. (1978) *J. Biol. Chem.* **253**, 2769–2776
- Rinderknecht, E. and Humbel, R. E. (1978) *FEBS Lett.* **89**, 283–286
- Wang, D., Nagpal, M. L., Lin, T., Shimasaki, S. and Ling, N. (1994) *Mol. Endocrinol.* **8**, 69–76
- Dombrowicz, D., Hooghe-Peters, E., Gothot, L., Sente, B., Vanhaelst, L., Closset, J. and Hennen, G. (1992) *Arch. Int. Physiol. Biochim. Biophys.* **100**, 303–308
- Moore, A., Chen, C. L. C., Davies, J. R. E. and Morris, I. D. (1993) *J. Mol. Endocrinol.* **11**, 319–324
- Liu, J. P., Baker, J., Perkins, A. S., Robertson, E. J. and Efstratiadis, A. (1993) *Cell* **75**, 59–72
- Maker, J., Liu, J. P., Robertson, E. J. and Efstratiadis, A. (1993) *Cell* **75**, 73–82
- Macauby, V. M. (1992) *Br. J. Cancer* **65**, 311–320
- Duguay, S. J. and Steiner, D. F. (1995) *J. Cell. Biochem.* **19B**, 243
- Duguay, S. J., Lai-Zhang, J. and Steiner, D. F. (1995) *J. Biol. Chem.* **270**, 17566–17574
- Kimura, C., Ohkubo, S., Ogi, K., Hosoya, M., Itoh, Y., Onda, H., Miyata, A., Jiang, L., Dahl, R. R. and Stibbs, H. H. (1990) *Biochem. Biophys. Res. Commun.* **166**, 81–85
- Arimura, A. (1992) *Regul. Pept.* **37**, 287–295
- Miyata, A., Arimura, A., Dahl, R. R., Minamino, N., Uchara, A., Jiang, L., Culler, M. D. and Coy, D. H. (1989) *Biochem. Biophys. Res. Commun.* **164**, 567–572

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